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TERMINAL DIFFERENTIATION REGULATED BY ALK5 IN PRENEOPLASTIC KERATINOCYTES

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ABSTRACT

Genetic studies investigating Transforming Growth Factor β (TGFβ) have revealed a dual role for TGFβ in cancer progression, with TGFβ acting as an early tumor suppressor and later acting as an oncogene. Contrary to this established paradigm, we have previously shown in a two-stage chemical carcinogenesis model that pharmacological inhibition of the TGFβ type I receptor (ALK5) by SB431542 (SB) inhibits papilloma formation and enhances malignant progression of papillomas that do form. Here we investigate altered terminal differentiation in premalignant keratinocytes as a potential mechanism by which ALK5 inhibition could decrease papilloma formation. SB treatment of *HRAS* expressing keratinocytes shows an increase in cornification that correlates with increased expression of terminal differentiation genes transglutaminase 1 (TGM1) and 3 (TGM3) and small proline-rich protein 1A (SPR1A) and 2H (SPR2H). Conversely, treatment with TGFβ1 decreases expression of these genes and inhibits cornification in *HRAS* expressing keratinocytes. These results are also observed *in vivo*, as mice expressing *HRAS* in basal keratinocytes that are treated with SB show increased epidermal thickness and increased expression of TGM1/3 and SPR1A/2H. Together, these results suggest that induction of terminal differentiation may be a potential mechanism by which pharmacological inhibition of TGFβ signaling inhibits early papilloma formation in a premalignant, *HRAS* expressing model.

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1. Introduction

1.1 The Epidermis

The epidermis is the outermost layer of the skin that serves as a protective barrier from physical and chemical damages, the mutating effects of UV light, infection by pathogens, and dehydration, and is essential to human survival. The epidermis is dynamic and constantly renews itself as dead cells are sloughed off and new cells are generated from epidermal stem cells. As cells transition outward from the basal layer and differentiate, they follow a program of biochemical and morphological changes that results in the formation of the stratum corneum, a hydrophobic, insoluble barrier of highly cross-linked proteins and lipids that form the outermost layer of the epidermis. [1] The process of epidermal differentiation is divided into four stages that correspond with different layers of the skin and expression of specific proteins. The first is the basal layer, which consists of a single layer of stem cells attached to a basal lamina by hemidesmosomes of $\alpha_6\beta_4$ integrins.[2] Keratin intermediate filaments Keratin 5 (K5) and Keratin 14 (K14) are expressed in the basal layer and compose the cytostructure of the cell. Basal cells are the only epidermal cells capable of proliferation but mitotic ability is lost when cells leave this layer. As cells transition to the spinous layer, they exhibit extensive $\alpha_3\beta_1$ desmosomal connections which give cells their namesake spinous appearance.[1,3] The basal to spinous transition is correlated with a downregulation of keratins 5 and 14 and upregulation of Keratin 1 and Keratin 10, keratin filaments which aggregate into thin bundles.[3] Theaerly stage differentiation marker involucrin is also expressed in the spinous layer and associates below the plasma membrane to later aid in cross-linking keratin bundles.[2] The spinous to granular transition is characterized by downregulation of Keratin 1 and Keratin 10 and upregulation of late stage differentiation proteins loricrin, filaggrin,

transglutaminases (TGMs) and small proline-rich proteins (SPRs) all of which are involved in bundling keratin filaments and cross-linking proteins. The granular layer is named for visible granules of loricrin and filaggrin and granules of lipids. During the transition to the cornified layer, granules of lipids are released into the extracellular space to form a waterproof seal between terminally differentiated keratinocytes. In this last transition, an influx of calcium activates transglutaminases to catalyze the formation of ε-(γ-glutamyl) lysine isopeptide bonds between keratin bundles and other proteins of the cornified envelope.3 The end product of terminal differentiation is the corneocyte, a flattened, nonviable squame composed of a dense protein matrix and sealed by extracellular lipids to form the stratum corneum.

While the specific mechanisms that signal basal cells to lose hemidesmosomal connections with the basal lamina and terminally differentiate are not entirely known, an increased calcium concentration signals keratinocytes to differentiate *in vivo* and *in vitro*.[4] An intracellular and extracellular calcium gradient exists across the epidermis with low concentrations in the basal layer and high concentrations in the stratum corneum. This calcium gradient is established by stage specific expression of phospholipase C (PLC) isoforms in the skin. While PLC γ1, γ2, and β3 are present in all layers of the epidermis,[5] PLCδ1 is upregulated in the spinous layer and generates increased diacylglycerol (DAG) and liberates calcium, stores. These actions are critical for activation of protein kinase C (PKC).[6] PKCs α, δ, ε, and ζ are expressed throughout differentiation, but expression of PKCη occurs in the granular layer. PKCη plays an essential role in the spinous to granular transition through downregulation of K1/10 and upregulation of loricrin, filagrin and transglutaminases.[3]

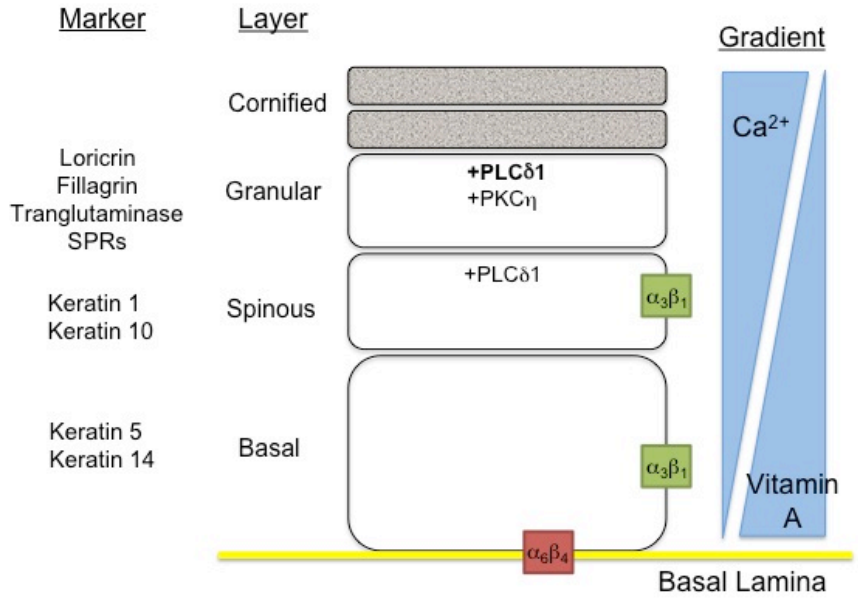


Figure 1-1: Differentiation in the epidermis
As keratinocytes transition from the basal layer outward, different protein markers are expressed. This transition is accompanied by an increasing calcium gradient and opposing vitamin A gradient.

Differentiation in epithelial tissues is a highly regulated process and alterations to this homeostasis can promote formation of epithelial cancers such as skin cancer. Skin cancer is the most common cancer in the United States[7] with about 1,000,000 new cases of skin cancer in 2010.[8] The two most common types of skin cancer are basal cell carcinoma (BCC), forming in the basal cells of the epidermis, and squamous cell carcinoma (SCC), which forms in more differentiated layers of squamous epithelia. BCCs usually remain contained within the basal layer while SCCs can possess a more malignant phenotype. BCC and SCC are collectively included in category of non-melanoma skin cancer and are much more common and benign than melanoma, a skin cancer that forms in melanocytes with a high rate of metastasis. Knowledge of how epithelial differentiation is regulated can enhance our understanding of how skin and other epithelial cancers form and progress.

1.2 The Ras Oncogene

Ras is a proto-oncogene that is involved in a wide variety of cellular processes such as cell proliferation, differentiation, survival, angiogenesis, migration, and differentiation.[9] These processes are highly regulated by a wide array of extracellular cytokines, hormones, and growth factors that transduce their signals via a complex network of intracellular signaling pathways. Ras is involved in many of these pathways and functions as a molecular switch that when activated, can promote growth, survival, angiogenesis, migration, and differentiation. The Ras gene's critical role in these cellular processes highlights its oncogenic potential, which is supported by the fact that Ras mutations are commonly found in about 20% of all human tumors[9,10], but incidence varies widely with tumor type. Pancreatic, colon and thyroid cancers have the highest incidence of Ras mutations. The particular Ras isoform that is mutated (H-Ras, N-Ras, or K-Ras) also varies widely between tumor type. Pancreatic cancers and adenocarcinomas of the lung contain mostly K-Ras mutations while colon and thyroid cancers show less specificity.[11]

To transduce downstream signaling, Ras must be converted from its inactive, guanine diphosphate (GDP) bound form to its active guanine triphosphate (GTP) bound form. Activation of Ras occurs predominantly through receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and Insulin-like growth factor 1 receptor (IGF-1R).[11] Dimerization of RTKs by extracellular growth factors results in auto-phosphorylation of specific tyrosine residues, which are recognized by the SH2 domain of adapter proteins such as Grb-2. Grb-2 contains two SH3 domains, which recruit guanine nucleotide exchange factors (GEFs) such as the mammalian Son of Sevenless (SOS1 and SOS2) to the plasma membrane. GEFs activate Ras by

catalyzing the dissociation of GDP allowing Ras to bind a new molecule of GTP and localize downstream effectors to the plasma membrane to initiate signaling.[9]

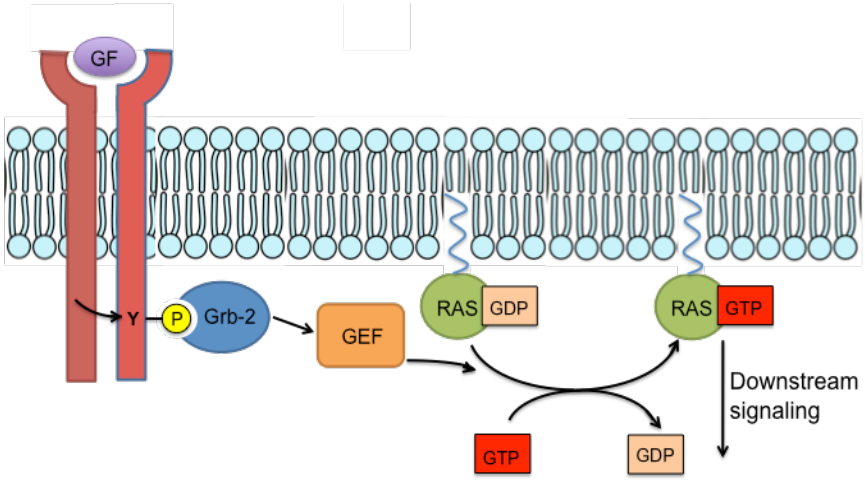


Figure 1-2:
Activation of Ras

Growth factor (GF) dimerizes receptor tyrosine kinases which results in auto-phosphorylation of tyrosine residues. GEF is recruited through the adapter protein Grb-2 and catalyzes exchange of GDP for GTP bound to Ras. Ras-GTP is activated for downstream signaling.

