REGULATION OF β-CATENIN BY GLI1 IN EPITHELIAL TRANSFORMATION

by

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A DISSERTATION

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ABSTRACT

Gli family members-mediated continuous Hedgehog (Hh) pathway activity plays a role in the growth of a number of human cancers, including the common malignancy of skin, basal cell carcinoma (BCC), and other highly aggressive tumor types. However, the mechanism by which Gli proteins act to promote the outgrowth of cancer in response to stimulation of Hh signaling is poorly understood. Identified as zinc finger transcriptional factors, the Gli family of proteins has been shown to be involved in multiple cellular processes through activating its target genes. Therefore, to study the function of Gli target genes is important for our understanding in the pathologies of Hh-associated cancers and would provide valuable knowledge for design of therapeutic strategies.

Gli1 can rapidly induce Snail transcripts in vitro, and co-expression of Snail with Gli1 was observed in anagen hair follicles and human skin tumors. Loss-of-function alleles of Snail inhibited transformation by Gli1 in RK3E epithelial cells, while enforced expression of wild type (WT) Snail promoted the outgrowth of transformed foci. In vivo, induction of Snail by a conditional Gli1 transgene was associated with cell proliferation in the interfollicular epidermis and with loss of E-cadherin in epithelial dysplasia. These observations identify Snail as an early responsive gene of Gli1 in the skin and as a limiting effector of Gli1-mediated epithelial transformation.
E-cadherin is normally complexed with β-catenin in adherens junctions. Loss of E-cadherin during developmental epithelial-mesenchymal transitions (EMT) may contribute to switching β-catenin from its role of cell-cell adhesion to its role of nuclear signaling transmission. In the current study, increased β-catenin was identified in Gli1-transformed cells \textit{in vitro} and in the E-cadherin-deficient, BCC-like lesions induced by Gli1 in transgenic mice. In addition to the observation that E-cadherin played a selective role of suppression during Gli1-mediated transformation \textit{in vitro}, we found that Snail loss-of-function was rescued by down-regulation of E-cadherin. Alleles of E-cadherin modulated transformation by Gli1 concordantly with their ability to promote or inhibit nuclear localization of β-catenin, and inhibition of Wnt pathway activity by dominant negative Tcf4 selectively blocked transformation by Gli1. Taken together, these data identify the Shh-Gli1 pathway as a key regulator to switch β-catenin to nuclear signaling in epithelial cells and cancers.
DEDICATION

I dedicate this dissertation to my grandma and to my family, who have been supportive all the way through my pursuing for the degree.
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<td>AML</td>
<td>acute myeloid leukaemia</td>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
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<td>BCC</td>
<td>basal cell carcinoma</td>
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<td>BMP</td>
<td>bone morphogenic protein</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CK1</td>
<td>casein kinase 1</td>
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<td>Dhh</td>
<td>Desert hedgehog</td>
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<td>Dsh</td>
<td>Dishevelled</td>
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<td>DOX</td>
<td>doxycycline</td>
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<td>EMT</td>
<td>epithelial mesenchymal transition</td>
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<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GSK3</td>
<td>glycogen synthase kinase 3β</td>
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<td>Fu</td>
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<td>Gli1-ER</td>
<td>Gli1-estrogen receptor fusion protein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HSCs</td>
<td>heamatopoetic stem cells</td>
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<td>Hh</td>
<td>Hedgehog</td>
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<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
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<td>IFT</td>
<td>intraflagellar transport</td>
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LIST OF ABBREVIATIONS (Continued)

K1 keratin 1
K14 keratin 14
K16 keratin 16
K17 keratin 17
MMP Matrix metalloproteases
MMLV-LTR Moloney murine leukemia virus long terminal repeat
NICD intracellular domain of Notch 1
NOD/SCID non-obese diabetic/severe combined immunodeficient mice
PDGF platelet-derived growth factor
Ptc Patched
Rb retinoblastoma
rtTAx X chromosome-linked reverse tet-responsive transactivator
SCC squamous cell carcinoma
Shh Sonic hedgehog
shRNAs short hairpin RNAs
Smo Smoothened
SuFu Suppressor of Fused
tet tetracycline
TGF-β transforming growth factor-β
VEGF vascular endothelial growth factor
WT wild-type
INTRODUCTION

Carcinogenesis is a multistage process Carcinogenesis is the process by which cancer develops. It is a multifaceted process that involves a large number of factors that disrupt normal regulation of cell proliferation, differentiation and programmed cell death (1).

Internal factors such as heredity, immunology, and hormones as well as external factors such as chemicals, viruses, diet, and radiation define the two groups of causal factors. Some of the chief causes of cancer are lifestyle factors such as diet, cigarette smoke, alcohol, and sun exposure. In particular, evidence shows that dietary factors and cigarette smoke are two major causes associated with about 50% of all human cancers (1).

Whatever the cause of cancer, to date a large amount of work indicates its development is a multi-stage process. According to the two-hit hypothesis proposed by Dr. Alfred Knudson based on studies on retinoblastoma (Rb), a germline mutation could result in hereditary predisposition to cancer, but a second hit of somatic mutation is necessary for tumorigenesis (1,2). In contrast to Rb, other tumor cells acquire multiple genetic alterations in the form of discrete changes such as point mutations, or large alterations like deletion or insertions (1,3). Studies on transgenic mouse models (e.g. transgenic Ras model for SCC) and on cultured cells also showed that there are often more than two rate-limiting steps required for the
development of cancer. Most common mutations analyzed in the germ line are subtle mutations (e.g. point mutations, small deletions or insertions), whereas alteration causing large rearrangement of the genome, such as amplifications or translocations, are usually identified in somatic cells.

The clonal evolution theory of Nowell states that development of cancer is an evolutionary process during which somatic mutations occur and accumulate (4). Germline mutations can contribute to the neoplastic process by predisposing to cancer in every cell. By natural selection, new variants of tumor cells can acquire a greater propagative advantage. This selection is one reason why cancers show diversity and heterogeneity. It also makes the development of effective treatment for cancers a difficult task (5,6).

Generally, cancer develops through four definable stages: initiation, promotion, progression and malignant conversion (5,7). The first stage, initiation, involves a somatic genetic alteration in a cell which originally has unlimited replicative potential and is blocked from terminal differentiation following the mutation. Even though the initial damage rarely results in cancer, this irreversible event makes the cell more susceptible and prone to becoming cancerous (8,9).

Promotion of carcinogenesis is the phase when the initiated cell undergoes clonal expansion mediated by acquired sustained growth signals, insensitivity to anti-growth signals or impaired apoptosis. During promotion, the expanded population of mutated cells could give rise to a benign tumor. This process is normally reversible, as benign tumors such as papillomas, can shrink or disappear (9).
The progression phase, also called premalignant progression, is less well understood compared with a substantial number of studies elucidating the mechanism of initiation and promotion. It is thought to be the stage of additional mutations and epigenetic alterations through which the promoted cell population becomes independent of external growth signals and resistant to growth inhibitors and apoptosis, thereby gaining a proliferative advantage over those unaffected cells (1,9).

Invasion and metastatic conversion are the final stages of carcinogenesis, when seeding and growth of satellite lesions occurs in distant sites from the primary tumor. Biochemical processes including increased cell motility, remodeling of the extracellular matrix, migration to specific secondary sites, and neoangiogenesis all contribute to the development of invasiveness and metastases (10-13).

A better understanding of the mechanism of cancer development will lead to more effective diagnostic strategies and to drug design against more promising therapeutic targets.

**Initiation of cancer: gatekeeper mutation in stem cells** Cancer has long been considered as a stem cell disease, since “the cell of origin” for cancer is thought to be a minority of cells representing stem-cell-like properties (8). This idea of cancer stem cells are supported by an extensive literature reporting that only a small subset of cancer cells isolated from malignancies such as leukemia and multiple myeloma is capable of unlimited proliferation (8,14-16). Dating back to 1960’s, it was observed that transplanting leukemia cells *in vivo* only resulted in 1-4% of spleen colony formation (16,17). Similar observations were also made for variety of solid cancer
types in both *in vitro* and *in vivo* (18,19). For example, less then 0.1% of lung cancer, ovarian cancer and neoblastoma cells are capable of forming colonies in soft agar (20). Lately, research on acute myeloid leukaemia (AML) has successfully identified a small group of the cancer cells that showed the ability to reconstitute tumors, and to transfer disease into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (21-23). Also, a small population of breast or brain tumor cells bearing specific cluster of differentiation (CD) molecules is able to trigger tumors in mice (24-27).

There are two major reasons underlying the hypothesis that cancer arises from a stem-cell-like origin. First, stem cells are known to have self-renewal ability and to generate different lineages of mature cells through differentiation. It gives stem cells the ability to overcome growth inhibitors and apoptosis factors that normally function in terminal differentiation. Stem cells also have a relatively slow turn-over compared with more committed cells in highly proliferating tissues. Therefore, there is greater chance for mutations to occur and accumulate in a single stem cell than in mature cells (8,9,14). Indeed, the cells capable of initiating human AML in NOD/SCID mice have a CD34+CD38– phenotype in most AML subtypes, and thus have a phenotype similar to normal hematopoetic stem cells (HSCs) (23). As the cell type mostly enriched in HSCs, CD34+CD38- cells were found to have typical leukemia-associated chromosomal abnormalities in chronic myeloid leukemia (28,29). Partially differentiated stem cell progenitors could also be targets for clonal expansion and transformation. Since these cells normally experience a relatively shorter period of
proliferation before terminal differentiation, mutations endowing them with the ability of self-renewal could be pre-requisite for their transformation (14).

Stem cell maintenance is a tightly controlled process. A significant body of work has shown that many pathways are associated with cell patterning, tissue regeneration and cancer development. These pathways could also regulate stem cell properties. For example, activation of Notch signaling by Jagged-1 in HSCs resulted in increased proliferation of primitive progenitors, suggesting a role of Notch in maintaining the multipotent characteristic of stem cells (30). In vitro studies showed evidence that Sonic hedgehog (Shh) signaling could promote the self-renewal activity in populations highly enriched for human HSCs (CD34+Lin–CD38–) when combined with other factors, like the bone morphogenic protein (BMP) (31). Overexpression of the Wnt downstream target β-catenin exhibited a positive influence on HSCs growth, while Wnt inhibitors could block the proliferation activity (32,33). Additionally, Wnt and Shh have been implicated in regulating stem cell and progenitor self-renewal in other tissues, such as skin, muscle and the neural system (8).

Deregulation of these pathways could possibly convert the normal self-renewal of stem cells to abnormal proliferation of cancer stem cells, thus causing initiation and growth of cancer. Cancer genetics studies on some types of familial cancer identified several gatekeeper pathways that could cause cancer predisposition. The known pathways include Shh-Gli in BCC, Wnt-APC in Colon cancer and Rb in retinoblastoma (5). Introduction of the concept of “gatekeeper” suggests that initiation of different types of cancer could be due to a mutation, when acquired by stem cells,
in a particular signaling pathway. In the future, further identification and characterization of gatekeeping genes would provide fundamental knowledge for understanding the initiation phase of particular types of cancer.

**Promotion and progression of cancer: unbalanced cell growth and cell death** Tumor cells evolve in a diverse and heterogeneous fashion when they encounter selective pressures within different microenvironment. A large body of work indicated that several critical processes are altered in various types of cancers during tumorigenesis. The list of essential alterations manifested in cancer cells include self-sufficiency in growth signals, insensitivity to antigrowth signals, inhibition of cell programmed death, limitless replicative potential, sustained angiogenesis, and tissue invasion (1).

These hallmark events underlie the molecular basis for neoplastic promotion and progression. Normal cell proliferation, differentiation, arrest of cell growth and apoptosis are precisely controlled by multiple signaling pathways. Therefore aberrant regulation of these pathways could result in escape of cells and permit unremitting clonal expansion to erode normal tissue architecture (5,6). How cancer cells acquire these rate-limiting alterations remains the critical question for researcher.

To enter the cell cycle, normal quiescent cells require mitogenic growth signals. Growth signaling is normally imparted when cells are within an appropriate environment. Tumor cells circumvent the barrier of restrained exogenous mitogen through growth signal autonomy. Targets for deregulation of growth signaling include extracellular growth signals (e.g. platelet-derived growth factor, PDGF), transcellular
transducers of signals (e.g. ligand-activated growth factor receptors and integrins), and intracellular processor of signals (e.g. Ras and a family of Ras effector molecules) (6,34). Taking the well-established ras proto-oncogene as an example, activation of Ras occurs in a large number of human cancers, mostly due to point mutations that maintain Ras in GTP-bound form (35). In the study of human colon cancers, up to 50% of the tumors bear mutant ras oncogene (3). Normal Ras functions are carried out by the Raf/MEK/ERK kinase cascade, the Ral GTPase pathway and the PI3K/AKT pathways. Deregulation of these pathways may result in activation of S phase genes and facilitate the degradation of pro-apoptotic proteins (36-39).

Superimposed upon the requirement for growth signals is a network of anti-growth factors which tightly monitor the transition of normal cells from G0 to G1 phase. Transforming growth factor-β (TGF-β) and interferons represent examples of this class of molecules (1). The tumor suppressor, Rb, and the c-Myc proto-oncoprotein are the two major effectors that mediate the anti-growth signals (6,34). Interestingly, through the E2F family of transcriptional factors as well as p19ARF and p53, Rb, and c-Myc all play dual roles in regulation of cell growth versus cell programmed death. The coupled control of these two processes enables normal cells to grow under a limited supply of growth factors. It also proposes Rb and c-Myc to be convenient targets for deregulation in tumor pathogenesis (34,40). Indeed, genetic alteration of the Rb gene itself, or abnormal posttranslational modification mediated by CDKs phosphorylation found in analysis of human cancers suggest a universal defects of this pathway in the development of cancer (41,42). For c-Myc, it
becomes oncogenic when it collaborates with survival signal pathways (e.g. Ras/PI3-K/AKT) or defects in apoptotic pathway (e.g. mutation in P53) (43-46).

The apoptotic machinery commits cells to the death pathway in response to abnormalities, such as DNA damage, growth factor signaling imbalance, survival factor insufficiency, or hypoxia (6). This process provides a universal mechanism to prevent a single proliferative lesion from leading to unrestrained cell growth. Therefore apoptosis is a critical barrier that cancer cells must circumvent to make sure their survival. Aberrant activation of anti-apoptotic factors and loss of proapoptotic regulators are the two most common defects found in cancer cells. One of the best studied examples is the p53 tumor suppressor protein. The p53 protein can initiate apoptosis by activating the proapoptotic Bax protein and subsequently releasing of cytochrome C, a potent catalyst of apoptosis, from mitochondria (47). Loss of function of p53 is implicated in more than 50% of human cancers (48). Overexpression of Bcl-2, an anti-apoptotic factor, was shown to cooperate with upregulated c-myc during lymphoma pathogenesis (43,49).

The complicated but precisely controlled cross-talks among growth signaling, anti-growth signaling and apoptotic pathways provide a basis for therapeutic targets.

**Metastases of cancer: EMT initiated eroding of normal tissue** Metastasis, the ultimate stage of carcinogenesis, is responsible for the majority of death due to cancer (5). Almost all the aggressive tumors show loss of contact inhibition, increase cell motility, sustained angiogenesis and invading ability into surrounding territories to enable proliferation and further invasive behavior at secondary sites (1).
For tumor derived from epithelial (i.e. carcinomas), the initiation of the invasive and metastatic phase is associated with epithelial mesenchymal transition (EMT). Well-known for its roles in development, EMT causes reorganization of the actin cytoskeleton and is normally involved in morphogenetic events during gastulation or the migration of neural crest cells. During the development of cancer, EMTs lead to the loss of polarity and cell-cell recognition and the gain of migratory capability. Thus the mechanism that underlies the regulation of cell-cell adhesion and cell-matrix interaction during development might reflect the potential targets for the metastatic evolution of tumor cells (10,50,51).

As key components of adherens junctions, E-cadherin and β-catenin are important in EMTs and in metastases of cancer. E-cadherin is a single-pass protein that mediates cell-cell adhesion through Ca\(^{2+}\)-dependent, homotypic interactions (52-57). Loss of E-cadherin has been analysed in many types of carcinomas (58-64). In cancerous cells, the intracellular domain mediating its connection to the cytoskeleton and also the juxtamembrane domain of E-cadherin containing the p120-catenin binding region appear to be targets of regulation (51). For example, mutations that abolish the p120 binding were found in colorectal carcinoma cell lines that exhibit disperse growth (65). Studies on β-catenin suggested that it is one of the molecules commonly involved in cancer and development. Regarded as a central player in the Wnt signaling pathway during development, mutations of β-catenin were also shown to be associated with several types of cancer (66-70). Observed mutations or alterations, usually resulting in stabilization of cytoplasmic β-catenin and
augmented association with Lef1/Tcf, suggesting that the development of these cancers is due to altered expression profile of β-catenin in membrane adherens junctions, the cytoplasm and the nuclei of cells (71-76).

Signals that define the surrounding cellular boundaries are also involved in the process that cells could acquire motile properties. Abnormal expression of Ephrins and their receptors, which mediate cell recognition in development, were found to be correlated with certain cancer types, e.g., ovarian carcinoma and bladder carcinoma (77,78). Matrix metalloproteases (MMP) are required for vascular endothelial growth factor (VEGF) activities during vascularization. Association of expression of metalloproteases with several kinds of tumors is in concert with the concept that angiogenesis is an essential event of the metastatic process (79-82).

Understanding the signaling pathway and molecules involved in regulation of EMT may provide insight into potential modulators of the metastatic process of cells. According to studies both in vitro and in vivo, pathways thought to be important for EMTs include tyrosine kinase receptor-mediated pathways (e.g. Ras), receptor tyrosine kinase signaling (e.g. fibroblast growth factor, FGF), TGFβ, Notch, Shh and small GTPases, e.g. Rho GTPases and their associated kinase ROCK (50,83). Taking Ras as the example, activation of Ras downstream effectors MEK and Rac was found to induce EMT in bladder carcinoma cells. Loss-of-function mutations of Ras could lead to the metastasis of transformed EpH4a cells, a mammary epithelial cell line, where activated Raf/MAPK was accompanied with the process (84).

