Requirement of the forkhead gene Foxe1, a target of sonic hedgehog signaling, in hair follicle morphogenesis

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The forkhead transcription factor FOXE1 is mutated in patients with Bamforth–Lazarus syndrome that exhibit hair follicle defects, suggesting a possible role for Foxe1 in hair follicle morphogenesis. Here, we report that Foxe1 is specifically expressed in the lower undifferentiated compartment of the hair follicle, at a time and site that parallel activation of the Shh signaling pathway. The Foxe1 protein is also expressed in human and mouse basal cell carcinoma in which hedgehog signaling is constitutively activated, whereas it is undetectable in normal epidermis and squamous cell carcinoma. Moreover, expression of a dominant-negative form of Gli2 in skin results in complete suppression of Foxe1 expression in the hair follicle, whereas transcriptionally active Gli2 stimulates activity of the Foxe1 promoter. Foxe1-null skin displays aberrant hair formation with the production of thinner and curly pelage hairs. Although the hair follicle internal structure is conserved and several lineage markers are properly expressed, the orderly downgrowth of follicles is strikingly disrupted, causing disorientation, misalignment and aberrantly shaped of hair follicles. Our findings provide a strong indication that the defect in Bamforth–Lazarus syndrome is due to altered FOXE1 function in the hair follicle, and is independent of systemic defects present in affected individuals. In addition, we establish Foxe1 as a downstream target of the Shh/Gli pathway in hair follicle morphogenesis, and as a crucial player for correct hair follicle orientation into the dermis and subcutis.

INTRODUCTION

The mammalian Forkhead Box (Fox) family of transcription factors consists of more than 50 proteins that are involved in biological processes such as tissue-specific transcription, cell fate determination during embryogenesis and cell survival (1).

Some forkhead genes have been associated with human developmental disorders, including immune, skeletal, circulatory, eye and skin defects (reviewed in 2). Mutations in the forkhead gene FOXE1/TTF-2 cause the Bamforth–Lazarus syndrome (OMIM 241850), a rare autosomal-recessive condition characterized by congenital hypothyroidism, thyroid dysgenesis, cleft palate and abnormal hair growth (3–5). During mouse embryogenesis, Foxe1 is expressed in the foregut endoderm, in the craniofaryngeal ectoderm and in the thyroid primordium (6,7). Foxe1 was first isolated as a thyroid-specific protein able to regulate transcription of the target genes, thyroglobulin and thyroid peroxidase, by binding to specific regulatory DNA sequences in their promoters (7–9). In vivo studies revealed that Foxe1 null mice either lack the thyroid gland or the gland is small and ectopic, but fully differentiated, indicating a crucial role of Foxe1 in thyroid morphogenesis (10). In these mice, the thyroid bud is unable to descend from the pharyngeal endoderm to its final...
destination, suggesting a defect in thyroid bud migration. In addition to defects in thyroid development, Foxe1-null embryos display severe cleft palate and die soon after birth for reasons that are incompletely understood. Thus, a functional Foxe1 protein is required for normal thyroid organogenesis and for secondary palate closure in humans and mice.

A consistent feature of Bamforth–Lazarus patients is sparse and spiky hair with reduced hair shaft diameter and loss of scale patterning, whereas nails and teeth are normal (3). Abnormal hair growth persists despite adequate thyroxin therapy (3). This finding indicates an intrinsic hair follicle abnormality suggesting that Foxe1 may be directly involved.

Hair follicle morphogenesis and terminal differentiation are highly regulated. Morphogenesis begins during embryogenesis in a region-specific manner and is governed by epithelial–mesenchymal interactions (11). In response to a mesenchymal cue, ectodermal cells form a hair follicle rather than epidermis and begin to proliferate and grow downward. In response to a subsequent ectodermal message, the underlying mesenchymal cells organize and become a specialized structure called dermal papilla. Finally, in response to a message from the dermal papilla, the developing hair follicle grows and differentiates. The hair follicle is composed of several epithelial layers with different degrees of differentiation. The most external epithelial layer, the outer root sheath (ORS), is the basal compartment of the hair follicle where undifferentiated cells are thought to migrate from the stem cell compartment to replenish the transit amplifying compartment in the matrix of the bulb (12–15). In the matrix, epithelial cells come in contact with the dermal papilla and are highly proliferative. When cells leave the matrix, they undergo one of at least seven programs of terminal differentiation, thus forming the various layers of the inner root sheath (IRS) and hair shaft. In postnatal skin, the hair follicle is perpetually renewed and undergoes cycles of proliferation (anagen), regression (catagen) and quiescence (telogen) (reviewed in (16–18)). In postnatal skin, the hair follicle is perpetually renewed and undergoes cycles of proliferation (anagen), regression (catagen) and quiescence (telogen) (reviewed in (16–18)).

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The molecular mechanisms downstream of the Shh/Gli signaling pathway that regulate hair follicle morphogenesis, hair cycling and BCC formation are largely unknown. Here, we report evidence that the forkhead domain transcription factor Foxe1 is a downstream target of Shh/Gli in hair follicle morphogenesis as well as in BCC. Importantly, Foxe1 is required for late stages of hair follicle morphogenesis and correct orientation of the hair follicle.

