A role of PDGFRα in basal cell carcinoma proliferation

Jingwu Xie*†‡, Michelle Aszterbaum§, Xiaoli Zhang*†, Jeannette M. Bonifas§, Christopher Zachary§, Ervin Epstein§, and Frank McCormick*

*Cancer Research Institute, and §Department of Dermatology, University of California, San Francisco, CA 94115; and †Sealy Center for Cancer Cell Biology and Environmental Health, Department of Pharmacology, University of Texas Medical Branch, Galveston, TX 77555-1048

Edited by Matthew P. Scott, Stanford University School of Medicine, Stanford, CA, and approved May 25, 2001 (received for review April 8, 2001)

Activation of the hedgehog pathway, through the loss of patched (PTC) or the activation of smoothened (SMO), occurs frequently in basal cell carcinoma (BCC), the most common human cancer. However, the molecular basis of this neoplastic effect is not understood. The downstream molecule Gli1 is known to mediate the biological effect of the pathway and is itself up-regulated in all BCCs. Gli1 can drive the production of BCCs in the mouse when overexpressed in the epidermis. Here we show that Gli1 can activate platelet-derived growth factor receptor α (PDGFRα) in C3H10T½ cells. Functional up-regulation of PDGFRα by Gli1 is accompanied by activation of the ras-ERK pathway, a pathway associated with cell proliferation. The relevance of this mechanism *in vivo* is supported by a high level expression of PDGFRα in BCCs of mice and humans. In the murine BCC cell line ASZ001, in which both copies of the PTC gene are inactivated, DNA synthesis and cell proliferation can be slowed by re-expression of PTC, which down-regulates PDGFRα expression, or by downstream inhibition of PDGFRα with neutralizing antibodies. Therefore, we conclude that increased expression of PDGFRα may be an important mechanism by which mutations in the hedgehog pathway cause BCCs.

Considerable insight into the role of the sonic hedgehog pathway in vertebrate development and human cancers has come from the discovery that mutations of the patched gene (*PTCH1*) are associated with a rare heritable human disorder—basal cell nevus syndrome (BCNS) (1, 2). BCNS patients have diverse phenotypic abnormalities, including tumors [e.g., basal cell carcinomas (BCCs) and medulloblastomas] and developmental anomalies (e.g., misshapen ribs, spina bifida occulta, and skull abnormalities) (3). Sporadic BCCs, the most common human cancer, consistently have abnormalities of the hedgehog pathway, and often have lost the function of patched (PTC) through *PTCH1* mutations and loss of the remaining allele (4–9). Similarly, sporadic medulloblastomas and trichoepitheliomas frequently have *PTCH1* mutations (9–12). Most *PTCH1* mutations cause a loss of PTCH1 protein function (1–2, 4–12). Mice that are heterozygous for a *PTCH1* null mutation exhibit a high risk of cancers such as medulloblastomas, rhabdomyosarcomas, and BCCs (13–15), confirming that *PTCH1* functions as a tumor suppressor. In addition to *PTCH1*, somatic mutations of smoothened (SMO), a putative seven-transmembrane-domain protein of the hedgehog pathway, occur in sporadic BCCs (16–18). Mutant SMO can transform cultured cells and can induce BCC-like tumors when expressed in the epidermis (16). This finding provides additional insight into the role of the sonic hedgehog pathway in BCCs and emphasizes the need to understand how this pathway works in normal and pathological cell proliferation.

The PTCH1 protein, a receptor for the secreted protein hedgehog (19, 20), is expressed in cell membrane of target tissues and is an important regulator of embryonic pattern formation (reviewed in ref. 21). PTCH1 represses SMO signaling, and relief of this repression by binding of sonic hedgehog to PTCH1 or after mutational inactivation of PTCH1 allows SMO signaling. PTCH1 cannot repress mutant SMO, resulting in uncontrolled SMO signaling (22). *PTCH1* itself is a target gene of the pathway. Thus, activation of the hedgehog pathway will increase the expression of PTCH1, which in turn will repress the pathway. Although the level of PTCH1 mRNA is increased in BCCs with *PTCH1* mutations, the protein is assumed to be inactive and is unable to control activation of the hedgehog pathway. Signaling events downstream of SMO are incompletely elucidated. Suppressor of fused [Su(Fu)] and protein kinase A (PKA) are intermediate molecules, and three *Gli* gene products transcription factors controlled by SMO signaling. Recent data indicate that Gli1 or Gli2 may mediate BCC formation (23–26).

To understand the molecular basis of hedgehog signaling-mediated tumor formation, we have used Gli1 as a biological probe to identify molecules that mediate BCC formation. Here we show that Gli1 can activate platelet-derived growth factor receptor α (PDGFRα) in C3H10T½ cells. Functional up-regulation of PDGFRα by Gli1 is accompanied by activation of the ras-ERK pathway, a pathway associated with cell proliferation. The relevance of this mechanism *in vivo* is supported by a high level expression of PDGFRα in BCCs of mice and humans. In the murine BCC cell line ASZ001, in which both copies of the *PTCH1* gene are inactivated, DNA synthesis and cell proliferation can be slowed by re-expression of PTCH1 possibly through down-regulating PDGFRα expression, or by downstream inhibition with PDGFRα-neutralizing antibodies. Therefore, we conclude that activation of PDGFRα may be an important mechanism by which mutations in the hedgehog pathway cause BCCs.

