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The Nude Mouse Skin Phenotype: The Role of *Foxn1* in Hair Follicle Development and Cycling

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The original nude mouse mutation has proven to be an incredibly valuable biomedical tool since its discovery in 1966. Initially its value was as a tool to study the immune system. The immunodeficiency in this mutant mouse made nude mice valuable as hosts for xenografts, primarily for cancer research. More recently, the most obvious clinical feature of this mutant mouse, lack of hair, has been capitalized on to define the role of *Foxn1* in normal and pathological skin and hair follicle physiology.

Key Words: Whn; *Hfh11^{nu}*; *nu*; baldness; skin.

INTRODUCTION

In 1966 S. P. Flanagan from the Institute of Animal Genetics, Edinburgh, Scotland, United Kingdom, described a phenotypically unique strain of hairless mice, that spontaneously arose in an albino strain that was named "nude" (Flanagan, 1966). Homozygote nude mice (*nu*/*nu*)[1] were characterized macroscopically by a more or less complete lack of fur development after birth. Flanagan also described increased postnatal mortality, decreased reproductivity, and in almost all mice, development of systemic toxoplasmosis, possibly due to an "inborn error of metabolism." Two years later it was discovered that *nu*/*nu* mice lacked a thymus, resulting in an impaired defense against pathogenic organisms (Pantelouris, 1968) (Fig. 1). Lack of fur development and athymia are not related to each other, since thymus restoration does not lead to hair growth (Eaton, 1976). Hence, nude is a pleiotropic mutation, leading to two independent phenotypic effects: (i) disturbed development of hair follicles and (ii) dysgenesis of the thymus, which is arrested in an early stage of development (Nehls *et al.*, 1996).

Because of their impaired T-cell function, *nu*/*nu* mice have been and still are extensively used for homo- and heterotransplants (xenografts) in oncological research (Rygaard, 1973; Sawada *et al.*, 1981, 1983; Sundberg, 1994). However, contrary to their extensive use, surprisingly few investigations have been published on the cutaneous abnormalities in nude mice (Flanagan, 1966; Rigdon and Packchanian, 1974; Köpf-Maier *et al.*, 1990). Once an evolutionarily conserved transcription factor [*Whn*, *Hfh11*, and now called *Foxn1* (Kaestner *et al.*, 2000)] was recognized as the nude gene, a renaissance in nude mouse skin research occurred (Nehls *et al.*, 1994). From many investigations it became clear that *Foxn1* is possibly involved in keratinocyte

[1]Abbreviations used: pp, postpartum; KGF/FGF7, keratinocyte growth factor/fibroblast growth factor 7; *Whn*, gene symbol for winged helix nude; *nu*, original gene symbol for the nude mutant gene locus; *Hfh11^{nu}*, third and currently obsolete gene symbol for the nude; *Foxn1^{nu}*, current accepted gene symbol for nude.

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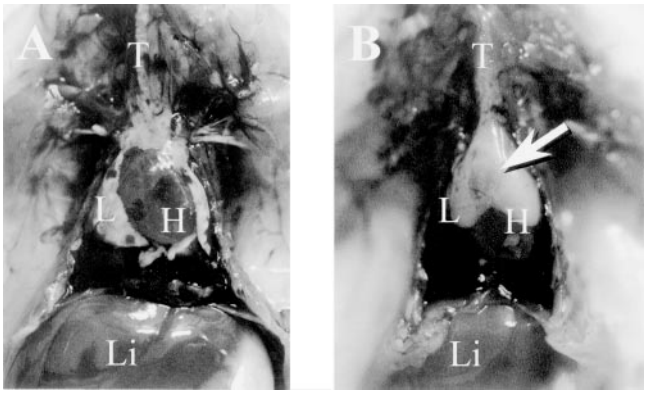

FIG. 1. *Foxn1*^{nu}/*Foxn1*^{nu} mice lack a thymus (arrow); necropsy of a NMRI *Foxn1*^{nu}/*Foxn1*^{nu} mouse (A) and a NMRI +/*Foxn1*^{nu} mouse (B) at day 42 pp; T, trachea; L, lung; H, heart; Li, liver.

differentiation, and only recently it was discovered that *Foxn1* is an important regulator of an acidic hair keratin gene, suggesting that nude represents the first inherited skin disorder that is caused by a loss rather than aberrant expression of a keratin gene (Meier *et al.*, 1999b).

