PHARMACOLOGICAL INHIBITION OF THE TGF-BETA TYPE I RECEPTOR REVEALS A DUAL ROLE OF TGF-BETA IN SKIN CARCINOGENESIS

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Abstract

Transforming growth factor β1 (TGFβ1) is a member of a large family of regulatory molecules that play both positive and negative roles in epithelial cancer. Pharmacological inhibitors of the Transforming Growth Factor β (TGFβ) type I receptor (ALK5) have shown promise in blocking growth of xenotransplanted cancer cell lines but the effect on a multistage cancer model is not known. In the present studies, the role of the ALK5 inhibitor SB431542 (SB) was investigated in a two-stage skin chemical carcinogenesis assay in order to determine the effect on tumor formation and progression. In this model, nearly all tumors have activating mutations in codon 61 of the HRas gene. We show that topical SB significantly reduced the total number, incidence and size of papillomas compared to 12-O-tetradecanoylphorbol 13-acetate (TPA) promotion alone, and this was linked to increased epidermal apoptosis, decreased proliferation and decreased cutaneous inflammation during promotion. In contrast, the frequency of conversion to squamous cell carcinoma (SCC) was two-fold higher in papillomas treated with SB. While there was no difference in tumor cell proliferation in early premalignant lesions, those that formed after SB treatment exhibited reduced squamous differentiation and an altered inflammatory microenvironment similar to SCC. In an inducible epidermal HRAS transgenic model, treatment with SB enhanced proliferation and cutaneous inflammation in skin, but decreased expression of keratin 1 and increased expression of simple epithelial keratin 18, markers of premalignant progression. In agreement with increased frequency of progression in the multistage model, SB treatment resulted in increased tumor formation with a more malignant phenotype following long-term HRAS induction. To further characterize the altered squamous differentiation associated with reduced tumor formation and increased malignant progression by ALK5 inhibition, an HRAS oncogene-induced model of preneoplastic keratinocytes was utilized. SB significantly enhanced HRAS-induced cornification which correlated with the increased expression of terminal differentiation genes transglutaminase 1 (TGM1) and 3 (TGM3) and small proline-rich protein 1A (SPR1A) and 2H (SPR2H) which cross-link structural proteins that make up the cornified envelope. There was a similar increase in cornified layers and TGM and SPR gene expression following SB treatment of mice expressing inducible epidermal HRAS. Alternatively, treatment of HRAS-expressing keratinocytes with TGFβ1 or overexpression of TGFβ1 and HRAS in an inducible epidermal transgenic model resulted in reduced expression of TGM1 and TGM3 and cornification in vitro. Papilloma (SP1) and squamous cell carcinoma (PAM2.12) cell lines were less responsive to TGFβ1 suppression of markers and marker induction by SB. Interestingly, a subpopulation of HRAS-expressing keratinocytes were resistant to induction of terminal differentiation by ALK5 inhibition. These cells were also resistant to senescence and rapidly immortalized, suggesting that they
represent a progressed phenotype. However, these cells were unable to grow in elevated calcium or produce tumors after grafting nude mice indicating that they were not fully malignant. Thus, selective responsiveness to differentiation may represent a mechanism by which blocked TGFβ signaling can inhibit the outgrowth of preneoplastic lesions but may cause a more progressed phenotype in a separate keratinocyte population. Collectively, these data support the concept that a large number of initiated keratinocytes are dependent on ALK5 signaling for enhanced tumor outgrowth. A second subset can form tumors in part through generation of an altered inflammatory microenvironment and through altered squamous differentiation induced by inhibition of ALK5. This latter group has a higher frequency of malignant conversion. In contrast to the current paradigm for TGFβ1 in carcinogenesis, these results demonstrate that cutaneous TGFβ signaling enables promotion of benign tumors but suppresses premalignant progression through context-dependent regulation of epidermal homeostasis and inflammation.
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Abbreviations

ALK5- activin receptor-like kinase 5, TGFβ type I receptor
BCC- basal cell carcinoma
BrdU- 5-bromo-2-deoxyuridine
CAK- CDK-activating kinase
CDK- cyclin-dependent kinase
CE- cornified envelope
DAB- 3,3’-diaminobenzidine
DAG- diacylglycerol
DMBA- 7,12-dimethylbenzanthracene
Dox- doxycycline
EMT- epithelial to mesenchymal transition
ERK- MAP-kinase
H&E- Hematoxylin and Eosin
HRas- mouse v-Ha-ras Harvey rat sarcoma viral oncogene homolog
HRAS- human v-Ha-ras Harvey rat sarcoma viral oncogene homolog (as expressed by tetORAS transgene)
HRas- mouse v-Ha-ras Harvey rat sarcoma viral oncogene homolog (protein)
HRAS- human v-Ha-ras Harvey rat sarcoma viral oncogene homolog (protein translated from tetORAS transgene)
KRas- mouse v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAP- latency-associated peptide
LTBP- latent TGFβ binding protein
MEK- MAP-kinase-kinase
MPO- myeloperoxidase
NRas- mouse neuroblastoma Ras viral (v-ras) oncogene homolog
PI- propidium iodide
PLC- phospholipase C
PKC- protein kinase C
PI3-kinase- phosphatidylinositol 3-kinase
qPCR- quantitative RT-PCR
Ras- protein subfamily of small GTPases
SA-β-gal- Senescence-associated β-galactosidase
SB- SB431542
SCC- squamous cell carcinoma
SPR1A- small proline rich protein 1A
SPR2H- small proline rich protein 2H
TβRI- TGFβ type I receptor
TβRII- TGFβ type II receptor
TGFβ1- transforming growth factor β1
TGM1- transglutaminase 1
TGM3- transglutaminase 3
TPA- 12-O-tetradecanoylphorbol-13-acetate
TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling
v-RasHA- viral Harvey Ras gene
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Chapter 1

Introduction

1.1 Skin carcinogenesis

1.1.1 Structure and physiology of skin

The skin is the largest organ of the integumentary system and assumes numerous functions. Perhaps the most obvious function of the skin is the protection it provides from ultraviolet (UV) light, chemicals, and thermal and mechanical insults. In the short term, this damage manifests as burns, scabs, and inflammation of the skin. However, a lifetime of this wear-and-tear can be seen through wrinkles, scars and changes in pigmentation. The skin allows mammals to regulate temperature by radiation, convection, conduction, and evaporation or sweating, essential for the normal functioning of the body’s internal organs. The UVB exposure stimulates synthesis of vitamin D$_3$ in the skin which is essential for calcium metabolism and absorption which has a direct effect on bone formation. The skin is a sensory organ for touch, pressure, pain and temperature. Besides function, the skin gives humans a visual identity, appearing in a wide range of colors from brown and nearly black to ivory and nearly white and blanketing the body in a way that gives unique characteristics and form to our species (1).

The skin is composed of three layers: the epidermis, dermis, and subcutaneous tissue (Figure 1-1). The epidermis is composed of keratinocytes, which self-regenerate to form a stratified squamous epithelium. The human epidermis is made up of the basal, spinous, granular, transitional and cornified layers (Figure 1-2). The basal layer is comprised of a single layer of cells that are highly irregular in shape and are bound to the basement membrane by hemidesmosomes. Interfollicular stem cells in the basal layer of the epidermis are the only epidermal cells that have the ability to synthesize DNA and undergo mitosis (2), and it is the differentiation of these cells that leads to upper epidermal layers (3). Melanocytes
are also present in the basal layer of the epidermis and are responsible for producing the pigment melanin. This pigment exists in a variety of colors from yellow, to brown, to black and may provide a protective function against ultraviolet radiation. The spinous layer is composed of polyhedral keratinocytes, contains desmosomes which join adjacent cells and produces fibrillar keratins which provide structural support. At the granular layer cells become progressively flattened but retain organelles and metabolic activity. So named due to the electron-dense keratohyalin granules and lipid-filled lamellar granules, this layer is important in the synthesis of proteins important in late-stage differentiation. In the transition layer that flanks both living and dead epidermis, cellular organelles are destroyed by proteases, DNase, RNase, acidic hydrolases and plasminogen activator. The lamellar granules produced in the granular layer release their lipid content into the extracellular space forming lipid sheets. This provides water-proofing for the skin and is a protective barrier for loss of fluids and ions as well as protection from the environment. Keratin filaments are also restructured in this layer and the cornified envelope, a covalently linked protein structure that forms beneath the plasma membrane, is formed. The cornified layer is composed of the polyhedron-shaped, flattened, dead cells from the transitional layer and is sealed together by lipids forming an impermeable, insoluble barrier. The dead cells of the cornified layer, called corneocytes, are eventually sloughed from the skin (4-6).

Keratins are the major structural protein of the epidermis and make up the cytoskeleton of these epithelial cells. They are intermediate filament (IF) proteins that form α-helical coiled-coil dimers that are assembled into 10nm keratin filaments. The α-helical rod segments have acidic and basic residues spaced four amino acids apart (7). This allows for the formation of ionic salt bridges that stabilize the α-helices (8). The rods also contain repeats of hydrophobic amino acids, which provide a hydrophobic seal and enables coiling of the IF polypeptides (9). Keratins are divided into two distinct classes, called type I and type II based on their isoelectric points and electrophoretic mobilities. Expressed as specific pairs of type I and type II proteins, they form obligatory heteropolymers. Therefore, keratin pairs tend to be similarly expressed in the skin (10).

Basal cells are distinguished by the expression of Keratin 5 (K5) and Keratin 14 (K14) (11) that make up the intracellular cytoskeleton. While the cells of the spinous layer are post-mitotic, they still remain metabolically-active, synthesizing two additional keratins, Keratin 1 (K1) and Keratin 10 (K10). However, expression of K5 and K14 is downregulated in this epidermal layer. Involucrin, a glutamine and lysine-rich protein is also synthesized by this layer and the granular layer, and is deposited on the inner surface of each cell (12). As spinous cells differentiate into granular cells, keratins and envelope proteins stop being produced. Instead, these cells synthesize the proteins filaggrin, a histidine-rich basic protein (13) and loricrin, a glycine-serine-cysteine-rich protein (14). The calcium-dependent enzyme transglutaminase (15) is activated by a calcium influx as the differentiating cells become more permeable.
This catalyzes the formation of ε-(γ-glutamyl) lysine isopeptide bonds, biochemically crosslinking the envelope proteins (12). Small proline-rich proteins (SPRs) are expressed in the granular and spinous layers. They have a high proline content (16) and are crosslinked to other proteins such as loricrin (17) by transglutaminases (18). The expression of transglutaminase and these covalently-crosslinked proteins stabilize keratinocytes into cornified envelopes, a hallmark of cells that have undergone epidermal differentiation (5). Quantification of keratinocyte differentiation can be achieved through boiling keratinocytes in an SDS/DTT solution; Due to the covalent crosslinked proteins, cornified cells are resistant to this treatment and appear as “ghost cells” when counted using a hemocytometer (19). As cells differentiate from the granular to corneum layers, they lose their nuclei, cytoplasm, and metabolic activity leaving the layers of keratin that look like flat flakes or sheets behind (20).

A calcium gradient exists in the epidermis in vivo, with low concentrations in the basal layer and higher concentrations in the suprabasal layer (Figure 1-2). Studies performed in vitro have demonstrated that the calcium concentration plays an important role in regulating keratinocyte differentiation. Maintaining keratinocytes in culture in low-calcium containing medium (0.05mM) results in cells that resemble those in the basal state, expressing keratins 5 and 14, while expressing a low level of differentiation markers such as keratins 1 and 10, loricrin, filaggrin, and involucrin. These cells continue to proliferate and remain undifferentiated. Keratinocytes can be induced to irreversibly withdraw from the cell cycle and differentiate by increasing the extracellular calcium concentration to 1.2mM by adding calcium to the media (21-23). The mechanism for calcium-induced differentiation comes from changes in phospholipase C (PLC) signaling (24, 25). While PLCγ1, -γ2 and –β3 can be detected in the basal layer, differentiating keratinocytes express PLCδ1 (24). As PLC expression increases with the calcium concentration, diacylglycerol (DAG) increases, which leads to activation of protein kinase C (PKC), a family of serine/threonine protein kinases, and key regulators of terminal differentiation and keratinocyte
maturation (25-28). There are five PKC isoforms expressed in the epidermis: -α, -δ, -ε, -ζ and -η. PKCα is activated by calcium and DAG, PKCδ and PKC-ε are calcium independent but also activated by DAG, and PKC-ζ is activated by neither (29). Activation of PKC signaling enhances the expression of late terminal differentiation markers including loricrin, filaggrin and transglutaminase, and suppresses keratins 1 and 10 (30). Furthermore, keratinocyte differentiation can be blocked by PKC inhibitors (30, 31). PKCα in particular is localized to the spinous layer (32) and has been shown to trigger terminal differentiation, specifically inducing expression of involucrin and transglutaminase (32, 33). This response is linked to a loss of cell matrix adhesion as basal cells become suprabasal (32). However, overexpression of PCKδ and PKCη rather than PKCα have been shown to be responsible for keratinocyte differentiation (34, 35).

Retinoids (and vitamin A-related compounds) are also regulators of keratinocyte differentiation, but in contrast to calcium, they suppress differentiation (Figure 1-2) (36, 37). The predominant receptors in keratinocytes both in vitro and in vivo are the retinoic acid receptor γ (RARγ) and the retinoid X receptor α (RXRα). These receptors exist as heterodimers (38), bind retinoic acids, and activate gene expression by interacting with cis-acting DNA response elements (39) or by interacting with other transcription factors (40, 41). In vitro, mutation of RARγ and RXRα blocked terminal differentiation and resulted in stratified layers of basal-like cells (42, 43) and this has also been shown in vivo using K14- (44) and K1- (45) driven expression of RARα. In vivo, acute retinoic acid treatment caused thickening of the epidermis and granular layer, and an increase in expression of transglutaminase, involucrin, and filaggrin, while the expression of loricrin is reduced. However, expression of keratins 1, 10 and 14 remain unchanged (46).

The dermis is composed of fibroblasts which synthesize collagen fiber bundles, elastin fiber strands, and matrix. It is made up of the papillary dermis that is adjacent to the epidermis and the deeper reticular dermis that borders adipose tissue. While the epidermis does not have a vascular supply or innervation, the dermis does and contains arterioles, capillary loops, venules, lymphatics and nerve endings. The subcutis or subcutaneous tissue is mainly composed of adipose tissue, hair follicles, and skeletal muscle, and eccrine glands may extend into this layer. The hair follicle is similar to skin in that it is composed of five epithelial cell layers that undergo keratinization. The primary function of eccrine sweat glands and ducts is to produce sweat. Evaporation of sweat from the skin is a means of lowering the body temperature as well as excretion of sodium and chloride ions, urea, and small molecular weight metabolites (6).

Langerhans cells (dendritic cells), the antigen-recognition and processing cells, are located in all layers of the epidermis. They express lymphocyte and macrophage surface markers, use their dendritic cytoplasmic processes to monitor the microenvironment of the skin, and present antigens to mount an immune response. The dermis is also home to a number of immune cells. Dermal dendritic cells, mast
cells, and a small number of cutaneous lymphocyte antigen (CLA)-positive memory T cells can all be found in the dermis. These immune cells aid in the skin’s defense against non-self and other injury (47).

1.1.2 Skin cancer

Cancer is a class of diseases that cause abnormal cell growth and tumor formation. Skin cancer can be caused by exposure to environmental/industrial toxicants, viruses, and ultraviolet (UV) light, genetics and lifestyle choices such as smoking, drinking and diet. However, 90% of non-melanoma skin cancers are associated with exposure to UV light (48). These exposures can cause damage to DNA which leads to the propagation of cells that have abnormal functions. The tumors that result from DNA damage can be either benign or malignant. Benign tumors grow locally without invading adjacent tissues, and are almost never life-threatening. Malignant tumors invade adjacent tissues and cause metastases, which are secondary tumors that form when cancer cells migrate to distant sites (49).

The World Health Organization finds that 30% of all human cancers are cancer of the skin, and one in five Americans will develop skin cancer in their lifetime (50). More than 2 million people are diagnosed with skin cancer annually in the United States (51). Furthermore, increased cancer mortality has been linked to a history of non-melanoma skin cancer (52). Non-melanoma skin cancers include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and are named based on the type and location of the affected cells. The more common BCC is a slow growing cancer that tends to appear in sun-exposed sites. It is the most common form of skin cancer and an estimated 2.8 million BCCs are diagnosed annually in the US (53). SCCs appear in similar places to BCC, but may develop in scars or skin ulcers in non-exposed regions. SCCs are a more aggressive cancer type, more likely to invade underlying fatty tissue and spread to lymph nodes. They are the second most common form of skin cancer with about 700,000 cases diagnosed each year in the US (53), resulting in 2,500 deaths in 2009 (54). In 2004, the total direct cost associated with the treatment of non-melanoma skin cancer was more than $1 billion (55), further highlighting the importance of preventing this disease.

Melanomas on the other hand originate from melanocytes and can occur anywhere on the skin, although they are most common on the trunk of the body in men and on legs in women. This cancer is much less common than BCC and SCC, but it is far more dangerous as it is more likely to spread before being identified (56). Melanoma accounts for only 3% of diagnosed skin cancers, but resulted in more than 75% of skin cancer-related deaths in 2002 (57). Keratoacanthomas are a less common skin cancer that closely resembles a well-differentiated SCC. However, these cancers often resolve spontaneously. Other less common cancers that account for less than 1% of non-melanoma skin cancers include Merkel cell carcinoma, Kaposi sarcoma, cutaneous lymphoma, skin adnexal tumors, and various types of sarcomas.
1.1.3 Chemically-induced skin carcinogenesis

About 90% of cancers in humans occur in epithelial cells. This includes breast, prostate, lung, kidney and skin cancers. Not only is the skin a highly accessible tissue, but studies in skin cancer have much relevance to these other cancers in epithelial tissues by elucidating fundamental cancer mechanisms and central processes involved in cancer progression. The two-stage skin carcinogenesis model is a highly developed, researched and reliable in vivo model that mimics the multistage nature of epithelial cancer progression in humans. This model can demonstrate the phenotypic changes of normal keratinocytes as they progress from a squamous papilloma to a squamous carcinoma. This system is composed of 3 different stages, initiation, promotion, and malignant conversion (58-60).