Shortly after activation, GTPase activating proteins (GAPS) stimulate Ras to hydrolyze GTP. GAPS are critical in attenuating growth factor signaling and consequently, mutations in GAPS can result in tumor formation. Neurofibromatosis, a disorder characterized by multiple tumors in nerves, is caused by a mutation in the GAP protein neurofibromatosis type 1 (NF1).[12]

Mutations in Ras at codons 12, 13 and 61 impair intrinsic and GAP catalyzed GTPase activity and result in an oncogenic, constitutively active form of Ras. Oncogenic Ras over stimulates a number of downstream pathways and promotes a variety of tumorigenic effects.

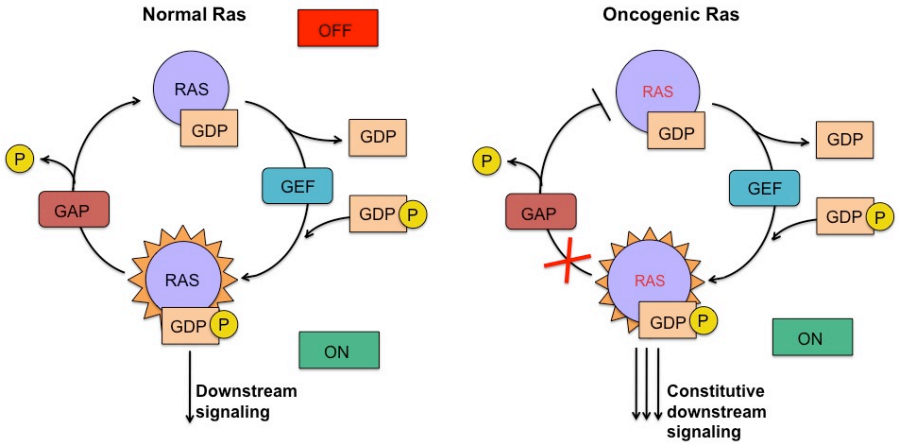


Figure 1-3: Oncogenic Ras

Mutation of Ras in codons 12, 13 or 61 results in a constitutively active form of Ras incapable of GTP hydrolysis.

The mitogen activated protein kinase (MAPK) pathway is one of the most studied signaling pathways activated by Ras. When bound to GTP, Ras recruits serine/threonine kinase RAF (MAP Kinase Kinase Kinase or MAPKKK) to the plasma membrane. RAF phosphorylates and activates MEK (MAP Kinase Kinase or MAPKK). MEK, a dual specificity kinase, phosphoryates threonine and tyrosine residues on ERK (MAPK), which then phosphorylates nuclear transcription factor targets ELK1 and JUN among others.[13] Through a combination of transcriptional events, ERK induces transcription of Cyclin-D1 which promotes cell cycle progression through inhibition of the retinoblastoma protein (pRb)[11], commonly referred to as the gatekeeper of cell cycle progression. Targets of ERK also induce transcription of Vascular Endothelial Growth Factor (VEGF), a potent stimulator of angiogenesis, and Matrix Metallopeptidase 9 (MMP-9), a protease that breaks down the extracellular matrix aiding in invasion.[11] Thus activation of the MAPK pathway by Ras can have many effects on tumorigenesis. Ras also signals through the phosphoinositide 3′-kinase (PI3K) pathway which activates Akt or Protein Kinase B (PKB).[11] Akt is a key mediator of cell survival signals and acts through a variety of pathways, notably through inhibition of the

pro-apoptotic protein BAD[14] and activation of nuclear factor kB (NF-kB) which inhibits apoptosis.[15] In addition to processes that promote tumorigenesis, Ras also induces epidermal differentiation by activation of phospholipase C (PLC). PLC activates PKC and releases calcium stores promoting differentiation in the epidermis.[3] Terminal differentiation is often associated with tumor suppressor pathways; thus this function of Ras may serve to counter overactive mitogenic signaling. Tumors that overexpress Ras may acquire additional mutations that interfere with the differentiation process.

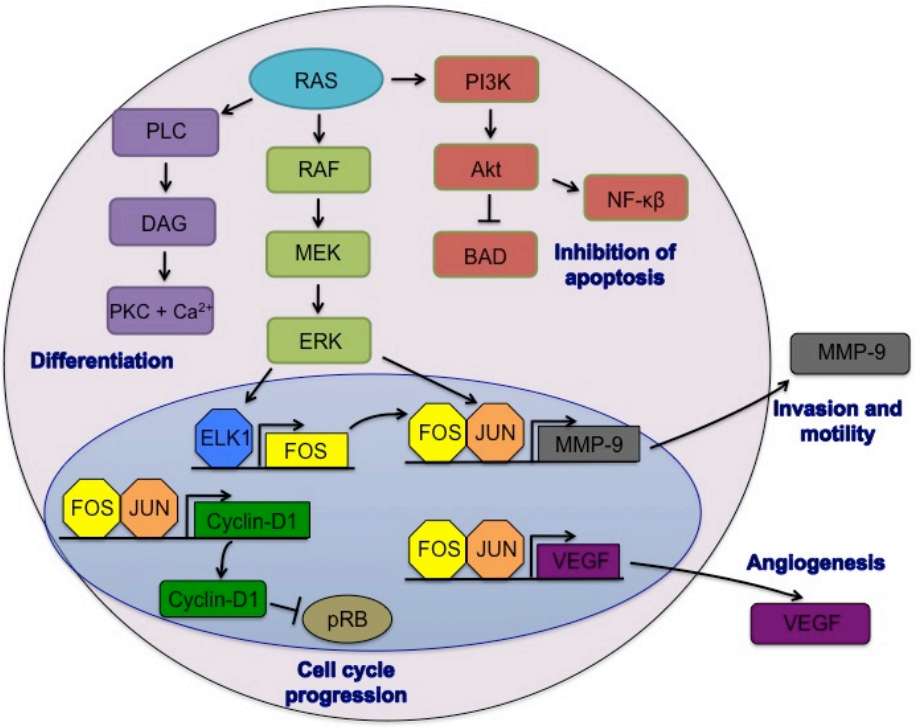


Figure 1-4: Ras Signaling

Ras activates a number of signaling pathways involved in tumorigenesis. Through the MAPK pathway, Ras induces transcription of transcription factors ELK1, FOS, and JUN. These transcription factors promote expression of proteins aiding in tumorigenesis: MMP-9 (invasion and motility), VEGF (increased angiogenesis) and Cyclin-D1 (cell cycle progression. Ras also signals through PLC to generate DAG and release calcium stores, resulting in activation of PKC and inducing differentiation. Through the P13K/Akt pathway, Ras inhibits apoptosis by activation of NFkB and inhibition of BAD.

1.4 The Two-Stage Chemical Skin Carcinogenesis Model

Although cancers are different in origin and result from a variety of mutations, there are operational stages of tumor formation and progression that are common to all cancers. It is widely believed that most human cancers result from an initial, mutational event in a stem cell population that when stimulated by external and internal signals, will clonally expand and acquire new genetic mutations over the course of time.[16] The two-stage chemical skin carcinogenesis model in mice mimics these operational stages of human epithelial cancers and provides a valuable model for studying the mechanisms by which tumors form and progress.[3] Initiation in the two-stage model is achieved by topical treatment of mouse skin with dimethylbenzanthracene (DMBA). Metabolism of DMBA yields DMBA-DNA adducts which intercalate into DNA during replication and cause mutations in keratinocytes. A common mutation is an activating mutation caused by an A → T transversion in codon 61 of *Ras*.[17] This alters the response of initiated keratinocytes to exogenous signals that cause epidermal proliferation and differentiation.

Tumor promotion, which is often caused by endogenous growth factors, hormones, or inflammation due to wound healing or UV damage, is mimicked in the model by topical treatment of mouse skin with 12-O-Tetradecanoylphorbol 13-acetate (TPA) at 10 days after DMBA initiation. TPA causes increased cell signaling, production of growth factors, and inflammation, all of which provide an environment that confers a growth advantage to initiated keratinocytes. With continued TPA promotion, initiated keratinoyctes clonally expand to form papillomas, or benign tumors within 10-15 weeks.3

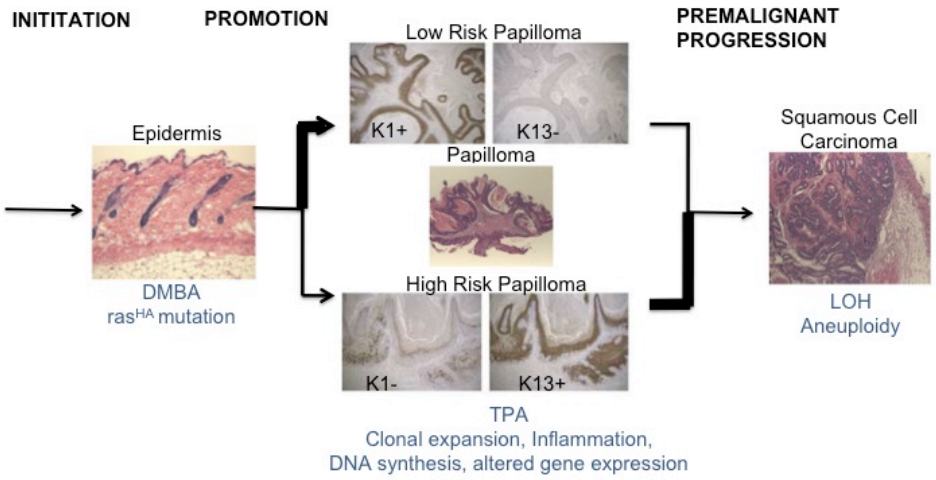
Within this group of papillomas, some will eventually regress upon discontinuation of promotion. Alternatively, continued promotion will cause a small subset of papillomas to acquire genetic changes such as loss of heterozygosity (LOH) in tumor suppressor genes and aneuploidy and these papillomas will convert to squamous cell carcinomas (SCCs), a malignant phenotype characterized by downward invasion and increased vascularization.[3,16] To some degree, it is possible to predict which papillomas will progress to SCC and which papillomas will regress. Papillomas can be characterized into two subgroups, high-risk and low-risk, based on the characteristics in Table 1-1.[3] While it is still speculative, it is hypothesized that high risk papillomas may arise from initiated epidermal stem cells that may contain additional mutations involved in tumorigenesis.[18]

Table 1-1: Markers for low risk and high risk papillomas

Property	**Low Risk**	**High Risk**
Histology	Benign	Benign
Sensitivity to strong promoter	Low	High
Persistence	TPA dependent	TPA independent
Keratin expression	K1+, K13-	K1-, K13+
Proliferation	Increased, basal	Increased, basal and suprabasal
TGFβ1 protein	Basal layer, strong	Basal layer, weak or lost

Figure 1-5: The two-stage chemical skin carcinogenesis model.

