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Foxn1 Is Required for Tissue Assembly and Desmosomal Cadherin Expression in the Hair Shaft

Sarah A. Johns,[†] Stephan Soullier,[‡] Penny Rashbass, and Vincent T. Cunliffe[*]

The mouse *nude* mutation inactivates the gene encoding the Foxn1 transcription factor, causing defective hair morphogenesis. Here, we show for the first time that Foxn1 is required for proper assembly of the hair medulla, and we identify Foxn1-regulated genes by transcript profiling. One such gene encodes the desmosomal cadherin, Dsc2. Significantly, Foxn1-dependent *Dsc2* expression is restricted to the hair medulla, and within these cells, Dsc2 protein is predominantly localized to specialized adhesion junctions between the cortex and the medulla. Our results reveal Foxn1 as an essential regulator of tissue assembly in the growing hair shaft and implicate Dsc2 as a downstream effector of this activity. *Developmental Dynamics 232:1062–1068, 2005.* © 2005 Wiley-Liss, Inc.

Key words: Foxn1; hair follicle; morphogenesis; desmosomal cadherin

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INTRODUCTION

The mammalian hair follicle provides an interesting model for the analysis of molecular and cellular mechanisms underlying tissue morphogenesis (Stenn and Paus, 2001; Fuchs et al., 2001). Within the follicle, the growing hair shaft comprises three major cell types, arranged in concentric layers around the hair's principal axis: a central core of medulla cells, a middle layer of keratin-rich cortex cells, and a superficial layer of flattened, overlapping cuticle cells. Little is known about the mechanisms that determine hair shape, its structural integrity, and the relative proportions of the three hair cell lineages in different hair types. However, some insights into these processes have come from the analysis of transgenic and mutant mice with coat defects (Nakamura et al., 2001). The *nude* mutation causes one of the most severe mutant coat phenotypes characterized to date (Kopf-Maier et al., 1990; Mecklenburg et al., 2001). Hair shafts erupt from the skin of *nu*/+ animals at postnatal day (P) 5.5, whereas they fail to do so in *nu*/*nu* animals. Instead, *nu*/*nu* mutant hair shafts bend and coil in the upper follicle, then break up into fragments. A sparse coat of short, bent hair shafts lacking cuticular scales eventually emerges onto the surface of the skin. In addition to severe alopecia, development of the thymic epithelium is also compromised in *nude* mice, leading to profound immunodeficiency.

The gene mutated at the *nude* locus encodes a transcription activator, Foxn1 (Nehls et al., 1994; Schuddekopf et al., 1996). In the hair follicle, *Foxn1* is transcribed primarily in the postmitotic precursors of the differentiating hair cortex and cuticle (Lee et al., 1999). In the thymus, Foxn1 expression is restricted to the thymic epithelium (Nehls et al., 1996). Experiments to identify genes whose

Centre for Developmental Genetics, School of Medicine and Biomedical Science, University of Sheffield, Firth Court, Western Bank, Sheffield, United Kingdom
Grant sponsor: The Wellcome Trust; Grant number: 052747.
[†]Dr. Johns' present address is Department of Animal and Plant Sciences, University of Sheffield, Alfred Denny Building, Western Bank, Sheffield, S10 2TN, UK.
[‡]Dr. Soullier's present address is Institut de Genetique Humaine, CNRS, UPR 1142, 141 rue de la Cardonille, 34396 Montpellier Cedex 05, France
[*]Correspondence to: Vincent T. Cunliffe, Centre for Developmental Genetics, School of Medicine and Biomedical Science, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, UK. E-mail: v.t.cunliffe@shef.ac.uk