Initiation in this model system represents a single genotoxic exposure or multiple exposures during one’s lifetime. In the chemical carcinogenesis assay, initiation is achieved by topically treating mice with a subcarcinogenic dose of a carcinogen, such as the polycyclic aromatic hydrocarbon DMBA (7,12-dimethylbenzathracene). DMBA requires bioactivation into highly reactive and electrophilic diol epoxide metabolites (61). This causes irreversible DNA damage that will be retained if not repaired in the proliferating keratinocytes located in either the basal layer or the bulge region of the hair follicle that contains skin “stem cells” (62, 63), producing an activating mutation in codon 61 of the HRas gene (64, 65). There is strong evidence that epithelial stem cells, which are located in the bulge region of the hair follicle and the basal layer of the epidermis (interfollicular stem cells), may contribute to chemically-induced skin cancer (66). This is demonstrated by the fact that this population of keratinocytes undergo mitosis and remain in the basal layer (67) and can retain carcinogen-DNA adducts (68). DMBA primarily causes an A to T transversion mutation. Other initiating agents can be used including benzo[a]pyrene, N-methyl-N-Nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU), cisplatinum, and β-propolactone, all of which cause activating Ras mutations (69). Mutations in the Ras gene can be detected 3-4 weeks following initiation.

One of the primary biological effects of initiation of epidermal cell is alterations in their response to differentiation signals. In normal keratinocytes in vitro, calcium elevation from 0.05mM to 1.4mM causes the proliferation of basal cells to cease and induces terminal differentiation and sloughing of squamous cells off of tissue culture dishes. However, initiation of keratinocytes with a chemical carcinogen alters this response. DMBA-treated keratinocytes that are switched from low to elevated calcium are resistant to calcium-induced terminal differentiation and form more colonies (70, 71) as do mouse epidermal keratinocytes exposed to other carcinogens (72). It has also been shown that keratinocytes infected with a Ras-oncogene expressing retrovirus are resistant to calcium or tumor promoter-induced terminal differentiation (73-76). This is accompanied by a decrease in keratins 1/10 and transglutaminase and an increase in loricrin and filaggrin (30). While Ras expression causes expression of
late terminal differentiation markers, differentiation is blocked, and this response is reversible as keratinocytes resume a basal phenotype when media calcium concentration is reduced (74). It appears that this *in vitro* observation is analogous to initiation *in vivo* that allows for the continued proliferation of a subpopulation of cells that are resistant to terminal differentiation signals. Cell lines generated from SCCs are also resistant to terminal differentiation, demonstrating that blocking terminal differentiation is a critical pathway for malignant conversion (77).

In humans, expansion of cells that carry the initiating DNA mutation is required for tumor formation. This may occur due to inflammation, hormones, growth factors, wound healing and exposure to UV light. The mouse carcinogenesis assay requires repeated exposure to a tumor promoting chemical to recreate this response. About two weeks following initiation, mice are treated with a tumor promoter such as 12-0-tetradecanoylphorbol-13-acetate (TPA) for approximately 25 weeks in order to elicit the formation of clonal outgrowths of the initiated cells, called benign papillomas. Other promoting agents include telocidin, okadaic acid, chrysarobin, benzoyl peroxide, ultraviolet irradiation, and wounding (69). The mutated keratinocyte population clonally expands due to a growth advantage over neighboring cells. *In vitro* analysis of tumor promoter treated keratinocytes show it is likely that differences in tumor formation are related to changes in cell selection that occurs during the promotion process. Both proliferative (78) and terminal differentiation (78-80) changes occur in the basal cells at the same time, although these processes are mutually exclusive, suggesting that heterogeneity may contribute to the selection process. In response to TPA treatment, some basal cells are induced to terminally differentiate, while others are stimulated to proliferate and resist differentiation, even following repeated exposures to TPA (81-83). The increase in proliferation leads to an increase in epidermal hyperplasia. This is accompanied by an increase in DNA synthesis, oxidative stress and inflammation, altered gene expression and blocked or delayed terminal differentiation (84). However, without additional promoter treatments, all of these responses would subside and the epidermis will regain a normal appearance within 2-3 weeks (85). A large number of biochemical changes occur following treatment with TPA. Some changes are very rapid, including changes in cyclic nucleotides and prostaglandin synthesis. RNA and protein synthesis, changes in transglutaminase and histidine activity, histone phosphorylation and phospholipid synthesis may take hours to be altered. However, DNA and histidine rich protein synthesis may not occur until up to 24 hours (69, 83). Interestingly, keratinocytes treated with TPA respond similarly to *Ras* overexpression following increased media calcium. A decrease in keratin 1/10 expression and increase in loricrin and filaggrin occurs with TPA treatment, although unlike *Ras* expression, TPA causes terminal differentiation when calcium concentration is increased (30).

The molecular mechanisms behind initiation and promotion must be discussed collectively. While DMBA treatment causes an “activating” mutation, this is not a strong enough stimulus to form tumors,
and when the skin is treated with tumor promoters alone, keratinocyte proliferation and hyperplasia are induced, but tumor formation will not occur. However, when keratinocytes bearing activating mutations are treated with tumor promoters, these mutations are then amplified by clonal expansion and this leads to tumor formation. The effects of mutating Ras are then amplified through the keratinocyte expansion caused by TPA treatment. Therefore, the mechanisms of tumor formation by initiation and promotion are not mutually exclusive. Treatment with DMBA that results in a HRas gene mutation causes a variety of downstream signals. Protein kinase C (PKC) activators are among the most widely recognized targets of Ras activation. As discussed above, phospholipase C (PLC) which catalyzes the formation of second messenger diacylglycerol (DAG), activates PKCα which has been identified as the specific isozyme involved in blocking terminal differentiation (86, 87). This is accompanied by tyrosine phosphorylation of PKCδ, although phosphorylation was not found when keratinocytes were treated with TPA (88). PKC signaling is important in both differentiation and tumor promotion, indicating that alterations to signaling will most likely impact both biological endpoints (89, 90). For example, mice that overexpress PKCδ are resistant to tumor formation (91) and mice lacking PKCα are more susceptible to tumor formation using the skin chemical carcinogenesis protocol. No effect has been observed in mice that overexpress PKCα in response to DMBA/TPA treatment, although TPA treatment alone caused an enhanced inflammatory response involving neutrophil infiltration (92). EGFR signaling is also important, as blocking this pathway may regulate cell cycle progression, terminal differentiation and migration which impairs growth of papillomas (93, 94).

There are a number of protein markers that have distinctive expression patterns during promotion and progression of skin tumors. Keratin 1/10 expression in the suprabasal layer of the epidermis continues during tumor promotion and papilloma formation. However, early loss of keratin 1/10 expression is characteristic of the gene expression patterns associated with SCCs and malignant conversion. Expression of keratin 13 (K13) is not present in normal or hyperplastic epidermis. However, K13 is found in papillomas, and early aberrant expression of K13 is characteristic of progressing papillomas (95, 96). Keratin 8/18 (K8/K18) are expressed in simple epithelial tissue and are not characteristic of adult stratified squamous epithelium or papillomas. Yet, keratin 8 has been noted in anaplastic areas of SCCs and has been documented as a marker for late stages of tumor progression (97, 98). Expression of loricrin is not detected in normal epidermis, although it can be detected in hyperplastic epidermis and papillomas. Expression is decreased in SCCs or more advanced carcinomas (84).

Following 25 weeks of promotion, papillomas can remain unchanged, regress, or progress toward malignancy. These papillomas would progress toward malignancy without further exogenous exposures, although the rate of progression would be very slow and most tumors would not undergo malignant
conversion during the normal lifespan of the animal. However, papillomas are capable of malignant conversion through genetic and epigenetic contributions to progression. Indeed, the papillomas undergo a pre-malignant progression that is characterized by major genetic abnormalities, aneuploidy, loss of heterozygosity, and dysplasia, and malignant conversion leads to the production of squamous cell carcinomas which are able to invade through the basement membrane, and migrate into the underlying stroma (99, 100). SCCs are highly vascularized lesions that invade downward and may be accompanied by metastases to organs such as the lung or lymph nodes. Rarely, SCCs progress to spindle cell carcinoma which is marked by fusiform development of rapidly proliferating cells and is poorly differentiated.

As described above, an activating Ras mutation has a very low frequency of conversion (about 5%) when acting alone. However, a variety of oncogenes have been identified as contributors to malignant conversion in vivo through grafting of cells onto nude mice. Two squamous papilloma cell lines have been very useful in this pursuit. The SP1 cell line was developed from papillomas in SENCAR mice initiated with DMBA and promoted with TPA (101). The 308 cell line was developed from DMBA-initiated adult Balb/c epidermis (102). Both of these cell lines have an activating HRas mutation which correlates with the in vivo initiation step of the multi-stage skin carcinogenesis model. When these HRas defective cells were also infected with the oncogene v-fos, papillomas converted to carcinomas (103). The mutated HRas oncogene, which is heterozygous in papillomas, is frequently homozygous in carcinomas. When v-RasHA is introduced into these cells, carcinomas form, suggesting that gene dosage is important in determining the neoplastic phenotype (104-106). Additional experiments were performed in p117 cells which were also derived from skin chemically induced to form papillomas. Retroviral vectors of p53(mut) and HER2/neu (ErbB-2) formed a different phenotype when grafted than that of Ras homozygosity; a less aggressive carcinoma that was not as invasive but still had highly dysplastic features that are similar to those of in situ carcinomas (107). Infection of c-myc (103), TGFα (108), v-jun and β-actin (99) did not cause malignant conversion in similar experiments. These experiments are summarized in Figure 1-3 and have been able to demonstrate which signaling pathways are associated with malignant progression and support a genetic basis for conversion (adapted from (99, 100)).
Not all papillomas are created equally. In fact, they can be grouped into two classes termed “high risk” and “low risk”. When skin is initiated and promoted, the majority of the papillomas that form do not progress to SCCs. These “low risk” papillomas are terminally benign. While “high risk” papillomas appear to be benign, they possess defining characteristics which correlate with a significantly higher frequency and rate of malignant conversion to SCC. Some of these characteristics are summarized in Table 1-1 (109, 110). The two-stage chemical carcinogenesis assay has been used to generate these data. Importantly, these characteristics are apparent very early on during tumor formation, at about 8-11 weeks following the commencement of TPA treatment.

Table 1-1: Markers distinguishing high risk from low risk papilloma at first clinical sign of tumor formation

<table>
<thead>
<tr>
<th>Property</th>
<th>Low-risk</th>
<th>High-risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Benign</td>
<td>Benign</td>
</tr>
<tr>
<td>Sensitivity to strong promoter</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Persistence</td>
<td>TPA dependent</td>
<td>TPA independent</td>
</tr>
<tr>
<td>Keratin expression</td>
<td>K1+, K13-</td>
<td>K1-, K13+</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Increased, basal</td>
<td>Increased, basal and suprabasal</td>
</tr>
<tr>
<td>TGFβ1 protein</td>
<td>Basal layer, strong</td>
<td>Basal layer, weak or lost</td>
</tr>
</tbody>
</table>

Tumor formation and progression is dependent on the strain of mouse used for the chemical carcinogenesis study. However, different stocks and strains of mice metabolize initiating agents similarly, and the formation and removal of covalent DNA adducts during initiation does not appear to differ between strains. Therefore, it is likely that the type and dose of promoting agent and the variation in the effects caused by treatment are responsible for changes in tumor formation. At the same dose of initiating...
and promoting agent, FVB/n mice form about 1-10 papillomas per mouse while BALB/c mice form 4.4.
BALB/c or SSIn mice are resistant to malignant conversion and the frequency of progression from papilloma to SCC is only about 1-10%. On the other hand, FVB/n mice can develop a much greater tumor burden and have up to 50% conversion of papillomas (84).

The types of questions that can be answered using the two stage chemical carcinogenesis assay are practically endless. This model can be used with genetically modified mice in order to determine the role of a particular gene in each stage of cancer development. Specific compartments such as the epidermis or dermis can be targeted with these genetic modifications. Chemical inhibitors can identify the role of a specific pathway during carcinogenesis. Therapeutic agents can be evaluated for efficacy and/or risks. However, this model does have some drawbacks. There is no direct equivalent for papillomas in humans. BCC and SCC in humans are more similar to the carcinomas in mice that develop following malignant conversion of papillomas. As explained earlier, the primary gene target for mutation by the initiating agent is HRas. While this mutation is present in human skin cancers, P53 is much more important target for gene mutations in non-melanoma skin cancers. However, HRas mutations are prevalent in other epithelial cancers including lung, colon, and pancreas. Furthermore, incidences of metastasis are very low for a number of mouse models, including the two-stage chemical carcinogenesis assay, inhibiting investigating of this stage of cancer progression (84).

1.1.4 The Ras Oncogene

The multi-stage skin cancer assay is dependent on the mutation of HRas (64) to mimic characteristics of human cancers. This gene was first identified in 1964 in a murine leukemia virus, named the Harvey-MSV retrovirus, that caused sarcomas in newborn mice (111). Other retroviruses were shown to carry the gene, including the Kirsten-MSV retrovirus (Ki-MSV) discovered in 1967 (112), the BALB-MSV retrovirus in 1974 (113) and the Rasheed strain of rat sarcoma virus in 1978 (114). In 1982, Der et. al. reported that DNA isolated from bladder and lung carcinoma had sequences homologous to the Ras oncogene (115). The identification of these Ras genes initiated the identification of homologs in human cancers. Following HRas and KRas, the third proto-oncogene homolog NRas was first identified in human neuroblastoma in 1983 (49). While point mutations can cause Ras overexpression, Ras gene amplification and activation of wild-type Ras protein by upstream activators can also cause aberrant signaling. Ras is activated in more than 15% of human tumors, with the highest frequency found in salivary gland, urinary tract and cervix. In addition to the mutation at codon Q61 following DMBA treatment, activating somatic mutations have also been found at G12, G13 and A59 (116). KRas mutations predominate in pancreas, biliary tract, large intestine, small intestine, and lung. NRas mutations are found with highest frequency in skin, nervous system, and hematopoietic and lymphoid tissue (117).
It is well documented that when an HRas mutation is introduced into normal mouse keratinocytes, papillomas form when these cells are grafted onto nude mice (64). However a Ras mutation in human keratinocytes is not enough to cause tumor formation in vivo, suggesting that other changes are required in order to transform human keratinocytes (118).

RAS proteins belong to a larger family of small GTP binding proteins, which are also called RAS-like GTPases or G proteins (guanine nucleotide-binding proteins). Like other G-proteins, RAS binds to GTP when active and GDP when inactive (Figure 1-4). Inactivation requires the help of specialized proteins called GTPase activating proteins or GAPS that accelerate the hydrolysis of GTP to GDP. Neurofibromin (NF1) and p120GAP are members of the GAP family. RAS activation is triggered by a guanine nucleotide exchange factor or GEF, which stimulates the release of GDP and catalyzes the replacement of GTP. There are three main classes of RAS GEFs that have a common CDC25 homology catalytic domain and an N-terminal RAS exchange motif. These include SOS (son of sevenless), RAS-GRF (RAS-Guanine Nucleotide Release Factors) and RAS-GRP (RAS guanine nucleotide releasing proteins). When the HRas gene is mutated at the three predominant residues G12, G13 and Q61, the intrinsic and GAP catalyzed hydrolysis of GTP is inhibited and RAS is maintained in a permanently active state (116, 119).

RAS proteins have almost identical structures and function similarly. Post-translational modification of RAS proteins includes: Prenylation, which involves enzymatic attachment of a farnesyl group to the cysteine of C-terminal CAXX and attachment of RAS to inner wall of cell membrane; proteolysis, where the precursor RAS-CAXX loses 3 C-terminal amino acids; methylation of the carboxyl of terminal cysteine; and palmitoylation, which is the covalent attachment of fatty acids to cysteine residues. It is the C-terminus where the RAS protein carries the covalently attached lipid tail composed of farnesyl, palmitoyl, or geranylgeranyl groups that enable these proteins to become anchored to the plasma membrane (120).
RAS plays a role in a variety of cellular processes including cell growth, survival, cell migration, differentiation and death. It is foreseeable that this can happen based on the number of signaling pathways that activated RAS triggers through effector pathways (Figure 1-5). RAS effectors are defined as proteins with a strong affinity to activated RAS and have a putative RAS binding domain. These proteins include: RAF, PI3K, TIAM, PKCζ, and AF6. Activated receptor tyrosine kinases are responsible for recruiting RAS-GEFs, which stimulates the activation of RAS. Because the activation of RAS is relatively short-lived, these signals must be converted into longer-lasting ones. The signals are sustained by altering downstream gene expression through a serine/threonine kinase phosphorylation cascade. RAS binds to and activates Raf (serine/threonine kinase) and promotes its association with the plasma membrane. The first kinase activated by RAS in the three component cascade is Raf (MAP-kinase-kinase-kinase). Once activated, it phosphorylates and activates MEK (MAP-kinase-kinase). MEK, a dual specificity kinase is capable of phosphorylating and activating ERK (MAP-kinase). Activated ERK has both cytosolic and nuclear targets and ultimately leads to regulation of key G1 phase cell cycle proteins such as Cyclin D. The phosphatidylinositol 3-kinase (PI 3-kinase) pathway that is responsible for cell survival and growth is also a key signaling pathway activated by RAS (117, 120). Activated PI3-kinase phosphorylates 3 inositol phospholipids to produce PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃ from PI, PI(4)P and PI(4,5)P₂, respectively. When PI(4,5)P₂ generates PI(3,4,5)P₃, this causes the phosphorylation and activation of PLC-γ, which cleaves PI(4,5)P₂ to generate IP₃ and diacylglycerol (DAG). IP₃ releases calcium from the endoplasmic reticulum and DAG activates PKC which causes cell growth. The PI3-kinase pathway that stimulates cell survival is regulated by protein kinase B or AKT. PI3-kinase binds to PI(3,4,5)P₃ which recruits phosphatidylinositol-dependent protein kinase (PDK1) and activates AKT. AKT promotes cell survival by inhibiting cell death activators and sometimes inhibiting transcription of genes that encode them (121).

