Transcriptional Regulation of bcl-2 Mediated by the Sonic Hedgehog Signaling Pathway through gli-1*

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Basal cell carcinomas (BCCs) express high levels of the antiapoptotic proto-oncogene, bcl-2, and we have shown that bcl-2 contributes to the malignant phenotype in a transgenic mouse model. The basis of bcl-2 transcriptional regulation in keratinocytes is unknown. The sonic hedgehog (SHH) signaling pathway is frequently altered in BCCs. Mediators of shh signaling include the downstream transactivator, gli-1, and transrepressor, gli-3. Seven candidate gli binding sites were identified in the bcl-2 promoter. Cotransfection of increasing amounts of gli-1 in keratinocytes resulted in a corresponding dose-dependent increase in bcl-2 promoter luciferase activity. Gli-1 was also able to up-regulate endogenous bcl-2. Gli-3 cotransfection resulted in no significant changes in bcl-2 promoter activity compared with control. Gli-3 has been demonstrated to be proteolytically processed into an N-terminal repressive form that can inhibit downstream transactivation by gli-1. Gli-3 mutants possessing only the N-terminal region or the C-terminal region were made and used in luciferase assays. The N terminus of gli-3 inhibited gli-1 transactivation of the bcl-2 promoter. Gel shift analysis and luciferase assays demonstrated that gli binding site 4 (–428 to –420), is important for gli transcriptional regulation. Skin samples from transgenic mice expressing an RU486 gli-1 transgene exhibited significantly higher levels of endogenous bcl-2 protein in epidermal keratinocytes as assessed by immunoblotting and immunohistochemistry. Together, these findings provide consistent evidence that gli proteins can transcriptionally regulate the bcl-2 promoter and that gli-3 can inhibit transactivation by gli-1. These studies further suggest that one consequence of the deregulation of shh signaling in BCC is the up-regulation of bcl-2.

Non-melanoma skin cancer is the most frequently diagnosed form of cancer in the United States and basal cell carcinomas (BCC) constitutes more than 75% of these neoplasms (1).

Recent evidence has established the importance of the shh pathway in the development of BCC (2–6). The sonic hedgehog (shh) pathway was first characterized in normal developmental processes, including the formation of the dorsal to ventral axis of the neural tube, the anterior to posterior axis of the limb bud, and the development of the foregut (7–9). Alterations in the sonic hedgehog pathway have been implicated in the development of malignancies other than basal cell carcinoma including medulloblastoma and rhabdomyosarcoma (3).

The sonic hedgehog receptor, patched (ptc), normally functions to inhibit shh signaling. Binding of shh to ptc relieves the inhibition, allowing for transduction to continue through smoothened (smo) and a large multiprotein complex in the cytosol (10). The downstream transcription factors, gli-1, -2, and -3 bind to the same DNA sequence (5′-GACCACCCA-3′) (11, 12). Gli-2 and Gli-3 have both transactivation and repressive domains, whereas gli-1 has been suggested to function only as a transactivator (13, 14). Protein kinase A is believed to be the common negative regulator of the pathway (10). It has been demonstrated that PKA activity indirectly induces cleavage of gli-3 into its repressor form, which can be inhibited by shh (13). The pathway is further complicated by the evidence that gli-3 may be required for transcriptional up-regulation of gli-1 (13).

BCC is associated with mutations in the shh pathway. Loss of heterozygosity is frequently found at the location of patched, 9q22, in patients suffering from nevoid basal cell carcinoma syndrome who are predisposed to developing BCCs, and in sporadic BCC tumors (2, 15). Premature protein termination and inactivating mutations of patched leads to increased expression of a mutant patched protein (2, 4). The loss of ptc leads to subsequent increased expression of smoothened, shh, and gli-1, because of the positive feedback loop initiated by gli-1 transactivation (5, 6, 16). This effect may be augmented by activating missense mutations in smo (17). The mutations in ptc and smo result in 50% of BCCs overexpressing shh and 98% overexpressing gli-1 (6). Transgenic mice with shh or gli-1 targeted to the basal layer of the epidermis developed features similar to BCCs (18, 19) and ptc−/− mice developed spontaneous BCCs upon UV radiation (20).

