Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation

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Although tissue microenvironments play critical roles in epithelial development and tumorigenesis, the factors mediating these effects are poorly understood. In this work, we used a genomic approach to identify factors produced by cells in the microenvironment of basal cell carcinoma (BCC) of the skin, one of the most common human cancers. The global gene expression programs of stromal cell cultures derived from human BCCs showed consistent, systematic differences from those derived from nontumor skin. The gene most consistently expressed at a higher level in BCC tumor stromal cells compared with those from nontumor skin was *GREMLIN 1*, which encodes a secreted antagonist of the bone morphogenetic protein (BMP) pathway. BMPs and their antagonists are known to play a crucial role in stem and progenitor cell biology as regulators of the balance between expansion and differentiation. Consistent with the hypothesis that BMP antagonists might have a similar role in cancer, we found *GREMLIN 1* expression in the stroma of human BCC tumors but not in normal skin *in vivo*. Furthermore, BMP 2 and 4 are expressed by BCC cells. *Ex vivo*, BMP inhibits, and Gremlin 1 promotes, proliferation of cultured BCC cells. We further found that *GREMLIN 1* is expressed by stromal cells in many carcinomas but not in the corresponding normal tissue counterparts that we examined. Our data suggest that BMP antagonists may be important constituents of tumor stroma, providing a favorable microenvironment for cancer cell survival and expansion in many cancers.

cancer biology | stem cell regulation | tissue microenvironment | tumor stroma

Tissue microenvironments play a critical role in specifying cellular niches in both the developing embryo and adult organisms (1, 2). In development, cell fate decisions are dictated not only by cell-autonomous signals but also by stimuli from the surrounding tissue microenvironment (3, 4). Similarly, in adult tissues that continue to renew throughout the lifetime of the organism, such as the skin, intestinal epithelium, and hematopoietic system, the self renewal and maturation of the stem cell population are regulated by specific molecular cues derived from the corresponding microenvironments (5–7). In the skin, hair follicle morphogenesis is regulated by signals coming from the dermal papilla, a specialized mesenchymal structure that signals to matrix stem cells located across the basement membrane (8, 9). Similarly, the modulation of stem cell activity in the intestine is also subject to cues derived from underlying mesenchymal cells that surround the crypt (10, 11). Hematopoietic stem cells are regulated in part by osteoblasts, cells that reside in the adjacent bone spicule (12, 13). In all of these cases, a crucial feature of the regulation of stem cell compartment size, location, and timing of self renewal is the production of critical factors by a specialized set of mesenchymal cells that create a customized microenvironment.

During carcinogenesis, an analogous system of specialized tissue microenvironment cells may also be important in specifying a "tumor cell niche" that supports a self-renewing population of tumor cells. Paradoxically, although uncontrolled proliferation and survival are the cardinal characteristics of cancer cells, it can be difficult to sustain these cells away from their corresponding microenvironment, either in culture or as explants (14). There is accumulating evidence that tumor stroma influences tumor development (15, 16). Genetic studies have shown that stromal cells are altered in some inherited cancer-susceptibility syndromes (17). In breast cancer, rearrangements at several loci have been noted exclusively in tumor-associated stromal cells (18). *In vivo* and *in vitro* experiments demonstrated that human prostatic epithelial cells showed dramatic changes both in histology and growth rate when grown with human fibroblast cells derived from prostatic carcinoma, suggesting that carcinoma-derived fibroblasts can stimulate tumorigenesis (19). Others have shown that coinjection of fibroblasts with tumor epithelial cells into mice can enhance tumor formation (20).

To identify factors produced by tumor stromal cells that contribute to the initiation or maintenance of the tumor, we used a genomic approach with basal cell carcinoma (BCC) of the skin, one of the most common human neoplasms, as our model system. Previous work with human autotransplants of BCC lesions has suggested that stromal cells in the tumor tissue play a crucial role in sustaining the tumor (21). Mouse models of the disease have shown that sustained activation of the Sonic Hedgehog pathway, a major genetic component of BCC, is maintained only in the context of the animal *in vivo*; when explanted in culture, tumor cells lose pathway activity (22).

We cultured stromal cells from BCC tumor and nontumor human skin and compared those two cell populations by cDNA microarray analysis. Antagonists of the bone morphogenetic protein (BMP) pathway were among the genes most consistently and significantly differentially expressed between the two populations. Given what is already known about the role of BMPs and their antagonists in regulating stem cell compartments in normal development and physiology, we hypothesized that a similar role could be played by BMPs and BMP antagonists in the context of the tumor.