**Figure 1-5:**
Ras effector pathways

Downstream activation by Ras occurs through a number of pathways including PI3K, Raf and PLCγ. This causes activation of many target genes that affect many biological endpoints. Therefore, it is understandable that deregulation of this gene would profoundly affect cell homeostasis.
1.2 TGFβ signaling

1.2.1 An overview of the TGFβ signaling pathway

The transforming growth factor (TGF) family is comprised of numerous structurally-related polypeptide growth factors. TGFs were first isolated from sarcoma-virus transformed 3T3 cells. When non-neoplastic normal rat kidney (NRK) cells were exposed to these TGFs, a change to a neoplastic (transformed) phenotype was observed and caused large colony formation in soft agar (122, 123). By high pressure liquid chromatography, two subsets TGFα and TGFβ were further purified and separated (124, 125). Colony formation in soft agar was dependent on TGFβ, but TGFα or epidermal growth factor (EGF) were required for this response (126). The bifunctional role of TGFβ became apparent early as this growth factor was identified as a potent inhibitor of anchorage-dependent fibroblast and human tumor cell growth and anchorage-independent human melanoma, lung carcinoma and breast carcinoma cell lines growth, but stimulated colony formation in the presence of other growth factors such as platelet-derived growth factor (PDGF) and EGF (127).

The founding member of this family, transforming growth factor β1 (TGFβ1) is a cytokine. Cytokines are small proteins that convey signals between cells acting as intercellular mediators and are classically involved in regulation of the immune system. Transforming growth factor β1 signaling controls cell proliferation, differentiation and apoptosis, and is involved in multiple biological processes, including development, inflammation and carcinogenesis. Following the identification of TGFβ1, an array of other growth factors that share structural homology were included in this superfamily under the subfamilies activin, growth and differentiation factor (GDF), and bone morphogenetic protein (BMP). Intermediate members include Nodal and Dorsalin, and distant members that have less homology include Müllerian inhibiting substance/ anti-Müllerian hormone (MIS/AMH) and Inhibin α (128).

Three mammalian TGFβ isoforms have been identified that share common receptors (129). TGFβ1, 2, and 3 are localized to three different human chromosomes 19q13, 1q41 and 14q24, respectively. While the amino acid sequences are similar, the active TGFβ cytokines have differences in the mature bioactive peptide region, as well as the latency-associated peptide. This may lead to variations in biological function (130). The TGFβ1 isoform in particular has been implicated in injury response, immune function, and in diseases including carcinogenesis. For this reason, our studies focus on the TGFβ1 ligand. The promoter of the TGFβ1 gene contains many regulatory sites that are activated by immediate early genes including c-Jun, c-Fos, and egr-1. These sites are also activated by oncogenes including abl, fos, jun, ras and src (131, 132) as well as virally transactivating proteins including HTVL1 tax, HBV-x protein (133), and CMV IE2 protein (134). Unlike the TGFβ2 and TGFβ3 isoforms, TGFβ1 does not have a classic TATAA box. Features of the promoter region give insight into the role of TGFβ1 during biological processes including repair of injury, in response to stress and virally mediated diseases,
and carcinogenesis. On the other hand, TATAA box containing TGFβ2 and TGFβ3 promoters have a common proximal cyclic AMP response element (CRE)-activating transcription factor (ATF) site that suggests hormonal and developmental control (135, 136). Furthermore, these observations of promoter content are supported by studies in mice in which TGFβ1, TGFβ2 and TGFβ3 are deleted. TGFβ1 (-/-) mice die at about 3 weeks of age from inflammation and autoimmune disease (137), TGFβ2 null mice displayed a wide range of developmental defects in the heart, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital tract (138), and TGFβ3 mice die perinatally from developmental defects in the lung and palate (139, 140).

The active form of TGFβ (25 kDa) exists as a dimer held together by hydrophobic interactions and an intersubunit disulfide bond (141). TGFβs, like other members of its family, are synthesized as a latent precursor protein that are 390-412 amino acids in size (142). The latent protein consists of an N-terminal pro-domain or latency-associated peptide (LAP) and C-terminal mature TGFβ protein (143, 144). Latent TGFβ can be activated in vitro by acid, alkali, heat, limited proteolysis, or incubation by glycosidases (128, 145). Activation in vivo is regulated by proteases including metalloproteases (146, 147) and the interaction of integrins with the proteases thrombin and MMP4 (148). However, digestion via the endopeptidase furin appears to be the most prevalent mechanism (149-151). The C-terminal domain is cleaved at a proteolytic site between amino acids 278 and 279 following N-glycosylation. Following cleavage of the LAP, TGFβ is activated and secreted from the cell (143, 144). However, most cells types secrete active TGFβ with the LAP that remains non-covalently bound to TGFβ. LAP renders TGFβ in a biologically inert form which is unable to bind betaglycan or signaling receptors (152). A large secretory glycoprotein called latent TGFβ-binding protein (LTBP) is disulfide linked to LAP and while not required for latency, this protein is implicated in the secretion, storage in the extracellular matrix, and activation of the TGFβ complex. The complex of LAP, mature protein, and LTBP are referred to as large latent TGFβ, while small latent TGFβ (65-75 kDa dimer) does not have bound LTBP (summarized in Figure 1-6) (153).
TGFβ transduces its signaling by binding to receptors with serine/threonine kinase activity. There are two families of TGFβ receptors based on structure and functional properties. While there are six different type II receptors in the TGFβ superfamily, only the TGFβ type II receptor (TβR-II) binds to TGFβ isoforms, with a higher affinity for TGFβ1 and -3 than TGFβ2 (154). The type I receptors have a higher level of sequence similarity, particularly in the kinase domain than the type II receptors. The type I receptors were historically named with neutral nomenclature as ALKs (activin receptor-like kinase). These receptors have also been named based on their physiological function in the table 1-2 below (128). Activin receptor-like kinase 5 (ALK5) is the predominant TGFβ type I receptor (TβR-I) that is activated by TGFβ through the type II receptor (155).
Table 1-2: TGFβ superfamily type I receptors

<table>
<thead>
<tr>
<th>Activin receptor-like kinase</th>
<th>Descriptive name</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK1</td>
<td>ACVRL-1</td>
<td>Activin, TGFβs</td>
</tr>
<tr>
<td>ALK2</td>
<td>ActR-IA</td>
<td>BMP, Activin, AMH, TGFβs</td>
</tr>
<tr>
<td>ALK3</td>
<td>BMPR-IA</td>
<td>BMPs</td>
</tr>
<tr>
<td>ALK4</td>
<td>ActR-1B</td>
<td>Activins, Nodal, Myostatin</td>
</tr>
<tr>
<td>ALK5</td>
<td>TβR-1</td>
<td>TGFβs, GDF9, Myostatin</td>
</tr>
<tr>
<td>ALK6</td>
<td>BMPR-IB</td>
<td>BMPs, GDFs, AMH</td>
</tr>
<tr>
<td>ALK7</td>
<td>ACVR1C</td>
<td>Nodal, Activin A/AB</td>
</tr>
</tbody>
</table>

The two types of serine/threonine TGFβ receptors are structurally similar as both the type I (55kDa) and type II (70 kDa) receptors have a cysteine-rich extracellular, hydrophobic transmembrane and intracellular domain, as well as a short C-terminal extension (128, 156). The extracellular domain is approximately 150 amino acids long, is N-glycosylated and contains 10 or more cysteines. A cluster of three cysteines (CCX_{4,5}C for type I, CXCX_{4,5}C for type II) are located near the transmembrane domain. The transmembrane region contains phosphorylation sites necessary for signal transduction (157). Phosphorylation of the type II receptor occurs at serine residues in the C-terminus (Ser549, Ser551) and in the transmembrane domain (Ser223, Ser226, Ser227) (158). Ser213 in the type II receptor transmembrane domain is phosphorylated independent of ligand activation (159). The N-terminal domain Ser165 of the type I receptor is phosphorylated by the type II receptor (158). The type I receptor (Figure 1-7) has additional structural features that allow for interaction with the type II receptor. The GS-box located in the juxtamembrane region within the intracellular domain is a highly conserved 30 amino acid region and is characterized by an TTSGSGSG sequence (160). Rich in glycine, serine and threonine residues, phosphorylation by the type I receptor also occurs at Thr185, Thr186, Ser187, Ser189, and Ser191 (158). Ligand-induced phosphorylation of these amino acids is essential for activation of signaling (158, 160, 161). Following the GS domain, the FKBP12 binding site is a Leu-Pro motif found in all type I receptors. Immunophilin FKBP12 is a cytoplasmic protein that is involved in inhibition of receptor phosphorylation (162, 163). FKBP12 is bound to the type I receptor in basal states and appears to be released following formation of the receptor complex (162, 164). The type I receptor also has a structural feature responsible for phosphorylation of Smad proteins. The L45 loop between β4 and β5 strands in the kinase domain of the type I receptor interacts with R-Smad proteins to cause phosphorylation of the C-terminal SSXS motif (165). Type II receptors have a very short cytoplasmic tail following the kinase domain, whereas the C-terminal extension of the type I receptor is nearly absent (128).
The TGFβ type I and type II transmembrane receptors have an intrinsic affinity for each other. When overexpressed in vitro, the TGFβ type I and II receptors can form heterodimeric complexes in the absence of TGFβ ligand (166, 167). In the basal state, the TGFβ type I receptor is not phosphorylated, but the type II receptor is phosphorylated on serine residues (160). Upon ligand activation, TGFβ binds to the type II receptor, which is then able to recruit the type I receptor. The dimeric structure of TGFβ ligands function to bring together heterodimeric receptors into a heterotetrameric complex (128). The type II receptor then phosphorylates the type I receptor at serine and threonine residues of the GS domain (158, 160, 161). It is the activated type I receptor that then phosphorylates the serines of the C-terminal SS(V/M)S motif and activates R-Smads (specifically Smad2 and -3) (168, 169). Smad proteins are major substrates of type I receptor kinases and key to the activation of signal transduction in the nucleus. R-Smads are expressed in most cells, although they are differentially controlled during development (170). Smad-2 and -3 are direct substrates of the TGFβ type I receptor kinase. Smad4, which participates in signaling by associating with receptor-regulated smads, heteroligomerizes with Smad2, and -3. The Smad complex comprising of Smads2, -3, and -4 are then able to translocate into the nucleus and activate transcriptional responses (128) as shown in Figure 1-8. Smad7 is an antagonistic protein that inhibits the signaling function of receptor-regulated Smads and Smad proteins associated with this complex (171, 172). Unlike Smad2 and -3, expression of Smad7 is highly regulated by extracellular signals (170).
Smad proteins can also be phosphorylated by other kinase pathways. This is exemplified by a number of target sites located on the MH1 domain of Smad2 and -3. The MH1 binds DNA and interacts with transcription factors, whereas TGFβ receptor phosphorylation occurs on the MH2 domain, or carboxy-terminal motif. PKC, MAPK, and CamKII (Ca2+/calmodulin-dependent protein kinase II) target sites are all located on this domain (173). PKC is indicated in Smad-mediated transcription as it can inhibit DNA binding of Smad3 through phosphorylation of the MH1 domain (174). ERK/MAPK (extracellular signaling-regulated kinase/mitogen-activated protein kinase) signaling, which is activated by tyrosine receptors or RAS phosphorylates the MH1 domain and linker segments of smad1, -2, and -3 (175, 176). One study found that RAS expression inhibits ligand induced nuclear translocation of activated Smads (175), although many other studies could not repeat this result (176-178). CamKII phosphorylates Smad2 and -3 in the linker and MH1 domain, and this can inhibit nuclear translocation. CamKII also affects hetero-oligomerization of Smad2 and Smad4 independently of TGFβ receptors (179).

After the discovery of Smad homologues from work in *Drosophila melanogaster* (180) and *Caenorhabditis elegans* (181), recognition of their function became paramount in identifying their importance in TGFβ signaling (128). Studies that investigated embryogenesis and targeted mutagenesis of these proteins to identify their function in TGFβ signaling were conducted. As reviewed by Weinstein, *et. al.* (182), Smad2(-/-) mice die at embryonic day 9.5 due to a failure to form the anterior-posterior axis (183, 184). Smad3(-/-) mice die between 1-8 months following birth due to defects in immune function and forelimb development (185, 186). Smad3 null mice also show accelerated cutaneous wound healing as well as reduction in the number of monocytes in the wound, indicating a role for Smad3 in wound healing and the local inflammatory response (187). Smad4(-/-) mice are embryonic lethal at day 6.5 and
show impaired gastrulation (188). With the generation of these transgenic mice, the role of the TGFβ1 pathway in carcinogenesis quickly became apparent and will be covered throughout this text.

Following activation of the TGFβ receptors by TGFβ, phosphorylation of Smad2 and -3, complexing with Smad4 and nuclear translocation, the Smad complex binds to Smad-binding DNA elements (SBE) in order to activate TGFβ1-induced transcription. Smad binding sites can be found on extracellular matrix proteins such as collagenase α1 (189) and collagenase α2 (190, 191), type I plasminogen activator inhibitor (PAI-1) (192, 193), Smad7 (194), and LTBP-1 (195). Transcription factors bind with high affinity to a DNA sequence (XBE), facilitate binding of Smad proteins, as well as recruit coactivators. Jun-B (196), lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF) (197, 198) and core binding factor A3 (CBFA3) (199) are all transcription factors for the Smad complex. Interestingly, the expression of these transcription factors is regulated by extracellular signaling such as c-Jun N-terminal kinase (JNK), Janus kinase 1/2 (JAK1/2), and Wnt/β-catenin which provides another level of transcriptional regulation (170). Mixer binds to DNA like other transcription factors, although it is a DNA-binding adaptor rather than regulator of transcription. This cofactor aids in the DNA contact of the activated Smad complex (200). Smad proteins can also recruit transcriptional coactivators and repressors. CREB-binding protein (CBP)/p300 are essential coactivators for TGFβ signaling (201). These coactivators interact with Smad1, -2, -3 and -4 on the MH2 domain (202-205). SMIF specifically interacts with Smad4 in the cytoplasm and accumulates in the nucleus (206), as does melanocyte specific gene (MSG1) (207), and both recruit CBP/p300. Since TGFβ inhibits the expression of a number of genes, this may be regulated by the recruitment of corepressors. TGFβ-interacting factor (TGIF) (208), Sloan-Kettering Institute proto-oncogene (SKI) (209) and SKI-related novel gene N (SnoN) (210) are all corepressors that bind to histone deacetylases. This causes chromatin condensation at the target genes to which they are bound and inhibits histone acetyltransferase activities and transcriptional activation through CBP/p300 (211). The regulation of TGFβ-induced transcription is summarized in Figure 1-9.
In addition to TGFβ signaling that occurs through Smad proteins, TGFβ receptors also activate Smad-independent pathways. ERK, JNK, and p38 MAPK kinase pathways can also be activated by TGFβ receptors (178). This is further supported by activation of RAS, ERKs and SAPKs (stress-activated protein kinase) by TGFβ in epithelial cells (212). TGFβ1 and BMP signaling activate TAK1 (TGFβ-activated kinase 1), a member of the MAP kinase kinase kinase family. TAB1, the TAK1 activator, appears to associate with XIAP (X-chromosome linked inhibitor of apoptosis protein) which may interact with BMP receptors (213). These Smad-independent signaling pathways can also positively regulate Smad protein phosphorylation. ERK1 can upregulate Smad2 transcription and phosphorylation, and this is inhibited by transfection of Smad2 mutants lacking an ERK phosphorylation site (176). In mammary epithelial cells TGFβ1 activated PI3K (phosphoinositide 3-kinase) and downstream AKT, and this was shown to be important for TGFβ-mediated epithelial to mesenchymal transition (EMT) (214).

Independent of Smads, protein phosphatase 2A (PP2A) can associate with the TGFβ type I receptor in response to TGFβ binding (215). This causes interaction between PP2A and p70S6K, a kinase that regulates translation and cell cycle progression, leading to dephosphorylation and inactivation of p70S6K (216). TGFβ1 is also responsible for activating Rho-like GTPases including RhoA, Rac and Cdc42, which are also important in EMT and for changes in the cytoskeleton (217, 218). These studies only begin to identify the complex signaling of the TGFβ superfamily, and shows that signaling is not as simple as the linear model shown in Figure 1-8. However, acknowledging the signaling crosstalk of these pathways with Smad proteins is important to consider when trying to understand the mechanisms of biological endpoints linked to TGFβ.

**Figure 1-9:**

**TGFβ-induced transcription**

Following translocation into the nucleus, the Smad complex (Smad2/3/4) bind to Smad binding elements (SBE) in cooperation with transcription factors (TF) that also bind to specific DNA sequences (XBE). The Smad complex interacts with essential coactivators CBP/p300 and others. Interaction with corepressors downregulated Smad-mediated transactivation. The coactivators establish formation of polymerase II (Pol II), TATA binding protein (TBP), TBP-associated factor (TAF) at the TATA box.
TGFβ1 has a myriad of functions in the cell that are context dependent including cell type, stage of differentiation, growth conditions, concentration of ligand, and presence of other growth factors. The TGFβ1 cytokine is most famous for its role as a potent growth inhibitor in epithelial, endothelial, and hematopoietic cells including macrophages, lymphocytes, and neutrophils (219, 220), underlying its role in inflammation and fibrosis. TGFβ1 was first shown to be growth inhibitory for keratinocytes in vitro (221) and increased epidermal proliferation was found with homozygous deletion of the TGFβ1 gene in vivo (222). Expression was also growth inhibitory in a TGFβ1 inducible model driven by the loricrin promoter in neonatal and adult epidermis (223). Mice with keratin 1 targeted expression of TGFβ1 in the suprabasal layer of the epidermis were not viable due to inadequate epidermal proliferation (224).

