Pharmacologic Inhibition of ALK5 Causes Selective Induction of Terminal Differentiation in Mouse Keratinocytes Expressing Oncogenic HRAS

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Abstract

TGFβ has both tumor suppressive and oncogenic roles in cancer development. We previously showed that SB431542 (SB), a small molecule inhibitor of the TGFβ type I receptor (ALK5) kinase, suppressed benign epidermal tumor formation but enhanced malignant conversion. Here, we show that SB treatment of primary K5rTA/tetOHRASV12G bitransgenic keratinocytes did not alter HRASV12G-induced keratinocyte hyperproliferation. However, continuous SB treatment significantly enhanced HRASV12G-induced cornified envelope formation and cell death linked to increased expression of enzymes transglutaminase (TGM) 1 and TGM3 and constituents of the cornified envelope small proline-rich protein (SPR) 1A and SPR2H. In contrast, TGFβ1 suppressed cornified envelope formation in HRASV12G keratinocytes. Similar results were obtained in HRASV12G transgenic mice treated topically with SB or by coexpressing TGFβ1 and HRASV12G in the epidermis. Despite significant cell death, SB-resistant HRASV12G keratinocytes repopulated the primary culture that had overcome HRas-induced senescence. These cells expressed reduced levels of p16ink4a and were growth stimulated by SB but remained sensitive to a calcium-induced growth arrest. Together these results suggest that differential responsiveness to cornification may represent a mechanism by which pharmacologic blockade of TGFβ signaling can inhibit the outgrowth of preneoplastic lesions but may cause a more progressed phenotype in a separate keratinocyte population. Mol Cancer Res; 9(6); 746–56. ©2011 AACR.

Introduction

TGFβ1 is a member of a large family of regulatory molecules that play both positive and negative roles in epithelial cancers. Genetic studies in the 2-stage skin carcinogenesis model have shown that overexpression of TGFβ (1) or related family members (2) suppresses benign tumor formation, whereas inactivation of TGFβ type I (3, 4) and/ or II receptor (5) signaling increases tumor development and malignant conversion. Similarly, Ferguson-Smith disease in humans which results in rapidly growing, locally invasive, squamous carcinoma-like skin tumors that spontaneously heal are a result of inactivating mutations in the TGFβ type I receptor (6). However, overexpression of TGFβ in mice also leads to outgrowth of a few poorly differentiated spindle cell carcinomas (7) and increased metastasis of benign papillomas (8).

In contrast to these genetic models, we have recently shown that pharmacologic inhibition of the TGFβ type I receptor (ALK5) during skin tumor promotion with SB431542 (SB) suppresses outgrowth of benign lesions, although this also enhances malignant conversion (9). Although it is generally accepted that the tumor suppressive function of TGFβ is linked to its role as a negative regulator of epithelial cell proliferation (10), this result suggests that the function of TGFβ signaling in the premalignant keratinocyte is more complex. In the 2-stage carcinogenesis model, cancer initiation occurs through mutational activation of the HRas gene (11). Keratinocytes expressing activated HRas are hyperproliferative in vitro (12) and exhibit abnormal responses to differentiation inducing signals, with suppression of the early differentiation program such as induction of keratin 1 and 10 but overexpression of late differentiation genes (13). However, previous studies have shown that these cells both produce and remain growth inhibited by exogenous TGFβ1 (12). To identify mechanisms underlying suppression of tumor formation by ALK5 inhibition, we examined the response of primary keratinocytes expressing an activated HRas oncogene to SB using hittransgenic primary keratinocytes from a doxycycline-inducible K5rTA/tetOR45S12G line. Here, we show that pharmacologic inhibition of ALK5 in HRASV12G-expressing primary keratinocytes causes not only cornification and cell death in the majority of keratinocytes but also the
secondary outgrowth of a cornification-resistant, nonsenescent subpopulation. These results suggest that the ability of SB to inhibit benign tumor formation but enhance malignant conversion in epidermal carcinogenesis is linked to distinct effects on different populations of initiated keratinocytes.