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Abbreviations: BCC, basal cell carcinoma; BMP, bone morphogenetic protein; ISH, *in situ* hybridization; IHC, immunohistochemistry.

Data deposition: The array data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5502).

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BMPs are important regulators of stem cell fate (23). In diverse settings, BMPs promote differentiation of stem cells, thus promoting exit from the stem cell compartment (12, 24). In the skin, conditional gene targeting of *BMPRIA* in mice has demonstrated that BMPRIA is required for proper differentiation of progenitor cells in the hair shaft (25, 26). The BMP inhibitor noggin is expressed by cells in the follicular mesenchyme, and mice lacking noggin display defects in hair follicle induction and morphogenesis (9, 27). High levels of *GREMLIN 1* transcript have been observed in mouse embryonic fibroblast cells that are capable of maintaining human embryonic stem cells in culture (28). These observations led us to investigate the hypothesis that BMP antagonists secreted by stromal cells in cancer tissues might be an important part of the specialized tumor microenvironment that allows continued proliferation and self renewal of cancer cells.

Results

Identification of Stromal Factors Important for BCC Tumorigenesis. To identify factors produced by tumor-associated stromal cells that contribute to the initiation or maintenance of BCC, we cultured stromal cells from fresh samples of human BCC or nontumor skin. The cells adhered readily to untreated plastic plates and were spindle-shaped and elongated. We used the Significance Analysis of Microarrays algorithm to identify genes differentially expressed between tumor- and nontumor-associated stromal cells (Fig. 1*A*; ref. 29). Fourteen genes were identified at an estimated false discovery rate of 5%, with 13 genes expressed at higher levels and one gene expressed at a lower level in the tumor-associated cells (Fig. 1*B* and Table 1, which is published as supporting information on the PNAS web site). Two of the 13 genes more highly expressed in BCC-derived stromal cells, *GREM1* (*GREMLIN 1*) and *FST* (*FOLLISTATIN*), both encode antagonists of the BMP pathway.

***GREMLIN 1* Expression Is Elevated in BCC, and BMPs Are Highly Expressed by BCC Tumors.** We analyzed expression of *GREMLIN 1 in vivo* in human tissue by quantitative RT-PCR analysis of independent samples of whole tissue from eight matched BCC and adjacent nontumor skin samples. *GREMLIN 1* transcripts were, indeed, expressed at higher levels in BCC tissue than in adjacent nontumor tissue from the same patient (Fig. 1*C*). We then performed *in situ* hybridization (ISH) in 15 paraffin-embedded BCC tissue samples and found detectable *GREMLIN 1* mRNA expression in 12 of 15 samples (80%). Expression was localized predominantly to stromal cells in the tumor, and immunohistochemistry (IHC) localized gremlin 1 protein to the stroma surrounding the tumor cell nests (Fig. 2 *B* and *D* and Fig. 6, which is published as supporting information on the PNAS web site). In contrast, no expression of *GREMLIN 1* RNA or protein was detected in normal skin (Fig. 2 *A* and *C*). Thirty-nine sections of normal skin from multiple anatomical sites, including arm (dorsal, ventral, posterior, and anterior), hand (dorsal and ventral), digits (posterior), palm, foot (dorsal and plantar), and leg (anterior, posterior, dorsal, and midline), were all negative for *GREMLIN 1* RNA, with only two exceptions: a few stromal cells surrounding a neuromuscular junction in one section of skin from below the knee, and a small number of stromal cells deep in the dermis of the foot dorsum (data not shown). These results indicate that *GREMLIN 1* RNA expression is below levels of detection or absent in the vast majority of normal human skin sites.

An implicit aspect of our hypothesis is that there exists a source of BMP in BCC tumors that needs to be antagonized to promote proliferation of tumor cells. We found that BMP 2 and 4 are, indeed, expressed in BCC tumor nests (Fig. 2 *E* and *F*). BMP antibody staining localized mostly to tumor cells, with macrophages occasionally demonstrating positive staining.