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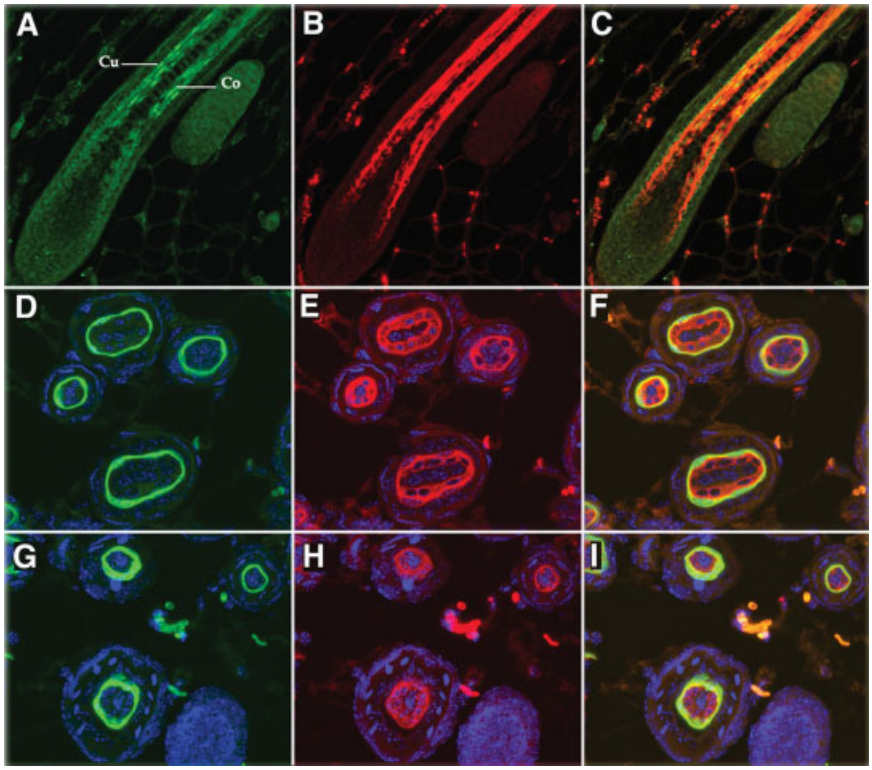

Fig. 1. Foxn1 protein is expressed in nascent hair cuticle and cortex but is dispensable for their specification. **A–C:** Longitudinal skin sections from wild-type mice stained with anti-Foxn1 (green) and anti-hair keratin (red) antibodies. Note absence of Foxn1 expression in hair medulla. **D–I:** Transverse sections through hair follicles of *nu*/+ (D–F) and *nu*/*nu* (G–I) littermates at postnatal day 7.5, showing expression of hair keratins in the cortex (Co in A) and cuticle (red, AE13; Cu in A) and specific expression of S100A3 in cuticle cells (green). Skin sections are oriented so that epidermis is toward the top and hair follicle bulbs are toward the bottom of each panel.

expression in the hair follicle requires Foxn1 indicate that some of the likely direct targets for Foxn1 in the hair cortex are hair-specific keratin genes and metallothionein-IV (Meier et al., 1999; Schlake et al., 2000; Schorpp et al., 2000; Schlake and Boehm, 2001). Another expression-profiling study of the embryonic thymus identified the PD-1 ligand as a likely downstream effector of Foxn1 function (Bleul and Boehm, 2001). Together, these results suggest that Foxn1 regulates a variety of target genes during development.

Overexpression of transcription factors can be a highly efficient route to the identification of low-abundance transcripts from downstream effector genes (Tada et al., 1998). We adopted this approach to investigate the in vivo function of *Foxn1* and compared the expression profiles of skin samples from *Foxn1*-overexpressing transgenic mice and from *nude* mutant mice. In this study, we identify several genes whose expression levels in the skin are strictly Foxn1 dose-dependent, one of which is the desmosomal cadherin gene *Dsc2*. We also investigated the cellular defects in *nude* hair follicles, and report that Foxn1 is re-

