Dissecting the Oncogenic Potential of Gli2: Deletion of an NH2-Terminal Fragment Alters Skin Tumor Phenotype

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ABSTRACT

Development of basal cell carcinomas (BCCs) in skin is associated with uncontrolled Sonic hedgehog (Shh) signaling, which operates primarily through the Gli family of transcription factors. Gli2 is a mediator of physiological Shh signaling in skin and is sufficient to produce BCCs when overexpressed by use of a Keratin 5 (K5) promoter. Analysis of Gli protein deletion mutants has identified an NH2-terminal transcription repressor domain in Gli2 but not Gli1. To assess the potential involvement of the Gli2 repressor domain in skin tumor development, we overexpressed the Gli2ΔN2 mutant in transgenic mice by use of the K5 promoter. K5-Gli2ΔN2 mice developed a variety of skin tumors resembling human trichoblastomas, cylindromas, basaloid follicular hamartomas, and rarely, BCCs. In striking contrast, K5-Gli2 mice overexpressing wild-type Gli2 developed only BCCs. Other differences between tumors arising in these two sets of transgenic mice included their gross appearance, growth rate, and predilection for specific body sites. However, the expression levels of Shh target genes, which reflect the magnitude of Shh pathway activation, were not dramatically different. Tumors from K5-Gli2ΔN2 mice, unlike human or mouse BCCs, gave rise to cell lines that constitutively expressed Shh target genes in vitro and were tumorigenic in nude mice. Interestingly, the phenotype of K5-Gli2ΔN2 mice was strikingly similar to that reported after K5 promoter-driven overexpression of GLI1, which lacks an NH2-terminal region homologous to the Gli2 repressor domain. These results underscore the qualitative difference in oncogenicity of GLI1 and Gli2 when overexpressed in skin, and reveal a previously unanticipated role for the Gli2 NH2 terminus in defining tumor phenotype.

INTRODUCTION

Shh3 regulates patterning and growth of a remarkable variety of tissues throughout embryogenesis (reviewed in Refs. 1, 2). Shh binds and reversibly inhibits the cell-surface receptor Ptc, which antagonizes the action of Smoothened (reviewed in Refs. 2, 3). Shh-mediated derepression of Smoothened results in up-regulation of Shh target genes, including Ptc1 and Gli1. Studies in Drosophila indicate that hedgehog modulates gene expression through the zinc-finger-containing transcription factor Ci, which can function as either a transcriptional activator or proteolytically cleaved repressor (reviewed in Ref. 4). In vertebrates, transcriptional responses to Shh and other hedgehog proteins are mediated by the three Ci homologues Gli1, Gli2, and Gli3 (reviewed in Refs. 2, 5, 6), which work in concert to modulate target gene expression. Gli1 and Gli2 appear to act primarily as transcriptional activators, whereas Gli3 functions largely as a repressor.

Although Gli1 mRNA is consistently up-regulated in target cells responding to Shh and in certain settings Gli1 can mimic responses to Shh (7–9), mice harboring functionally null Gli1 alleles are born without detectable abnormalities and are viable and fertile (10). In contrast, disruption of Gli2 function results in developmental defects involving multiple Shh target tissues (11–15), strongly suggesting an obligatory role for Gli2 as a primary effector of Shh signaling. Hair follicle morphogenesis is severely impaired in Shh (16–18) and Gli24 mutants, but not in Gli1 or Gli3 mutants. These findings strongly suggest that the physiological effector mediating responses to Shh in normal skin is Gli2.

Although precise spatial and temporal activation of Shh signaling is required during normal embryogenesis, constitutive activation of this pathway is associated with cancer development. Patients with nevoid basal cell carcinoma syndrome exhibit a variety of developmental abnormalities and have a markedly increased incidence of BCCs and several other neoplasms (reviewed in Ref. 19). These individuals harbor germ-line PTCH1 mutations and have lost the remaining normal PTCH1 allele in BCCs (20, 21), resulting in derepression of SMO and hence constitutive SHH pathway activation. PTCH1 mutations have also been found in >50% of sporadic BCCs (20–24), and SHH target genes GLI1 and PTCH1 are up-regulated in nearly all BCCs examined (22, 25, 26), suggesting that uncontrolled activation of the SHH pathway plays a central role in the development of these tumors.