Members of a zinc finger transcriptional factor family, slug and snail, are
central in developmental EMTs, including the migration of neural crest and the delamination of early mesodermal cells during gastrulation (85). In epithelial cells like MDCK, Snail induces EMTs by repressing the transcription of E-cadherin (86,87). The negative correlation between the expression of Snail and E-cadherin observed in invasive tumor cell lines and also in some types of invasive cancers reinforces the idea that Snail is a repressor of E-cadherin and a positive modulator of the invasion and metastases of tumor cells (51).

**Wnt signaling in development and cancer** As reviewed previously, the essential difference between cancer and developmental biology is: development is about regular and reproducible cellular processes, cancer is about irregular and unpredictable chains of events. However from an evolutionary point of view development and cancer share strategic similarities, as both processes involve in fixation of one or several abnormalities of pathways to form different organisms or different types of cancers. Thus the shared molecular features between cancer and development can be attributed to the fact that these processes are two sides of the same coin. Indeed, five signaling pathways including Wnt/Frizzled, Hedgehog/Patched (Hh/Ptch), TGFβ/BMPs, Delta/Notch, and RTKs, are thought to represent the core machinery underlying both development and cancer (51,88).

Based on the current view of Wnt signaling, a protein complex composed of the scaffolding protein Axin, the adenomatous polyposis coli (APC) tumor suppressor, casein kinase1 (CK1), and glycogen synthase kinase 3β (GSK3β) acts to degrade β-catenin through the β-TrCP mediated ubiquitination pathway. Binding of Wnt
ligand to the cell-surface receptor complex Frizzled/LRP recruits Dishevelled (Dsh) and induces phosphorylation of the intracellular domain of LRP mediated by CK1 as well as by GSK3β, leading to the ultimate disruption of the large protein complex and to stabilization of the cytoplasm pool of β-catenin. Association of β-catenin with members of the Lef1/Tcf family of transcription factors in the nucleus activates transcription for a variety of Wnt target genes (Figure 1) (89-91).

By shifting from adherens junction in the cytoplasm to the nuclei, β-catenin is proposed to be a pivotal factor that couples loss of cell adhesion to Wnt signaling, (92). Although Wnt signaling is not currently shown to be a major regulator that determines β-catenin association with other proteins, recent studies show that other signaling pathways regulate β-catenin function in Wnt signaling versus cell adhesion (90,92,93). Upon phosphorylation of Y142 of β-catenin, Bcl9-2 competitively binds the α-catenin-binding region, preventing incorporation of β-catenin into adhesion complexes (94). As a mammalian homolog of Drosiphilla Legless, Bcl9-2 can be shuttled to the cytoplasm where it binds to β-catenin, while in the nucleus it binds to Pygopus to promote Tcf/Lef1 mediated transactivation (95,96). To date, Wnt signaling are shown to be involved in different cellular activities through its target genes: cell adhesion (e.g. Claudin1, E-cadherin), cell proliferation (e.g. Cyclin D1, c-Myc), and cell migration (e.g. Twist) (97-101).

During development, the Wnt signaling pathway plays roles in a wide range of embryonic patterning events. Current evidence indicates that the Wnt cascade is the most important force in controlling cell fate along the crypt-villus axis of the intestine.
This process is largely performed through β-catenin/Tcf/Lef1 driving cell proliferation in the crypt progenitor compartment, since abrogation of Wnt signaling by knocking out Tcf4 in mouse results in depletion of stem cells. The Wnt signal gradient also controls expression of EphB/ephrinB receptors and ligands, results in the establishment of crypt-villus boundaries (102,103). In epidermal development, Wnt signaling promotes the proliferation of resting stem cells in the bulge, promoting the generation of different cell lineages of hair follicle, sebaceous glands, as well as the interfollicular epidermis (104). Constitutive expression or conditional induction of an active form of β-catenin shows a dramatic phenotype of inducing hair follicle morphogenesis in adult interfollicular epidermis (105,106). Tcf3, one of the four family members, is implicated specifically in bulge stem cells. Upon expression in interfollicular epidermis, Tcf3 inhibited terminal differentiation of keratinocytes and promoted features of bulge cells (107). Recent studies indicate that Wnt signaling is involved in maintaining virtually all the defined human adult stem cells (108,109). Amplification of progenitor cells were also shown to be responses to stimulated Wnt signaling or introduction of activated β-catenin in the stem cell systems of blood, muscle, and mammary gland, while antagonism or inhibition of Wnt were associated with decreased proliferation of these cells (110). In summary the Wnt cascade controls the fate of cells that derive from the stem cells in different progenitor compartments.

In concert with the profound influence it exerts in development, deregulation of the Wnt pathway is correlated with various types of cancer. The best studied
example of this correlation seems to reside in colorectal cancer. In a hereditary cancer syndrome termed familial adenomatous polyposis (FAP), defective APC alleles were implicated in the syndrome, characterized by development of large numbers of colon polyps or adenomas. The evolution of adenocarcinomas usually occurs when additional mutations in oncogenes or tumor suppressor genes, like p53, are acquired by APC deficient epithelial cells (111,112). Loss of APC occurs in most sporadic colorectal cancers, suggesting it as a gatekeeper in the pathogenesis of colon cancer (3). Inactivation of APC leads to constitutive Wnt nuclear signaling through the transactivation complex of β-catenin/Tcf/Lef1, which trigger a series of cellular activities programmed by its targets. DNA microarray analysis of the target gene profile was carried out in colorectal cell lines conditionally expressing a dominant-negative Tcf4 (113). Linking cancer phenotype and stem cell properties, the profile turned out to be similar in colorectal cancer cells as in crypt stem and progenitor cells. The almost invariable correlation between colorectal cancer and aberrant activation of the Wnt pathway thus reflects that the adenoma cells are transformed counterparts of the crypt progenitor cells. Abnormality of the Wnt cascade also appears to relate to other types of cancers. Overexpressing β-catenin or γ-catenin transgene led to hair follicle tumors or acute myeloid leukemia, respectively in vivo (105,106,114).

**Hh signaling in development and cancer** Hh signaling, like the Wnt cascade, plays a critical role during development and tumorigenesis. The hh gene was first identified as secreted protein essential for segmental specification in the *Drosophila*...
embryo (115-119). In mammals, there are three hh genes, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) (115,119).

Ptch, a twelve-span transmembrane receptor and a member of seven-transmembrane receptor family, Smoothened (Smo), are the two crucial factors that respond to Hh signals. To initiate signaling, active peptides of Hh are produced following autoprocessing and dual lipid modification at its N-and C-terminals (120). Activity of Smo is normally inhibited by Ptch. Hh binding to Ptch stimulates Smo activity and induces transcriptional activation of Hh target genes by Drosophila Ci or mammalian Gli proteins (Figure 2) (121-123). Although biochemical studies suggested a heteromeric receptor model in which dissociation of Smo from Ptch is induced by direct interaction of Hh with a Smo/Ptch complex, recent studies in vivo indicated the two proteins residing in different locations (118,124,125).

The mechanism by which Hh signaling can be transmitted to nuclei is not completely understood. Studies in Drosophila draw an incomplete picture with multiple molecules including serine/threonine protein kinase Fused (Fu), Suppressor of Fused (SuFu), the kinesin-like protein Costal-2 (Cos2), and several kinases modulating the activity of Ci. In the absence of Hh ligand, the Ci/ Fu/ SuFu complex is anchored to the cytoplasm via Cos2. In this complex, full-length Ci is phosphorylated by PKA, GSK3β and CK1 and then processed into a 75 kD Ci repressor form (CiR), which acts in the nucleus to shut off the transcription of Hh targets. Upon stimulation by Hh protein, Smo accumulates at the cell membrane and recruits the Cos-2 complex with higher efficiency directly through its phosphorylated
cytoplasmic tail. This recruitment step appears to enable the release of Ci from the Cos-2 complex, probably by activation of Fu kinase and phosphorylation of SuFu, and allows the stabilisation of full-length Ci and the formation of the Ci activator form, of which a small fraction translocates to the nucleus to activate HH-target genes (126-129).

Hh signals regulate cell proliferation and differentiation in diverse tissues, including the central nervous system, limbs, somites, skeleton and skin (126). For example, Shh signaling has a central role in hair follicle growth by regulating both epithelial and mesenchymal components of the hair follicle. Shh knockout mice show normal follicle spacing, but arrested development of embryonic hair follicles. The arrested hair follicles demonstrate both a decrease in epithelial proliferation and a failure of the underlying mesenchyme to form dermal papilla. Shh expression is restricted to the distal portion of the growing hair follicle where it seems to induce its targets Ptch and Gli1 in both the proliferating hair progenitor cells and the adjacent dermal papilla (130-133). Recent studies proposed that Shh signaling could potentiate postnatal hair morphogenesis (134). Additionally, increasing data suggest the Hh pathway plays a role in maintenance of stem cells. Both in vitro and in vivo studies demonstrate that loss of Hh signaling through inactivation of Smo inhibits neural stem cell proliferation (135,136). Similar effects have also been observed in HSC and follicle stem cells residing in Drosophila ovary (137,138).

The roles of Hh signaling in stem cell renewal and cell patterning imply the connection between the pathway and the initiation and growth of cancers. This
connection was initially established by the identification of Gli1 as a transforming oncogene and by identification of heterozygous mutations in the tumor suppressor, Ptc, as the cause of Gorlin’s syndrome (115,139,140). This syndrome is associated with an increased incidence of BCC, medulloblastoma, and rhabdomyosarcoma. Mutations of Ptc are also observed in sporadic forms of these cancers. Inappropriate activation of proto-oncogene Smo by sporadic mutations was also found to be associated with a similar range of cancers. Indeed, overexpressing Shh or Smo transgene in mouse skin resulted in formation of BCC and other hair follicle tumors (132,141). Utilizing an Hh antagonist, cyclopamine, or neutralizing antibodies, the Hh pathway was shown to be involved in a diverse array of cancer types that are not related to Gorlin’s syndrome (142-145). These cancers include small-cell lung cancer and carcinomas of the oesophagus, stomach, pancreas, biliary tract, and prostate (142-145). In the pathogenesis of these cancers, high-level activation of Hh pathway targets occurs only in cancer cells even though the Hh family proteins are also expressed in normal cells. This observation suggested that the limiting factor of carcinogenesis is the response to ligand, rather than expression of ligand itself.

Understanding Gli proteins as mediators of the Hh signaling pathway In mammals, all the transcriptional responses to the Hh signaling are mediated by the Gli family of transcription factors. The first Gli gene identified was Gli1, based on its amplification in a glioblastoma (146). The mammalian family of Gli proteins has three members, Gli1, Gli2 and Gli3, all of which share high homology to the Drosophila Ci in the zinc-finger domain (126). The highly conserved DNA binding
region comprises 5 zinc finger domains of the C2-H2 class, through which all Gli proteins recognize the consensus sequence GACCACCCA to trigger Hh-induced responses (147,148). Besides the zinc-finger domain, the Gli proteins contain multiple PKA sites and also a conserved transactivation domain at the C-terminus. Gli1, in contrast to Gli2 and Gli3, is shown to lack an N-terminal repression domain (Figure 3) (126). Evidence suggests that Gli1 and also Gli2 mainly function as the activator of HH-target genes, while Gli3 acts mainly as repressor, although some Gli3 activator function is also involved in induction of target gene transcription (149,150).

Our current understanding of the mechanisms of Hh signaling benefits from studies in Drosohpila (Figure 2), but increasing data suggest that divergent strategies were adopted by mammals (151-153). SuFu has been shown to play a key role in negatively regulating the activity of Gli proteins by sequestering them in the cytoplasm, although its Drosophila counterpart is a dispensable factor (151-153). Consistent with the negative regulation of Gli by SuFu, loss of SuFu function results in phenotypes that resemble to, in some aspects, loss of Ptc1, including predisposition to medulloblastoma in human and hair follicle tumors in mice (152,154). Previous reports have demonstrated that SuFu acts as an modulator for nuclear-cytoplasmic shuttling of Gli1 by sequestering the proteins in the cytoplasm (155). SuFu has also been implicated in repressing Gli target genes by recruiting a transcriptional silencing complex with histone deacetylase activity consisting of SAP18 and SIN3, to target promoters (156).

In contrast to Gli2 and Gli3, Gli1 is mainly regulated at the transcriptional
level, and represents a reliable marker of pathway activity (157-159). Gli2 and also Gli3 are considered transcriptional regulators of Gli1 by directly binding to consensus Gli binding sites in the Gli1 promoter (160,161). Modification of the N-terminus of Gli2 and Gli3 may also be a critical step in the regulation of the transcriptional activity of Gli proteins. This region is likely to harbor a repressor domain, since removal of a large portion of the N-terminus results in constitutively active Gli2 and Gli3 proteins (162).

Extensive genetic screens in mice have uncovered several recessive mutations in intraflagellar transport (IFT) genes leading to deficient embryonic development in the neural tube, similar to those observed in Shh or Smo mutant embryos (163-165). Furthermore, the absence of functional IFT proteins leads to a significant decrease in the Gli3 repressor fragment, identifying a role of IFT proteins in the formation of Gli3 repressor. These observations suggest that IFT controls both activation and repression activities of mammalian Gli (166,167). IFT proteins are essential for the growth and maintenance of flagella and primary cilia. As mammalian Cos2 orthologs seem to be dispensable in regulation of Hh signaling, primary cilia, instead, seem likely to be involved transmission of signal (168).

A series of gain and loss-of-function approaches indicate that individual Gli proteins are sufficient and/or essential factors in Hh-associated tumorigenesis. Gli1 is capable of transforming RK3E cells, a rat kidney epithelial cell line immortalized by adenovirus E1A (139). Keratin 5-promoter driven expression of Gli1 in the epidermis of transgenic mice leads to development of BCC and several types of hair follicle
tumors (169). Gli appears to be a limiting factor for proliferation of tumor cells, since loss-of-function studies show that primary prostate carcinoma cells cease to proliferate when treated with Gli1 siRNA (170). Overexpression of Gli1 is sufficient to convert low-metastatic prostate carcinoma cells into highly metastatic lines, which suggest a potential role of Gli1 in tumor metastases (145). Despite the fact that Gli1 is dispensable for mouse development, it is required for the development of medulloblastoma in Pch+/− mice, mediating critical downstream processes in Hh-associated brain tumors (171). Similar to Gli1, Gli2 mRNA levels are elevated in BCC lesions compared to normal skin. Keratin5-Gli2 transgenic mice develop tumors with histological and molecular characteristics of BCC (172). A conditional tetracycline-regulated Gli2 mouse model showed that Gli2 was also demonstrated required for tumor maintenance (173).

**Identifying Gli targets in Hh-induced cancer** Aberrant Hh-Gli signaling has been shown to be associated with a number of human cancers, which together account for about 25% of human cancer death (142-145). But substantial gaps still remain in our understanding of the downstream mechanisms underlying the pathogenesis of these cancers. The relative lack of understanding of Gli target genes has largely hampered the progress in this field. Recent experiments using a combination of global gene expression profiling, promoter assays and genetic studies of model organisms have led to the identification of direct Hh-Gli target genes, the expression of which is likely to be a critical parameter in initiation, progression or else metastatic conversion of tumor cells.
In various cell types, activation of Hh activity increases the expression of key regulators of the cell cycle, therefore promoting the transition from a quiescent to a proliferative state (174,175). Hh signaling promotes transcription of Cyclin E and Cyclin D during development in Drosophila (176). Upregulation of Cyclin E through direct binding of Ci to the Cyclin E promoter mediates the ability of Hh to induce DNA replication. Although it is not as clear in mammals, these observations suggest a potential direct regulatory relationship between Gli and principal cell-cycle regulators. In an analysis of the expression profiles from Gli transformed cells, Cyclin D2 expression was upregulated by Gli1 and also a Gli-binding site was identified in Cyclin D2 promoter region by electrophoretic mobility shift assays (177). Cyclin D2 induction depends on functional Gli2 and Gli3 protein, suggesting that activation of Hh-signaling directly increases Cyclin D2 expression via the latent transcriptional activators Gli2 and Gli3 (158).

BCCs express high levels of the antiapoptotic proto-oncogene, Bcl-2, and also Bcl-2 contributes to the malignant phenotype in a transgenic mouse model (178). This observation led to the discovery that Gli1 and Gli2 act as activator for the expression of Bcl-2 in epidermal cells. Using electrophoretic mobility shift assay and luciferase assay, recent studies identified a Gli-binding site that is important for Bcl-2 transcriptional regulation. The same report also showed that Gli1 was able to induce endogenous Bcl-2 in both cultured epithelial cells and the epidermal keratinocytes of transgenic mice. This upregulation of Bcl-2 by Gli1 could be inhibited upon introduction of the repressor form of Gli-3 (178). These results provided evidence that
Gli transcription factors may enable the tumor cells to acquire anti-apoptotic ability, resulting in promotion of cell survival.

Hh has also been implicated in the epithelial ventromedial somite wall to induce the connective tissue mesenchyme of the sclerotome, an example of EMT (179-181). This observation proposed a potential role of the Hh-Gli pathway in control of the metastatic behavior of cancer cells. Indeed, our studies utilizing a conditional Gli1-estrogen receptor fusion protein (Gli1-ER) showed that Gli1 can rapidly and directly induce expression of Snail (182). Snail and Gli1 transcripts were prominent in human BCCs, and in adjacent normal anagen hair follicles. Furthermore, invasive and metastatic growth of various prostate cancer cell lines can be significantly reduced by exposing cells to the Hh-pathway antagonist cyclopamine, while overexpression of Gli can convert cells with low metastatic potential into highly malignant cells, where prominent expression of Snail was induced (145). Gli consensus DNA binding sequences were identified in the 5' regions of osteopontin and plakoglobin suggesting that these genes represent immediate downstream targets. Gel shift analysis confirmed the ability of the Gli1 protein to bind these sequences (177). Both plakoglobin and osteopontin have been shown to regulate the extracellular matrix remodeling, an essential step in the metastatic process (183-185). Loss of plakoglobin or increase of osteopontin is usually observed in cancers with high metastatic features.

Taken together, these studies suggest that Gli oncogenes are involved in multiple tumorigenic processes through direct regulation of their targets: stimulation
of cell proliferation, inhibition of apoptosis, promotion of invasiveness and metastasis (Figure 4). Given the broad spectrum of biological activities controlled by Hh-Gli signaling, future studies aiming at the genome-wide identification of direct Gli target genes will unravel additional routes by which aberrant Hh-signaling contributes to cancers.