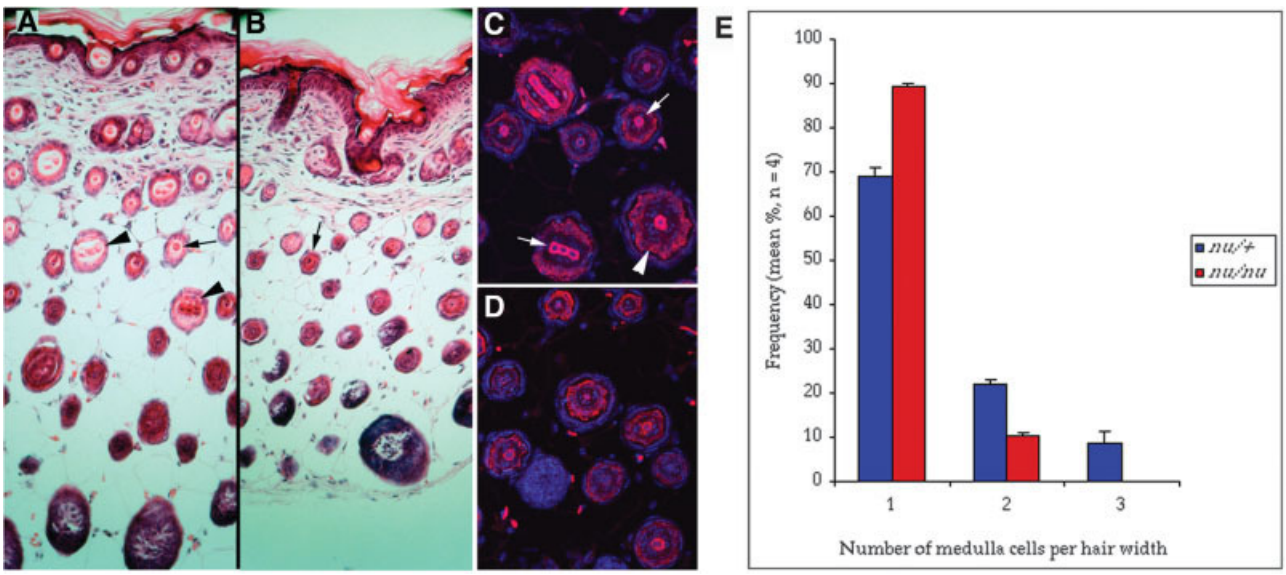

Fig. 2. Foxn1 promotes the assembly of septulate hair shafts. **A,B:** Hematoxylin–eosin stained skin sections from postnatal day (P) 7.5 *nu*/+ (A) and *nu*/*nu* (B) littermates, showing septate (arrows, one column of medulla cells) and septulate (arrowheads, multiple columns of medulla cells) hair shafts in transverse cross-section. B: Note a deficiency of septulate hairs. Similar deficiencies of septulate hair shafts were also observed in *nu*/*nu*mutants analyzed at P10.5. **C,D:** Immunostaining P7.5 *nu*/+ (C) and *nu*/*nu* (D) skin sections shows expression of trichohyalin in the hair medulla (arrows in C) and IRS (arrowhead in C) of both *nu*/+ and *nu*/*nu* animals and also reveals a deficiency of septulate hair shafts in *nu*/*nu* skin. **E:** Quantitative analysis of medulla cell numbers per follicle cross-section in P7.5 *nu*/+ and *nu*/*nu* mice. Four animals of each genotype were analyzed, with between 50 and 120 follicle cross-sections scored per animal. Results are expressed as percentages of the number of cross-sections analyzed per animal ± SEM. In A–D, sections are oriented as in Figure 1.

quired for proper assembly of the hair medulla. Our results indicate that reduced expression of cell adhesion machinery such as Dsc2 in the hair medulla is a significant factor leading to the hair shaft defects in *nude* mutants.

RESULTS AND DISCUSSION

Foxn1 Gene Product Is Dispensable for Hair Cell Type Specification but Essential for Hair Shaft Assembly

In the mid-anagen hair follicle, Foxn1 protein is specifically localized to the nuclei of differentiating, postmitotic cortex and cuticle cells (Fig. 1), which is consistent with its proposed role in mediating keratinization of these cell types (Meier et al., 1999). However, Foxn1 is not required for the onset of either hair cuticle or cortex differentiation, because both differentiated cell types can readily be distinguished in anagen hair follicles of *nu/+* and *nu/nu* littermates (Fig. 1D–I). We compared the structure of the hair medulla in *nu/+* and *nu/nu* hair follicle cross-sections, which reveals a marked deficiency of septulate hairs with multiple columns of medulla cells in *nu/nu* skin, suggesting that Foxn1 is required for the efficient formation of medulla with a septulate organization (Fig. 2A–E). The observed frequencies of septate and septulate hairs in *nu/+* skin are similar to previous measurements in wild-type animals (Sundberg and Hogan, 1994). Because Foxn1 protein is undetectable in the medulla of wild-type hair shafts (Fig. 1), these observations suggest that interactions between Foxn1-expressing cortex and adjacent medulla promote assembly of the medulla. Although medulla assembly is defective in *nu/nu* mutant hair shafts, medulla cells still express the differentiation marker trichohyalin (Fig. 2C,D), confirming that this cell type can still be specified in the absence of *Foxn1* function.

