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Complete rescue of the nude mutant phenotype by a wild-type *Foxn1* transgene

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Abstract. In this paper we describe the production and analysis of mice carrying a 110-kb transgene that encompasses the wild-type *Foxn1* genomic locus. Mutations in *Foxn1* cause the nude phenotype. We show that in the hair follicles, transgenic mice with increased *Foxn1* gene dosage exhibited increased *Foxn1* expression that was restricted correctly to the nascent, post-mitotic cells of the differentiating hair cortex and hair cuticle lineages. We also demonstrate for the first time that a *Foxn1* transgene rescues completely both the hair follicle and the thymus defects in animals that are also homozygous for the nude mutation at the endogenous *Foxn1* locus, causing the development of a full coat of hair and a normal population of peripheral blood T lymphocytes. We conclude that sufficient *cis*-acting regulatory information resides within this 110-kb transgene to direct reliable and appropriate tissue-specific expression of the *Foxn1* gene.

Introduction

The *Foxn1* gene is required for normal development of the hair follicles and thymus in mice, since mutations in this gene cause the nude mutant phenotype (Flanagan 1966; Pantelouris 1968; Nehls et al. 1994). Homozygous nude mice lack both a coat of hair and a functional thymus. Hair follicles are specified in nude mutants, and their development is initiated correctly, but the developing hair rudiments subsequently exhibit impaired keratinization and morphological defects in cuticle formation (Flanagan 1966; Kopf-Maier et al. 1990; Meier et al. 1999). As a result of defective hair differentiation, fragments of broken hair shaft accumulate in the upper portion of each hair follicle, giving this region of the follicle an abnormally distended and mis-shapen appearance. In the nude mutant thymus, epithelial cells fail to differentiate normally, which prevents normal T lymphocyte development in this organ and so causes severe immunodeficiency (Nehls et al. 1996).

The Foxn1 protein is a member of the forkhead family of transcription factors and possesses both a winged-helix DNA-binding domain and a potent transcription activating domain (Nehls et al. 1994; Schlake et al. 1997; Schuddekopf et al. 1996). In the hair follicle, the *Foxn1* gene is transcribed predominantly in post-mitotic precursors of the hair cuticle and cortex (Lee et al. 1999), a pattern of expression that requires Bone Morphogenetic Protein (Bmp) signaling (Kulessa et al. 2000); however, it is not yet known if *Foxn1* is a direct target of this signaling pathway. Taken together, the mutant phenotype and the *Foxn1* expression pattern imply that Foxn1 may function at an early stage in the differentiation of hair cortex and cuticle cells.

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A cosmid-derived transgene containing 26 kb of mouse genomic DNA that encompassed the coding exons of *Foxn1* plus 8.5 kb of 5′-flanking sequence and 3 kb of 3′-flanking sequence was able only to rescue partially the phenotypic effects of homozygosity for the nude mutation at the endogenous *Foxn1* locus (Kurooka et al. 1996). Homozygous nude mutant animals that also carried this transgene exhibited an incomplete coat of hair, suggesting that expression of the transgene was variable in the hair follicles, possibly owing to the absence of critical *cis*-regulatory elements determining the transcriptional stability or efficiency of the *Foxn1* transgene in hair follicle cells. Moreover, peripheral T lymphocytes were completely absent from these transgenic animals, and thymus development was arrested as in non-transgenic nude animals, indicating that critical regulatory elements required for thymus-specific *Foxn1* expression were absent in the transgene employed. In order to begin to dissect the developmental mechanisms regulating *Foxn1* expression in the hair follicle and thymus, we sought to define a region of genomic DNA encompassing the murine *Foxn1* locus that caused the complete transgenic rescue of both hair follicle and thymus phenotypes in animals homozygous for the nude mutation at the endogenous *Foxn1* genomic locus. In this paper we describe transgenic mice carrying a 110-kb *Foxn1* transgene that fulfills these requirements.

Materials and methods

Mice. The nude mutation was maintained on a Balb/c background from a stock originally supplied by Harlan, UK. Homozygous nude mutants could be distinguished from littermates shortly after birth by the absence of whiskers. The identity of nude homozygotes was confirmed by PCR-based sequence analysis of exon 3 of *Foxn1* in genomic DNA samples.

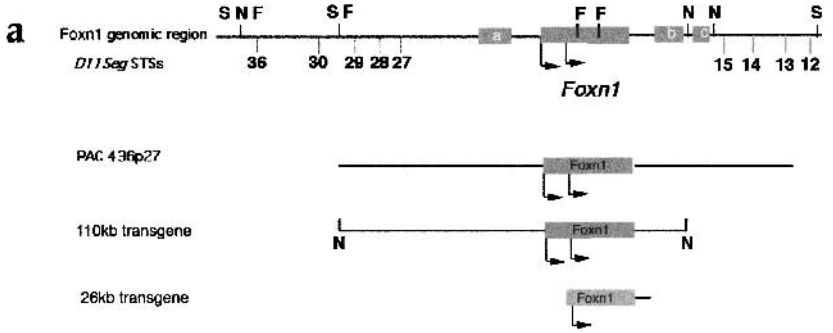
Histology, histochemistry, and immunocytochemistry. Skin tissue was dissected from the anterior back region of sex- and age-matched animals and fixed in 4% paraformaldehyde in Phosphate-Buffered Saline (PBS) overnight at 4°C. Samples were dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin wax. Histological sections of 8 μm thickness were taken onto Vectabond-subbed slides, dehydrated, and cleared, then stained with Hematoxylin and Eosin and mounted in DPX. Cell types were identified by reference to histological texts under a compound microscope.