**RESULTS**

**Foxe1 is expressed in the undifferentiated compartment of the hair follicle**

Mutations in the forkhead protein FOXE1 are associated in humans with a developmental syndrome involving abnormal hair growth. To test whether Foxe1 is directly involved in hair follicle morphogenesis and/or maintenance, we first determined whether Foxe1 is expressed in skin. For this purpose, we isolated total RNA from various adult murine tissues and performed RT–PCR with Foxe1-specific oligonucleotides. This analysis revealed robust Foxe1 mRNA expression in adult and newborn skin, whereas none of the other tested tissues expressed Foxe1 (Fig. 1A). Northern blot analysis confirmed Foxe1 mRNA expression in skin (data not shown).

To establish the localization of Foxe1 mRNA in skin, in situ hybridization analysis was performed on sections of 10-day-old mouse skin with DIG-labeled cRNA specific for mouse Foxe1. Foxe1 mRNA was readily detectable in the lower portion of the ORS in the hair follicle, whereas there was no expression in the upper part of the hair follicle or in the epidermis (Fig. 1B and data not shown). As control in situ hybridization with specific probes for keratin 17 and caspase 14, specific markers of the entire ORS and of the epidermis, respectively, gave the expected expression pattern (data not shown).

Immunohistochemistry analysis of mouse skin using anti-Foxe1 polyclonal antibodies resulted in nuclear staining in the lower ORS (Fig. 1C). Although the ORS surrounds the entire follicle and is continuous with the basal layer of the interfollicular epidermis, Foxe1 expression was restricted to
ors of the hair follicle (see arrow). probe for mouse skin sections at postnatal day 10 (P10) using a DIG-labeled antisense sense probe gave no detectable signal under the same conditions (not shown). Scale bars: 20 in other hair follicle compartments, in the epidermis (epi) or in the dermis (de). with ORS and IRS markers (data not shown). No Foxe1 expression is detected in the undifferentiated compartment as shown by staining of adjacent sections which Foxe1 is not expressed. In (E) Foxe1 expressing cells are located in the lower ORS at P5 and in the hair follicle. Arrows indicate sites of Foxe1 expression. Arrowheads in (D) indicate immature hair follicle in the lower portion of the ORS, around and just above the hair bulb, as well as in the dermal papilla (27,40). Our findings indicate that Foxe1 is a target of the Shh/Gli pathway.

In postnatal skin, Shh expression ceases in telogen (quiescent stage), and its up-regulation in early anagen (growing stage) is thought to be crucial for hair follicle growth (23,24). Interestingly, Foxe1 expression displayed similar changes in expression during the hair cycle. In contrast to other ORS markers, such as K14 and p63, Foxe1 protein expression was lost in the first telogen period (Fig. 2A). Foxe1 expression was re-activated in early anagen and maintained in the following anagen steps and in catagen, whereas it was lost again in the subsequent telogen period, which lasts several weeks (data not shown). Interestingly in early anagen Foxe1 expression was localized in a cone of cells below the developing hair bulb (Fig. 1E), similar to what was observed at beginning of hair follicle morphogenesis. At later stages in the fully developed follicles, Foxe1 was again expressed primarily in the lower ORS (data not shown).

Thus, the temporal and spatial expression of Foxe1 in the hair follicle correlates with activation of the Shh signaling pathway.

In Shh and Gli2 null mice, hair follicle morphogenesis is arrested before the stage in which Foxe1 is expressed (20,21,27); thus, analysis of Foxe1 expression in the absence of these genes could not be performed. To test whether Shh/Gli signaling is required for Foxe1 expression in vivo, immunohistochemical analysis using anti-Foxe1-specific antibodies was performed in transgenic mice expressing Gli2ΔC4, a transactivation domain mutant of Gli2 that inhibits Gli function in a dominant-negative manner (41). Gli2ΔC4 transgenic mice display short and malformed hair follicles, but express multiple markers for hair follicle cell lineages (42), H. Sheng and A.A. Dlugosz, manuscript in preparation). Although Foxe1 expression was readily detected in wild-type littermates, no expression could be detected in transgenic mice expressing Gli2ΔC4 (Fig. 2B). In contrast, K14 expression, used as control of ORS integrity, was unaffected in the presence of Gli dominant-negative mutant.

**Foxe1 expression is dependent on the Shh/Gli signaling pathway**

*Shhh* gene is expressed in the proliferating epithelial cells at the lower tip of the developing hair follicle (38,39). Similarly *Gli1*, *Gli2* and *Ptc1* are expressed in partially overlapping domains in the lower epithelial portion of the hair bulb and ORS, as well as in the dermal papilla (27,40). Our findings indicate that Foxe1 protein is specifically expressed in the lower ORS surrounding the hair bulb raised the possibility that Foxe1 is a target of the Shh/Gli pathway.

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Taken together, these findings indicate that in skin Foxe1 is expressed in a temporal and spatial highly regulated fashion, which parallels Shh activation, and that Shh/Gli signaling is required for Foxe1 expression in the hair follicle.