The consistent up-regulation of Shh target genes in BCCs suggests that heightened activity of Shh transcriptional effectors plays a central role in BCC development. Given the requirement for Gli2 in physiological Shh signaling in embryonic hair follicles, we proposed that Gli2 may also have a crucial function in pathological, constitutive Shh signaling in BCCs. To test this hypothesis, we engineered transgenic mice expressing Gli2 driven by a K5 promoter (27). K5-Gli2 mice spontaneously developed multiple BCCs (28), suggesting an important role for Gli2 in BCC formation triggered by upstream activation of the Shh pathway. Interestingly, overexpression of Gli1 using the same K5 transgenic cassette resulted in a distinct phenotype, characterized by the appearance of multiple types of skin tumors, with a minority being BCCs (29).

On the basis of assays measuring transcriptional activity by use of an 8xGli-binding site luciferase reporter, Gli2 was shown to contain an NH2-terminal repressor domain that is not present in Gli1 (30). To test the involvement of the Gli2 NH2 terminus in BCC tumorigenesis, we overexpressed the Gli2ΔN2 mutant in skin, using the K5 promoter. Although differing in several respects from K5-Gli2 mice (28), K5-Gli2ΔN2 mice resembled transgenic mice in which GLI1 was driven by the same promoter (29). Our findings strongly implicate the NH2-terminal repressor domain of Gli2 in the distinct tumor phenotypes produced by skin-targeted overexpression of Gli2 versus GLI1.

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2 The abbreviations used are: Shh, Sonic hedgehog; Ptc, Patched; BCC, basal cell carcinoma; K5, keratin 5.

3 P. Mill et al., manuscript in preparation.
**MATERIALS AND METHODS**

**Transfection and Reporter Assays.** SV40-transformed human keratinocytes (Ref. 31; obtained from Dr. Richard Schlegel, Georgetown University, Washington, DC), were subcultured into 6-well plates at a density of 2 × 10^5 cells/well the day before transfection. Cells were cotransfected with reporter plasmid (0.3 μg) containing eight copies of a 30-bp sequence containing the Gli binding site (GACACCA) from the mouse Hnf3β (Foxa2) promoter, a β-crystalline minimal promoter, and luciferase cDNA (32); control plasmid (SV-β-gal; 0.1 μg); and effecter plasmid (0.6 μg), either pDNA3.1HisB (vector control), pDNA3.1HisBGli2, or pDNA3.1HisBGli2ΔN2 (30). Transfections were performed with 3 μl of FuGENE6 reagent (Roche) according to the manufacturer’s protocol. Cells were harvested 48 h after transfection, and luciferase levels were determined as described previously (33). β-Galactosidase activity was used to normalize for transfection efficiency.

**Generation and Identification of K5-Gli2ΔN2 Transgenic Mice.** Gli2ΔN2 cDNA containing an NH2-terminal His tag was released from pDNA3.1HisBGli2ΔN2 by digestion with BsrBI and NotI, subcloned into the SnaBI site of the bovine K5 transgenic cassette kindly provided by Dr. Jose Jorcano (CIEMAT, Madrid, Spain; Ref. 27), and verified by sequencing. The K5-Gli2ΔN2 transgene was released by use of BssHII, purified, and microinjected into (C57BL/6 × SJL) F2 mouse eggs by personnel at the University of Michigan Transgenic Core. The same genetic background was used to produce K5-Gli2 mice (containing a Gli2 NH2-terminal FLAG epitope tag) that we described previously (28), and C57BL/6 × CBA F2 oocytes were used by Tofgar et al. (29) to produce Gli2ΔN2 mice. Transgenic mice were identified by PCR analysis of tail DNA using vector-specific primers (5′-CCCATATGTCTTCCGGATGTCG-3′ and 5′-AAGCGCATCTTCCGCTTACATTGCAGG-3′). For Northern blotting, 10 μg of each total RNA sample were separated on a 1.2% agarose formaldehyde gel, transferred to Zeta-Probe nylon membrane (Bio-Rad), and hybridized overnight at 42°C to 32P-labeled cDNA probes (labeled by random priming) in hybridization buffer, as recommended (50% formamide, 0.12 μM NaHPO4, (pH 7.2), 0.25 μM NaCl, 7% SDS, and 1 mM EDTA) by the manufacturer. Probes were as follows: Pch1, 841-bp 5′ EcoRI fragment (kindly provided by Dr. Andrew McMahon, Harvard University, Boston, MA); Gli1, 3′ PCR product spanning nucleotides 2655–3321; Gli2, a 1-kb fragment (34). Blots were washed at a final stringency of 2× SSC/0.1% SDS at 60°C, exposed to phosphorimager screens, and analyzed using a Storm 840 Phosphorimaging System (Molecular Dynamics). Quantitative PCR was performed using real-time TaqMan technology, and products were analyzed on a Model 7700 Sequence Detector (Applied Biosystems). Two PCR primers and a hybridization probe labeled with a reporter dye, 6-carboxyfluorescein, on the 3′ nucleotide were used (sequences provided on request). Fifty-microliter reactions contained 50 ng of total RNA, 12.5 units of murine leukemia virus reverse transcriptase, 1.25 units of AmpliTaq Gold DNA polymerase, 0.2 units of RNase inhibitor, 1× PCR reaction buffer containing 5 mM magnesium chloride, 165 ng (or 500 ng) of each primer, 300 μM deoxynucleotidetriphosphates, and 100 ng (300 nm) of TaqMan probe. Ct values, corresponding to the cycle number at which the fluorescent emission monitored in real-time reaches a threshold of 10 SD above the mean baseline emission, from cycles 3 up to 15 were measured. Cycling parameters were 30 min at 48°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Immunoprecipitation and Western Blot Analysis.** Protein extracts were prepared by homogenizing skin or tumor samples in lysis buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 120 mM NaCl, 1% NP40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol] in an Omni- Probe tissue homogenizer. Protein concentration was determined using the Bio-Rad DC protein assay. For immunoprecipitation, 200 μg of protein extracts were incubated with 10 μl of rabbit anti-His polyclonal antibody (M-21; Santa Cruz Biotechnology) and 30 μl (1:1 slurry in lysis buffer) of protein G-agarose beads (Invitrogen Life Technologies) at 4°C overnight with shaking. The proteins were eluted in 30 μl of 2× SDS loading buffer. For Western blotting, 30 μg of protein lysate or 30 μl of protein eluate from immunoprecipitation were separated on 7–5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Schleicher & Schuell). The membranes were blocked with 5% nonfat dry milk in PBS, probed with 1:2000 rabbit anti-His antibody (M-21) for 1 h at room temperature, washed, and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Corp.).