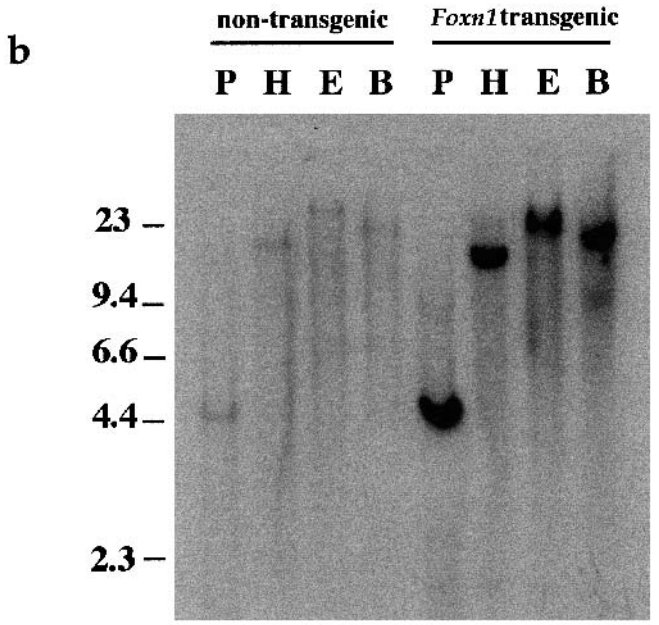
For immunostaining wax-embedded skin sections, tissues were mounted on Vectabond-subbed slides, then cleared in xylene, dehydrated through an ethanol series, and rinsed sequentially in distilled water followed by PBS.

To prepare sections for immunostaining with the AE13 monoclonal antibody (Lynch et al. 1986), the anti-Foxn1 (Lee et al. 1999) or anti-phospho-histone H3 polyclonal rabbit antisera (Santa Cruz Biotechnology), slides were boiled for 15 min in citrate buffer (Vector Laboratories, Burlingame, Calif.), then cooled to below 60°C over a period of 20 min. After rinsing all slides in PBS, we blocked tissues with 3% heat-inactivated goat serum (HIGS) in PBS/0.1% Triton X-100, for 30 min at room temperature in a humidified chamber. Slides were then drained, and primary antibody was added after dilution in 3% HIGS/PBS/0.1% Triton X-100 (1:50 for anti-Foxn1 and AE13; 1:1000 for anti-phospho-H3). Slides were

then incubated overnight at 4°C in a humidified chamber. Slides were washed for 10 min twice in PBS/0.1% Triton X-100, prior to incubation with fluorescein or Cy3-labeled secondary antibodies at the dilutions specified by the manufacturers (Vector laboratories, Sigma) in 3% HIGS/PBS/0.1% Triton X-100. Specimens were mounted in Vectashield (Vector Laboratories) and analyzed with a Leica confocal microscope.

Isolation of genomic clones encompassing the Foxn1 gene, preparation of DNA for oocyte microinjection, and generation of transgenic mice. A DNA fragment comprising exon 3 of the *Foxn1* gene was amplified from C57B1/6J genomic DNA by the Polymerase Chain Reaction (PCR), with the following primers: 5′-GCATGCTAACTTCAGCTGCTCGTCGT-3′ (exon 3 forward primer), 5′-GAATTTGGTTGTGTTCCTGGCTGGGGTAAG-3′ (exon 3 reverse primer). This exon 3 probe was used to screen the RPCI21 mouse genomic library constructed in the pPAC4 vector (supplied by MRC Human Genome Mapping Project Resource Centre, Cambridge, UK, originally from the Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, N.Y.). Positive clones were isolated by alkaline lysis miniprep, typed for Sequence Tagged Site (STS) content by PCR, and restriction mapped with frequent cutter restriction enzymes. Rare cutter restriction maps were generated by using Pulsed Field Gel Electrophoresis (PFGE) and Southern blotting. These maps were compared with the restriction maps of the genomic region, and clones that were un-rearranged were used for further studies. The P1 Artificial Chromosome (PAC) clone 436p24 was unrearranged and encompassed over 170 kb between STS markers *D11Seg14* and *D11Seg29,* which included the *Foxn1* gene. PAC DNA maxipreps were purified by using Qiagen columns. A 110-kb *Not*I fragment from 436p24 that contained the entire *Foxn1* locus plus 74 kb of 5′ flanking sequence and 21 kb of 3′ flanking sequence was isolated from 436p24 by preparative





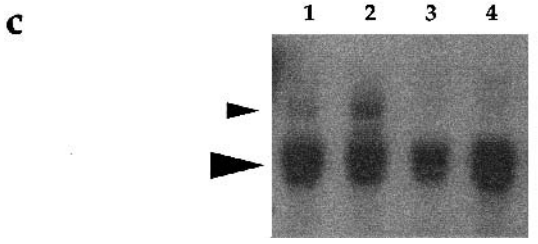


Fig. 1. Production of transgenic mice with increased *Foxn1* gene dosage and expression. **(a)** Long-range restriction and SSLP maps of the genomic region encompassing the *Foxn1* locus, together with maps of the 436p24 PAC clone and purified DNA fragment obtained by *Not*I digestion that was used for transgenesis. The locations of flanking genes are indicated on the physical map as follows: Sodium/dicarboxylate transporter (a), retinal 4 gene (b), aldolase C (c). The positions of the two putative promoters responsible for transcription initiation at exons 1a and 1b of the *Foxn1* gene are marked with arrows. A map of the 26-kb *Foxn1*-encoding genomic DNA fragment in the cosmid previously used for transgenesis by Kurooka et al. (1996) is also shown for comparison (S: *Sac*II, N: *Not*I, F: *Sfi*I). **(b)** Southern blot of restriction-digested genomic DNA from Foxn1TG1 and non-transgenic littermates (15 μg per lane) hybridized to an exon 3 probe from the *Foxn1* gene. (P: *Pst*I; H: *Hin*dIII; E: *Eco*RI; B: *Bam*HI). Positions of DNA markers with sizes in kb are given on the left. **(c)** Ribonuclease protection analysis for expression of *Foxn1* (small arrowhead) and γ-actin control (large arrowhead) mRNA in 1: non-transgenic back skin; 2: Foxn1TG1 back skin; 3: non-transgenic heart; 4: non-transgenic kidney.