Surprisingly, TGFβ1 appeared to be growth stimulatory in quiescent epidermal cells with keratin 10 driven overexpression of TGFβ1 although there were no histological changes. Yet, this model showed growth inhibition in the basal keratinocytes following treatment with TPA, as expected (225), showing the complexity of predicting the function TGFβ1 due to differences in context. Changes to cell growth in particular have been shown to be mediated by direct effects on molecules that regulate the cell cycle including G1 phase cyclins, cyclin-dependent kinases (Cdks), Cdk-activating kinase (CAK), Cdc25A, and Cdk inhibitors as shown in Figure 1-10. However, in fibroblast cells, TGFβ appeared to stimulate growth by an indirect mechanism (187, 226).

TGFβ1 can inhibit cyclin D/cdk4/6 complexes and cyclin E/cdk2 complexes, resulting in hypophosphorylation of pRbs and decreased transcriptional activity by E2F. When the E2F transcription factor is inhibited from binding to target genes, transcription of genes required for G1 to S phase transition are downregulated (227). Growth inhibitory effects of TGFβ1 are associated with increased expression of the cyclin-cdk inhibitors p15\(^{Ink4B}\), p21\(^{Cip1}\) and p27\(^{Kip1}\). Following treatment with TGFβ in HaCaT keratinocytes and CCL64 mink lung epithelial cells, p15\(^{Ink4B}\) is induced and binds to cdk4/6, which inhibits the ability of cdk4/6 to bind cyclin D and induce transcription (228). It appears that p27\(^{Kip1}\) and p21\(^{Cip1}\) also play an important role in TGFβ1-induced growth inhibition by serving as a “back up plan” for G1 growth arrest. In the absence of p15\(^{Ink4B}\), TGFβ1 can regulate new synthesis, localization and protein levels of the cyclin-dependent kinase inhibitors p27\(^{Kip1}\) and p21\(^{Cip1}\) as has been shown in a human melanoma cell line (229). p21\(^{Cip1}\) in particular is regulated by TGFβ1 in a p-53 independent manner (230) that requires activation of RAS/MAPK signaling in untransformed epithelial cells (231), HaCaT cells (232), and mouse fibroblasts (233). However, p27\(^{Kip1}\) is required for TGFβ1-induced growth inhibition, as demonstrated by reversal of growth arrest following treatment with the E1A oncprotein that binds and blocks p27\(^{Kip1}\) in mink lung epithelial cells (234). In lung epithelial cells, p15\(^{Ink4B}\) (cytoplasm) and p27\(^{Kip1}\) (nuclear) coordinately inhibit cell cycle progression by binding to cyclin D-cdk4/6 and cyclin E-cdk2 complexes, respectively (235). Further, while there appears to be no direct effect of TGFβ signaling on
the phosphorylation state of pRB in human keratinocytes (236), growth inhibition of mink lung epithelial cells was linked to suppression of pRB phosphorylation implying a direct effect (237).

<table>
<thead>
<tr>
<th>Early G1</th>
<th>Late G1</th>
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<tbody>
<tr>
<td>Cyclin D</td>
<td>Cyclin D</td>
<td>Id</td>
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<tr>
<td>Cdk4/6</td>
<td>Cdk4/6</td>
<td>Rb</td>
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<tr>
<td>c-myc</td>
<td>p21, p27</td>
<td>E2F</td>
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<tr>
<td>p15INKB</td>
<td>cdc25</td>
<td>Rb</td>
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<td>CAK</td>
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<td>Cdk2</td>
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<td>Cyclin E</td>
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**Figure 1-10:** Role of TGFβ1 in cell cycle progression

TGFβ1 controls cell cycle progression and growth inhibition in epithelial cells through regulation of molecules involved in the cell cycle including cyclins, cyclin-dependent kinases (Cdks), Cdk-activating kinase (CAK), cdk inhibitors and retinoblastoma (Rb). Cell cycle phases in which regulation occurs are noted.

Cell cycle regulation by TGFβ1 is more complex than a single set of signaling pathways. For example, the proto-oncogene and transcription factor c-myc is expressed in the basal layer of the epidermis as well as the bulge region (238). Decreased expression of c-myc by TGFβ1 inhibits cell cycle progression through direct binding of Smad3 to a repressive Smad binding element on the c-myc promoter (239). It is known that c-myc can inactivate p27^Kip1 and p21^Cip1, induce the CDK-activating phosphatase Cdc25, and deregulate cyclin E expression (240). C-myc is down-regulated by TGFβ1 in a variety of cell types including keratinocytes (236) and serves as a useful marker as a target gene for TGFβ1 signaling. Id proteins (inhibition of DNA binding or differentiation) are important in promoting cell cycle progression. Id2 sequesters pRB releasing E2F and allowing progression through the S-phase (241). Id2 and Id3 are also phosphorylated by CDK2 in late G1 which inhibits bHLH activation of p15^INK4B, p16^INK4A and p21^CIP1 (242, 243). Repression of Id proteins by TGFβ1 via Smad3 further regulates the cell cycle (244, 245).
1.2.2 Role of TGFβ1 in skin carcinogenesis

TGFβ1 plays a dual role in cancer development in human and experimental mouse models, acting as a tumor suppressor and an oncogene (155, 155, 246, 247). Alterations and mutations to the TGFβ1 signaling pathway are present in many human cancer types and models for overexpression of TGFβ1 signaling correlate well with characteristics of human cancers. In fact, there is a significant correlation between TGFβ1 expression in tumors and poor prognosis for cancer patients (248). In a retrospective study of human malignant breast (249), colon (250), prostate (251) and pancreatic cancers (252), the majority of the tumors stained positively for TGFβ1. Increased serum and plasma TGFβ1 levels were also found in patients with colorectal cancer (253, 254). Human colon cancers with increased TGFβ1 levels are 18 times more likely to recur and metastasize than tumors with low expression levels (255) and a similar result was found in infiltrating duct carcinoma of the breast (256). Collectively, increased levels of TGFβ1 in the tumors or serum correlated with cancer progression and poor prognosis. However, blocked signaling due to changes in receptor function or Smad proteins have also be associated with tumor formation. Mutations in the type I receptor have been found in breast, pancreatic, biliary and cervical cancers. Investigation of the type II receptor has identified an even larger pool, including mutations found in colon, gastric, endometrial, prostate, breast, lung, hepatic, pancreatic, cervical, glioma, and head and neck. Mutations in Smad2 have also been identified in human colorectal, lung, and hepatocellular cancers (257).

The two stage chemical carcinogenesis protocol has provided an extremely useful model for understanding the role of the TGFβ1 signaling pathway in tumor formation and progression (Figure 1-11). In normal skin, TGFβ1 is a potent growth inhibitor, with tumor suppressing activities toward the formation of benign lesions. Transgenic mice that express TGFβ1 transgene (258) or TGFβ superfamily members (259) in the epidermis have reduced papilloma formation in skin chemical carcinogenesis studies. As described above, TGFβ1 halts signaling pathways responsible for proliferation and growth, suppressing the formation of benign tumors. It has been shown that the regulation of cell cycle progression by TGFβ1 is a mechanism linked to its role as a tumor suppressor (260). Interestingly, TGFβ1 also maintains gene stability independent of cellular proliferation pathways. Keratinocytes lacking expression of the TGFβ1 cytokine have increased gene amplification in an assay for genomic stability (261) and in an in vitro model of premalignant lesions (262), and this is reversed by the addition of exogenous TGFβ1 (261, 262). In vitro analysis shows that TGFβ1 also regulates epidermal homeostasis and potential inhibition of tumor formation by inducing apoptosis in human keratinocytes (263). In vivo, TGFβ1 overexpression in mammary epithelium (264) and increased Smad phosphorylation in prostate (265) is associated with an increase in apoptotic cells. Mice that have K14 promoter driven expression of TGFβ1 type II receptor knockout maintain epidermal homeostasis by inducing an increase in apoptosis.
that is coupled with hyperproliferation (266). Keratinocytes from these transgenic mice infected with the v-Ras<sup>HA</sup> retrovirus are refractory to TGFβ1-mediated apoptosis, and these cells form poorly differentiated SCCs when grafted onto nude mice (266).

**Figure 1-11: Dissecting how alterations to TGFβ1 signaling impacts tumor formation and progression**

The two stage chemical carcinogenesis assay has been used with a variety of transgenic mouse models to determine the role of TGFβ1 signaling in cancer. Arrow on the left represents an increase/decrease in papilloma or SCC formation. Model systems that overexpress TGFβ1 are in green; TGFβ1 signaling repression shown in red. Model systems that overexpress TGFβ1 are in green; TGFβ1 signaling repression shown in red.

Considering these data, papillomas lacking full expression of TGFβ1 progress more rapidly to squamous cell carcinoma (222, 267, 268). Grafts of TGFβ1 null keratinocytes virally transduced with the v-Ras<sup>HA</sup> retrovirus convert more rapidly than wildtype v-Ras<sup>HA</sup> keratinocytes (268), and transgenic mice expressing dominant negative TβRII subjected to the two stage carcinogenesis protocol show an earlier appearance of papillomas and progression to carcinomas that was absent in wildtype mice (269-271). Keratinocytes from K14-TβRII knockout mice infected with the v-Ras<sup>HA</sup> retrovirus are refractory to TGFβ1-mediated apoptosis, and these cells form poorly differentiated SCCs when grafted onto nude mice (266). However, smad3 knockout mice treated with DMBA/TPA show reduced papilloma and SCC formation (272). Similar to this study, TGFβ1(+/−) mice had fewer papillomas, yet reduced TGFβ1 cytokine caused a higher incidence of malignant conversion (273). These data show that components of TGFβ1 signaling inhibit tumor formation as expected, although this loss of signaling causes enhanced malignant conversion. However, in these model systems, the TGFβ1 signaling pathway, receptors, or
Smad proteins are lost at the earliest stages during tumor development. Alterations to cell proliferation by loss of TGFβ1 signaling may cause more DNA damage, altering the phenotype of these papillomas that then have a growth advantage (270, 274).

Paradoxically, carcinomas often secrete excess TGFβ1, which acts as an oncogene by increasing invasion and metastasis (247). In studies performed by Cui et al. using transgenic mice that express the TGFβ1 transgene, papillomas that did form using the skin chemical carcinogenesis protocol had enhanced malignant conversion and increased incidence of spindle cell carcinoma (258). TGFβ1 overexpression via the loricrin promoter in papillomas also rapidly induced metastasis, although induction of the transgene early in the tumor promotion phase suppressed papilloma formation (275). Epigenetic mechanisms may contribute to the ability of TGFβ1 to instigate conversion, including involvement of TGFβ1 with the AP-1/ERK signaling pathway (196). Overexpression of Smad7 but not Smad6 in a premalignant keratinocyte model caused a rapid progression to SCC that was accompanied by inhibition of Smad2 and Smad3 nuclear localization (276). Other genetic deletions namely in the INK4a locus that contains p16INK4A, p19arf and p15INK4B have been consistently found in more progressed tumors and may interact with TGFβ1 signaling to cause a more invasive phenotype (277, 278). It is also likely that the cells exposed and responding to TGFβ1 in premalignant lesions are very different from the epidermal cells that form papillomas following promotion. This population may become refractory to TGFβ1 signaling or possibly provide autocrine maintenance of TGFβ signaling (279, 280). These observations may help to explain a mechanism for the tumor suppressor-to-oncogene switch, by which tumors become unresponsive to the growth inhibitory effects of TGFβ1 and rather promote progression and metastasis.

TGFβ1 is known to contribute to some of the mechanisms that cause malignant conversion. Tumor growth is dependent on the availability of oxygen and nutrients that are supplied by the vasculature. In order to continue growth, the tumors must be able to initiate growth of new blood vessels, a process called angiogenesis. This response is dependent on expression of angiogenesis initiating signals such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) that bind tyrosine kinase receptors on endothelial cells (281). A variety of mouse models have demonstrated the importance of the TGFβ1 signaling pathway in normal angiogenesis that occurs during development. TGFβ1 null mice have defective haematopoiesis and endothelial differentiation that results in retarded development of blood vessel growth (282). Also, both TβRI (283) and TβRII (284) null mice are embryonic lethal and have defects in vasculogenesis. The application of TGFβ1 to chicken chorioallantoic membrane (a model system that allows for observation of a developing chicken embryo) induced the formation of large blood vessels (285) and a similar response was seen with treatment of endothelial cells (286). Similar to regulation of blood vessel formation in normal tissue, the TGFβ pathway regulates this response during tumor formation. Overexpression of TGFβ1 in tumor cells grafted
onto nude mice showed increased angiogenesis that was absent in normal tumor cells (287, 288). A multistage cancer model using dominant negative TβRII mice had an earlier appearance of tumors, in greater numbers, and enhanced metastasis that was correlated with increased angiogenesis due to increased expression of VEGF and decreased expression of thrombospondin-1 (269). TGFβ1 may have a paracrine effect on angiogenesis by increasing VEGF expression in transformed fibroblasts and epithelial cells, but not directly on endothelial cells (289, 290). Matrix metalloproteinase-2 and -9 (MMP2/9) proteolytically cleave and activate TGFβ1 and are required for angiogenesis (146) and tissue inhibitors of metalloproteinases (TIMPs) inhibit this response (291). Similarly, human prostate tumors (251), non-small cell lung carcinoma (292), and hepatocellular carcinoma (293) with overproduction of TGFβ1 have increased tumor grade and vascularization.

Epithelial to mesenchymal transition (EMT) is a reversible process that causes morphological changes in epithelial cells to those of mesenchymal cells. In terms of cancer progression, this transition causes a loss of cell to cell adhesions, including the expression of E-cadherin (294) and increased cell motility, which supports a more invasive phenotype (295). Numerous studies have been done to demonstrate that TGFβ1 plays an important role in causing this switch (294, 296, 297). In vitro, treatment of Ras-transformed mammary epithelial cells with TGFβ1 causes an epithelial to mesenchymal switch, resulting in cells that have a fibroblastoid appearance and are invasive. Further, while normal epithelial cells respond to TGFβ1 by undergoing growth arrest, the Ras-transduced cells are resistant (279, 298). Dominant negative TGFβ1 type II receptor transfection into a malignant metastatic keratinocyte cell line inhibited EMT when cells were injected into nude mice (294). In contrast, DN-Smad4/ Ras-infected keratinocytes showed that loss of Smad4 increased RAS/ERK activity and secretion of urokinase-type plasminogen activator (uPA), and enhanced motility and epithelial to mesenchymal transition (299). A two-stage chemical carcinogenesis study showed that overexpression of TGFβ1 in keratinocytes elicits an EMT response which correlated with a higher incidence of spindle cell carcinoma (258). Importantly, this progression phenotype has been identified in human cancers. The earliest reports of this phenotype in human esophagus and skin cancer show that these SCCs have a fibroblastoid appearance and produce collagen (300). Poorly differentiated skin carcinomas in humans have shown mesenchymal markers, such as vimentin, (301) and short survival and HER2 overexpression correlated with vimentin expression in human breast carcinomas (302), indicating that this switch is characteristic of more progressed tumors.

The EMT phenotype enhances invasion and metastasis by creating a tumor microenvironment where cell-cell adhesion molecules and tethering of cells to surrounding tissue are lost. In the same way that TGFβ1 is responsible for the EMT switch, TGFβ1 is also responsible for creating a more invasive phenotype (303, 304), solidifying the role of this cytokine as an oncogene. In vitro, a nontumorigenic keratinocyte cell line is growth arrested when treated with TGFβ1, but transformed keratinocytes are
induced to undergo EMT. This is associated with progression to spindle cell carcinoma, migration and invasion (305). TGFβ1 also enhances the expression of molecules that are responsible for extracellular matrix degradation such as urokinase (uPA), plasminogen activator inhibitor (PAI-1) and matrix metalloproteinases (MMPs) (306) in the transformed but not the nontumorigenic keratinocytes (305). Changes in TGFβ1 signaling can also impact how fibroblasts alter tumor invasion and metastasis, highlighting the importance of stromal-epithelial crosstalk in cancer progression (307, 308). When the TGFβ type II receptor was conditionally inactivated in mouse fibroblasts, intraepithelial neoplasia in prostate and invasive squamous cell carcinoma in the forestomach was observed (309). Grafted tumors that arose from mammary carcinoma cells and TβRII deficient fibroblasts were more invasive than tumors created with control fibroblasts (310). This shows that increased tumorigenesis was observed in adjacent epithelium as a result of stromal alterations. Figure 1-12 summarizes these data, emphasizing the role of TGFβ1 in tumor formation and progression and the mechanisms that contribute to these changes.

1.2.3 Role of TGFβ1 in inflammation

As with most TGFβ1 responses, this cytokine plays a dual role in regulating the immune system. TGFβ1 can both stimulate and inhibit the activities of different immune cells and this maintains tissue homeostasis following exposure to various stimuli that result in an inflammatory response. Mice with deletion of TGFβ1 or overexpression have revealed the dual effect of TGFβ1 on the inflammatory response. At around 3 weeks following birth, TGFβ1 knockout mice exhibited severe wasting including ruffled coat, hunched posture, conjunctivitis and skin irritation. Tissue necrosis was found in the heart, stomach, liver, lung, pancreas, salivary gland, and striated muscle (311, 312). Inflammatory cell
infiltration of lymphocytes and macrophages, but not neutrophils was detected in these lesions (311) although others report that neutrophil infiltration was found in stomach lesions (312). However, keratin 5 promoter overexpression of TGFβ1 in head and neck epithelia caused severe inflammation, hyperproliferation and angiogenesis, potentially due to a negative feedback loop involving the upregulation of Smad7 (313). A similar result was observed in epidermal overexpression of TGFβ1. These mice exhibit psoriasis-like plaques and substantial neutrophil, macrophage and T lymphocyte infiltration into the epidermis (314). Chronic expression in the epidermis caused alopecia characterized by epidermal and follicular hyperplasia, apoptosis, and inflammation (315). This highlights the potential for dramatically different tissue specific effects of TGFβ1 signaling.