Identification of Downstream Effectors of Foxn1 Function by Transcript Profiling on cDNA Microarrays

To identify candidate genes encoding proteins involved in Foxn1-dependent cell interactions within the hair shaft, a microarray-based expression screen was carried out. To maximize the sensitivity of the screen, we took advantage of the Foxn1TG1 transgenic line in which Foxn1 was overexpressed in the hair cuticle and cortex (Cunliffe et al., 2002) and looked for transcripts that were detectable in Foxn1TG1 skin but absent or much reduced in skin of *nu/nu* animals. An array of cDNAs from the NIA mouse embryonic cDNA set (Tanaka et al., 2000) was cohybridized with labeled cDNA probes synthesized from Foxn1TG1 and *nu/nu* skin mRNA. Differentially expressed genes were identified, and their degree of differential expression was quantified (Fig. 3A). Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) then confirmed independently the sensitivity of these genes to the available dose of functional Foxn1 protein (Fig. 3B). To validate the RT-PCR conditions used for detection of Foxn1 dose-dependent transcripts, assays for *mHa3* and *MT-IV* expression were performed (Fig. 3B), along with the use of three separate loading controls (*Hsc73*, *Gapdh*, and *Arbp*). Only genes with expression profiles that were Foxn1 dose-dependent were retained for further study. These genes include *TPD52l1* (accession no. C87174), *Stat1* (AA408197), *eIF2B-delta* (AW537268), a novel RING-finger protein *Frg4* (*Foxn1-regulated gene 4*; AW543469), *desmocollin 2* (*Dsc2*, AA409377), *Cupidin* (AA407944), and *cytokeratins 1–18* (AW538107) and *2–8* (C77408). All of these genes were more abundantly expressed in *Foxn1TG1:nu/+* mice than in *nu/nu* mice when analyzed by RT-PCR, in full confirmation of the results obtained from the microarray analysis. Of interest, whereas most genes exhibited a level of transcription that increased progressively as the dose of functional Foxn1 protein increased, the expression level of *Cupidin* was similar in both *Foxn1TG1:nu/+* and *nu/+* mice.

Foxn1 Is Required for Expression of the Desmosomal Cadherin *Dsc2* in the Hair Medulla

Of the Foxn1-dependent genes identified in our study, the desmosomal cadherin *Dsc2* appeared to be a strong candidate for mediating the observed effect of Foxn1 on the assembly of the hair medulla (Garrod et al., 1996; Green and Gaudry, 2000). Therefore, we compared the expression pattern of *Dsc2* mRNA and localization of Dsc2 protein in the hair follicles of *nu/+* and *nu/nu* mice. In *nu/+* skin, strong expression of *Dsc2* mRNA is restricted to the hair medulla (Fig. 4A). Moreover, Dsc2 protein accumulates specifically in the specialized A-ridges of *nu/+* hair shafts (Fig. 4I,O, arrowheads; Trigg, 1972). Lower levels of Dsc2 protein can also be detected at the interfaces between vertically adjacent medulla cells (Fig. 4G,I, arrow). In contrast, *Dsc2* transcription is dramatically reduced in *nu/nu* hair shafts (Fig. 4B). Furthermore, only trace amounts of Dsc2 protein can be detected at the cortex–medulla interface, and there is an absence of prominent A-ridges in *nu/nu* shafts (Fig. 4J–L,P–R). Close comparison of *nu/+* and *nu/nu* hair shafts also reveals defective proximodistal alignment of medulla cells in the absence of Foxn1 function (evident as irregular kinks in the medulla, Fig. 4G–L). In contrast to the effect of the *nude* mutation on *Dsc2*, the medulla cells of *nu/nu* hair shafts still express trichohyalin (Fig. 2C,D), indicating that only particular aspects of the medulla differentiation program are affected by loss of *Foxn1* function.

