Evidence That the Satin Hair Mutant Gene Foxq1 Is among Multiple and Functionally Diverse Regulatory Targets for Hoxc13 during Hair Follicle Differentiation

Christopher S. Potter1,2, Ron L. Peterson1,2, Jeremy L. Barth1,2, Nathanael D. Pruet1,2, Donna F. Jacobs3, Michael J. Kern6, W. Scott Argraves6, John P. Sundberg7, and Alexander Awgulewitsch1,2

From the 1Departments of Medicine and 2Cell Biology, Medical University of South Carolina, Charleston, South Carolina 29425, 3Division of Integrative Expression Profiling, Novartis Institutes of Biomedical Research, Cambridge, Massachusetts 02139, and 4the Jackson Laboratory, Bar Harbor, Maine 04609

It is increasingly evident that the molecular mechanisms underlying hair follicle differentiation and cycling recapitulate principles of embryonic patterning and organ regeneration. Here we used Hoxc13-overexpressing transgenic mice (also known as GC13 mice), known to develop severe hair growth defects and alopecia, as a tool for defining pathways of hair follicle differentiation. Gene array analysis performed with RNA from postnatal skin revealed differential expression of distinct subsets of genes specific for cells of the three major hair shaft compartments (cuticle, cortex, and medulla) and their precursors. This finding correlates well with the structural defects observed in each of these compartments and implicates Hoxc13 in diverse pathways of hair follicle differentiation. The group of medulla-specific genes was particularly intriguing because this included the developmentally regulated transcription factor-encoding gene Foxq1 that is altered in the medulla-defective satin mouse hair mutant. We provide evidence that Foxq1 is a downstream target for Hoxc13 based on DNA binding studies as well as co-transfection and chromatin immunoprecipitation assays. Expression of additional medulla-specific genes down-regulated upon overexpression of Hoxc13 requires functional Foxq1 as their expression is ablated in hair follicles of satin mice. Combined, these results demonstrate that Hoxc13 and Foxq1 control medulla differentiation through a common regulatory pathway. The apparent regulatory interactions between members of the mammalian Hox and Fox gene families shown here may establish a paradigm for “cross-talk” between these two conserved regulatory gene families in different developmental contexts including embryonic patterning as well as organ development and renewal.

Recent progress in defining the molecular framework underlying normal and pathological development of skin and hair increasingly implicates the Hox family of transcriptional controller genes as key regulatory molecules (1). The hair follicle is of particular interest because both morphogenesis and cyclical renewal of this complex miniature organ employ many of the same genetic control mechanisms as required for embryonic patterning (2, 3). Follicular morphogenesis is initiated during embryonic development and progresses in successive rostro-caudal waves across the epithelium (4), thus resulting in a fixed number of follicles at various stages of differentiation at birth (5). Furthermore, hair follicles and fibers display a great degree of regionally specified morphological heterogeneity defining hair follicle type (6), and there exists additional variation among follicles of the same type so that probably no two follicles of an individual are identical (7). In keeping with the role of the Hox gene system to define regional identities along the anterior-posterior axis during axial and paraxial patterning including limb development (8–10), it has been proposed that this system might be employed to establish territorial specificity of the skin and its appendages (11). This concept is supported by the observation of distinct anterior-posterior expression domains for various Hox genes in developing skin as well as hair and feather follicles (11–16). In parallel to this regionally restricted expression, certain members of the Hox family apparently are distinctly expressed in all follicles, as it applies to Hoxc13 (17) and probably several other members of this gene family (1). This universal follicular expression suggests essential functional roles during follicle morphogenesis and/or cycling. Support for this concept was provided by the lack of hair in Hoxc13 gene-targeted mice (17) and the delayed hair growth and subsequent loss of hair in Hoxc13-overexpressing transgenic mice (Tg(Hoxc13)61B1Aaw, MGI: 3574566, also referred to as GC13 mice (18)), essentially affecting all body regions in both cases.

In the progressive growth phase, anagen, the Hoxc13 expression pattern in the hair follicle bulb, originates in a conspicuous region of the matrix above the proliferative cell compartment (see Fig. 1 (18)). This expression domain includes a portion of the outer root sheath (ORS)3 layer lining the upper dermal

* This work was supported in part by National Institutes of Health Grants AR47204-04 and RR00173 (to A. A. and J. P. S.) and in part by the Medical University of South Carolina Institutional Research Funds of 2004–2005. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Medicine, Medical University of South Carolina, 96 Jonathan Lucas St., Suite 912 CSB, Charleston, SC 29425. Tel.: 843-792-8946; Fax: 843-792-7121; E-mail: awgulewa@musc.edu.

3 The abbreviations used are: ORS, outer root sheath; IRS, inner root sheath; TEM, transmission electron microscopy; SEM, scanning electron microscopy; PBS, phosphate-buffered saline; PPD, post-partum day; Q-PCR, quantitative-PCR; EMSA, electrophoretic motility shift assay; hd, homeodomain; kb, kilobases; ChIP, chromatin immunoprecipitation; ISH, in situ hybridization; oligos, oligonucleotides; KAP, keratin-associated protein; TALE, three-amino acid loop extension.
Foxq1 Is a Hoxc13 Regulatory Target in Hair

The germative layer is suggested to harbor stem cells that give rise to the diverse lineages differentiating into the individual hair follicle compartments (19), a proposition that has recently been supported by clonal labeling during hair follicle growth (20). This finding combined with the distinctly layered expression patterns of specific hair keratins in the matrix of human hair follicles suggest a high level of organization in the matrix where the compartment-specific lineages originate at distinct proximo-distal levels (19, 20). According to this model, Hoxc13 expression in the upper internal ORS is consistent with a potential role in determining the fate of presumptive progenitors to differentiate along medulla and cortex-specific pathways (see Fig. 1). Furthermore, the continued expression of both mouse Hoxc13 and its human ortholog in differentiating cells of the medulla, cortex, cuticle of the hair shaft, and inner root sheath (IRS), and the companion layer overlaps well with the compartment-specific patterns of various hair keratin genes and suggests a role in their regulation (17–19). This idea is supported by *in vitro* DNA binding data showing sequence-specific interaction of human HOXC13 with cognate binding sites present in the promoter regions of human cortex-specific hair keratin genes *hHa2* and *hHa5* (21) as well as in the mouse *Krt16-5* promoter (22).