THE SKIN PHENOTYPE OF NUDE MICE

At birth, BALB/C *nu*/*nu* mice macroscopically lack vibrissae, in contrast to heterozygote and wildtype mice. However, hair bulbs of vibrissae are well developed 24 h after birth, and short and curly vibrissae finally appear on day 6 postpartum (pp) (Rigdon and Packchanian, 1974) (Fig. 2).

The most obvious abnormality in *nu*/*nu* mice is the lack of fur development (Fig. 3). However, at the time of birth, no histological abnormalities can be found (Flanagan, 1966). In contrast to heterozygote controls or wildtype mice, no hairs emerge from the dorsal skin of *nu*/*nu* mice by day 5 pp. Hair shafts bend and coil when entering the hair canal, subsequently dilating the infundibulum during the final hair follicle morphogenesis. By day 8 pp most hair canals are dilated and contain cornified debris as well as a small and curly keratinized hair shaft that does not penetrate the epidermis (Flanagan, 1966; Rigdon and Packchanian, 1974) (Fig. 4). Proliferation of stratified squamous epithelium of the root sheaths is a compensatory response to hair shaft penetration laterally (Rigdon and Packchanian, 1974). Finally, some fragments of hairs appear penetrating the epidermis on the head, the neck, and the front extremities of *nu*/*nu* mice by day 10 pp (Flanagan, 1966; Rigdon and Packchanian, 1974; Köpf-Maier, *et al.*, 1990), when in heterozygote or wildtype animals a dense hair coat has already developed. Hair shafts of nude mice exhibit multiple fractures and are twisted or locally thickened (Fig. 5). Ultrastructural analyses revealed that the cuticle of the inner root sheath and the cuticle of the hair shaft are filled up by abnormal globular aggregates, that the hair cortex is fragmented into irregular cornified material, and that the hair medulla is partially lacking (Köpf-Maier *et al.*, 1990). Despite the tremendous alterations of the hair shaft infundibulum of the hair follicles, the bulb region remains relatively unaltered. Although the number of hair bulbs was described to be decreased in some areas of the body (Rigdon and Packchanian, 1974), in general nude mice exhibit the same number of hair bulbs as normally haired mice (Köpf-Maier *et al.*, 1990). Dermal/follicular papilla fibroblasts and keratinocytes of the hair follicle matrix are essentially unaltered (Köpf-Maier *et al.*, 1990). Keratinocytes with pycnotic and fragmented nuclei, indicating keratinocyte degeneration, have been reported in some hair bulbs of *nu*/*nu* mice between day 10 and day 20 pp (Rigdon and Packchanian, 1974). Since catagen develops spontaneously during this period, its association with the genotype is equivocal. Moreover, mild dermal infiltrations of leukocytes have been described in association with keratinocyte degeneration (Rigdon and Packchanian, 1974) and can occasionally be observed in combination with folliculitis in nude

FIG. 2. Head of a NMRI *Foxn1*^{nu}/*Foxn1*^{nu} nude mouse, day 42 pp; vibrissae are small, curled, and crinkled.

FIG. 3. NMRI +/*Foxn1*^{nu} (left) and NMRI *Foxn1*^{nu}/*Foxn1*^{nu} (right) littermates at day 19 pp. Heterozygote mice have a normal dense hair coat, whereas homozygote animals lack a macroscopically visible hair coat and are much smaller in size.

mice. Follicular rupture, however, is a very rare event (Mecklenburg, unpublished observation). Whereas Flanagan (1966) originally described the sebaceous glands to be abnormally located at the base of the hair canal, no abnormalities were observed by other authors (Rigdon and Packchanian, 1974). In the NMRI congenic strain, we have found that the sebaceous glands are morphologically unaltered in mutant mice. The sebaceous glands are slightly displaced laterally by the bending hair shaft and dilated infundibulum, which may give the appearance that they are abnormally located (Mecklenburg *et al.*, unpublished observation).