Foxn1: Programming Tissue Assembly by Means of Coordinated Synthesis of Cytoskeletal and Cell Adhesion Machinery

We demonstrate here for the first time that the Foxn1 transcription factor is specifically required for assembly of the hair medulla, as well as for mediating specific expression of *Dsc2* in the hair medulla. Surprisingly, we find that Foxn1 protein is specifically localized to the hair cortex and cuticle and undetectable in medulla cells. Although it is possible that Foxn1 protein is present in the medulla at levels below the threshold of detection, this seems unlikely as neither in situ hybridization for *Foxn1* mRNA, nor histochemical analysis of a *lacZ* expres-

sion cassette inserted into the *Foxn1* locus previously detected Foxn1 expression in the hair medulla (Meier et al., 1999; Lee et al., 1999; Schlake and Boehm, 2001). A previous microarray study identified expression of *Dsc2* as being Foxn1-dependent in the skin (Schlake and Boehm, 2001); however, precisely which cell types within the skin expressed *Dsc2* in response to Foxn1 activity was not determined in that study. Our results clearly show that Dsc2 is specifically expressed in the hair medulla, and they suggest that Foxn1 promotes expression of a signal in the hair cortex and cuticle that induces medulla cells to up-regulate expression of *Dsc2*. Several different intercellular signaling pathways are known to regulate cell adhesion in the skin (Jamora and Fuchs, 2002). Moreover, Foxn1 modulates expression of at least one signaling molecule, transforming growth factor-alpha, in keratinocytes (Prowse et al. 1999). In addition, several lines of evidence now support the view that desmosomes themselves can act as intercellular signaling complexes (reviewed by Green and Gaudry, 2000), and it is possible, therefore, that *Foxn1* regulates expression of desmosomal components in cortex cells that promote expression and/or stable accumulation of desmosomal components in the adjacent medulla. The reduced expression of keratin intermediate filaments in the hair cortex of *nude* mutants may also influence the intercellular signaling properties of desmosomal

A

Gene name	Accession Number	Mean Fold Change (Foxn1TG1/nu)
TPD52l1	C87174	5.8 +/- 1.1
Stat 1	AA408197	2.6 +/- 1.1
eIF2B-delta	AW537268	4.2 +/- 0.7
Frg4	AW543469	3.6 +/- 0.6
Dsc2	AA409377	3.4 +/- 0.5
Cupidin	AA407944	3.1 +/- 0.6
Keratin 1-18	AW538107	2.9 +/- 0.6
Keratin 2-8	C77408	2.6 +/- 0.6