Currently it is not clear whether Hoxc13 acts primarily as a downstream effector of other regulatory molecules in the presumptive transcriptional control of these hair keratin genes or whether it might act also further upstream, *i.e.* whether it may influence the expression of other regulatory genes. The remarkable overlap of the Hoxc13 expression pattern with matrix-specific activity domains of several signaling molecules and transcription factors, including Wnt5a, Catnb, Bmp4, Fgf5, Tcf/Lef1, Foxn1, Foxq1, and Mx2 (for review, see Ref. 23) supports the idea of Hoxc13 being engaged in both mechanisms.

To address this question we determined the differential gene expression profile in postnatal skin of GC13 mice by DNA microarray analysis. The results identified genes of different functional categories as potential downstream targets for Hoxc13 in distinct follicle compartments. Genes specifically expressed in the medulla formed the largest subgroup, including Foxq1 as the most conspicuous member of this group. Foxq1sa is the mutated allele underlying the satin hair phenotype (24), a mouse mutation with a defect in the hair follicle medulla. Our data demonstrate that Foxq1 is a downstream target for Hoxc13 during medulla differentiation, thus demonstrating a functional link in mammals between two conserved families of regulatory genes essential for embryonic patterning and organ development.

### MATERIALS AND METHODS

**Transmission and Scanning Electron Microscopy (TEM and SEM)**—Dorsal skin from two severely affected GC13 transgenic mice and from two age- and gender-matched FVB/NTac controls was removed after euthanasia by CO₂ asphyxiation using Institutional Animal Care and Use Committee-approved methods and cut into 1-mm strips in rostro-caudal orientation. Samples were fixed overnight in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2, at 4 °C and transferred into PBS for overnight shipment. Upon receipt tissues were post-fixed overnight in 1% osmium tetroxide, PBS and processed for embedding, sectioning and TEM analysis as described in detail (25). For SEM, parallel skin samples of about 1 cm² taken from the same animals were processed and analyzed essentially as described (25).

**Gene Array Analysis**—Total RNA (10 μg) from post-partum day (PPD) 5 normal and GC13 skin of the trunk was converted into biotinylated, fragmented cRNA according to the Affymetrix protocol. Purification of biotinylated cRNA, hybridization in triplicate to MG-U74Avt2 GeneChips (Affymetrix, San Diego, CA) containing probes for interrogating ~12,500 presumptive genes, and normalization of hybridization data were performed as previously described (26). Comparative expression analysis was conducted using the DNA Chip Analyzer tool (dChip (27, 28)). Differential expression was scored by the following inclusion criteria; (i) genes had to be identified as “present” by GeneChip® MAS 5.0 software in at least 2 of the 3 replicate arrays, (ii) p ≤ 0.01 for the t test (unpaired, 2-tailed, assuming unequal variance), and (iii) changes in average intensity values of ≥2-fold. False discovery rate, estimated as the median number of genes discovered by iterative comparison of randomized sample groupings (29, 30), approximated 0.00 for these criteria. Significant gene ontologies (p < 0.005) were identified by dChip, which derives a probability score (p value) based on hypergeometric distribution of ontology terms (28).

**Real-time Quantitative (Q)-PCR**—Total RNA from PPD 5 normal and GC13 skin was isolated by a CsCl step gradient, and polyadenylated RNA was extracted using the GenElute mRNA Miniprep Kit (Sigma). Complete cDNA was synthesized using a Marathon cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). For standardization, cDNA samples were quantified using a PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR). Q-PCR was performed on an ABI PRISM 7000 sequence detection system using ABI Sybr Green PCR mixture and 20 μM primers exactly as described by the manufacturer. Standard primers for β₂-microglobulin were used as controls and for normalization. Forward (F) and reverse (R) PCR primers specific for the genes tested (Table 1) were (5’→3’): Krt2-1, F-CAGATTGCTGCGTGACCTTCC and R-TCCAGGGCCAGCTTGTTT; Krt2-18, F-CCAGCTTTACCTGTTGGAGAC and R-CCTGTCATAAGGGAAGCCG; Krt2-35, F-GACCAAGGGTAGCTGTGGCA and R-GAGGTTTTGGCCGGGAGGT; Krt5-4, F-TGGCCACACATCTCCAGCA and R-CAGGCTGTAAAGGGTACCGGA; Dsc2, F-CCAGGATATGGATGGCCAGT and R-ATGCACTTTGCGAAGCGC; Krtap5-4, F-TGGCCACACATCTCCAGCA and R-CAGGCTGTAAAGGGTACCGGA; Twist2, F-TTGTGGTTCCTCATGACCTCCC and R-GTCCTGTAGGGTCTCTTCT; Foxq1, F-ACTTCTCCCAGTATAGGCTTT and R-GGAAGGGCAGGCGAACATAG. Cycling conditions for
standard reactions using 200 pg of cDNA were 50 °C for 2 min, 95 °C for 10 min, and (95 °C for 15 s and 60 °C for 1 min) × 50 cycles.