Initiation is achieved by a c-HaRas mutation caused by DMBA. Initiated epidermis is promoted with TPA treatment causing inflammation, DNA synthesis, altered gene expression, and clonal expansion into benign papillomas, which are categorized as low risk or high risk. The thickness of lines represents relative frequency of event, thus during promotion, more low risk papillomas are formed than high-risk papillomas. Upon acquiring additional genetic changes such as LOH or aneuploidy, a greater frequency of high-risk papillomas will progress to SCCs.



This multistage model can be used in combination with genetic or pharmacological changes in the skin to investigate their effects on tumor promotion and progression. While this model system has been highly developed and studied for over 50 years, there are similarities and differences to human cancer that must be considered when evaluating its relevance. An advantage of this model is that it recreates the stages of cancer progression observed in human cancers, and accomplishes this with a sub carcinogenic dose of initiator and low dose of promoter. This mimics the exposure to low doses of carcinogens and tumor promoters that humans experience throughout their life.[19]

However, a difference between this model and human skin cancers is that while the two-stage model involves a benign papilloma stage at which tumors can progress or regress, there is no correlate for a papilloma stage in human skin cancers. While human skin cancers require both initiation and promotion, they progress directly to SCCs or BCCs and do not form terminally benign papillomas. Furthermore, most human skin cancers are caused by DNA damage by UV light rather than chemical carcinogens. While a *Ras* mutation is chemically induced in the two-stage model system, *RAS* mutations are not commonly found in human skin cancers. A more important mutation in human skin cancers occurs in *p53*, a tumor suppressor critical for the cellular response to UV induced DNA damage.[20] However, activation of components in the Ras signaling pathway are more commonly found in skin cancers, and *RAS* mutations are also prevalent in other human epithelial cancers such as lung, colon and pancreatic cancers.[9] While these drawbacks must be considered when using this model, it is highly useful for investigating the basic mechanisms of cancer progression and the effects of potential anti-cancer therapies over the multiple stages of cancer.

In the Glick lab, we also use a transgenic mouse model of conditional *HRAS* expression as a genetic equivalent of the initiation stage in the two-stage chemical carcinogenesis model. This serves extremely useful for *in vitro* studies by providing a premalignant (*HRAS* expressing) keratinocytes and short term *in vivo* studies. Because transgenic mice that express oncogenic *Ras* during development or in tissues other than the epidermis often show lethal phenotypes, a conditional, tissue specific expression system is used to selectively turn on *Ras* expression in the epidermis. Double transgenic mice containing the K5rtTA and tetO*RASV12G* transgenes express the human *HRAS* transgene under the control of the Keratin 5 promoter, specifically in basal keratinocytes upon treatment with doxycyline (dox). The

K5rtTA transgene contains a bovine keratin gene promoter that is active in the basal layer of the epidermis, and drives the expression of tetracycline transactivator protein rTA. When rTA is activated by tetracycline or doxycycline (by addition to culture media *in vitro* or topical application *in vivo*), rTA/dox binds to the heptameric tetO binding site and induces transcription of the HRAS oncogene.[21] Keratinocytes can be isolated from the epidermis of these mice and cultured *in vitro* with doxycyline containing media to induce Ras expression, providing an *in vitro* model of preneoplastic keratinocytes that can be used to study cancer progression.

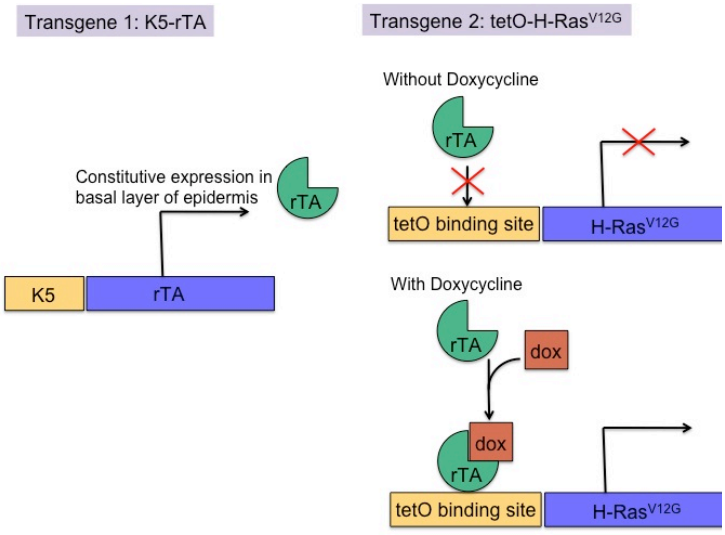


Figure 1-6:
Doxycycline inducible model for Ras expression in double transgenic mice.

The K5rtTA transgene constitutively expresses tetracycline transactivator (rTA) under the control of the K5 promoter in basal cells of the epidermis. Treatment with doxycyline activates rTA to bind the tetO binding site of the tetO-H-Ras^{V12G} transgene, activating transcription of H-Ras^{V12G}.

1.5 TGFβ

Transforming Growth Factor β (TGFβ) is a growth factor and cytokine involved in a variety of cellular processes, including cell growth, differentiation, inflammation, apoptosis, and development. TGFβ1 signals through the serine/threonine kinase receptors of the TGFβ receptor family by binding the extracellular domain and dimerizing the type II receptor (TβR-II) and the type I receptor (Activin receptor like kinase, ALK5). When TGFβ induces the formation of a receptor complex, TβR-II phosphorylates ALK5 on multiple threonine/serine residues on the cytoplasmic tail and activates ALK5's kinase domain.

Phosphorylation of receptor Smads (R-Smads) Smad2 and Smad3 by ALK5 allows them to bind to Smad4, a co-smad, which facilitates formation of a Smad2/3/4 complex. This complex translocates into the nucleus where it can activate transcription of target genes.

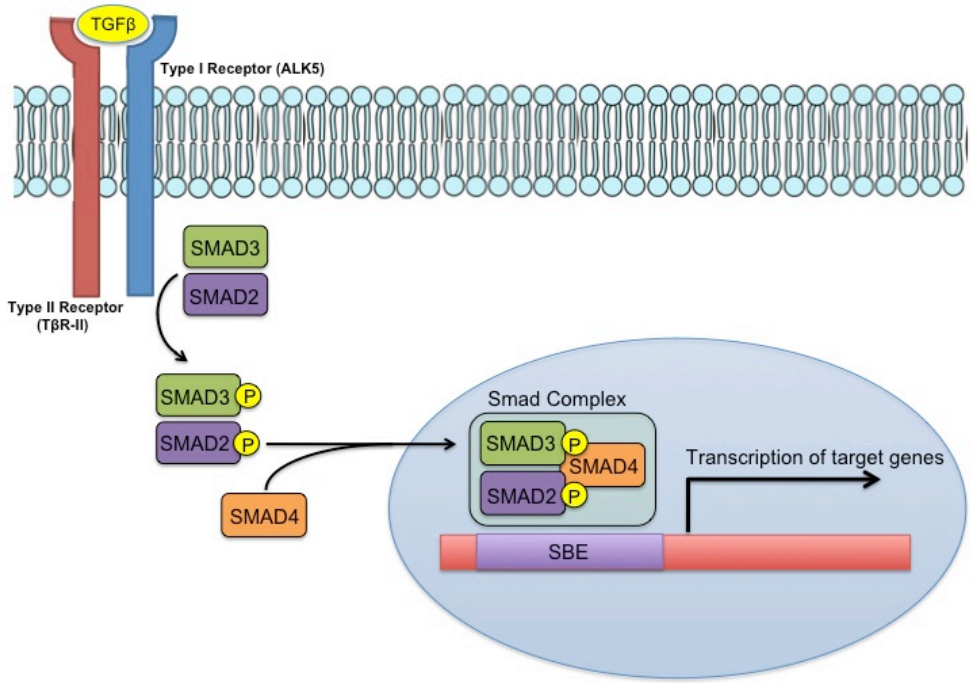


Figure 1-7: TGFβ Signaling

Binding of TGFβ1 to its receptor transduces an intracellular signal through phosphorylation of R-smads Smad2 and Smad3. p-Smad2 and p-Smad3 complex with Smad-4 which promotes nuclear translocation. The Smad complex acts as a transcription factor by binding to the Smad Binding Element (SBE) in the promoter sequence of target genes and induces gene expression.

1.6 TGFβ and cancer

TGFβ has been extensively studied in cancer with paradoxical roles as both a tumor suppressor and tumor promoter.[22,23,24,25,26,27] The current paradigm of TGFβ signaling in cancer shows that in early stages of cancer, TGFβ acts as a tumor suppressor by inhibiting cell cycle progression and cell growth. At later stages, TGFβ acts as a tumor promoter when

tumors become refractory to TGFβ signaling through a defect in the signaling pathway. While some tumor cells do not respond to TGFβ, they secrete excess TGFβ that exerts its tumor promoting effects on nearby cells, aiding in angiogenesis, metastasis, and immune evasion.