B

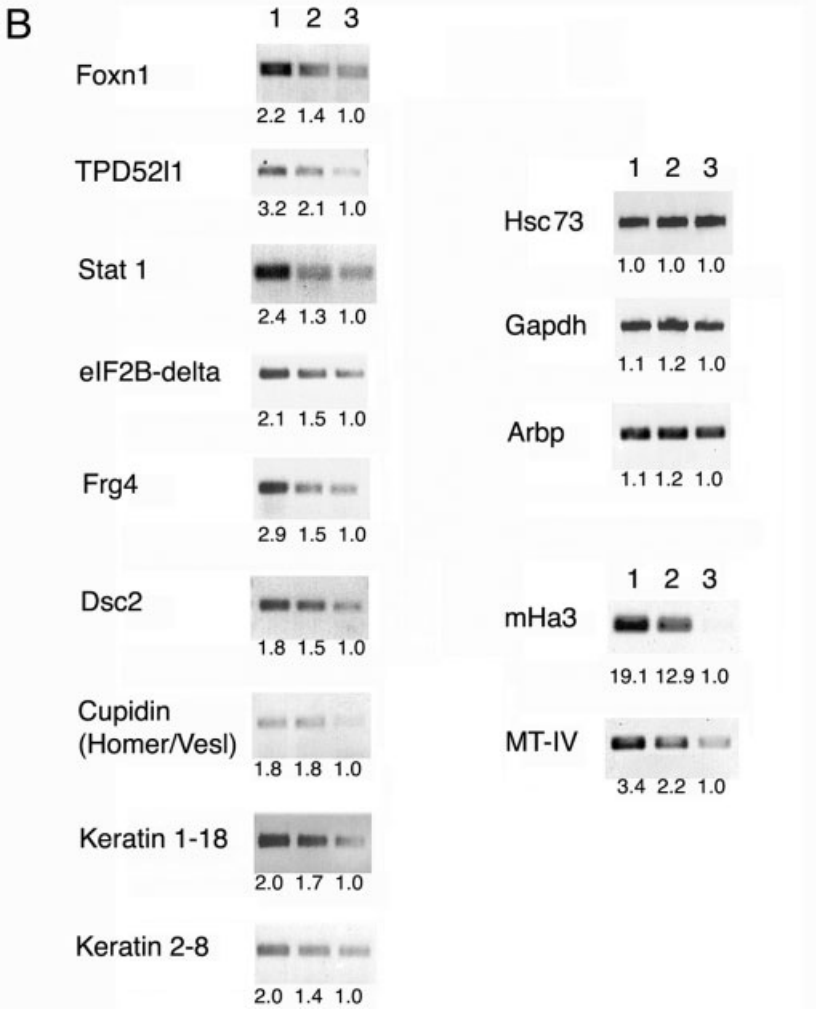

Fig. 3. Identification of Foxn1-dependent transcripts in postnatal day (P) 7.5 anterior back skin. **A:** Quantification of expression level differences for Foxn1-dependent transcripts in *Foxn1TG1:nu*/+ and *nu*/*nu* skin samples using microarrays. Results are expressed as fold-differences in transcript levels between the two genotypes ± SEM. For each gene, n = 6 except for *Stat1*, where n = 5. **B:** Semiquantitative reverse transcriptase-polymerase chain reaction (PCR) analysis of Foxn1-dependent transcripts in skin. Lane 1, P7.5 *Foxn1TG1:nu*/+; lane 2, P7.5 *nu*/+; lane 3, P7.5 *nu*/*nu*. *Hsc73*, *Gapdh*, and *Arbp* exhibited essentially equivalent expression levels in all samples. *mHa3* and *MT-IV* were previously identified as Foxn1-dependent transcripts (Schlake and Boehm, 2001). Expression level differences between genotypes were quantitated densitometrically and are expressed as relative quantities of PCR product generated, immediately below the data for each gene.