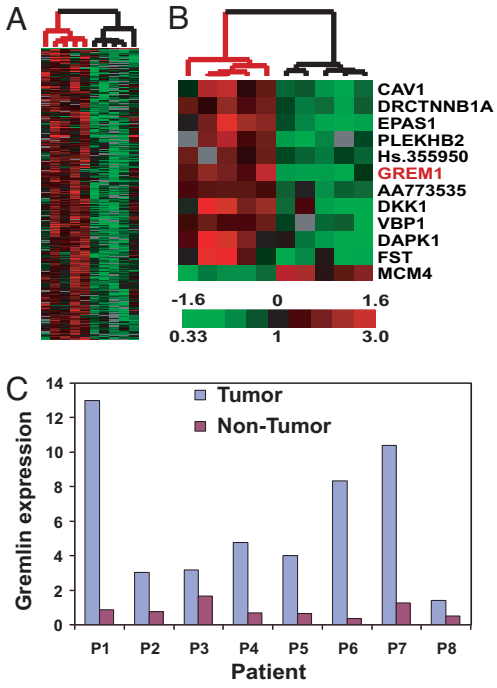

Fig. 1. *GREMLIN 1* mRNA is elevated in BCC tumor-derived stromal cultures *ex vivo* and in BCC tumors *in vivo*. (*A* and *B*) Gene expression in stromal cells from BCC tumor and nontumor skin. Each row in the heat map represents a gene, and each column represents a sample; cultures derived from BCC and nontumor skin are indicated by red and black branches, respectively. (*A*) Hierarchical clustering of samples based on expression of 403 array elements selected for differential expression by two-class Significance Analysis of Microarrays analysis at a false discovery rate of 15%. The level of expression of each gene in each stromal cell sample is relative to the mean level of expression of that gene across all samples and is represented by using a red-green color scale. (*B*) Hierarchical clustering of samples according to their expression of a more stringently selected set of genes (false discovery rate of 5%). (*C*) RT-PCR analysis of whole-tissue samples of BCC tumor or adjacent nontumor skin from eight patients. The relative level of *GREMLIN 1* RNA in each sample was normalized to *GAPDH* for that sample.

To better characterize the stromal cell population that expressed *GREMLIN 1* in BCC tumors, we analyzed adjacent serial sections of tumor by ISH for *GREMLIN 1* and IHC for various cell lineage markers: vimentin (characteristic of mesenchymal cells), CD45 (hematopoietic lineage), CD31 (endothelial cells), desmin (smooth muscle cells), cytokeratins (epithelial cells), and glial fibrillary acid protein (astrocytes and Schwann cells). *GREMLIN 1*-expressing cells were also strongly positive for vimentin, mostly or entirely negative for CD45 and desmin, and completely negative for CD31, keratins, and glial acid fibrillary protein (GFAP) (Fig. 2 *G–N*).

A Functional Response to gremlin 1 in Cultured Human Skin Epithelial Cells. We reasoned that if the functional role of gremlin 1 in maintaining a tumor cell niche was analogous to its role in the normal skin progenitor cell niche, gremlin 1 might be capable of inhibiting differentiation and promoting expansion of keratinocytes. To directly examine the effects of gremlin 1 on BCC tumor cells, cells isolated from fresh BCC tumors were cultured in the presence of recombinant human BMP 4, recombinant mouse gremlin 1, or both, and allowed to expand for 7 days. The resulting cell populations were compared by using quantitative RT-PCR to characterize their differentiation state (Fig. 3*A*). Compared with untreated controls, cells maintained in BMP 4 exhibited elevated mRNA levels of *SPRR1A*, *SPRR1B*, *SPRR3*, and *SPRR4*, established markers of differentiated keratinocytes.