Foxe1 expression in human and mouse BCC

BCC is a locally invasive tumor of the skin thought to derive from cells of the undifferentiated compartment of the hair follicle, and to be a consequence of inappropriate, sustained activation of the Shh signaling pathway (29–33,35,37). To determine whether Foxe1 is expressed under conditions in which the Shh signaling pathway is aberrantly activated, we tested whether FOXE1 was expressed in BCC as compared to normal human skin. Skin sections were immunostained with Foxe1-specific antibodies. Although FOXE1 protein expression was low to undetectable in normal human epidermis, intense nuclear staining was found in BCC (Fig. 3A and data not shown). Fourteen independent BCC samples were tested and all were positive for Foxe1 expression. In contrast, no expression was detected in normal skin or in squamous cell carcinoma (SCC), an epidermal-derived cancer, in which Shh signaling is not activated (reviewed in 43), suggesting that FOXE1 expression is a specific feature of BCC (Fig. 3A).

BCC is very uncommon in mice, but can be induced by constitutive activation of Shh signaling (28,35–37). Transgenic mice overexpressing the Shh effector Gli2 in keratinocytes develop multiple BCCs (37). Immunohistochemical analysis of BCC derived from Gli2 overexpressing transgenic mice showed that Foxe1 was highly expressed in tumors cells, but not in overlying epidermis or in age-matched control skin (Fig. 3A and data not shown), in parallel with previously reported expression of Gli2, Gli1 and Ptch 1 (37). Induced expression of Foxe1 gene in mouse BCC was confirmed at the RNA level by real-time RT–PCR (data not shown).

To further dissect Foxe1 responses to Shh signaling in keratinocytes, we studied skin tumor cell lines derived from transgenic mice expressing a constitutively active form of Gli2 under the control of keratin 5 promoter (Gli2ΔN2), which displays constitutive activation of the Shh pathway in vitro and in vivo, on the basis of up-regulation of Gli1 and Ptch1 mRNA (34,41). Foxe1 expression was analyzed and compared in Gli2ΔN2 expressing keratinocyte cell lines versus a similarly treated wild-type keratinocyte cell line, designated WT-7. Although Foxe1 expression at the RNA level was very low in WT-7 cells, it was strongly up-regulated in two independent K5-Gli2ΔN2 tumor cell lines (G2N2c and Tb3a) (Fig. 3B). Interestingly, Foxe1 expression was induced to a similar extent as the classical

Figure 2. Foxe1 expression in the hair follicle is dependent on the Shh/Gli signaling. (A) Foxe1 expression ceases in the resting phase of the hair cycle. Paraffin sections of CD1 mice in the resting phase of the hair cycle (P21) were probed with polyclonal antibodies to Foxe1 (left panel), to the transcription factor p63 (middle panel) or to keratin 14 (K14; right panel) using an alkaline phosphatase reporter system. Foxe1 expression is undetectable during the resting phase of the hair cycle, whereas expression of the other ORS markers is strongly positive. Scale bars: 50 μm. (B) Immunolocalization of Foxe1 in skin of transgenic mice expressing a dominant-negative form of Gli (Gli2ΔC4) (42). Paraffin sections of mouse skin at P10 were isolated from Gli2ΔC4 or from wild-type mice (wt), and stained with anti-Foxe1 antibodies. Although in wt sections Foxe1 expression is localized in the ORS, no expression is detected in the ORS of transgenic mice carrying Gli2ΔC4. Staining with keratin 14 antibodies (K14) was used as positive control to detect the ORS and the basal layer of the epidermis. Scale bars: 50 μm.
Shh downstream targets Gli1 and Ptch1. A strong Foxe1 induction in Gli2 expression was observed in cells derived from both human and mouse origin. In contrast, SCC did not express Foxe1 at any detectable level. Foxe1 mRNA levels were quantified by real time RT–PCR in wild-type keratinocytes derived from a control mouse (wt) or in trichoblastoma cells derived from K5-Gli2ΔN2 transgenic mice (G2N2c and Tb3a) (34). Foxel mRNA is strongly up-regulated in cells overexpressing Gli2 in parallel with up-regulation of Gli1 and Ptch1, two known targets of the Shh/Gli signaling. Values were normalized to GAPDH mRNA expression. Foxe1 protein is strongly induced in trichoblastoma as compared with wild-type cells. An aliquot of 30 μg of total cell extract obtained from the rat thyroid cell line FRTL-5 used as positive control (FRTL-5), from mouse primary keratinocytes (I Ker.), from wt, as well as from G2N2c and Tb3a trichoblastoma cells were run on a 12% SDS–PAGE gel, and immunoblotted using anti-Foxe1 antibodies. Molecular weight (in kDa) is shown on the left side. Membranes were probed again with an antibody to ERK1 as a loading control.