Mutations of components of the TGFβ signaling pathway, TβR-II, Smad-4 and Smad-2 have been found in human cancers and act as classic tumor suppressors.[28,29,30] Additionally, chemical carcinogenesis models in mice have used genetic alterations to mediators of the TGFβ pathway to characterize the role of TGFβ as an early tumor suppressor. In chemical carcinogenesis experiments, mice that overexpress TGFβ in suprabasal keratinocytes show reduced skin tumor formation[31,32] while $TGF\beta^{+/-}$ mice form more liver and lung tumors compared to TGFβ WT mice.[33] These changes in tumor formation are likely a result of TGFβ's role as a potent growth inhibitor of keratinocytes and have been linked to cell cycle regulation and growth suppression by TGFβ.

While in early tumor formation, TGFβ acts as a tumor suppressor, it is commonly found that late-stage, advanced tumors secrete high levels of TGFβ and are correlated with poor patient prognosis. Many breast cancer cell lines secrete bioactive TGFβ,[34] and immunostaining of breast carcinomas shows a correlation between increased bioactive TGFβ and invasiveness.[35,36] In chemical carcinogenesis assays, it is found that overexpression of TGFβ1 in keratinocytes initially reduces papilloma formation, but papillomas that do form progress rapidly to spindle cell carcinomas.[32] In these late stage effects TGFβ can promote malignant progression through actions on additional cell types apart from epithelial cells and influence changes to cell-matrix interactions that mediate survival[37], enhance angiogenesis[38],

or suppress immunosurveillance.[39]

Furthermore, transgenic models with defects in the TGFβ signaling pathway targeted to epithelial cells show an increase in malignant conversion. Mice with a conditional knockout of the type I receptor[40] targeted to the basal keratinocytes in head and neck epithelia formed head and neck SCC in a chemical carcinogenesis protocol compared to control littermates who did not develop tumors. In a similar chemical carcinogenesis protocol, expression of a dominant negative Type II receptor in basal keratinocytes significantly increased SCC formation[41]. On the other hand, Smad3 knockout mice have shown reduced formation of papillomas and SCCs[42] indicating that the role of TGFβ in malignant conversion is complex and not easily predictable.

These genetic studies generally show that an excess of extracellular bioactive TGFβ and functional TGFβ signaling at the time of an initiating event prevents the expansion of initiated cells, yet may select for a more malignant papilloma phenotype. Additionally loss of a responsiveness to TGFβ signaling in combination with excess TGFβ in the tumor microenvironment aids in tumor development and increased metastasis.

As TGFβ regulates a wide variety of cellular processes, different alterations to the pathway may involve a balance of positive and negative effects towards tumorigenesis and progression. In a study by Pérez-Lorenzo et al., $TGF\beta^{+/-}$ mice subjected to the two-stage chemical skin carcinogenesis protocol showed an increase in benign tumors yet decrease in malignant conversion.[43] As compared to other studies that targeted TGFβ mutations to specific tissues, this study involved a TGFβ mutation in every cell and produced very different results. This study highlights that the wide variety of cellular processes regulated by TGFB may result in varied and often contradictory responses in chemical carcinogenesis

experiments depending on where mutations are localized.

1.7 Pharmacological Inhibition of the TGFβ pathway

The well-established link between TGFβ and cancer makes it a potential target for pharmaceutical intervention, and consequently small molecule inhibitors of the TGFβ type I receptor have been developed as potential anti-cancer therapeutics. Genetic models have shown that TGFβ acts as an early tumor suppressor and late stage tumor promoter, however these studies cannot be directly compared to pharmacological studies. Genetic models that manipulate the TGFβ signaling pathways are more precise in targeting specific cells and also lack off-target effects on other proteins. On the other hand, pharmacological interventions may affect multiple cell types and are selective though not specific, yet pharmacological therapies are ultimately more relevant to potential clinical applications. Given the wide variety of effects on cellular processes induced by TGFβ and the variability of these effects depending on cell type and tumor stage, it is important to consider the safety and efficacy of pharmacological inhibitors as potential therapies in a multistage model of cancer.

In a few recent studies, pharmacological inhibitors of ALK5 have shown promise in inhibiting tumor growth *in vivo* and inhibiting a malignant phenotype *in vitro*. SD-208, a selective ALK5 inhibitor has been shown to inhibit the growth and metastasis of mouse mammary carcinomas[44] and gliomas.[45] Similarly, an *in vitro* study using the ALK5 inhibitor SB431542 shows a reduction in epithelial to mesenchymal transition, cell motility, migration and invasion, and VEGF secretion.[46] While these studies show promise for TGFβ blockade as a possible cancer target, another study using SB-525334 found that ALK5 inhibition significantly decreased the incidence and size of uterine leiomyoma in Eker rats, yet also

promoted growth of epithelial lesions in the kidneys forming renal cell carcinomas. [47]

SB431542 (SB) (4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H- imidazol-2-yl]-benzamide) is a small molecule inhibitor of the TGFβ type I receptor developed as a potential anticancer therapeutic.[48,49,50] SB competitively binds in the ATP binding pocket of the kinase domain of ALK5 and inhibits the kinase activity of ALK5 with an IC_{50} of 92 nM as measured by TGFβ-induced fibronectin mRNA formation in A498 cells. SB has also been shown to inhibit activin receptor ALK4 and nodal receptor ALK7, but with less specificity than ALK5. SB has no effects on components of the ERK, JNK, or p38 MAP kinase pathways. [51]

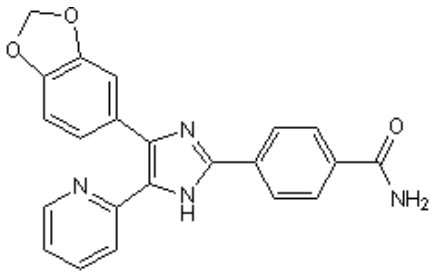


Figure 1-8: SB431542 (SB) (4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H- imidazol-2-yl]-benzamide)

In a recent study from the Glick lab, Markell *et al*. have shown in the multi-stage skin cancer model that long-term treatment with SB acts contrary to the TGFβ paradigm established in genetic studies. Unexpectedly, treatment of mice with SB in a chemical carcinogenesis protocol initially suppressed papilloma formation, and later increased the frequency of conversion in the papillomas that do form[52], suggesting that the role of ALK5 in cancer progression may be more complex than predicted by previous genetic studies. Papillomas from each treatment group were also investigated for differences in malignancy markers Keratins 1 and 13. Keratin 1 is a differentiation marker expressed in the suprabasal layer that is present in low-risk papillomas and lost in high-risk papillomas. Additionally, high-risk papillomas express Keratin 13, a keratin not normally expressed in the skin and a marker for increased progression. We have found that low-risk papillomas (K1+, K13-) form

in the absence of SB treatment, while a greater proportion of papillomas formed with SB treatment are high-risk (K1-, K13+). Based on these observations, we hypothesize that SB (or ALK5 inhibition) acts on certain subpopulations of keratinocytes that differentially respond to SB treatment. While SB suppresses the outgrowth of keratinocytes that form low-risk papillomas, it exerts different effects on a subpopulation of keratinocytes that are prone to form high-risk papillomas. Following this work that has shown the biological effect of SB treatment on tumor progression, this thesis will investigate the mechanism by which SB could inhibit the outgrowth of low risk papillomas. Determining this mechanism may provide a better understanding of how pharmacological inhibition of the TGFβ pathway affects cancer progression.

1.8 Hypothesis and Aims

Though the results from Markell *et al.* could be related to a variety of cellular processes regulated by TGFβ, this thesis will focus on the role of TGFβ signaling on terminal differentiation in a preneoplastic model of skin carcinogenesis to investigate a possible mechanism for reduced papilloma formation and enhanced malignant conversion by pharmacological ALK5 inhibition. The aims of this thesis are to: 1.) Investigate altered terminal differentiation *in vitro* and *in vivo* using a pharmacological inhibitor of ALK5 in HRas expressing keratinocytes and 2.) confirm these results in a genetic model using adenovirus expression of TGFβ signaling components. We hypothesize that SB increases terminal differentiation by up-regulating genes required for cornification, and that this increase in terminal differentiation by SB may explain the biological outcome of inhibiting early tumorigenesis.

2. Materials and Methods

2.1 Isolation of Primary Keratinocytes

All work with mice followed procedures approved by the Pennsylvania State University's Institutional Animal Care and Use Committee. Double transgenic newborn pups containing the K5rtTA and tetO*RASV12G* transgenes were obtained from a heterozygous K5rtTA and homozygous tetO*RASV12G* cross. Genotyping with the Keratin 5 promoter was performed to select for pups containing both transgenes. Pups were euthanized by CO_2 exposure and whole skin was isolated and floated on 0.25% trypsin (Gibco Invitrogen, Carlsbad, CA) overnight. The epidermis was removed from the dermis and minced in high (1.4 mM) calcium media (Formula # 98-0216DJ, Gibco Invitrogen, Carlsbad, CA). The minced epidermis was filtered through a cell strainer and centrifuged to pellet keratinocytes, which were resuspended in 10% DMSO in high (1.4 mM) calcium media. The cells were frozen at -80°C overnight and then transferred to liquid nitrogen storage to be plated for future experiments.

2.2 Adenovirus Infection

Frozen cells were resuspended in 0.2 mM calcium and plated at 2 newborn skins (mouse equivalents) per 6 well tray. Media was changed to low calcium (0.05mM) the following day and cells were grown to complete confluence by day 3 at 37°C/ 7% CO_2. Doxycyline (1 ug/mL) was added to the media on day 3 and maintained for 48 hours to induce conditional HRAS expression in the K5rtTA/tetO*RASV12G* transgenes. Cells were infected with adenovirus on day 4 (ALK5, NCI, 19.2 x 10^{10} pfu/mL; DN-TBRII, NCI, 5 x 10^{10} pfu/mL; lac-Z 17.1 x 10^{10} pfu/mL). Cells were washed with PBS and incubated with 300

μL of 0.05 mM calcium media containing 2.5 ug/mL polybrene and adenovirus at a multiplicity of infection of 5 (5 viral particles per cell). Cells were incubated at 37°C, 7% CO_2 for 30 minutes and rocked once halfway through the infection. After 30 minutes, the well volume was brought to 2 mL with 0.05 mM calcium media. The TGFβ treatment group was treated with 1 ng/mL TGFβ after infection.