MEDICAL SCIENCES

Materials and Methods

Transient Reporter Analysis. The serum response element (SRE) cis-reporting plasmid was purchased from Stratagene. Murine PDGFRα promoter (a gift from C. Wang, University of Illinois at Chicago) was cloned into pGL3 basic luciferase-reporting plasmid (Promega). Transfection of C3H10T½ cells was performed according to Murone *et al.* (22). In the reporter assay, cells were incubated with low serum medium (0.05% FBS) for 36 h. U0126 was purchased from Promega. In the PDGFRα promoter reporter analysis, cells were incubated with 0.05% or 10% FBS for 48 h, and reporter gene activity was determined (22).

Immunoprecipitation and Western Blot Analysis. Immunoprecipitation of PDGFRα was performed with 4×10 cm plates of cells stably expressing Gli1 after retrovirus infection. Cells were lysed for 20 min on ice in RIPA buffer (150 mM NaCl/1% NP-40/0.5% sodium deoxycholate/0.1% SDS/50 mM Tris, pH 8.0) with protease inhibitors (complete–mini, Roche Molecular Biochemicals). Cell debris was removed by centrifugation at $10{,}000 \times g$, and the supernatant precleared with protein A-Sepharose (Sigma). Lysates were incubated with an antibody to PDGFRα (Upstate Biotechnology, Lake Placid, NY) for 2 h,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PTC, patched; SMO, smoothened; PDGFRα, platelet-derived growth factor-receptor α; BCC, basal cell carcinoma; SRE, serum response element; PKA, protein kinase A; Su(Fu), suppressor of fused; GFP, green fluorescent protein; MEK-1, mitogen-activated protein kinase kinase-1.

‡To whom reprint requests should be addressed. E-mail: jinxie@utmb.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

followed by protein A-Sepharose (Sigma) for 30 min. The pellet was washed with lysis buffer (four times, 10 min each). The yielded protein was analyzed by Western blot analysis. Appropriate antibodies were used in Western blot analysis (PDGFRα from R & D Systems; ERK and phospho-ERK from New England Biolabs; and phosphotyrosine-specific antibody 4G10 from Upstate Biotechnology). The signals were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia).

Immunohistostaining. Standard three-step immunoperoxidase staining was performed on 4-μm-thick sections of paraffin-embedded human BCCs and PTC heterozygote mouse BCCs (27). For optimal antigen retrieval, sections were boiled in 10 mM citrate buffer, pH 6.0. Sections were incubated for 2 h at 25°C with rabbit polyclonal antibody raised against the carboxyl terminus of PDGFRα of human and mouse origin (dilution of 1 μg/ml; catalog no. SC-338, Santa Cruz Biotechnology) or rabbit polyclonal antibody for human PDGF-A (dilution of 15 μg/ml; catalog no. AB-20-NA, R & D Systems). The specificity of the PDGFRα antibody was tested by preincubation with the specific blocking peptide (catalog no. SC-338P, Santa Cruz Biotechnology).

Cell Proliferation Assay, Cell Sorting, and Colony Formation Assay. BrdUrd labeling was performed by using an *in situ* cell proliferation kit (Roche Molecular Biochemicals). Neutralizing antibodies were purchased from R & D Systems. The percentage of BrdUrd-positive cells was obtained by counting more than 2,000 cells under the fluorescence microscope. ASZ001 cells were transfected with pEGFP (from CLONTECH) and another plasmid [PTCH1, Su(Fu), PKA, or the empty vector]. Forty-eight hours after transfection, cells were harvested and divided into two parts. One portion of transfected cells was sorted with green fluorescent protein (GFP) in our core facility. The GFP-positive cells were used to check the protein level of PDGFRα by Western blot analysis. The other portion was used to assay colony formation. One thousand cells from each transfection were plated onto a 10-cm Petri dish. The next day, 1 mg/ml G418 was added, and the medium was changed twice a week. The cell colonies (with more than 10 cells) were visualized by 0.5% crystal violet in 20% ethanol at 16 days after transfection. Transfection of each construct was duplicated in one experiment, and the experiment has been repeated three times.

Results

Gli1 Activates the ras-ERK Pathway in Cultured Cells. In human BCCs, Gli1, but not Gli2 or Gli3, is consistently up-regulated, indicating that Gli1 is probably the major downstream mediator for tumor formation (24, 25). To help elucidate the molecular mechanism by which activated hedgehog signaling induces BCC formation, we examined the effect of Gli1 on several major signal pathways in the hedgehog-responsive cell line C3H10T½ by using reporter gene analysis. Fig. 1*A* shows that Gli1 activated SRE reporter activity over 5-fold, but did not significantly change the reporter activities of N-FAT, CREB, NF-κB, or TCF-binding elements (data not shown). Because overexpression of Su(Fu) and PKA are known to inhibit the transcriptional activity of Gli1 (28–31), we tested its effect on Gli1-induced SRE activation and found that, indeed, both of them inhibit the Gli1 effect in this assay (Fig. 1*A*). This experiment shows the specificity of Gli1 effect on SRE. Because the ras-ERK pathway is important for SRE activation and is associated with cell proliferation (32–33), we also tested whether inhibitors of the ras-ERK pathway could block Gli1-induced SRE activation. Coexpression of rasN17, dominant-negative mitogen-activated protein kinase kinase-1 (MEK-1), or MEK-inhibitor U0126 (not shown) prevented Gli1-induced SRE activation (Fig. 1*A*), suggesting that the effect of Gli1 on the SRE was through a molecule upstream of ras.