**Figure 3.** Foxe1 expression is induced in BCC. (A) Immunolocalization of Foxe1 in human and mouse skin tumors. Paraffin sections of human SCC and BCC, as well as of mouse BCC derived from transgenic mice expressing Gli2, were probed with polyclonal antibodies to Foxe1 using an alkaline phosphatase reporter system. This analysis revealed aberrant Foxe1 expression in BCC cells derived from both human and mouse origin. In contrast, SCC did not express Foxe1 at any detectable level. (B) Foxe1 mRNA levels were quantified by real time RT–PCR in wild-type keratinocytes derived from a control mouse (wt) or in trichoblastoma cells derived from K5-Gli2ΔN2 transgenic mice (G2N2c and Tb3a) (34). Foxe1 mRNA is strongly up-regulated in cells overexpressing Gli2 in parallel with up-regulation of Gli1 and Ptch1, two known targets of the Shh/Gli signaling. Values were normalized to GAPDH mRNA expression. (C) Foxe1 protein is strongly induced in trichoblastoma as compared with wild-type cells. An aliquot of 30 μg of total cell extract obtained from the rat thyroid cell line FRTL-5 used as positive control (FRTL-5), from mouse primary keratinocytes (I Ker.), from wt, as well as from G2N2c and Tb3a trichoblastoma cells were run on a 12% SDS–PAGE gel, and immunoblotted using anti-Foxe1 antibodies. Molecular weight (in kDa) is shown on the left side. Membranes were probed again with an antibody to ERK1 as a loading control.

Shh downstream targets Gli1 and Ptch1. A strong Foxe1 induction in Gli2ΔN2 keratinocytes was also observed at the protein level by immunoblotting using Foxe1-specific antibodies (Fig. 3C). Although mouse primary keratinocytes and WT-7 cells did not express Foxe1 protein at any detectable levels, Foxe1 expression was readily detectable in cells expressing Gli2ΔN2.

**Regulation of Foxe1 promoter by Shh/Gli signaling**

To determine whether Foxe1 promoter was responsive to Shh/Gli signaling, we cloned a 2.39 kb rat genomic fragment comprising 1.8 kb of the promoter region and the entire 5′-UTR (0.59 kb), upstream of the luciferase gene used as reporter. Consistent with the expression data, Foxe1 promoter displayed a strong activity in G2N2c cells as compared with the promoter-less construct and to a thymidine kinase (TK) promoter (Fig. 4A). To test whether the Foxe1 promoter activity was dependent on Gli, the promoter was co-transfected with the Gli dominant-negative mutant Gli2ΔC4. In the presence of Gli2ΔC4, Foxe1 promoter was strongly inhibited (up to 80%) in a dose-dependent manner (Fig. 4A). In contrast, TK promoter was not affected by expression of Gli2ΔC4.

To further identify the Foxe1 promoter region responsive to Gli, various promoter deletions were generated and tested for their susceptibility to Gli2ΔC4 (Fig. 4B). Progressive deletions of the promoter region enhanced transcriptional activity, but displayed similar inhibition by Gli2ΔC4 (Fig. 4C). The smallest region tested, comprising only 0.19 kb of genomic region upstream of the transcription start site, as well as the 5′-UTR region, was still strongly inhibited by Gli2ΔC4 (70% inhibition at the highest Gli2ΔC4 concentration).

The Foxe1 promoter sequence was analyzed for Gli consensus binding sites (GACCACCCA) (44–46) by visual inspection and by bioinformatics analysis using MatInspector (GenomatixSuite 3.1.0) (47), TRANSFAC (TRANSFAC Professional 8.2) (48) and by comparison between rat and human promoters using rVISTA (49). No putative binding sites for Gli could be found in D1600 Foxe1 promoter even at low stringency, whereas a putative binding site (GGCCACCCA) was located at position –1.1 kb in the rat and mouse
To construct reporter plasmids for transient transfection value is set to 1. (<i>relative luciferase activity corrected for transfection efficiency. The control Xba</i> mutant was generated by restriction enzyme digestion with 1.8 kb promoter region (thin bar) and 5Gli2 promoter activity was measured in the presence or in the absence of TK. Although TK promoter activity was measured in the presence or in the absence of Gli2 promoter, but not in the human promoter. However, consistent with the deletion experiment, mutation of the Gli-binding site (GGCCAGGCCA) did not affect the response to Gli2ΔC4 (data not shown).

![Graph A](https://academic.oup.com/hmg/article-abstract/13/21/2595/587489)