2.3 Cornified Envelope (CE) Assay

Keratinocytes were isolated as described above and plated in 60 mm^2 dishes in 0.2 mM calcium. The following day, the media was changed to 0.05 mM calcium and the next day cells were treated with 1 μg/mL dox and/or 0.5 μM SB. To perform the CE assay, media containing cornified/floating cells was collected in addition to 2 PBS washes and centrifuged for 5 minutes at 800 rpm. The cell pellet was resuspended in 100 μL of 2% sodium dodecyl sulfate and 20mM DTT and boiled at 90°C for 10 minutes. This treatment solubilizes any floating cells that are not cornified. A hemocytometer was used to view and count the cornified cells, which appear as rounded "ghost cells." (Figure 2-1) Attached cells were trypsinized with 0.5 mL of 0.25% trypsin (Gibco Invitrogen, Carlsbad, CA), incubated for 10 minutes at 37°C,7% CO_2, collected, and counted using a Z1 Coulter particle counter (Beckman Coulter). The number of cornified cells was expressed as a ratio to the number of attached cells.

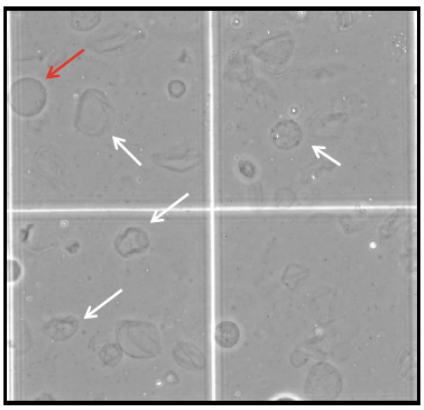


Figure 2-1: Cornified Envelope Assay

Following boiling with 2% SDS/ 20mM DTT solution, cornified cells appear as irregularly shaped "ghost cells" (white arrows). Four quadrants were counted under a hemocytometer for each sample.

2.4 RNA Isolation and qPCR

Cells were washed twice with cold PBS and 0.5 mL Trizol (Invitrogen) was added to each well. Plates were rocked for 10 minutes at room temperature (RT), and then transferred to microfuge tubes. Tubes were vortexed, and incubated for 10 minutes at RT. 100 μL chloroform was added to each tube and tubes were shaken vigorously to fully mix the aqueous and organic layers. Tubes were incubated at RT for 5 minutes and then centrifuged at 9000 rpm for 30 minutes at 4°C. The aqueous phase was transferred to a new tube and 250 μL of isopropanol was added. RNA was precipitated overnight at -80°C. Tubes were centrifuged at 9000 rpm for 30 minutes at 4°C. Isopropanol was decanted, the tubes were air-dried and the RNA pellet was resuspended in 200 μL DEPC water, 20 μL sodium acetate (pH 5.2) and 550 μL 95% ethanol was added to each tube and vortexed. RNA was precipitated overnight at -80°C. Tubes were centrifuged at 12000 rpm for 30 minutes at 4°C. Ethanol was decanted, the pellet was washed with 1 mL 70% ethanol, and tubes were centrifuged at 10000 rpm for 15 minutes at 4°C. The ethanol was aspirated and the RNA pellet was resuspended in 30 μL DEPC water. RNA was quantitated by measuring absorbance at 260 nm with a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

RNA was treated with Turbo DNA-*free* (AM-1907, Applied Biosystems/Ambion, Austin, TX) to remove any DNA contamination. cDNA was reverse transcribed from DNAse treated RNA with the High Capacity cDNA reverse transcription kit (Applied Biosystems/Ambion). Quantitative RT-PCR (qPCR) was performed on samples using the MyIQ system (BioRad Laboratories, Hercules, CA) and PerfeCTa SYBR Green SuperMix for iQ (#95053-500, Quanta Biosciences, Gaithersburg, MD). 18s rRNA was used as a

housekeeping gene.. The following qPCR program was used: 50°C, 2min.; 95°C, 10min.; (95°C, 15sec.; 60°C, 1min. x 40 cycles).

Table 2-1: Primer Sequences

Gene	Forward Sequence	Reverse Sequence
18srRNA	TCGATGCTCTTAGCTGAGTGTCCC	TATTCCTAGCTGCGGTATCCAGGC
SPRR1A	CCATTGCCTTGTGCTACCAA	TCAGGAGCCCTTGAAGATGG
SPRR2H	CTTCCCTCCAAAGCCATTCA	TGAAGCGCTGAGGAAGGACTA
TGM1	ACACAACCTAAACCTACGCGTCCA	ACATATTCTTGCCGCCAGTCCTCA
TGM3	CGCAACATCTTCGAGGAATC	TCCTTCCACACTTCGTGGACAA

2.5 Protein Isolation and Western Blotting

Cells were washed twice with PBS and protein was extracted with 200 μL of 0.5% NP40 Lysis Buffer (0.5% IGEPAL CA-630, 250mM NaCl, 50mM Tris HCl, pH 7.4). Protease and phosphatase inhibitors were added to the NP40 lysis buffer at 1 uL/mL at the following concentrations: DTT (1M), Sodium OrthoVanidate pH=10 (200mM), NaF (1M), PMSF (200mM), Aprotinin (1mg/mL), Leupeptin (5mg/mL), Pepstatin (1mg/mL), and β-glycerophosphate (0.24mg/mL). Wells were scraped with a cell scraper and the liquid was transferred into microfuge tubes. The tubes were rotated for 1 hour at 4°C and then centrifuged at 14000 rpm for 15 minutes to pellet cellular debris. The supernatant was transferred to a new tube and samples were stored at -80°C. Protein was quantitated using the Bio-Rad protein assay (#500-0006, Bio-Rad) which measures absorbance at 595 nm.

Samples of 20 μg protein were combined with an equal volume of loading buffer (Laemmli Protein Loading Dye, SDS, and Beta-mercaptoethanol) and boiled at 99°C for 5 minutes. Samples were loaded onto a 7.5% agarose gel and electrophoresed at 53V through

the stacking phase and 140V through the rest of the gel until the dye front reached the end of the gel. Protein in the gel was transferred to a 0.2 μM nitrocellulose membrane at 100V for 75 minutes. The blot was stained with Ponceau stain to check for effective transfer. The blot was washed with TBS-tween (0.1% Tween-20/Tris-buffered saline) and blocked with 5% milk/TBS-tween for 1 hour at RT. After rinsing with TBS-tween, the primary antibody in 3% bovine serum albumin/TBS-Tween was added and rocked with the blot at 4°C overnight. Following 6 x 10 minute washes, the secondary antibody in 5% milk/TBS-Tween was added to the blot and rocked for 1 hour at RT. A chemiluminescent substrate (ECL, Pierce, Rockford, IL, 0.3 mL Stable Peroxide solution, 0.3 mL of Luminol/Enhancer) was added to the blots and exposed to film. The film was developed on a Konica SRX-101A processor, scanned into Adobe Photoshop 7.0, and converted to grayscale.

The following antibodies and concentrations were used: Smad 2/3 (1:2000) (Cell Signaling Technology, Inc., Danvers, MA); p-Smad2 (1:1000) (Cell Signaling); H-ras, (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); GAPDH (1:1000) (Cell Signaling) and β-actin (1:20,000) (Millipore), and HRP-conjugated secondary antibody (1:2000) (BioRad).

2.6 Animal Studies

Involucrin tTA (InvtTA) x tetO*RASV12G* mice were maintained on 10μg/mL doxycycline water which prevents induction of HRAS expression. When mice were seven weeks old, dox water was replaced with water in which nothing was added and HRAS expression was induced. Mice were topically treated with 200 μL of acetone or 10.0 μM SB in 200μl of acetone every other day for 5 days. Mice were sacrificed 24 h after the last SB treatment and tissue sections were harvested and fixed in 10% neutral buffered formalin or

70% ethanol. Tissue was then paraffin embedded and 5 μm sections were prepared and mounted on slides. Epidermal thickness was measured at 200x magnification with 30 measurements per slide.

Statistical Analysis:

One-way ANOVA and Tukey's Multiple Comparison post-test were used to test significance of multiple groups within an experiment. *Student's t* test was used to compare two groups only, and the significance of the difference was described.

3. Results

3.1 SB431542 inhibits smad-2 phosphorylation *in vitro*.

We first show that SB treatment of keratinocytes can specifically inhibit Smad2 phosphorylation without off-target effects. Since ALK5 has a kinase domain similar to MAPKKs p38 and ERK, we validated that SB was specific for ALK5 inhibition in FVB/n keratinocytes over a range of doses and found that SB did not affect p38 and ERK activity. (Figure 1A). To confirm that inhibition was maintained for long-term *in vitro* experiments, we evaluated inhibition of Smad2 phosphorylation over 48 hours. Figure 1B shows that 0.5 μM SB treatment of FVB/n keratinocytes inhibits Smad2 phosphorylation at 0.5 hours and maintains inhibition through 48 hours. Since our experiments also use inducible K5rtTA/tetO*RASV12G* keratinocytes that conditionally express HRAS upon dox treatment, we first confirmed that HRAS expression is induced by 250 ng/mL and 1 μg/mL of dox, and that HRAS expression is not observed in single transgenic mice expressing tetORAS only. (Figure 1C). We found that in both control and HRAS expressing keratinocytes, SB inhibits Smad2 phosphorylation at 1 and 4 days of SB treatment (Figure 1D).