In Situ Hybridization—Freshly dissected scapular skin from euthanized (CO2 asphyxiation) GC13 and FVB/NTac mice at PPD 5 was fixed in 4% paraformaldehyde, PBS at 4 °C overnight and cryo-preserved by overnight incubation in 30% sucrose, PBS before embedding into Tissue-Tek® OCT compound and storage at −80 °C. Probes specific for genes tested (Table 1) were generated by PCR using total cDNA derived from FVB/NTac mice at PPD 5 and the following forward (F)- and reverse (R-) primers: Dsc2, F-CCAGAAGCTGTCAAGACTAC, R-CTCCATAGATGTCATCTGGT; Krtap5-4, F-GCCAGTGTAATAATCTGAG, R-CCACAGAGCTAGAAAAG; Krt-2-I, F-CGCTGAAGTTTGTTTCTCA, R-GAAAGCCTAGA-TCTGAAA; Krt2-16, F-CTGCCAGCACACAAACCT, R-GGCCACATGGAGCTAAGA; Krt2-18, F-CCACTCTCCTCTGC-CTTCT, R-GCAAGACCTGTCTCAAG; Krt2-35, F-AGCGAGCTCTAAGATGTCATCTGGT; Foxn1, F-TCCAGACCTTGCACCCCAAT, R-TGCTATGTCTCCTCAGCAAGCTCT, R-CGTTGAGGCAAATAGGAGG; Wnt5a, F-GCACGCATCC-TCATGAACTTAC, R-CCATCCCCTGAGGTCTTGT. Annealed and labeled oligos were incubated with Hoxc13 protein overnight (22) and a TNT reaction enhancer was used to substitute for the expression plasmid as a negative control. Approximately 24 h after transfection, cells were harvested, luciferase and β-galactosidase assays were performed, and values were normalized as previously described (22).

An enhanced green fluorescent protein expression plasmid was used to substitute for the expression plasmid as a negative control. Approximately 24 h after transfection, cells were harvested, luciferase and β-galactosidase assays were performed, and values were normalized as previously described (22).

Each dish of 3T3 cells was transfected with 0.5 and 0.3 µg of reporter and expression vector, respectively, in addition to 0.2 µg of a β-galactosidase expression vector. C2C12 cells were transfected with double the amount of each plasmid. An enhanced green fluorescent protein expression plasmid was used to substitute for the expression plasmid as a negative control. Approximately 24 h after transfection, cells were harvested, luciferase and β-galactosidase assays were performed, and values were normalized as previously described (22). Each
transfection was performed in triplicate with at least three independent experiments.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed using methods from Ren and Dynlacht (35) with the following modifications. C2C12 mouse myoblast cells were plated at a density $3 \times 10^5$ in 100-mm plates. After 48 h cells were transfected with a Hoxc13 expression vector where the Hoxc13 coding sequence was fused to a FLAG epitope at the amino terminus. After an additional 24 h cells were resuspended using trypsin/EDTA (Sigma) and cross-linked using formaldehyde solution. Glycine (2.5 m) was added to stop the cross-linking reaction. Cells were lysed to extract chromatin and sonicated to generate DNA fragments of $\sim 500$ bp. Immunoprecipitation of FLAG-tagged protein-DNA complexes was achieved by incubating the lysate overnight at 4 °C with EZview Red anti-FLAG M2 affinity gel (Sigma). After 2 washes with PBS the FLAG-tagged complexes were eluted with 2.5 m glycine, pH 3.5. Cross-links were reversed with 1% SDS, and protein was digested with proteinase K. DNA was purified by phenol/chloroform extraction. Precipitated Foxq1 DNA was detected by PCR using Foxq1-specific primer sets A and B (A-fwd, 5'-TCTTCTTCTTCTTCTTC, A-rev, 5'-ACTTCATCCGTACACCTC; B-fwd, 5'-GGCTGGAAACCTTCTATTG; B-rev, 5'-AATTAGCCAGTTGCAAGGTGT). Primer sets specific for Prxl (5’-CTCGAGTTACCTGCACCTCTG; 5’-AGGACTGAGGAGATCTTCTG) and Smad6 (5’-GACGGGTGGGCGTACTGGGGAGCA; 5’-GAG-TAACCCGGTGACACCTTG) genomic regions not containing Hoxc13 consensus binding sequences were used as controls. Annealing for all primers was at 60 °C using standard conditions.

RESULTS

Structural Definition of Hair Defects in Hoxc13-overexpressing Mice—Mouse Hoxc13 and its human ortholog are expressed in all three hair-forming compartments including hair shaft cuticle, cortex, and medulla as well as in the cuticle of the IRS and the companion layer (Fig. 1 (17, 18, 21, 22)). Accordingly, the follicular overexpression in GC13 transgenic mice may affect all of these compartments, and characterization of resulting hair defects has the potential to detect parallels to genetically defined hair mutants exhibiting similar defects. Mouse pelage hair fibers consist of four distinct types, guard, awl, auchenne, and zigzag hair, with the zigzag hair being the most common of truncal hair (6). With GC13 mutant mice, these hair types were difficult to distinguish when viewing skin and hair under SEM because most of the hair fragments of were lysed to extract chromatin and sonicated to generate DNA were transfected with a Hoxc13 expression vector where the Hoxc13 coding sequence was fused to a FLAG epitope at the amino terminus. After an additional 24 h cells were resuspended using trypsin/EDTA (Sigma) and cross-linked using formaldehyde solution. Glycine (2.5 m) was added to stop the cross-linking reaction. Cells were lysed to extract chromatin and sonicated to generate DNA fragments of $\sim 500$ bp. Immunoprecipitation of FLAG-tagged protein-DNA complexes was achieved by incubating the lysate overnight at 4 °C with EZview Red anti-FLAG M2 affinity gel (Sigma). After 2 washes with PBS the FLAG-tagged complexes were eluted with 2.5 m glycine, pH 3.5. Cross-links were reversed with 1% SDS, and protein was digested with proteinase K. DNA was purified by phenol/chloroform extraction. Precipitated Foxq1 DNA was detected by PCR using Foxq1-specific primer sets A and B (A-fwd, 5'-TCTTCTTCTTCTTCTTC, A-rev, 5'-ACTTCATCCGTACACCTC; B-fwd, 5'-GGCTGGAAACCTTCTATTG; B-rev, 5'-AATTAGCCAGTTGCAAGGTGT). Primer sets specific for Prxl (5’-CTCGAGTTACCTGCACCTCTG; 5’-AGGACTGAGGAGATCTTCTG) and Smad6 (5’-GACGGGTGGGCGTACTGGGGAGCA; 5’-GAG-TAACCCGGTGACACCTTG) genomic regions not containing Hoxc13 consensus binding sequences were used as controls. Annealing for all primers was at 60 °C using standard conditions.