The bcl-2 family is important in the regulation of apoptosis (21). bcl-2 is composed of three exons, with an untranslated first exon and two introns of 220 bp and a large 370-kb intron (22). Studies show that bcl-2 has two promoter regions. P2 is located immediately 5′ to the open reading frame in exon II and contains both a TATA and CAAT box (22). This site is responsible for a small percentage of transcripts in cells such as B cells (22). The second promoter, P1, is located in exon I in a
GC-rich region and does not contain a TATA nor a CAAT box (22). PI contains several Sp1 binding sites and has transcription initiation sites (24). Transcription factors previously reported to be involved in the regulation of bcl-2 include: Brn3a in neuronal cells (23), WT1 (24, 25), Aiolos in T-cells (26), HIV Tat (27), and FAX8 (28). In addition to these positive regulatory sites, the promoter also contains pie1 binding sites, which serve as negative regulatory regions and are thought to be important in pre-B cell selection (29, 30).

We have previously demonstrated that the members of the bcl-2 family are expressed in specific regions of the epidermis. Bcl-2 is exclusively expressed in the basal layer of the epidermis and undergoes a 2–3-fold increase in expression in BCCs (31, 32). In contrast, bcl-2 is undetectable in squamous cell carcinomas (31, 32). The increased expression of bcl-2 may contribute to the malignant phenotype through inhibition of cell death (33, 34).

Transcriptional regulators of bcl-2 expression in keratinocytes have yet to be identified. Here we demonstrate a link between the shh signaling pathway and bcl-2 expression in keratinocytes. Seven candidate gli binding sites were identified in the bcl-2 promoter. Gli binding site 4 was shown to be important in the transcriptional regulation by gli. Additionally it was demonstrated that a balance between gli-1 transactivation and gli-3 repression controls bcl-2 levels in keratinocytes. These observations suggest that the overexpression of gli-1 in basal cell carcinomas may contribute to the development of BCC by transcriptionally up-regulating bcl-2, thus inhibiting cell death.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The pGL3-2.8 bcl-2 promoter construct is illustrated in Fig. 1. The sequence depicted in Fig. 1 can be found in GenBank™, accession numbers X51898 and M13994. A three-step cloning strategy was used. Briefly, a 308-bp product was PCR amplified with primer generated XhoI and HindIII from a 7.8-kb genomic HindIII fragment of the Bcl-2 gene. This 308-bp product covers a region just 5' to the Accl site to just 5' of the initiation codon. This product was cloned into the XhoI and HindIII sites of the pGL3 basic luciferase vector (Promega, Madison, WI) to produce pGL3-308 bcl-2. Next, a 456-bp XhoI AccI restriction fragment was cloned into pGL3-308 bcl-2 producing pGL3-748 bcl-2, which lacks gli binding sites 1 through 6. Finally, a 2100-bp XhoI fragment was cloned into pGL3-748 bcl-2 producing pGL3-2.8 bcl-2. The remaining bcl-2 luciferase deletion constructs were made using unique restriction sites in the promoter as observed in Fig. 6 and blunt-end ligation. pGL3-2.8 bcl-2; pGL3-87; pGL3-84; pGL3-486; pGL3-387; pGL3-2100; pGL3-1175; pGL3-748 were run on a 4% acrylamide gel in 0.5× TBE at 100 V that was prerun for 30 min, 1 l of cold competitor oligonucleotide for 30 min on ice. 1 l of the hot probe was added and incubated for 30 min on ice. The reactions were run on a 4% acrylamide gel in 0.5× TBE at 100 V that was prerun for 5 min. 1 l of binding buffer, 1 l of water was added to the sample load for electrophoresis. 1 l of cold competitor oligonucleotide for 30 min on ice. 1 l of the hot probe was added and incubated for 30 min on ice. The reactions were run on a 4% acrylamide gel in 0.5× TBE at 100 V that was prerun for 5 min.
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Fig. 1. Potential gli binding sites on the bcl-2 promoter and homology to the gli consensus sequence. PatSearch version 1.1 from Transfac was used to search the 2.9-kb region of the bcl-2 promoter for potential gli binding sites. A, location of the potential gli binding sites (numbers 1–7) in relation to the structure of the gene. P1 and P2 represent the two transcriptional start sites. ORF represents the open reading frame, which begins in exon 2 and ends in exon 3. The distance between P1 and the beginning of the promoter region is 1420 base pairs, whereas the distance between the ORF and the beginning of the promoter is 2852 base pairs. B, position of the binding sites on the promoter in relation to the P1 transcriptional start site and the sequence homology to the gli consensus binding sequence, GACCACCCA.