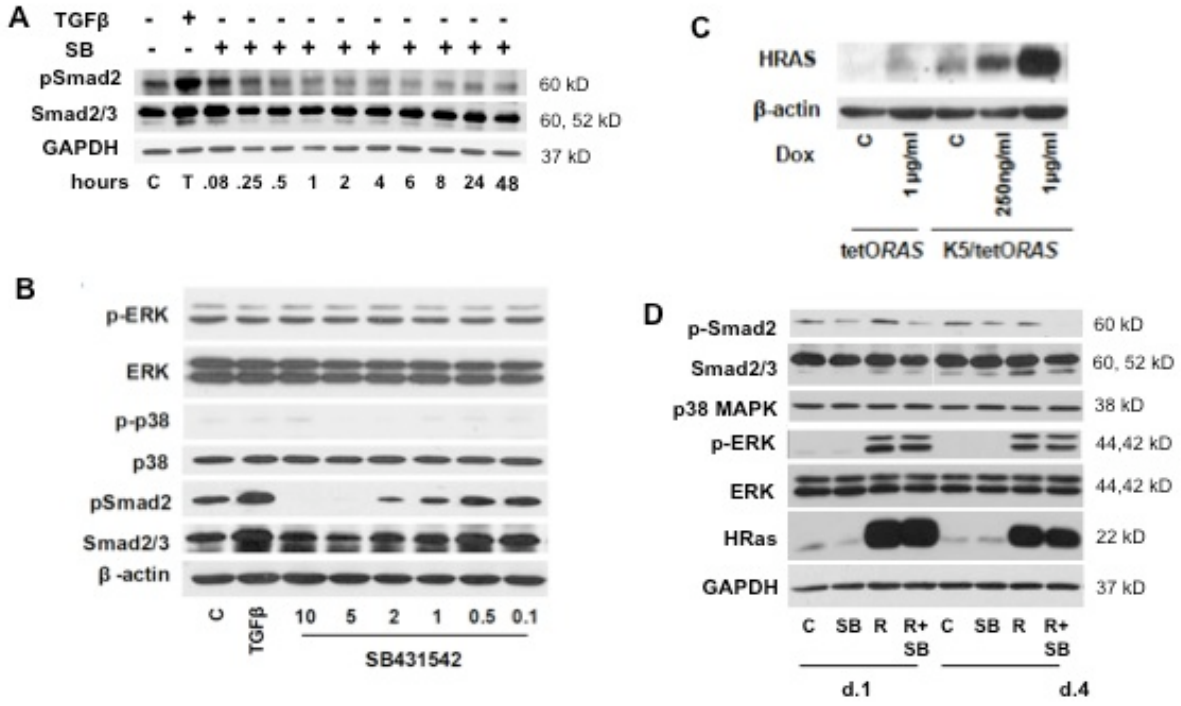


Figure 3-1. SB inhibits Smad-2 phosphorylation *in vitro*.

(A) An initial 24h TGFβ treatment (T) shows an induction of endogenous TGFβ signaling while SB treatment at different time points shows inhibition of baseline TGFβ signaling. SB inhibits Smad2 phosphorylation at 0.5h and maintains this inhibition for 48h. **(B)** SB inhibits phosphorylation of Smad2 over a range of doses and does not affect ERK or p38 activity. **(C)** Keratinocytes isolated from a double transgenic K5rtTA/tetoRASV12G line show a dose dependent expression of HRAS upon treatment with dox. As a control, single transgenic tetoRASV12G keratinocytes show no response to dox. **(D)** Continuous treatment of K5rtTA/tetoRASV12G with SB for 4 days shows that SB inhibits Smad2 phosphorylation in HRAS (R) expressing keratinocytes. HRAS expression (R) does not affect overall Smad2/3 levels, p-Smad2 levels, p38 MAPK levels, or p-p38 MAPK levels (not shown) but increases levels of p-ERK.

3.2 SB431542 increases and TGFβ1 decreases cornification in HRAS expressing keratinocytes.

As TGFβ is a potent growth inhibitor of keratinocytes, we first determined if the ALK5 inhibitor SB affected keratinocyte proliferation. After 5 days of SB treatment, there was no change in cell number in normal keratinocytes, but there was a 50% decrease in cell number of HRAS expressing keratinocytes (Figure 3-2A). Figure 3-2B shows reduced confluence in HRAS + SB keratinocytes as compared to HRAS alone. The decrease in cell number at day 5 of SB treatment occurred after a number of cornified cells were observed to detach from the dish suggesting cell death by cornification was responsible for the decrease in cell number (Figure 3-2C). To quantitate the increase in cornification, a cornified envelope assay was performed to determine the ratio of cornified cells to attached cells at day 5. SB treatment alone did not increase cornification, but SB treatment with HRAS expression increased the ratio of cornified cells to attached cells (Figure 3-2D). Conversely, TGFβ1 treatment resulted in a decrease in cornification in HRAS expressing keratinocytes (Figure 3-2D). Together these results indicate that the TGFβ pathway regulates terminal differentiation in HRas expressing keratinoyctes.

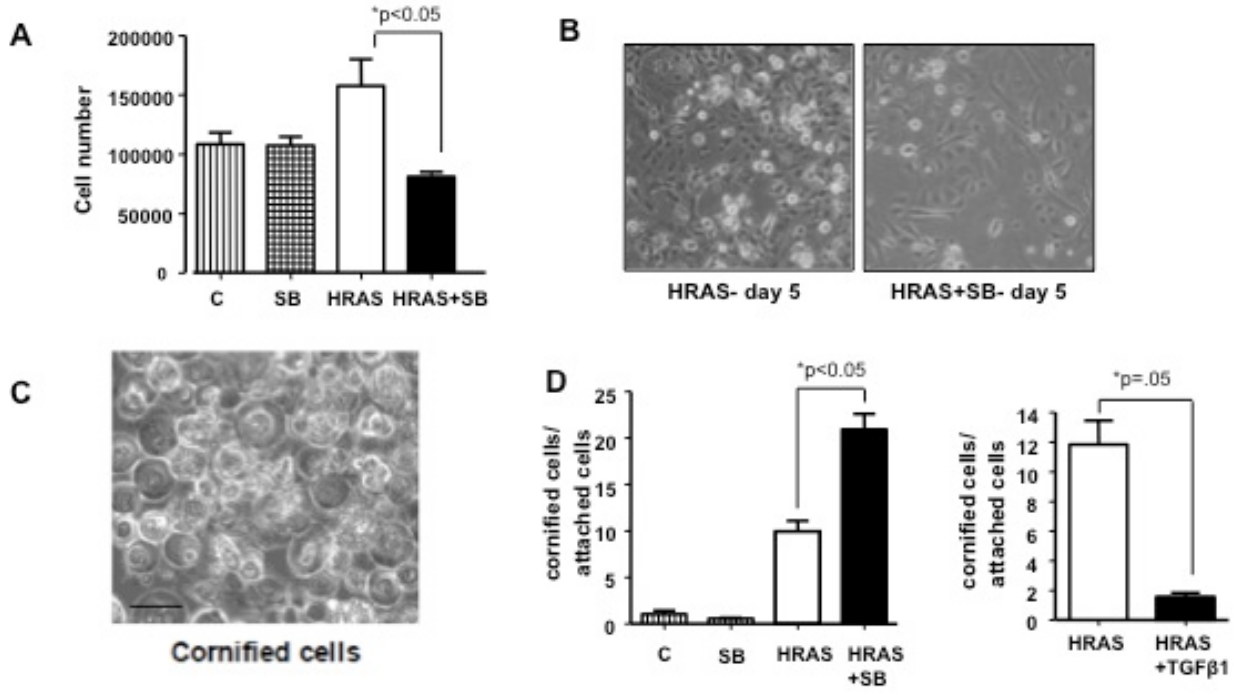


Figure 3-2. SB treatment causes increased cornification of RAS expressing keratinocytes

(A) SB shows no affect on cell number in keratinocytes expressing endogenous RAS levels, while SB treatment in keratinocytes overexpressing *HRAS* results in a 50% decrease in cell number. Experiment repeated. **(B)** Observation of *HRAS* expressing keratinocytes at day 5 reveals reduced confluence with SB treatment. Magnification, 200x. **(C)** Cornified cells observed upon SB treatment in *HRAS* expressing keratinocytes. Magnification, 200x. Scale bar = 50 μm. (**D)** A cornified envelope assay reveals a significant increase in cornified cells upon *HRAS* expression, and a further significant increase upon the addition of SB to *HRAS* expressing keratinocytes. Treatment with TGFβ in *HRAS* expressing keratinocytes shows the opposite effect with a decrease in cornified cells. n=3 and performed 3 times.

3.3 **ALK5 regulates expression of terminal differentiation genes *in vitro.***

After establishing that pharmacological inhibition of ALK5 increases terminal differentiation of HRAS expressing keratinocytes, we investigated if this response is linked to altered expression of genes involved in cornified envelope formation, specifically small proline-rich proteins SPR1A and SPR2H and transglutaminases TGM1 and TGM3. Transcript levels of these terminal differentiation markers was quantified by qPCR in control or HRAS keratinocytes after short-term treatment with either SB or TGFβ1. While no significant change in gene expression was detected from SB treatment of control keratinocytes, SB treatment in HRAS keratinocytes increased TGM3 and SPR2H expression and significantly increased TGM1 and SPR1A and expression. (Figure 3-3A) These results correlated well with the observed increase in cornification with SB treatment of HRAS keratinocytes. Conversely, TGFβ1 treatment reduced expression of TGM1 and TGM3, also correlating with the decreased cornification observed in HRAS keratinocytes with TGFβ1 treatment. (Figure 3-3B) To further investigate the role of ALK5 in terminal differentiation, we used a genetic model in which HRAS inducible keratinocytes were infected with adenoviruses overexpressing a constitutively active ALK5 or a dominant negative type II receptor (DNTβRII). ALK5 overexpression significantly decreased TGM1 expression and DNTβRII overexpression significantly increased TGM1 expression. (Figure 3-3C) Collectively, these results further support the conclusion the TGFβ signaling regulates cornification of HRAS expressing keratinocytes.