The nude gene does not directly interfere with the cyclic activity of the hair follicles (Flanagan, 1966). Flanagan noticed that during the third week pp there is a reduction of skin thickness, corresponding to the catagen stage of normal skin. During catagen the follicles in nude mice shorten and build club hairs as in normally haired skin (Flanagan, 1966). However, the infundibuli of nude mouse hair follicles remain widened (Fig.6). Hair follicle ostia widen and finally the distorted hair shaft is released as a new hair shaft emerges. During the new anagen phase, nude mice develop macroscopically visible sparse and fine hairs. These hairs are lost again during the next catagen wave at about 6 weeks of age. It was reported that the elderly (more than 4 months old) nude mice hair growth pattern continues to follow the juvenile pattern with a strict hair growth wave, whereas this synchronicity is well known to be lost in normally haired mice (Eaton, 1976). Hair cycle analyses of different allelic mutations or the same mutation on multiple congenic strains have not confirmed this observation (Militzer, 2001). Apparently, the hair growth pattern does not differ in homo- and heterozygote nude mice.

Epidermal alterations have also been described in nude mice (Köpf-Maier *et al.*, 1990). Epidermal keratinocytes are irregularly shaped and the lamellae of the stratum corneum are irregular and detach from one another (Köpf-Maier *et*

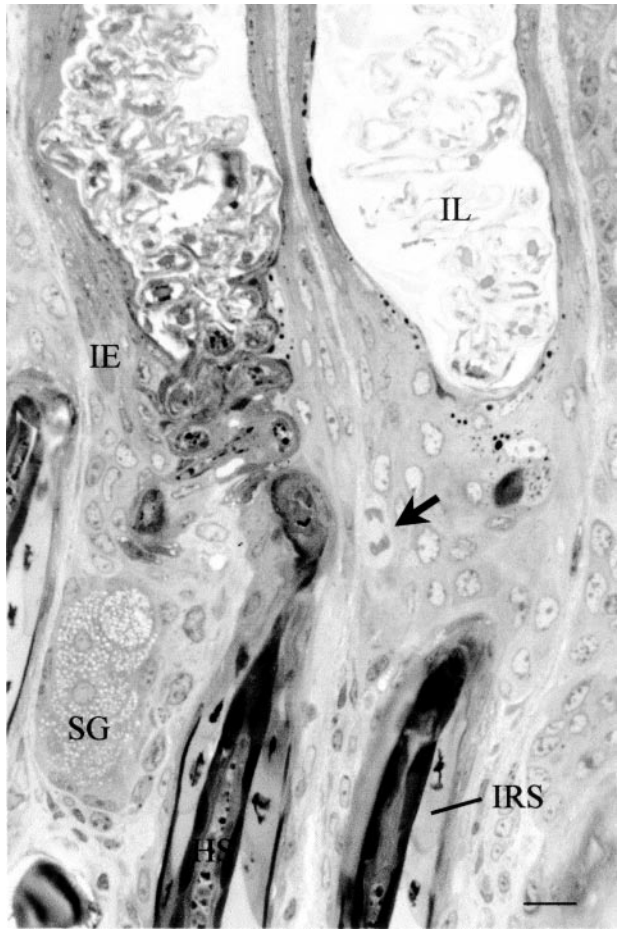

FIG. 4. Dorsal skin from a NMRI *Foxn1*^{nu}/*Foxn1*^{nu} mouse, day 16 pp; the hair shaft (HS) bends as it loses support by the inner root sheath (IRS) at the level where the sebaceous gland (SG) is located. The infundibular lumen (IL) is filled with a distorted hair shaft; note the mitotic keratinocyte (arrow) in the infundibular epithelium (IE), indicating epithelial proliferation; semithin plastic section stained with toluidin blue; bar, 15 μm.