**Materials and Methods**

**Cell culture**

Primary mouse keratinocytes were isolated and cultured according to a standard protocol (14) from newborn transgenic littermates. tetORASV12G (15) mice that express HRASV12G when induced by the bovine keratin 5 promoter (K5rtTA; ref. 16) were obtained from the NCI (National Cancer Institute) mouse repository and newborn mice of each genotype identified by PCR were obtained (National Cancer Institute) mouse repository and newborn mice. HRASV12G mice were treated with 1 ng/mL TGF1 for indicated times (R&D Systems). For all long-term experiments, media was changed every other day. Photomicrographs of keratinocytes were made using an inverted Olympus CKV41 microscope, UPlan Fl objectives, SPOT RT-KE Mono-IR camera, and SPOT Software 4.5 (Diagnostics Instruments, Inc.).

**Measurement of cell proliferation**

For cell growth curves, K5rtTA/tetORASV12G keratinocytes were preplated, plated in high calcium (1.4 mmol/L) for 2 hours to remove contaminating fibroblasts which attach faster than keratinocytes, and then seeded at a density of 200,000 cells in 24-well culture trays. On day 2 postplating, keratinocytes were treated with doxycycline and/or SB in 0.05 mmol/L Ca2+ media and media was replaced every other day. Triplicate samples of each treatment group were counted twice at indicated time points using a Z1 Coulter particle counter (Beckman Coulter). For 2-color analysis of cell cycle, keratinocytes were pulsed with 40 μmol/L 5-bromo-2-deoxyuridine (BrdU; Becton Dickinson) 1 hour before harvesting, fixed in 70% ethanol, and then stained with anti-BrdU-FITC antibody (ABFM-18; Phoenix Flow Systems) and propidium iodide (PI; Invitrogen). Cells were analyzed using an EPICS-XL-MCL flow cytometer (Beckman Coulter) and the percentage of cells at each phase of the cell cycle determined with FlowJo Flow cytometry analysis software.

**Cornified envelope assay**

A protocol adapted from published methods (17) was used to quantify cornified envelope formation. Floating/cornified cells were boiled at 90°C for 10 minutes in 2% SDS/20 mmol/L DTT, as cornified cells are resistant to this treatment. Phase contrast microscopy was used to view and count cornified cells under a hemocytometer which appear as "ghost cells" (18). The attached cells were counted using a Z1 Coulter particle counter (Beckman Coulter).

**Measurement of senescence**

Keratinocytes were plated in 12-well tissue culture plates and on day 3 postplating, they were treated with doxycycline and/or SB for 11 days. Senescence-associated β-galactosidase (SA-βgal) staining was carried out as described (12) and an inverted Olympus CKX41 microscope (20× microscope frame) was used. Positive cells were expressed as a percentage of total cells for each treatment group. Three different fields from each well were counted and triplicate samples were analyzed for each treatment group.

**Calcium resistance**

To determine responsiveness to increased calcium concentrations, passedaged/immortalized K5rtTA/tetORA5SV12G keratinocytes at 5,000 cells/60 mm dish were seeded in 0.05 mmol/L Ca2+ media with 1 μg/mL doxycycline and 0.5 μmol/L SB. After 48 hours, the calcium concentration was increased to 0.5 mmol/L and treatment continued for 9 days. Calcium-resistant colonies were counted after the dishes were fixed and stained in hematoxylin.

**RNA and protein analysis**

Whole skin was homogenized using a Qiagen TissueLyzer (Qiagen) in TRIzol (Invitrogen). Quantitative RT-PCR (qPCR) was done for the indicated genes and normalized to 18S rRNA expression using the MyIQ System (BioRad Laboratories) and PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences). All PCR samples were normalized to GAPDH (glyceraldehydes-3-phosphate dehydrogenase). Primer sequences were obtained from published studies or using Primer 3 (19) software with Genbank sequence information. Protein was isolated with 0.5% NP-40 lysis buffer (0.5% IGEPAL CA-630, 250 mmol/L NaCl, 50 mmol/L Tris HCl, pH 7.4) with protease and phosphatase inhibitors. Antibodies directed against Smad2/3, p-Smad2, p-ERK (extracellular signal–regulated kinase), ERK, p15, GAPDH (Cell Signaling Technology, Inc.); HRAS, p38, p16, p21 (Santa Cruz Biotechnology); p-p38 (New England Biolabs); and β-actin (Millipore) were used.