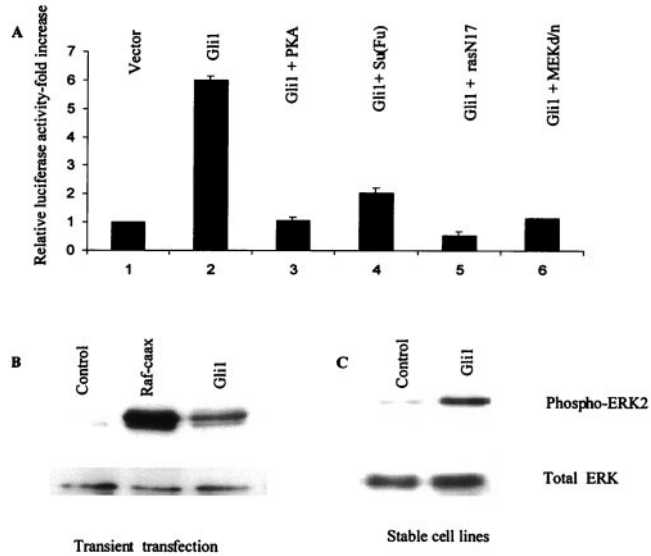

Fig. 1. Activation of the ras-ERK pathway by Gli1 in C3H10T½ cells. (*A*) Activation of SRE reporter by Gli1. C3H10T½ cells were transiently transfected with a luciferase reporting plasmid driven by the SRE and various expressing constructs as indicated. pRL-TK *Renilla* luciferase-reporting construct (Promega) was cotransfected to each sample to normalize transfection efficiency. Cells were cultured in 0.05% FBS medium for 36 h and the luciferase activity was measured. The relative luciferase activity is shown with the control sample as 1. All experiments are expressed as mean ± SD of duplicate samples from three independent experiments. Dominant-negative TCF4 used as a control did not change Gli1 activity on the SRE reporter (data not shown). Under the same condition, we noticed that Gli1 only slightly increased the AP-1 reporter activity (80–90%), suggesting that activation of AP-1 reporter is a later event. (*B*) Increased phosphorylation of ERK2 by Gli1. C3H10T½ cells were transiently transfected with ERK2 and another plasmid as indicated and maintained in 0.05% FBS medium for 36 h. (*C*) Stable cell lines were made with retrovirus infection of C3H10T½ cells. Phosphorylation of ERK2 in these cells was immunodetected by the phospho-specific antibody from New England Biolabs.

To confirm that ras-ERK is responsive to Gli1, we tested the effect of Gli1 on the level of phospho-ERK2, an indicator of ERK activation (33). Gli1 and ERK plasmids were cotransfected into C3H10T½ cells, and the levels of phospho-ERK2 and total ERK were assessed by specific antibodies. Gli1 expression did increase phospho-ERK2 in transient (Fig. 1*B*) and stable (Fig. 1*C*) transfections. In addition, we observed that rasN17 expression reduced Gli1-induced ERK2 phosphorylation (data not shown), just as it inhibited Gli1-induced SRE activation. Thus, both our SRE assay and phospho-ERK2 analyses indicate that Gli1 can activate the ras-ERK pathway.

PDGFRα Mediates Gli1 Effect on ERK Activation. Because receptor kinases are a major source for activation of the ras-ERK pathway (33), we used antibodies against phosphotyrosine to identify candidate receptor tyrosine kinases that might be responsible for ras-ERK activation. Phosphoproteins that comigrate approximately with PDGF receptor were identified (data not shown). We then examined the protein level of PDGF receptors after Gli1 expression and found that PDGFRα protein was highly expressed in Gli1-expressing C3H10T½ cells, but not in the control cells (Fig. 2*A Left*). Furthermore, we found a high level of PDGFRα phosphorylation in cells expressing Gli1, indicating that the protein is activated under these conditions (Fig. 2*A Right*). Confirming these data, we found a high level of PDGFRα mRNA in Gli1-expressing cells (Fig. 2*B*). Gli1 up-regulates murine PDGFRα promoter (34) activity more than 3-fold, and