RESULTS

Structural Definition of Hair Defects in Hoxc13-overexpressing Mice—Mouse Hoxc13 and its human ortholog are expressed in all three hair-forming compartments including hair shaft cuticle, cortex, and medulla as well as in the cuticle of the IRS and the companion layer (Fig. 1 (17, 18, 21, 22)). Accordingly, the follicular overexpression in GC13 transgenic mice may affect all of these compartments, and characterization of resulting hair defects has the potential to detect parallels to genetically defined hair mutants exhibiting similar defects. Mouse pelage hair fibers consist of four distinct types, guard, awl, auchenne, and zigzag hair, with the zigzag hair being the most common of truncal hair (6). With GC13 mutant mice, these hair types were difficult to distinguish when viewing skin and hair under SEM because most of the hair

![Figure 2. Hair from adult (4 months) GC13 mice exhibits multiple structural defects.](image)

**TABLE 1**

| Gene symbol | Gene name | Accession number | Hybridization data* | FC (array) | p value | Validation |
|-------------|-----------|------------------|---------------------|-----------|---------|------------|
| Kr2-1       | Keratin complex 2, basic, gene 1 | M10937 | Control 440 (P) | Mutant 1129 (P) | 2.57 | 0.0062 | Epidermis 3.0 |
| Kr2-18      | Keratin complex 2, basic, gene 18 | AF021836 | Control 472 (P) | Mutant 197 (P) | -2.39 | 0.0002 | Precortex -2.5 |
| Kr2-35      | Keratin complex 2, basic, gene 35 | AA791234 | Control 388 (P) | Mutant 150 (P) | -2.58 | 0.0009 | Cuticle -3.8 |
| Krtap5-4    | Keratin associated protein 5-4 | AA739024 | Control 288 (P) | Mutant 96 (P) | -2.99 | 0.0000 | Cuticle; IRS -5.5 |
| Dsc2        | Desmocollin 2 | AW228162 | Control 76 (P) | Mutant 18 (P) | -5.59 | 0.0079 | Medulla -4.3 |
| Kr2-16      | Keratin complex 2, basic, gene 16 | X65505 | Control 36 (P) | Mutant 4 (A) | -9.08 | 0.0007 | Medulla -8.9 |
| Twist2      | Twist homolog 2 | U36584 | Control 45 (P) | Mutant 114 (P) | 2.53 | 0.0058 | Medulla 2.5 |
| Foxq1       | Forkhead box Q1 | AF010405 | Control 42 (P) | Mutant 17 (A) | -2.45 | 0.0020 | Medulla -4.0 |
| Foxn1       | Forkhead box N1 | X81593 | Control 16 (P) | Mutant 5 (A) | -3.20 | 0.0037 | Precortex -1.8 |

*Numbers are the averages of normalized replicate hybridization values; majority detection calls (present (P) and absent (A)) are shown in parentheses.

![Figure 2. Hair from adult (4 months) GC13 mice exhibits multiple structural defects.](image)
fibers were twisted and had an indistinct or poor quality cuticle (Fig. 2, A and B) compared with the straight appearance of hair from normal mice that exhibited a well defined and regular cuticular septation (Fig. 2, A’ and B’). Furthermore, although normal mouse skin harbored a dense mass of hair fibers emerging sharply from the epidermis via the follicular osteum (Fig. 2A’), the mutant skin had few hair fibers emerging from ostea with a rosette-like mass of laminated cornified material surrounding the fiber (Fig. 2A). These changes are consistent with cutaneous orthokeratotic hyperkeratosis indicative of IRS defects. TEM analysis of the skin from two sets of mice revealed follicular hyperplasia compared with control mice (data not shown), which is consistent with earlier histopathologic results (18). This hyperplasia was accompanied by a marked increase in intermediate filaments within follicular keratinocytes. The IRS had large numbers of intermediate filaments that formed an intricate basket weave-like pattern near the hair fiber (Fig. 2C) not seen in the control (Fig. 2C’). Furthermore, we recently reported that the highly regular septation seen in the medulla of normal hair shafts is severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36). This abnormality turns out to be very interesting since there exist several mouse hair keratin mutations that severely disrupted in the mutant (36).
Foxq1 Is a Hoxc13 Regulatory Target in Hair