Fig. 2. Transfection of increasing concentrations of gli-1, but not gli-3, results in a dose-dependent increase in bcl-2 luciferase activity. Primary keratinocytes were transfected with 1, 3, or 5 μg of pcDNA gli-1 or gli-3, 2 μg of pGL3-2.8 bcl-2, and 0.5 μg of Renilla luciferase. Nonspecific vector control pcDNA was used to normalize to a total of 7.5 μg. Protein lysates were prepared after 24 h and quantitated using a luminometer. Results were normalized for transfection efficiency using Renilla luciferase. The experiment was performed in triplicate and repeated 3 times.
using mouse bcl-2 antibody from BD Pharmingen (San Diego, CA) and α-actin, Sigma.

RNA was isolated from skin biopsies using the RNeasy Mini kit from Qiagen (Valencia, CA) as per the supplier's protocol. 1 μg was treated with RQ1 RNase-free DNase (Promega) according to the supplier’s protocol. Synthesis of cDNA was done using Superscript RNase H-reverse transcriptase (Invitrogen), 50 ng of oligo(dT)$_{15}$ primer (Promega), PCR nucleotide mixture, and recombinant RNasin ribonuclease inhibitor (Promega). 5 μl of cDNA was used for the PCR using Taq DNA polymerase (Promega) and oligonucleotide primers specific for gli-1 for 40 cycles. Reaction products were electrophoresed on a 2% SeKem LE-agarose gel.

**RESULTS**

**Identification of Candidate Gli Binding Sites in the bcl-2 Promoter**—The observation that both gli-1 and bcl-2 are overexpressed in basal cell carcinomas prompted us to search the bcl-2 promoter for potential gli binding sites. Gli-1, -2, and -3 bind to the DNA consensus sequence 5’-GACCACCCA-3’ (11,
PatSearch version 1.1 from Transfac was used to locate potential regions of gli binding. Data base analysis revealed seven potential sites (Fig. 1). Sites 1 through 5 are located upstream of the P1 transcriptional start site, whereas site 6 is located downstream of P1 in exon 2 and site 7 is located slightly upstream of the P2 transcriptional start site. The homology of each gli binding site to the canonical consensus sequence 5'-GACCACCCA-3' varied from 55% (site 2) to 89% (sites 1 and 4).

**Gli-1 Positively Regulates bcl-2 Transcription**—Gli-1 has been shown to contain only a transactivation domain and is considered the primary regulator of shh-induced transactivation (13, 14). Luciferase assays were used to determine whether gli-1 and gli-3 are able to regulate bcl-2 transcription. A bcl-2 reporter construct (pGL3–2.8 bcl-2) was made by ligating a 2.8-kb fragment of the bcl-2 promoter to the firefly luciferase gene. Assays were done in primary keratinocyte cultures obtained from neonatal human foreskin.

Increasing concentrations of gli-1 pcDNA transfected with the pGL3–2.8 bcl-2 promoter construct resulted in a dose-dependent increase in promoter activity. In contrast, increasing concentrations of gli-3 pcDNA resulted in no apparent change in steady-state promoter activity (Fig. 2). Reporter luciferase values were normalized for transfection efficiency using values obtained with the Renilla luciferase construct. Similar results were obtained using NIH 3T3 fibroblasts (data not shown).

**The N-terminal Domain of Gli-3 Inhibits Gli-1 Transactivation**—To determine whether gli-3 is able to inhibit gli-1 transactivation of the bcl-2 promoter, luciferase assays were done in primary keratinocytes using 2 μg each of gli-1 and gli-3 in combination. Interestingly, gli-1 co-transfected with gli-3 dem-
onstrated no significant changes compared with gli-1 alone (Fig. 3), suggesting that gli-3 may not be processed into its N-terminal repressive form upon transfection into the primary keratinocytes. These observations prompted us to make gli-3 mutants containing either a truncated N terminus (gli-3\textsuperscript{H9004N}pcDNA) lacking the repressive domain or a truncated C terminus (gli-3\textsuperscript{H9004C}pcDNA), similar to the physiological repressive form of gli-3 (Fig. 4). Luciferase assays were performed in primary keratinocytes as above with gli-1 pcDNA combined with equal concentrations of either gli-3\textsuperscript{H9004N} pcDNA or gli-3\textsuperscript{H9004C}pcDNA. Conditions were normalized by adding nonspecific vector control DNA and through the use of Renilla luciferase.