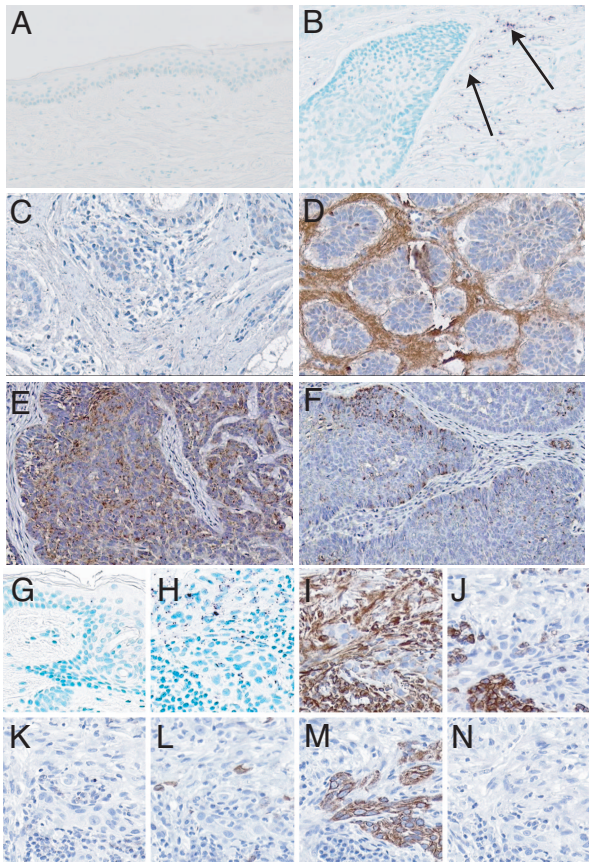

Fig. 2. Expression of *GREMLIN 1* and BMP 2 and 4 in BCC tumor tissues. (*A* and *B*) ISH for *GREMLIN 1* RNA in normal scalp (*A*) and BCC tumor skin (*B*). *GREMLIN 1* is expressed by stromal cells surrounding the tumor (indicated by arrows) but is undetectable in normal scalp. Positive signal appears as dark purple dots. (*C* and *D*) IHC for gremlin 1 protein in normal scalp (*C*) and BCC tumor skin (*D*). Positive signal appears as diffuse brown staining. (*E* and *F*) IHC with antibodies against BMP 2 (*E*) and 4 (*F*) in large sections of human BCC. Signal is represented by brown color. (*G–N*) serial sections of a BCC tumor showing that *GREMLIN 1*-expressing cells have properties of fibroblasts. (*G* and *H*) RNA ISH for *GREMLIN 1* RNA in nontumor (*G*) and tumor (*H*) skin. *GREMLIN 1* expression is indicated by dark purple dots. (*I–N*) IHC for cell lineage markers vimentin (*I*), CD45 (*J*), CD31 (*K*), desmin (*L*), pancytokeratin (*M*), and GFAP (*N*). Signal is represented by brown color.

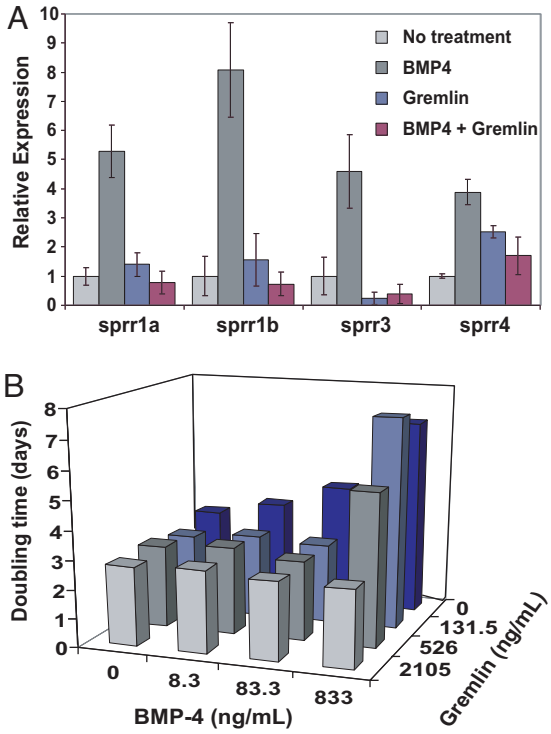

Fig. 3. Effects of BMP and gremlin 1 on BCC cell differentiation and expansion *in vitro*. (*A*) Cells were cultured from a human BCC tumor and then treated for 7 days in culture with recombinant human BMP 4 (833 ng/ml), recombinant mouse gremlin 1 (2,105 ng/ml), or both. Populations were then compared by using quantitative RT-PCR to detect the levels of *SPRR1A*, *SPRR1B*, *SPRR3*, and *SPRR4* transcripts. (*B*) Cells were cultured *in vitro* from human BCCs then treated with varying concentrations of gremlin 1 or BMP 4 protein for 7 days. Cells were counted by using a hemacytometer, with triplicate counts taken for each measurement; each measurement is the average of duplicate experiments.

Gremlin 1 strongly attenuated this effect. Gremlin 1 protein alone, in the absence of exogenously added BMP 4, had little effect on *SPRR* expression. [Note that basal media contains no detectable BMP (data not shown).]

Gremlin 1 also antagonized BMP-mediated repression of cell proliferation. Primary BCC keratinocytes were cultured and cell growth assessed in the presence of varying concentrations of recombinant human BMP 4 and recombinant mouse gremlin 1 (Fig. 3*B*). The doubling time of these cells in culture with no added BMP or gremlin 1 was 3.1 (+/− 0.1) days. Addition of gremlin 1 in the absence of added BMP 4 did not significantly affect growth rate, even at the highest concentration of gremlin (2.105 μg/ml). In the absence of gremlin 1, doubling time increased steadily with increasing BMP 4 concentration, reaching a maximum of 7.4 (+/− 0.1) days, 2.4 times the baseline doubling rate. At the highest level of BMP 4, increasing the concentration of gremlin 1 protein steadily lowered the doubling time back to baseline. These results indicated that BMP 4 inhibits the expansion of BCC cell populations in culture, and that gremlin 1 attenuates this inhibition.