named for their satiny sheen, which is similar to that of the GC13 hair coat and is in both cases due to the abnormal light refraction secondary to hair medulla defects (data not shown). Perhaps the most salient feature of the abnormally differentiated medulla in hair of Foxq1<sup>sa</sup>/Foxq1<sup>sa</sup> mice is the near absence of air spaces resulting in the loss of septation typical for this structure (6). This is accompanied by a lack of cortical ridges and abnormal keratinization (6). The Foxq1<sup>sa</sup> allele carries an intragenic deletion resulting in a frameshift and a truncated protein product (24). To examine how expression of Hoxc13 and Foxq1 itself might be affected by these changes, we performed ISH on dorsal interscapular skin of Foxq1<sup>sa</sup>/Foxq1<sup>sa</sup> mice. Although Hoxc13 expression, normally seen in the lower medulla of FVB mice (Fig. 6A), was difficult to discern in the medulla of satin hair, expression clearly continued to be present in medulla precursor cells capping the dermal papilla (Fig. 6C). This suggests that activation of Hoxc13 in medulla precursor cells does not require functional Foxq1 to be expressed in the same region. Expression of the Foxq1<sup>sa</sup> allele is restricted to the same area in Foxq1<sup>sa</sup>/Foxq1<sup>sa</sup> mice, thus showing that transcription of the defective allele is still initiated in a spatially appropriate manner (Fig. 6D). By contrast, expression of medulla-specific differentiation markers down-regulated in GC13 mice, including Krt2-16 and Dsc2, was no longer detectable in the hair of Foxq1<sup>sa</sup>/Foxq1<sup>sa</sup> mice (Fig. 6, F and H), indicating that their activity was either directly or indirectly dependent on functional Foxq1.

Evidence for Interaction of Hoxc13 with Foxq1 Promoter Region—The previous results are consistent with the concept of a regulatory relationship between Hoxc13 and Foxq1. In support of this idea, we found multiple copies of putative Hoxc13 binding sites in the Foxq1 promoter and 3′-flanking sequences (Fig. 7A). The consensus binding sequence (5′-TT(A/T)ATNpurp-3′) has recently been defined for human HOXC13 (21). An overall amino acid sequence similarity of 98% between the mouse and human orthologs and 100% identity between the homeodomains of these two proteins suggests that this consensus sequence is relevant for mouse Hoxc13. We have recently shown that Hoxc13 does indeed interact specifically with presumptive cognate binding sites present in the Crisp1 promoter region (36), which was one of the medulla-specific genes down-regulated in hair of GC13 mice. In vitro DNA binding studies using EMSAs revealed sequence-specific interaction between Hoxc13 and double-stranded oligos corresponding to two of its three most proximal putative binding sites clustered in the Foxq1 promoter region (Fig. 7, A and B). Hoxc13-oligo complex formation was inhibited by HOXC13-specific antisera (21), which reacts specifically with mouse Hoxc13 in both supershift and immunohistochemical assays (22, 36), whereas anti-tyrosinase-related protein 1 antiserum (see “Materials and Methods”) used as a control did not affect complex formation (Fig. 7B).
To determine whether these Hoxc13-oligo interactions are dependent on the DNA binding function of the Hoxc13 hd, we performed a second set of EMSAs using poly histidine-tagged Hoxc13 (Hoxc13-hd) compared with His-tagged Hoxc13 in which the hd was deleted (Hoxc13Δhd-his). The results show a lack of specific complex formation between Hoxc13Δhd-his and oligos 2411 and 2188 (Fig. 7C, lanes 9 and 19) compared with Hoxc13-his (Fig. 7C). Combined, these data suggest that Hoxc13 specifically interacts with at least two of the cognate binding sites found in the Foxq1 promoter region in vitro.

Further evidence for presumptive regulatory interactions between Hoxc13 and the Foxq1 promoter region was obtained by transient co-transfection assays in NIH3T3 cells and in C2C12 myoblasts. A Foxq1-luciferase reporter gene construct (Foxq1-luc) was utilized that included the two bona fide Hoxc13 binding sites and 12 additional putative Hoxc13 sites within 9 kb of Foxq1 upstream sequences (Fig. 8A). Surprisingly, in contrast to the repression of Foxq1 gene expression by endogenous Hoxc13, co-transfection of this reporter with a Hoxc13 expression vector resulted in ~10- and 5-fold up-regulation of reporter gene activity in 3T3 and C2C12 cells, respectively, compared with control assays without Hoxc13 expression vector (Fig. 8B). Similar results were observed with the vector expressing His-tagged Hoxc13 (Fig. 8C). To determine how truncation of the promoter region affects this activation, we sequentially removed segments of 4 and 8.5 kb in Foxq1ΔB-luc and Foxq1ΔE-luc, respectively (Fig. 8A). Remarkably, Foxq1ΔB-luc showed a 3-fold up-regulation compared with Foxq1-luc, thus suggesting the presence of repressor functions in the deleted region. On the other hand, Foxq1ΔE, in which most of the promoter region was deleted, resulted in complete loss of activity. Furthermore, although co-transfection of Foxq1-luc with Hoxc13Δhd-his versus Hoxc13-his expectedly resulted in a clear reduction of luciferase activity (Fig. 8C), this reduction was only about 50%, thus indicating that part of the Hoxc13-mediated activation of Foxq1-luc was hd-independent. hd-independent transcriptional activation has been reported previously, a particularly relevant case being the regulation of the Bmp4 promoter through Hoxa13 in which one of the three separate activation domains is functional in a hd-independent manner, whereas the other two require the hd (44).

To provide evidence for in vivo occupancy of the Foxq1 promoter region by Hoxc13, we performed ChIP assays with chromatin extracted from C2C12 cells that were transfected with a FLAG epitope-tagged Hoxc13 expression vector. Testing of precipitated chromatin by PCR using two primer sets, Foxq1(A) and Foxq1(B), specific for the proximal Foxq1 promoter region that included the presumptive Hoxc13 binding sites tested by EMSA, resulted in the amplification of Foxq1-specific DNA fragments in both cases (Fig. 9). Control reactions performed with precipitated chromatin and primers specific for exon 1 of Smad6 and the 5′-untranslated region of Prx1 that do not harbor Hoxc13 binding site consensus sequences remained negative (Fig. 9).