**Animal studies**

Crosses of involucrin rtA (20) and tetORASV12G mice were maintained on 10 μg/mL doxycycline water, which was switched to water alone to induce HRASV12G expression. Seven-week-old mice were treated with 200 μL of acetone and/or 200 μL of 10.0 μmol/L SB every other day for 5 days, and skin was harvested 24 hours after the last SB treatment. Nontransgenic, K5rtTA/tetOTGFβ1 (21), K5rtTA/tetORASV12G, and K5rtTA/tetORASV12G/tetOTGFβ1 mice were treated with doxycycline for 2 days in chow (1 g/kg) to induce expression of HRASV12G and/or TGFβ1. All transgenic mice are on an FVB background. All animals were kept under a controlled environment of temperature and humidity and a 12-hour light–dark
cycle. Animal studies were conducted under approved IACUC protocols.

**Results**

**ALK5 inhibition induces and TGFβ1 suppresses cornification of keratinocytes expressing an HRAS oncogene**

We first tested whether SB could suppress endogenous TGFβ1 signaling in keratinocytes expressing human oncogenic HRASV12G. Primary keratinocytes were isolated from bitransgenic K5rtTA/tetO HRASV12G mice and treated with doxycycline to induce HRASV12G in the presence or absence of SB. Figure 1 shows that 24 hours after the addition of doxycycline, there was a large increase in expression of HRAS as detected by immunoblotting and this was accompanied by a corresponding increase in levels of p-extracellular signal-regulated kinase (ERK) 1/2 but expression of p-p38 mitogen-activated protein kinase (MAPK) was not detected. Although there was no major increase in total or p-Smad2 levels after induction of HRASV12G, addition of SB caused an immediate reduction of p-Smad2 and this was maintained with continuous treatment.

Because TGFβ1 is a potent growth inhibitor for keratinocytes expressing v-HRas (12), it was anticipated that inhibition of autocrine TGFβ signaling would increase proliferation. However, Figure 2A shows that there was no effect of SB on the fraction of HRASV12G expressing keratinocytes in G1, S, or G2-M phases of the cell cycle as measured by 2-color flow cytometry. In addition, SB did not enhance proliferation of keratinocytes expressing reduced levels of HRASV12G produced using a suboptimal concentration of doxycycline (data not shown). Consistent with these results, induction of HRASV12G in keratinocytes caused an increase in cell number over time relative to control keratinocytes, but SB caused no additional increase in cell number within the first 5 days. However, after 5 days of continuous exposure to SB, the number of attached HRASV12G keratinocytes rapidly decreased below that of control keratinocytes with a 50% decrease in attached cell number compared with HRASV12G alone (Fig. 2B). HRASV12G keratinocytes that remained attached to the culture dish had a more spindle-shaped morphology than HRASV12G alone (Fig. 2C). Importantly, there was no effect of SB on normal keratinocytes that did not express HRASV12G.

Although flow cytometric analysis did not show a significant increase in the sub-G1 fraction prior to 5 days of SB treatment, there was a significant increase in the number of floating cells with a morphology consistent with that of a cornified envelope, the insoluble product of keratinocyte terminal differentiation characterized by extensive protein cross-linking (ref. 22; Fig. 2D). Because cornified envelopes are resistant to SDS/DTT and boiling, we used this method to quantify the fraction of cornified envelope in cultures of HRASV12G keratinocytes treated with SB. Consistent with earlier studies showing aberrant terminal differentiation in v-HRas–expressing mouse keratinocytes (13), expression of HRASG12V caused an increase in cornified envelopes relative to control keratinocytes. Treatment with SB significantly increased the number of cornified envelopes produced by HRASV12G keratinocytes but had no effect on control keratinocytes (Fig. 2E). Figure 2F shows that TGFβ1 treatment of HRASV12G keratinocytes significantly reduced the level of cornified envelopes indicating that the effect of SB was due to inhibition of TGFβ signaling rather than an off-target effect. Together these results suggest that autocrine TGFβ signaling does not act as a negative regulator of cell proliferation in these premalignant keratinocytes but is directly linked to terminal differentiation.

**TGFβ signaling regulates expression of cornified envelope genes**

We examined expression of genes encoding specific cornified envelope proteins and transglutaminases (TGM1 and TGM3) that cause cross-linking to form the cornified envelope. TGFβ1 inhibited the induction of TGM1 and reduced baseline TGM3 expression by HRASV12G (Fig. 3A), but there was no effect on expression of small proline-rich (SPR) genes SPR1A and SPR2H (data not shown). Conversely, inhibition of TGFβ signaling with SB increased expression of TGM1 and TGM3 and reduced baseline TGM3 expression by HRASV12G (Fig. 3B). These results are consistent with earlier studies showing aberrant terminal differentiation in v-HRas–expressing mouse keratinocytes (13).