**Tissue Harvests, Immunostaining, and in Situ Hybridization.** Skin samples were fixed overnight in neutral-buffered formalin or Carnoy’s fixative (10% glacial acetic acid, 30% chloroform, 60% absolute ethanol), transferred to 70% ethanol, processed, embedded in paraffin, sectioned at 5 μm, and stained with H&E. For immunohistochemistry, formalin-fixed sections were boiled in 0.01 M citrate buffer (pH 6) for 10 min and reacted with antibodies against mouse K5 (1:2000; Covance) or K17 (1:2000; gift from Dr. Pierre Coulombe, Johns Hopkins University, Baltimore, MD). Sections fixed with Carnoy’s fixative were used for mouse K6 (1:1000; Covance) immunostaining. All sections were blocked with 10% normal goat serum for 30 min, incubated with primary antibody diluted in 2 mL BSA for 1 h at room temperature, rinsed in PBS, incubated with biotinylated secondary antibody (1:10000; Vector) for 30 min, visualized using the Vectastain ABC kit (Vector) and 3,3′-diaminobenzidine as substrate, and counterstained with hematoxylin. For Bcl-2 immunostaining, formalin-fixed sections were treated with 0.01 M citrate buffer (pH 6), blocked with TNK [100 mM Tris (pH 7.6), 500 mM NaCl, 10 mM KCl, 2% BSA, 0.1% Triton X-100, 1.5% normal goat serum] for 1 h, and incubated at 4°C overnight with anti-Bcl-2 (PharMingen) diluted 1:1000 in TNK. The remainder of the immunostaining protocol was the same as described above. In situ hybridization was performed on frozen sections, using digoxigenin-labeled probes as described previously (15). The Gli2 probe detects both endogenous Gli2 and Gli2ΔN2 mRNA produced by the transgene.