**Questions addressed in this dissertation** Snail, a transcriptional repressor of E-cadherin, was demonstrated in previous work as a potential direct target of Gli1 (145,182). Although Snail is clearly important in EMT, the role of Snail in transformation by Gli1 had not been examined. Our work provides evidence that Snail acts as a limiting factor for Gli1-induced transformation in a context of cultured epithelial cells. *Snail* was identified as an early Gli1 response gene in the epithelial cells and interfollicular epidermis in *vivo*, where *Snail* transcription was turned on within 3-6 hours upon induction of Gli1. Our correlation of loss of E-cadherin with induction of Snail led us to ask whether E-cadherin functions as a repressor of epithelial transformation by Gli1 and to examine the role of β-catenin in this activity of E-cadherin.
**Figure 1** The canonical Wnt signaling pathway. In the absence of Wnt ligand (left panel: No Wnt), β-catenin is in a complex with Axin, APC, CK1 and GSK3β, and is targeted for degradation through phosphorylation. β-catenin also binds to cadherin in adherens junction (AJ) and regulates cell-cell adhesion. In the presence of Wnt ligand (right panel: + Wnt), LRP/Frizzled recruits Dsh and inhibits GSK3β. β-catenin is uncoupled from the complex and translocate to the nucleus, where it binds Tcf/Lef1 and turns on Wnt target genes.
Figure 1
Figure 2  The Shh-Gli signaling pathway. In the absence of Shh signaling pathway (left panel: no Shh), Smo activity is inhibited by Ptch. Gli proteins are sequestered in the complex of primary cilia, Kinases and Sufu, where they get phosphorylated and are processed to the repressor form of Gli (GliR). GliR represses transcription of target genes. In the presence of shh signaling (right panel: + Shh), Smo activity is stimulated. Smo may interact with primary cilia and disrupt the degradation complex, thus allows the activation forms of Gli to translocate to nucleus and activates transcription. Sufu may act as a co-repressor of GliR in the nucleus.
Figure 3  Structures of the Gli family of zinc finger transcriptional factors. The highly conserved DNA binding region comprises 5 zinc finger domains of the C2-H2 class (red). Besides the zinc-finger domain, the Gli proteins contain multiple PKA sites (green), but Gli1 contains three sites instead of four. The conserved transactivation domains locate at the C-terminus (pink). Gli1, in contrast to Gli2 and Gli3, is shown to lack an N-terminal repression domain.
Figure 4  Gli proteins are involved in multiple tumorigenic processes. Upon constitutive Shh signaling in a number of cancers, Gli proteins may act through their direct regulation of their targets to stimulate cell proliferation, to antagonize apoptosis, and to promote invasiveness and metastasis. Gli may also involved in stem cell renewal.
Constitutive Shh signaling

Gli

- Promote cell proliferation (Cyclin D)
- Inhibit apoptosis (Bcl2)
- Metastasis (Snail)
- Stem cell renewal (??)

Figure 4
SNAIL INDUCTION IS AN EARLY RESPONSE TO GLI1 THAT DETERMINES THE EFFICIENCY OF EPITHELIAL TRANSFORMATION

by

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Abstract

*Gli* family members mediate constitutive Hedgehog signaling in the common skin cancer, basal cell carcinoma. *Snail/Snail* is rapidly induced by Gli1 *in vitro*, and is co-expressed with Gli1 in human hair follicles and skin tumors. In the current study we generated a dominant negative allele of *Snail*, SnaZFD, composed of the zinc finger domain and flanking sequence. In promoter-reporter assays, SnaZFD blocked the activity of wild type Snail on the E-cadherin promoter. Snail loss-of-function mediated by SnaZFD or by one of several short hairpin RNAs inhibited transformation of RK3E epithelial cells by Gli1. Conversely, enforced expression of Snail promoted transformation *in vitro* by Gli1, but not by other genes that were tested, including Notch1, ErbB2, and N-Ras. As observed for Gli1, wild type Snail repressed E-cadherin in RK3E cells and induced blebbing of the cytoplasmic membrane. Induction of a conditional Gli1 transgene in the basal keratinocytes of mouse skin led to rapid upregulation of Snail transcripts and to cell proliferation in the interfollicular epidermis. Established Gli1-induced skin lesions exhibited molecular similarities to BCC, including loss of E-cadherin. The results identify Snail as a Gli1-inducible effector of transformation *in vitro*, and an early Gli1-responsive gene in the skin.
**Introduction**

Genetic alterations that deregulate cell fate pathways play an early, critical role in the genesis of specific forms of carcinoma (Vogelstein and Kinzler, 2004). Mutations that lead to constitutive Sonic Hedgehog (Shh) signaling, such as inactivation of the Ptch1 tumor suppressor, are a consistent step in development of cutaneous basal cell carcinoma (BCC), the most common malignancy in Caucasians, and may play a role in other tumor types (Goodrich et al., 1997; Callahan and Oro, 2001; Ruiz i Altaba et al., 2002; Berman et al., 2003; Watkins et al., 2003; McMahon et al., 2003; Pasca di Magliano and Hebrok, 2003; Karhadkar et al., 2004). Although Shh pathway defects are rate-limiting in human BCC, current mouse models exhibit a significant lag between Shh pathway activation and the outgrowth of skin tumors, indicating that defects in other pathways play an important role (Aszterbaum et al., 1999; Nilsson et al., 2000; Grachtchouk et al., 2000; Oro and Higgins, 2003; Hutchin et al., 2005). Conditional inactivation of the cell fate determinant Notch1 in the skin leads to BCC-like lesions, and p53 mutation is frequent in human BCC (Ling et al., 2001; Nicolas et al., 2003). An accurate model of BCC will incorporate these known alterations and will require identification of other genetic or epigenetic changes, the temporal order of alterations, the cell types in which they occur, and the effects of specific mutations on stem cell renewal, cell proliferation, differentiation, and the apoptotic response.

Unlike many other tumor types, BCCs are not associated with an identifiable precursor lesion, and the cell type of origin remains unclear (Miller, 1995). BCC may originate in stem cell populations found in the follicle bulge and the basal layer of the interfollicular epidermis (Blanpain et al., 2004; Tumbar et al., 2004). An origin of BCC
within interfollicular epidermis is suggested by induction of BCC-like lesions in the embryonic skin of mice transgenic for Shh or Smo, a seven-transmembrane protein that transduces the Shh signal and exhibits gain-of-function mutations in BCC (Oro et al., 1997; Xie et al., 1998). However, a conditional Gli2 model showed early tumors associated with the follicle bulge (Hutchin et al., 2005).

An extensive literature points to the Gli family of zinc finger transcription factors as mediators of Shh signaling in development and in tumors (Ruiz i Altaba et al., 2002; McMahon et al., 2003; Pasca di Magliano and Hebrok, 2003). Indeed, either Gli1 or Gli2 can induce BCC in transgenic mice (Nilsson et al., 2000; Grachtchouk et al., 2000; Oro and Higgins, 2003). Gli genes are thought to regulate multiple cellular processes relevant to transformation, including cell cycle progression, apoptosis, and others (Louro et al., 1999; Yoon et al., 2002; Louro et al., 2002; Duman-Scheel et al., 2002; Bigelow et al., 2004; Callahan et al., 2004). However, few downstream effectors have been extensively characterized.

As one of its multiple roles in development, Shh signals to the epithelial ventromedial somite wall to induce the connective tissue mesenchyme of the sclerotome, an example of epithelial-mesenchymal transition (EMT) (Fan and Tessier-Lavigne, 1994; Hay, 1995; Thiery, 2003). We previously utilized a conditional Gli1-estrogen receptor fusion protein (Gli1-ER) to show that Gli1 can rapidly and directly induce expression of Snail, a regulator of EMTs in embryonic development and in tumor progression (Louro et al., 2002). Snail and Gli1 transcripts were prominent in human BCCs, and in adjacent normal anagen hair follicles. Furthermore, when expressed in AT2 prostate cancer cells with low metastatic potential, Gli1 induced prominent expression of Snail and a more
metastatic phenotype (Karhadkar et al., 2004). Conversely, treatment of Snail-positive, highly metastatic AT6 prostate cancer cells with the Shh pathway inhibitor cyclopamine inhibited both Snail expression and the metastatic phenotype.

In the current study, we used tetracycline (tet)-inducible strategies to correlate Gli1 and Snail expression in vitro and in vivo. In the interfollicular epidermis, Gli1 induced expression of Snail and cell proliferation within 6-12 hours of induction. By four weeks, superficial lesions with molecular and morphologic similarities to BCC were observed. A functional role for Snail in vivo was suggested by loss of E-cadherin in Gli1-induced skin lesions. Studies performed in an epithelial model in vitro supported our previous observation that Snail is directly regulated by Gli1 (Louro et al., 2002), and indicated that Snail is a limiting factor in Gli1-induced transformation that recapitulates the cell morphologic changes induced by Gli1.

**Results**

**Rapid induction of Snail by Gli1 in vitro.** Previously, we utilized an E1a-immortalized, epithelial line derived from rat kidney, termed RK3E, to identify a gene expression profile for Gli1 (Ruppert et al., 1991; Louro et al., 2002). Unlike control RK3E cells and RK3E cells transformed by c-MYC, RAS, or KLF4/GKLF, Gli1-transformed cell lines showed prominent expression of Snail. Direct regulation was suggested by rapid induction of Snail transcripts upon exposure of Gli1-ER cells to tamoxifen, even in the presence of the protein translation inhibitor cycloheximide. Although induced by Gli1, the functional significance of Snail to Gli1-induced transformation remained unclear.
To further examine Snail as a potential transcriptional target of Gli1, we isolated clones of RK3E cells in which a human Gli1 allele is induced by tet (Figure 1a). Immunoblot analysis identified several clones with a low background of Gli1 in uninduced cells and prominent expression after exposure to tet for 6 hours (left panel). Expression of transcripts corresponding to Snail and the well-established Gli1 target gene, Ptch1, were examined by semi-quantitative reverse transcription and PCR (RT-PCR, right panel). Analysis of multiple clones revealed consistent regulation of Snail and Ptch1. When RTOG10 cells were similarly analyzed over a timecourse, Snail and Ptch1 were co-regulated and induced by 1-3 hours, consistent with direct regulation of these by Gli1 (Figure 1b) (Agren et al., 2004).

When expressed in a variety of epithelial cell types, Snail inhibits expression of E-cadherin and induces features of EMT (Cano et al., 2000; Batlle et al., 2000). We generated puromycin-resistant RK3E cell populations by retroviral transduction of pBABE-puro Snail (termed Snail cells) or pBABE-puro vector (termed Vector cells). Snail cells and Vector cells exhibited similar growth rates at subconfluence, a similar frequency of apoptosis as indicated by condensed nuclei, and were similarly contact inhibited at confluence (data not shown, and see below). In contrast to Vector cells, Snail cells showed reduced expression of E-cadherin (Figure 1c), and phase-contrast microscopy revealed prominent blebbing of the cytoplasmic membrane (Figure 1d, middle panel). This phenotype was previously described in other cell types and was attributed to impaired mechanical stability of actin filaments underlying the cell membrane (Cunningham et al., 1997; Flanagan et al., 2001). Like Snail cells, Gli1 cells have reduced expression of E-cadherin (Figure 1e) and exhibit blebbing (clones Gli-A
and Gli-C; e.g., Figure 1d, bottom panel).

The typical features of EMT, such as acquisition of a spindled morphology, were not apparent in Snail cells (Figure 1d). Nor was cell migration altered, as indicated by a wound healing assay in vitro (data not shown) (Cano et al., 2000). Absence of these features may be attributed to expression of E1a, which strongly promotes the epithelial phenotype (Frisch, 1994), or to lower levels of Snail protein in these cells compared with previous studies (Cano et al., 2000; Batlle et al., 2000). Transgene expression in Snail cells was not detected by immunoblot or indirect immunofluorescence using anti-Snail antibody (Santa Cruz), or else 12CA5 antibody against the C-terminal hemagglutinin [HA] epitope. Nor was protein expression observed when Snail cells were transformed with Gli1 or RAS, treated with the proteasome inhibitor MG132, or when GSK3β was inactivated with LiCl (Zhou et al., 2004; Yook et al., 2005). In these experiments, Cos-7 cells transiently expressing the same HA-tagged transgene served as a positive control, and yielded a strong signal on immunoblots (data not shown). Thus, the level of transgene-derived Snail in RK3E cells and transformed derivatives is low, even though its activity is evident as described below.

Characterization of dominant negative Snail. Mayor and colleagues generated truncated alleles of Xenopus Snail that functioned as dominant negatives (Aybar et al., 2003). To examine a role of Snail in mammalian cells, we generated fragments of mouse Snail and tested these for inhibition of the wild type protein (Figure 2a-b). Of several alleles tested in HEK293 cells, only SnaΔZFD and SnaZFD could specifically antagonize the activity of Snail on the E-cadherin promoter. Titration experiments identified SnaZFD as the more potent of the two, as E-Cadherin transcription was
restored using 0.5 μg of plasmid, equal to the input of wild type Snail (Figure 2b, compare lanes 3 and 6). SnaZFD was likewise more active than SnaΔZFD when the input was twice that of wild-type (compare lanes 4 and 7), which was sufficient to saturate the effect (compare lanes 4 and 5). Consistent with inhibition of Snail by the truncated proteins, co-expression of the two was not additive or synergistic (Figure 2b, lane 8). Induction of E-cadherin promoter activity beyond levels obtained without exogenous Snail suggested the presence of an endogenous repressor (Figure 2b, compare lanes 3, 4, 5, 7, and 8 to lane 1). Indeed, Snail is expressed in HEK293 cells (Figure 2c). The activity of truncated Snail alleles on the E-cadherin promoter, their lack of activity on the Renilla control (data not shown), and their overall similarity to the Xenopus dominant negative alleles previously characterized are consistent with their function as dominant negatives.

**Induction of endogenous Snail is limiting for Gli1-induced transformation in vitro.**

To determine if Snail has a role in transformation by Gli1, we expressed SnaZFD under control of the Moloney murine leukemia virus long terminal repeat (MMLV-LTR). Following lipid-mediated co-transfection with Gli1 expression vector, SnaZFD repressed the outgrowth of transformed foci by 69% (mean number of foci/dish: Gli1+Vector, 87; Gli1+SnaZFD, 27; Figure 2d, column 1, compare rows 1 and 2; and Figure 2e).

In multiple transformation assays, expression of Snail alone did not induce foci (Figure 2d, column 4, row 3). However, induction of Snail appeared to be a limiting factor in Gli1-induced transformation, as co-transfection of Snail with Gli1 induced focus formation by 5.5-fold (mean number of foci/dish: Gli1+Vector, 87; Gli1+Snail, 475; Figure 2d, column 1, compare rows 1 and 3; and Figure 2e).
As a complementary approach, we used sequential retroviral transduction to determine the transforming efficiency of Gli1 or N-RAS retroviral supernatants when applied to pooled populations of puromycin-resistant cells transduced with pBABE-puro (Vector cells) or pBABE-puro-Snail (Snail cells; described above, Figure 1d). In three independent experiments, each performed in duplicate, Gli1 induced an average of 5.4-fold more foci in Snail cells compared with Vector cells (mean foci/dish, 514 vs. 95), whereas the two cell lines showed a similar number of foci in response to N-RAS (92 vs. 87 foci/dish; Supplement Figure 1). Thus Snail cooperates with Gli1 but not with N-Ras.

Snail and SnaZFD may modulate Gli1 transformation in a pathway-specific fashion, or have a nonspecific effect on cell proliferation. To address this issue, we transfected these constructs into RK3E cells in combination with the intracellular domain of Notch 1 (NICD), previously reported to transform RK3E cells (Ascano et al., 2003), or an activated allele of ErbB2 that transforms these cells more efficiently than the wild type allele (unpublished data, MHK). Neither Snail nor SnaZFD altered the transforming efficiency of the controls (Figure 2d, columns 2 and 3; and Figure 2e). Thus, Snail specifically modulates transformation by Gli1.

To further address the possibility that Snail and SnaZFD may alter cell growth in a non-specific fashion, colony size and number were determined following transfection of MMLV-LTR plasmids into RK3E. Puromycin selection was applied 24 hours post-transfection, and colonies were analyzed at 7 days (Figure 2d, column 5). As indicated by colony forming efficiency, colony size, and cell density within the colonies, neither Snail nor SnaZFD exhibited an apparent growth phenotype in nontransformed
RK3E. The colony assay supports the similar growth properties noted above for RK3E-Snail and RK3E-Vector cells (Figure 1c-d). In contrast to these results, transfection of Gli-C or Gli-A with SnaZFD produced fewer colonies compared with Vector (data not shown). Overall, the results shown in Figure 2 d-e indicate that Snail promotes transformation in an oncogene-specific fashion, and is largely dispensable for transformation by NICD and ErbB2.

If the activity of SnaZFD in Gli1 transformation assays is due to interference with Snail, it might restore E-cadherin in Gli1 cells. We therefore examined E-cadherin by indirect immunofluorescence following transient transfection of E-cadherin-low Gli-C cells with the pcDNA3.1-SnaZFD vector (Figure 2f). For these studies, an electroporation protocol was optimized to enable transfection of >95% of cells, as indicated by a green fluorescent protein (GFP) control (not shown). Gli-C cells transduced with a vector control exhibited diffuse cytoplasmic staining and discontinuous, punctate membrane staining, but rarely exhibited continuous staining along the circumference of the cell (Figure 2f, Vector). In contrast, transfection with SnaZFD yielded cells with continuous staining along the cell periphery in nearly all fields examined (Figure 2f, SnaZFD). Immunoblot analysis revealed ~2-fold induction of E-cadherin by SnaZFD (not shown), consistent with restoration of E-cadherin expression in a subset of cells.

**Snail short hairpin RNAs (shRNAs) restore E-cadherin in Gli1 cells and suppress RK3E transformation by Gli1.** While inhibition of Gli1 transformation by SnaZFD is consistent with gain-of-function data indicating a role for Snail in this process, the dominant negative allele contains a conserved DNA binding domain that might compete
with other E-box binding proteins (Bolos et al., 2003). To further address a specific role for Snail, we generated six Snail shRNA expression constructs (pSna\textsuperscript{sh1-6}) and a control (pSi\textsuperscript{Ctl}) by insertion of annealed oligonucleotides downstream of a U6 promoter (Table 1). As described above for SnaZFD, each was co-transfected with Gli1 into RK3E cells (Figure 3a-b, Table 1). Four constructs (pSna\textsuperscript{sh2-5}) dramatically decreased focus formation, one (pSna\textsuperscript{sh6}) was weakly inhibitory, and one (pSna\textsuperscript{sh1}) had no effect.