A number of chronic inflammatory diseases such as inflammatory bowel and Crohn’s disease, chronic pancreatitis and reflux oesophagitis (316) as well as chronic wounding (317) are associated with increased incidence of cancer. These conditions are often accompanied by a decrease in TGFβ1 expression which reduces the anti-inflammatory properties that allow for resolution of inflammation (318-320). Similar to the inflammatory response in these chronic diseases, the two-stage chemical carcinogenesis assay uses tumor promoters such as TPA to produce chronic inflammation and amplification of mutated epidermal cells. Following treatment with tumor promoters, TGFβ1 mRNA levels increase (321, 322). Treatment of mouse skin with TPA has shown that constitutive expression of TGFβ1 results in reduced TPA-induced hyperplasia (225) while TGFβ1(+/-) (323) and TGFβ1(-/-) (324) mice have an increased inflammatory response following TPA treatment. These experiments show that TGFβ1 acts as an anti-inflammatory molecule in this model. However, it is difficult to define the role of the inflammatory response. An inflammatory infiltrate can induce DNA damage by generating free radicals, promote angiogenesis and tissue remodeling, and suppress the antitumor adaptive immune response. However, the very nature of the immune system is to prevent and protect against the expansion of damaged cells (325). Considering that TGFβ1 normally functions as a growth inhibitor in normal skin, reducing the expression of this cytokine in the presence of an inflammatory stimulus could lead to cancer development, potentially via reduced immunosurveillance.

In the presence of stimuli such as environmental pathogens, ultraviolet radiation or wounding, the innate immune response is the immediate responder. The innate response is quick but non-specific and does not provide long-lasting protection. Leukocytes are the primary innate immune cells and include: natural killer cells and mast cells and phagocytic leukocytes including macrophages, neutrophils and dendritic cells (47). TGFβ1 also induces resident and infiltrating cells to begin secreting more TGFβ1 (326). In this scenario, TGFβ1 acts as a pro-inflammatory cytokine in the skin. TGFβ1 stimulates the chemotaxis of innate immune cells including neutrophils, monocytes and mast cells. TGFβ1 is a potent chemoattractant for neutrophils (327, 328), although others demonstrated that neutrophil degranulation
and activation, but not chemotaxis is inhibited by TGFβ1, and this was reversed by pharmacological inhibition of the TGFβ1 type I receptor (329). TGFβ1 is a chemoattractant to peripheral blood monocytes and stimulates the production of various growth factors. The high expression of TGFβ1 receptors on these monocytes is consistent with these observations (330). Mast cells also accumulate at site of inflammation. TGFβ1 induced the migration of mouse mast cells which could be inhibited by a TGFβ1 neutralizing antibody. This was consistent with the presence of type I and III receptors on mast cells (331). It has also been demonstrated that TGFβ3 is a more potent chemoattractant for human mast cells than TGFβ1 or -2, and migration is mediated by the presence of the TGFβ type I and II receptors, but not type III (332). This is highlighted in wounded (187) and irradiated (333) Smad3(-/-) mice where the innate immune and inflammatory response is diminished. However, TGFβ1 also regulates mast cell homeostasis as maturation and activation is blocked independent of Smad3 (334).

Unlike the innate response, the adaptive immune response is responsible for specificity through the infiltration of antigen-presenting cells like Langerhans and dendritic cells. The antigens are presented to naïve and central memory T cells which allows for an efficient response in the target tissue (47). Langerhans cells require TGFβ1 for differentiation and migration (335, 336). However, inhibition of TGFβ1 signaling improves dendritic cell function as TGFβ regulates the antigen presentation function of these cells by inhibiting the expression of major histocompatibility complex (MHC) class II and costimulatory molecules (335, 337). While TGFβ1 generates a pro-inflammatory phenotype following an insult, it is also responsible for suppression and resolution of inflammation. Natural killer (NK) cells recognize and kill foreign cells, participating in the innate immune response. Again, TGFβ1 is immunosuppressive by inhibiting the production, cytolytic activity and cytokine production of these cells (338, 339). The role of TGFβ1 in controlling T cell function has been studied extensively. TGFβ1 inhibits CD4+ and CD8+ T cells (311, 312, 340) and T cell proliferation via IL-2 dependent (341) and independent (342) mechanisms. TGFβ1 stimulates the generation of regulatory T cells (Tregs) in vitro, promoting immunosuppression (343, 344) although other studies do not indicate TGFβ as being essential for Treg development (345).

The functions of TGFβ1 during tumor formation are very similar to its role in resolving an acute insult. As addressed above, this is demonstrated through a number of immune cells. TGFβ1 reduces the cytotoxicity and cytokine production of NK cells in orthotopic murine head and neck SCC (346) and lung and colorectal cancer patients (347). Langerhans cell migration is inhibited by TGFβ1 and this is associated with progressed tumors that have evaded immunological destruction (348). TGFβ1 is also responsible for suppressing T cells in the tumor microenvironment as well as T cell effector molecules including interferon-γ and perforin (349, 350). This is exemplified by the use of a tumor cell line transfected with TGFβ1 cDNA that did not stimulate cytotoxic T lymphocyte responses in vitro or in vivo.
promoting tumor growth (351) and when TGFβ1 signaling was blocked, helper and cytotoxic T cells were shown to inhibit malignant tumor development (352). Overall, tumor progression seems to be enhanced by reduced immunosurveillance due to TGFβ1 signaling, agreeing with the observation that high risk papillomas have reduced levels of pro-inflammatory genes and CD3+ cells, showing that reduced immunosurveillance may be associated with tumor progression (109).

1.2.4 Role of TGFβ1 in senescence

Alterations in TGFβ1 signaling affect other mechanisms of tumor suppression, such as “senescence”, that may contribute to tumor development. Senescence was first described by Hayflick and Moorehead as the limited lifespan of human diploid cells in vitro. This G1 growth arrest is observed in aged cells, potentially as a safeguard against replication of genetically altered cells. These cells are metabolically active, but lose their ability to synthesize DNA (353). The mechanism of “replicative senescence” is dependent on telomere shortening which triggers a damage response that halts cell cycle progression and inhibits further cell division. This type of senescence may be related to aging in humans (354). It is now known that senescence can be caused by oncogene activation, oxidative stress, DNA damage, chemotherapeutic drugs, and cell culture conditions (355). It is proposed that oncogene-induced senescence (OIS) is a mechanism by which premalignant or benign neoplasms are suppressed from becoming malignant tumors. This oncogenic signaling paradoxically elicits G1 growth arrest (356).

Studies that investigate changes in oncogene-induced senescence often use model systems that overexpress the Ras oncogene. Growth arrest and senescence is induced in both fibroblasts (357-362) and keratinocytes (363) in vitro with retroviral Ras infection or conditional and/or tissue specific overexpression. Interestingly, senescence has been identified in premalignant lesions, but not malignant tumors, indicating that the more progressed tumors are refractory to this growth arrest (364). Premature senescence can be caused by activation of tumor suppressor genes that are often inactivated in cancers. Cells that undergo senescence are large, flat, vacuolated and express senescence-associated acidic β-galactosidase (SA-β-gal) (365). This marker reflects increased lysosomal content although the biochemical basis is unknown and this enables detection of β-galactosidase at a suboptimal pH (366). Senescence is irreversible and a mechanism for this involves alterations in the chromatin that results in permanent and stable repression of genes responsible for proliferation. Senescence-associated heterochromatic foci (SAHF) resemble heterochromatin and can be visualized by the appearance of DAPI-stained clusters both in vitro and in vivo (367-369).

Although senescence can occur due to a variety of stimuli, two pathways in particular are responsible for senescence: p16INK4a-RB and p19ARF-p53 (370, 371). In Ras retrovirus infected keratinocytes (363, 372, 373), senescent fibroblasts (374, 375) and human uroepithelial cells (376), both
p16\(^{INK4A}/RB\) and p53 tumor suppressor pathways are known to show increased activity and/or levels in senescent cells. In agreement, mice that are defective in CDKN2A \(p16^{INK4a}/p19^{ARF}\) have increased susceptibility to spontaneous and chemically induced carcinogenesis (377, 378). Therefore, increased expression of \(p16^{INK4A}\), \(p19^{ARF}\) and p53 are known markers of OIS. Other markers of oncogene-induced senescence include increased expression of p21 (379), which cooperates with \(p16^{INK4A}\) to maintain RB in an unphosphorylated and inactivated form, inhibiting cell cycle progression. Decoy receptor 2 (Dcr2) and differentiating embryo-chondrocyte expressed (DEC1) expression levels correlated with SA-\(\beta\)-gal and SAHF, and have been useful in identifying OIS \textit{in vivo} (364).

While senescence has been widely confirmed \textit{in vitro} (380, 381), confirmation of an \textit{in vivo} tumor suppressor mechanism has been more difficult to substantiate. The benign tumor melanocytic nevi, also known as moles, are clonal proliferations of cells that have a mutation to evoke a proliferative response. Nevi have been shown to senesce as indicated by SA-\(\beta\)-gal, increased p16 expression and proliferative arrest (382, 383). OIS is also linked to T-cell lymphomas (367) and prostate cancer (384) in mice. Furthermore, senescence was induced in mice overexpressing \(K\)\(R\)as2 at levels similar to tumors, while lower levels of expression caused proliferation in mammary tumors (385). In a clinical setting, studies have shown that tissue specimens taken from human breast tumors (386) and primary murine lymphomas (387) that have undergone chemotherapy display features of senescence, and this is related to improved prognosis.

Previous studies have shown that TGF\(\beta\)1 expression is upregulated in mouse epidermal keratinocytes infected with a \(v\)-\(R\)as\(^{\text{Ha}}\) retrovirus (73). Additionally, these cells undergo hyperproliferation followed by \(G_1\) growth arrest and senescence following 8-15 days in culture which is accompanied by an increase in \(p16^{INK4A}\), \(p19^{ARF}\) and p53. Both \(v\)-\(R\)as\(^{\text{Ha}}\) infected TGF\(\beta\)1 null and dominant negative T\(\beta\)RII keratinocytes resisted growth arrest and did not senesce. The molecular basis for this response in DN-T\(\beta\)RII keratinocytes involved decreased expression of p15 and \(p16^{INK4A}\), constitutive RB phosphorylation and increased cdk4 and -2 kinase activity (363). These data show a role for TGF\(\beta\)1 in the induction of senescence in mouse keratinocytes. This response may lead to tumor suppression by TGF\(\beta\)1 and explain how defects in TGF\(\beta\)1 signaling could contribute to premalignant progression in cancer. Using Smad3 null keratinocytes and smad adenoviral infection of \(v\)-\(R\)as\(^{\text{Ha}}\) transduced keratinocytes, it has also been shown that Smad3, but not Smad2 or Smad4, mediates the TGF\(\beta\)1-induced senescence response (373). Cripto-1, a member of the epidermal growth factor-CFC family of cytokines, can block phosphorylation of Smad2/3 and TGF\(\beta\)1 receptor binding and is expressed in a wide variety of human cancers. Shukla \textit{et al.} showed that when \(v\)-\(R\)as\(^{\text{Ha}}\) infected keratinocytes are treated with TGF\(\beta\)1, senescence is induced, but this response is inhibited by Cripto-1 (388). \textit{In vivo} studies demonstrate that long term treatment with
TGFβ1 caused A549 lung adenocarcinoma cells to senescence, and did not yield tumors in nude mice (389).

Collectively, evidence shows that senescence is not only an in vitro phenomenon, but a tumor suppressor pathway that occurs in vivo. Not only can this biological endpoint help to identify proper cancer staging and treatment as it occurs only in premalignant lesions, it could also be useful as a marker of chemotherapeutic drug efficacy. Learning how the cell signals to induce this response could be important as a therapy to ensure that tumors do not progress, and evidence thus far has demonstrated that the TGFβ1 signaling pathway plays an important role in this tumor suppressive function.

1.2.5 Role of TGFβ1 in terminal differentiation

Terminal differentiation is another potential mechanism that may lead to further understanding how TGFβ pathway inhibition contributes to tumor progression. Terminal differentiation has been discussed in terms of the normal structure and physiology of the skin and the importance of this biological process in skin tumor formation and progression. Unlike senescent cells, terminally-differentiated keratinocytes are metabolically-inactive and slough off culture dishes or the skin, rather than being retained. There are numerous markers for different stages of terminal differentiation, each of which correlate with specific epidermal layers. The deepest of these layers is the stratum basale, which houses the proliferating cells of the epidermis and expresses keratins 5 and 14. Following a rise in calcium concentration, markers of an earlier stage of differentiation in the stratum spinosum keratins 1 and 10 are expressed 8-24 hours after treatment. K1/10 are subsequently suppressed as proteins involved in cornified envelope formation, such as loricrin, filaggrin, involucrin, and transglutaminases are more prevalent in terminally differentiated cells 24-48 hours after calcium elevation (23). This model of differentiation has been documented in both humans (390, 391) and mice (392) in vivo.

Only a few studies have demonstrated a link between terminal differentiation and TGFβ1 signaling in keratinocytes. TGFβ1 and -2 have been shown to not only be ineffective at inducing differentiation, but inhibitory in stratified human epidermal cells (393, 394). Previous studies have shown that TGFβ1 suppresses the expression of IFN-γ and TPA-induced transglutaminase 1, which is an enzyme responsible for protein crosslinking of the cornified envelope (395, 396), but experiments investigating terminal differentiation were not performed. Calcium-induced terminal differentiation was also inhibited by TGFβ (397) suggesting that modulation by TGFβ1 requires the induction of differentiation by a variety of stimuli to yield a response. However, TPA-induced terminal differentiation as measured by cornified envelopes was decreased in TGFβ1(+/−) keratinocytes and this correlated with a decrease in PKC activity. Others showed that TGFβ1 suppressed TPA stimulation of PKC activation (398) although this was not related to terminal differentiation. This may indicate that terminal differentiation with these stimuli may
be regulated by both calcium dependent and independent mechanisms of PKC activation. Treatment of keratinocytes with the ALK5 inhibitor SB431542 also caused a significant decrease in PKC activity (399). It has also been shown that Cripto-1, which blocks TGFβ1 signaling, inhibits induction of keratin 1 and blocks calcium-induced terminal differentiation in primary keratinocytes. These results are strikingly similar to the decrease in keratin 1 mRNA and protein levels following calcium-induced differentiation with SB431542 treatment or DN-TβRII infection. Inhibiting TGFβ1 signaling via SB431542, DN-TβRII infection, and Cripto-1 all induced expression of keratin 8, which is a characteristic of tumor progression. These data not only suggest that Cripto-1 plays an important role in altering differentiation in keratinocytes and promoting malignant conversion, but also identifies the TGFβ1 signaling pathway as the key to these biological responses (388). As a mechanism for this response, Cl(-) intracellular channel 4 (CLIC4) translocates to the nucleus in response to treatment with TGFβ1 or TPA causing cell cycle arrest and gene expression that is associated with terminal differentiation (400).

1.2.6 Small molecule inhibitors of TGFβ signaling

Pharmacological inhibitors of the TGFβ type I receptor kinase have become important molecules for anti-cancer therapeutics. TGFβ receptors in particular are amendable to small molecule inhibition, with ALK5 being the most heavily understood in terms of characterization of domain structure, biochemical activity and biological function (401). Pharmaceuticals and in particular, small molecule inhibitors, have been identified through the use of three technologies important in identifying lead molecules: high-throughput screening (HTS), target-hopping, and virtual screening. The biotechnology and pharmaceutical industries have used HTS as a lead-generation tool. In order to analyze many compounds, liquid handling robotics, homogenous assay technologies (402), data-handling software and huge target compound libraries are required (403). Target-hopping, as the name implies, utilizes chemical, biological and structural information of one protein kinase family to determine the activity of a compound on a similar target protein kinase family. The effect of an inhibitor on one kinase is applied to other desired target kinases that share similar binding sites (404, 405). Finally, virtual screening is a computational searching tool that utilizes an in silico method to screen for kinase targets, enhancing the speed of identifying hits and decreasing expensive laboratory work (406).

Identification of ALK5 inhibitors has utilized all three methods. The use of the p38α kinase was a jumping-off point for ALK5 inhibitor development by target-hopping. A potent SAPKα/p38α and SAPKβ/p38-β2 kinase inhibitor SB-203580 (compound 1, Figure 1-13) belongs to a class of pyridinyl imidazoles and functions by binding to the ATP-binding pocket of the kinase. A threonine residue at the ATP-binding site, at the entrance of the hydrophobic pocket, allows this inhibitor to competitively bind. However, it has been shown that a methionine residue, such as that found with SAPK1γ/JNK1, SAPK3
and SAPK4, does not allow inhibition of kinase activity by SB-203580 (407, 408). Using site directed mutagenesis of a variety of receptors, it was determined that inhibition by SB-203580 could be achieved if this residue was no larger than that of threonine, which includes serine, alanine or glycine. This knowledge was then applied to the TGFβ1 type I receptor, which has a serine residue at the ATP-binding region. This study showed that phosphorylation of the TGFβ1 type I receptor could indeed be inhibited by the pyridinyl imidazole SAPK/p38 kinase inhibitor and this was dependent on the residue at the ATP-binding site (407).

Compounds similar to SB-203580 were subsequently identified by HTS. Two separate screenings of compound libraries for small molecules that inhibit the ability of the TGFβ type I receptor to phosphorylate Smad3 were conducted by GlaxoSmithKline (409). Understandably, compounds that contained an imidazole ring and had previously been identified as p38α inhibitors were discovered. These hits also contained a 4-pyridyl nitrogen like SB-203580 which was shown to form a hydrogen bonding interaction with the hinge region of p38α, although another compound (2; SKF-104365) with a 2-pyridyl nitrogen group inhibited the TGFβ1 type I receptor with a higher potency than p38α (409). In a screening done by Lilly Research Laboratories, the 2-pyridyl group was again identified as being important for ALK5 inhibition, although this inhibitor (3; LY-36497) contained a pyrazole ring instead of imidazole. It showed potency and selectivity in inhibiting phosphorylation of the type I receptor with weak inhibition of p38α (410).