In summary, although we currently lack sufficient information to explain the opposite effect of Hoxc13 on in vivo Foxq1 gene expression compared with the results of transient transfection assays, taken together the data derived from EMSAs, co-transfection, and ChIP assays do suggest a role for Hoxc13 in the transcriptional regulation of Foxq1.

**DISCUSSION**

**Functional Complexity of Hoxc13 in Hair**—Despite the great heterogeneity of the tissue used in the present study, i.e. total skin from the trunk of 5-day-old Hoxc13-overexpressing mice, which consists of a wide variety of cell types at different stages of differentiation, we succeeded in identifying a considerable number of presumptive Hoxc13 target genes. During the validation process involving Q-PCR, co-localization of expression with Hoxc13 in hair and in silico analysis of promoter regions for the presence of putative Hoxc13 binding sites, many of these emerged as potential direct targets for Hoxc13 regulation in differentiating hair follicles. Consistent with previous data published by this group and others (18, 21, 22, 36), the data pre-
presented here suggest that Hoxc13 regulates the expression of various sets of "structural" genes (i.e. genes encoding hair keratins, KAPs, and desmosomal cadherins) that are required for proper terminal differentiation of distinct hair follicle compartments.

Two of the four KAP genes found to be down-regulated, Krtap8-2 and Krtap6-3 (see the supplemental list of differentially expressed genes), were also isolated by us in a previous screen for differentially expressed genes in postnatal GC13 skin by using a different method, i.e. cDNA suppression subtraction hybridization (18). Both KAP genes were identified as members of a novel KAP gene domain on MMU16 in a region of conserved linkage to HSA21q22.11 (18, 22), and most of the cDNAs isolated by suppression subtraction hybridization in the previous study corresponded to this domain (18, 22), a bias that was not observed in the DNA microarray data set presented here. The up-regulation of Krt2-1 in the epidermis (Table 1) was also noted in the previous screen (18), and although this finding is consistent with the epidermal hyperproliferation and ichthyosiform condition observed in GC13 mice, a potential mechanistic link to the overexpression of Hoxc13 in hair follicles is currently obscured since epidermal Hoxc13 expression was not detectable by ISH in both normal and GC13 mice (data not shown).

In addition to implicating Hoxc13 in the regulation of structural genes, we have validated Hoxc13-induced expression changes for two upstream regulatory transcription factors (Foxq1 and Foxn1, Table 1) and have obtained differential expression data for several other regulatory factors including Tcf3, Sox18, and Smad7 (supplemental list of differentially expressed genes). These regulatory genes are distinctly expressed in the hair follicle matrix (Refs. 24 and 45–49; for review, see Ref. 23), and many, including Hoxc13, are conspicuously expressed in the internal ORS layer surrounding the dermal papilla, also known as germinative layer (Fig. 1), which recently has been demonstrated to harbor restricted self-renewing stem cells (20). These cells are organized in distinct sectors arranged symmetrically on either side of the dermal papilla, and their position along the proximal-distal axis determines the specific compartment lineage of transient amplifying or differentiating cells originating from these precursors (20, 19). According to this model and assuming that the presumptive regulatory tar-
**Foxq1 Is a Hoxc13 Regulatory Target in Hair**

**Figure 8.** Subregions of Foxq1 promoter are differentially responsive to Hoxc13. A, maps of Foxq1-luc, Foxq1ΔB-luc, and Foxq1ΔE-luc containing −9, 5, and 0.5 kb, respectively, of Foxq1 promoter region. B, co-transfection assays with Foxq1-luc and Hoxc13 expression vector in NIH3T3 cells and C2C12 myofibroblasts indicate an ~5–10-fold up-regulation of reporter gene activity compared with control assays without Hoxc13 expression vector. C, co-transfection of Foxq1ΔB-luc with Hoxc13-his expression vector reveals an ~3-fold enhancement of expression compared with Foxq1-luc, whereas removal of most of the Foxq1 promoter region in Foxq1ΔE-luc, including all of the presumptive Hoxc13 binding sites, results in complete loss of luciferase activity. Interestingly, co-transfection of Foxq1-luc with Hoxc13-hd-his, in which the homeodomain was deleted, resulted in only a 50% reduction of expression compared with co-transfection with Hoxc13-his.

The disorganized expression of targets as we have done in the case of Foxq1, Hoxc13 is likely to have an important function in the organization of the matrix and its lineage trajectories, at least as far as this affects the organization of the upper lineages contributing to the hair shaft compartments. The confounding aspect of Foxq1 in vivo is that in co-transfection assays Foxq1 was activated, whereas down-regulation in GC13 versus normal mice suggests that Hoxc13 acts as a repressor of Foxq1 in vivo in hair follicles. These opposing effects are likely reconcilable with each other when considering that plasmid-based reporter gene activation in cultured cells is unlikely to reflect an authentic response of the corresponding gene in its native cellular and nuclear environment. The absence of appropriate chromatin structure, lack of potentially critical control elements not included in the reporter construct, and importantly, cell type-specific differences in co-factor supply are only some of many issues that have to be considered when interpreting these results. Even if the same co-factors were present in diverse cell types, the regulation of targets still may be altered by signaling cascades. For example, Hox-Pbx cofactor complexes may be switched from transcriptional repressor to activator in response to protein kinase A signaling or cell aggregation.