To correlate shRNA modulation of Gli1 transformation and repression of Snail, constructs were analyzed for their ability to reduce Snail mRNA (Figure 3c) or to restore E-cadherin expression (Figure 3d) in Gli1 cells. In contrast to transient expression of pSi\textsuperscript{Ctl} or the non-inhibitory pSna\textsuperscript{sh1}, cells expressing the transformation-inhibitory constructs pSna\textsuperscript{sh2} and pSna\textsuperscript{sh3} exhibited increased peripheral expression of E-cadherin by immunofluorescence (Figure 3a, compare to untransformed RK3E, bottom). RT-PCR indicated that Snail transcripts were reduced by 33% or 41%, respectively, in cells expressing the Gli1 inhibitory constructs pSna\textsuperscript{sh4} and pSna\textsuperscript{sh5}, in correlation with their relative Gli1-inhibitory activities (Figure 3c, Table 1). Immunoblot analysis showed that expression of E-cadherin, unlike β-actin, was increased in Gli1 cells by two active shRNAs but not by the inactive pSna\textsuperscript{sh1} (Figure 3d). In summary, Snail shRNAs that inhibited transformation by Gli1 also inhibited Snail and induced expression of E-cadherin. Thus both gain- and loss-of-function studies indicate that Snail induction is limiting for Gli1-induced transformation.

**Conditional expression of Gli1 in the skin.** Expression of Gli1 or Gli2 in mouse skin induces outgrowth of BCC-like tumors (Nilsson et al., 2000; Grachtchouk et al., 2000; Oro and Higgins, 2003; Hutchin et al., 2005). To examine early events following
induction of Gli1, we employed the same tet-on strategy that we used for analysis of KLF4 (Foster et al., 2005) (Figure 4a). In this approach, an X chromosome-linked reverse tet-responsive transactivator (rtTAX) is expressed under control of a keratin 14 (K14) promoter, and administration of doxycycline (dox) results in rapid activation of tet response element (TRE)-linked transgenes.

Founders were identified using a PCR assay in which the endogenous mouse alleles served as an internal reference (Figure 4b). Each of 5 founders was crossed to K14-rtTA animals. The progeny were induced with dox and monitored for a skin phenotype.

**Gli1 rapidly induces hyperplastic lesions in the skin.** Three of the five TRE-Gli1 lines showed dorsal hair loss and outgrowth of discrete skin lesions after 4 weeks of induction (Figure 4c). Grossly, lesions affected the back, neck, and ears, and appeared as clusters of flattened or slightly raised plaques, with each plaque measuring one to several mm in diameter. Lesions were yellowish in color, and microscopic analysis showed nests of basaloid cells budding from the epidermis, with extension into the dermis (Figure 4c). There was no difference in latency when 4 week inductions were initiated in mid-gestation, in 14 day-old pups, at 6 weeks, or in older adults. Thus no specific role of the hair follicle cycle was apparent (not shown). This result is in contrast to the restriction of Gli1 activity to anagen phase skin in mice with expression of Gli1 under control of a constitutive Keratin 14 promoter (Oro and Higgins, 2003). This difference between the two models may be attributed to different expression strategies and/or distinct genetic backgrounds. In the studies shown below we utilized TRE-Gli1 line #10.

Immunostaining identified similarities between Gli1-induced skin lesions, hair
 Established lesions showed uniform expression of K17, a marker of follicles and BCCs, and recently implicated as a direct transcriptional target of Gli family members (Callahan et al., 2004) (Figure 5a-b). K16 marks hyperproliferative skin and is typically positive in squamous cell carcinoma (SCC), but negative in BCC. This cytokeratin was low in normal epithelial cells (Figure 5c), and likewise low in basaloid cells composing most of the lesion, but was positive in maturing keratinocytes overlying the hyperproliferative cells (Figure 5d). K1, which is normally induced upon commitment to terminal differentiation (i.e., in the parabasal cells of normal dorsal skin, Figure 5e), stained only the most superficial layer of cells within lesions, indicating a delay of differentiation, and did not stain the basaloid cells budding into dermis (Figure 5f). K14 marked the basal layer in normal skin (Figure 5g) and was uniformly positive within lesions (Figure 5h).

We utilized anti-HA antibody and indirect immunofluorescence to localize the Gli1 protein within lesions (Figure 6a). Gli1 was expressed in the interfollicular hyperproliferative epithelium, and in the outer root sheath of follicular epithelial cells. Consistent with the tight control of expression in this system (Foster et al., 2005), Gli1 was not detected without induction (Figure 6b). The strictly conditional nature of Gli1 expression in these animals is consistent with the absence of any skin phenotype in uninduced animals, and with the overall excellent health and fecundity of the transgenic lines. Primary keratinocytes obtained from bitransgenic mice exhibited prominent staining of the cytoplasm and nucleus following treatment with dox (Figure 6, c-e). At subconfluence in calcium-low culture media, these cells failed to exhibit any morphologic, proliferative, or apoptotic response to Gli1 (data not shown). The absence
of any apparent phenotype of Gli1 in these cells is consistent with the limited effect of Gli1 when expressed alone in other primary rodent cells (Ruppert et al., 1991). The failure of Gli1 to affect keratinocyte morphology or growth rate at subconfluence recapitulates results obtained when Shh was expressed in primary human keratinocytes (Fan and Khavari, 1999). Our studies do not preclude more subtle effects of Gli1 such as alteration of cell cycle occupancy following a differentiation stimulus, or induction of increased cell density at confluence, both of which were observed for Shh.

While these rapidly-induced, diffuse basaloid lesions exhibit certain molecular and morphologic similarities to BCC, they failed to form the large, focal tumors observed following longer term expression of Gli1 or Gli2 (Nilsson et al., 2000; Grachtchouk et al., 2000; Hutchin et al., 2005). We therefore sought to modify the phenotype by introducing genetic alterations observed in human tumors. We introduced deficient alleles of \( p53 \) and \( Ptch1 \), respectively, to obtain \( K14-rtTA_x\);TRE-Gli1;\( p53^{+/−} \) and \( K14-rtTA_x\);TRE-Gli1;\( Ptc1^{+/−} \) mice. In \( p53^{+/−} \) animals, Gli1-induced lesions were significantly enhanced, with a 2.3-fold increased in thickness (mean 194 mm vs 83.3 mm, \( P<0.0001 \); Supplement Figure 2). These enhanced lesions had prominent neovascularization, perhaps due to the increased growth potential of mesenchymal cells in \( p53^{+/−} \) animals, and/or to proliferative signals emanating from the neoplastic cells. In contrast to \( p53 \), the Gli1-induced skin phenotype was not altered in \( Ptc1^{+/−} \) animals (data not shown).

**Gli1 rapidly induces Snail transcripts and cell cycle progression in the interfollicular epidermis.** To temporally correlate activation of Gli1 with induction of Snail *in vivo*, we examined expression of the human Gli1 transgene and endogenous Snail and K17,
and monitored BrdU incorporation at intervals following administration of dox to bitransgenic mice (Figure 7). For these studies, littermates were induced at 40 days of age, when the hair follicle cycle is still synchronized and in the telogen phase.

Prior to induction there was no detectable expression of Gli1, Snail, or K17 in the interfollicular epidermis, and, of these, only K17 was present in normal follicle cells (Figure 7a, e, i, m). In the 6-12 hr interval following induction with dox, interfollicular cells concomitantly upregulated Gli1 and Snail, and incorporated more BrdU (Figure 7b, f, j). These increased until 48 hrs (Figure 7, a-l). Sections from t = 6 hr mice stained like t = 0 hr (not shown). Telogen follicle cells were slower to respond, showing increased Gli1, Snail, and BrdU signals only at 48 hrs. Compared to Snail and BrdU incorporation, K17 was slower to respond in the interfollicular cells, and became prominent only at 48 hrs. Thus, K17 may not be directly regulated by Gli1 in this setting, or its expression in interfollicular cells is restricted (Figure 7, m-p).

To determine if Snail is active during outgrowth of skin lesions, we examined E-cadherin in tissue sections from these same animals and from an animal with established lesions. Prior to induction, E-cadherin was similarly expressed in interfollicular cells and follicle cells (Figure 8a). E-cadherin largely persisted through the first 48 hrs of induction, although at this timepoint expression was lower in the interfollicular epidermis compared with adjacent follicle cells on the same slide (Figure 8b). This differential effect is consistent with the more rapid induction of Gli1, Snail, and BrdU in interfollicular epidermis compared to telogen follicles (Figure 7). More strikingly, E-cadherin was markedly reduced in established lesions, particularly in dysplastic basaloïd cells infiltrating the dermis (arrowheads, Figure 8c). In the
interfollicular cells of these animals, E-cadherin staining was lower where lesion thickness was greater (Figure 8c). These results recapitulate the loss of E-cadherin expression in infiltrating human BCC (Pizarro et al., 1994), and indicate that Snail may repress E-cadherin in response to Gli1 in vivo.

Discussion
Conditional expression of Gli1 in RK3E cells and in the basal keratinocytes of mouse skin demonstrated a temporal correlation of Shh pathway activity with Snail expression, and identified Snail as an early response to Gli1. Using a Snail dominant negative allele, multiple Snail shRNAs, and enforced expression of wild-type Snail, we identified Snail as a critical downstream effector of Gli1. Co-transfection of Gli1 with SnaZFD, Vector, and Snail represents a type of allelic series, with progressively increasing Snail activity. At the extremes, co-expression of SnaZFD vs. wild type Snail altered Gli1 transforming activity by 18-fold. Snail appeared specific in its ability to cooperate with Gli1, indicating that the mechanism of transformation by Gli1 is distinct from that of N-RAS, ErbB2, or Notch1. Such specificity is consistent with the induction of Snail by Gli1, but not by other genes such as KLF4 or c-MYC (Louro et al., 2002; Karhadkar et al., 2004). Our studies indicate that, in epithelial cells, the Shh signaling pathway extends to Snail to effect transformation in vitro. This signaling is a potential mechanism of E-cadherin repression in human BCC and other tumor types (Pizarro et al., 1994; Louro et al., 2002; Karhadkar et al., 2004).

EMT occurs at gastrulation and during formation of the neural tube, somites, and cardiac valves, and is essential for the establishment of complex three-dimensional...
tissues from epithelial precursors (Hay, 1995; Nieto, 2002; Thiery, 2003). Unlike epithelial cells, mesenchymal cells can invade and migrate through the extracellular matrix. EMT is partially or completely recapitulated invasive human carcinomas, such as BCC, and is believed to be a late event in tumor progression, when cells finally exit the epithelial compartment and become invasive and/or metastatic. EMT is regulated by signals that normally control cell fate, including the TGFβ, Notch, and Shh pathways (Hay, 1995; Thiery, 2003). During normal development and tumor progression, EMT is often linked to Snail family members such as Snail or Slug (Snai2) (Nieto, 2002).

In addition to their role in EMT, Snail family members function as regulators of proliferation or apoptosis (Cano et al., 2000; Batlle et al., 2000; Vega et al., 2004; Bachelder et al., 2005; Savagner et al., 2005; Jamora et al., 2005). Although expression in some cell types in vitro induces growth arrest, in other settings Snail induces proliferation and/or inhibits cell death. When expressed in the basal keratinocytes of transgenic mice under control of a K14 promoter, Snail activated the RAS-Mitogen-activated protein kinase pathway, repressed expression of components of the hemidesmosomes and the underlying basement membrane, and induced proliferation (Jamora et al., 2005). GSK3β represses Snail in keratinocytes (Bachelder et al., 2005), and Slug is an effector of cell migration during wound healing in the skin (Savagner et al., 2005). Thus, the normal function of Snail family members extends beyond development to include homeostasis and healing in adult epithelium.

Both Shh and Snail are implicated in folliculogenesis. Shh-deficient mouse embryos initiate hair follicle morphogenesis, but follicles arrest at an early stage due to impaired epithelial proliferation and failure to form a dermal papilla (St Jacques et al.,
1998; Chiang et al., 1999; Callahan and Oro, 2001). In addition, Shh and Gli1 are expressed in the distal portion of adult anagen follicles, probably to enable rapid downward expansion (Ghali et al., 1999; Oro and Higgins, 2003). Gli1 signaling through Snail may contribute in several respects to this process, promoting epithelial proliferation and remodeling of the basement membrane and dermis. We previously identified transcripts of Snail, Gli1, and multiple Gli1 target genes in morphologically normal anagen follicles adjacent to human BCCs (Louro et al., 2002). Similarly, Snail is transiently induced in proliferating epithelial cells of the embryonic hair bud, through the action of TGFβ2 (Jamora et al., 2005).

Our observation that Snail, like Gli1, induces membrane blebbing may help explain the minimal morphological alteration of RK3E cells upon transformation by Gli1 (Ruppert et al., 1991). In contrast to Gli1 cells, RK3E cells transformed by RAS or KLF4 show prominent membrane extensions and markedly increased spindling and refractility. Membrane blebbing has been ascribed to the inability of cells to produce more typical membrane extensions, possibly because of reduced viscosity of the peripheral actin gel (Cunningham et al., 1997; Flanagan et al., 2001; Straight et al., 2003). For melanoma cells, a tumor type that consistently expresses Snail (Poser et al., 2001), this phenotype is attributed to deficiency in the cross-linking protein Filamin A/Actin-binding protein. Our results link Snail expression to membrane blebbing, which may result from reduced expression of E-cadherin and/or from another activity of Snail (Ohkubo and Ozawa, 2004). Whether this indicates a role of Snail in regulation of Filamin A or other components of the cortical actin gel warrants further study.

Previously it was shown that constitutive Shh or Smo transgenes induced
BCC-like lesions in the interfollicular epithelium of embryonic mouse skin (Oro et al., 1997; Xie et al., 1998). Our results demonstrate that Gli1 can rapidly convert interfollicular keratinocytes to proliferating, Snail-positive cells with morphological and molecular similarities to BCC. The tet-on Gli1 mouse showed clearly that adult, mammalian interfollicular epidermis, a setting relevant to human BCC tumorigenesis, is competent to rapidly respond to Shh pathway activity.

Under regulation of the same K14-rtTA transgene as used in the current study, KLF4 induced SCC-like lesions, with nuclear hyperchromicity and pleomorphism of superficially invading cells (Foster et al., 2005). The distinct phenotypes induced by Gli1 or KLF4 in the skin are consistent with expression studies and genetic data linking these genes to BCC and SCC tumorigenesis, respectively (Dahmane et al., 1997; Foster et al., 1999; Foster et al., 2000; Foster et al., 2005). Unlike Gli1-induced lesions, KLF4-induced lesions were negative for K17 and diffusely positive for K1 and K16. In contrast to the BCC phenotype induced by constitutive Gli1 transgenes (Nilsson et al., 2000; Oro and Higgins, 2003), the limited growth induced by short-term expression of Gli1 in our study suggests that other alterations are needed for progression to BCC. Such alterations may involve other Shh pathway members, such as Gli2 or Gli3, or BCC tumor suppressors such as Notch1. In addition, it will be important to examine the role of Snail in Shh-pathway mediated tumor progression in vivo, using gene deletion or conditional expression of Snail antagonists such as SnaZFD or Snail shRNAs.

Materials and Methods

Plasmid Construction  For tet-on induction in RK3E cells, an HA-tagged allele of human, wild type Gli1 was released from pBluescript (Louro et al., 2002) using XbaI and
HindIII, blunted with T4 polymerase, and cloned into the EcoRV site of pcDNA4/TO (Invitrogen).

For generation of TRE-Gli1 transgenic mice, the HA-Gli1 cDNA was linker-adapted and inserted into the XbaI site of pXP2-TRE-hGFAT (a gift from J. E. Kudlow). For microinjection, a 5.7 kb fragment was released using SalI and ClaI.

SnaZFD, encoding bases 366-841 of Genbank accession M95604, was amplified from first strand cDNA of mouse NIH3T3 cells. The forward primer (5’cgcggatccgctctggccaacatggctctccttggag 3’) contained a BamHI site (underlined) followed by translation initiator sequences (italics) and a region upstream of the ZFD. The reverse primer (5’ ccggaaatcagatgccacggag 3’) contained an EcoRI site (underlined) and hybridized to the Snail 3’ UTR. SnaΔZFD, encoding the region up to base 488 of M95604, was released from pcDNA3-mmSnail-HA (Batlle et al., 2000) using BamHI and SspI. For use in transient transfection assays, the fragments were inserted into the pcDNA3.1+ (Invitrogen) and verified by sequencing. For stable expression, inserts were transferred to the MMLV-LTR vector pBABE-puro (Morgenstern and Land, 1990).

Employing recombinant PCR, alanine 648 (GCC) in the wild type ErbB2 cDNA (NM_004448) was altered to encode cysteine (TGC) (di Fiore et al., 1987; Kraus et al., 1987). The Xho I-adapted ErbB2 coding sequence was cloned into the Sal I site of pBABE-puro to yield pBpuro-ErbB2A648C. The mouse Notch1 cDNA (NICD), a gift of C. Jane McGlade (McGill and McGlade, 2003), was inserted into pBABE-puro.

Duplexes encoding Snail shRNAs (Table 1) were cloned into pSilencer™ 2.1-U6 neo (Ambion), transfected in XL1-Blue cells (Stratagene), and verified by sequencing.
pSi$^{+}\text{ctl}$ encodes a hairpin transcript without identity or perfect complementarity to Snail or other cellular transcripts in Genbank (release 147.0), and was constructed by reversal of sequence in pSna$^{sh4}$.

**Cell transfection and retroviral transduction**  pcDNA4/TO-HA-Gli1 and pcDNA6/TR (Invitrogen) were linearized with ScaI and SapI, respectively, purified from an agarose gel (Qiagen), and transfected at a 1:6 mass ratio into RK3E cells by electroporation (parameters available upon request). Colonies were selected in blasticidin (1.0 µg/ml) and zeocin (Invitrogen, 10.0 µg/ml). At 2 weeks post-transfection, individual clones were transferred to 24-well plates and expanded for further analysis.