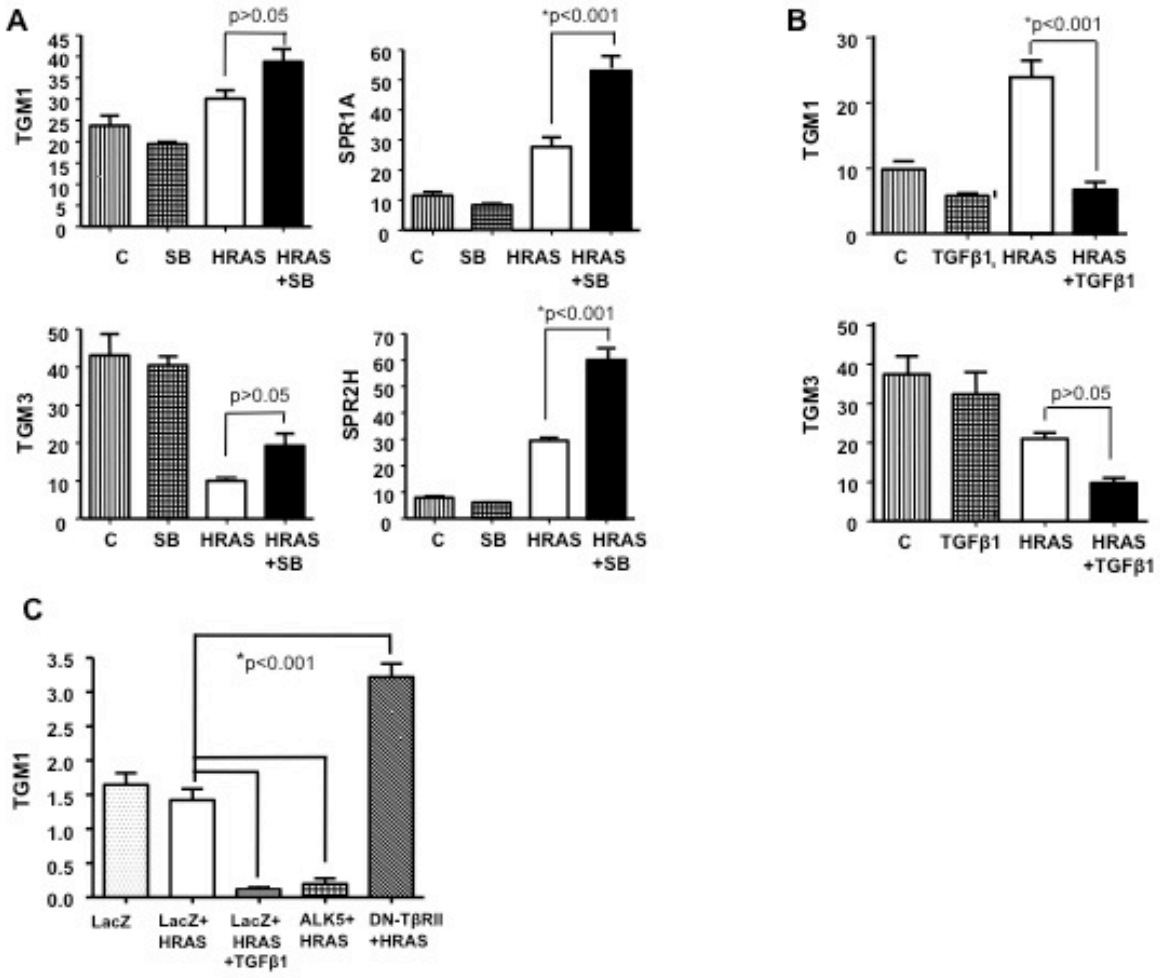


Figure 3-3. ALK5 regulates terminal differentiation markers *in vitro.*

(A) In *HRAS* expressing keratinocytes, SB increases expression of terminal differentiation genes TGM1/3 and significantly increases expression of SPR1A/2H**.** n=4. Experiment repeated. **(B)** TGFβ treatment of *HRAS* expressing keratinocytes significantly decreased expression of TGM1/3**.** n=4. Experiment repeated. **(C)** Adenovirus infection of ALK5 and DN-TβRII in *HRAS* expressing keratinocytes also changes TGM1 expression. Ad-ALK5 significantly decreases TGM1 expression while DN-TβRII significantly increases expression of TGM1. n=3. Experiment repeated.

3.4 SB increases cornification *in vivo*.

To investigate if similar changes in cornification are observed *in vivo*, InvtTA/tetO*RASV12G* mice were used to determine the effects of topical SB treatment. InvtTA/tetO*RASV12G* mice are induced to express HRAS when dox is removed from their drinking water. 7-week-old mice were given water in which nothing was added to induce *HRAS* expression and were then topically treated with 10.0μM SB in 200μl acetone for 5 days. Mice were sacrificed 24 hours following the last SB treatment and histological sections were taken to measure changes in cornification. Tissue sections from SB treated mice expressing HRAS showed a thickened cornified layer as compared to HRAS mice treated with acetone (Figure 3-4A). Measurement of the cornified layer revealed this difference to be almost 2-fold greater in SB treated skin (Figure 3-4B). qPCR reveals increased expression of terminal differentiation genes with significant increase in TGM1 and SPR1A with similar trends in TGM3 and SPR2H. (Figure 3-4C) These results provide an *in vivo* correlate for SB-induced cornification in *HRAS* expressing keratinocytes.

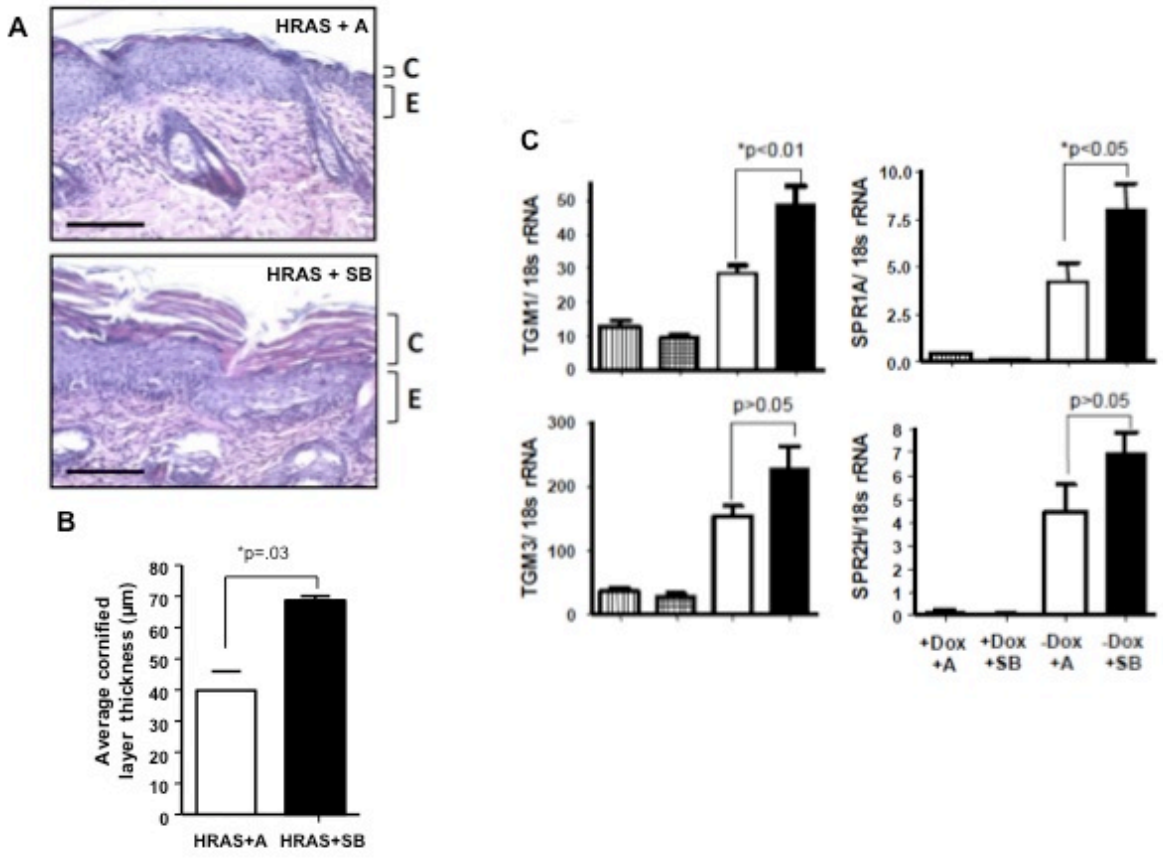


Figure 3-4. SB increases cornification *in vivo*.

(A) InvtTA/tetORAS mice were removed from dox to express *HRAS* and treated with 200 µL of acetone (A) or SB. Representative pictures show that SB treatment increases thickness of cornified layer (C) thickness. Epidermis is denoted by (E). Magnification 200x, Scale bar = 50 µm. **(B)** Changes in cornified thickness measured at magnification 200x, 30 measurements/slide. n=5. **(C)** Changes in mRNA expression is observed in SB treated InvtTA/tetoRAS mice. SB treatment significantly increases expression of TGM1 and SPR1A with similar trends observed in TGM3 and SPR2H.

4. Discussion

TGFβ has been extensively studied in cancer with dual roles as a tumor suppressor and oncogene and consequently, small molecule inhibitors of the TGFβ pathway have been developed for potential use in cancer treatment. While some of these inhibitors have shown promise in inhibiting tumor formation, we have previously shown that treatment with the ALK5 inhibitor SB in a two-stage chemical carcinogenesis assay inhibits papilloma formation but enhances malignant conversion to SCC.[52]

Here we used a model of conditional *HRAS* expression to investigate the effects of ALK5 inhibition *in vitro*. To recapitulate the two-stage chemical carcinogenesis assay in which DMBA causes an activating mutation in *RAS*, we used a conditional model of *HRAS* expression and verified that doxycycline induces *HRAS* expression in a dose-dependent manner. We also verified that SB inhibits baseline and *HRAS*-induced ALK5 phosphorylation of Smad2 and that this inhibition was maintained for the time period between SB treatments (48 hours). Consistent with other studies using ALK5 inhibitors, we showed that SB has chemopreventive effects *in vitro* by induction of cell death by terminal differentiation in preneoplastic keratinocytes. We confirmed that this response is specific to TGFβ signaling as TGFβ treatment of keratinoyctes produced the opposing response of a decrease in cornification. Additionally, to confirm that these are not off-target effects of SB, we reproduced these results in a genetic model using adenoviral expression of DN-TβRII and ALK5. We further showed that the cornification response is associated with regulation of differentiation genes TGMs 1/3 and SPR1A/2H.