The gli-3\textsuperscript{H9004N} pcDNA construct, lacking a repression domain, resulted in increased luciferase activity, whereas gli-3\textsuperscript{H9004C} pcDNA alone was similar to gli-3 pcDNA transfection (Fig. 3). When gli-1 and gli-3\textsuperscript{H9004N} pcDNA constructs were used in combination bcl-2 promoter activity was increased over that observed using gli-1 alone (Fig. 3). However, the N-terminal repressive form of gli-3 (gli-3\textsuperscript{H9004C}pcDNA) was able to inhibit transactivation by gli-1 (Fig. 3) (p = 0.039) compared with gli-1 alone. Similar results were obtained in NIH 3T3 cells (data not shown). These observations suggest the importance of the cleavage event of gli-3 to produce a repressive form capable of inhibiting transactivation by gli-1.

**Determination of Gli Binding Sites in the bcl-2 Promoter**

Electrophoretic mobility shift assays were performed to determine whether the gli transcriptional regulatory proteins could interact with any of the candidate gli binding elements present in the bcl-2 promoter. Gli-1 and gli-3 protein were prepared by in vitro transcription/translation using rabbit reticulocyte lysates. \textsuperscript{35}S-Labeled protein was run on SDS-PAGE to determine whether the correct sized protein was produced (Fig. 5A). Gel shift assays were done using 3\textmu g of cold translated protein incubated with probes corresponding to a positive control previously reported to bind to gli (35), a mutant oligonucleotide (−), and the seven probes corresponding to the potential gli binding sites in the bcl-2 promoter. The reactions were incubated with either excess cold competitor or mutant competitor before the addition of hot labeled oligonucleotide.

Results indicate that both the gli-1 and gli-3 proteins were able to bind to the positive control, but not the mutant oligonucleotide. The shift could be competed with an excess of cold competitor. Shifted bands were also observed with the oligonucleotides corresponding to binding sites 4 and 6. These were
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also able to be competed with cold competitor, but not a mutant (Fig. 5B). Site 4 has an 89% homology to the consensus sequence differing with an adenine at position 8, whereas site 6 has a 78% homology, with a cytosine at position 1 and an adenine at position 7 (Fig. 1).

To verify the results obtained through in vitro transcribed/translated protein, gel shift analysis was performed with a bacterially expressed protein. The DNA binding domain of gli-3 was expressed as a GST fusion protein and 0.5 μg of GST-gli3DBD protein was used in similar analysis as explained above with GST alone as a negative control. Results verify those obtained with gli-1 and gli-3 TNT protein (data not shown).

Gli Binding Site 4 Is Responsible for Gli-1 Transactivation—To further specify which region of the bcl-2 promoter mediated transcriptional regulation by gli-1 a series of bcl-2 luciferase mutants were made using unique restriction sites in the bcl-2 promoter (Fig. 6). Luciferase assays were performed as described, in primary keratinocytes. Results were compared with those obtained for gli-1 pCDNA with the full-length pGL3–2.8 bcl-2 construct.

Deletion of the regions between −1324 to −897 (pGL3-2.8 bcl-2ΔB) and −399 to −350 (pGL3-2.8 bcl-2Δ5) had no significant effect on luciferase activity compared with the full-length pGL3-2.8 bcl-2 construct (Fig. 7), suggesting that sites 1 and 5 are not important for gli transactivation. However, deletion of the region −897 to −350 (pGL3-2.8 bcl-2Δ2-5) and −1324 to −350 (pGL3-2.8 bcl-2Δ1-5), which includes gli binding site 4, decreased luciferase activity in half compared with the pGL3-2.8 bcl-2 (Fig. 7) (p = 0.051 and 0.035, respectively). Further deletion of the region between −350 to +677, which includes gli binding site 6 (pGL3-748 bcl-2) decreased luciferase activity to basal levels (Fig. 7) (p = 0.002).