***GREMLIN 1* Is Expressed by Stromal Cells in Diverse Human Carcinomas.** *GREMLIN 1* is highly expressed in the fibroblasts of most BCCs and undetectable in most normal skin sites. Evidence that BMPs regulate stem cell expansion in many tissues (skin, intestine, and blood) raised the possibility that expression of gremlin 1 may be an important feature of the tumor microenvironment in other cancers (12, 24, 25). We therefore examined *GREMLIN 1* RNA expression in a total of 774 tumors, including melanoma and carcinomas of the liver, testis, ovary, uterus, kidney, thyroid, prostate, head and neck, bladder, breast, lung, colon, pancreas, and esophagus (n = 11–260 samples of each) by ISH to tissue microarrays. *GREMLIN 1* was expressed by stromal cells in at least 50% of samples in carcinoma of the bladder, breast, lung, colon, pancreas, and esophagus, and in at least 25% of prostate and head and neck cancers (Fig. 4). Expression of *GREMLIN 1* was exclusively localized to the stromal cells, with the exception of some breast and prostate samples, which showed limited expression in the tumor cells themselves.

We also examined large sections of breast, pancreas, lung, and intestine, both tumor and nontumor. *GREMLIN 1* expression was undetectable in normal and benign breast tissue. In a series of 165 samples of pancreas, including normal tissue and benign and malignant lesions, we detected *GREMLIN 1* RNA in only 5% (2/37) of normal samples, compared with 71.5% of pancreatic tumors (68/95) (Fig. 7, which is published as supporting information on the PNAS web site). *GREMLIN 1* expression was also detected in 45% (15/33) of benign pancreatic disease samples, including pancreatitis, benign neuroendocrine tumors,

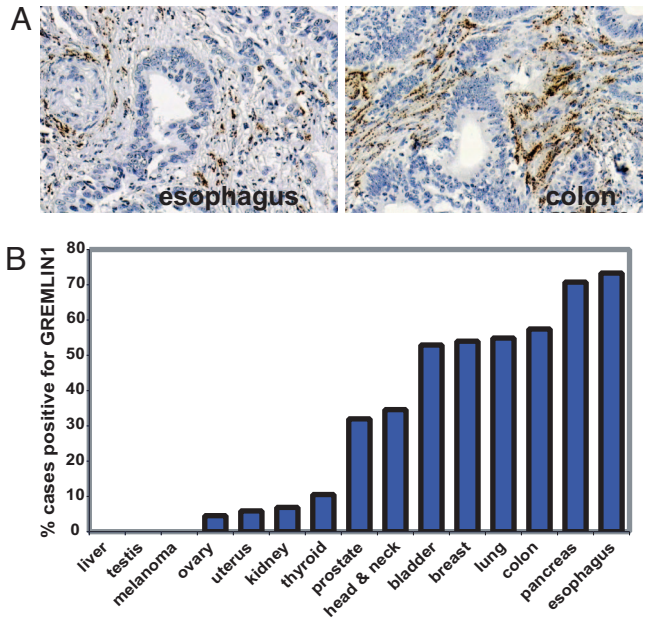

Fig. 4. *GREMLIN 1* RNA is widely expressed in cancer. (*A*) Representative images for ISH in other carcinomas. Tissue microarrays representing 774 human cancers of diverse tissue types were analyzed for expression of *GREMLIN 1* RNA. Positive signal is denoted by dark brown staining. (*B*) Tabulation of results for all tumor types examined for which *n* was >10.