**Generation of Established Cell Lines and Tumorsphere Assays.** Portions of tumors were removed from sacrificed animals and were washed twice with medium (HiCa/10× pen-strep) prepared using modified S-MEM (Invitrogen Life Technologies), 8% FCS (Gemini Bioproducts), penicillin (200 units/ml), streptomycin (200 μg/ml), and 1.4 mM CaCl2. Tumors were minced and digested using 0.35% type I collagenase (Worthington Biochemical Corp.) in DMEM containing 5% FCS, 200 units/ml penicillin, and 200 μg/ml streptomycin for 2 h at 37°C with occasional agitation. The remaining clumps of epithelial cells were further disrupted by triturating with a 10-ml pipette, washed twice with HiCa/10× pen-strep medium, and plated in type I collagen-coated tissue culture wells in modified S-MEM containing 8% FCS (Gemini Bioproducts), penicillin (200 units/ml), 1.4 mM CaCl2, and 1 ng/ml keratinocyte growth factor (R&D Systems). After 24–36 h, the cells were switched to Lo/K/C medium [S-MEM, 8% Ca2+–depleted FCS (35), 0.05 mM CaCl2, 1 ng/ml keratinocyte growth factor, 50 ng/ml cholera toxin (Calbiochem), 20 units/ml penicillin, and 20 μg/ml streptomycin] and fed every 2–3 days. Cells were subcultured periodically until homogeneous, robust cell lines emerged. Spontaneously immortalized control cell lines were established from Glirosa26 (36) mouse skin keratinocyte cultures (35) that were grown in Lo/K/C for several weeks, during which the majority of cells underwent crisis. After allowing for expansion of the surviving cells in the original dishes, cultures were passaged repeatedly until homogeneous cell lines were established. To test for tumorigenicity, cell lines grown in T-175 flasks were trypsinized and injected s.c. (5 × 10^6 cells in 100 μl of HiCa medium) into 10–11-week-old nude mice (nu/nu-nuBR; Charles River), which were examined once or twice per week and harvested 25 days after injection.

**RESULTS**

**Increased Activity of Gli2ΔN2 Deletion Mutant in Cultured Keratinocytes and Skin-targeted Overexpression in Transgenic Mice.** Full-length mouse Gli2 is a relatively weak transcriptional activator in rat neural MNS70 cells, and removal of the NH2-terminal 279 amino acids to produce the Gli2ΔN2 mutant enhances transcriptional activity up to 15-fold (30). We obtained similar results with
cultured keratinocytes: Gli reporter activity was up to 10-fold higher in cells transfected with Gli2ΔN2 than wild-type Gli2 expression vector (Fig. 1A). These findings support the concept that the NH2 terminus of Gli2 contains a domain that acts as a transcriptional repressor in a variety of cultured cell types.

We have previously shown that overexpression of full-length Gli2 in skin by use of a transgene containing the bovine K5 promoter (K5-Gli2) results in the exclusive appearance of BCCs (28). To examine the role of the Gli2 repressor domain in BCC biology, we overexpressed Gli2ΔN2 in mouse skin, using the same K5 transgenic cassette (Fig. 1B). The K5 promoter was selected because it is active in the epidermal basal layer and outer root sheath of hair follicles, which contains stem cells (37) that are believed to be tumor precursors (38). Eighteen transgenic founders were identified by PCR-based genotyping, and 5 of these developed multiple skin tumors. We verified transgene expression by reverse transcription-PCR using RNA from skin of several founders exhibiting a skin phenotype (Fig. 1C). Using an antibody against the His epitope tag in immunoblots, we detected exogenous Gli2ΔN2 protein with the expected molecular weight in lysates obtained from tumors but not normal-appearing skin (Fig. 1D). In an effort to increase sensitivity, we also immunoprecipitated cell lysates that were then examined by immunoblotting. Although this resulted in a stronger band from tumor lysates, Gli2ΔN2 protein was still not clearly detected in normal skin of transgenic mice. These findings suggest that tumors can develop only when cells express a sufficiently high level of Gli2ΔN2.

Development of Multiple Different Tumor Types in K5-Gli2ΔN2 Mice: Gross Appearance. The gross phenotype of K5-Gli2ΔN2 mice was distinct from that of K5-Gli2 mice, with major differences, including (a) earlier development of visible skin abnormalities, (b) appearance of multiple tumor types, (c) highly variable tumor growth rates, and (d) predilection for different body sites (Table 1). Among the five founders who developed tumors, each exhibited a phenotype by 14 days after birth and developed multiple tumors between 3 weeks and 5 months of age. Two founders 391 and 116, were runted and had substantially less hair than their littermates. By ~3 weeks of age, founder 391 had developed multiple tumors and expired at 4 weeks. Tumors were detected on founder 116 at ~4 weeks of age, and by 8 weeks almost the entire skin contained numerous tumors (Fig. 2C). The phenotypes of founders 426 and 427 were very similar: both showed linear regions completely devoid of hair by 1 week of age (Fig. 2A) and developed multiple rapidly growing tumors in these areas by 5–6 weeks of age. The fifth founder, founder 83, exhibited a small hairless area evident within the first week, but did not develop tumors until 5 months of age (Fig. 2D). In contrast to the early (within 1–2 weeks) initial appearance of skin abnormalities in K5-Gli2ΔN2 mice, K5-Gli2 mice rarely developed skin changes before 3–4 weeks of age.