Numerous studies in recent years revealed a continuously broadening spectrum of regulatory mechanisms affecting Hox-target gene interaction including sequence-specific binding of Hox proteins as monomers (55) and in association with one or several cofactors (56). The most thoroughly characterized cofactors known to selectively and cooperatively increase binding affinity and specificity of Hox proteins belong to the class of...
TALE (three-amino acid loop extension) homeodomain proteins and include members of the Pbx, Meis, and Prep groups (56, 57). Potential cofactor requirements for Hoxc13 are currently unknown. The in vitro cofactor-independent binding of human and mouse Hoxc13 to HOXC13 consensus binding sequences present in the promoter regions of several hair keratin and KAP genes (21, 22) as well as Crisp1 (36) and Foxq1 (Fig. 7, A and B) does not preclude Hoxc13-cofactor interaction in vivo. Examination of the human hHa5 and hHa2 hair keratin gene promoter regions known to drive reporter gene expression in PtK2 cells upon co-transfection with a HOXC13 expression vector in a homeodomain-dependent manner (21) identified perfect matches to both Meis/Prep heterodimer and Pbx consensus binding sequences (56) located upstream of multiple monomeric HOXC13 sites (21). However, although expression of Pbx1-4, Meis1a, Meis1b, and Meis2 as well as Prep1 and Prep2 was indicated in the bulb of human anagen hair follicles by reverse transcriptase PCR, immunofluorescence studies localized the corresponding proteins primarily in the IRS and mid- to lower cortex, and none of them showed nuclear colocalization with HOXC13 (58). In light of these results, the significance of multiple putative Pbx1 as well as composite Pbx1/Meis1 and Meis1/Prep1 sites found to be interdigitated with the Hoxc13 binding sites in the Foxq1 genomic 5′- and 3′-flanking regions (data not shown) is currently uncertain.

The circumstance that none of the putative Hoxc13 binding sites (Fig. 7A) was found to form a potential heterodimer site with any of the TALE cofactors might lend support to the concept of a monomeric role for Hoxc13. Although there is generally little information about the regulation of Hox target genes in a cofactor-independent manner, data showing repression of the Drosophila spalt gene through multiple Ubx monomeric binding sites have led to the proposition that regulation of some Hox target genes evolved via the accumulation of multiple monomeric binding sites (59). Because Hox proteins generally bind with low affinity to monomeric sites, the cumulative effects of interactions with multiple low affinity binding sites may range from relatively high levels of expression to complete repression of a target gene depending on Hox protein concentration. The quantitative changes in Foxq1 expression observed in GC13 versus normal mice (Table 1; Fig. 5, E and F) are consistent with this model. This type of modulated response offers greater adaptability and, thus, greater system stability than a simple on-off switch type of regulation.

Recent evidence that Hox-mediated transcriptional responses depend on compartment-specific contextual information (60, 43) provides a further argument for reconciling the opposing effects of Hoxc13 on Foxq1 regulation in in vivo versus cultured cells. According to this model, the contextual co-factors do not control the binding properties of Hox-TALE protein complexes but modulate the transcriptional output depending on whether cognate binding sites required for their recruitment are present in cis-regulatory regions of target genes (43). Although this has not yet been shown in mammals, it is likely that cell-type-specific co-factors will be of universal relevance in determining the quality of Hox-controlled transcriptional responses.

Acknowledgments—We thank Dr. J. Schweizer for HOXC13 antiserum, Drs. D. Kurtz and D. Watson for advice on ChIP assays, and K. Silva and L. Bechthold for expert assistance with SEM and TEM studies. The use of the Medical University of South Carolina (MUSC) Proteogenomic Facility was supported by the MUSC Research Resource Facilities program and National Institutes of Health Grants (R24CA95841 to W. S. A.), RR-16434 and C06-RR015455.

REFERENCES

1. Awgulewitsch, A. (2003) Naturwissenschaften 90, 193–211
2. Oro, A. E., and Scott, M. P. (1998) Cell 95, 575–578
3. Schmidt-Ullrich, R., and Paus, R. (2005) BioEssays 27, 247–261
4. Mann, S. J. (1962) Anatom. Rec. 145, 135–142
5. Stenn, K. (1988) Cell 62, 271–282
6. Sundberg, J. P., and Hogan, M. E. (1994) in (Sundberg, J. P., ed) pp. 541–572, Urban and Schwarzenberg, Baltimore-Munich
7. Duboule, D. (1992) BioEssays 14, 534–558
8. Mcginnis, W., and Krumlauf, R. (1992) Cell 68, 283–302
9. Bauers, D. (1999) BioEssays 14, 375–384
10. Capcacci, M. R. (1997) Cold Spring Harbor Symp. Quant. Biol. 62, 273–281
11. Chuong, C. M., Oliver, G., Ting, S. A., Jegalian, B. G., Chen, H. M., and De Robertis, E. M. (1990) Development 110, 1021–1030
12. Biberich, C. J., Rubble, F. H., and Stenn, K. S. (1991) Ann. N. Y. Acad. Sci. 642, 346–354
13. Chuong, C. M. (1993) BioEssays 15, 513–521
14. Chuong, C. M., and Noveen, A. (1999) J. Investig. Dermatol. Symp. Proc. 4, 307–311
15. Kanzler, B., Viallet, J. P., Le Mouellic, H., Boncinelli, E., Duboule, D., and Dhouailly, D. (1994) Int. J. Dev. Biol. 38, 633–640
16. Reid, A. I., and Gaunt, S. J. (2002) Int. J. Dev. Biol. 46, 209–215
17. Godwin, A. R., and Capecchi, M. R. (1998) Genes Dev. 12, 11–20
18. Tkatchenko, A. V., Visconti, R. P., Shang, L., Papenbrock, T., Pruet, N. D., Ito, T., Ogawa, M., and Awgulewitsch, A. (2001) Development 128, 1547–1558
Foxq1 Is a Hoxc13 Regulatory Target in Hair