A few other studies have previously investigated the effects of TGFβ signaling on terminal differentiation. It has been shown that while TGFβ alone does not influence differentiation in Normal Human Epidermal Keratinocytes (NHEK) cells, it inhibits Interferon-γ induced differentiation.[53] TGFβ has also been shown to inhibit TPA induced differentiation in keratinocytes which is correlated with decreased expression of the late stage differentiation marker TGM1.[54] These results suggest that TGFβ is able to inhibit terminal differentiation in the presence of agents that normally induce terminal differentiation such as TPA and Interferon-γ. We observed induction of keratinocyte differentiation by *HRAS* expression and only observed alterations to terminal differentiation by TGFβ and SB in the presence of *HRAS* expression, indicating that like TPA and Interferon-γ, *HRAS* induction of terminal differentiation may be required to observe the differentiation effects of TGFβ.

It has been shown that Ras expression can induce aberrant terminal differentiation of keratinocytes *in vitro*.[55] The normal course of differentiation in the skin involves an early upregulation of K1 and K10 in the basal to spinous transition. PKC is a key player in the spinous to granular transition responsible for downregulation of K1 and K10 and concurrent upregulation of late stage differentiation genes TGM1/3, loricrin, fillagrin, and SPRs. Ras has been shown to activate PKC-δ1 by tyrosine phosphorylation causing downregulation of K1/10 and late-stage gene upregulation. Activation of PKC by Ras may bypass early-stage program of differentiation by suppression of early stage differentiation and induction of late stage differentiation.

We have shown that ALK5 inhibition enhances the differentiation response to *HRAS* and that this response is also associated with upregulation of late stage differentiation genes TGM1/3 and SPR1A/2H. It is plausible that TGFβ acts on some of the same pathways as Ras

to inhibit late-stage differentiation and that treatment with the ALK5 inhibitor SB may allow for an exaggerated differentiation response by *HRAS*. It has been shown *in vitro* that TGFβ can regulate TGM1 expression induced by TPA treatment and that this response can be reversed by Smad7, an inhibitor of Smad2 and Smad3 activation.[53] This suggests that the response is mediated through Smad-dependent rather than Smad-independent pathways. While the exact mechanism by which TGFB regulates TGM1 expression is not known, the TGM1 promoter contains two AP1 binding sites[56] which could be involved in regulation of TGM1 expression. AP-1 activity is influenced by both Ras/PKC and TGFβ signaling[57,58] which suggests that a balance of positive and negative regulation of AP-1 by Ras and TGFβ could explain alterations in terminal differentiation. A decrease in AP-1 activity by ALK5 inhibition may induce the expression of TGM1 and other terminal differentiation genes. Further work investigating Ras and TGFβ regulation of the promoter region of TGM1 and other late stage differentiation genes could confirm this hypothesis.

Our results showing that SB induces terminal differentiation in *HRAS* expressing keratinocytes may provide a mechanism by which SB can inhibit early papilloma formation by initiating the program of terminal differentiation in cells that would otherwise form papillomas. While other studies investigating the efficacy of ALK5 inhibitors have shown potential chemopreventative effects, ours is the first to demonstrate increased terminal differentiation as a mechanism for reduced tumor formation. It has been previously shown that TGFβ can inhibit differentiation in epidermal keratinocytes induced by TPA and interferon-γ. Here, we have demonstrated a similar pattern in Ras induced differentiation. The regulation of terminal differentiation by an interaction of Ras and TGFβ signaling may

inform future chemical carcinogenesis studies on the role of TGFβ in tumor formation and progression.

While SB's induction of terminal differentiation may highlight a potential chemopreventive strategy, it is unclear whether pharmacological inhibition of ALK5 will be clinically useful. In the two-stage chemical carcinogenesis model study investigating the effects of SB *in vivo,* characterization of papillomas from different treatment groups revealed genetic and categorical differences, with the formation of predominantly low-risk papillomas in the control and the delayed formation of high-risk papillomas with SB treatment.[52] This suggests that SB may suppress the outgrowth of certain initiated keratinocytes that are predisposed to form early, low-risk papillomas and may require ALK5 signaling for tumor outgrowth. While inhibiting these low-risk, ALK5 dependent papillomas, SB may also select for a high-risk phenotype that forms tumors despite ALK5 inhibition.

Interestingly, Markell *et al*. observed that while the first 5 days of SB treatment of HRAS expressing keratinocytes resulted in cornification, at day 11, the dish was repopulated by keratinocytes resistant to SB induced differentiation and growth stimulated by SB. Further characterization of these cells revealed decreased senescence in a β-galactosidase assay and reduced levels of senescence marker p16. However, these cells were growth arrested by elevated calcium (0.05 mM) and failed to form tumors when xenografted onto nude mice indicating they do not possess a malignant phenotype.[59] The re-emergence of this population resistant to senescence and differentiation after SB induced cornification of other keratinocytes in culture provides an interesting correlate to the results from the two-stage chemical carcinogenesis model. These results suggest that a bidirectional response of

keratinocytes to ALK5 inhibition may be responsible for two distinct tumor pathways observed in the chemical carcinogenesis assay.

While SB may induce terminal differentiation of initiated keratinocytes which form low-risk papillomas leading to the overall decrease in papillomas observed, long-term treatment may eventually select for a subpopulation of differentiation and senescence resistant keratinocytes which are more prone to form high risk tumors. Further work to characterize these resistant cells and any role they may have in malignant conversion could provide insight into the mechanism of the dual role of TGFβ in cancer. While SB and more generally ALK5 inhibition could potentially be used in cancer therapy, more work must be done to evaluate the safety and efficacy of such strategies.

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57 Mordasky Markell L, Masiuk KE**,** Blazanin, N. Glick, AB. Pharmacological Inhibition of ALK5 Causes a Cornification Response in Mouse Keratinocytes Expressing Oncogenic HRas. (Accepted to Molecular Cancer Research with revisions)

Curriculum Vitae
Katelyn Masiuk

EDUCATION

The Pennsylvania State University **University Park, PA**
- Bachelor of Science, Biochemistry and Molecular Biology May 2011
- Schreyer Honors College Scholar

RESEARCH EXPERIENCE

Glick Laboratory, Pennsylvania State University **University Park, PA**
Research Assistant & Independent Studies September 2008-May 2011
- Investigate the role of TGFβ type I receptor in regulating the progression of epithelial cancers from a benign to malignant phenotype utilizing transgenic mice

Albrecht-Kossel Institute for Neuroregeneration, University of Rostock, Rostock, Germany
Research Intern Summer 2010
- Assay neural differentiation markers and neuron function to optimize cell culture techniques for growth and differentiation of human neural progenitor cells on a 3D protein scaffold

PUBLICATIONS

Mordasky Markell L, Pérez-Lorenzo R, **Masiuk KE**, Kennett MJ, Glick AB. Use of a TGFbeta type I receptor inhibitor in mouse skin carcinogenesis reveals a dual role for TGFbeta signaling in tumor promotion and progression. Carcinogenesis. 2010;31(12):2127-35.

Mordasky Markell L, **Masiuk KE,** Blazanin, N. Glick, AB. Pharmacological Inhibition of ALK5 Causes a Cornification Response in Mouse Keratinocytes Expressing Oncogenic HRas. (Accepted to Molecular Cancer Research with revisions)

ABSTRACTS

Masiuk, KE, Mordasky Markell, L, Blazanin, N, Glick, AB. (2011) Pharmacological inhibition of the TGFβ type I receptor induces premalignant keratinocyte terminal differentiation. The Toxicologist 104 (1): # 222

Awarded 1st Place Dermal Toxicology Specialty Section Abstract Award

Mordasky Markell, L., Pérez -Lorenzo, R., **Masiuk, KE.,** Glick AB. (2010) Use of a TGFb1 Type I Receptor Inhibitor in Mouse Skin Carcinogenesis Reveals a Dual Role for TGFb1 Signaling in Tumor Promotion and Progression. International Skin Carcinogenesis Conference. State College, PA.

Mordasky Markell, L., Pérez -Lorenzo, R., **Masiuk, KE.,** Kwon, H., Glick AB. (2010) Pharmacological Inhibition of TGFβ1 Signaling Enhances Malignant Progression of Chemically Induced Skin Cancers through Changes in Inflammatory Response. The Toxicologist 103 (1): # 671.

HONORS AND AWARDS

- Student Marshal, Biochemistry and Molecular Biology, Spring 2011 Commencement

- **Grants**
  - Schreyer Honors College Summer Research Grant, Summer 2009, Summer 2010
  - Deutscher Akademischer Austausch Dienst (DAAD) RISE Grant, Summer 2010
  - Eberly College of Science Travel Grant, Spring 2010
  - Schreyer Ambassador Travel Grant, Spring 2010, Summer 2010.

- **Scholarships**
  - Homer F. Braddock Scholarship 2007-2011
  - Schreyer Honors College Academic Excellence Scholarship 2007-2011
  - National Merit Scholar 2007

EMPLOYMENT

PSUKnowHow Tutoring,
University Park, PA
Chemistry Review Session Leader September 2010-January 2011
Chemistry, Biology, Biochemistry Tutor

COMMUNITY INVOLVEMENT

Schreyer Signature Speaker Series **University Park, PA**
Committee Member August 2008–December 2009
- Responsible for organizing a lecture and bringing a speaker to campus that embodies the values of academic excellence, a global perspective, and civic engagement

Schreyer Honors College Orientation **University Park, PA**
Mentor March 2009 - August 2009
- Lead a group of twelve first year honors students in a three-day orientation

Schreyer Honors College Student Council **University Park, PA**
Tour guide and Panelist September 2007- May 2008
- Lead tours and serve on question and answer panels at recruitment events for prospective students and their parents

Penn State Dance Marathon (THON): Springfield THON Organization
University Park, PA
Organization member September 2007- Present
- Provide emotional and financial support for families afflicted by pediatric cancer