Interestingly, tumors in K5-Gli2ΔN2 mice frequently arose in locations different from those in K5-Gli2 mice. Most of the tumors that developed in K5-Gli2ΔN2 mice were located in dorsal skin overlying the trunk, with a few lesions on the head and limbs and two arising in footpads (Fig. 2B). No grossly visible tumors were detected in the skin on the ears of any of the K5-Gli2ΔN2 transgenic founders, and only one founder developed lesions on the tail. In striking contrast, the tail, ears, and dorsal paws were sites at which the great majority of tumors arose in K5-Gli2 mice (see Fig. 1 in Ref. 28), whereas grossly evident tumors were never observed in footpads of K5-Gli2 mice. In addition, although BCCs arising in pigmented K5-Gli2 mice were frequently dark brown or black because of melanin accumulation (see Fig. 1 in Ref. 28), this was uncommon in tumors arising in K5-Gli2ΔN2 mice (Fig. 2). Finally, the growth rate of tumors in K5-Gli2ΔN2 mice was highly variable. This was most apparent when comparing different tumors on the same animal as illustrated in Fig. 2, E–H. During a 10-day interval, there were no detectable changes in the sizes of tumors located on the limb (Fig. 2, E and F), whereas tumors on the side of the trunk enlarged dramatically during the same time interval (Fig. 2, G and H). In striking contrast, nearly all tumors arising in K5-Gli2 mice had a similar gross appearance, and they all grew at a relatively slow rate, consistent with the behavior of BCCs in humans.

Table 1 Comparison of tumor phenotype in K5 promoter-targeted Gli2ΔN2 and Gli2 transgenic mice

<table>
<thead>
<tr>
<th>K5-Gli2ΔN2</th>
<th>K5-Gli2</th>
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<tbody>
<tr>
<td>Tumor types</td>
<td>Multiple, few BCCs</td>
</tr>
<tr>
<td>Location</td>
<td>Trunk &gt; head &gt; extremities</td>
</tr>
<tr>
<td>Growth rate</td>
<td>Variable</td>
</tr>
<tr>
<td>Phenotype first evident</td>
<td>1-2 weeks</td>
</tr>
<tr>
<td>Pigmented tumors</td>
<td>Minority</td>
</tr>
<tr>
<td>Tumorigenic cell lines</td>
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Fig. 1. HEIGHTENED TRANSCRIPTIONAL ACTIVITY OF Gli2ΔN2 IN VITRO, TRANSGENIC DESIGN, AND CONFIRMATION OF Gli2ΔN2 EXPRESSION IN VIVO. A, Gli2 contains an NH2-terminal repressor domain. Reporter assays were performed in keratinocytes transfected with an bGli binding-site luciferase reporter and empty vector (Control), effector plasmid with full-length Gli2 (Gli2), or an NH2-terminal Gli2 deletion mutant (Gli2ΔN2). Cotransfection with a lacZ expression plasmid was used to normalize transfection efficiency, and cell lysates were prepared 48 h after transfection. Columns represent mean values from two independent dishes, with the range indicated by error bars. B, transgenic construct containing 5.3-kb bovine K5 promoter, rabbit b-globin intron, NH2-terminal His-tagged mouse Gli2ΔN2 cDNA, and composite rabbit b-globin/SV40 poly(A) sequence. C, reverse transcription-PCR performed using RNA from three control (Lanes 1–3) and four transgenic (Lanes 4–7) mouse skin samples using transgene-specific and b-actin primers. Note absence of detectable transgene expression in sample from transgenic mouse without a visible phenotype (Lane 7). RT, reverse transcriptase. D, anti-His tag immunoblot of lysates and immunoprecipitations (IP) performed with control mouse skin (Lanes 1 and 4), transgenic mouse skin (Lanes 2 and 5), or transgenic skin tumor (Lanes 3 and 6).
Histopathology and Marker Analysis. The differences in behavior of individual tumors arising in K5-Gli2ΔN2 mice suggested that they may be a less homogeneous population than the BCCs that developed in K5-Gli2 mice. In fact, examination of H&E-stained sections revealed a multiplicity of tumor types in the skin of K5-Gli2ΔN2 mice. The majority of tumors were histologically similar to human trichoblastomas, particularly the “rippled pattern” variant (39, 40). They contained numerous mitotic cells (Fig. 3A, inset), and large lesions had necrotic centers (arrow in Fig. 3A). A minority of tumor nodules contained pigment (arrowheads in Fig. 3A). Despite the massive size of many of these tumors, they did not invade surrounding tissues. A second tumor type appeared grossly similar to BCCs arising in K5-Gli2 mice and grew at a similarly slow rate, but the histological features were consistent with a distinct tumor type called cylindroma (Fig. 3B). These lesions contain nests of basaloid cells closely approximating each other to resemble pieces of a jigsaw puzzle. The third type of lesion commonly seen in K5-Gli2ΔN2 mice (Fig. 3C) was histologically similar to human basaloid follicular hamartoma (41). These lesions were composed of frond-like epithelial downgrowths with cellular nests and strands, resembling relatively undifferentiated hair follicle epithelium. To our surprise, only three BCCs (for example, see Fig. 3D) were identified in the five transgenic founders with tumors. Various other tumor types were observed (Fig. 3E) that were difficult to classify according to histological criteria used for identifying human skin tumors. These were generically classified as benign hair follicle tumors, with some exhibiting features of trichoadenoma or trichoepithelioma. K5-Gli2ΔN2 mice also developed keratinized cysts within the dermis (Fig. 3F). It is notable that despite the large number and heterogeneity of tumors arising in K5-Gli2ΔN2 mice, squamous neoplasms (papillomas, squamous cell carcinomas, or keratoacanthomas) were never observed.