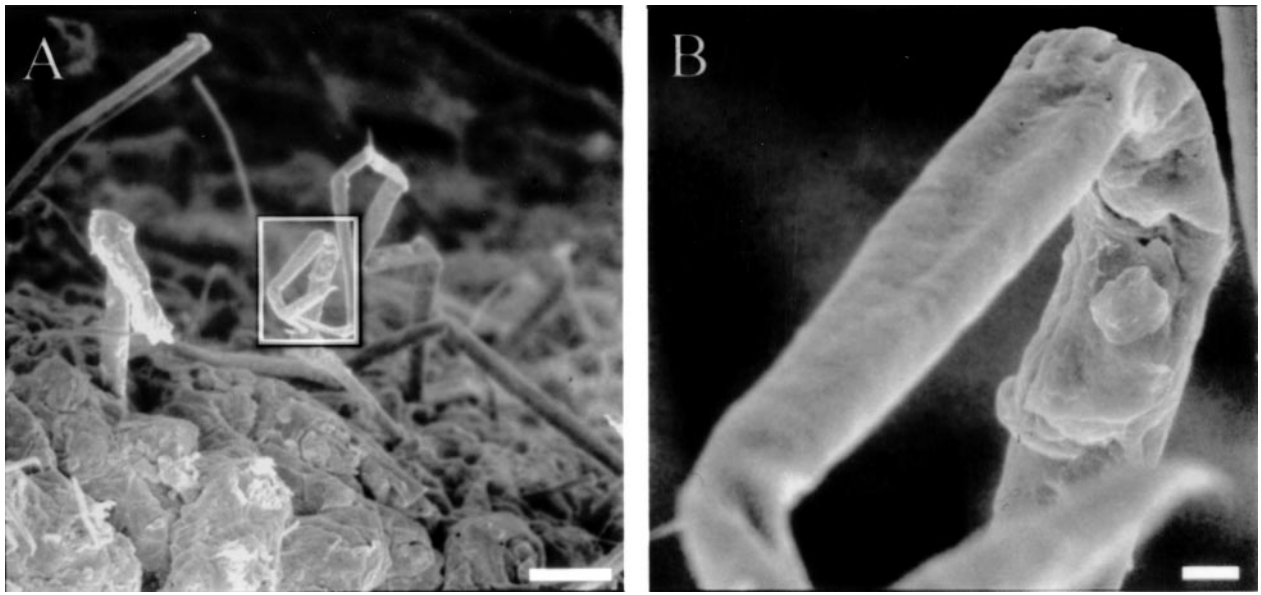

FIG. 5. Scanning electron microscopy from an 8-week-old female B6.Cg *Foxn1*^{nu}/*Foxn1*^{nu} mouse reveals few twisted hair shafts emerging from the skin surface (A; bar, 100 μm). Closer examination of the bent area boxed in A reveals that the shaft is flattened at the bend and that it lacks a cuticle (B; bar, 10 μm).

al., 1990). The number of tonofilaments in keratinocytes of the stratum granulosum and the stratum basale are reduced and hemidesmosomes, with only a few inserting tonofilaments, are expressed irregularly with large gaps between them (Köpf-Maier *et al.*, 1990). Targeted mutated mice that lack *Foxn1* and that are phenotypically comparable to nude mice exhibit a thickened epidermis with suprabasal expression of keratin 5 and increased accumulation of involucrin (Lee *et al.*, 1999). This is a common compensatory mechanism observed in numerous mutant mice in which chronic alopecia is a major phenotype (Sundberg, unpublished observations).

GENETICS

The mouse nude locus was localized on mouse Chromosome 11 (Takahashi *et al.*, 1992; Lisitsyn *et al.*, 1994) 45 cM from the centromere (http://www.informatics.jax.org) and by positional cloning *nude* mice were found to contain a mutation in the *Whn* (winged-helix-nude) gene that encodes for a transcription factor (whn or Hfh11) of the evolutionarily conserved winged-helix domain family (Nehls *et al.*, 1994; Segre *et al.*, 1995). This gene is now called the *Foxn1* gene according to the Fox (Forkhead box) Nomenclature Committee (Kaestner *et al.*, 2000). There are five allelic mouse mutations that have a similar phenotype (*Foxn1*^{nu}, *Foxn1*$^{nu\text{-}Bc}$, *Foxn1*$^{nu\text{-}str}$, *Foxn1*$^{nu\text{-}Y}$, and *Foxn1*$^{nu\text{-}StL}$). Defects in the *Foxn1* gene have been well characterized in all except *Foxn1*$^{nu\text{-}str}$. A nucleotide deletion in the *Foxn1*^{nu} mutation (original, spontaneous nude mouse) causes a frameshift resulting in loss of the DNA-binding domain of this transcription factor (Nehls *et al.*, 1994; Segre *et al.*, 1995). *FOXN1*$^{nu\text{-}Bc}$ has aberrant splicing of the *Foxn1* mRNA due to a transposon insertion in an intron upstream of the first coding exon (Hoffmann *et al.*, 1998). *Foxn1*$^{nu\text{-}Y}$ contains a missense mutation (R320C) in exon 7 leading to a nonfunctional FOXN1 protein (Schlake *et al.*, 2000a). The phenotype of *Foxn1*$^{nu\text{-}StL}$ is caused by a 2-bp insertion in exon 7 leading to a translational frameshift and loss of the activation domain (Schorpp *et al.*, 2000). Mutations in the *Foxn1* gene have also been detected in humans and rats exhibiting a nude mouse-like phenotype (Nehls *et al.*, 1994; Segre *et al.*, 1995; Schüddekopf *et al.*, 1996; Frank *et al.*, 1999). Finally, targeted disruption of the *Foxn1* gene (*Foxn1*$^{tm/Boe}$) leads to a typical nude mouse phenotype including athymia and macroscopic hairlessness (Nehls *et al.*, 1996). Transgenic overexpression of the *Foxn1* gene in nude mice partially corrects the skin phenotype (Kurooka *et al.*, 1996).