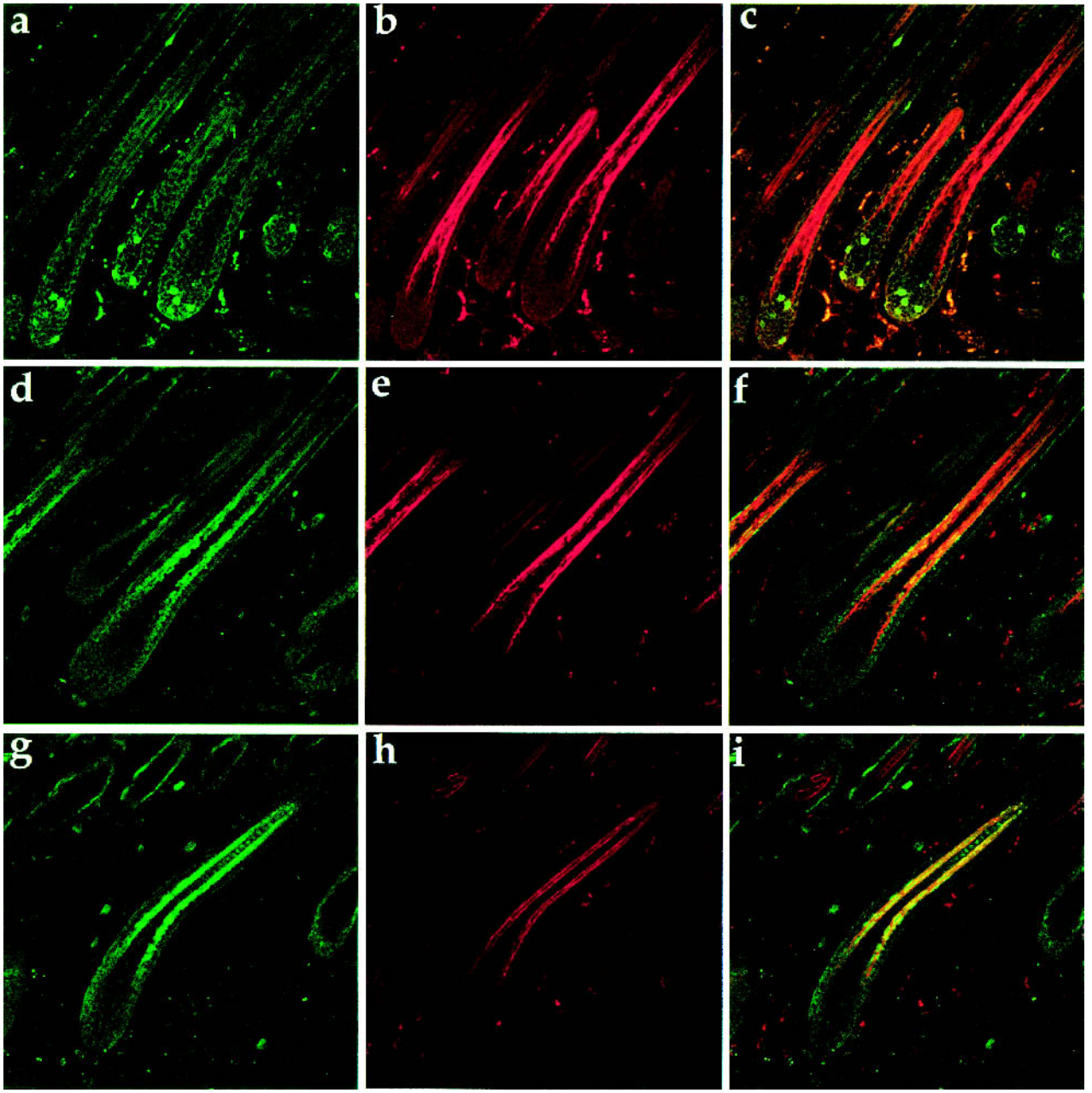


Fig. 2. Foxn1 protein is localized to nuclei of differentiating, postmitotic precursors of the hair cortex and hair cuticle of wild-type mice and is over-expressed in the same domain in Foxn1TG1 mice. Confocal images of longitudinal sections through anterior backskin of non-transgenic **(a–f)** and Foxn1TG1-transgenic **(g–i)** littermates at P7.5. Antibodies used in immunostaining are: anti-phospho-H3 (green: a, c); anti-Foxn1 (green: d, f, g, i); and anti-hair keratin AE13 (red: b, c, e, f, h, i). Image in c is merge of a and b; image in f is merge of d and e; image in i is merge of g and h.