Gli1 and H-Ras expression plasmids used for *in vitro* transformation assays were described previously (Ruppert et al., 1991). Focus assays were performed in 10 cm dishes, without selection, following Lipofectamine-mediated plasmid transfection (Invitrogen) (Ruppert et al., 1991). For analysis of Snail, SnaZFD and shRNAs, 2.0 µg of Gli1, NICD, or ErbB2 vector were co-transfected with 8.0 µg of the indicated plasmid. Retroviral transduction of RK3E and selection in puromycin were previously described (Foster et al., 1999; Louro et al., 2002). Colony assays following plasmid transfection utilized selection in puromycin. Transformation by retroviral transduction used the MMLV-LTR vector pLJD-HA-Gli1 (Louro et al., 2002), pCTV4-N-Ras (a gift of Robert Kay), and pLJD (a control). Phase contrast microscopic images were captured using an Axiovert 25 inverted microscope equipped with an Axiocam digital camera (Zeiss).

**Transient transfection and luciferase reporter assays** Twenty-four hrs before transfection, HEK293 cells were plated at $2 \times 10^5$ cells per well in 6-well plates.
Transfection mixtures included 0.5µg of E-cadherin promoter-luciferase reporter plasmid (Batlle et al., 2000), 0.1 µg of Renilla luciferase reporter, 0.5µg of pcDNA3.0-mmSnail-HA (Batlle et al., 2000) and the indicated amount of pcDNA3.1-SnaZFD, pcDNA3.1-SnaΔZFD, or pcDNA3.1 (Invitrogen). pcDNA3.1 was used to standardize the total amount of DNA. TransIT®-LT1 Reagent (6µl, Mirus) was used according to the manufacturer’s instructions. Extracts were prepared in Passive Lysis Buffer and luciferase activities were determined 48 hrs post-transfection.

**Generation and analysis of transgenic mice**

C57BL/6 (B6) X SJL (J) F2 fertilized ova were treated by microinjection in the Transgenic Animal/Embryonic Stem Cell Core Facility at the University of Alabama at Birmingham. Transgenic founders were identified by PCR. PCR primers for genotyping were 5’cctgtttgggatgctggatgg3’ and 5’ggcctcagctccctggagca3’. TRE-Gli1 founders were crossed to B6;J F2 animals, and these lines were subsequently crossed to mice transgenic for X chromosome-linked alleles of K14-rtTA. Following fixation and embedding in paraffin, hematoxylin- and eosin-staining was performed by the Tissue Procurement Core Facility of the University of Alabama at Birmingham. The described gross or microscopic results were observed in five or more animals, with complete penetrance.

To generate TRE-Gli1 mice with the genotypes indicated for K14-rtTA\textsubscript{X} and p53, the three crosses used (male X female) were: p53\textsuperscript{+/−} X rtTA\textsubscript{X}\textsuperscript{+/−}, TRE-Gli1\textsuperscript{+/−};rtTA\textsubscript{X}\textsuperscript{+/Y} X rtTA\textsubscript{X}\textsuperscript{+/+}, and p53\textsuperscript{+/−}; rtTA\textsubscript{X}\textsuperscript{+/Y} X TRE-Gli1\textsuperscript{+/−};rtTA\textsubscript{X}\textsuperscript{+/+}. Oligonucleotides, cycling parameters, and electrophoretic parameters for genotyping are available upon request. Dox (Sigma) was administered at 2.0 mg/ml in 5% sucrose water in amber bottles, and was changed three times per week.
**Immunostaining** Keratins were detected in paraffin sections as described (Foster et al., 2005). K17 polyclonal antibody, a gift from Pierre Coulombe, was used at 1:2000 (McGowan and Coulombe, 1998). Immunodetection was performed using the brown chromogen diaminobenzidine (BioGenex).

E-cadherin was detected in Gli1 cells at 72 hrs post-transfection, or in RK3E cells, using primary antibody at 0.5 µg/ml (BD Biosciences #610181) and a red fluorescent secondary antibody as described (Pandya et al., 2004). For analysis of transfected cells, 20 random fields were examined at 400-fold magnification, and images corresponding to representative fields were recorded using epifluorescence. HA-Gli1 was similarly detected in primary keratinocytes using 12CA5 (Roche). For detection of HA-Gli1 in vivo, mouse skin cryosections were fixed for 10 minutes at room temperature in 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed in PBS, and blocked in 20% goat serum in binding buffer (Pandya et al., 2004). The rat anti-HA antibody 3F10 (Roche) was used at 0.5 µg/ml in 2% goat serum, and bound antibody was detected using a red fluorescent secondary antibody (Molecular Probes #A11017). Nuclei were stained in DAPI and digital images were captured as described (Pandya et al., 2004).

**mRNA expression studies** For RT-PCR analysis of cultured cells, total RNA was extracted using RNeasy (Qiagen). RT reactions contained 3.0 µg of total RNA per sample, oligo-dT(12-18) at 50 µg/ml, and SuperScript™ II RT (Invitrogen). First-strand product was diluted to 200 µl, and 1-3 µl were used as PCR template. Primers are shown in Supplement Table 1. PCR reactions were terminated at intervals between 18 and 30 cycles, and products were detected by scanning of ethidium-bromide stained gels using a Typhoon 8600 (GE Biosystems). For selected samples, variation of input cDNA
was used to ensure the semi-quantitative nature of the reactions.

mRNA *in situ* hybridization analysis of paraffin-embedded sections of mouse skin was performed using digoxigenin (dig)-labeled transcripts as described (Bardelli et al., 2003). Templates for *in vitro* transcription were isolated from total mRNA of mouse NIH3T3 cells by RT-PCR, and PCR primers incorporated a T7 RNA polymerase binding site. Forward and reverse primers for Gli1 were as described (Louro et al., 2002). For antisense Snail, primers were: 5’ cgtagagctgacctcgctgtccgat 3’ and 5’ ggatctaatctcgctgtccgat3’. For the Snail sense control probe, primers were: 5’ggatctaatctcgctgtccgat3’ and 5’ttcagagcgcccaggtgctagt3’. Hybridized transcripts were detected using enzyme-antibody conjugates and the alkaline phosphatase substrate BCIP/NBT Blue (Sigma #B3804). Counterstaining was performed using Nuclear Fast Red (Sigma #N3020).

**Immunoblot analysis** Cells were lysed in Laemmli buffer and extracts were quantitated as described (Sheffield et al., 1987). An immunoaffinity purified, rabbit antibody raised against residues 410-427 of human Gli1 was used at 1.7 µg/ml (Geneka/Active Motif). E-cadherin antibody (BD Biosciences #610181) was used at 0.1 µg/ml, and β-Actin antibody (Sigma #A5316) was used at 0.26 µg/ml. Bound antibodies were detected using the ECL method (Amersham Biosciences).

**In vivo BrdU incorporation** BrdU reagent (Zymed #40286619, 1.0 ml/100 gr body weight) was injected into the peritoneum 2 hrs before sacrificing. BrdU was detected by immunostaining of paraffin sections as directed by Zymed (#93-3943).
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**Figure 1** Regulation of Snail and E-cadherin by Gli1 *in vitro*.  

**(a)** RK3E epithelial cells were stably transduced with plasmids conferring tet-inducible Gli1 expression as described in the Materials and Methods. Cell lines were expanded from single colonies, induced with tet or vehicle control for 6 hours, and examined for Gli1 expression by immunoblot (left panel). A smaller, unidentified species detected by the antibody served as a loading control (LC). Semi-quantitative RT-PCR was used to detect the Gli1 target genes Ptch1 and Snail (right panel).  

**(b)** The time-course of Ptch1 and Snail induction was examined by RT-PCR analysis of a tet-on Gli1 cell line. Gapdh served as a control.  

**(c)** RK3E cells were stably transduced with pBpuro-Snail (Snail cells) or Vector control retrovirus (Vector cells). RT-PCR was used to detect expression of the Snail transgene, endogenous E-cadherin and Gapdh.  

**(d)** Morphology of Snail cells, Vector cells, and Gli1-transformed RK3E cells (Gli1 cells) by phase contrast microscopy. Arrows indicate examples of Snail cells with blebbing of the cytoplasmic membrane. Additional examples are shown in greater detail in the inset. Scale bars, 50µm (Vector, Snail) or 20µm (Gli1).  

**(e)** Immunoblot analysis utilized parental RK3E and transformed cell lines previously derived from oncogene-transformed foci (186). The filter was queried sequentially with the indicated antibodies. β-actin served as a loading control.
Figure 1
Figure 2  Characterization of dominant negative alleles of Snail. (a) Schematic of wild type mouse Snail, SnaΔZFD and SnaZFD. The approximate position of GSK3β phosphorylation sites is indicated (asterisk) (Zhou et al., 2004; Yook et al., 2005). (b) Truncated Snail alleles were analyzed for interference with Snail-mediated repression of E-cadherin promoter activity. Plasmids were transfected into HEK293 cells, and cell extracts were analyzed using the Dual Luciferase Reporter Assay. Three experiments were performed in triplicate and standard error bars are shown. (c) Endogenous Snail in HEK293 cells was detected by RT-PCR. No product was detected without addition of RT. (d) Snail and SnaZFD were analyzed for modulation of oncogene transforming activity by lipofectamine-mediated co-transfection of the indicated plasmids (Construct 1, Construct 2) into RK3E cells. Wright-stained petri dishes contained foci of transformed cells on a monolayer of untransformed RK3E (Transformation assay, columns 1-4), or else colonies of puromycin-resistant cells that survived 7 days in selective culture media (Colony assay, column 5). (e) Transformation efficiency was plotted on a log_{10} scale. Results for Gli1 represent 3 independent experiments performed in triplicate, and standard error bars are shown. Results for NICD and ErbB2 represent one experiment performed in triplicate, and standard deviation bars are shown. (f) Gli-C cells were electroporated with the indicated expression plasmid. A GFP control vector indicated successful transfection of nearly all cells (not shown). E-cadherin expression (red) was visualized 48 hrs post-transfection by indirect immunofluorescence. Nuclei were stained with DAPI (blue). Scale bar, 10µ.
Figure 2

(a) Sna\textsuperscript{\textregistered}ZFD and Snail proteins were detected by Western blotting in HEK293 cells. (b) E-Cadherin promoter activity was measured in HEK293 cells transfected with varying concentrations of Snail and Sna\textsuperscript{\textregistered}ZFD. (c) HEK293 cells were transfected with constructs containing Snail and/or Sna\textsuperscript{\textregistered}ZFD and subjected to a transformation assay. (d) Representative images from the transformation and colony assays. (e) Transformation efficiency was quantified for different constructs. (f) Immunofluorescence images of cells expressing Sna\textsuperscript{\textregistered}ZFD and Snail proteins.
**Figure 3** Inhibition of Gli1-mediated transformation by Snail shRNAs.  

(a) shRNA expression vectors were tested for modulation of Gli1 transforming activity (left) and for ability to alter expression or localization of E-cadherin in Gli-C cells (right). RK3E cells served as a positive control for E-cadherin (bottom right). The transformation assays shown were performed once in triplicate. Similar results were obtained in an independent experiment, performed in duplicate, that utilized distinct shRNA constructs. For immunostaining assays, Gli1-transformed cells were transduced by electroporation with the indicated shRNA expression vector and examined at 72 hrs post-transfection. 

(b) Quantitation of the transformation assays shown in panel a. Standard deviation bars are shown. 

(c) RT-PCR analysis of Snail transcripts in Gli1 cells following transient transfection of shRNA constructs. 

(d) Immunoblot analysis of E-cadherin in Gli1 cells transfected with the indicated construct. β-actin served as a control for loading.
Figure 3
Figure 4  Tet-inducible Gli1 transgenic animals exhibit hyperproliferative skin lesions. 

(a) Schematic of the HA-tagged, human Gli1 transgene, showing the tet response element (TRE), the minimal CMV promoter (PminCMV), and the SV40 intron and polyadenylation signal. Restriction sites used for generation of the microinjection fragment are shown (SalI, ClaI). (b) PCR analysis used one pair of conserved primers, derived from exons 6 and 7, to detect both the mouse gene and the human transgene. Control DNAs were mouse genomic DNA alone or else mouse DNA admixed with a molar excess of Gli1 cDNA. (c) Histology of the skin following induction of Gli1 for 4 weeks in transgenic lines derived from 3 independent founders. A mouse of line 10 served as a control and exhibited morphologically normal skin (No dox). Scale bar, 50µ.
Figure 4
**Figure 5** Immunostaining of Gli1-induced lesions. Dox was administered to wild type (Wt) or TRE-Gli1 mice for 4 weeks, and the indicated antibodies were applied to sections of dorsal skin. Arrowheads indicate the dermo-epidermal junction (DEJ). Scale bar, 50 µ.
Figure 5
**Figure 6** Expression of Gli1 in tissues and cells by indirect immunofluorescence. Antibody to the aminoterminal HA epitope was used to localize Gli1. All panels show merged red (antibody) and blue (nuclei) images. **(a)** Frozen section of a Gli1-induced skin lesion following 4 weeks of dox. Arrowheads indicate the DEJ. **(b)** Control skin from an animal that was not induced with dox. **(c)** Primary keratinocytes from a K14-rtTA<sub>X</sub>;TRE-Gli1 newborn mouse were induced with dox overnight. **(d)** Uninduced keratinocytes from a K14-rtTA<sub>X</sub>;TRE-Gli1 mouse. **(e)** Further magnification of the image shown in **c**. Scale bars, 50µ.
Figure 6
Figure 7 Skin alterations in the period immediately following induction of Gli1. Dox was added to the drinking water of 40 day old K14-rtTA\textsubscript{X};TRE-Gli1 littermates for the indicated interval, and the telogen-phase skin was analyzed. Expression of Gli1 \textit{(a-d)} and Snail \textit{(e-h)} were monitored by \textit{in situ} hybridization using anti-sense RNA probes. A Snail sense probe served as negative control and exhibited no signal in any section (not shown). BrdU incorporation \textit{(i-l)} and K17 expression \textit{(m-p)} were determined by immunostaining. Sections taken at 6 hrs were similar to the zero timepoint, indicating that Gli1, Snail, and BrdU were co-induced at 6-12 hrs. Arrowheads point to the DEJ. Insets \textit{(e-h)} show the epidermis at higher magnification. Scale bars, 50\textmu m.
Figure 7
Figure 8  Analysis of E-cadherin expression during Gli1-induced neoplastic progression.

(a-c) K14-rTAE;TRE-Gli1 mice were induced with dox for the indicated interval. Sections of skin corresponding to no treatment (No Dox); t=6, 12, or 24 hr; or t=42 days were stained in parallel with antibody to E-cadherin. Staining is indicated by a brown precipitate. (c) At 42d, adjacent areas from a single tissue section are shown, corresponding to less involved skin (left panel) or more involved skin (right panel). Arrowheads indicate epithelium with reduced staining. No signal was observed using as control a normal mouse IgG at the same concentration (not shown). Asterisks indicate hair follicles, and arrows indicate the DEJ. Scale bar, 100µ.
Supplement Figure 1 Snail-transduced RK3E cells are more susceptible to transformation by Gli1. (a) Pooled populations of stably transduced RK3E cells (Construct 1) were treated with the indicated retroviral supernatant (Construct 2) and then incubated at confluence to detect focus formation. Three weeks after transduction, cells were fixed and stained as in Figure 2d. The experiment was performed 3 times in duplicate. (b) Relative transforming efficiencies were calculated after counting of foci. Standard error bars are shown.
Supplement Figure 1

(a) Construct 1

Vector  |  Snail
-------|-------
Vector  |       |
Construct 2
Gli1     |       |
N-Ras    |       |

(b) Transformation efficiency (%)

<table>
<thead>
<tr>
<th>Construct 1:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
</tr>
<tr>
<td>Snail</td>
</tr>
</tbody>
</table>

Gli1  |  N-Ras
-----|------
0     | 0
Supplement Figure 2 p53 deficiency cooperates with Gli1 to promote outgrowth of lesions. (a) Littermates of the indicated genotype were induced with dox for six weeks. Neovascularization of lesions is indicated by arrowheads. (b) Following acquisition of digital images, lesion thickness was measured using tools within Axiovision software (Zeiss). The two-tailed P value was calculated using the t-test. Standard deviation bars are shown. Scale bar, 50µ.
Supplement Figure 2
<table>
<thead>
<tr>
<th>ShRNA</th>
<th>Targeted region(^a)</th>
<th>Insert sequence(^b) (BamHI-Sense-loop-antisense-terminator-HindIII)</th>
<th>Reduction of Gli1 transformation (%)</th>
</tr>
</thead>
<tbody>
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<td>sh1</td>
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<tr>
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<tr>
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<tr>
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<td>GATCCCGccctccggaagttgactctTCAAGAGAtaaaacgtcaactttccgcgaaggTTTTTTGGAAA</td>
<td>control</td>
</tr>
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</table>

\(^a\)Numbering system refers to rat Snail (Genbank accession NM_053805), where the protein coding region is 81-875.  
\(^b\)The strand shown corresponds to the final RNA Polymerase III transcript, with the +1 base in bold. Restriction sites, hairpin loop sequences, and terminator sequences are upper case.
### Supplement Table 1  Oligonucleotides for RT-PCR

<table>
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<tr>
<th>Transcript</th>
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<th>Reverse primer (5’-3’)</th>
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</thead>
<tbody>
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<td>TTCAGAGCGCCCAGGCTGAGGTACT</td>
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<tr>
<td>Snail (rat)</td>
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<td>GGCTGAGGTACTCCTTATTAC</td>
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<tr>
<td>Snail (human)</td>
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<td>CACGCTGGCACTGTTACTTCT</td>
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<td>Ptc1 (rat)</td>
<td>GAGACCAACGTGGAGGAGCTGT</td>
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<td>Gapdh (rat, human)</td>
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<tr>
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<td>ATCTAAAGCTTCACAAGCTGGA</td>
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GLI1 ACTS THROUGH SNAIL AND E-CADHERIN TO PROMOTE NUCLEAR SIGNALING BY β-CATENIN

by

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AND J. MICHAEL RUPPERT

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Abstract

The Hedgehog pathway transcription factor Gli1 induces transformation of epithelial cells in part through induction of Snail, a represser of E-cadherin. E-cadherin is normally complexed with β-catenin at the cell membrane. Loss of E-cadherin during developmental epithelial-mesenchymal transitions can switch β-catenin from its role at adherens junctions to its role in nuclear transcription. However, during tumor progression it is unclear which pathways might trigger this switch. In the current study, gain- and loss-of-function approaches identified E-cadherin as a selective suppressor of transformation by Gli1, and Snail loss-of-function was rescued by down-regulation of E-cadherin. Gli1 induced a loss of β-catenin at the cell membrane and promoted expression in the nucleus. Wild-type or mutant alleles of E-cadherin modulated transformation by Gli1 concordantly with their ability to promote or inhibit nuclear localization of β-catenin, and inhibition of Wnt pathway activity by dominant negative Tcf4 selectively blocked transformation by Gli1. Consistent with these in vitro studies, a mouse model of Gli1-induced skin tumors showed unphosphoryated β-catenin in tumor cells infiltrating the dermis. These studies identify E-cadherin as a selective suppressor of transformation by Gli1 and point to the Shh-Gli1 pathway as a key regulator of the β-catenin switch in epithelial cells and cancers.
Introduction

The Sonic Hedgehog (Shh) signaling pathway plays a central role in cell fate determination and developmental patterning. Deregulated SHH signaling is implicated in the initiation, progression, and/or maintenance of diverse human tumor types, including those with mutations in SHH pathway components such as cutaneous basal cell carcinoma (BCC) (Toftgard, 2000; Taipale and Beachy, 2001; Pasca and Hebrok, 2003; Karhadkar et al., 2004; Xie, 2005; Sanchez et al., 2005; Hooper and Scott, 2005; Kayed et al., 2006). Key effectors of Shh signaling include the three zinc finger proteins of the Gli family. Gli1 and Gli2 regulate transcription of specific target genes to promote cellular transformation. In cultured RK3E epithelial cells and in mouse skin, Gli1 rapidly induces transcription of Snail, a zinc finger protein that represses transcription of E-cadherin and promotes the epithelial-mesenchymal transition (EMT) (Cano et al., 2000; Batlle et al., 2000; Louro et al., 2002; Li et al., 2006). Snail short-hairpin RNAs (shRNAs) or a dominant-negative (DN) allele blocked transformation of RK3E cells by Gli1, while exogenous Snail cooperated with Gli1 to induce transformation (Li et al., 2006). In contrast to these effects on Gli1, wild-type (WT) or DN Snail had no effect on the transforming activity of several control oncogenes including ErbB2, Notch1, or N-Ras.