**Figure 4.** Foxe1 promoter is responsive to Gli. (A) Luciferase activity was measured in G2N2c after transient transfection with a reporter plasmid carrying the Foxe1 promoter in the presence or in the absence of a dominant-negative form of Gli2 (Gli2ΔC4) in the indicated amounts (μg). Similarly, TK promoter activity was measured in the presence or in the absence of Gli2ΔC4. Although TK promoter was unresponsive, Foxe1 promoter was inhibited by Gli2ΔC4 in a dose-dependent manner. Results are presented as relative luciferase activity corrected for transfection efficiency. The control (pcDNA3.1) transfection value is set to 1. (B) Schematic presentation of Foxe1 promoter deletion constructs. To construct reporter plasmids for Foxe1 promoter, a 2.39 kb rat genomic fragment including a 5′ upstream 1.8 kb promoter region (thin bar) and 5′-UTR (0.59 kb; thick bar) was cloned in front of the luciferase gene pGL3-Luc (Promega). Deletion mutants were generated by restriction enzyme digestion with Xbal–KpnI, generating a deletion of 500 bp (Δ500), BsrXI–KpnI generating a deletion of 1100 bp (Δ1100) and with SacI generating a deletion of 1600 bp (Δ1600). The size of each DNA fragment is indicated below the wt promoter. (C) Deletions of the Foxe1 promoter were tested in the absence (light gray bars), or in the presence of Gli2ΔC4 at either 0.5 μg (dark gray bars) or 1 μg (black bars). Results are presented as relative luciferase activity corrected for transfection efficiency. The wild-type promoter (wt) transfection value is set to 1. The data table is presented below the graph. Each condition was tested in triplicate wells, and standard deviations are indicated. Transient transfection assay shown are representative of at least three independent experiments.

Taken together, these data suggest that Foxe1 gene expression is regulated by the Shh/Gli pathway at the transcriptional level, and that regulation of the Foxe1 promoter is likely to be indirect.

**Foxe1-null skin displays aberrant hair follicle morphogenesis**

To assess the role of Foxe1 in hair follicle morphogenesis and maintenance, we analyzed the skin of mice carrying a homozygous disruption of Foxe1 (Foxe1−/−) (10). Histological and immunohistochemical analyses of dorsal skin sections of Foxe1−/− mice at birth did not reveal any differences compared to wild-type skin (data not shown). Thus, Foxe1 is not required for early stages of hair follicle morphogenesis, as also suggested by its late expression.

As Foxe1−/− mice die at birth, we grafted Foxe1−/− newborn skin onto the backs of immunodeficient mice to examine later stages of hair follicle morphogenesis. Thirty immunodeficient mice received grafts from either Foxe1−/− mice or wild-type littermates. Hair appeared within 7–10 days in grafts from wild-type newborns, and reached maximum length in about 2 weeks (Fig. 5A). A similar time course was observed in Foxe1−/− grafts; however, in this case the hair coat was sparse, thin and kinky as compared to wild-type ones, with hairs pointing in different directions at different angles (Fig. 5B). To determine whether there was a defect at the level of the hair shaft, hairs were plucked and observed under the dissecting microscope. The mouse coat contains four hair types—guard, awl, auchene and zigzag—that differ in several properties (50,51). The hair types are distinguished by their length and by the structure of the medulla (the central cylinder of the hair), which normally contains cells arranged in columns. Zigzag hairs, the most abundant hair type, possess a single column of cells, whereas the remaining hair types have two or more columns side by side. Wild-type hair from the graft appeared normal and had the expected ratio among hair types (Fig. 5C and data not shown). In contrast, Foxe1−/− hairs were kinky and curly, unusually thin, with a C- or S-shaped curvature, and an irregular septation between cells, making it difficult to distinguish among the different hair types (Fig. 5D). Quantitative analysis of more than a thousand hair shafts revealed a 50% reduction in hair consisting of 3–5 cell layers (auchene/awl), with a concomitant increase of single cell hairs, which appeared unusually thin, lacked sharp bends and were irregularly septulated.

Histological analysis revealed remarkable changes in Foxe1−/− skin. At 2 weeks after grafting, wild-type hair follicles were uniformly sloped and organized in an orderly parallel array (Fig. 6A). In contrast, in Foxe1−/− skin, hair follicles were clearly misaligned, variably angled and somewhat smaller than wild-type ones (Fig. 6B, see arrows). The presence of oblique and transverse sections among longitudinal sections of hair follicles indicates that follicles curved in and out of the plane of section. Foxe1 immunostaining was used as control for the presence (Fig. 6C) or absence (Fig. 6D) of Foxe1 protein in wild-type and Foxe1−/− grafts, respectively. Interestingly, the differentiation program of Foxe1−/− skin appeared normal as judged by
immunostaining with several differentiation markers such as K17 (staining the ORS, medulla of the hair shaft and the matrix) and trichohyalin (a marker of IRS), as well as K14 (a marker of ORS and basal layer of the epidermis), K6a (a marker of ORS inner layer), Hb2 (a marker of hair cuticle), and markers of the epidermis such as K1 (a marker of the spinous layer), involucrin (a marker of the granular layer), filaggrin, loricrin (a marker of the granular layer and stratum corneum) (Fig. 6E–H and data not shown). Proliferation was also unaffected at least as judged by expression of the proliferation marker Ki67, which was similarly expressed in Foxe1–/– and wild-type grafts at 3 weeks after grafting and examined under the microscope at the same magnification (20×). Wild-type hairs (C) were classified according to Dry (50). In this classification, pelage hair can be differentiated by having one zigzag (z), two guard (g), or two or more awl (aw) and aulcine (au) rows of air cells. In contrast, hair categories from Foxe1–/– coat (D) were not easily distinguishable due to their aberrant shape and septation.