PFGE and purified with Gelase (Epicentre Technologies, Madison, Wisconsin). The purified, linear, 110-kb *Not*I DNA fragment was resuspended to 1 pg/nl in microinjection buffer (10 mM Tris pH 7.5, 0.1 mM EDTA). DNA was injected into pronuclei of fertilized eggs from (C57Bl/6J × CBA) F_1 crosses (Hogan et al. 1994). This fragment also contained two genes that closely flanked the *Foxn1* gene, the Na^+-dicarboxylate cotransporter gene located 11 kb upstream and the retinal 4 gene located 8.5 kb downstream of *Foxn1* (Chen et al. 1998; Higashide et al. 1996; Sekine et al. 1997). RT-PCR analysis confirmed that expression of the Na^+-dicarboxylate cotransporter gene is restricted to the kidney, and expression of the retinal 4 gene is restricted to the retina (data not shown). As neither gene was expressed in either transgenic or nontransgenic skin, both were discounted as contributing to the effects of the transgenes described herein.

Characterization of transgenes in genomic DNA and breeding of Foxn1*-transgenic mice.* Genomic DNA was prepared from tail biopsy samples from pups at 3 weeks of age and was analyzed for the presence of transgene sequences. For PAC-derived transgene identification, advantage was taken of the fact that the end of the 110-kb *Not*I fragment of PAC 436p24, which lies upstream of the *Foxn1* gene, terminates in the SP6 promoter sequence derived from the PAC vector. Therefore, genomic DNA sequence immediately adjacent to this SP6 sequence in 436p24 was determined, and a primer corresponding to sequence approximately 400 bp downstream was used, together with the SP6 primer, to test for transgene sequences in genomic DNA samples by PCR amplification. The primer sequences were as follows: Forward (SP6) primer: 5′-CATACGATTTAGGTGACACTATAG-3′; reverse (*Foxn1*) primer: 5′-

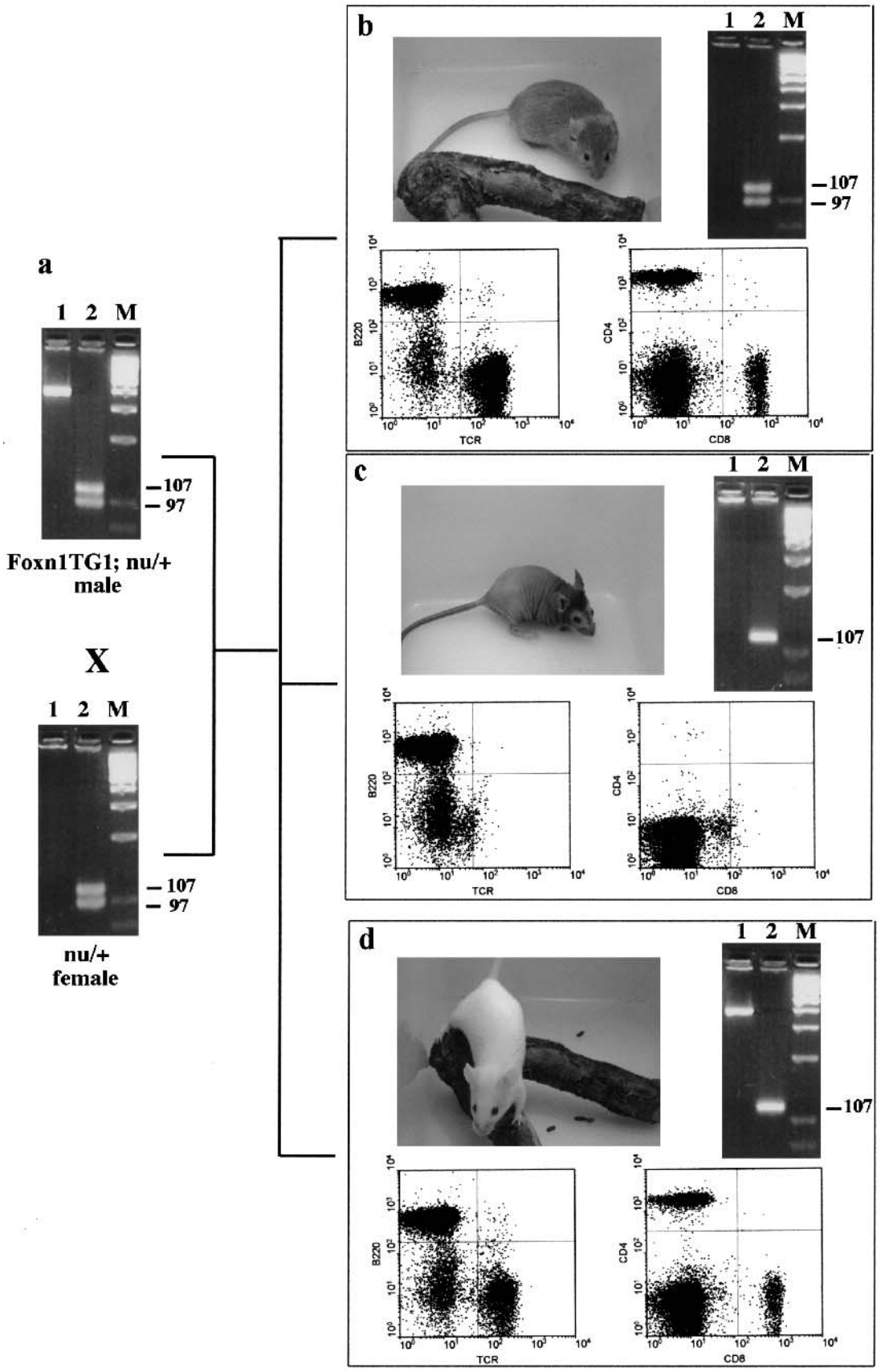

a
1 2 M
–107
– 97
Foxn1TG1; nu/+
male
X
1 2 M
– 107
– 97
nu/+
female
b
1 2 M
–107
– 97
B220
TCR
CD4
CD8
c
1 2 M
–107
B220
TCR
CD4
CD8
d
1 2 M
–107
B220
TCR
CD4
CD8

Fig. 3.