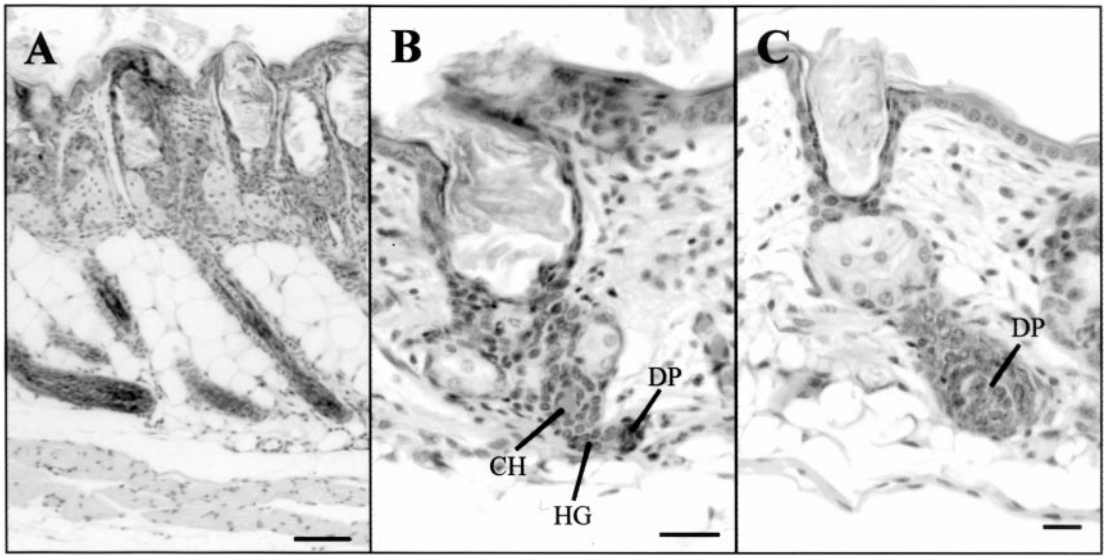

FIG. 6. Sections of NMRI *Foxn1*^{nu}/*Foxn1*^{nu} dorsal mouse skin at day 16 pp (early catagen) (A), day 25 pp (telogen) (B), and day 25 pp (early anagen) (C); CH, club hair; HG, hair germ; DP, dermal papilla; bar, 25 (A), 10 (B), 6.25 μm (C). Production of a weak, twisted hair shaft results in progressive dilation of the infundibulum encompassed by increasing amounts of cornified debris. H&E stained paraffin sections.

PATHOGENESIS OF THE NUDE PHENOTYPE

The hair follicle abnormalities in nude mice do not depend on a functional thymus (Eaton, 1976). Flanagan (1966), originally stated that the nude phenotype in the skin results from abnormal keratinization, possibly due to an inadequate synthesis of keratin precursors. Hormonal changes have also been considered as a cause of the nude phenotype because decreased concentrations of estradiol, progesterone, thyroxin, and prolactin were found in female nude mice (Pierpaoli *et al.*, 1976; Köpf-Maier and Mboneko, 1990).