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**Statistical analysis**

One-way ANOVA and Tukey’s multiple comparison posttest were used to test significance of multiple groups within an experiment. Student’s t test was used to compare 2 groups only, and the significance of the difference was described.
Figure 2. SB enhances terminal differentiation in HRASV12G-expressing keratinocytes but does not affect cell proliferation. 

A, two-color flow cytometric analysis of cell proliferation after 4 days of SB treatment shows no significant effect on cell cycle of either control or HRASV12G keratinocytes. Cells were stained with BrdU/PI and analyzed according to Materials and Methods.

B, attached cell number of HRASV12G keratinocytes is significantly reduced after 5 days of continuous treatment with SB. n = 3, N = 2. 

C, photomicrographs showing effect of SB on bitransgenic cell monolayers after 5 days. HRASV12G keratinocytes alone (left) and HRASV12G + SB (right) on day 5. Magnification 100×. 

D, photomicrograph of floating cornified cells from SB-treated HRASV12G keratinocytes. Magnification 200×, scale bar represents 50 μm. 

E, enhanced formation of cornified envelopes in keratinocytes expressing HRASV12G treated with SB. Floating cells counted in triplicate by cornified envelope assay and expressed relative to attached cells on day 5. n = 3, N = 3. 

F, TGFβ1 blocks cornified envelope formation in HRASV12G-expressing keratinocytes. HRASV12G was induced for 3 days followed by an additional 2 days in the presence or absence of 1 ng/mL TGFβ1. Cornified cells were measured as described in Materials and Methods and expressed relative to total attached cells. All keratinocytes were cultured and treated in 0.05 mmol/L Ca2+ media. n = 3, N = 2. C, control; HRAS, HRASV12G expression induced by Dox. Values with * are significantly different at P < 0.05. Error bars represent SEM.
significantly increased the expression of SPR1A and SPR2H (Fig. 3B). No change in keratin 1 or involucrin expression was detected between these treatment groups. Figure 3C shows that similar to TGFβ1 treatment, infection of HRASV12G-expressing keratinocytes with a constitutively active ALK5 adenovirus reduces TGM1 gene expression. Conversely, infection with an adenovirus expressing a dominant-negative TGFβ type II receptor (DNTβRII) increases TGM1 expression. To further test the ability of TGFβ1 to suppress aberrant differentiation in HRASV12G-expressing keratinocytes, we used a different model in which primary keratinocytes were transduced with a replication-defective retrovirus expressing v-HRas. Previous studies have shown that v-HRas suppresses calcium-mediated induction of early differentiation markers such as keratin 1 but increases expression of late differentiation markers (13).
Figure 3D shows that TGFβ1 reduced expression of the cornified envelope protein filaggrin in v-HRas–expressing keratinocytes in both proliferative (0.05 mmol/L Ca²⁺) and differentiation-inducing media (0.5 mmol/L Ca²⁺).

To determine whether inhibition of the TGFβ pathway could enhance terminal differentiation of premalignant keratinocytes in vivo, we used bitransgenic involucrin tTA/tetO RASV12G mice in part due to rapid mortality of mice expressing HRASV12G in the basal layer. Bitransgenic mice were taken off doxycycline to induce HRASV12G and were then treated topically with SB or acetone for 5 days. SB treatment caused a significant increase in the thickness of cornified layers compared with acetone alone (Fig. 4A and B), and this correlated with a significant increase in the expression of TGM1 and SPR1A (Fig. 4C). Coinduction of TGFβ1 with HRASV12G in triple transgenic mice reduces expression of TGM1 and TGM3 compared with HRASV12G alone (Fig. 4D).