AATCTCATTCCGTTACGCAG-3′. PCR-positive animals were then crossed with CBA or C57Bl/6J animals to establish transgenic lines. Increased copy number and structural integrity of the *Foxn1* gene in transgenic DNA was confirmed by Southern analysis (Sambrook et al. 1989).

Ribonuclease protection assay for Foxn1 *mRNA.* Transgenic and non-transgenic animals were sacrificed at postnatal day 31 when the hair follicles had entered their second anagen period. Total RNA was extracted from skin and other tissues, and 3 μg was assayed for expression of *Foxn1* and γ-actin mRNAs by ribonuclease protection assay (Sambrook et al. 1989). *Foxn1* mRNA was detected with an antisense probe complementary to exon 3 sequences, whereas γ-actin was detected with a probe complementary to the 3′-untranslated region. *Foxn1* mRNA expression levels were quantitated relative to the γ-actin internal standard mRNA, by using the Quantity One software (BioRad) to analyze digitized TIFF image files of autoradiograms.

Rescue of nude mutant phenotypes with a wild-type Foxn1 transgene. In order to test whether the expression and function of *Foxn1* in the 110-kb PAC-derived transgene was correctly targeted to the hair follicles and thymus, the phenotype of homozygous nude mutants was compared with that of Foxn1-transgenic mice that were also homozygous for the nude mutation at the endogenous *Foxn1* locus. These mice were produced by using the following genotyping and breeding strategies, which enabled wild-type and nude mutant alleles at the endogenous *Foxn1* locus to be distinguished both from each other and from the wild-type *Foxn1* transgene.

The *Foxn1* transgene present in the Foxn1TG1 line can be uniquely distinguished from the endogenous *Foxn1* locus by PCR amplification of the PAC-specific DNA fragment as described above.

To distinguish nude mutant and wild-type alleles at the endogenous *Foxn1* locus by PCR, the Simple Sequence Length Polymorphic (SSLP) marker *DBhm148* was used. *DBhm148* lies just outside of the region of DNA that is defined by the 110-kb *Foxn1* transgene and occurs in two polymorphic forms: (i) a 97-bp form found in C57Bl/6J and (ii) a 107-bp form found in Balb/c, CBA, and 129/Sv (Nehls et al. 1995). Moreover, since *DBhm148* maps to <0.5 cM of *Foxn1* on Chr 11 and is therefore tightly linked to the endogenous *Foxn1* gene, it could be used as a surrogate polymorphic marker for the endogenous *Foxn1* locus on Chr 11. Heterozygous *nu*/+ animals were produced with a genotype in which the 107-bp *DBhm148* allele co-segregated with the nude mutation (on a Chr 11 of Balb/c origin), and the 97-bp *DBhm148* allele co-segregated with the wild-type *Foxn1* allele (on a Chr 11 of C57Bl/6J wild-type origin). These animals were then crossed to individuals of identical genotype at the endogenous *Foxn1* locus that also carried the Foxn1TG1 transgene. All progeny were then genotyped for the presence of the Foxn1TG1 transgene and for the presence of the 97-bp and 107-bp alleles of *DBhm148.* Homozygosity for the 107-bp allele of *DBhm148* consistently indicated homozygosity for the nude mutation on Chr 11, whereas homozygosity for the 97-bp *DBhm148* allele indicated homozygosity for the wild-type *Foxn1* allele on Chr 11. Rescued animals were designated as Foxn1TG1: *nu/nu* individuals.

DBhm148 primers used in these experiments were: Forward: 5′-AGG GGA AGT CCT GTA TGG ACA-3′. Reverse: 5′-ACC AAC CTC GAT AGA GCC ATC-3′.

Results

Construction of transgenic mice carrying a 110-kb transgene that spans the wild-type Foxn1 *gene.* Previous studies indicated that a cosmid containing 26 kb of genomic DNA from the wild-type *Foxn1* locus could only partially rescue the phenotype caused by the nude mutation (Kurooka et al. 1996). These results suggested that critical *cis*-acting regulatory elements for the *Foxn1* transcription unit lay outside the limits of the cosmid used for the experiment. Therefore, to attempt to fully recapitulate *Foxn1* expression with a *Foxn1* transgene, we isolated a 110-kb fragment of mouse genomic DNA from a *Foxn1*-containing PAC genomic clone by *Not*I digestion and PFGE, and used it to produce transgenic mice. This 110-kb fragment encompassed the complete *Foxn1* gene and, in addition, contained 75 kb of 5′ flanking DNA and 21 kb of 3′-flanking DNA (Fig. 1a). Three transgenic founders (Foxn1TG1, FoxnTG4, and Foxn1TG5) were produced and backcrossed to CBA or C57Bl/6J mice to establish lines from each founder. In the experiments described below, Foxn1TG1 animals were used to determine whether the 110-kb transgene directed appropriate expression of *Foxn1* in skin and thymus and thus rescued fully the nude mutant phenotype.