To further verify if gli binding sites 4 and 6 are responsible for gli transactivation, the full-length bcl-2 promoter between the region of −230 to +600, which contains site 6, was ligated to the pGL3-basic promoter (pGL3-2.8 bcl-2Δ1-5/7). This region did not demonstrate a significant increase in luciferase activity when co-transfected with the gli-1 expression construct compared with vector control (Fig. 7). This suggests that site 6 is not the principle mediator of transcriptional regulation by gli-1.

The region between −435 to −198, containing site 4, was ligated to the pGL3-SV40 minimal promoter (pGL3SV40 bcl-2-4) and similar luciferase assays were performed in primary keratinocytes. Co-transfection with gli-1 induced luciferase activity 20-fold compared with vector control (Fig. 8). PCR-based site-directed mutagenesis was performed on site 4, mutating the potential gli-binding site 5′-GACCACAA-3′ to 5′-GAT-CTTAAA-3′ (pGL3SV40 bcl-2mut4). The 5-base pair mutation reduced luciferase activity to 5-fold over vector control (Fig. 8). These observations combined with the results obtained with gel shift analysis suggest that the region responsible for gli transactivation is mediated mainly by binding site 4.

Up-regulation of Endogenous bcl-2 Expression in Gli-1 Transgenic Mouse Epidermis—Immunohistochemical analysis of the epidermis from K14p65GL/gli-1 transgenic mice induced with RU486 demonstrated a high level of bcl-2 protein compared with control littermate epidermis (Fig. 9). To further examine the effect of increased gli-1 expression in mammalian skin, 2-mm mouse ear biopsies were obtained from a K14p65GL control littermate and K14p65GL/gli-1 mice induced with RU486. Immunoblot analysis demonstrated consistently higher levels of bcl-2 protein in lysates from RU486-induced K14p65GL/gli-1 mice compared with those from uninduced K14p65GL/gli-1 mice compared with control littermate epidermis (Fig. 9). To further examine the effect of increased gli-1 expression in mammalian skin, 2-mm mouse ear biopsies were obtained from a K14p65GL control littermate and K14p65GL/gli-1 mice induced with RU486. Immunoblot analysis demonstrated consistently higher levels of bcl-2 protein in lysates from RU486-induced K14p65GL/gli-1 mice compared with those from control littermates.

Fig. 9. Immunostaining for Bcl-2 in mouse epidermis. A, sections of skin from control littermate (A) and from K14GLp65/gli-1 transgenic mice (B) induced by RU486 were immunostained for endogenous bcl-2 and examined by light microscopy. Substantially higher levels of bcl-2 protein were observed in the epidermis of the K14GLp65/gli-1 mice compared with that from control litters.

Fig. 10. Western blot analysis of tissue sample from Gli-1 transgenic mice. A, results from the immunohistochemical studies were further validated using immunoblotting of protein extracts from control littermate and K14GLp65/gli-1 transgenic mice induced RU486. Protein levels are significantly higher in epidermal samples from K14p65GL/gli-1 mice compared with control littermates. B, reverse transcriptase-PCR assessment of gli-1 expression in transgenic mice. Only the K14p65GL/gli-1 mice induced with RU486 show the predicted 350-bp gli-1 band in ethidium bromide-stained agarose gels (lanes 1 and 2). No detectable transcript was observed in amplified template from control littermate and K14p65GL/gli-1 transgenic mice induced with RU486. Protein levels are estimated using molecular weight standards (M) were used to estimate the size of the amplified bands.

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control littermate mice (Fig. 10A). Lysates from uninduced skin showed levels of bcl-2 protein comparable with those from induced control littermates (not shown). Matched skin biopsies from the above mice were also assessed for gli-1 mRNA expression using reverse transcriptase-PCR. The predicted 350-bp amplified gli-1 fragment was observed only in RU486-induced K14p65GL/gli-1 tissue (Fig. 10B).

**DISCUSSION**

Studies in our laboratory and others have previously documented that cutaneous BCCs consistently express high levels of the anti-apoptotic bcl-2 protein (31, 32). It has more recently been established that alterations in the shh signaling pathway occur in virtually all BCCs of the skin (2–6). Transgenic animal models have demonstrated that both bcl-2 and shh pathway members can contribute to multistep skin carcinogenesis in vivo (18–20, 33). It was therefore of interest to determine whether there might exist a relationship between alterations in shh signaling and up-regulation of bcl-2 in keratinocytes. This issue was of particular interest in that transcriptional regulators of bcl-2 in the epidermis have not yet been defined.