19. Langbein, L., and Schweizer, J. (2005) *Int. Rev. Cytol.* **243**, 1–78
20. Legué, E., and Nicolas, J. F. (2005) *Development* **132**, 4143–4154
21. Jave-Suarez, L. F., Winter, H., Langbein, L., Rogers, M. A., and Schweizer, J. (2002) *J. Biol. Chem.* **277**, 3718–3726
22. Pruett, N. D., Tkatchenko, T. V., Jave-Suarez, L., Jacobs, D. F., Potter, C. S., Tkatchenko, A. V., Schweizer, J., and Awgulewitsch, A. (2004) *J. Biol. Chem.* **279**, 51524–51533
23. Fuchs, E., Merrill, B. J., Jamora, C., and DasGupta, R. (2001) *Dev. Cell* **1**, 13–25
24. Hong, H. K., Noveroske, J. K., Headon, D. J., Liu, T., Sy, M. S., Justice, M. J., and Chakravarti, A. (2005) in *Systematic Characterization of Mouse Mutations* (Sundberg, J. P., and Boggess, D., eds) pp. 121–129, CRC Press, Inc., Boca Raton, FL
25. Bechtold, L. S. (2000) in *Genesis* (Legue´, E., and Nicolas, J. F. (2005)) pp. 3718–3726
26. Peterson, R. L., Wang, L., Albert, L., Marchese, E., Wong, A., Mounts, W. M., Hayes, L., Bouchard, P., Keith, J., and Dorner, A. J. (2002) *Pharmacogenomics* **2**, 283–399
27. Li, C., and Hung Wong, W. (2001) *Genome Biology* **2**, RESEARCH0032
28. Li, C., and Wong, W. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 31–36
29. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D., and Lander, E. S. (1999) *Science* **286**, 531–537
30. Tusher, V. G., Tibshirani, R., and Chu, G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5116–5121
31. Murtaugh, L. C., Chyung, J. H., and Lassar, A. B. (1999) *Genes Dev.* **13**, 225–237
32. Frank, S., and Zoll, B. (1998) *DNA Cell Biol.* **17**, 679–688
33. Kozak, M. (1983) *Microbiol. Rev.* **47**, 1–45
34. Norris, R. A., and Kern, M. J. (2001) *J. Biol. Chem.* **276**, 26829–26837
35. Ren, B., and Dynlacht, B. D. (2004) *Methods Enzymol.* **376**, 304–315
36. Peterson, R. L., Tkatchenko, T. V., Pruett, N. D., Potter, C. S., Jacobs, D. F., and Awgulewitsch, A. (2005) *J. Invest. Dermatol. Symp. Proc.* **10**, 238–242
37. Langbein, L., Rogers, M. A., Praetzel, S., Winter, H., and Schweizer, J. (2003) *J. Invest. Dermatol.* **120**, 512–522
38. Langbein, L., Rogers, M. A., Praetzel, S., Aoki, N., Winter, H., and Schweizer, J. (2002) *J. Invest. Dermatol.* **118**, 789–799
39. Wood, L., Mills, M., Hatzenbuhler, N., and Vogeli, G. (1990) *J. Biol. Chem.* **265**, 21375–21380
40. Schlake, T. (2005) *Development* **132**, 2981–2990
41. Johns, S. A., Soulier, S., Rashbass, P., and Cunliffe, V. T. (2005) *Dev. Dyn.* **232**, 1062–1068
42. Lin, K. K., Chudova, D., Hatfield, G. W., Smyth, P., and Andersen, B. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 15955–15960
43. Merabt, S., Pradel, J., and Graba, Y. (2005) *Trends Genet.* **21**, 477–480
44. Suzuki, M., Ueno, N., and Kuroiwa, A. (2003) *J. Biol. Chem.* **278**, 30148–30156
45. Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H., and Boehm, T. (1994) *Nature* **372**, 103–107
46. DasGupta, R., and Fuchs, E. (1999) *Development* **126**, 4557–4568
47. Merrill, B. J., Gat, U., DasGupta, R., and Fuchs, E. (2001) *Genes Dev.* **15**, 1688–1705
48. Pennisi, D., Bowles, J., Nagy, A., Muscat, G., and Koopman, P. (2000) *Mol. Cell. Biol.* **20**, 9331–9336
49. Liu, X., Alexander, V., Vijayachandra, K., Bhogte, E., Diamond, I., and Glick, A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 9139–9144
50. Auber, L. (1952) *Trans. R. Soc. Edinb.* **62**, 191–254
51. Liu, X. Y., Dangel, A. W., Kelley, R. I., Zhao, W., Denny, P., Botcherby, M., Cattanach, B., Peters, J., Hunsicker, P. R., Mallon, A. M., Strivens, M. A., Bate, R., Miller, W., Rhodes, M., Brown, S. D., and Herman, G. E. (1999) *Nat. Genet.* **22**, 182–187
52. Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. L., and Dunn, A. R. (1993) *Cell* **73**, 249–261
53. Chen, K., Manga, P., and Orlow, S. J. (2002) *Mol. Biol. Cell* **13**, 1953–1964
54. Saleh, M., Rambaldi, I., Yang, X. J., and Featherstone, M. S. (2000) *Mol. Cell. Biol.* **20**, 8623–8633
55. Pinsonneault, J., Florence, B., Vaessen, H., and McGinnis, W. (1997) *EMBO J.* **16**, 2032–2042
56. Mann, R. S., and Affolter, M. (1998) *Curr. Opin. Genet. Dev.* **8**, 423–429
57. Williams, T. M., Williams, M. E., and Innis, J. W. (2005) *Dev. Biol.* **277**, 457–471
58. Jave-Suárez, L. F., and Schweizer, J. (2006) *Arch. Dermatol. Res.* **297**, 372–376
59. Galant, R., Walsh, C. M., and Carroll, S. B. (2002) *Development* **129**, 3115–3126
60. Gebelein, B., McKay, D. J., and Mann, R. S. (2004) *Nature* **431**, 653–659