Comparison of *Foxn1* genomic DNA sequences in non-transgenic and Foxn1TG1 animals demonstrated that the Foxn1TG1 transgene was unrearranged since *Foxn1*-specific DNA sequences in transgenic animals exhibited the same pattern of fragmentation, with four different restriction enzymes, as that of non-transgenic animals (Fig. 1b). Ribonuclease protection analysis and quantification by densitometry revealed that expression of *Foxn1* mRNA was fourfold more abundant in Foxn1-transgenic skin than in non-transgenic skin (Fig. 1c). Thus, increased *Foxn1* gene dosage resulted in elevated levels of *Foxn1* mRNA in the skin. Next, the expression of Foxn1 protein in the hair follicles of P6.5 Foxn1TG1 transgenic and non-transgenic littermates was compared. Figure 2 shows that Foxn1 protein was localized to the nuclei of newly forming hair cortex and cuticle cells in both Foxn1TG1 transgenic and non-transgenic littermates. These Foxn1-positive cells had begun their program of terminal differentiation, as judged by their strong expression of hair keratins, and they were also postmitotic, since phosphorylated histone H3-positive mitotic nuclei were restricted to cells in the hair bulb that lay beneath the zone of Foxn1 and hair keratin expression. However, the Foxn1 signal in Foxn1TG1 transgenic follicles was consistently stronger in these cells than that detectable in non-transgenic follicles, demonstrating that Foxn1TG1 transgenic animals over-expressed Foxn1 in the correct hair cell types. No misexpression of Foxn1 in any inappropriate cell type was observed in Foxn1TG1 transgenic tissue.

Complete rescue of the phenotype of homozygous nude mutants with a 110-kb transgene spanning the wild-type Foxn1 *gene.* To confirm that the Foxn1TG1 transgene was fully functional and expressed in the cell types in which its function is required, we tested the ability of this transgene to rescue the phenotypic effects of homozygosity for the nude mutation at the endogeneous locus on Chr 11. Mice were produced by genotyping and breeding strategies that enabled wild-type and nude mutant alleles at the endogenous locus to be distinguished from each other and from the wild-type *Foxn1* transgene (see Materials and methods; Fig. 3).

Fig. 3. The breeding scheme used to demonstrate rescue of nude phenotypes by the Foxn1TG1 transgene. Genotypes of parents are illustrated on the left of the figure with agarose gel images of PCR products amplified from genomic DNA. Lanes 1: Foxn1TG1-specific PCR product; Lanes 2: *DBhm148* SSLP PCR products; M: 100-bp ladder. The 107-bp *DBhm148* allele is tightly linked to the nude mutation, whereas the 97-bp *DBhm148* allele is linked to the wild-type *Foxn1* locus, but *DBhm148* lies outside of the region encompased by the 110-kb Foxn1TG1 transgene. Three of the relevant classes of progeny are shown in boxes b, c, d to the right of the figure. In each box, genotyping data and corresponding phenotypes of progeny are shown. Photographs of animals show coat phenotype, whereas FACS profiles illustrate expression of T- and B-lymphoid markers on peripheral blood leukocytes (PBL) from the same animal. The FACS plot on the lower left of each box shows expression of the B-lymphocyte marker B220 and the T-lymphocyte marker TCR (T-cell Receptor); the FACS plot on the lower right shows expression of the T-lymphocyte markers CD4 and CD8. Thus, in **(a)** a Foxn1TG1; *nu*/+ male was crossed to a *nu*/+ female to produce progeny that included: **(b)** a *nu*/+ heterozygous, non-transgenic animal with wild-type phenotype; **(c)** a *nu/nu* homozygous, non-transgenic animal with nude mutant phenotype; **(d)** a *nu/nu* homozygous, Foxn1TG1 transgenic with wild-type phenotype. The frequencies of each class of progeny obtained from such crosses are presented in Table 1.

Table 1. Phenotype frequencies in progeny of crosses between Foxn1TG1: *nu*/+ males and *nu*/+ females. Foxn1TG1: *nu*/+ males were crossed to *nu*/+ females, and progeny were scored for coat phenotype (wild-type or nude), then genotyped by PCR to detect Foxn1TG1 transgene sequences (Tg+/Tg−). Percentages are given in brackets.

	wild-type; Tg+	wild-type; Tg−	nude; Tg+	nude; Tg−
Number observed	54	30	0	7
Totals	84 (92%)		7 (8%)	
Number expected, if no rescue occurs	68 (75%)		23 (25%)	
Number expected, if rescue occurs	80 (87.5%)		11 (12.5%)	

First, animals were bred that were heterozygous for the nude mutation and either carried or lacked the Foxn1TG1 transgene (Fig. 3a). Animals with these two genotypes were then crossed and the frequencies of wild-type and nude mutant phenotypes in the progeny were scored (Table 1). Only 8% of the resulting progeny exhibited the nude mutant phenotype (7/91 mice; Table 1) compared with expectations of 25% nude mutant progeny if the transgene was nonfunctional or 12.5% nude mutant progeny if the transgene was fully functional. Importantly, none of the phenotypically nude animals were transgenic, whereas all Foxn1-transgenic animals had a complete coat of hair and a fully functional thymus, including individuals that were genotypically nude homozygotes at the endogenous *Foxn1* locus (Fig. 3d; designated Foxn1TG1: *nu/nu*). The proportions of T-lymphocytes and B-lymphocytes in peripheral blood, as well as the proportions of CD4-positive and CD8-positive T-lymphocytes, were measured by FACS analysis of peripheral blood lymphocytes in adult progeny. FACS analyses of peripheral blood from rescued Foxn1TG1: *nu/nu* animals were indistinguishable from those of non-transgenic *nu*/+ heterozygotes, whereas homozygous nude mutants lacked all peripheral T cells (Fig. 3b–d). These observations demonstrated that thymus function in Foxn1TG1: *nu/nu* animals was completely restored by the presence of the Foxn1TG1 transgene. Finally, when rescued Foxn1TG1: *nu/nu* animals were crossed with homozygous nude mutants, only two classes of progeny were produced in a 1:1 ratio: non-transgenic, phenotypically nude animals and Foxn1TG1: *nu/nu* phenotypically fully rescued animals (data not shown).