The expression patterns of several protein markers support the concept that skin tumors arising in K5-Gli2ΔN2 mice were derived from hair follicle epithelium, which is believed to contain progenitor cells for a variety of tumor types, including trichoblastomas and BCCs (42). Nearly all tumor cells in trichoblastomas contained K5 (Fig. 4A), which is usually detected in the basal layer of the epidermis and the outermost cell layers of the hair follicle (outer root sheath). K17,
usually restricted to the outer root sheath and hair shaft precursor cells in normal adult skin (43, 44), was also detected in nearly all cells of trichoblastomas (Fig. 4B). Nearly all cells in other tumor types seen in K5-Gli2ΔN2 mice were also immunoreactive for K5 and K17 (not shown). K6 is found in cells of the innermost (differentiating) layer of the outer root sheath (45) and in hyperplastic epidermis, and was detected in isolated cells or cell clusters within a subpopulation of tumor nests (not shown). Bcl-2, a useful diagnostic marker for human BCCs and trichoblastomas, was expressed diffusely throughout tumor nodules (Fig. 4C).

Up-Regulation of Gli2ΔN2 Transgene and Shh Target Genes in Tumors. To assess whether there was a correlation between transgene expression levels, Shh pathway activity, and tumor phenotype, we isolated RNA from multiple tumors and skin for Northern blot analysis. In transgenic mouse skin without visible tumors, levels of Gli2ΔN2 mRNA were minimal or undetectable (Fig. 5A, Lane 9). One sample from normal-appearing skin that contained microscopic tumors in adjacent tissue expressed a low level of Gli2ΔN2 mRNA (Fig. 5A, Lane 7). All tumor samples contained substantial amounts of transgene mRNA, with large tumors generally expressing higher levels than relatively small tumors (Fig. 5A). Transgene expression in a single founder could thus vary from low or undetectable (in normal-appearing skin) to very high (in large tumors), revealing a strong association between transgene expression and tumorigenicity. Comparable results were obtained by immunoblotting, with much higher levels of Gli2ΔN2 protein detected in tumor samples than in normal-appearing skin (Fig. 1D). We examined the abundance of Gli1 and Ptch1 mRNA to assess the level of Shh pathway activation and found a strong correlation with transgene expression levels (Fig. 5A). These findings are consistent with the concept that differences in the level of Shh pathway activity in K5-Gli2ΔN2 mice can lead to distinct tumor phenotypes in skin. Given the dramatically increased transcriptional activity of Gli2ΔN2 compared with Gli2 in cultured cells, additional RNA analysis was performed using TaqMan PCR to quantify Shh target gene expression in control skin and tumors derived from K5-Gli2ΔN2 and K5-Gli2 mice. Although Gli1, Ptch1, and Ptch2 mRNA all appeared higher in K5-Gli2ΔN2 tumors than those from K5-Gli2 mice, only Gli1 expression was significantly elevated, and the increase was <2-fold (Fig. 5B). These data suggest that the distinct tumor phenotypes in K5-Gli2 and K5-Gli2ΔN2 transgenic mice are
not likely attributable to differences in the overall level of Shh pathway activation. In situ analysis revealed that Gli2/\(Gli2\)N2 mRNA, as well as Gli1 and Ptch1 mRNA, was expressed specifically in tumor epithelium (Fig. 5C).