Based on the combined dysgenesis of two ectodermal derivatives, the thymus and the skin, it was concluded that a defect in the embryonal ectoderm might underlie the nude phenotype, even though this should simultaneously lead to defects in the ear canal, in neural crest formation, and in the development of teeth and nails (Köpf-Maier *et al.*, 1990). After *Foxn1* was designated the gene responsible for the nude phenotype (Nehls *et al.*, 1994), research focused on the distribution of the FOXN1 protein and its effects in keratinocyte biology. The FOXN1 protein is evolutionarily highly conserved (Schlake *et al.*, 1997) and its analogue in *Drosophila* spp. possesses essential functions throughout development (Sugimura *et al.*, 2000; Strodicke *et al.*, 2000). *Foxn1*-like transcription factor genes have been maintained in single copy throughout chordate evolution (Schlake *et al.*, 2000a). In mammals, *Foxn1* expression is restricted mainly to the skin and the thymus, although it has also been found in the developing nails, nasal passages, tongue, palate, and teeth (Nehls *et al.*, 1996; Lee *et al.*, 1999). Moreover, *Foxn1* transcription has been described in the normal human kidney and thyroid gland (Pierpaoli and Sorkin, 1972; Gattenlohner *et al.*, 1999). Human and mouse FOXN1 proteins have 85% sequence homology (Schlake *et al.*, 1997). They contain a winged helix N-terminal DNA-binding domain and a C-terminal transcription activating domain (Schüddekopf *et al.*, 1996). Separation of both domains leads to a loss of function, while function is regained after they are linked noncovalently, suggesting that structural integrity and physical proximity of both domains are necessary for transactivation (Schlake *et al.*, 2000b). The DNA-binding domain folds into a variant of the helix-turn-helix motif and is made up of three α-helices and two characteristic large loops, or "wings" (Kaestner *et al.*, 2000). This winged helix DNA-binding domain has been shown to specifically bind *in vitro* to an 11-bp consensus sequence, 5′-A A/G N G A C G C T A/T T, containing the invariant tetranucleotide 5′-ACGC (Schlake *et al.*, 1997).

The FOXN1 protein is expressed exclusively in epithelial cells. This is in line with observations from hair reconstitution grafting assays: If wildtype dermal papilla cells are recombined with nude keratinocytes, hair follicles of the

nude phenotype develop, suggesting that *Foxn1* activity is specific to epithelial cells (Brisette *et al.* 1996). *Foxn1* expression in the thymus is also restricted to epithelial cells and has been shown to be necessary for the development of the mature thymic epithelium (Blackburn *et al.*, 1996; Brisette *et al.*, 1996; Lee *et al.*, 1999). Expression of the *Foxn1* gene, the subsequent *Foxn1* mRNA, and the FOXN1 protein can be detected as early as day 13 of gestation in the developing nasal region (Lee *et al.*, 1999). On the 16th day of gestation, *Foxn1* is expressed in the suprabasal epidermis. It cannot be found in the hair bud, the first stage of hair follicle development, but becomes detectable in a conical region above the bulbar matrix. Within the more mature hair follicle and in all anagen hair follicles, *Foxn1* is transcribed in the supramatrical region, in the hair shaft, and in the inner and outer root sheath (Lee *et al.*, 1999).

FOXN1 is possibly involved in regulating the balance between epithelial cell growth and differentiation (Brisette *et al.*, 1996; Lee *et al.*, 1999). This is in line with several observations that keratinocytes from nude mice have an increased propensity to differentiate abnormally and that the FOXN1 protein can specifically suppress the expression of differentiation-responsive genes in keratinocytes (Krueger *et al.*, 1980; Brisette *et al.*, 1996). Even in the hair follicle, expression of the *Foxn1* gene and its subsequent translation appear to correlate with the onset of terminal differentiation, although (FOXN1) has occasionally been found in some proliferating cells of the basal epidermis, the outer root sheath, and the hair follicle matrix (Lee *et al.*, 1999). During hair follicle regression (catagen), *Foxn1* expression is lacking in the regressing epithelial compartment but remains in keratinocytes surrounding the developing club hair and is retained in some cells of the isthmus region during telogen (Lee *et al.*, 1999). Studies with transgenic mice overexpressing *Foxn1* from an involucrin promoter at late stages of differentiation further suggest that *Foxn1* is involved in regulating the switch from proliferating to postmitotic epithelial cells. Epithelial cell proliferation is enhanced and their terminal differentiation is disrupted in the absence of FOXN1, leading to epidermal thickening and persistent anagen (Prowse *et al.*, 1999).