**DISCUSSION**

Hair follicle morphogenesis and the hair cycle are highly regulated processes in which induction and silencing of gene expression are finely tuned. The regulation of gene expression in terminally differentiating hair follicle cells is well characterized (52–59). In contrast, little is known about gene expression control in cells of the ORS, where undifferentiated progenitors cells are thought to migrate from the stem cell compartment to replenish the transit-amplifying compartment. We show here that the forkhead protein Foxe1 is expressed in the lower ORS surrounding the bulb, is a downstream target of the Shh signaling, and participates in late stages of hair follicle morphogenesis, as lack of Foxe1 causes aberrant downgrowth of the hair follicle in the deep dermis.

Macroscopically, the hair defect in Foxe1-deficient skin recapitulates the hair phenotype (sparse and spiky hair) of patients affected by Bamforth–Lazarus syndrome, which are homozygous for loss-of-function mutations affecting the Foxe1 DNA-binding domain (3–5). Our expression data, coupled with grafting experiments, provide a strong
indication that the defect is due to altered Foxe1 function in the hair follicle and is independent of systemic defects present in affected individuals. In addition, we show that hair defects are due to aberrant hair follicle morphogenesis caused by an inability of the hair follicle to descend correctly into the dermis and subcutis—a process that leads to misaligned and aberrantly shaped hair follicles. In contrast, cell proliferation and terminal differentiation of Foxe1−/− hair follicles appear unaffected.

Hair follicle defects similar to those found in Foxe1−/− skin occur in mice in which the TGF-alpha/EGF-receptor signaling is disrupted (60–64). A major difference between these animal models is that, though TGF-alpha null mice display defects in pelage hair as well as in whiskers hair, defects in Foxe1 null mice are confined to pelage hair (A. Brancaccio and C. Missero, unpublished data). A similar selectivity was also reported in Shh and Gli2 defective mice (27), even though both molecules are expressed in the whisker follicles. These observations suggest that some molecular mechanisms in hair follicle morphogenesis are distinct between pelage and whisker follicles, and that the Shh/Gli/Fox pathway is essential only in pelage follicle morphogenesis, whereas TGF-alpha/EGFR signaling is required for both. A genetic interaction between the EGF pathway and Foxe1 has not been explored fully; however, Foxe1 is unlikely to control TGF-alpha expression, as they do not co-localize in the hair follicle. In addition, exogenous expression of Foxe1 in mouse primary keratinocytes by retroviral infection does not alter the expression levels of TGF-alpha, EGF or EGF receptor (A. Brancaccio and C. Missero, unpublished data). The EGF receptor is expressed in the ORS, raising the possibility that Foxe1 may be controlled by the TGF-alpha signaling pathway. In this context, the absence of Foxe1 may disrupt a proliferative signal required for ORS and/or bulb growth, which in turn may result in aberrant downgrowth. However, obvious proliferation defects could not be detected in Foxe1−/− skin either at birth or at 2 weeks after grafting, suggesting that the molecular defect underlying abnormal hair follicle morphogenesis may be more complex. In line with this hypothesis is the phenotype of Shh-null mice, which display severe defects in hair follicle morphogenesis and downgrowth that are not simply explained by proliferation defects. Indeed, when grafted to nude mice, Shh mutant skin gives rise to large abnormal follicles with a high rate of proliferation in some cells; however, these abortive follicles display impaired downgrowth (20,21).

Foxe1 has a very restricted spatial and temporal pattern of expression in the hair follicle, being present specifically in the lower ORS surrounding the bulb, an undifferentiated portion of the hair follicle, which may contain migrating stem cells (16). Interestingly, Foxe1 expression ceases in telogen and is then induced in the new anagen. This peculiar pattern of expression overlaps—at least in part—with Shh and its targets genes Ptch1 and Gli, which are also expressed in the lower hair follicle (40), and similarly to Foxe1 are not expressed in telogen. Of the three Gli transcription factors involved in Shh-responsive gene expression, Gli2 is the key mediator of Shh in mouse skin (27). Here, we present genetic evidences demonstrating that Foxe1 is a downstream target of Shh/Gli signaling: (i) Foxe1 expression is completely suppressed in transgenic mice expressing a dominant-negative Gli in skin; (ii) Foxe1 is aberrantly expressed in human and mouse BCC where the Shh signaling is constitutively activated; (iii) Foxe1 is strongly up-regulated in keratinocytes expressing an activated form of Gli2; (iv) activity of the Foxe1 promoter is much stronger in keratinocytes carrying the activated form of Gli2 than in wild-type keratinocytes; and (v) a dominant-negative Gli mutant inhibits Foxe1 promoter activity.