Establishment of Tumorigenic Cell Lines from K5-Gli2N2 Tumors. In contrast to many other epithelial malignancies, human BCCs grow slowly and rarely metastasize (reviewed in Ref. 46). The indolent nature of these tumors is associated with an inability to establish BCC cell lines that retain their tumorigenicity in nude mouse xenografts; this has been a major impediment to progress in the field. The rapid growth of many tumors arising in K5-Gli2\(\Delta N2\) mice prompted our attempts to generate cell lines from several of these lesions. We established a total of 10 cell lines from four different tumors. Analysis of the first two cell lines that were established revealed continued Gli2/\(Gli2\)N2 mRNA and protein expression (Fig. 6).

Moreover, the Shh pathway was constitutively activated in these cells in vitro, based on up-regulation of Gli1 and Ptch1 mRNA (Fig. 6).

These cell lines were highly tumorigenic when injected s.c. into nude mice.
mice, with tumors first evident 7–10 days after injection. The rapid growth rate and histology were similar to those of the original tumor, a trichoblastoma, from which these lines were generated (compare Figs. 6E and 3A). These findings strongly suggest that mouse trichoblastomas are biologically distinct from BCCs and further strengthen the concept that the oncogenic potential of Gli2ΔN2 differs from that of Gli2.

**DISCUSSION**

The consistent up-regulation of Shh target genes in nearly all human BCCs examined suggests a central role for Shh transcriptional effectors in tumorigenesis. In keeping with this hypothesis, skin-targeted overexpression of Gli2 or GLI1 by use of a K5 promoter is sufficient to produce BCCs in transgenic mice, although K5-GLI1 mice preferentially developed other types of skin tumors. In this report, we have mimicked the K5-GLI1 tumor phenotype by skin-targeted overexpression of a Gli2 mutant, Gli2ΔN2. Our findings reveal a previously unsuspected role for the NH2-terminal domain of Gli2 in defining skin tumor phenotype.

Several transgenic models have been generated to examine the involvement of deregulated Shh signaling in cutaneous tumorigenesis. Skin-targeted overexpression of SHH by use of a K14 promoter (47), or the gain-of-function M2SMO mutant by use of a K5 promoter (48), resulted in development of basaloid proliferations in skin of late-stage embryos or newborn mice. Pch1<sup>1<sup>1/2</sup></sup> mutant mice develop visible skin tumors 6–9 months after exposure to ionizing or UV radiation (49); both of these agents also enhance human BCC development. The tumor phenotype of K5-GLI1 mice reported by Nilsson et al. (29) is particularly interesting in light of our studies with Gli2 and the Gli2ΔN2 mutant. K5-GLI1 transgenics develop several types of skin tumors, including trichoblastomas, cylindromas, trichoepitheliomas, and BCCs (29), whereas K5-Gli2 mice develop only BCCs (28). These divergent tumor spectra suggest that Gli2 and GLI1 are not equivalent in terms of their oncogenic potential, which is in keeping with results of studies exploring the function of these molecules in mouse embryogenesis (10).

The qualitative difference in tumorigenicity of Gli2 and Gli2ΔN2 in skin was unexpected. Given the increased in vitro transcriptional activity of Gli2ΔN2 and its ability (unlike full-length Gli2) to activate Shh target genes in the dorsal neural tube in vivo (30), we anticipated that Gli2ΔN2 would be substantially more potent than Gli2 at activating Shh signaling in skin. However, comparison of Shh target gene expression in tumors from K5-Gli2ΔN2 and K5-Gli2 mice failed to reveal significant differences with the notable exception of Gli1, which was approximately twice as abundant in K5-Gli2ΔN2 tumors (Fig. 5B). These findings underscore the importance of cellular context when examining responses to Gli proteins and raise the interesting possibility that the multiplicity of tumor types in K5-Gli2ΔN2 mice is indirectly attributable to modestly enhanced expression of endogenous Gli1. This could also be partly responsible for the greater potency of Gli2ΔN2 in reporter assays (Fig. 1 and Ref. 30) that do not distinguish between exogenous and endogenous Gli molecules. In keeping with this hypothesis, the mouse Gli1 promoter contains several Gli binding sites and has been shown to be activated by Gli3 (50); it would presumably be activated by Gli2 and Gli2ΔN2 as well.

An alternative explanation for our findings would attribute differences in tumor phenotype to qualitative differences in the oncogenic potential of Gli2ΔN2 and full-length Gli2 that are independent of effects on Gli transcriptional activity. This possibility implies an important regulatory function for the Gli2 NH2 terminus that may involve direct interaction with other signaling molecules, and its presence in K5-Gli2 mice may either promote BCC development or repress formation of other follicle-derived tumor types seen in K5-Gli2ΔN2 and K5-GLI1 mice. Support for, or against, this hypothesis could be obtained by assessing the phenotype of K5 promoter-driven transgenic mice expressing a Gli2-GLI1 chimera containing the Gli2 NH2-terminal fragment that is deleted in Gli2ΔN2.