E-cadherin is a type I integral membrane protein that localizes to adherens junctions and mediates Ca$^{2+}$-dependent, cell-cell adhesion (Semb and Christofori, 1998; Nelson and Nusse, 2004; Cavallaro and Christofori, 2004; Gumbiner, 2005; Junghans et al., 2005; Brembeck et al., 2006). The C-terminus of E-cadherin is linked to $\alpha$-catenin and the actin cytoskeleton through tight association with $\beta$-catenin, the mammalian homolog of Drosophila armadillo. In addition to its role in cell-cell
adhesion, β-catenin plays a central role as a nuclear effector of Wnt signals that induce cell proliferation and transformation. Wnt signals stabilize β-catenin by inhibiting GSK3β, which normally phosphorylates the N-terminus of β-catenin and targets it for degradation (Cadigan and Nusse, 1997; Polakis, 2000; Nelson and Nusse, 2004; Bienz, 2005; Brembeck et al., 2006). The increased β-catenin then translocates to the nucleus where it associates with the DNA binding proteins Lef/Tcf and other factors. In this complex, the C-terminus of β-catenin mediates the transactivation of Wnt pathway targets such as Cyclin D1.

The stability and function of the E-cadherin–β-catenin complex is regulated by diverse signaling pathways via phosphorylation (Nelson and Nusse, 2004; Brembeck et al., 2006). Participation of β-catenin in cell-cell adhesion or Wnt signaling is determined by competitive binding of β-catenin to α-catenin and the actin network versus Wnt signaling components in the cytoplasm (Gottardi and Gumbiner, 2004; Bienz, 2005; Brembeck et al., 2006). Switching of β-catenin from cell adhesion to Wnt signaling can be induced by tyrosine phosphorylation of β-catenin, which promotes binding to BCL9-2 instead of α-catenin. Likewise, suppression of E-cadherin by Snail can induce switching of β-catenin during gastrulation (Ciruna and Rossant, 2001). In response to localized Wnt signals in the primitive streak, FGF induces Snail, leading to repression of E-cadherin and rapid accumulation of cytosolic β-catenin levels. By this mechanism FGF induces an EMT that is essential for invagination and morphogenesis of the mesoderm. By analogy, loss of E-cadherin during tumor progression could likewise promote Wnt signaling (Nelson and Nusse, 2004; Bienz, 2005). However, specific contexts in which the cadherin-bound pool of β-catenin is released for signaling in the nucleus remains poorly understood.
E-cadherin loss-of-function in tumors or cell lines is not often associated with increased β-catenin signaling, nor is cell-cell adhesion consistently altered by Wnt signals (Caca et al., 1999; van de et al., 2001; Nelson and Nusse, 2004; Gottardi and Gumbiner, 2004; Gumbiner, 2005).

Several observations indicate that Wnt signaling can be a downstream effector of Shh. In *Drosophila*, *wingless* is directly regulated by *Ci* and mediates hedgehog effects in the imaginal disc (Vonohen et al., 1997; Von Ohlen and Hooper, 1997). In *Xenopus* animal caps, induction of Wnt8 and Wnt11 is important for Gli2-mediated alteration of the posterior mesoderm, as antagonists of Wnt signaling inhibit the Gli2-induced phenotype (Mullor et al., 2001). Human BCCs, which frequently show mutations in Shh pathway components such as Ptc1 or Smo (Toftgard, 2000; Pasca and Hebrok, 2003; Hooper and Scott, 2005), show increased levels of Wnt2b and Wnt5a (Bonifas et al., 2001). Consistent with a role for Wnts in the response to Shh, many BCCs show increased levels of nuclear β-catenin (Yamazaki et al., 2001; El Bahrawy et al., 2003; Saldanha et al., 2004). As observed for Shh pathway genes such as Gli1, Gli2 or Smo, β-catenin likewise promotes follicle-like tumors or BCCs in transgenic mice (Gat et al., 1998; Nicolas et al., 2003; Silva-Vargas et al., 2005). Although Shh and Wnt signals are coordinated at multiple levels, downstream effectors of growth or transformation by the Shh/Gli pathway remain incompletely understood, including the possible role of Wnt/β-catenin signaling.

In the current study we used a DN Tcf4 to determine that canonical Wnt signaling is required for in vitro transformation by Gli1. Nuclear β-catenin was increased in Gli1-transformed cells in vitro and in the E-cadherin-deficient, BCC-like lesions induced by Gli1 in transgenic mice. A role of E-cadherin in this deregulation was suggested by ability of WT E-cadherin to suppress levels of nuclear β-catenin and
to suppress transformation by Gli1. These suppressive effects of E-cadherin required binding of β-catenin. In addition we used a cell adhesion-deficient, IL2Rα--E-cadherin chimera that contains the extracellular and transmembrane domains of the IL2R fused to the cytoplasmic domain of E-cadherin. In RK3E cells this construct induced the accumulation of cytosolic β-catenin on its own and, in the presence of Gli1, promoted nuclear localization of β-catenin and malignant transformation. These studies identify E-cadherin as a selective suppressor of transformation by Gli1 and suggest a model in which signaling by Gli1 and Snail can induce the β-catenin switch during epithelial transformation.

Results

DN Tcf4 inhibits transformation by Gli1 but not by other oncogenes  

Tcf alleles lacking the N-terminal β-catenin binding sequence act as DN s and have been used repeatedly to inhibit Wnt signaling (Molenaar et al., 1996; van de Wetering et al., 2002). To test for a role of Wnt signaling during epithelial transformation by Gli1, we utilized a DN Tcf4 allele, Tcf4ΔN31. This allele blocks transformation of RK3E by activated β-catenin or by WT γ-catenin but permits transformation by activated RAS, which served as a control (Kolligs et al., 1999; Kolligs et al., 2000). As compared to parental RK3E cells, Gli1-induced foci in RK3E-Tcf4ΔN31 were reduced by 85% (mean no. of foci/dish: RK3E-Tcf4ΔN31, 12.8; RK3E, 85.7; Figure 1a and b). Quantitation was performed on stained dishes by counting foci > 0.5 mm in diameter. The few foci induced by Gli1 in Tcf4ΔN31 cells (compare Gli1 and Vector columns in Figure 1a) are comparable in size and number to those induced by activated β-catenin in these cells (Kolligs et al., 1999).

The controls ErbB2 or H-Ras induced transformation similarly in the two cell lines (Figure 1a and b). ErbB2 induced 31 and 29 foci/dish, while H-Ras induced 93
and 97 foci/dish in RK3E and RK3E-Tcf4\(\text{AN31}\), respectively. In summary, Tcf4\(\text{AN31}\) similarly suppresses transformation by Gli1, \(\beta\)-catenin or \(\gamma\)-catenin, indicating that Wnt signaling is probably essential for Gli1-mediated transformation.

**Rapid induction of Wnt2b by Gli1**

Multiple Wnt ligands were previously shown to be expressed in Gli1-induced skin tumors and in human BCCs (Bonifas et al., 2001; Mullor et al., 2001). Of these, we found that *Wnt2b* transcripts are upregulated in RK3E cells analyzed 10 days after retroviral transduction of a Gli1 vector (Louro et al., 2002). To better define the temporal relationship between induction of Gli1 and Wnt2b, we used RTOG10 cells, a clone of RK3E that contains a tetracycline (tet)-inducible Gli1 allele (Li et al., 2006). By semi-quantitative RT-PCR, transcripts of *Wnt2b* were induced between 3 and 6 hours following addition of tet to the media (Figure 1c, lanes 1-4). Compared to RK3E cells, Wnt2b was also increased in Gli-C cells, a Gli1-transformed clone of RK3E (lanes 5-6). Snail served as a positive control and was strongly induced at 3 hrs as shown previously (Li et al., 2006), and Gapdh served as a control for loading. The slower induction of Wnt2b compared with Snail could reflect the greater length of Wnt2b (\(~17\) kb) compared to Snail (\(~4.5\) kb) or may indicate that Wnt2b is an indirect target of Gli1. Nevertheless, these results show that Gli1 can rapidly induce a Wnt family member that is also upregulated in human BCC (Bonifas et al., 2001).

**Gli1 induces the accumulation of transcriptionally active \(\beta\)-catenin in cell nuclei**

To determine whether or not Gli1 can alter the localization of \(\beta\)-catenin, we first analyzed Gli-C cells and RK3E cells by indirect immunofluorescence. In RK3E cells, \(\beta\)-catenin was at the cell periphery, and did not appreciably overlap the DAPI-stained nuclei (Figure 2a, see \(\beta\)-cat/DAPI panel) or the actin cytoskeletal network (see \(\beta\)-cat/phalloidin panel). In contrast, Gli-C cells showed reduced
staining at the cell periphery, and instead showed prominent, diffuse staining of the cytoplasm and nucleus, similarly as observed previously for colorectal cancer cells with activated Wnt signaling (Kolligs et al., 2000) (Figure 2a, lower panels).

Immunoblot analysis of whole cell extracts of RK3E and Gli-C cells showed that overall levels of β-catenin were similar in these two lines (data not shown). We used immunoblot analysis to analyze the nuclear and cytoplasmic pools of β-catenin (Figure 2b). Compared to RK3E cells, Gli-C cells showed increased nuclear β-catenin consistent with the immunostaining analysis above. The cytoplasmic protein β-tubulin and the nuclear Lamin B1 served as controls and demonstrated efficient separation of the cytosolic and nuclear fractions, respectively.

To test whether this increased nuclear β-catenin is active in transcription, we used a Tcf-responsive reporter (Topflash) or a mutated control (Fopflash). When transfected into RK3E or Gli-C cells and normalized to a Renilla luciferase control, Tcf-specific Firefly reporter activity was observed only in Gli-C cells (Figure 2c). Increased Tcf activity was not a consequence of malignant transformation because no activity was observed in RK3E cells transformed by Notch1. We also examined whether transient expression of Gli1 could induce Tcf activity in HEK293 cells. At higher levels (0.5-1.0 µg) the vector pcDNA3.1-Gli1 specifically induced Topflash reporter activity (Figure 2d).

Several studies have demonstrated increased nuclear β-catenin in human BCCs (Yamazaki et al., 2001; El Bahrawy et al., 2003; Saldanha et al., 2004). Using a recently developed model in which BCC is induced by tet-on Gli1 (Li et al., 2006), we examined paraffin sections using an antibody to the active, unphosphorylated form of β-catenin (van Noort et al., 2002). The superficial epithelium stained only weakly compared to the more infiltrative basaloid cells deeper within the skin (Figure 2e).
This pattern is complementary to the pattern of E-cadherin in these lesions, which is greatly suppressed in cells deeper within the dermis (Li et al., 2006). In contrast to active β-catenin, antibody to total β-catenin gave more uniform staining of the superficial and deeper lesions (not shown). Thus, reduced E-cadherin may identify BCC cells with increased Wnt pathway activity.

**Enforced expression of E-cadherin suppresses Gli1-mediated transformation** We wished to investigate the possibility that Snail-mediated loss of E-cadherin contributes to Wnt signaling and therefore to transformation by Gli1 (Ciruna and Rossant, 2001; Nelson and Nusse, 2004). If repression of E-cadherin contributes to activation of β-catenin during transformation by Gli1, then enforced expression of E-cadherin should block transformation by a Wnt-dependent gene such as Gli1, but have little effect on a Wnt-independent gene such as ErbB2 (see Figure 1a). We expressed WT mouse E-cadherin under control of the MMLV LTR. By immunoblot with E-cad antibody, RK3E cells stably transduced with this retroviral vector showed increased expression of a species that co-migrates with endogenous rat E-cadherin (Figure 3a). RK3E-E-cad cells appeared identical to Vector control cells with respect to cell morphology, and grew at a similar rate in culture (not shown). To assess the effect of enforced expression of E-cadherin on transformation of RK3E, we co-transfected vectors for Gli1 or ErbB2 with either E-cadherin or a Vector control (Figure 3b and c). Counting of foci in replicate dishes demonstrated that E-cadherin suppressed Gli1-mediated transformation by 83%, (mean no. of foci/dish: Gli1+ Vector, 17.8; Gli1+ E-cadherin, 3), with no effect on ErbB2 (mean no. of foci/dish: ErbB2+ Vector, 16; ErbB2+ E-cadherin, 16.7). Thus, like Tcf4ΔN31, E-cadherin functions as a selective suppressor of transformation by Gli1.
**E-cadherin loss-of-function promotes transformation by Gli1**

We previously showed that exogenous Snail can cooperate with Gli1 to induce transformation, indicating that induction of endogenous Snail by Gli1 is not saturating. To further investigate a role of E-cadherin as a selective regulator of transformation by Gli1, we generated shRNA vectors that could suppress rat E-cadherin (Table 1). These constructs, or a control (pSiCtl) that should not target any transcript, were transfected into RK3E cells and to colonies of G418-resistant cells were selected. Immunoblot analysis of early passage cells derived from pooled colonies showed that E-cadsh1 or E-cadsh2 suppressed the endogenous E-cadherin in these cells (Figure 4a). As the size and number of colonies obtained using pSiCtl and the E-cad-specific hairpin constructs were similar (not shown), E-cadherin knockdown has little or no effect on cell growth rates. The growth-neutral phenotype of these shRNAs is consistent with results obtained when E-cadherin was suppressed in RK3E cells by exogenous Snail (Li et al., 2006).

To determine the effect of E-cadherin knockdown on transformation, we co-transfected these constructs with Gli1 or ErbB2 vectors. As compared with pSiCtl, E-cadsh1 and E-cadsh2 each promoted the outgrowth of Gli1-transformed cells (mean no. of foci/dish: Gli+ pSiCtl, 21.6; Gli1+ E-cadsh1, 51.2; Gli1+ E-cadsh2, 43.4; Figure 4b and c). In contrast, the efficiency of ErbB2-induced transformation was relatively unchanged (Figure 4b and c).

**Snail loss-of-function is rescued by down-regulation of E-cadherin**

We previously used Snail shRNAs to block transformation by Gli1, and showed that these shRNAs induce E-cadherin in Gli-C cells (Li et al., 2006). When co-transfected with an equal amount of SnaSH3 vector (Li et al., 2006), E-cadSH2 and E-cadSH1 promoted Gli1 transformation by 3-fold and 5-fold relative to pSiCtl (mean number of foci/dish:
Gli1+Sna^sh3+pSi^Ctl, 6; Gli1+Sna^sh3+E-cad^sh2, 20.8; Gli1+Sna^sh3+E-cad^sh1, 30.8; Figure 5a and b).  Consistent with targeting of E-cadherin by the shRNAs, their effects were greater when endogenous Snail was absent (compare Figures 4c and 5b).  E-cadherin shRNAs not only promoted transformation in the presence of Snail loss-of-function, but yielded transformation efficiencies similar to (E-cad^sh2) or greater than (E-cad^sh1) the levels observed when endogenous Snail induction by Gli1 was permitted (mean number of foci/dish: Gli1/pSi^Ctl, 21.6; Figure 5a and b).  Although Snail has other cellular targets that are likely important for its activities, these results suggest that suppression of E-cadherin is the major mechanism by which Snail contributes to transformation by Gli1.

**The transformation suppressor activity of E-cadherin requires β-catenin binding**

To better understand how E-cadherin functions as a suppressor of Gli1-mediated transformation we generated retroviral constructs for two alleles of E-cadherin (Figure 6a).  E-cad^Δβ-cat contains a 35 amino acid deletion in the C-terminus that abolishes the interaction with β-catenin.  However this allele showed increased cell-cell adhesion compared with WT E-cadherin (Gottardi et al., 2001).  The second allele, E-cad^cyto, fuses the E-cadherin cytoplasmic domain to the extracellular and transmembrane domains of the IL2R (Gottardi et al., 2001).  Similar constructs have been shown to be inactive for the cell-cell adhesion function of E-cadherin.  E-cad^cyto and similar alleles can also inhibit Wnt signaling, perhaps by trapping β-catenin in the cytosol (Gottardi et al., 2001; Blache et al., 2004; Margulis et al., 2005).  The allele we used was previously shown to inhibit growth of SW480 colorectal carcinoma cells that have constitutive Wnt signaling resulting from APC loss-of-function (Gottardi et al., 2001).