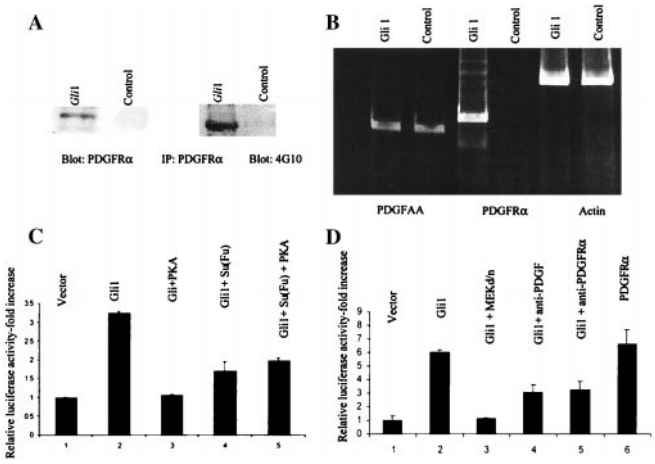

Fig. 2. Increased expression of PDGFRα by Gli1 in C3H10T½ cells. (*A*) Functional up-regulation of PDGFRα protein in Gli1-expressing cells. Western blot analysis was used to detect the protein level in the total cell lysates, and the phosphorylation of PDGFRα was assessed by immunoprecipitation (IP) of the lysates with PDGFRα antibody followed by Western blot detection with phosphotyrosine-specific antibody 4G10. (*B*) Increased PDGFRα mRNA in Gli1-expressing cells. Reverse transcription–PCR was performed by using RAP-PCR kit (Stratagene) to check the mRNA levels of PDGFRα and its ligand PDGF-A. (*C*) Gli1 activates PDGFRα promoter activity. The murine PDGFRα promoter activity was measured 48 h after transfection of C3H10T½ cells in the presence of Gli1, empty vector alone, or coexpression of Gli1 with other constructs as indicated. (*D*) PDGFRα mediates Gli1 effects on SRE activation. Gli1-induced SRE activation was examined in the presence of neutralizing antibodies for PDGF-A or PDGFRα, PDGFRα expression alone or after coexpression of Gli1 with the dominant-negative form of MEK1.

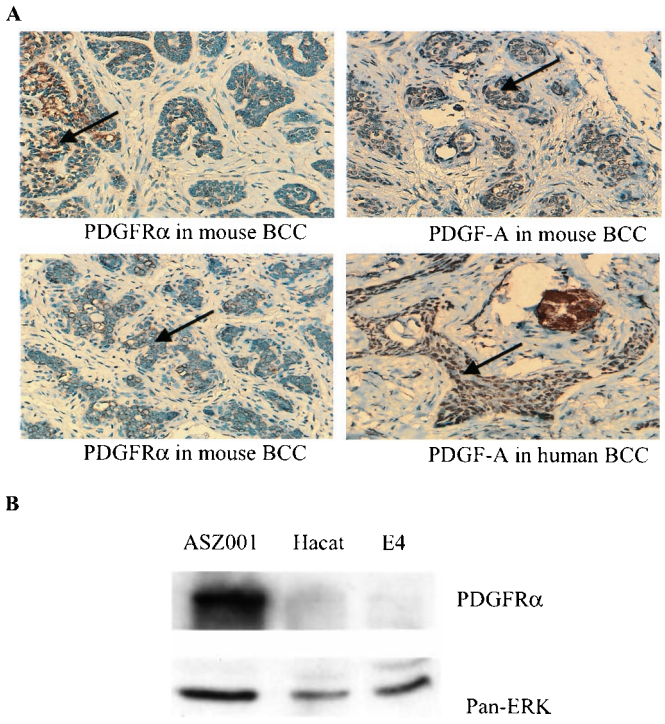

Fig. 3. Expression of PDGFRα and PDGF-A in primary BCCs. (*A*) Detection of PDGFRα in mouse BCCs was performed according to standard procedures (27) by using the antibody to PDGFRα (Santa Cruz Biotechnology, C-20). Positive staining was brown and the tissue section was counterstained with hematoxylin and eosin (blue). Detection of PDGF-A in mouse BCCs and human BCCs was done with PDGF-A polyclonal antibodies (R & D Systems). Arrows indicate the site of positive staining in the tumor. Only weak staining can be seen in the stroma. (*B*) Western blot analysis of PDGFRα in a BCC cell line ASZ001, a squamous cell carcinoma cell line E4 (a gift from Allan Balmain, University of California, San Francisco), and a human keratoinocyte cell line HaCat (a gift from Norbert Fusenig, German Cancer Research Center, Heidelberg, Germany).

this effect was specifically inhibited by coexpression of PKA or Su(Fu) (Fig. 2*C*). These data indicate that PDGFRα gene can be regulated by Gli1.

Based on these data, we hypothesized that Gli1 expression can result in up-regulation of PDGFRα that is activated by its ligand PDGF-A, leading to activation of the ras-ERK pathway. Indeed, we observed that PDGF-A was transcribed in C3H10T½ cells regardless of Gli1 expression (Fig. 2*B*; and by ELISA, data not shown). To test our model further, we used PDGF-A- and PDGFRα neutralizing antibodies in the SRE reporter analysis. Fig. 2*D* shows that the addition of neutralizing antibodies led to significant reduction in Gli1-induced SRE activity (Fig. 2*D*). In addition, expression of PDGFRα alone was sufficient to cause SRE activation (Fig. 2*D*). These data indicate that PDGFRα is the molecule that mediates Gli1-induced SRE activation.