In addition to the disparate tumor phenotypes in K5-Gli2 and K5-Gli2ΔN2 mice, there are several other distinctions (Table 1). One of the most striking is the predisposition of tumors in K5-Gli2 mice to arise in certain locations on the body, including the tail, ears, and dorsal paws. This finding suggests strong regional differences in the susceptibility of skin to form tumors in response to K5 promoter-driven Gli2, but not Gli2ΔN2 or GLI1. Whether this is related to differences in transgene expression levels or reflects an intrinsic difference in responsiveness of keratinocytes residing in these regions is not known. The increased occurrence of human BCCs in certain locations is frequently attributed to the mutagenic effects of increased sun exposure, but regional differences in susceptibility to human BCC development may also exist.

The focal appearance of tumors in K5-Gli2ΔN2 and K5-Gli2 mice is also notable given the fact that the K5 promoter is active throughout the epidermal basal layer and outer root sheath of hair follicles (27, 51). Focal tumor development is not restricted to K5-Gli2 founders and thus is unlikely to be the result of mosaicism. Because transgenic expression can exhibit substantial cell-to-cell variability in the same animal (52–55), we propose that focal tumors reflect outgrowth of a relatively small number of cells with the sufficiently high transgene expression levels needed to drive tumorigenesis. This concept is supported by our (a) Northern data, in which Gli2ΔN2 transgene expression was markedly higher in all tumor samples than in unaf- fected transgenic skin (Fig. 5A); (b) in situ analysis, where only tumor cells were found to express high levels of transgene mRNA (Fig. 5C); and (c) immunoblot analysis (Fig. 1). Similarly, Gli2 mRNA was readily detected in BCCs arising in K5-Gli2 mice, whereas expression of Gli2 in the adjacent epidermal basal layer was below the level of detection (Fig. 2E in Ref. 28). Although sufficiently high expression of positive-acting Gli transcription factors is likely to play a central role in the genesis of certain follicle-derived skin tumors, the downstream target genes driving this process and how they alter keratinocyte biology are not yet known.

Despite the appearance of multiple tumor types in K5-Gli2ΔN2 mice, none of them resembled squamous neoplasms, which are common in other transgenic models overexpressing a variety of growth factors, receptor tyrosine kinases, or oncogenes in skin (reviewed in Refs. 56–58). Cutaneous squamous cell carcinomas, like other epithelial malignancies, develop through a series of morphological stages that are accompanied by multiple genetic alterations (59, 60). In contrast, BCC precursor lesions have not been identified, and there is no evidence of neoplastic progression in this tumor type. Constitutive Shh signaling is detected even in microscopic human BCCs and, based on several mouse models, may be sufficient for the development and maintenance of these tumors. Mechanism-based approaches to BCC treatment and/or prevention may thus require only effective inhibition of the Shh pathway.

The development of multiple tumor types in K5-Gli2ΔN2 mice strengthens the notion that deregulation of Shh signaling can give rise to a variety of appendage-derived tumors in skin (reviewed in Ref. 61), but the basis for tumor heterogeneity in this mouse model is not known. One possibility is that different levels of Shh pathway activity, even in the same progenitor cell, will yield different tumors. A similar proposal was put forth to explain tumor heterogeneity in K5-GLI1 mice (29). Although the Northern results presented in Fig. 5A are consistent with this interpretation, direct support for this hypothesis awaits the development of an inducible mouse model in which Gli
transgene expression can be maintained at different levels and the resultant tumor phenotypes evaluated. Another possibility is that spontaneous mutations are occurring in initially homogeneous K-GLI2ΔN2 tumors, driving the outgrowth of some cells into histologically distinct tumor phenotypes. Although this possibility cannot formally be excluded, the simultaneous appearance of multiple tumor types in young mice argues against it. In addition, at least in the case of BCCs, tumor phenotype in both humans and K5-GLI2 mice is remarkably stable. Finally, the different tumors arising in K5-GLI2ΔN2 mice may result from expansion of progenitor cells at different stages of hair follicle maturation. This hypothesis can be tested by overexpressing GLI2ΔN2 in cutaneous keratinocyes by use of promoters with a more restricted expression pattern than K5, a strategy previously used with the v-raf oncogene to probe the relationship between target cell and tumor type (62).

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GLI2 NH2 TERMINUS AND SKIN TUMOR PHENOTYPE


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