During mouse embryonic development, a number of forkhead genes is regulated by Shh, and some of these genes have been shown to be direct transcriptional targets of Shh/Gli signaling (44,65–68). We show that the Foxe1 promoter is responsive to Shh/Gli signaling as it is strongly inhibited by the dominant-negative form of Gli. Very recently, FOXE1 mRNA and promoter have been reported to be induced by wild-type Gli2 overexpression in HaCaT cells, a human keratinocyte cell line (69). A homologous portion of the human and rat promoters was analyzed and found to be responsive to Gli2 ([69] and present study), reinforcing the notion that Gli2 affects Foxe1 transcription by controlling directly or indirectly its proximal promoter region. Five Gli-binding sites with partial homology with the previously reported Gli-binding consensus motif GACCACCA (45) were found in the human promoter, however, none of these potential binding sites were conserved in the rat promoter sequence. We further dissected the rat Foxe1 promoter region responding to Gli and found that it comprises a very small region of the promoter surrounding the transcription start site and the 5′-UTR, where no putative Gli-binding sites could be identified by bioinformatics analysis. Foxe1 promoter is TATA-less and several putative cis-acting elements, such as AP1-, AP2-, E2F- and SP1-binding sites, are found in this region, which are conserved among human, mouse and rat sequences. Further experiments will be required to identify which of these sites mediate Gli transcription effect on the Foxe1 minimal promoter, or whether Gli binds directly to the promoter in a non-canonical binding site. Interestingly, Foxe1 displays a narrower pattern of expression as compared to Shh and Ptch1, suggesting that Foxe1 expression may be refined by mechanisms other than Shh signaling.

Although Foxe1 has a very restricted spatial and temporal pattern of expression in the hair follicle and its loss results in aberrant hair follicle morphogenesis, deregulated expression of Foxe1 is associated with BCC in humans and mice. Foxe1 mRNA has been reported to be expressed at the RNA level in a panel of human BCCs (69). These results are consistent with our findings that the Foxe1 protein is aberrantly expressed in human BCC, and also in a mouse model of BCC induced by overexpression of the transcription factor Gli2, strengthening the notion that Foxe1 is a target of Shh signaling and is likely to play a role in BCC formation. While Foxe1 role in BCC formation remains to be explored, our results point to a possible role of Foxe1 in contributing to local invasion of surrounding tissues by facilitating downgrowth of neoplastic tissue in the dermis in parallel with its physiological role in the downgrowth of the hair follicle.
MATERIALS AND METHODS

Cell cultures, transfection and reporter assays

Mouse primary keratinocytes were isolated from 2-day-old Swiss CD1 mice and cultured under low calcium conditions (0.05 mm) in the presence of 4% Ca2+-chelated fetal bovine serum (Invitrogen), and EGFR (BD Bioscience), as previously described (70, 71). WT-7, G2Nc2 and Tb3A keratinocytes were cultured under low calcium conditions in the presence of 8% Ca2+-chelated fetal bovine serum, and KGF (Sigma) as described (34). Confluent mouse primary keratinocytes were transfected in 12-well plates 5 days after plating, whereas keratinocyte cell line were subcultured in 12-well plates and transfected 2 days after plating under subconfluent conditions. Transient transfections were performed using 4 μL of Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Cells were transfected with reporter plasmids (0.5 μg) Foxe1 promoter-Luc vector, pGL3 (control plasmid), or a TK minimal promoter-pGL3 (72); effector plasmid Gli2AC4 (0.5 and 1 μg) (41, 42); and pCMV-Renilla (20 ng). Cells were harvested 48 h after transfection, and luciferase levels were determined with Dual-Luciferase Reporter assay kit (Promega) following the manufacturer’s protocol. Renilla luciferase activity was used to normalize for transfection efficiency. To construct reporter plasmids for Foxe1 promoter, a 2.39 kb rat genomic fragment (54) upstream 1.80 Kb promoter region and Foxe1 5’-UTR (0.59 kb) was cloned blunt in Smal in front of the luciferase gene pGL3-Luc (Promega). Deletion mutants were generated by cutting and ligating pGL3-Foxe1 promoter with Sacl (Δ1600), BstXI–KpnI (Δ1100) and Xbal–KpnI (Δ500).

RNA isolation and analysis

RNA was extracted from CD1 mouse tissues using Trizol-LS Reagent (Invitrogen) and treated with RNase-free DNase I (Promega). cDNA was synthesized using Superscript II (Invitrogen) and random primers. Specific cDNA was amplified with Taq DNA polymerase (Invitrogen) with the following oligonucleotide primers: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): GAPDHf (GTAT...C), GAPDHr (CATG...G), Foxe1f (GACT...G) and Foxe1r (GATC...A); for Hox11: Hox11f (GTAT...G) and Hox11r (GATC...A); for Gli2: Gli2f (CCGC...G) and Gli2r (GTTG...G); for Foxe1: Foxe1f (GACA...G) and Foxe1r (GATC...A); and for Ptch1: Ptch1f (AGAC...A) and Ptch1r (AGAC...A). Two-step real-time RT–PCR was performed using the SYBR® Green PCR Core Kit (Applied Biosystem). For real-time RT–PCR oligonucleotide primers were as follows: GAPDHf (GTAT...G), GAPDHr (TTCC...G), Foxe1f (GACA...G) and Foxe1r (GATC...A); and for Ti: Ti1f (CATT...G) and Ti1r (GACT...G). Amplification was carried out under the following conditions: 95°C for 15 s, 60°C for 1 min, 72°C for 1 min, for 40 cycles. The amplified products were confirmed by melting curve analysis and gel electrophoresis.