In an independent virtual screening by Biogen Idec, compound 3, named HTS-466284, was also identified, demonstrating the overlap and potential usefulness of merging these technologies. Compound 1 was used as a starting point and 87 hits were collected following a filter of 200,000 compounds. This compound was shown to be ATP-competitive and inhibited TGFβ-induced PAI-luciferase activity (411). Other small molecule inhibitors have been identified that have a similar 2-pyridyl group with either a dihydropyrrolopyrazole (compound 4) or imidazole (compounds 5 and 6) ring. Overall, these studies identified the importance of the 2-pyridyl group in contributing to potency and selectivity of the TGFβ type I receptor inhibitors. Further, identification of the X-ray crystal structure of the TGFβ type I receptor (412) has revealed how each of these inhibitors can bind to the inactive conformation of the kinase and compete with ATP binding when the receptor kinase is in the activated state (410, 411, 413).
More recently, the development of these ALK5 inhibitors has been built upon in order to create small molecule inhibitors that have the potential for clinical use. It is desirable to create effective pharmaceutical therapies that are less toxic than chemotherapy which is often used at maximal doses with limited success. Therefore, the chemical structure of the dihydropyrrolopyrazole ALK5 small molecule inhibitors has been altered in order to make them orally bioavailable. One compound is 3-[6-(2-Morpholin-4-yl-ethoxy)-naphthalen-1-yl]-2-pyridin-2-yl-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole (1; Figure 1-14) and was found to be highly inhibitory against 50 kinases, with weak kinase inhibition for Lck, p38α, MKK6, Fyn and JNK3 at very high concentrations. It was the most potent of the series as tested with an in vivo target inhibition assay of Smad2 phosphorylation. This ALK5 inhibitor was shown to have low metabolism levels (<60% in human and rat microsomes), demonstrating that this compound is likely to have adequate in vivo exposure. In vivo evaluation of efficacy showed that this inhibitor, administered by oral gavage in saline solution, significantly delayed tumor growth in xenografted mice with human MX-1 breast carcinoma cells which had previously been shown to be sensitive to TGFβ1.
A new class of benzimidazole substituted dihydopyrrolopyrazoles molecules that are selective versus the TGFβ type II receptor and MLK-7 kinase, a kinase in the MAP kinase signaling pathway were also developed for oral bioavailability. 6-(2-Pyridin-2-yl-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazol-3-yl)-1H-benzoimidazole (2; Figure 1-14) was tested in the p3TP-Lux reporter and NIH3T3 proliferation assays in vitro. Both assays demonstrated that the ALK5 inhibitor blocked TGFβ signaling and growth. In a tumor xenograft model, Calu6 human anaplastic carcinoma lung cells treated with the inhibitor had significantly reduced Smad2 phosphorylation (415).

Due to the fact that ALK5 has such sequence homology with ALK4 and ALK7, it is difficult to make these small molecule inhibitors specific. Although the sequence identity between ALKs ranges from 41-68%, the ATP binding sites hold the highest homology (401). Small molecule inhibitors 5 and 6 have been shown to cross-react with ALK4 and ALK7, although neither appear to affect signaling through ALK1, ALK2, ALK3, or ALK6 (416, 417). While ALK4, 5 and 7 transduce signaling via Smad2/3-dependent pathway, BMP signaling occurs via the Smad1/5/8-dependent pathway (Figure 1-15). Understanding the role of ALKs 4, 5 and 7 in signaling is important to fully understanding the ramifications of potentially inhibiting these receptors though non-specific effects.

Figure 1-15: Activation of TGFβ family type I receptors

This generalized schematic shows that the ligands activin, TGFβ, and Nodal all phosphorylate Smad2 and -3, while BMP signaling occurs via Smad1/5/8. This figure emphasizes other signaling pathways that may be altered by treatment with SB431542.

ALK4, or Activin Receptor 1B was first identified as a type I receptor for Activins (418). Nodal and its related proteins were also found to bind to ALK4. However, this interaction is dependent on Cripto, an epidermal growth factor-CFC (EGF-CFC) family member (419). ALK4 has been implicated in mouse development, including egg cylinder structure before gastrulation and formation of the primitive
streak (420). This receptor is expressed throughout the hair follicles and interfollicular epidermis in neonatal skin (421), and it is restricted to the epidermis in adult mouse skin (422). Dominant negative keratinocyte ALK4 mice displayed perturbed wound healing due to altered epithelialization. Hair follicle development was normal but delayed, and the structure of the epidermis, dermis and appendages was normal even though smad2 phosphorylation was inhibited (423, 423). Furthermore, ALK4 expression was decreased in breast cancer tissue compared to normal breast tissue (424), indicating a role for ALK4 as a growth inhibitor. Interestingly, ALK4 plays a role in the inflammatory response, with increased expression in alveolar cells, fibroblasts and infiltrating cells during allergy induced airway inflammation (425).

ALK5 is best known for its ability to mediate TGFβ signaling by complexing with the TGFβ type II receptor (426, 427). As reviewed above, ALK5 phosphorylates Smad2 and Smad3 and regulates a wide variety of downstream endpoints. However, it has recently been reported that growth differentiation factor 9 (GDF9) (428) and myostatin (429) can signal through ALK5 when complexed with BMPRII and ActRIIB, respectively. Like ALK4, ALK5 is also involved in embryonic development. ALK5 null mice are not viable due to vascular defects in the yolk sac. Endothelial cells of these mice also showed increased proliferation and abnormal migratory behavior (283). While ALK4 seems to play a role in inflammation, there is little evidence thus far indicating ALK5 in the development of the immune response. ALK5 knockout mice display normal hematopoiesis, and conditional knockout of ALK5 in hematopoietic stem cells caused normal differentiation of hematopoietic progenitor cells (430).

Little has been known about the ligands, receptor binding and function of ALK7 until recently. Three ligands, Nodal, activin AB, and activin B have been characterized as binding to ALK7. ALK7 appears to bind to the type II receptor Activin Receptor IIB, mediating Nodal signaling during embryogenesis (431). The ligands activin AB and activin B bind to Activin Receptor IIA and ALK7, transducing signaling in a pancreatic β cell line MIB6 (432). Importantly, ALK7 has been shown to activate smad2 and smad3 (433, 434) and MAP kinases such as ERK and JNK (435), and p38 (436). While Nodal is important in embryo development, ALK7 null mice are viable and fertile, showing none of the abnormalities present in mice lacking Nodal, indicating that ALK7 does not play an essential role in Nodal signaling during development (437). However, studies have shown in a variety of in vitro models that ALK7 is important for regulation of proliferation and apoptosis. Overexpression of ALK7 induced apoptosis in human hepatoma cell lines (436), and inhibited cell proliferation and increased apoptosis in human trophoblast (438) and epithelial ovarian cancer cell lines (439). These Nodal induced effects were then blocked by dominant negative mutants of ALK7, Smad2 and Smad3 (438, 439) showing this receptor and intermediates are essential for regulation of proliferation and apoptosis. Studies in human trophoblast indicate that alterations to cell proliferation are most likely through the induction of
G1 arrest that may be mediated by the p27 pathway (438). Apoptosis is induced by caspase 3 and caspase 9 though ALK7 (436, 438, 439). In vivo animal models have not been used to further explore the involvement of ALK7 in adult physiological process and will be required in order to make better predictions about how inhibition of this receptor may alter epidermal homeostasis and carcinogenesis.

A number of disease pathologies are due to an overexpression of TGFβ1 (440), and the potential for therapeutics that inhibit TGFβ signaling have been explored. Interest in ALK5 small molecule inhibitors began as a treatment for fibrotic diseases, in which the TGFβ1 pathway is also a proposed mediator. Fibrotic skin diseases, such as scleroderma, occur due to a failure to terminate the wound-healing response and are characterized by early inflammation and vascular injury, followed by fibrosis of skin and other organs (441). Fibroblasts from lesional tissue secrete TGFβ1, have enhance expression of surface receptors for TGFβ1 (442), and have higher levels of phosphorylation of Smad2 and Smad3 (443). Connective Tissue Growth Factor (CTGF) is an early response gene product and cysteine rich mitogenic peptide, secreted by fibroblasts after activation by TGFβ. CTGF causes increased proliferation and migration of fibroblasts. It induces transformation into myofibroblasts, which stimulates collagen contraction and promotes collagen maturation into a highly cross-linked dense matrix (forms a scar). CTGF elevates the synthesis of extracellular matrix components including fibronectin, type I collagen, laminin, and glycosaminoglycans, while decreasing degradation of extracellular matrix due to direct inhibition of protease activity and stimulation of the synthesis of protease inhibitors. In the tight skin mouse model of scleroderma crossed with TGFβ1(+/−) mice, dermal fibrosis is reduced and a decrease in dermal thickness was observed (444). Lung and dermal fibrosis was also reduced following treatment with an anti-TGFβ antibody in bleomycin-induced models of scleroderma (445, 446). Studies have shown that small molecule inhibition of ALK5 reduced expression of fibrotic markers in dermal fibroblasts of patients with diffuse cutaneous systemic sclerosis (447-449). Therefore TGFβ is considered to play a fundamental role in fibrosis and is a target of therapeutics for preventing or arresting the fibrotic process (442, 450).

Chronic renal diseases including diabetic nephropathy, hypertension and glomerulonephritis are linked to overexpression of TGFβ signaling. This causes fibrosis and reduced kidney filtration function through a number of TGFβ1 dependent mechanism (451). In vitro, TGFβ1 caused an increase in extracellular matrix proteins fibronectin and type IV collagen in glomerular epithelial cells (452) and a decrease in matrix turnover by reduced activity of plasminogen activators and increased expression of plasminogen activator inhibitor (PAI-1) (453). Transgenic mice that overexpressed TGFβ1 in the liver developed progressive glomerulosclerosis (454) and a similar result was observed in rat kidney following in vivo transfection of the TGFβ1 gene (455, 456). Small molecule inhibitors of ALK5 have been effective in treating renal diseases. GW788388 (4-(4-[3-(Pyridin-2-yl)-1H-pyrazol-4-yl] pyridin-2-yl)-N-
(tetrahydro-2Hpyran-4-yl) benzamide) (457) is an ALK5 inhibitor that blocks both the TGFβ type I and type II receptors, but not the related bone morphogenic protein type II receptor (458). GW788388 significantly reduced PAI-1 and type I collagen in renal epithelial cells and fibronectin in NMuMG cells at mRNA and protein levels. Renal fibrosis and glomerulopathy was attenuated in the db/db mouse model of spontaneous diabetic nephropathy (type II diabetes mellitus) following 5 weeks oral treatment with GW788388 and this correlated with reduced expression of genes involved in extracellular matrix deposition (458). Similar results were achieved with SB-525334 where treatment of A498 renal epithelial carcinoma cells inhibited TGFβ1-induced increases in PAI-1 and procollagen α1 mRNA. Reduced expression of these fibrotic markers was also observed following ALK5 inhibition in the acute puromycin aminonucleoside rat model of renal disease (459).

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that primarily affects synovial joints and can lead to the erosion of cartilage causing deformities and disabilities (460). Increased levels of TGFβ1 (461), TGFβ type II receptor, latent TGFβ binding protein (462), and TGFβ type I receptor (463) were found in the synovial fluid of patients with RA. This rise in levels was also associated with fibrosis and active inflammation (462). Synovial fibroblasts in particular express molecules involved in tissue remodeling (463, 464) and inflammatory cytokines (464), resulting in joint destruction. In a mouse model of chronic erosive polyarthritis, treatment with the TGFβ1 and TGFβ2 neutralizing antibody 1D11.16 blocked leukocyte recruitment and the tissue pathology associated with tissue destruction (465). Administration of an ALK5 small molecule inhibitor (HTS466284) reduced symptoms of collagen-induced rheumatoid arthritis in vitro and in vivo through alterations in cell proliferation, inflammation and angiogenesis (466). However, it is important to note that other observations have shown that treatment with TGFβ1 systemically can suppress joint erosion in polyarthritic rats demonstrating that it may be important to inhibit TGFβ1 signaling only at the site of inflammation (467).

Psoriasis is a chronic, inflammatory, autoimmune skin disease. It is a T-cell mediated disease, characterized by epidermal hyperplasia, parakeratosis, angiogenesis and the skin displays alterations in growth and differentiation (468). Transgenic mice that overexpress TGFβ1 in the basal layer of the epidermis via the keratin 5 promoter exhibit a skin phenotype that resembles psoriasis. Skin inflammation increased over the mouse lifetime, and the skin became scaly with focal erytheroderma. Inflammatory cell infiltration including neutrophils, macrophages, T lymphocytes, angiogenesis, and degradation of the basement membrane was found, demonstrating that the overexpression of TGFβ1 greatly contributes to this disease pathogenesis (314). The inflammatory phenotype was associated with the upregulation of a variety of cytokines that alter the inflammatory response, extracellular matrix production and angiogenesis (314, 469). Increased serum (470) and plasma (471, 472) levels of TGFβ1 corresponded
with increased severity of psoriasis in humans. Although there is sufficient evidence that TGFβ1 plays an important role in psoriasis, studies have not investigated therapeutics that inhibit this signaling pathway.

TGFβ1 signaling is also implicated in inflammatory bowel diseases (IBD) such as Crohn’s disease and ulcerative colitis (473). These diseases can be distinguished by sustained activation of a mucosal immune response due to gastrointestinal tract microflora or antigens from dietary sources (474). Ulceration and submucosal fibrosis, accompanied by infiltration of lymphocytes and macrophages to all layers of the bowel wall can be found with Crohn’s disease, while inflammation and infiltration of lymphocytes and granulocytes tends to be restricted to the mucosal layers with ulcerative colitis (475). Transgenic mice with a homozygous mutation in the TGFβ1 gene suffer from a mixed inflammatory cell response that causes necrosis in the stomach (312). Similarly, Smad3 knockout mice exhibit inflammation and abscess formation at mucosal surfaces, in addition to resistance of T cells to inhibition by TGFβ1 and impaired chemotactic response of neutrophils to TGFβ1 (185). Paradoxically, in both humans (476, 477) and mouse models (478) of colitis, increased expression of TGFβ and Smad7 have been detected, although phosphorylation of Smad2/3 is defective (476, 479). While mRNA levels of Smad7 were unchanged between normal and diseased intestinal samples, Smad7 was regulated post-transcriptionally though differences in acetylation and reduced proteosomal degradation (480). It appears that endogenous TGFβ1 levels control IBD by negatively regulating the inflammatory response and therapeutics have targeted the downregulation of Smad7 in order to aid in the resolution of gut inflammation (479, 481). Small molecule inhibitors of TGFβ1 signaling have not been documented in the literature as therapeutics for IBD. Yet, as with cancer, TGFβ1 signaling in IBD can be context and cell type dependent. Therefore, it is possible that inhibiting receptor function may behave similarly to Smad7 therapeutics.

ALK5 inhibitors may also have potential as anti-cancer agents. As already discussed, TGFβ1 is excessively produced in tumors as a response to tissue perturbation (482) and oncogene activation (483). Yet, inhibiting this signaling pathway as a therapy for cancer prevention seems counterintuitive. TGFβ1 acts as a tumor suppressor and induces differentiation and apoptosis in normal epithelial cells. However, during cancer progression TGFβ1 appears to act as a tumor promoter through changes in the tumor microenvironment and tumor-type specific properties. By inhibiting TGFβ signaling, it is hypothesized that anti-cancer effects can be achieved.

As proof of concept for carcinogenesis, studies have highlighted the therapeutic potential of antagonizing the TGFβ signaling pathway for this endpoint. Earlier work that initiated an interest in targeting the TGFβ signaling pathway focused on the effect of inhibition by TGFβ receptor agonists and transgenic mouse models in order to prevent epithelial carcinogenesis. While the TGFβ type II receptor has been proposed to be a tumor suppressor, evidence shows that inhibiting receptor activity actually inhibits tumor formation and progression. Transgenic mice expressing a TGFβ antagonist of the soluble
type II TGFβ receptor:FC fusion protein through the regulation by the mammary-selective MMTV-LTR promoter were also used to determine the effect of long term inhibition of TGFβ signaling. When crossed with the MMTV-neu transgenic mouse model of metastatic breast cancer and in a tail vein metastasis assay, these mice were resistant to the development of metastases at multiple organ sites compared to wild-type controls. Additionally, the transgenic mice did not exhibit the pathology characteristic of TGFβ1 null mice when exposed to the antagonist long-term, suggesting that neutralization of TGFβ may be selectively inhibiting metastasis, while allowing TGFβ in the unaffected tissues to act normally in its protective and homeostatic function (484). In a short term study using a similar model, reduced apoptosis, tumor cell motility, intravasation, and lung metastasis was seen when the Fc:TβRII transgenic mouse was crossed with the mouse mammary tumor virus promoter coupled with the polyomavirus middle T antigen (MMTV-PyT) transgenic mouse. Antagonism by the fusion protein also inhibited metastasis in transplanted 4T1 and EMT-6 mammary tumors in syngeneic BALB/c mice (485). T-cell specific blockade of TGFβ signaling by CD4-dn-TβRII mice leads to reduced tumor formation when injected with the metastatic murine melanoma line B16-F10 and the murine lymphoma line EL-4 when observed for 90 days (352).