Unlike WT E-cadherin, E-cad^Δβ-cat did not block transformation by Gli1 (mean
no. of foci/dish: Gli1 + Vector, 21; Gli1 + E-cad\(^{\Delta\beta\text{-cat}}\), 20.6; Gli1 + E-cad, 3; Figure 6b and c). This result indicates that β-catenin binding is required for suppression of Gli1 by E-cadherin.

Although we anticipated that E-cad\(^{\text{cyto}}\) would inhibit β-catenin nuclear localization and block transformation by Gli1, E-cad\(^{\text{cyto}}\) induced 2.9-fold more foci when co-transfected with Gli1 (mean foci/dish, 61.2 vs. 21; Figure 6a and b). To determine whether E-cad\(^{\text{cyto}}\) promoted cell growth independently of its effects on Gli1, we transduced RK3E cells with the constructs shown in Figure 6d and plated drug-resistant cells at 1000 cells per dish. After 9 days colonies were stained to assess cell growth (Figure 6d). Cell morphology was examined by phase contrast microscopy (Figure 6e). As expected, Gli1 promoted cell growth and altered cell morphology toward a transformed phenotype. However, E-cad\(^{\text{cyto}}\) and E-cad\(^{\Delta\beta\text{-cat}}\) cells appeared similar to the Vector control. Thus, the ability of E-cad\(^{\text{cyto}}\) to potentiate Gli1 transformation is not due to a Gli1-independent growth phenotype.

**Gli1 activity correlates with nuclear localization of β-catenin**

We determined how each of three modulators of Gli1-mediated transformation (E-cadherin, E-cad\(^{\text{cyto}}\), and Snail) affects β-catenin in the presence or absence of exogenous Gli1. RK3E cells were transduced with E-cad, E-cad\(^{\text{cyto}}\), Snail or a control retrovirus, selected in culture, and then transduced with a Gli1 retrovirus. To analyze β-catenin, nuclear and cytosol extracts were prepared 14 days later (Figure 7a). Gli1 promoted nuclear localization of β-catenin in settings where it is active in transformation, including Vector cells, E-cad\(^{\text{cyto}}\) cells, and Snail cells (compare upper and middle panels, lanes 5, 7, and 8). In E-cadherin cells, where Gli1 transforming activity is suppressed, nuclear localization of β-catenin was not increased (compare upper and middle panels, lane 6). Similarly, E-cad\(^{\text{cyto}}\) and Snail augment transformation by Gli1 and
concomitantly increased nuclear β-catenin (compare lanes 5, 7, and 8, upper panels). In the absence of Gli1 β-catenin levels were similar in most lines (middle panels, lanes 1, 2, 4, 5, 6 and 8), except E-cad$^{ESY}$ cells, which accumulated β-catenin in the cytoplasm (middle panel, lane 3) and nucleus (middle panel, compare lanes 5 and 7). When transduced with Gli1, E-cad$^{ESY}$ cells showed similar accumulation of β-catenin in the cytoplasm and nucleus (upper panel, compare lanes 3 and 1; compare lanes 7 and 5). Thus E-cad$^{ESY}$ increased β-catenin independently of Gli1, and this pool of β-catenin was available for signaling in response to Gli1. Since Snail alone had no effect on localization of β-catenin in the absence of Gli1, other Gli1 target genes may play a role in this signaling.

In cells expressing E-cadherin and E-cad$^{ESY}$, Gli1 protein levels were similar to Vector cells in both the cytosol and nucleus (Figure 7a, lower panels). Surprisingly, Snail cells showed increased Gli1 in the nucleus and cytoplasm (lower panel, lanes 4 and 8). Thus Snail promotes the accumulation of Gli1. This effect is independent of E-cadherin activity as shown in lanes 2, 3, 6, and 7.

We next examined Wnt target genes in Gli-C and RK3E cells using semi-quantitative RT-PCR (Figure 7b). Cyclin D1 and Twist2 were increased in Gli-C cells, while c-Myc was slightly reduced (Figure 7b). Indeed, c-Myc is not upregulated by β-catenin in RK3E cells (Kolligs et al., 1999), and the expression profile of RK3E cells transformed by c-Myc or Gli1 are distinct (Louro et al., 2002). As γ-catenin/plakoglobin was found to induce c-Myc in RK3E and to require c-Myc for transformation (Kolligs et al., 2000; Barker and Clevers, 2000), our results suggest that Gli1 signals through β-catenin rather than γ-catenin in RK3E cells.
Discussion

Gli1 induces *Snail* transcripts in vitro in RK3E cells (Louro et al., 2002), a finding confirmed in cultured prostatic epithelial cells (Karhadkar et al., 2004). Using a tet-on Gli1 mouse model of BCC, *Snail* transcripts were upregulated in mouse skin just hours after induction of Gli1 by doxycycline (Li et al., 2006). Loss- and gain-of-function studies showed that endogenous Snail is required for efficient transformation by Gli1 and may be a limiting factor, as exogenous Snail promoted Gli1 transformation.

In the current study we analyzed the role of a Snail-regulated gene, E-cadherin, in transformation by Gli1. Consistent with suppression of Gli1 by Snail shRNAs (Li et al., 2006), enforced expression of E-cadherin blocked transformation by Gli1 but had no effect on a control, ErbB2. When Snail induction was blocked by shRNAs, E-cadherin shRNAs rescued Gli1 activity. Thus, suppression of E-cadherin is likely a major mechanism by which Snail promotes transformation. E-cadherin suppresses Gli1 activity by binding β-catenin, as E-cad Δβ-catenin had no effect. β-catenin signaling or nuclear localization was promoted by Gli1 in several settings and was required for transformation by Gli1, but not by ErbB2 or H-Ras, as indicated by use of DN Tcf4 cells.

Snail also promoted nuclear expression of Gli1, indicating that Snail has at least two activities relevant to Gli1 function: transcriptional repression of E-cadherin and post-transcriptional induction of Gli1. Ability of Snail to independently regulate E-cadherin and Gli1 was supported by the lack of any effect on Gli1 expression by WT E-cadherin or E-cad Δcyto. Taken together, these studies support potential roles for Snail, E-cadherin, and β-catenin in human tumors that show activation of the Shh-Gli pathway. As shown by gain- and loss-of-function of
Snail (Li et al., 2006) and E-cadherin (this work), Gli1 fails to completely suppress E-cadherin during transformation of RK3E, suggesting that other mechanisms that induce Snail or suppress E-cadherin might cooperate with Gli1 during tumor progression.

E-cadherin has been implicated as a candidate tumor suppressor in multiple forms of human carcinoma, including BCC (Birchmeier and Behrens, 1994; Bracke et al., 1996; Semb and Christofori, 1998; Li et al., 2006). While E-cadherin’s role as a suppressor of invasion by tumor cells is well-established (Vleminckx et al., 1991; Frixen et al., 1991; Birchmeier and Behrens, 1994; Bracke et al., 1996; Perl et al., 1998; Margulis et al., 2005), its ability to suppress tumor cell growth or oncogene-induced transformation is less studied (Gottardi et al., 2001). The current study identifies E-cadherin as a selective suppressor of transformation by Gli1. We did not observe any suppressive effect on transformation by ErbB2, consistent with the ability of Snail to cooperate with Gli1 but not the other oncogenes tested (Li et al., 2006). Consistent with its role as an oncogene-selective transformation suppressor, endogenous E-cadherin levels in cells transformed by H-Ras or KLF4 are comparable to parental RK3E cells (Li et al., 2006 and data not shown).

The Shh and Wnt pathways share several regulatory components and interact at multiple levels (Taipale and Beachy, 2001; Nusse, 2003). For example, β-catenin and the Shh pathway component Suppressor of Fused have a direct protein-protein interaction (Taylor et al., 2004). GSK3β acts in the Wnt pathway in opposition to Wnt ligands and also suppresses Hedgehog signaling, including the Gli1-target gene Snail (Jia et al., 2002; Price and Kalderon, 2002; Zhou et al., 2004; Yook et al., 2005; Price, 2006; Tempe et al., 2006). Shh also induces other Wnt pathway components such as sFRP2 (Lee et al., 2000).
These pathways coordinate several developmental transitions in mammals. During somitogenesis, Wnt signals regulate Gli transcript levels (Borycki et al., 2000). Subsequently, notochord-derived Shh and ectoderm/neural tube-derived Wnt act on the epithelial somites to induce EMT, leading to specification and differentiation of somite muscle progenitor lineages (Pownall et al., 2002). Similarly, Wnt and Shh signaling play essential roles in the development of hair follicles in the skin (St Jacques et al., 1998; Chiang et al., 1999; Huelsken et al., 2001; Jamora et al., 2003). Wnt signals initiate de novo hair bud and follicle formation, while Shh signals potentiate the Wnt effects and are also required for the subsequent proliferation/migration of bud or follicle cells (Gat et al., 1998; Alonso and Fuchs, 2003; Van Mater et al., 2003; Silva-Vargas et al., 2005).

Although Wnt signals appear to function prior to Shh in these settings, our studies support the idea that Shh-Gli signaling can feed back into the Wnt pathway through Snail and E-cadherin, promoting cell division by maintaining or amplifying the initiating Wnt signal (Figure 7c). In addition to induction of Snail, Gli1 induces transcription of at least one Wnt family member, Wnt2b. As Snail alone did not alter β-catenin localization, these two Gli1 target genes may synergize to switch β-catenin from its role in cell adhesion to a role in nuclear signaling. In summary, our studies identify Shh-Gli1 expressing epithelial cells as a setting in which Snail-induced EMT and increased cell growth are linked by the dual roles of β-catenin. Hedgehog-Gli1 regulation of the β-catenin switch may be important in normal and disease states such as somitogenesis in the embryo, in hair follicle morphogenesis and cycling, and in the genesis of human carcinomas that show activation of the SHH pathway.
Materials and Methods

Expression vectors WT E-cadherin (Genbank X06115) was released from pEM2 (Nagafuchi et al., 1987) by digestion with StuI and EcoRV. The blunted 3.3 kb fragment was ligated to BstXI adaptors and then inserted into the same site of the MMLV retroviral vector pCTV3B, which confers resistance to hygromycin (Whitehead et al., 1995). E-cad\textsuperscript{cyto} and E-cad\textsuperscript{\Delta\beta\text{-cat}} plasmids were provided by Cara Gottardi (Northwestern Univ.) and the inserts were cloned into the pBABE-puro, which confers resistance to puromycin (Morgenstern and Land, 1990). For targeting of rat E-cadherin, hairpin fragments (Table 1) were ligated to pSilencer\textsuperscript{TM} 2.1-U6 neo (Ambion), electroporated into XL1-Blue cells (Stratagene), and verified by sequencing as described (Li et al., 2006). The constructs pSn\textsuperscript{a\text{sh3}}, encoding an shRNA against Snail, and pSi\textsuperscript{C\text{tl}}, containing a hairpin sequence without similarity to mammalian cDNAs, were previously reported (Li et al., 2006).

Cell culture and transfection To generate RK3E cells stably expressing E-cadherin, E-cad\textsuperscript{cyto}, and E-cad\textsuperscript{\Delta\beta\text{-cat}}, retroviral transduction and antibiotic selection was performed as described (Foster et al., 1999; Louro et al., 2002). Vector cells and Snail cells were previously generated by the same methods using pBABE-puro or pBABE-puro-Snail (Li et al., 2006). To introduce Gli1, each of these lines were transduced with the neo vector pLJD-HA-Gli1 (Louro et al., 2002). In order to maintain representation of the starting cells, all lines were derived from \textgreater 100,000 transduced cells and maintained as populations by mass selection in antibiotics. RK3E-Tcf4\text{\DeltaN31} cells were generated previously by Fearon and colleagues by retroviral transduction of an early passage of RK3E cells provided by our laboratory, followed by mass selection in G418 (Kolligs et al., 1999).
For analysis of transformation, focus assays were performed following lipid-mediated transfection of 10.0 µg of plasmid into 70% confluent cells as previously described (Li et al., 2006). Cells were fixed and incubated in Wright’s stain at 2-3 weeks post-transfection, and foci that were greater than 0.5 mm in diameter were counted. Expression vectors for Gli1 (pLTR6) (Ruppert et al., 1991), ErbB2 (Li et al., 2006) or H-Ras (pHO6T1) were transfected directly into RK3E or RK3E-Tcf4ΔN31 cells. For co-transfection experiments 2.0 µg of Gli1 or ErbB2 vector were co-transfected with a total of 8.0 µg of the indicated plasmid(s). For colony morphology analysis cells were plated in 10 cm tissue culture dishes and selected for 9 days in G418 (400 µg/ml) or puromycin (80 µg/ml). Phase contrast microscopic images were captured using an Axiovert 25 inverted microscope equipped with an Axiocam digital camera (Zeiss).

For analysis of shRNA-mediated E-cadherin knockdown, RK3E cells were transfected by electroporation of pSilencer constructs (protocol available upon request). Populations of cells were selected for 2 weeks in 400 µg/ml of G418 and whole cell extracts were prepared for immunoblot analysis.

**mRNA expression studies** Semi-quantitative RT-PCR analysis was performed on cultured RK3E, Gli-C, a clone derived from a focus of Gli1-transformed RK3E cells, and RTOG10, a tet-on Gli1 line (Li et al., 2006). Total RNA was extracted using RNeasy (Qiagen). RT reactions included 5.0 µg of total RNA, oligo-dT(12-18) at 50 µg/ml, and SuperScript™ II RT (Invitrogen) in a final volume of 50µl. 1/50 of the first strand products served as PCR templates. Primers are shown in Table 2. In order to analyze products before the yields were saturating, PCR reactions were terminated at intervals between 17 and 30 cycles and analyzed by agarose gel electrophoresis.
**Subcellular fractionation and immunoblot analysis** Fractionation was performed by sequential extraction of cytosolic (using low salt) and nuclear proteins (using high salt) in non-ionic detergent as described (Jian et al., 2006). Antibodies to β-catenin (BD Biosciences #610153, used at 0.5 µg/ml), β-tubulin (Sigma #T0198, 0.55 µg/ml), Lamin B1 (GeneTex #GTX16048, 0.1 µg/ml) and Gli1 (Li et al., 2006) were used to detect proteins. For analysis of E-cadherin, whole cell extracts were prepared by application of hot Laemmli buffer to cells. SDS extracts were quantitated by amido black staining of spotted samples (Sheffield et al., 1987). E-cadherin antibody (BD Biosciences #610181) was used at 0.1 µg/ml, and β-Actin antibody (Sigma #A5316) was used at 0.26 µg/ml. Bound antibodies were detected by chemiluminescence (Pierce).

**Immunostaining and digital imaging** For localization of β-catenin by indirect immunofluorescence, β-catenin primary antibody (BD Biosciences #610153, used at 0.17 µg/ml) and a fluorescent secondary antibody (Molecular Probes #A-11032) were used. Cells were counterstained with phalloidin (Molecular probes #A-12379) and DAPI and digital images were captured as described (Li et al., 2006).

To detect active β-catenin paraffin sections were stained with β-catenin antibody (Millipore #05-665, used at 10 µg/ml). Normal mouse IgG at the same concentration served as a negative control (Dako #X-0910). Bound antibody was detected using horse-radish peroxidase and the chromogenic substrate diaminobenzidine as described (Huang et al., 2005).

**Luciferase reporter assays** RK3E, Gli-C and Notch-1 cells were plated at 2.0 x 10^5 cells per well in 6-well plates at twenty-four hrs before transfection. Transfection cocktails included 0.5 µg of pGL3-OT or pGL3-OF (gifts of Bert Vogelstein), and 0.05-0.1 µg of the Renilla luciferase reporter pRL-TK. HEK293 cells were treated
similarly except that pcDNA3.1-HA-Gli1 or empty vector was included as indicated in the Figure. TransIT®-LT1 Reagent (Mirus) was used according to the manufacturer’s instructions and normalized luciferase activities were determined 48 hrs post-transfection using the Dual-Luciferase Reporter Assay System (Promega).

Acknowledgements

We thank Eric Fearon and Frank Kolligs for Tcf4ΔN31 cells, and Bert Vogelstein, M. Takeichi, Robert Kay and Cara J. Gottardi for plasmids. This work was supported by grants CA094030 and CA065686 from the U.S. National Cancer Institute.

References


an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol* **166**: 37-47.