Figure 4. TGFβ signaling pathway regulates expression of terminal differentiation markers in vivo. A, photomicrographs showing increased thickness of the cornified layer in bitransgenic involucrin tTA/tetO RASV12G mice treated with SB (10.0 μmol/L in 200 μL acetone) for 5 days. Mice were removed from Dox to induce HRASV12G expression. Magnification 200×, scale bar represents 50 μm. n = 5. C, cornified layer; E, epidermis. B, quantification of cornified layer thickness, measured at magnification 200×, 30 measurements per slide. n = 5. C, topical SB (10.0 μmol/L) treatment of involucrin tTA/tetO RASV12G mice significantly increases TGM1 and SPR1A gene expression, with similar trends for TGM3 and SPR2H. n = 5. D, coinduction of TGFβ1 with HRASV12G in triple transgenic mice reduces expression of TGM1 and TGM3 compared with HRASV12G alone. n = 5. A, acetone; HRAS, HRASV12G expression induced by Dox removal; TGFβ, tetOTGFβ. Values with * are significantly different at P < 0.05. Error bars represent SEM.
significant increase in TGM1 and SPR1A mRNA levels (Fig. 4C). Although it is unclear why expression of HRASV12G causes an increase in TGM3 in vivo but reduced expression in vitro, SB treatment caused a similar but not statistically significant increase in TGM3 and SPR2H. To test the opposite response, we coinduced HRASV12G and TGFβ1 in triple transgenic mice containing tetORASV12G and tetOTGFβ1 transgenes. TGFβ1 reduced expression of TGM3 with a similar trend for TGM1 (Fig. 4D). Expression of TGFβ1 alone also inhibited baseline TGM1 expression in vivo (Fig. 4D) similar to its effects on TGM1 expression in vitro (Fig. 3A). Although oncogenic HRASV12G induced SPR expression, TGFβ1 did not alter this response and no change was detected with TGFβ1 expression alone compared with control (data not shown). Taken together, these results indicate that TGFβ1 signaling suppresses cornification of keratinocytes expressing oncogenic HRAS, whereas pharmacologic disruption of the TGFβ pathway with SB enhances cornification.

**HRASV12G keratinocytes resistant to SB-induced cornification have an altered senescence phenotype**

Although there was a significant induction of cornification in the SB-treated HRASV12G keratinocytes, some remained which were resistant to this effect and these cells reconstituted the culture over time in the presence of continuous SB and doxycycline treatment. Figure 5A and B show that the large drop in attached cells which occurred in the SB-treated HRASV12G keratinocytes at 5 to 6 days was followed by a rapid recovery such that by 11 days, the culture became nearly confluent with no significant difference in cell number between HRASV12G treated with and without SB. These cells were keratinocyte in origin, as they expressed keratin 1 (data not shown). A similar increase in cell number did not occur in SB-treated control

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**Figure 5.** Continued proliferation of HRASV12G keratinocytes resistant to SB-induced cornification. A, growth curve showing expansion of SB-resistant cells and recovery of attached cell numbers after 11 days continuous treatment. n = 3, N = 2. B, photomicrographs of bitransgenic monolayers cultured for 11 days with and without HRASV12G expression and SB, showing loss of attached cells at day 6, regrowth by day 11, and lack of effect of SB on keratinocytes not expressing oncogenic HRas. C, control, bitransgenic keratinocytes without Dox or SB; HRAS, HRASV12G expression induced by Dox.
senescence (12), whereas cells that did not undergo cornification and continued to proliferate in the presence of SB were only 30% SA-βgal positive. Similarly, when v-HRAs transduced BALB/c keratinocytes, which have a pronounced senescence response (23) were treated with SB for 11 days, there was a reduced senescence response as measured by SA-βgal (Fig. 6A). The senescence-associated induction of the tumor suppressor p16ink4a was reduced in HRASV12G keratinocytes treated with SB (Fig. 6B), but there was little effect on expression of the cyclin-dependent kinase inhibitor p21waf1. Similarly, when v-HRAs keratinocytes were treated continuously with SB, there was a dose-dependent reduction in p16ink4a relative to untreated senescent cultures with no change in p21waf1 (Fig. 6C). Treatment of day 11 senescent keratinocytes with SB for an additional 4 days did not cause a significant reduction in p16ink4a levels suggesting that the effect of SB on expression of this tumor suppressor was indirect (data not shown) or once activated, its expression was independent of TGFβ signaling. These nonsenescent keratinocytes were still sensitive to SB, as p-Smad2 levels were reduced relative to control and expression of PAI-1, a well-characterized TGFβ1-responsive gene (24), was inhibited by SB to the same extent in senescence-resistant cells as primary bitransgenic cells after a 24-hour treatment (Supplementary Fig. S1A and B). To test if the cornification- and senescence-resistant cells represented a subpopulation of HRASV12G-expressing keratinocytes, we plated primary bitransgenic keratinocytes at low density before switching to doxycycline media with or without SB for 11 days. Figure 7A shows that after 11 days of SB treatment, large colonies of densely packed cells formed only in the SB-treated HRASV12G keratinocytes in contrast to a thin lawn of cells with the other treatment groups. To determine the growth properties of these cells, we subcultured a mass population following 14 days in doxycycline and SB and passaged twice. Cells were grown in culture media without doxycycline or SB for 2 days and then tested for responsiveness to TGFβ1 and SB with and without HRASV12G. Figure 7B shows that TGFβ1 inhibited the growth of these keratinocytes indicating no gross selection for resistance to this growth factor. As expected, induction of HRASV12G with doxycycline-stimulated proliferation as with the initial primary culture, but in contrast to primary cultures, SB now stimulated proliferation of these cells with and without HRASV12G. To test whether these senescence-resistant keratinocytes had undergone malignant conversion, we determined their ability to form colonies under differentiation-inducing conditions of elevated calcium, a hallmark of malignant conversion of primary keratinocytes expressing oncogenic Ras (25, 26). When plated at clonal density and cultured with doxycycline and SB in low calcium proliferation media (0.05 mmol/L Ca2+), these keratinocytes readily formed colonies (Fig. 7C). No colonies formed in the absence of either doxycycline or SB indicating continued dependence on oncogenic HRas and blocked TGFβ signaling (not shown). When cells were switched from proliferation to differentiation media (0.5 mmol/L Ca2+) containing doxycycline and SB, colony formation