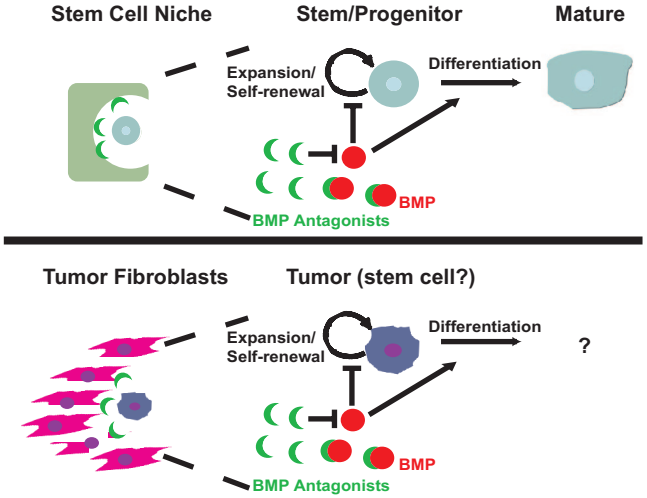

Fig. 5. A possible parallel for the role of BMPs and BMP antagonists in the tumor stem cell niche and normal stem cell niche. BMPs can induce differentiation and block expansion of normal stem and progenitor cells. (*Upper*) BMP antagonists can reverse this effect and favor expansion. Signals that are critical for stem cell self renewal, such as BMP antagonists, derive from the stem cell niche. BMPs may often come from epithelial or stem cells. (*Lower*) In tumor biology, BMPs can also inhibit expansion of tumor cells. BMP antagonists secreted by the tumor cell niche may reverse this inhibition, allowing tumor cell expansion to continue.

and benign adenomas. In normal lung tissue, we observed *GREMLIN 1* RNA in only a few smooth muscle cells. In large sections of both adenocarcinoma of the lung and adjacent normal lung tissue, there was no detectable *GREMLIN 1* mRNA in the normal lung, whereas the tumor stroma and not the tumor cells themselves showed expression of *GREMLIN 1* mRNA (Fig. 8, which is published as supporting information on the PNAS web site). In normal intestine, no *GREMLIN 1* expression was observed except in the lamina propria, in what appear by morphology to be smooth muscle cells (data not shown).

Discussion

We found that the BMP antagonist *GREMLIN 1* is frequently expressed by stromal cells in the microenvironment of human carcinomas, including BCC, and can enhance cell expansion and block differentiation *in vitro*. Carcinomas are histologically complex tissues comprising not only tumor cells but also fibroblasts, smooth muscle cells, endothelial cells, adipocytes, and leukocytes, as well as components of the extracellular matrix. Interactions with these cells and factors in the tumor microenvironment, or tumor cell niche, may also play a critical role in the initiation and progression of cancer (15, 16, 30). We used a genomic approach to identify factors differentially expressed by BCC-associated fibroblasts compared with their nontumor-associated counterparts. Global gene expression profiling of these two cell populations revealed intrinsic, systematic differences in gene expression programs. We chose to focus on one gene in particular, the BMP antagonist *GREMLIN 1*. In many settings, BMPs promote differentiation of stem cells, thus promoting exit from the stem cell compartment (23, 31). These observations led us to hypothesize that BMP antagonists may define a niche for a self-renewing population in some cancers.

In this model, the role of BMPs and their antagonists in regulating a self-renewing tumor cell compartment parallels their role in regulating the normal stem cell compartment (Fig. 5). In normal physiology, factors (including the BMP antagonists) that support "stemness" of stem cells are often provided by a stem cell "niche," a molecular microenvironment defined by a localized population of cells that regulates the size of the stem cell compartment (32, 33). Our data suggest a directly analogous model for the tumor context in which the tumor cells require BMP antagonists coming from the tumor fibroblasts (another specialized stromal compartment) to maintain their expansion.

Our results represent a dramatic example of the differences between stromal cells in cancer and those in the normal tissue counterpart. Elevated expression of *GREMLIN 1* has previously been documented in a small subset of cells in normal skin, the putative epithelial stem cells, compared with other normal skin epithelial cells (34). In our study, *GREMLIN 1* RNA was expressed in stromal cells of nearly all BCC samples examined, but undetectable in the vast majority of normal skin sites. The cells that express *GREMLIN 1* have the appearance and immunohistochemical characteristics of fibroblasts and not cells of epithelial, lymphocytic, endothelial, smooth muscle, or glial origin.

How is this distinct, specialized tumor stromal compartment initially established? Does the gremlin 1-rich tumor niche develop in response to signals derived from the tumor? If so, the presence of gremlin 1-expressing fibroblasts could be the product of either *de novo* differentiation, recruitment from a distant site, or preferential expansion of an otherwise rare population in response to molecular signals from the tumor cells. In an alternative model, the chronology is reversed, that is, a specialized niche favorable to tumor initiation and expansion may be established before the tumor can form, perhaps as a result of clonal expansion of a mutant or epigenetically modified clone of fibroblasts. Indeed, the familiar focal, patchy alterations in skin pigmentation and texture, hair morphology, and vascularization seen in aging, sun-exposed skin are consistent with preexisting local clonal fields of altered cells (35). Whatever events lead to the accumulation of gremlin 1-expressing fibroblasts in diverse carcinomas, the ability of tumor-derived fibroblasts to maintain this distinctive expression program even after many generations of culture *ex vivo*, away from the influence of their tumor

counterpart, suggests this maintenance is specified by a stable genetic or epigenetic program.