The ability of the 110-kb *Foxn1* transgene to rescue the nude skin phenotype was also assessed histologically (Fig. 4). In comparison with the skin of *nu/nu* mutants, all follicles in the skin of rescued Foxn1TG1: *nu/nu* animals were normal in histological appearance and lacked the swollen, distended morphology characteristic of the upper regions of nude mutant hair follicles (Fig. 4 a–d). In addition, the developing hair rudiments of rescued Foxn1TG1: *nu/nu* animals exhibited strong expression of hair keratin in all the cells of the well-developed cortex and cuticle lineages, in contrast to the much reduced expression of hair keratins that was observed in the poorly differentiating cortex and cuticle structures of nude mutant hair follicles (Fig. 4 e–h).

Taken together, these results demonstrate that the 110-kb genomic DNA fragment that encompasses the wild-type *Foxn1* gene completely rescues the phenotypic effects of homozygosity for the nude mutation, and, therefore, this transgene encodes all the regulatory elements required to determine the faithful and reliable expression of *Foxn1* in hair follicles and the thymus.

Discussion

The use of BAC and PAC genomic clones in transgenic studies represents a powerful advance in the analysis of mammalian gene function and regulation. BACs and PACs are much more stable and less prone to chimerism and shearing during transgenesis than are Yeast Artificial Chromosomes (YACs), and PAC/BAC clones more than 200 kb in size can be used in transgenic studies. To date, relatively few studies have been described that demonstrate the use of BAC/PAC clones for functional complementation of mouse mutations (see Giraldo and Montoliu 2001, for review) but the potential of this technology for aiding the functional characterization of mammalian genes and the analysis of *cis*-regulatory elements governing gene expression is considerable.

The limitations of using small cosmids for molecular complementation of mouse mutations are well exemplified by the previous description of a partial rescue of the nude mutant phenotype by a *Foxn1*-containing cosmid (Kurooka et al. 1996). These results suggested that critical *cis*-regulatory components of the *Foxn1* chromatin domain were absent from the transgene, leading to reduced and/or variable expression in skin and no significant expression in thymus. In contrast, we found that the Foxn1TG1: *nu/nu* animals analyzed in this study possessed a complete coat of hair and a fully functional thymus, demonstrating that the 110-kb *Foxn1* transgene was reliably and efficiently expressed in the correct cell types of the hair follicles and thymus. By using the SSLP marker *DBhm148,* which (a) was tightly linked to the *Foxn1* locus, (b) existed in polymorphic forms that enabled nude and wild-type alleles of the endogenous *Foxn1* to be discriminated, and (c) lay just outside the genomic DNA fragment used for complementation, Foxn1TG1: *nu/nu* animals could be identified unambiguously by genotype and distinguished from the Foxn1TG1: *nu*/+ genotype without the need for extensive modification of the transgene fragment, which could have compromised its function. The inclusion of some prokaryotic DNA sequences in transgenic constructs is known to interfere with transgene expression (Ramirez et al. 2001). Therefore, we removed almost all of the PAC vector backbone from the *Foxn1*-containing DNA fragment used for pronuclear injection, except for an SP6 promoter sequence that was used to tag the transgene at one end, which enabled the transgene to be distinguished from the endogenous *Foxn1* locus in PCR assays. It is possible that this technical refinement further improved the expression characteristics of the 110-kb *Foxn1*-containing transgene.

A previous study of *Foxn1* transcription in skin and thymus demonstrated that this gene is subject to alternative splicing and the use of alternative promoters because *Foxn1*-derived cDNA clones contained one of two alternative exons at their 5′ end, each initiated from distinct promoters (Schorpp et al. 1997). This study found that cDNAs encoding exon 1b were detected only in skin, whereas exon 1a-encoding cDNAs were recovered from both skin and thymus. Importantly, exon 1a lies outside of the 26 kb of genomic DNA present in the cosmid previously used for transgenesis, but within the 110 kb genomic fragment utilized in our studies. Since our results demonstrate that *cis*-regulatory elements lying within 110 kb of genomic DNA around *Foxn1* are sufficient to drive *Foxn1* expression in both the thymus and skin, they support the suggestion that the promoter responsible for transcription of exon 1a is one such element. Intercellular signaling by Bone Morphogenetic Proteins (Bmps) has recently been shown to be required for *Foxn1* expression in the hair follicles (Kulessa et al. 2000), which suggests that Bmp-responsive *cis*-regulatory elements are likely to reside within the 110-kb fragment used for transgenesis.