A High Level of PDGFRα Expression Is Observed in BCCs of Mice and Humans. Our *in vitro* data in C3H10T½ cells suggest that functional up-regulation of PDGFRα could be an important consequence after Gli1 up-regulation in BCCs *in vivo*. To explore this possibility, we first examined PDGFRα expression in BCCs derived from *PTC1*$^{+/-}$ mice (15). Previous report indicates that the hedgehog pathway is activated in these tumors (15). The remaining copy of the PTC gene is inactivated during tumor formation, often through loss of the wild-type allele (15). Immunohistostaining indicated high expression of PDGFRα in these tumors (Fig. 3*A*), and in one tumor, staining was stronger at the periphery than in the center of the tumor (Fig. 3*A*). This immunohistostaing was confirmed by Western blot analysis in our mouse BCC cell line (Fig. 3*B*). Furthermore, we detected a high level of the ligand PDGF-A in the tumor and weak expression in the stroma (Fig. 3*A*). Therefore, in this well-defined genetic system in which the hedgehog pathway is constitutively activated, PDGFRα expression is very high, suggesting *in vivo* regulation of PDGFRα by the hedgehog pathway.

To extend our mouse findings, we next assessed PDGFRα expression in human BCCs. Most human BCCs, like the mouse BCCs, do stain with anti-PDGFRα (Fig. 4*A*). Most of this protein was distributed in the tumor nest (Fig. 4*A*, 11 of 14). In three BCCs, strong staining of PDGFRα in the stroma was observed (Fig. 4*A*), a finding that has been reported (35). By Western blot analysis, we detected PDGFRα protein expression in 10 of 11 BCCs, whereas it was undetectable in the control epidermis (Fig. 4*B*). In the tumors with PDGFRα expression, a high level of phosphorylated ERK was observed (data not shown). Thus, the Western blot analysis data are consistent with the immunohistostaining results—high expression of PDGFRα is common in human BCCs. These analyses indicate that PDGFRα is highly expressed in human as well as in mouse BCCs. Because other factors may also affect the expression of PDGFRα (36), a comprehensive analysis of human BCCs is required for understanding the heterogeneous staining among different BCCs. Furthermore, we examined PDGF-A expression in primary human BCCs by immunohistostaining because activation of PDGFRα requires its ligand, PDGF-A. We found that all BCCs examined ($n = 5$) were positive for PDGF-A (Fig. 3*A*). The high levels of PDGFRα and of its ligand PDGF-A in the tumor imply their importance for BCC development.

Cell Proliferation of BCC Cells Is Inhibited by *PTCH1* or Inhibition of PDGFRα Function. Because no human BCC cell lines are available, we have generated a cell line, ASZ001, from a Gli1-expressing

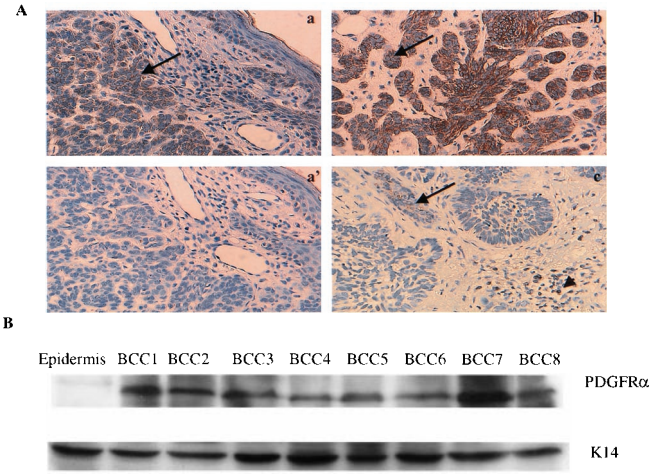

Fig. 4. Expression of PDGFRα in primary human BCCs. (*A*) Detection of PDGFRα expression in human BCCs was carried out by using PDGFRα antibody (Santa Cruz Biotechnology; C-20). The PDGFRα peptide with which the antibody was made (Santa Cruz Biotechnology) was used to test the staining specificity (negative staining shown in *a'*). Arrows indicate the positive staining of the antibody. The staining is often in the tumor nest but sometimes in the stroma (see arrowhead). (*B*) Western blot analysis of PDGFRα expression in primary human BCCs was performed by using homogenized primary BCCs or control epidermis. The PDGFRα antibody was from R & D Systems, and K14 antibody was used as control.