The addition of gremlin 1 alone to basal media was not enough to sustain long-term culture of BCC-derived cells. Thus, future work is needed to define additional supporting factors present in the tumor cell niche. As a preliminary step, we have used RT-PCR to examine the expression of a number of other reported BMP antagonists, including *TSG1*, *FOLLISTATIN*, *NOGGIN*, and *CHORDIN*, in whole tissue samples of human BCC and matched nontumor tissue. Like *GREMLIN 1*, both *TSG1* and *CHORDIN* were typically expressed at higher levels in tumors compared with nontumor controls (Fig. 9, which is published as supporting information on the PNAS web site). Further characterization of other factors in the tumor cell niche, combined with the identification of signals derived from basal cell tumor cells, will help elucidate the reciprocal crosstalk that occurs between the tumor and its microenvironment. Along with *GREMLIN 1*, other genes that were elevated in BCC tumor-associated fibroblasts included a number of components of the Wnt signaling pathway, such as *DICKKOPF HOMOLOG 1* (*DKK1*), a secreted protein inhibitor of the Wnt signaling pathway. The Wnt proteins (along with BMPs) are targets of the Sonic Hedgehog pathway (36). In one report of Wnt pathway activity in BCC, the pattern of nuclear β-catenin showed increased staining at the periphery of tumor nests, as well as some staining in tumor-adjacent fibroblasts (37). Additional experiments will be useful in uncovering the connections between Wnt, Sonic Hedgehog, and BMP signaling in BCC.

We have shown that BMP inhibits expansion of BCC cells in culture, and that gremlin 1 can overcome this inhibition. The mechanism of gremlin 1/BMP action and downstream signaling events, however, remains unclear. Although we have not definitively addressed whether the effects of gremlin are mediated exclusively through the BMP pathway, our data on cultured cells from BCC tumors suggest that this is likely, because gremlin 1 had no appreciable effect on cell expansion unless BMP was present. Although we did observe BMP in some of the tumor cells *in vivo*, the tumor cells *in vitro* showed a response to gremlin 1 only in the presence of exogenous BMP. Thus, the level of BMP production by the cultured tumor cells was not high enough to produce a clear effect at the plated cell density, possibly because of the effects of dilution by the media or by loss of normal cell–cell interaction normally seen *in vivo*.

The expression of *GREMLIN 1* by stromal cells in diverse human carcinomas, in contrast to its rare expression in corresponding normal tissues, suggests that expression by cells in the tumor microenvironment of factors that regulate the self renewal of the tumor cells may be a general feature of human cancer. Inhibiting these critical molecular signals from the tumor microenvironment may thus be a useful therapeutic strategy. The potential parallels between stem cell–microenvironment interactions in normal development and cancer should provide fertile ground for further investigations.

Materials and Methods

Primary Human Cells. Stromal cells were isolated from discarded skin tissue from the Dermatology Clinic with approval from the Institutional Review Board (Stanford University Medical Center). Fat was removed by using a sterile scalpel and forceps; tissue was minced into ≈1-mm cubes. Incubation in a six-well dish without medium at 37°C for 10 min allowed for adhesion of the tissue to the plate. Fresh media containing DMEM, 10% FBS, and penicillin-streptomycin were added, and samples were maintained at 37°C and 5% CO_2. Media were replaced every 2 days. Outgrowth of spindle-shaped cells was typically apparent after 5–15 days in culture and had a success rate of ≈60%. When the cells were near confluence, they were subcultured with 0.25% trypsin-EDTA. Cultures were expanded until sufficient for RNA isolations (typically four passages).

Human BCC keratinocyte cultures were derived from fresh skin tissue as described (38). A small crosssectional piece of each sample was cut and fixed in 10% buffered formalin for histological confirmation. The remaining tissue was placed overnight in 5 mg/ml dispase (Gibco, Carlsbad, CA) at 4°C. The next day, epidermis was separated from dermis with dissecting forceps, minced by using sterile forceps and scalpel, and incubated in 0.05% trypsin-EDTA at 37°C for 15 min, with occasional mixing to disperse cells. After neutralization with HBSS containing 15% FBS, cells were spun down at 900 rpm in a Beckman Allegra GR centrifuge for 5 min, then resuspended in Keratinocyte serum-free media supplemented with EGF, bovine pituitary extract, and penicillin-streptomycin (Gibco). Cells were plated onto 12-well collagen I-coated plates (BD Biosciences, Franklin Lakes, NJ) and incubated at 37°C in 5% CO_2. Media were replaced every 2 days. Contamination from fibroblasts or normal keratinocytes was avoided by subjecting the culture to differential trypsinization and a transient increase in calcium concentration, respectively (39).