We show here that in the well-developed anagen hair follicle, Foxn1 protein is localized to the nuclei of newly born, postmitotic hair cuticle and hair cortex cells, suggesting that its principal role is to regulate the differentiation of these cell types from an early stage. Foxn1 is required for the expression of a subset of hair keratin genes in the developing hair (Meier et al. 1999; Schlake et al. 2000), and, as indicated from the results presented here, loss of *Foxn1* function causes a general reduction in the levels of hair

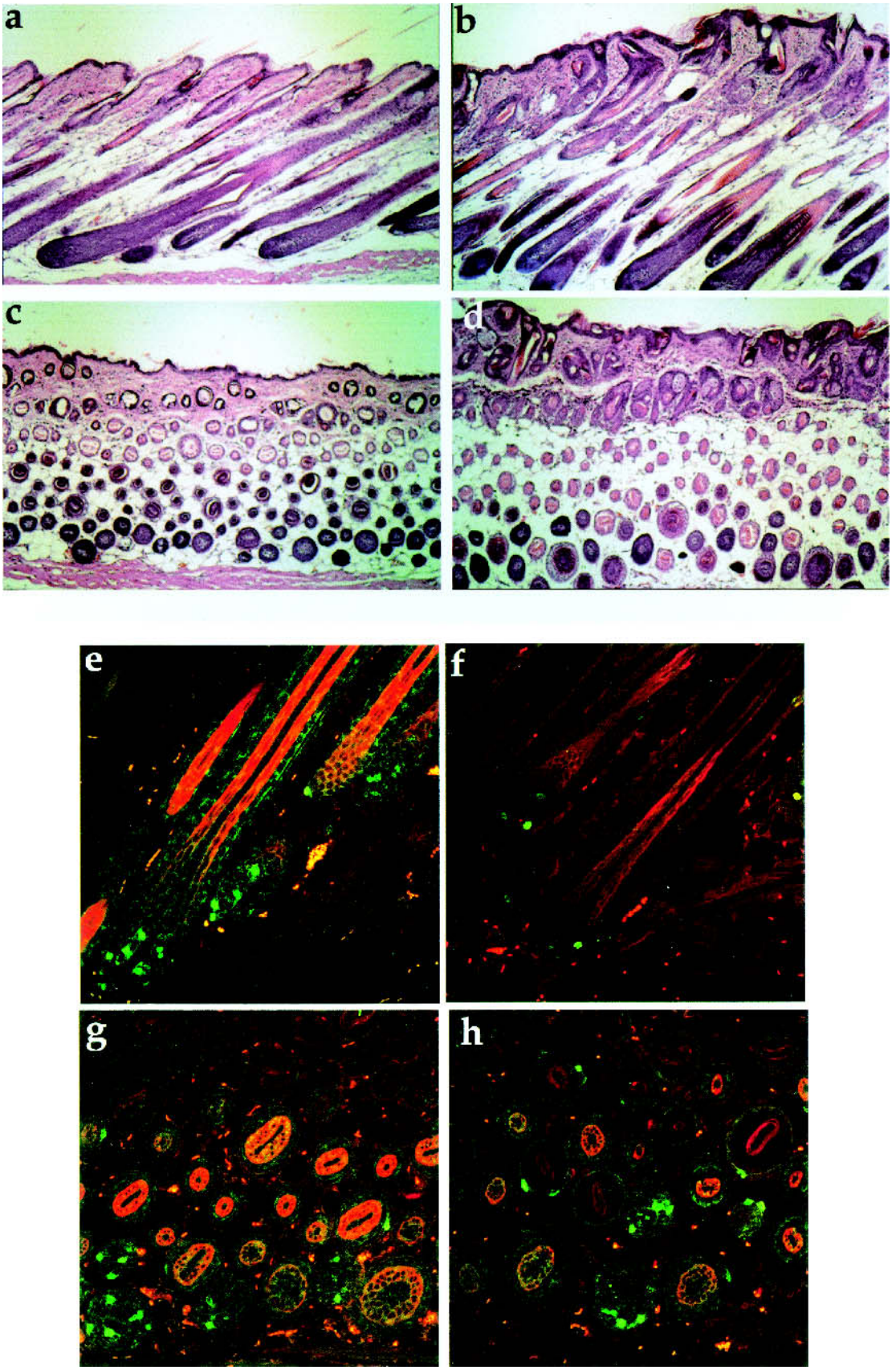


Fig. 4. Phenotypic comparison of longitudinal and transverse cross-sections through hair follicles of **(a, c, e, g)** rescued, Foxn1TG1 transgenic littermates (*nu/nu* homozygous at endogenous *Foxn1* locus), and **(b, d, f, h)** homozygous nude mutants at day P36.5. Dorsal surface of skin is uppermost. (a–d) low power views of skin sections stained with hematoxylin and eosin. (e–h) high power confocal images of hair follicles immunostained with anti-phospho-H3 (green) and anti-hair keratin AE13 (red).

keratin proteins that can be detected in the differentiating hair cortex and cuticle cells. Hair keratin genes are, therefore, good candidates for direct regulation by Foxn1 (Schlake et al. 2000). In the thymus, a recent microarray-based search for *Foxn1*-responsive genes identified a relatively small number of candidate target genes, including the PD-1 ligand-encoding gene, which were expressed in the embryonic thymic epithelium of wild-type mice, but absent in that of nude mice (Bleul and Boehm 2001). Intriguingly, none of the hair keratin genes implicated as Foxn1 targets in the hair follicle were identified as such in the thymus, suggesting that Foxn1 target gene specificity may itself be differentially regulated in these two tissues. Since the increased *Foxn1* gene dosage in Foxn1TG1 transgenic animals caused over-expression of Foxn1 in only the correct follicle cell types and no mis-expression was observed, the transgenic mice described in this paper may be a useful tool for future microarray-based gene expression analyses aimed at uncovering new *Foxn1* target genes. In particular, these animals should be useful in identifying *Foxn1*-responsive genes whose expression level may be below the threshold of detection in wild-type tissues, but more abundant, and therefore more easily detected, in tissues that over-express Foxn1.

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