However, reduced TGFβ1 signaling has also shown detrimental effects on inhibiting tumor progression in vivo. Stable transfection of the dominant negative-TβRII cDNA which is truncated and kinase-defective in a human oral carcinoma cell line showed decreased growth inhibition when treated with TGFβ1 and reduced differentiation, a marker of a more aggressive phenotype, when transplanted into the mouth of athymic mice. Invasion and metastasis to the lungs and lymphatic system were also observed with the type II receptor knockdown (486). Transgenic mice that express the dominant negative TGFβ type II receptor under the control of the mouse mammary tumor virus promoter (MMTV-DNTβRII) spontaneously developed mammary tumors, mostly carcinomas in situ, although they also showed reduced tumor invasion (487). A similar result was also seen with MMTV-TβRII mice treated with DMBA, although there was no difference in spontaneous tumorigenesis. These mice showed enhanced incidence and multiplicity of lung and mammary tumors (488). However, expression of TGFβ in MMTV-TGFβ1 mice caused suppression of mammary tumorigenesis when subjected to DMBA (489). In a liver carcinogenesis model where mice were treated with the chemical carcinogen diethylnitrosamine, mice heterozygous for the deletion of the TGFβ type II receptor gene (490) and DN-TβRII transgenic mice that overexpress the transgene in hepatocytes (491) had enhanced tumorigenesis in the liver compared to wild-type mice.

Much less attention has been given to understanding the role of the TGFβ type I receptor in carcinogenesis. An inducible head and neck-specific TGFβ type I receptor knockout mouse model has been use to better understand the role of this receptor in squamous cell carcinoma. Upon tumor induction
with DMBA, 45% of the knockout mice developed SCCs starting 16 weeks following treatment, while no tumors were observed in wildtype mice. The mechanism for this difference was due to a decrease in apoptosis, increase in proliferation and activation of the PI3K-Akt pathway in tumor bearing knockout mice (492). A similar result occurred with conditional deletion of TβRI (TβRI floxed mice crossed with neurofilament H Cre mice). 35% of knockout mice developed spontaneous squamous cell carcinomas in periorbital and or perianal regions. To confirm tumorigenicity, these tumors were transplanted onto athymic nude mice, resulting in 62% tumor formation. Further analysis indicated that this result was related to immune system evasion, with increased expression of interleukin-13 receptors which correlates with clinically advanced head and neck SCCs (493). In another model of epithelial cancer, TβR1(+/-) mice were crossed with APC(Min/+), mice which causes intestinal tumorigenesis and multiple intestinal adenomas, mimicking human familial adenomatosis polyposis coli. Twice as many intestinal tumors and adenocarcinomas of the colon developed with TβRI knockdown which may be a result of increased tumor cell proliferation and cyclin D1 expression (494). Expression of TβRI also resulted in poorly differentiated oral tumors compared to more differentiated lesions and normal epithelium (495). Since the type II receptor can complex with other type I receptors, targeting a specific type I receptor can potentially lead to more distinct biological outcomes, rather than an array of off target effects. ActRI can complex with the type I receptors ALK2, ALK3, or ALK1 (496, 497). Signaling through TβRII/ALK1 complexes activates Smad1, Smad5, and Smad8, whereas signaling through the TβRII/ALK5 complex results in phosphorylation of Smad2 and Smad3. Furthermore, targeting the type I receptor eliminates changes in Smad independent signaling that may occur through the type II receptor.

However, due to the dual nature of the TGFβ1 signaling pathway, responsible for both tumor suppression and oncogenic activities, use of these pharmaceuticals can be quite challenging. The small molecule inhibitor A-83-01 inhibited epithelial to mesenchymal transition, which could potentially prevent the progression of epithelial cancers (498). Intracranial SMA-560 gliomas in syngeneic VM/Dk mice treated with ALK5 inhibitor SD-208 show increased survival, although this cannot be correlated with changes in angiogenesis, proliferation, or apoptosis. Instead, this response correlated with immune cell infiltration (499). In vivo studies show that inhibition via i.p. injection 1-12 hours post-treatment in malignant mesothelioma tumors (500) and via oral exposure at 2 hours post-exposure in mammary fat-pad tumors (500, 501) inhibits Smad2 phosphorylation. The ALK5 inhibitors SD-208 and SM16 have also been shown to inhibit growth and metastasis of mouse mammary carcinoma (502, 503). Using the ALK-5 inhibitor SB-525334, treatment promoted an increase incidence of epithelial renal cell carcinoma in Eker rats, although the incidence and volume of mesenchymal tumors were inhibited (504).
Whereas TGFβ antibodies and various levels of TGFβ blockade have been explored to determine the role of TGFβ1 signaling in skin carcinogenesis, the use of ALK5 inhibitors allows for inhibition of signaling of all isoforms of TGFβ. Small molecule inhibitors are orally and topically available and fairly inexpensive to administer. Small molecule inhibitors that target the TGFβ type I receptor kinase are ATP mimetics, and competitively bind to the ATP binding site of the kinases. ALK5 inhibitors are inherently not as specific as other pharmaceuticals such as antisense or antibody therapies. However, they are designed to be specific for the phosphorylation of Smad2 and Smad3. Yet, these studies do not address long-term treatment with these pharmacological agents. While this class of drugs are not specific for the ALK5, there is evidence that activin signaling may be similar to TGFβ, acting as both a tumor suppressor in tumor formation and an oncogene in advanced cancers (505-507). Some signaling molecules can also be transduced through both ALK5 and ALK4, such as the case with Smad2/3 and Cripto-1 (388, 508). By targeting all 3 kinases, we are able to block all signaling that occurs via upstream precursors. Also, by targeting the type I receptor, we are able to ensure that signaling of independent pathways that originate from the type II receptor are not being affected.

1.2.7 SB431542

SB431542 (SB) is a small molecule inhibitor that was first identified through the screening of the internal compound collection for inhibitors of the ALK5 receptor at GlaxoSmithKline Pharmaceuticals. SB421542 was identified using a high-throughput screen with a flash-plate-based assay (homogenous radiometric assay), that allows for detection of Smad3 phosphorylation as an endpoint readout. This assay resulted from the optimization of an internal compound collection for inhibitors of ALK5 at GlaxoSmithKline.

Figure 1.16: Chemical structure of SB431542

4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide resulted from the optimization of an internal compound collection for inhibitors of ALK5 at GlaxoSmithKline.
was developed using the kinase domain of ALK5, as well as the C-terminal and full length Smad3 protein (as the immobilized substrate) which were expressed as N-terminal glutathione S-transferase (GST) fusion proteins in a baculovirus expression system. These proteins were then coated on the flash-plates in the presence of \([\gamma-^{33}]\text{ATP}\) which was detected in order to quantify alterations in Smad3 phosphorylation by ALK5 (409). Several p38 kinase inhibitors were identified. However, in order to identify small molecule inhibitors that were specific for ALK5, a screening hit SB-202620 (1; Figure 1-16) was used with a target-hopping strategy to further increase potency. This molecule has a triarylimidazole template and 4-pyridyl substituent, making it an equipotent inhibitor of ALK5 and p38. Replacing the 4-pyridyl with the 2-pyridyl substituent, as described with the SKF-104365 compound (2; Figure 1-13, 1-16), then reduced the inhibitory activity of p38, although ALK5 inhibition was lost slightly. Introduction of a 3,4 methyl-enedioxyphenyl ring was found to be better at inhibiting Smad3 in the ALK5 kinase assay (3; Figure 1-16), although this was less pronounced in a cellular assay for changes in TGF\(\beta\)1-induced fibronectin mRNA (509). Since the carboxylic acid functional group of this molecule is known to limit cell permeability, carboxamide was substituted which resulted in the most potent inhibitor of the series (4; Figure 1-16) (409). This commercially available inhibitor (4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide) was later named SB431542.

In the initial small molecule identification studies, SB showed the greatest potency in inhibiting ALK5 as measured by TGF\(\beta\)-induced fibronectin mRNA formation in A498 cells (IC\(_{50}\)=94nM) and had no effect on p38 kinase activity (409). It shows selectivity for the kinase activity of ALK5, and to a lesser extent activin receptor ALK4 and the nodal receptor ALK7. This inhibitor has no direct effect on Smad-independent signaling such as ERK, JNK or p38 MAP kinase pathways, nor the Activin and BMP receptors ALK2 and ALK6, respectively (416). It acts as a competitive ATP binding site kinase inhibitor and has been shown to inhibit the \textit{in vitro} phosphorylation of Smad3 (409) and -2 (510). While some small molecule inhibitors directed toward ALK5 are also inhibitory to the TGF\(\beta\) type II receptor (compounds 3 and 4, Figure 1-13), SB431542 is inactive. This is explained by the fact that when a substituted aryl group is located at the 4-position of the dihydropyrrolopyrazole, pyrazole ring or imidazole rings, neither the TGF\(\beta\) type II receptor nor p38\(\alpha\) is inhibited (401). Pharmacokinetic profiling of SB431542 has been performed \textit{in vivo}. Femoral vein catheters in Sprague-Dawley rats were used to infuse SB. Clearance of SB431542 was 37.5 ± 12.2 ml min\(^{-1}\) kg\(^{-1}\) and the half-life was 28.5 ± 16.1 minutes following intravenous injection (458).

In connection to the role of ALK5 in fibrotic diseases, SB431542 was shown to inhibit the mRNA expression of extracellular matrix markers fibronectin, collagen I\(\alpha\)1 and PAI-1 (511). Studies have shown that SB431542 inhibits TGF\(\beta\)1-induced transcription and gene expression, apoptosis, and growth suppression. It also diminishes the tumor-promoting effects of TGF\(\beta\)1 through inhibition of
epithelial to mesenchymal transition, cell motility, migration and invasion in human cell lines (512). In mouse mammary cell lines, SB431542 induces apoptosis, which may be protective against tumor formation and metastasis (513). It also reduced cell proliferation, angiogenesis, and motility in human malignant glioma cell lines, indicating a potential therapy for malignant gliomas (514). Yet, Shukla et al. show that treatment of keratinocytes with SB431542 can induce expression of keratin 8 and block keratin 1, alteration that are characteristic of a progressive tumor phenotype (388). Pharmacological inhibition of the TGFβ type I receptor has been shown to regulate tumor progression in vivo, altering the CD8+ antitumor response (500), cytotoxic T lymphocytes (500, 502, 503), and natural killer cells and macrophages (515). These studies demonstrate the importance of the TGFβ1 type I receptor as an immunomodulator in cancer progression.
1.3 Hypothesis and Aims

TGFβ1 is a cytokine, a potent growth inhibitor in epithelial cells and is important in the regulation of development, inflammation and carcinogenesis. TGFβ transduces its signaling by binding to and activating the type II receptor. The type II receptor binds and activates ALK5 which is the predominant TGFβ1 type I receptor. ALK5 then phosphorylates the Smad2 and Smad3 proteins, which bind to Smad4 and translocates into the nucleus to induce signal transduction (128). TGFβ1 plays a dual role in cancer acting as both a tumor suppressor and oncogene, and the use of the two-stage skin chemical carcinogenesis assay has been vital in dissecting how alterations to TGFβ1 signaling can impact tumorigenesis (246, 247). In this protocol the epidermis is treated once with the carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA) to cause an initiating mutation in the HRas gene. This is followed by repeated application of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) which results in the clonal expansion of mutated cells (84). Previous studies investigating the TGFβ1 signaling pathway in cancer have shown variable and sometimes unpredictable results. Transgenic mice that express the TGFβ1 transgene (258) or TGFβ superfamily members (259) in the epidermis have reduced papilloma formation, although the same is observed in TGFβ1(+/−) (273) and Smad3 (272) knockout mice. On the other hand, both TGFβ1 overexpression (258) and TGFβ1 heterozygosity (273) resulted in enhanced malignant progression. Yet, Smad3 knockout mice exhibited reduced malignant conversion (272). Investigation of the TGFβ type II receptor show that mice dominant negative for the receptor have an earlier appearance of both papillomas and carcinomas (269-271). Studies have not previously investigated the role of ALK5 in a multi-stage model of carcinogenesis.

Considering its role in carcinogenesis and other diseases, the TGFβ signaling pathway is a possible target for therapeutics. In response, pharmaceutical companies are developing small molecule inhibitors of ALK5 like SB431542 (SB) that are potent in blocking the TGFβ signaling pathway, with potential use as anti-cancer and anti-fibrotic agents. As an anti-fibrotic drug, ALK5 have shown promise through the use of in vitro (459), in vivo (458) and ex vivo (516-518) experiments. ALK5 inhibitors have also been investigated for anti-cancer effects in vivo showing that treatment increased survival (519) and inhibited growth and metastasis (500, 501), although another study showed that ALK5 inhibition increased the incidence of epithelial renal cell carcinoma (504). Furthermore, genetic studies show that deletion of ALK5 frequently resulted in enhanced malignant progression in other epithelial cancers (492-495). However, studies have not investigated pharmacological ALK5 inhibition in an in vivo model of tumor formation and progression. While some research has investigated the role of the TGFβ type I receptor in carcinogenesis, little work has sought to elucidate the role of this receptor following long-term suppression of receptor activity. We hypothesize that long-term pharmacological inhibition of the TGFβ type I receptor inhibits tumor formation but accelerates malignant conversion in vivo.
In Chapter 2, mRNA, protein and immunofluorescence analysis are used to validate the use of SB for topical treatment of mouse skin. To determine the effects of pharmacological ALK5 inhibition by SB on tumor number, incidence and size, the two-stage skin chemical carcinogenesis assay is used. Proliferation, apoptosis and the inflammatory response are analyzed in order to determine a mechanism for altered tumorigenesis. Additionally, an inducible model of epidermal HRAS expression is used to support the results showing altered differentiation and inflammatory responses of epidermal cells to ALK5 inhibition. These studies provide a rational foundation for determining the efficacy and risks involved in targeting the TGFβ signaling pathway as a potential anti-cancer/anti-fibrotic therapy.

Chapter 3 uses in vitro and in vivo techniques to further elucidate the mechanisms by which pharmacological ALK5 inhibition alters skin carcinogenesis. An inducible oncogenic HRAS keratinocyte model is established in order to examine the role of TGFβ signaling in terminal differentiation as a mechanism for altered tumor formation. Changes in gene expression of differentiation markers, proliferation and cornification following treatment with SB and TGFβ1 are investigated in HRAS-expressing keratinocytes and skin as well as papilloma and squamous cell carcinoma cell lines. A subset of keratinocytes resistant to terminal differentiation is also characterized. Others have shown that TGFβ1 inhibits terminal differentiation in keratinocytes induced to differentiate (395-397). Additionally, cells that are inhibited from undergoing terminal differentiation demonstrate characteristics of malignant conversion (77, 520, 521). Therefore, these studies will be important in identifying a mechanism by which pharmacological ALK5 inhibition can alter tumor formation and malignant progression through terminal differentiation.

Due to the cancer stage specific responses of TGFβ1, elucidating the effect of ALK5 pharmacological inhibition both in vivo and in vitro will help identify potential risks involved with targeting the TGFβ pathway for therapeutics.
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Chapter 2

Use of a TGFβ Type I Receptor Inhibitor in Mouse Skin Carcinogenesis Reveals a Dual Role for TGFβ Signaling in Tumor Promotion and Progression

2.1 Abstract

Pharmacological inhibitors of the Transforming Growth Factor β (TGFβ) type I receptor (ALK5) have shown promise in blocking growth of xenotransplanted cancer cell lines but the effect on a multistage cancer model is not known. To test this we treated mouse skin with SB431542 (SB), a well-characterized ALK5 inhibitor, during a 2-stage skin carcinogenesis assay. Topical SB431542 significantly reduced the total number, incidence and size of papillomas compared to 12-O-tetradecanoylphorbol 13-acetate (TPA) promotion alone, and this was linked to increased epidermal apoptosis, decreased proliferation and decreased cutaneous inflammation during promotion. In contrast, the frequency of conversion to squamous cell carcinoma (SCC) was two-fold higher in papillomas treated with SB431542. While there was no difference in tumor cell proliferation in early premalignant lesions, those that formed after SB431542 treatment exhibited reduced squamous differentiation and an altered inflammatory microenvironment similar to SCC. In an inducible epidermal HRAS transgenic model, treatment with SB431542 enhanced proliferation and cutaneous inflammation in skin, but decreased expression of keratin 1 and increased expression of simple epithelial keratin 18, markers of premalignant progression. In agreement with increase frequency of progression in the multistage model, SB431542 treatment resulted in increased tumor formation with a more malignant phenotype following long-term HRAS induction. In contrast to the current paradigm for TGFβ1 in carcinogenesis, these results demonstrate that cutaneous TGFβ signaling enables promotion of benign tumors but suppresses premalignant progression through context dependent regulation of epidermal homeostasis and inflammation.

2.2 Introduction

Transforming growth factor β1 (TGFβ1) is a member of a large family of regulatory molecules that play both positive and negative roles in epithelial cancer. Most epithelial cells produce TGFβ1 and respond to it through a heterodimeric receptor complex composed of the TGFβ type II and TGFβ type I (ALK5) receptor (1). Small molecule inhibitors of ALK5 have been developed for therapeutic use in cancer and other diseases where TGFβ overexpression is linked to disease phenotype. A commercially available small molecule inhibitor of the TGFβ type I receptor, SB431542 (SB) (4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide), shows selectivity for the kinase activity of ALK5, and to a lesser extent other members of the TGFβ superfamily including the activin signaling receptor ALK4 and the nodal receptor ALK7. It acts as a competitive ATP binding site kinase
inhibitor and has been shown to inhibit the *in vitro* phosphorylation of Smad3 (2) and Smad2 (3). This inhibitor has no direct effect on Smad-independent pathways such as ERK, JNK or p38 MAP kinase pathways (4).

Recent *in vitro* and *in vivo* studies with ALK5 inhibitors have shown promise in preclinical models for inhibition of malignant tumor growth (5-10) and suppression of fibrotic diseases (11-14), but studies in genetically altered mouse models indicate that disruption of this pathway could enhance malignancy (15-18). Further, the complex role of TGFβ1 signaling in epithelial carcinogenesis suggests ALK5 inhibitors could have both inhibitory and promoting effects on cancer, but this has not been tested in a long-term multistage cancer model which could be informative to clinical studies. The mouse 2-stage chemical carcinogenesis model in which the epidermis is treated once with the carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA) followed by repeated application of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) has been extensively used to determine how the TGFβ signaling pathway affects cancer development. In this model nearly all tumors that form have activating mutations in codon 61 of the *HRas* gene, but only a small fraction of benign lesions progress to squamous cell carcinoma (SCC) (19, 20). Both overexpression of TGFβ1 (21) or TGFβ superfamily members (22) in the epidermis or reduction of TGFβ1 expression (23) and genetic deletion of Smad3 (24) suppress formation of benign tumors. In contrast, reduction of TGFβ1 levels or inactivation of TGFβ receptor signaling in the epidermis enhances conversion to SCC (23, 25).