![Figure 6](image)

Figure 6. Reduced senescence in HRas-expressing keratinocytes treated long term with SB. A, HRASV12G and v-HRas expressing BALB/c keratinocytes treated for 11 days with SB have reduced percentage of senescent cells compared with untreated HRas-expressing controls. n = 3, N = 2. B, senescence-associated increase in p16ink4a is blocked in HRASV12G keratinocytes cultured in SB. Blot was probed for p21waf1 followed by p16ink4a without stripping. N = 2. C, dose-dependent inhibition of p16ink4a protein expression by SB in v-HRas-infected keratinocytes. Retrovirally infected BALB/c keratinocytes were treated with the indicated dose of SB continuously for 11 days prior to isolation of cell extract for immunoblotting. No change in p16ink4a was observed (not shown). β-Actin was used as a loading control. N = 2, C, control; R, HRASV12G expression induced by Dox. Values with * are significantly different at P < 0.05. Error bars represent SEM.
did not occur, even though induction of HRASV12G in these cells was sufficient to suppress keratin 1 induction (not shown). In addition, when senescence-resistant keratinocytes were skin grafted onto athymic nude mice (14) predosed with doxycycline, 0 of 7 grafts formed tumors, even with topical SB treatment. Thus, these cells do not have characteristics of fully malignant keratinocytes but exhibit altered growth and differentiation responses to inhibition of TGFβ1 signaling.

Discussion

TGFβ1 signaling has a well-characterized role in chemical carcinogenesis of the mouse epidermis. Recently, we showed that, in contrast to the paradigm of TGFβ as a tumor suppressor derived from genetic models, pharmacologic inhibition of TGFβ signaling with the small molecule ALK5 inhibitor SB suppressed benign tumor formation and enhanced malignant conversion. Here, we have used primary keratinocytes expressing inducible oncogenic HRASV12G to study potential mechanisms through which ALK5 inhibition could produce these dual responses in vivo. Our study provides support for the novel hypothesis that subpopulations of HRASV12G-expressing keratinocytes have distinct responses to inhibition of TGFβ signaling and these dual responses are linked to tumorigenic potential.

In chemical carcinogenic studies in the mouse skin, mutational activation of HRas is an essential first step in tumor formation. Keratinocytes expressing oncogenic HRas have abnormal responses to terminal differentiation signals (27, 28). The end product of keratinocyte differentiation is the cornified envelope, a highly insoluble complex of cross-linked proteins that is essential for the barrier function of the epidermis. As keratinocytes leave the basement membrane and withdraw from the cell cycle, they express specific proteins that are involved in formation of the cornified envelope including involucrin, filaggrin, specific suprabasal keratins, and small proline-rich proteins as well as transglutaminases that cause cross-linking of these structural proteins (22). In vivo studies show that oncogenic HRas blocks calcium-mediated induction of the early differentiation markers keratin 1 and 10 but causes overexpression of proteins associated with later stages of cornification including loricrin, filaggrin, and transglutaminase (13). We observed increased cornified envelope formation in HRASV12G keratinocytes but this also was balanced by hyperproliferation such that cell loss was not observed. Although it is generally accepted that exogenous TGFβ1 acts as a growth inhibitor for normal and preneoplastic keratinocytes and other epithelial cells, we found that inhibition of endogenous TGFβ signaling with SB had no effect on cell cycle or proliferation of HRASV12G keratinocytes. Rather, there was a significant increase in cornified envelope formation accompanied by loss of attached cells and increased expression of genes associated with cornified envelope formation. This indicates that the majority of HRASV12G-expressing keratinocytes were being driven toward cornification without self-renewal when ALK5 signaling