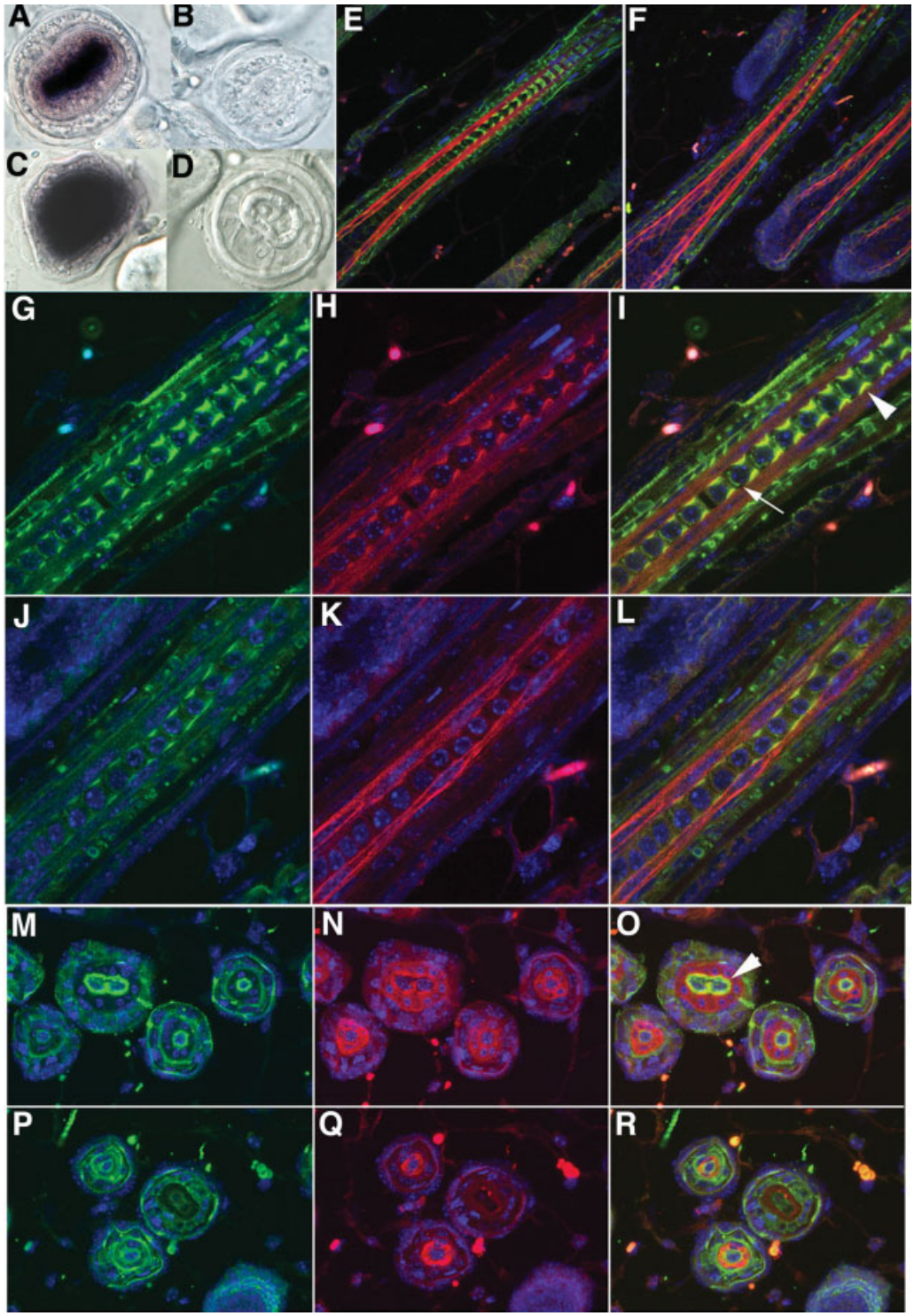

Fig. 4. A–R: Foxn1 is required for *Dsc2* expression in the hair medulla. Transverse (A–D,M–R) and longitudinal (E–L) sections through postnatal day (P) 7.5 hair follicles in anterior back skin of *nu*/+ (A,C,E,G–I,M–O) and *nu*/*nu* (B,D,F,J–L,P–R) littermates. **A,B:** In situ hybridization for *Dsc2* mRNA, revealing strong Foxn1-dependent *Dsc2* expression in hair medulla. **C,D:** In situ hybridization for hair keratin mHa3 mRNA, confirming strong Foxn1-dependent mHa3 expression in differentiating hair cuticle and cortex. **E–R:** Immunostaining of hair follicles with anti-Dsc2 (green) and anti-hair keratin (red) antibodies. TOPRO-3 staining of nuclei (blue) shows normal nuclear morphology in hair cell lineages. Dsc2 protein is localized to the specialized A-ridges of medulla cells (E,G–I,M–O and arrowheads in I,O). Low levels of Dsc2 protein can also be detected between vertically adjacent medulla cells (arrow, I). Dsc2 protein levels in the hair shaft are greatly reduced by the *nude* mutation, with very little detectable Dsc2 protein present in the A-ridges (F,J–L,P–R). Skin sections are oriented as in Figure 1.

complexes, with consequent effects on adjacent medulla cells. It will be of great interest to investigate these possibilities further and to define more precisely the properties of the Foxn1-dependent signal that mediates upregulation of *Dsc2* expression in the hair medulla.

Desmosome-mediated cell adhesion is essential for both formation and maintaining the integrity of many tissues, including skin and hair follicles, and involves the establishment of stable interactions between desmocollin and desmoglein molecules on adjacent cells (Garrod et al., 1996; Green and Gaudry, 2000). Thus, in the hair shaft, interactions between Dsc2 and a desmoglein isoform are likely to mediate cortex–medulla and possibly medulla–medulla cell adhesion events, which enable the efficient assembly of septulate hair shafts. Our results unveil a remarkable Foxn1-dependent enrichment of Dsc2 protein in the A-ridges at the cortex–medulla interface, providing the first molecular evidence that these structures are likely to be sites of strong cell adhesion in the hair shaft. It is tempting to speculate that this regularly repeating, discontinuous distribution of Dsc2 along the length of the nascent hair shaft contributes to its combined high mechanical strength and flexibility.

In epithelial cells, other desmosomal proteins facilitate interactions of desmosomes with the keratin-intermediate filaments that influence cell shape. It is all the more remarkable, therefore, that Foxn1 is also required for expression of multiple hair keratin genes in the differentiating hair shaft (Meier et al., 1999). When taken together with these observations, our findings implicate Foxn1 as the coordinator of a gene expression program in the hair shaft, which generates changes in cytoarchitecture and cell adhesion that are required for shaft assembly. Other recent expression profiling experiments have demonstrated that genes encoding additional keratin-associated proteins were expressed in the skin in a Foxn1-dependent manner (Schlake and Boehm, 2001), although the expression domains within the skin were not defined for any of these genes. Our transcript profiling experiments identified several other Foxn1-regulated gene products in the skin that may also control cytoarchitecture, such as the EVH1 domain-containing cupidin, the partner keratins 1–18 and 2–8, and the coiled-coil protein Tpd52l1. Further analysis of these genes will help to clarify their relationship to Foxn1 function and the mechanisms of hair formation. Finally, the possibility that Foxn1 coordinates cytoarchitectural transformation and adhesion behavior in the thymus, now merits detailed investigation.