In this study, the first evidence of transcriptional regulation of bcl-2 in epidermal keratinocytes by gli-1 and gli-3 is provided. Gli-1 is considered the positive transcriptional transactivator in the shh pathway. Our studies showed that gli-1 was able to positively regulate bcl-2 transcription in primary keratinocytes in a dose-dependent manner, however, gli-3 showed no significant changes in luciferase activity compared with control. Gli-1 was also able to induce endogenous bcl-2 expression in gli-1-inducible transgenic mice. Gli-3 is processed by an, as yet unidentified, protease resulting in the generation of an N-terminal cleaved product that functions as a transcriptional repressor. This repressor form of gli-3 is capable of inhibiting transactivation by gli-1 (13, 14). The observations of no significant effect of the full-length gli-3 on bcl-2 promoter activity prompted us to create gli-3 mutants that lacked either the repressive domain (gli-3ΔN pcDNA) or the transcriptional domain (gli-3ΔC pcDNA). Luciferase assays with these constructs revealed that the repressive form of gli-3, gli-3ΔC, but not the construct lacking the repressive domain, gli-3ΔN, was able to inhibit gli-1-induced transactivation.

The gli DNA-binding consensus site is CAGCAGCAGC. Downstream gli targets have not been extensively characterized. Recent gli targets identified through DNA/protein interactions include HNF-3B, cyclin D2, IGFBP-6, osteopontin, and plakoglobin (36, 37). The binding sites in these promoters each include HNF-3B, cyclin D2, IGFBP-6, osteopontin, and plakoglobin (36). The binding sites in these promoters each include HNF-3B, cyclin D2, IGFBP-6, osteopontin, and plakoglobin (36).

These observations agree with the associated activities of gli-1 and gli-3 and suggest that the overexpression of bcl-2 in BCCs is a consequence of the deregulation of the shh pathway. Gli-1 is overexpressed in other malignancies including glioblastoma and medulloblastoma (3). Interestingly, it has been discovered that bcl-2 overexpression is also observed in these malignancies (38). These findings suggest the possibility that a common consequence of gli-1 deregulation may be the up-regulation of bcl-2 expression in specific malignancies.

Interestingly, the shh pathway has been implicated in the regulation of developmental cell death. The phenotype of shh-deficient mice includes loss of bronchial arch structures resulting from disordered cell death (39). Mimicking the loss of shh by blocking the shh signal with a hybridoma antibody resulted in craniofacial and brain abnormalities associated with an increase in apoptosis (39). Together these observations suggest that the observed derepression of shh signaling in BCC, such as that resulting from inactivating ptc mutations, may result in an inappropriate viability advantage in tumor cells. Furthermore, on the basis of the findings reported here it is reasonable to assume that this enhanced viability would, at least in part, be mediated by deregulation of bcl-2 expression.

The shh pathway is necessary for normal morphogenesis of the hair follicle. Sonic hedgehog expression is induced in the epithelium by fibroblast growth factors and bone morphogenic proteins, which subsequently induces the expression of patched in the underlying mesenchyme and the formation of the dermal papillae (40, 41). Shh-deficient mice exhibit reduced hair growth resulting from impaired formation of dermal papillae (41). Conversely, adenoviral shh-infected mouse epidermis has been shown to exhibit an increased rate of hair growth (42). Gli-1 is normally expressed in the outer root sheath of the hair follicle and a subpopulation of mesenchymal cells in the bulge region (43). Notably, bcl-2 protein is also expressed in the outer root sheath and in dermal papillae (31, 44). It has been suggested that basal cell carcinomas arise from stem cells located in the hair follicle region (45), implying that a deregulation of the shh pathway in the hair follicle may result in the development of BCC. On the basis of these observations, it seems reasonable to consider that bcl-2 is an important transcriptional target in the shh signaling pathway.

In summary, our observations are consistent with the interpretation that one consequence of deregulated hedgehog signaling in BCC is the up-regulation of the bcl-2 anti-apoptotic proto-oncogene. This effect can be mediated directly at the level of the bcl-2 promoter by gli-1 transcriptional activation and is likely to be of importance in the pathogenesis of these malignancies.

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