Figure 7. SB-resistant cells arise from a subpopulation of HRASV12G keratinocytes stimulated to proliferate by SB. A, HRASV12G keratinocytes plated at low density and treated with SB (2.0 μmol/L) for 11 days form large colonies, whereas cells expressing HRASV12G or treated with SB alone do not. N = 3. B, passaged resistant cells are growth stimulated by reexpression of HRASV12G and SB. A mass culture of SB-resistant cells was passaged twice before plating in media without supplements for 2 days followed by treatment as indicated, and attached cell number was determined at the indicated days. There was a significant increase in cell number in cells expressing HRASV12G + SB compared with HRASV12G expression alone. n = 3. N = 2. C, passaged HRASV12G + SB-resistant keratinocytes can grow clonally in proliferation media (0.05 mmol/L Ca²⁺) with continued Dox and SB treatment (left) but are unable to form colonies in differentiation media (0.5 mmol/L Ca²⁺ media, right). No colonies formed in either growth condition when cells were cultured with Dox or SB alone or without any treatment. Passaged cells were seeded at clonal density in the presence of Dox and SB to allow attachment and then switched to the indicated conditions. N = 4. HRAS, HRASV12G expression induced by Dox; TGFβ1, 1 ng/mL. Values with * are significantly different at P < 0.05. Error bars represent SEM.
was blocked. Conversely, our in vitro and in vivo studies show that TGFβ1 signaling can block cornified envelope formation and genes associated with formation of the cornified envelope in HRASV12G-expressing keratinocytes. Although some studies have not shown any effects of TGFβ1 on terminal differentiation in normal human keratinocytes (29), our results are consistent with other studies showing that TGFβ1 can suppress squamous differentiation in normal and transformed human and mouse keratinocytes (30–33) and indicate that at this stage, TGFβ effects on differentiation rather than proliferation predominate. Taken together our studies suggest that one mechanism underlying the ability of SB to suppress benign papilloma formation in 2-stage skin carcinogenesis is through enhanced terminal differentiation of epidermal keratinocytes containing a mutated HRas gene.

Although SB enhanced terminal differentiation and cell death in keratinocytes expressing HRASV12G, a subpopulation of these cells were resistant to this effect and continued to proliferate. Further analysis showed that the growth of these cells was stimulated when TGFβ1 signaling was blocked with SB suggesting that TGFβ signaling was now linked to negative regulation of the cell cycle. These cells also had reduced expression of the senescence marker SA-βgal and the tumor suppressor p16ink4a suggesting that they were also resistant to the senescence phenotype associated with oncogenic Ras expression in primary mouse keratinocytes. These results are consistent with previous studies which showed that TGFβ1−/−, Smad3−/−, or DNTBRII-expressing primary keratinocytes could overcome a v-HRas–induced senescence response (12, 34) and that TGFβ1 can induce p16ink4a expression in v-HRas keratinocytes (35). In this study however, treatment of senescent keratinocytes with SB did not reduce p16ink4a expression and ALK5 inhibition for proliferation, and could not proliferate under differentiation-inducing conditions indicating that they were not fully transformed. Nevertheless, proliferation and reduced senescence following long-term SB treatment of keratinocytes harboring activated HRASV12G provide a mechanism for a more progressed phenotype that may be dependent on interaction with the tumor microenvironment or further mutations for full malignancy.

Although a number of different small molecule ALK5 inhibitors can suppress phenotypic changes associated with malignancy in vitro (9, 40) and in vivo (41), this is the first study to demonstrate potential suppressive effects of ALK5 inhibition through induction of terminal differentiation in a premalignant epithelial cell as well as bidirectional responses to ALK5 inhibition within a primary cell population. Although this expands the potential therapeutic potential for ALK5 inhibitors, it also highlights the potential difficulties associated with long-term use.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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