EXPERIMENTAL PROCEDURES

Mice

The *nude* mutation was maintained on a Balb/c background. Foxn1TG1 animals were crossed to *nude* mutant mice and the progeny intercrossed to produce litters that included Foxn1TG1 and non-transgenic *nu*/+ and *nu*/*nu* siblings, as previously described (Cunliffe et al., 2002).

Histology, Histochemistry, Immunohistochemistry, and In Situ Hybridization

Skin samples were analyzed by histological and immunohistochemical techniques as described (Cunliffe et al., 2002). Primary antibodies were applied at the following dilutions: mouse anti-hair keratin, 1:25 (AE13, Lynch et al., 1986); mouse anti-trichohyalin, 1:10 (O'Guin et al., 1992); rabbit anti-Foxn1, 1:50 (Lee et al., 1999); rabbit anti-S100a3, 1:2,000 (Kizawa et al., 1998); rabbit anti-Dsc2, 1:200 (North et al., 1999). After applying secondary antibodies, samples were counterstained with TO-PRO-3 (Molecular Probes) to reveal nuclear morphology and analyzed by confocal microscopy. In situ hybridization was performed using digoxigenin-labeled probes (Hogan et al. 1994).

Transcript Profiling on NIA cDNA Microarrays

Back skin was dissected from four P7.5 mice of each of the *Foxn1TG1:nu*/+ and the *nu*/*nu* genotypes and frozen in liquid nitrogen. Total RNA was isolated using QIAShredder columns (Qiagen) and Absolutely RNA RT-PCR kits (Stratagene). The 100 μg of total RNA isolated from two individuals of identical genotype were then combined to yield a pool of 200 μg (4 μg/μl) of RNA, which was reverse transcribed with Superscript II RT (GIBCO BRL). The resulting cDNA was labeled using the BioPrime DNA Labeling System (GIBCO BRL), FluoroLink Cy5-dCTP or FluoroLink Cy3-dCTP (1 mM stock; Amersham Pharmacia Biotech). Cy5 and Cy3 probes were combined for cohybridization to glass slides containing duplicate samples of cDNA clones from the NIA Mouse 15K embryonic cDNA clone set (Tanaka et al., 2000). Within an experiment, Cy5 and Cy3 probe syntheses were done independently three times, using separate aliquots of cDNA template corresponding to each of the two genotypes, and the three Cy5/Cy3 probe combinations were then cohybridized to three separate microarrays. The entire procedure was performed twice with completely new biological material and slides, providing a total of six experimental repetitions of each probe synthesis and cohybridization. Posthybridization analysis was carried out with the GeneTac scanner using GNSL and ScanAlyse software. Cy3/Cy5 ratios were calculated using the ScanAlyse software after subtracting local background signals from each spot signal. Signal intensities for all six slides were then normalized to the transcript signals from *Hsc73*, whose abundance was insensitive to Foxn1 function. Mean Cy3/Cy5 ratios were derived for each gene, and the standard errors of the mean (SEM) were calculated.

Semiquantitative RT-PCR Analysis of *Foxn1*-Dependent Gene Expression

RNA samples were isolated from different animals to those used to generate probes for microarray experiments. The 4 μg samples of total RNA from back skin tissue of P7.5 animals of the *Foxn1TG1:nu*/+, *nu*/+, and the *nu*/*nu* genotypes were used to synthesize cDNA. cDNA was then amplified by PCR for 25 cycles with gene-specific primers, and products were analyzed on a 1% agarose gel. For each of the genes studied, 25 cycles of amplifica-

tion were found to be both within an approximately linear range of responsiveness to varying amounts of input cDNA and adequate for PCR product detection. For each gene, the mean fold changes in mRNA expression levels were quantitated, relative to the *Hsc73* internal standard, using the Quantity One software (Bio-Rad) to analyze digitized TIFF image files of the synthesized PCR products. Each RT-PCR experiment was repeated twice, with similar results each time.

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