Figure 1 Inhibition of Gli1-mediated transformation by DN Tcf4 (a) Parental RK3E or RK3E-Tcf4ΔN31 cells were transfected with Gli1 or ErbB2 vectors. Cells were fixed and stained at two weeks post-transfection. Background was determined by transfection of pBABE-puro (Vector). (b) Transformation efficiency was determined by counting foci. Two experiments were performed in duplicate and standard error (SE) bars are shown. (c) Induction of Wnt2b in response to Gli1 in RK3E cells. Semi-quantitative RT-PCR analysis was used to detect Wnt2b in RK3E cells that conditionally express Gli1 in response to tet (RTOG10 cells), in parental RK3E cells, and in Gli1-transformed RK3E cells. Snail is a direct transcriptional target of Gli1 (Louro et al., 2002; Li et al., 2006) and served as a positive control. Gapdh served as a control for mRNA input.
Figure 1
**Figure 2** Activation of β-catenin signaling in response to Gli1 *in vitro* and *in vivo.*

(a) β-catenin (β-cat, shown in red) was detected in RK3E and Gli-C by indirect immunofluorescence. Nuclei were stained with DAPI (shown in blue) and the cytoplasm was stained with phalloidin (shown in green). Merged images are shown in the columns to the right. Scale bars, 5 µ. (b) Cytosolic and nuclear proteins were isolated from the indicated cell lines by sequential extraction as described in the Materials and Methods. β-cat was detected by immunoblotting. As a control for loading and to demonstrate successful fractionation, filters were reprobed with antibodies to β-tubulin (β-tub), a cytoplasmic protein, and Lamin B1, a nuclear protein. (c) Topflash (WT Tcf4 binding sites) or Fopflash (mutated Tcf4 binding sites) reporter constructs were transfected into RK3E cells or cells transformed by Gli1 (Gli-C) or Notch1. Data were normalized using a Renilla luciferase internal control, and Fopflash activity in RK3E cells was arbitrarily set to 1.0. Two experiments were performed in triplicate. Bars, SE. (d) HEK293 cells were transfected with Topflash or Fopflash, a Renilla luciferase control, and the indicated amount of a Gli1 vector. After normalization to the Renilla control, the Fopflash activity for Vector-transfected cells (0.0 µg Gli1) was set to 1.0. Bars, SE. (e) Antibody that detects the active, unphosphorylated form of β-cat was used to stain paraffin sections of the BCC-like skin lesions from Gli1 transgenic mice (Li et al., 2006). Staining was detected using a brown chromogen. Normal mouse IgG served as negative control and arrows indicate the dermo-epidermal junction. Scale bar, 100 µ.
Figure 2
Figure 3 Suppression of Gli1-mediated transformation by E-cadherin. (a) E-cadherin (E-cad) was detected by immunoblot analysis following retroviral transduction of an E-cadherin vector or a Vector control and selection in culture. β-actin served as a control for loading. (b) The indicated plasmids (Construct 1, Construct 2) were co-transfected into RK3E cells and transformed foci were scored at 2-3 weeks. Assays were performed twice in triplicate. (c) Quantitation of the assays shown in panel b. Bars, SE.
Figure 3
Figure 4. E-cadherin loss-of-function promotes Gli1-mediated transformation. (a) Immunoblot analysis of E-cadherin in cells expressing the indicated shRNA. RK3E cells were transfected by electroporation with pSilencer vectors that express shRNAs under control of the U6 promoter. Cells were selected in G418 prior to preparation of whole cell extracts. β-actin served as a control for loading. (b) Gli1 or ErbB2 vectors (Construct 1) were transfected into RK3E cells together with shRNA vectors (Construct 2). Transformed foci were scored at 2-3 weeks. Assays were performed twice in triplicate. (c) Quantitation of the assays shown in panel b. Bars, SE.
Figure 4
Figure 5. E-cadherin loss-of-function is sufficient to promote Gli1-mediated transformation when Snail is suppressed. (a) The indicated quantities of Gli1 and shRNA vectors were transfected into RK3E cells, and transformed foci were scored at 2-3 weeks. Assays were performed twice in triplicate. (b) Quantitation of the assays shown in panel a. Bars, SE.
Figure 5
Figure 6. Analysis of functionally impaired mutants of E-cadherin in cell growth and transformation. (a) RK3E cells were transduced by retroviruses encoding E-cad^cyto, E-cad_{Alp-cat} or empty Vector, and E-cadherin was detected by immunoblot. As a control for loading, Lamin B1 was bound to the same filter. Molecular weight (MW) markers are indicated on the right. (b) The indicated plasmids were transfected into RK3E using Lipofectamine, and transformed foci were scored at 2-3 weeks. One experiment was performed using five replicates. (c) Quantitation of the transformation assays shown in panel b. Bars, SD. (d) To determine the effect of transgenes on cell growth and morphology, cells stably transduced with the indicated constructs were plated at 1 x 10^3 cells per dish and colonies were selected for 9 days. To assess colony size and number, cells were fixed and incubated in Wright’s stain. (e) The morphology of live cells was examined by phase contrast microscopy. Arrowheads indicate the edge of an area of Gli1 cells with a transformed morphology. Scale bars, 50 µ.
Figure 6
Figure 7. Modulation of $\beta$-catenin nuclear localization during transformation by Gli1. (a) RK3E cells were transduced with the retroviral constructs Vector, E-cad, E-cad$^{cyto}$, or Snail. These cells were cultured in selective media and then transduced with a Gli1 retroviral vector. Cells stably expressing Gli1 [(+) Gli1] were compared to cells carrying only the first transgene [(−) Gli1]. Cytosolic and nuclear fractions were analyzed by immunoblot using the antibodies indicated to the left. $\beta$-tubulin or Lamin B1 was utilized as loading controls for cytosolic or nuclear extracts, respectively (Control). (b) Analysis of Wnt pathway responsive transcripts. Semi-quantitative RT-PCR assays were performed as described in the Materials and Methods. Gapdh served as a control for mRNA input. (c) Regulation of Wnt signaling by Gli1 in epithelial cells. Arrows indicate inductions, and crossbars denote repression or reduced activity. The interactions shown are from the current work or else were previously reported as indicated in the text.
### Figure 7

#### (a) Western Blot Analysis

<table>
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#### (b) Gene Expression

- RK3E
- Gli-C
- Cyclin D1
- Twist2
- c-Myc
- Gapdh

#### (c) Schematic Diagram

- Wnt2b
- Snail
- Gsk3\(\beta\)
- \(\beta\)-catenin
- E-cadherin
- Lef1/Tcf
- Cyclin D1
- Twist2
- Sufu

---

**Figure 7**
Table 1. Structure of E-cadherin shRNAs.

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<sup>a</sup>Numbering system refers to rat E-cadherin (Genbank AB017696), where the protein coding region is 127-2787.

<sup>b</sup>The strand shown corresponds to the final RNA Polymerase III transcript, with the +1 base in bold. Restriction sites, hairpin loop sequences, and terminator sequences are upper case.
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SUMMARY DISCUSSION

The Shh pathway is one of the core signalling pathways that determine embryonic pattern formation and tissue homeostasis. Deregulation of this pathway, including constitutive activation of positive regulators (e.g. Smo) and inhibition of negative modulators (e.g. Ptc), can result in not only in developmental phenotypes but can also initiate or promote growth of various types of human cancers(132,143-145,186). The Gli family of transcriptional factors has long been regarded as the major effectors of Shh-induced cancers. Of the known Gli1 target genes, Snail is one of the most intriguing, particularly with regard to epithelial tumors (i.e. carcinomas). Utilizing microarray analysis, we identified a gene expression profile of Gli1 (182). Snail was prominent among genes that were dramatically upregulated in Gli1-transformed RK3E cells. Further analysis of a Gli1-ER conditional cell line exhibited a rapid induction of Snail transcripts, even when protein synthesis was blocked, supporting a direct mechanism of regulation. Gli1 and Snail transcripts were both prominent in human BCCs and in anagen follicles of adjacent skin. These discoveries provided evidence that the EMT inducer Snail is a direct target of Gli1 and suggested that induction of Snail may contribute to Gli1-induced tumor formation. Although these descriptive studies were compelling, a functional role for Snail was not demonstrated.

In the first part of this dissertation, we carried on series of studies to identify the role of Snail in transformation mediated by Gli1. First, we demonstrated Gli1 regulation of Snail in a mammalian epithelial cell model, RK3E cells. Snail was rapidly induced by Gli1 at both mRNA (1 hour) and protein levels (6 hours) in a new
Gli1 conditional RK3E line with a tet-inducible Gli1 transgene. The coordinate upregulation of Snail and Ptch1 (a known direct target of Gli1) supports direct regulation of Snail by Gli1. When expressed in RK3E cells, Snail repressed E-cadherin transcripts. Snail has also been show to promote the keratinocytes of K14-driven Snail transgenic mice, while in vitro studies suggested its role as regulator of cell death (86,187-191). But we didn’t observe alteration of cell growth or cell death in RK3E cells containing a Snail expression vector (192). Interestingly, although the loss of epithelial markers was demonstrated in these cells, the typical morphological changes of EMT were not observed. Instead, we found prominent blebbing of the cytoplasmic membrane, a phenotype previously described in several melanoma cell lines that also show Snail (193,194). We attribute this absence of EMT to our use of a different epithelial model, since previous studies used MDCK cells (86). Membrane blebbing has been described as a consequence of reduced stability of the actin cytoskeleton in the periphery of cells. The phenotype observed in our model could be due to aberrant regulation of cytoskeletal components by Snail, possibly representing an alternative activity of Snail in some cell lines. Future studies are required to extend our observation of Snail affects on the cytoskeleton.

We generated partially truncated mouse Snail alleles, either at the C- or the N-terminals. The two alleles, termed SnaΔZFD and SnaZFD, showed dominant negative effects and antagonized endogenous Snail or an exogenous transgene. Similar constructs of Xenopus Snail were reported to have dominant negative activity (195). SnaZFD appears to be the more active one of the two, probably by competing with endogenous Snail for the DNA-binding sites of E-cadherin. SnaΔZFD, although
low potent, is also capable of rescuing suppression of E-cadherin by WT Snail.

By analysis of the dominant negatives, we showed Snail is an important downstream mediator and probably a limiting factor in Gli1 transformation. When SnaZFD was co-expressed with Gli1, it abolished most of the transforming ability of the oncogene. We didn’t observe these effects using SnaΔZFD, consistent with its weaker overall activity. Independently, we showed that enforced WT Snail cooperates with Gli1 to induce a dramatic increase of transformed foci. As controls for specificity, we co-transfected SnaZFD or else WT Snail with N-RAS, ErbB2, or Notch1. In contrast to Gli1, no effects were observed for these controls. The role of Snail was confirmed by shRNAs against Snail, which potently inhibited transformation when they were co-introduced with Gli1. As for dominant negatives, shRNAs have no effects on ErbB2 or Notch1. Therefore, both loss-of-function and gain-of-function of Snail demonstrated its selective role in transformation of Gli1, consistent with their phenotypic effects. SnaZFD and Snail shRNAs restored E-cadherin in Gli1 cells. These data show that ongoing expression of Gli1 and Snail are required for suppression of E-cadherin (63,145,182).

Shh is the major pathway that control cell cycle and differentiation of hair follicles (130,133,196). Thus the role of aberrant Shh pathway activation in the development of BCCs and other follicular neoplasm has been extensively studied in recent years. Lesions resembling human BCCs were detected in the skin of transgenic mice overexpressing Smo or Shh (132,141). Mice expressing human Gli1 or Gli2 developed tumors similar to human BCC, and Gli1 also induced benign neoplasms resembling human trichoepitheliomas, cylindromas and trichoblastomas (169,172). The Ptch+/- mice developed BCC-like lesions after a long-term exposure to UVR. These animal models supported the role of Shh signalling in carcinogenesis of BCCs,
however do not distinguish between initiation versus promotion, and the ability of Shh signaling to initiate growth in adult skin was unknown. We employed the tet-on strategy to generate a mouse model bearing the conditional Gli1 expression constructs, and successfully established TRE-Gli1 lines showing an increased incorporation of BrdU in interfollicular skin in as little as 6 hours of induction. The resulting BCC-like lesions (induced by 4 weeks) shared histological and morphological characteristics with human BCCs, albeit they appeared to be more diffused and superficial rather than the focal, invasion tumors observed after several months in constitutive Gli1 or Gli2 models (169,172,173). Consistent with the observation that up to 50% of studied sporadic BCCs showed \( p53 \) mutations, we showed enhanced thickness and neovascularization following introduction of a deficient \( p53 \) allele. Other genetic or epigenetic alteration may be required for the progression of BCC. One possibility is that Notch1 loss-of-function is required for the full BCC phenotype(197).

The origin of BCC is somewhat controversial, but one possibility is that it results from Shh-dependent clonal expansion of follicular stem cell-like cells (beachy, 41, 59). However, it is thought BCC may arise from locations outside the hair follicles (132,141). Our results suggested that Gli1 signalling through Snail may redirect interfollicular keratinocytes to a follicle/BCC fate, probably with stem cell-like properties. This process may also attribute to morphogenesis of follicles in normal skin. The Wnt signalling pathway has been linked to the ability of skin epithelial cells to acquire stem cell-like features (104,106). Recently, TGF\( \beta2 \) and BMP signalings was shown to be important in forming hair follicles, through Snail or Noggin respectively (100,191). Further work is required to understand the cross-talk of different morphogens and downstream events that guide hair follicle morphogenesis.

In the second part of this dissertation, we extended our research of Snail, a
critical factor downstream of Gli1, to its downstream target E-cadherin. Previous literature and our results above showed that loss of E-cadherin is correlated with multiple forms of human cancers, suggesting its role of candidate tumor suppressor (59,198). However, functional studies demonstrated a role of E-cadherin as a suppressor of transformation or tumor cell growth are few (199). We showed that overexpression of the WT E-cadherin blocked Gli1-induced transformation in RK3E cells. This loss-of-transformation effect induced by E-cadherin was not observed for the control oncogene ErbB2. Therefore, we consider E-cadherin as a selective suppressor of Gli1 during epithelial transformation. Further investigation showed that Gli1 can transform when both E-cadherin and Snail are suppressed by shRNAs. Therefore E-cadherin suppression is the major activity of Snail. Thus we showed that Snail-mediated suppression of E-cadherin plays a major role in Shh-Gli1 induced transformation.

Utilizing two mutated allele of E-cadherin, termed E-cad\textsuperscript{\Delta\beta\text{-cat}} and E-cad\textsuperscript{cyto}, we mapped its activity as a suppressor of Gli1. The \(\beta\)-catenin binding region was required for E-cadherin to suppress Gli1, since E-cad\textsuperscript{\Delta\beta\text{-cat}} failed to inhibit focus formation. In contrast, E-cad\textsuperscript{cyto} promoted Gli1-mediated transformation, probably by stabilizing \(\beta\)-catenin. As for Snail, both loss-of function and gain-of-function of E-cadherin affects Gli1 but not other oncogenes, supporting the idea that E-cadherin is a selective tumor suppressor in the context of Shh signalling.

In other cells, E-cad\textsuperscript{cyto} and similar constructs were previously shown to inhibit Wnt signalling, perhaps by trapping \(\beta\)-catenin in the cytosol (200-202). Although \(\beta\)-catenin clearly has dual functions in cell adhesion (by associating with the C-terminal of E-cadherin, \(\alpha\)-catenin and the actin cytoskeleton) and in transmitting Wnt signals to nuclei (in association with Tcf/Lef), E-cadherin loss-of-function has
rarely been associated with increased Wnt signaling (66,203). In Gli1 transformed cells, we demonstrated increased β-catenin signalling or nuclear localization in several distinct assays *in vitro*, and accumulation of the active, unphosphorylated form of β-catenin in the infiltrative, hyperproliferative basaloid cells within the skin of the TRE-Gli1 mouse model. Also, activated β-catenin was complimentary to expression of E-cadherin in these skin lesions.

Additionally, we showed that activated β-catenin signalling is required for Gli1-induced transformation, as a dominant negative allele of Tcf4 blocked about 90% of the outgrowth of foci. This data supported the hypothesis that β-catenin is a downstream effector for Shh-Gli signalling. Taken together, our results suggested that E-cadherin loss-of-function in Shh-induced tumors or cell lines is associated with increased β-catenin signalling. In both development and in tumors, Shh-Gli1 signalling may represent an additional pathway that manipulates β-catenin to choose nuclear signalling rather than cell-cell adhesion.

Using an efficient strategy for fractionating cell extracts, we showed that the abilities of E-cadherin, E-cad<sup>cyto</sup> and Snail to modulate Gli1-mediated transformation are concordant with the levels of β-catenin accumulated in cell nuclei. In the presence of Gli1, E-cadherin suppressed the level of nuclear β-catenin. E-cad<sup>cyto</sup> induced prominent accumulation of β-catenin in both the cytoplasm and nuclear fractions. This E-cad<sup>cyto</sup>-promoted accumulation of β-catenin was an independent event from Gli1, although coexpression with Gli1 results in redistribution of this pool of β-catenin to the nuclei. E-cad<sup>cyto</sup> behaves very differently in RK3E than in other cell lines (204-206). In RK3E, E-cad<sup>cyto</sup> is capable of promoting β-catenin signalling, possibly through inhibition of GSK3β-mediated phosphorylation. But more work is
required to unravel the mechanism underlying this event.

Surprisingly, we showed that Snail not only promoted the nuclear pool of β-catenin, but also nuclear expression of Gli1. This feed back regulation of Gli1 was revealed to be independent from the effect of Snail on E-cadherin, since WT E-cadherin or E-cad<sup>cyto</sup> alone did not show any effect of Gli1 expression level. This distinct role of Snail is also consistent with its ability to dramatically promote outgrowth of Gli1-induced foci (192). Thus, Snail may also be involved in post-transcriptional regulation of Gli1, although the mechanism remains unclear. We cannot exclude the possibility that other genes that induce Snail or suppress E-cadherin might cooperate with Gli1 during tumor progression, since Gli1 fails to completely suppress E-cadherin during transformation of RK3E. Multiple pathways are thought to regulate Snail in different context, e.g. TGFβ2, Notch, and FGF (92,93,191). Whether or how these pathways possibly interact with Shh-Gli signalling to promote cancer would be an intriguing question to further investigate.

Shh and Wnt are two signalling pathways that are coordinated at multiple levels. Following the discovery of Ci as a direct regulator of wg in Drosophila, several observations indicate that members of Wnt family can be induced by Shh, e.g. in human BCCs (134,207). Consistent with a role for Wnts in the response to Shh, many BCCs show increased levels of nuclear β-catenin (208-210). Shh and Wnt pathways also share regulation by GSK3β (211,212). As the two pathways contribute to the core network for cell fate determination and patterning, they are coordinated in several developmental events, e.g. somitogenesis and hair follicle morphogenesis (91,100,106,130,134,196,213).

Our investigation of targets of Gli1 showed that Wnt2b was rapidly induced in “tet-on” Gli1 cells. This is consistent with the observation of upregulated Wnt ligands
in human BCCs (182,214). Our studies support the idea that Shh-Gli signalling can coordinate with the Wnt pathway through Snail and E-cadherin, promoting cell division by maintaining or amplifying the initiating Wnt signal (section 2, Figure 7c). Snail and Wnt2b may synergize to promote β-catenin nuclear signaling or even transformation \textit{in vitro}, possibilities yet to be examined. In summary, our studies demonstrate potential roles of Snail, E-cadherin, Wnt signalling and β-catenin in the Shh response and suggest similar role in human tumors that show activation of the Shh-Gli pathway. Our model of Shh-Gli1 regulation of the β-catenin in the epithelial transformation may be applicable to several development settings. It may also be generally applied to tumor types showing aberrant Shh-Gli signalling.
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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL
NOTICE OF APPROVAL

DATE: April 3, 2006

TO: J. Michael Ruppert, M.D., Ph.D.
WTI-570 3300
FAX: 975-5650

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Role of GLI in Tumor Progression
Sponsor: NIH
Animal Project Number: 060306040

On March 29, 2006, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
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<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>400</td>
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Animal use is scheduled for review one year from March 2006. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 060306040 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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