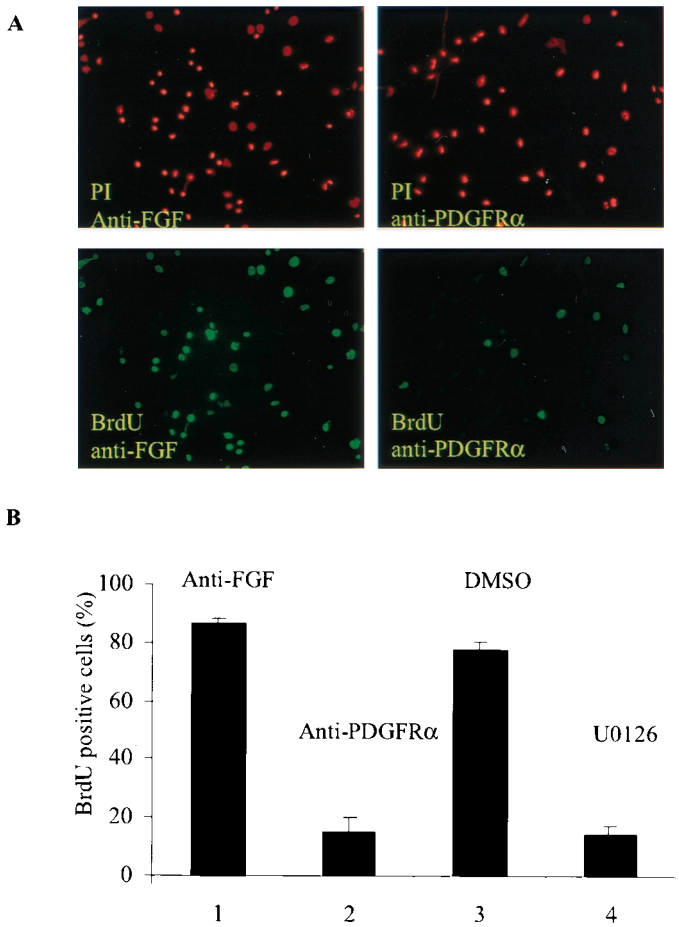

Fig. 5. Inhibition of cell proliferation by PDGFRα neutralizing antibody in ASZ001 cells. (*A*) ASZ001 cells were cultured in keratinocyte growth medium CF145 (Cascade Biologics, Portland, OR) without growth supplements. Cells were incubated with BrdUrd (Roche Molecular Biochemicals) in the presence of PDGFRα antibody (R & D Systems) or the control fibroblast growth factor (FGF) antibody (R & D Systems). Propidium iodide (PI; Roche Molecular Biochemicals) was used to stain nucleus and FITC-conjugated anti-BrdUrd IgG was used to detect BrdUrd incorporated into cells. (*B*) A summary of DNA synthesis in ASZ001 cells, including cells in the presence of MEK inhibitor U0126. In contrast, PDGFRα-neutralizing antibody did not affect DNA synthesis of a squamous cell carcinoma cell line E4 (data not shown here).

mouse BCC tumor induced by UV radiation in a $PTC^{+/-}$ mouse (15). Both copies of the *PTC* gene are lost in the ASZ001 cells, thus resembling the *PTCH1* status in human BCCs. This cell line does express a high level of PDGFRα (Fig. 3*B*). Furthermore, the ASZ001 cell line, unlike other keratinocytes tested, can grow in the absence of keratinocyte growth supplements (which contain many growth factors), and is therefore amenable to manipulation of the ras-ERK pathway. We treated the cells with anti-PDGFRα neutralizing antibody and measured DNA synthesis by BrdUrd labeling. As shown in Fig. 5, incubation of the cells with PDGFRα-neutralizing antibody for 36 h reduced DNA synthesis by 70% (Fig. 5 *A* and *B*). In contrast, fibroblast growth factor (FGF)-neutralizing antibody (Fig. 5*A*) or purified goat IgG (data not shown) did not affect DNA synthesis in this cell line. In addition, we found that the MEK inhibitor U0126 inhibited DNA synthesis by over 70% (Fig. 5*B*). Therefore, increased expression of PDGFRα and activation of the ERK pathway appears to be important for cell proliferation in the ASZ001 cells.

To test for a direct link between the hedgehog pathway and PDGFRα, we introduced *PTCH1* into ASZ001 cells in which both copies of *PTC* are lost. PDGFRα protein level was reduced after expression of *PTCH1* (Fig. 6*A*), indicating that PDGFRα expression can be regulated by manipulating the hedgehog pathway. This reduced expression of PDGFRα is correlated with reduced cell proliferation in a colony-formation assay (Fig. 6*B*). Thus, down-regulation of the hedgehog pathway can reduce the expression of PDGFRα and can inhibit cell proliferation. In conclusion, we have discovered that PDGFRα can be regulated by Gli1 and that PDGFRα mediates Gli1-induced SRE activation. The expression of PDGFRα is up-regulated in BCCs of mice and humans. In the mouse BCC cell line ASZ001, perturbation of PDGFRα function, whether directly by neutralizing antibodies or indirectly by PTCH1, leads to decreased cell proliferation. Therefore, up-regulation of PDGFRα appears to be an important mechanism by which hedgehog signaling induces basal cell carcinomas.

Discussion

The hedgehog-PTC signaling pathway is important for carcinogenesis, as shown by the presence of constitutional mutations of *PTCH1* in the basal cell nevus syndrome, and mutations in PTCH1 and SMO in sporadic cancers, particularly basal cell carcinomas. Further understanding of BCCs at the molecular level requires identification of the molecules that link the hedgehog pathway to tumor formation. Our data indicate that PDGFRα activation is one important mechanism by which abnormal hedgehog signaling causes the continued cell proliferation of basal cell carcinomas. In C3H10T½ cells, we demonstrated that PDGFRα mediates the effect of Gli1 on activation of ERK, and Gli1 itself can activate the promoter activity of PDGFRα, suggesting that PDGFRα might be a target gene of Gli1. Furthermore, we found a high level of PDGFRα expression in BCCs of mice and humans, indicating that up-regulation of PDGFRα is common in BCCs. In the PTC1 null BCC cell line ASZ001, we demonstrated that inhibition of the hedgehog pathway by PTCH1 reduced the level of PDGFRα and inhibited cell proliferation, which is consistent with the data that inhibition of PDGFRα function with neutralizing antibodies