Histology and immunostaining

Skin samples were fixed in 4% paraformaldehyde in PBS overnight at 4°C, embedded in paraffin. Wild-type skin was determined using the Bio-Rad DC protein assay. Upon addition of 5% 2-mercaptoethanol, 30 μg of protein lysates were run on a 12% SDS–PAGE gel and transferred onto Immobilon-P transfer membrane (Millipore). Membrane was then blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS), probed with anti-Foxe1 polyclonal antibodies (1:500) (6) for 1 h, washed and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:2000) and detected by chemiluminescence (Amersham). Reprobing of the same filter was performed by incubating membrane in stripping buffer (2% SDS, 100 mM 2-mercaptoethanol in 62.5 mM Tris–HCl pH 6.8). After extensive washing, the membrane was incubated with anti-ERK1-specific antibodies (1:200; Santa Cruz) as loading control.

In situ hybridization

In situ hybridization was performed as previously described (6). Briefly, skin was fixed in 4% fresh paraformaldehyde overnight at 4°C, washed in PBS, incubated in 30% sucrose/ PBS overnight at 4°C and embedded in OCT compound (Sakura). Sections of 16 μm were treated with proteinase-K 20 μg/ml for 15 min and hybridized with DIG-riboprobe in 50% formalin overnight at 60°C. After extensive washings, slides were incubated with anti-DIG-alkaline phosphatase antibodies (Roche), washed and incubated in NBT/BCIP solution (Roche), 1 mM levamisole (Sigma) for 6–12 h. Hybridization was performed with 1 μg/ml of digoxigenin-labeled Foxe1 cRNA probe corresponding to a Not1–BanHI cDNA fragment comprising 442 bp of coding sequence (lacking the forkhead domain) and 326 bp of 3′-UTR, cloned into Bluescript KS+ (6). Probes for keratin 17 and caspase 14 were generated as previously described (73, 74). Antisense and sense probes were transcribed from the T3 and T7 promoters, respectively, using a DIG labeling kit (Roche) as described by manufacturer’s instructions.

Immunoblotting analysis

Protein extracts were prepared by lysing subconfluent 100 mm dishes in 2× SDS loading buffer. Protein concentration was determined using the Bio-Rad DC protein assay. Upon addition of 5% 2-mercaptoethanol, 30 μg of protein lysates were run on a 12% SDS–PAGE gel and transferred onto Immobilon-P transfer membrane (Millipore). Membrane was then blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS), probed with anti-Foxe1 polyclonal antibodies (1:500) (6) for 1 h, washed and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:2000) and detected by chemiluminescence (Amersham). Reprobing of the same filter was performed by incubating membrane in stripping buffer (2% SDS, 100 mM 2-mercaptoethanol in 62.5 mM Tris–HCl pH 6.8). After extensive washing, the membrane was incubated with anti-ERK1-specific antibodies (1:200; Santa Cruz) as loading control.

Histology and immunostaining

Skin samples were fixed in 4% paraformaldehyde in PBS overnight at 4°C, embedded in paraffin. Wild-type skin was obtained from Swiss CD-1 mice at different ages unless otherwise indicated. Mouse BCC samples were obtained from transgenic mice expressing wild-type Gli2 under the control of the bovine K5 promoter (37). Mouse skins carrying a dominant-negative form of Gli were obtained from transgenic mice expressing Gli2ΔC4 under the control of the bovine K5 promoter (42). Mouse tissue samples were obtained according to a protocol approved by the University of Michigan IRB. For immunohistochemistry, 7 μm thick sections were deparaffinized, boiled in 10 mM sodium citrate (pH 6.0) for 20 min, and stained with the Vectastain kit according to the manufacturer’s instructions (Vector Laboratories). For immunofluorescence, relevant Texas Red-conjugated goat antibodies (1:100; Jackson Laboratories) were used to detect primary antibodies. Before the last wash, DNA was stained with DAPI at 100 ng/ml in PBS (5 min). Slides were mounted using Vectashield as mounting reagent (Vector Laboratories). Slides were examined under an Axioplan 2
imaging microscope (Zeiss). We used the following primary antibodies: Foxe1 (diluted 1:500) (6), keratin 14 (1:10 000; Covance-Babco), trichohyalin (1:1000; donated by George Rogers), keratin 17 (1:200; donated by Pierre Coulombe).

Skin grafting

Full-thickness skin grafting was performed as described by Sundberg et al. (75) with minor modifications. A patch of full-thickness skin (~10 × 10 mm²) was removed from the back of an anaesthetized nu/nu CD-1 mouse, and was replaced by a patch of full-thickness skin from newborn Foxe1-null mouse or a wild-type littermate. Each graft was secured by sterile Vaseline gauze and silicon transplantation chamber. The chambers were removed 4–5 days after grafting and graft biopsies and hair were harvested 2–3 weeks after grafting. Skin from 14 wild-type and 16 Foxe1-null mice was grafted. All experiments involving mice were conducted according to IACUC procedures.

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