Foxn1 targeted genes are believed to (i) promote the differentiation of *Foxn1*-expressing keratinocytes and (ii) stimulate cell proliferation of neighboring cells via a paracrine mechanism (Prowse *et al.*, 1999). Changes in gene expression are indeed associated with FOXN1 malfunction. Recently, the gene for a novel serine protease was shown to be overexpressed in nude mouse skin. However, its upregulation is probably an indirect consequence of the differentiation defect in the nude mouse hair follicle rather than a direct effect of FOXN1 signaling. The role of this novel gene in skin physiology and pathology has not been clarified to date (Meier *et al.*, 1999a). *Foxn1* mRNA and the mouse ortholog of human acidic hair Keration gene 3 (*KRTHA3*, hereafter *mHA3*) mRNA are coexpressed in hair follicles, nails, and papillae of the tongue. In nude mice *mHa3* expression is completely absent in pelage hair follicles, indicating that *Foxn1* malfunction leads to a loss of expression of keratin genes (Meier *et al.*, 1999b; Schlake *et al.*, 2000b). The delayed appearance of vibrissae in $Foxn1^{nu}/Foxn1^{nu}$ mice could be explained by the remaining low-level expression of *mHa3* in vibrissae (Meier *et al.*, 1999b). Investigations in human HeLa cells suggest that FOXN1 is indeed a transcriptional regulator of hair keratin genes. It influences the expression not only of the acidic hair keratin *mHa3*, but also that of the mouse orthologs of the human hair Keratin genes, *KRTHA1, KRTHA2, KRTHA4, KRTHB3, KRTHB4, KRTHB5,* and *KRTHB6* (Schorpp *et al.*, 2000). Investigations of a novel nude allele ($Foxn1^{nu\text{-}StL}$) revealed that the FOXN1 protein of nude mice is not able to enter the nucleus and that obviously a highly complex set of transcriptional control mechanisms for hair keratin genes exists (Schorpp *et al.*, 2000).

When Sawada *et al.* (1987) investigated the effect of Cyclosporin A, an immunosuppressive fungal metabolite (Borel *et al.*, 1976) that is able to induce hypertrichosis in humans (Wysocki and Daley, 1987), on human tumors transplanted onto nude mice, they observed an increase in hair growth. Topical (0.2/1.0/2.0/2.5%), oral (10 to 160 mg/kg/day), or subcutaneous (500 mg/kg/day) application of Cyclosporin A for 7 days induced macroscopically visible hair growth of slightly crinkled long hair in $Foxn1^{nu}/Foxn1^{nu}$ mice (Sawada *et al.*, 1987; Watanabe *et al.*, 1991). However, morphological differences between Cyclosporin A-treated $Foxn1^{nu}/Foxn1^{nu}$ mice and wildtype (+/+), normal haired controls were not detected histologically (Watanabe *et al.*, 1991). Hair growth induction was dose-dependent, ceased after withdrawal, and had no effect in older mice (Sawada *et al.*, 1987; Watanabe *et al*, 1991). Even in isolated cultured $Foxn1^{nu}/Foxn1^{nu}$ mice vibrissae, Cyclosporin A (4.0 to 8.0 μM) stimulated hair growth, whereas this was not the case in vibrissae derived from normal +/+ mice (Buhl *et al.*, 1990). The mechanism by which Cyclosporin A affects hair growth in nude mice is still obscure. It has been speculated that Cycolsporin A might influence the process of keratinization or that it interferes with the skin immune system, which might be able to influence the hair growth cycle itself (Watanabe *et al.*, 1991). It has also been described that Cyclosporin A lengthens the anagen phase of the hair cycle, thus resulting in thicker and longer hair shafts that finally penetrate the

epidermis (Hozumi *et al.*, 1994). Dose-related induction of keratinocyte hyperplasia has also been described as an untoward effect of Cyclosporin A in human oral mucosa and in dog skin (Seibel *et al.*, 1989; Bennett and Christian, 1985).

It has also been noted that keratinocyte growth factor [KGF, also known as fibroblast growth factor 7, FGF7 (Finch *et al.*, 1989)], injected intraperitoneally or subcutaneously stimulates hair growth in nude mice. The morphology of hair follicles normalizes and the rate of proliferation in follicular keratinocytes increases under KGF treatment (Danilenko *et al.*, 1995).

The lack of a certain hair keratin and the reduction of a number of hair keratins might indeed explain the skin phenotype that occurs in nude mice. Hence the nude phenotype might represent the first example of an inherited skin disorder that is caused by loss of expression rather than production of an abnormal protein as suggested by Meier *et al.* (1999b). However, the biological role of *Foxn1* is far from being completely understood. Future research will have to clarify (i) growth retardation in nude mice (Fig. 3), (ii) how Cyclosporin A and KGF/FGF7 influence the nude phenotype, (iii) whether *Foxn1* influences the hair growth cycle, and (iv) what functions *Foxn1* has in hair follicle biology beyond its role in the regulation of keratin gene expression.

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