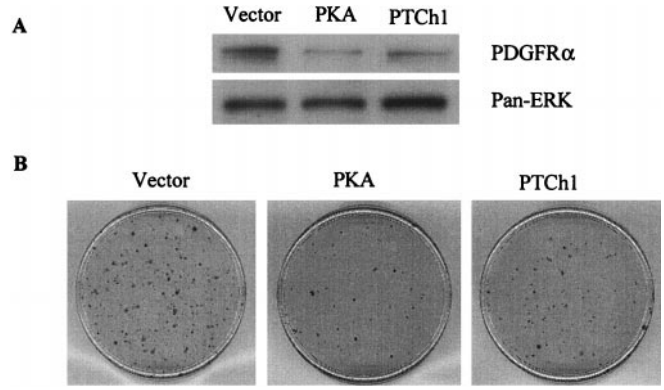

Fig. 6. Regulation of PDGFRα by down-regulating the hedgehog pathway with PKA or PTCH1 in ASZ001 cells. Cells were transfected with GFP plasmid and other constructs as indicated. GFP-positive cells were enriched after cell sorting, and the PDGFRα level was detected by Western blot analysis with a PDGFRα antibody (R & D Systems). (*A*) The reduced protein level of PDGFRα was seen in cells with expression of PTCH1 and PKA. A small portion of the transfected cells was used for colony-formation analysis. (*B*) Colonies were visualized 16 days after transfection by using 0.05% crystal violet in 20% ethanol. Most colonies derived from PKA- and PTCH1-transfected cells are small, suggesting a slow proliferation in these cells. This result is consistent with the reduced expression of PDGFRα in the cells. Transfection of each construct was duplicated in each experiment and the experiment has been repeated three times with consistent results.

blocked DNA synthesis. Therefore, we believe that activation of PDGFRα is at least one important mechanism by which hedgehog signaling mediates tumor formation. These data thus detail the molecular basis of hedgehog signaling-mediated tumor formation. From our model, we predict that pharmaceutical inhibitors of PDGFRα would inhibit BCC development in *PTC1*$^{+/-}$ mice (15).

Furthermore, our data indicate that activation of PDGFRα can be used as a readout to understand how signals from SMO are transduced to downstream Gli molecules. At present, signaling events from SMO to Gli1/Gli2 are poorly understood. It was shown in our transient reporter gene assays (Figs. 1*A* and 2*C*) that both PKA and Su(Fu) inhibited Gli1 activity (28–31). However, PKA, but not Su(Fu) (data not shown here), blocked cell proliferation of ASZ001 cells in which Gli1 is up-regulated, suggesting that Su(Fu) alone is not sufficient to inhibit endogenous Gli1 activity.

Ci, the Gli homologue in *Drosophila*, regulates PTC by way of Gli1 binding sites (37). Three Gli1 binding sites are present within 1 kb of the predicted transcription start site. However, the regulation of target genes in mammals seems more complicated. Similar to *Drosophila* PTC, human PTC (PTCH1) is a known target of the hedgehog pathway, and Gli1 can activate *PTCH1* promoter activity (refs. 28–31, and our observations). After sequence analysis, we only found one Gli1 binding site (base pairs −1120) in the human *PTCH1* promoter (within 3 kb of the transcription start site) and no Gli1 binding sites in the 900 bp upstream of the murine PDGFRα promoter. Two putative Gli1 binding sites are present in human PDGFRα promoter (within 3 kb upstream of ATG). Because transcriptional up-regulation of *PTCH1* is a typical signature of the hedgehog pathway activation and Gli1 can specifically activate PTC1 in mouse embryos (reviewed in ref. 25), Gli1 may exert its effects through atypical binding sites. The cis-elements through which Gli1 regulates PTCH1 and PDGFRα have not yet been identified.

We thank Drs. Rosemary Akhurst, Allan Balmain, Yan Cheng, Norbert Fusenig, Neil Giese, Carl-Henrik Heldin, Martin McMahon, Monica Nister, Pablo Rodriguez-Viciana, Rune Toftgård, Bert Vogelstein, and Cuiwei Wang for providing reagents, and members of the McCormick lab for discussion. This study was supported by Concern Foundation (J.X.), John Sealy Memorial Endowment Fund for Biomedical Research (to J.X.), the Wood Foundation (F.M.), and the National Cancer Insitute (E.H.E.), and by gifts from the Michael Rainen Family Foundation and from Patricia Hughes (to E.H.E.).

1. Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr., & Scott, M. P. (1996) *Science* **272,** 1668–1671.
2. Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Undèn, A. B., Gillies, S., *et al.* (1996) *Cell* **85,** 841–851.
3. Gorlin, R. J. (1987) *Medicine (Baltimore)* **66,** 98–113.
4. Gailani, M. R., Stahle-Backdahl, M., Leffell, D. J., Glynn, M., Zaphiropoulos, P. G., Undèn, A. B., Dean, M., Brash, D. E., Bale, A. E. & Toftgård, R. (1996) *Nat. Genet.* **14,** 78–81.
5. Undèn, A. B., Holmberg, E., Lundh-Rozell, B., Stahle-Backdahl, M., Zaphiropoulos, P. G., Toftgård, R. & Vorechovsky, I. (1996) *Cancer Res.* **56,** 4562–4565.
6. Wolter, M., Reifenberger, J., Sommer, C., Ruzicka, T. & Reifenberger, G. (1997) *Cancer Res.* **57,** 2581–2585.
7. Aszterbaum, M., Rothman, A., Johnson, R. L., Fisher, M., Xie, J., Bonifas, J. M., Zhang, X., Scott, M. P. & Epstein, E. H., Jr. (1998) *J. Invest. Dermatol.* **110,** 885–888.
8. Daya-Grosjean, L. & Sarasin, A. (2000) *Mutat. Res.* **450,** 193–199.
9. Raffel, C., Jenkins, R. B., Frederick, L., Hebrink, D., Alderete, B., Fults, D. W. & James, C. D. (1997) *Cancer Res.* **57,** 842–845.
10. Xie, J., Johnson, R. L., Zhang, X., Bare, J. W., Waldman, F. M., Cogen, P. H., Menon, A. G., Warren, R. S., Chen, L. C., Scott, M. P. & Epstein, E. H., Jr. (1997) *Cancer Res.* **57,** 2369–2372.
11. Vorechovsky, I., Tingby, O., Hartman, M., Stromberg, B., Nister, M., Collins, V. P. & Toftgård, R. (1997) *Oncogene* **15,** 361–366.
12. Vorechovsky, I., Undèn, A. B., Sandstedt, B., Toftgård, R. & Stahle-Backdahl, M. (1997) *Cancer Res.* **57,** 4677–4681.
13. Goodrich, L. V., Milenkovic, L., Higgins, K. M. & Scott, M. P. (1997) *Science* **277,** 1109–1113.
14. Hahn, H., Wojnowski, L., Zimmer, A. M., Hall, J., Miller, G. & Zimmer, A. (1998) *Nat. Med.* **4,** 619–622.
15. Aszterbaum, M., Epstein, J., Oro, A., Douglas, V., LeBoit, P. E., Scott, M. P. & Epstein, E. H., Jr. (1999) *Nat. Med.* **5,** 1285–1291.
16. Xie, J., Murone, M., Luoh, S.-M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. -W., Hynes, M., Goddard, A., *et al.* (1998) *Nature (London)* **391,** 90–92.
17. Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P. & Reifenberger, G. (1998) *Cancer Res.* **58,** 1798–1803.
18. Lam, C. W., Xie, J., To, K. F., Ng, H. K., Lee, K. C., Yuen, N. W., Lim, P. L., Chan, L. Y., Tong, S. F. & McCormick, F. (1999) *Oncogene* **18,** 833–836.
19. Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. & Tabin, C. J. (1996) *Nature (London)* **384,** 176–179.
20. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., *et al.* (1996) *Nature (London)* **384,** 129–134.
21. Ingham, P. W. (1998) *EMBO J.* **17,** 3505–3511.
22. Murone, M., Rosenthal, A. & de Sauvage, F. J. (1999) *Curr. Biol.* **9,** 76–84.
23. Ruiz i Altaba, A. (1999) *Nat. Cell Biol.* **1,** E147–E148.
24. Dahmane, N., Lee, J., Robins, P., Heller, P. & Ruiz i Altaba, A. (1997) *Nature (London)* **389,** 876–881.
25. Green, J., Leigh, I. M., Poulsom, R. & Quinn, A. G. (1998) *Br. J. Dermatol.* **139,** 911–915.
26. Nilsson, M., Undèn, A. B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P. G., Toftgård, R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3438–3443. (First Published March 21, 2000; 10.1073/pnas.050467397)
27. Shi, S. R., Key, M. E. & Kalra, K. L. (1991) *J. Histochem. Cytochem.* **39,** 741–748.
28. Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Undèn, A. B., Sandstedt, B., Toftgård, R. & Zaphiropoulos, P. G. (1999) *Nat. Cell Biol.* **1,** 312–319.
29. Stone, D. M., Murone, M., Luoh, S., Ye, W., Armanini, M. P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F. J. & Rosenthal, A. (1999) *J. Cell Sci.* **112,** 4437–4448.
30. Ding, Q., Fukami, S., Meng, X., Nishizaki, Y., Zhang, X., Sasaki, H., Dlugosz, A., Nakafuku, M. & Hui, C. C. (1999) *Curr. Biol.* **9,** 1119–1122.
31. Kaesler, S., Luscher, B. & Ruther, U. (2000) *Biol. Chem.* **381,** 545–551.
32. Treisman, R. (1995) *EMBO J.* **14,** 4905–4913.
33. McCormick, F. (1994) *Curr. Opin. Genet. Dev.* **4,** 71–76.
34. Wang, C. & Stiles, C. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7061–7065.
35. Ponten, F., Ren, Z., Nister, M., Westermark, B. & Ponten, J. (1994) *J. Invest. Dermatol.* **102,** 304–309.
36. Coin, P. G., Lindroos, P. M., Bird, G. S. J., Osornio-Vargas, A. R., Roggli, V. L. & Bonner, J. C. (1996) *J. Immunol.* **156,** 4797–4806.
37. Alexandre, C., Jacinto, A. & Ingham P. W. (1996) *Genes Dev.* **10,** 2003–2013.