Microarray Procedures. Construction of human cDNA microarrays with ≈42,000 elements, representing ≈24,000 genes, and hybridizations was as described (40). Forty-eight hours before RNA harvest of stromal cultures, cells were washed three times in prewarmed PBS and then maintained in low serum media containing DMEM and 0.1% FBS. mRNA was harvested by using the FastTrack kit (Invitrogen, Carlsbad, CA). Universal Human Reference RNA (Stratagene, La Jolla, CA) was used as reference for array experiments.

Arrays were scanned with a GenePix 4000A scanner and images analyzed with GenePix 3.0 (Axon Instruments, Union City, CA). Microarray data were stored in the Stanford Microarray Database (41). All microarray data are available at the web site http://microarray-pubs.stanford.edu/Gremlin1_BCC.

Data Analysis. We considered only genes for which the cognate array element had a fluorescent signal at least 1.5-fold greater than the local background signal in both channels. Significance Analysis of Microarrays (29) was then used to identify a set of genes whose expression levels were significantly different between five tumor- and five nontumor-derived stromal cell cultures at a false discovery rate of 15% or 5%. Resulting expression patterns were organized by hierarchical clustering (42).

ISH. Digoxigenin-labeled sense and antisense riboprobes for *GREMLIN 1* were synthesized by using T7 polymerase-directed *in vitro* transcription of linearized plasmid DNA (IMAGE clone 7262108) by using the DIG RNA Labeling Kit (Roche Diagnostics). ISH on paraffin sections was performed by using a biotinyl tyramide amplification procedure, essentially as described (43). Results were considered specific when a strong pattern of distinct punctate staining was seen for the antisense probe, and little or no staining was observed for the corresponding sense probe. Tissue microarrays of tumor samples were made as described (44).

IHC. IHC staining for Gremlin 1 was performed with Dako Envision Plus (Glostrup, Denmark). Anti-gremlin 1 antibody (Imgenex, San Diego, CA) was used at 1:10 dilution. IHC for BMPs was performed by using Vectastain ELITE ABC Rabbit IgG (Vector Laboratories, Burlingame, CA). Anti-BMP 2 and 4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:50 dilution. IHC for cell lineage specific markers was performed by using the Vectastain ELITE ABC Mouse IgG kit with antibodies against Vimentin (1:200), CD31 (1:30), CD45 (1:100), GFAP (1:100), Desmin (1:100), and pancytokeratin

(1:100; Dako). In all cases, antigen retrieval consisted of a microwave step in 10 mM citrate buffer. Nuclei were stained with hematoxylin.

As positive and negative controls, each antibody was also tested on a tissue microarray containing a large variety of normal and tumor human tissue samples to confirm the nominal specificity. ISH and IHC images were acquired with the BLISS Microscope System (Bacus Laboratories, Lombard, IL).

***In Vitro* Expansion and Differentiation Assays.** To assess the effects of gremlin 1 and BMP proteins on expansion of cells *in vitro*, BCC-derived cells were maintained in keratinocyte growth media containing bovine pituitary extract, human EGF, bovine insulin, hydrocortisone, gentamicin, and amphotericin B (Clonetics, San Diego, CA). Cells were incubated with recombinant mouse gremlin 1 and/or recombinant human BMP 2 or 4 (R&D Systems, Minneapolis, MN) at the concentrations indicated. Cell number was assessed by using triplicate counts with a hemacytometer, or RNA was collected for RT-PCR analysis.

Quantitative RT-PCR. Total RNA was isolated from whole tissue, either tumor or adjacent nontumor tissue from the same patient, by using the RNeasy Fibrous Tissue Mini kit (Qiagen, Chatsworth, CA) and a rotor homogenizer. Total RNA was isolated from cultured cells by using RNeasy Mini (Qiagen). First-strand DNA was generated from mRNA by using the SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR (TaqMan) was performed by using ABI 7300 (Applied Biosystems, Foster City, CA) with duplicate experimental samples for each sample and each probe/primer set. *GAPDH* was used for normalizing PCR results.

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