Here we have determined how topical SB431542 treatment of DMBA initiated skin during the promotion phase affects squamous tumor formation and progression. In contrast to the generally accepted paradigm of TGFβ1 as a tumor suppressor during early stages of tumor formation and a promoter of malignant progression and metastasis, our results show that pharmacological inhibition of cutaneous TGFβ signaling blocks benign tumor formation. However the tumors that form have an enhanced frequency of conversion to SCC. This differential effect on benign and premalignant lesions is associated with potent effects of ALK5 inhibition on the squamous epithelial phenotype and the inflammatory microenvironment associated with tumor formation and progression.

### 2.3 Materials and Methods

#### 2.3.1 Animal Studies:

For two-stage chemical carcinogenesis studies, female FVB/n mice in the resting phase of the hair cycle (7 weeks of age) were initiated with a single topical dose of 50 µg of DMBA (Sigma, St Louis, MO). Ten days after initiation, mice were treated with 5 µg of TPA (Calbiochem, La Jolla, CA) in 200 µL of acetone twice a week and/or 10.0µM SB431542 (Sigma, St. Louis, MO) in 200 µl acetone for 25 weeks. This concentration of SB431542 was chosen based on previous work showing that this inhibitor
effectively reduces phosphorylation of Smad2 following activation of TGFβ1 signaling in the K5rTA x tetOTGFβ1 mouse model (26). After 25 weeks the tumors were allowed to develop up to 52 weeks. Mice were euthanized by overexposure to carbon dioxide as squamous cell carcinoma formation occurred to adhere to Institutional protocols. The number of papillomas per mouse (> 1mm in diameter) was calculated by dividing the total number of papillomas at each time point by the maximum number of mice developing tumors. Tumors from each mouse were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned and H&E stained. A pathologist examined H&E sections of papillomas and suspected carcinomas. In a similar experiment, 7 week-old FVB/n female mice were initiated with 50µg DMBA and 10 days later treated with 5µg TPA and/or 10.0µM SB431542. Developing papillomas were harvested at 12 weeks of tumor promotion, 4h after the last TPA/SB treatment.

For tumor promotion experiments, mice were treated twice daily for 2 days with SB, and then with TPA-4h/SB-4h or TPA-24h/SB-12h (acute). For the chronic tumor promotion experiment, female FVB/n mice were treated twice/weekly with TPA and thrice/weekly with SB and tissue was harvested 48h following the last SB treatment and 24h following the last TPA treatment.

To induce oncogenic HRAS expression in the epidermis bitransgenic Involucrin tTA x tetORAS mice were generated from a cross of the Involucrin tTA line (27) and the tetORASV12G line (28). Seven week old mice were removed from water containing 10 µg/ml doxycycline and treated with 200 µL of acetone and/or 200 µL of 10.0µM SB431542 every other day for 5 days. Skin was harvested 24h after the last SB treatment.

Tumors and skin from each mouse were fixed in 10% neutral buffered formalin or 70% ethanol and then paraffin embedded. Sectioned tissue was cut at 5µm and placed on charged slides to ensure adhesion during the staining process. All animals were injected intraperitoneally with bromodeoxyuridine (BrdU) 1h before sacrifice (6mg in 300µl PBS per mouse). All animals were kept under a controlled environment of temperature and humidity and a 12h light/dark cycle. Animal studies were conducted under approved Institutional protocols.

2.3.2 Tissue Analysis:

2.3.2.1 Histopathology analysis:

Histopathological analysis of tumors and measurement of epidermal thickness was done on Hematoxylin and Eosin (H&E) stained sections of neutral buffered formalin-fixed tissues. Sections were deparafinized, rehydrated and incubated for 30 minutes in Gill’s Hematoxylin. Slides were then rinsed for 10 minutes in running tap water and incubated in Eosin for 30 seconds, followed by rehydration. The number of cell layers in the epidermis was counted every 20 basal cells for each section.
Photomicrographs of tissue sections were made using an Olympus BX61Epi-Fluorescence Microscope, Olympus DP71 camera, UPlan Fl objectives and DP-BSW Basic Software.

2.3.2.2 Proliferation analysis:

Cell proliferation was measured using anti-BrdU immunohistochemistry (Becton Dickson, Franklin Lakes, NJ) and was performed in skin, papillomas and SCCs. Sections were deparaffinized, rehydrated and incubated with 4N HCl for permeabilization. Endogenous peroxidase was blocked with 3% H₂O₂ in distilled water. Sections were blocked with 10% normal goat serum for 20 minutes at room temperature and subsequently incubated with primary mouse anti-BrdU antibody (Becton Dickinson) applied to the sections at a 1:50 dilution and incubated overnight at 4°C. Slides were then incubated with secondary biotinylated goat anti-mouse IgG (Jackson ImmunoResearch) diluted 1:1000 for 40 minutes at room temperature followed by incubation in avidin biotin horseradish peroxidase (ABC kit, Vector Laboratories) for 30 minutes at room temperature. 3,3’-diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories, Burlingame, CA) was used following the manufacturer’s recommended procedures for detection of positively labeled cells. The sections were counterstained with Gill’s hematoxylin and visualized under a light microscope. Proliferation was expressed as the percentage of BrdU positive cells per basal cells in ethanol-fixed sections, 5 fields/section at 400x magnification. Photomicrographs of tissue sections were made using an Olympus BX61Epi-Fluorescence Microscope, Olympus DP71 camera, UPlan Fl objectives and DP-BSW Basic Software.

2.3.2.3 Apoptosis analysis:

Apoptotic cells were identified using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (TUNEL) in ethanol-fixed tissues. Sections were deparaffinized, hydrated and incubated with proteinase K at room temperature for 15 minutes, followed by 5 minutes incubation with 3% H₂O₂ at room temperature. Sections were then incubated for 5 minutes with TdT reaction buffer (25 mM Tris-HCl, 200mM Sodium Cacodylate, 0.25 mg/mL BSA, 1mM CoCl₂) followed by incubation with terminal deoxyribonucleotidyltransferase enzyme (New England Biolabs, Ipswich, MA) and digoxigenin-11-uridine-5'-triphosphate (Roche, Basel, Switzerland) in the TdT reaction buffer. After incubation at 37°C for 1 hour in a humidified box, the reaction was stopped with 1X Trevigen TdT Stop Wash Buffer by application to each section and incubating for 5 minutes at room temperature. Sections were blocked with 10% normal rabbit serum for 20 minutes at room temperature and subsequently incubated with sheep anti-digoxigenin (Roche) applied to the sections at a 1:800 dilution and incubated for 30 minutes at room temperature. Slides were then incubated with secondary biotinylated rabbit anti-sheep IgG (Vector) diluted 1:2000 for 20 minutes at room temperature followed by incubation in avidin biotin horseradish
peroxidase (ABC kit, Vector Laboratories) for 30 minutes at room temperature. 3,3’-diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories) was used following the manufacturer’s recommended procedures for detection of positively labeled cells. The sections were counterstained with Gill’s hematoxylin and visualized under a light microscope. Apoptotic cells were expressed as a percentage of total cells from 5 fields/section at 400x magnification.

2.3.2.4 Inflammatory cell analysis:

Neutrophils were detected using an anti-myeloperoxidase (MPO) antibody (Dako, Denmark) in skin, papillomas and SCCs. Sections were deparafinized, rehydrated and endogenous peroxidase was blocked with 3% H₂O₂ in methanol for 20 minutes at room temperature. Sections were blocked with 5% normal goat serum for 30 minutes at room temperature and subsequently incubated with primary mouse rabbit anti-human MPO (DAKO, Denmark) applied to the sections at a 1:500 dilution and incubated 30 minutes at room temperature. Slides were then incubated with secondary biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:2000 for 30 minutes at room temperature followed by incubation in avidin biotin horseradish peroxidase (ABC kit, Vector Laboratories) for 30 minutes at room temperature. 3,3’-diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories) was used following the manufacturer’s recommended procedures for detection of positively labeled cells. The sections were counterstained with Gill’s hematoxylin and visualized under a light microscope. The average number of positive cells per field was scored, 5 fields per section at 400x magnification. Photomicrographs of tissue sections were made using an Olympus BX61Epi-Fluorescence Microscope, Olympus DP71 camera, UPlan Fl objectives and DP-BSW Basic Software.

An anti-F4/80 (Serotec, Raleigh, NC) (29) antibody was used for indirect immunofluorescence in ethanol-fixed sections to detect macrophages in tumor-promoter treated skin. Sections were deparafinized, rehydrated and antigen retrieval performed using antigen unmasking solution (Vector Laboratories) heating slides twice for 5 minutes at 20% power. Sections were blocked with 10% normal goat serum for 30 minutes at room temperature and M.O.M. Mouse IgG Blocking reagent (Vector Laboratories) for 1 hour at room temperature. Sections were then incubated with M.O.M diluent protein concentrate for 5 minutes, and primary anti-F4/80 antibody in the M.O.M diluent protein concentrate was applied to the sections at a 1:100 dilution and incubated 30 minutes at room temperature. Slides were then incubated with secondary biotinylated goat anti-rat IgG (Jackson ImmunoResearch) diluted 1:250 for 30 minutes at room temperature and streptavidin bound AlexaFluor 488 (1:200 dilution) for 30 minutes at 4°C protected from light. Sections were counterstained with TO-PRO3 (1:5000 dilution) for 20 minutes at 4°C protected from light. Sequential scans of indirect immunofluorescence stained sections were imaged using an Olympus FV300 Laser Scanning Confocal Microscope, Inverted Olympus IX-70 microscope,
using Fluoview 300 Version 4.3b software. Alexa Fluor 488 was excited using a 488nm Argon ion laser and collected through 510nm LP and 530nm SP filters. TOPRO-3 was passed through a 660nm LP filter after 633nm HeNe excitation. A 400X UplanFl objective was used with 2x zoom. Images were saved as a 24-bit TIFF file.

### 2.3.2.5 Keratin analysis:

Immunohistochemistry for loricrin, keratins 1, 13 (30) and 8 (31) were used in ethanol-fixed sections of skin (23) in order to detect early markers for malignant conversion in papillomas. Sections were deparafinized, rehydrated and endogenous peroxidase was blocked with 3% H$_2$O$_2$ in distilled water for 5 minutes at room temperature. Sections were blocked with 10% normal goat serum for 20 minutes at room temperature and subsequently incubated with primary keratin 1 (1:2000), keratin 10 (1:500), keratin 8 (1:5), keratin 13 (1:1500), and loricrin (1:1000) antibodies and incubated overnight at 4°C. Slides were then incubated with secondary biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:1000 for 40 minutes at room temperature followed by incubation in avidin biotin horseradish peroxidase (ABC kit, Vector Laboratories) for 40 minutes at room temperature. 3,3’-diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories, Burlingame, CA) was used following the manufacturer’s recommended procedures for detection of positively labeled cells. The sections were counterstained with Gill’s hematoxylin and visualized under a light microscope. Staining was scored by two different people in 12 papillomas per treatment group and 5 animals for Involucrin/tetORAS transgenic mice. Photomicrographs of tissue sections were made using an Olympus BX61Epi-Fluorescence Microscope, Olympus DP71 camera, UPlan Fl objectives and DP-BSW Basic Software.

### 2.3.2.6 TGFβ signaling pathway analysis:

Rabbit anti-phospho-Smad2 (Millipore, Billerica, MA) (23) antibody was used for indirect immunofluorescence in ethanol-fixed sections to detect changes in TGFβ pathway activation in TPA and SB treated skin. Sections were deparafinized, rehydrated and antigen retrieval performed using antigen unmasking solution (Vector Laboratories) heating slides twice for 5 minutes at 20% power. Sections were blocked with 10% normal goat serum for 30 minutes at room temperature. Sections were then incubated with primary anti-phospho Smad2 at a 1:50 dilution for 1 hour at room temperature. Slides were then incubated with secondary biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 for 30 minutes at room temperature and streptavidin bound AlexaFluor 488 (1:200 dilution) for 30 minutes at 4°C protected from light. Sections were counterstained with TO-PRO3 (1:5000 dilution) for 20 minutes at 4°C protected from light. Sequential scans of indirect immunofluorescence stained sections were imaged using an Olympus FV300 Laser Scanning Confocal Microscope, Inverted Olympus IX-70 microscope,
using Fluoview 300 Version 4.3b software. Alexa Fluor 488 was excited using a 488nm Argon ion laser and collected through 510nm LP and 530nm SP filters. TOPRO-3 was passed through a 660nm LP filter after 633nm HeNe excitation. A 400X UplanFl objective was used with 2x zoom. Images were saved as a 24-bit TIFF file.

2.3.3 RNA Analysis:
Whole skin was homogenized using a Qiagen Tissuelyzer (Qiagen, Valencia, CA) in Trizol (Invitrogen, Carlsbad, CA). RNA was quantitated using a Beckman Coulter DU-800 Spectrophotometer at wavelengths 260nm for quantitation and 280nm for detection of protein contamination. All samples were treated for DNA contamination using the Turbo DNA-free (AM-1907, Applied Biosystems/Ambion, Austin, TX) according to manufacturer’s recommended procedures. cDNA was made from RNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems/Ambion). Quantitative RT-PCR (qPCR) was done for the indicated genes using the MyIQ system (BioRad Laboratories, Hercules, CA) and PerfeCTa SYBR Green SuperMix for iQ (#95053-500, Quanta Biosciences, Gaithersburg, MD). The following conditions were used for qPCR: 50°C, 2min.; 95°C, 10min.; (95°C, 15sec.; 60°C, 1min. x 40 cycles). All values were normalized to GAPDH. Primer sequences were obtained from published studies or using Primer 3 (32) software with Genebank sequence information.

Table 2-1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad7</td>
<td>TCTCAGGCATTCTCCTCGGAAGTCAA</td>
<td>AAGGTACACAGCATCTGGACAGCCT</td>
</tr>
<tr>
<td>LTBP-1</td>
<td>TGAATGCTTTGCTTGTTGGAG</td>
<td>GCAGTTGAGGAGCCCTCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAATGGTGAGTTGCTGTCAGCG</td>
<td>TGGCAACAATCTCCACTTCCAC</td>
</tr>
<tr>
<td>KC</td>
<td>TGGGCTGGAATACCTCAAGAACAT</td>
<td>TGTGGCTATGACTTCGTTTGGGT</td>
</tr>
<tr>
<td>MIP2</td>
<td>CTGCCGGCTCTCTCAGTCTGCACTG</td>
<td>GCCTTGCTTGTTCAGTATCTTTTGG</td>
</tr>
<tr>
<td>S100A8</td>
<td>ATGCCGGCTCAGAACCTGGAGAA</td>
<td>TGGGCTGTCTTTTGAGATGC</td>
</tr>
<tr>
<td>S100A9</td>
<td>TCATCGACACCTCTCAGCTCAA</td>
<td>TTACTTCCACAGCCTTTC</td>
</tr>
<tr>
<td>Keratin 1</td>
<td>ACATGGAAGCAAGGTGTTGAGGAGT</td>
<td>TGGTCACGAACCTTCCCCAGG</td>
</tr>
<tr>
<td>Keratin 8</td>
<td>TATGGAGGATTGCAGACCCCTGCTG</td>
<td>TCTGGCTTGGAGGCCTTTCAATCT</td>
</tr>
<tr>
<td>Keratin 18</td>
<td>TCAAGGTGAAGCTTCAGAGCAGAGA</td>
<td>ACGGTTAGTTGCTTCTGCACAGT</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>GCAACATGTGGAGAATCTCCTACCAGAA</td>
<td>GACGTCAAAGACAGCCACTC</td>
</tr>
</tbody>
</table>

Changes in gene expression of TGFβ1 target genes, neutrophil chemokines and markers for malignant conversion were investigated using the following primer sequences.
2.3.4 Protein analysis:

Following treatment, keratinocytes were washed twice with PBS, scraped off dishes, and lysed with 200µl of 0.5% NP-40 lysis buffer (0.5% IGEPAL CA-630, 250mM NaCl, 50mM Tris HCl, pH 7.4) with 1µl/ml each protease and phosphatase inhibitors including DTT (1M), Sodium OrthoVanadate pH=10 (200mM), NaF (1M), PMSF (200mM), Aprotinin (1mg/mL), Leupeptin (5mg/mL), Pepstatin (1mg/mL), and β-glycerophosphate (0.24mg/ml). Cells were rotated at 4°C for 1 hour, spun at 14,000 rpm for 15 minutes at 4°C, and the supernatant was transferred to new tubes. Whole skin was homogenized using a Qiagen Tissuelyzer (Qiagen, Valencia, CA) in 0.5% NP-40 lysis buffer (0.5% IGEPAL CA-630, 250mM NaCl, 50mM Tris HCl, pH 7.4) with 1µl/ml each protease and phosphatase inhibitors freshly added to lysis buffer including DTT (1M), Sodium OrthoVanadate pH=10 (200mM), NaF (1M), PMSF (200mM), Aprotinin (1mg/mL), Leupeptin (5mg/mL), Pepstatin (1mg/mL), and β-glycerophosphate (24.4mg/ml). Homogenized tissue was rotated at 4°C for 1 hour and spun at 14,000 rpm for 15 minutes at 4°C. The supernatant was transferred to new tubes.

Protein was quantitated using the Bio-Rad protein assay (#500-0006, Bio-Rad) based on the Bradford Method and measured at 595nm wavelength. SDS-PAGE was performed using 20µl protein per sample and transferred to a nitrocellulose membrane. Transfer efficiency and equal loading was verified by Ponceau-S staining. Following blocking in 5% non-fat dry milk in 0.1% Tween-20/Tris-buffered saline (TBS-Tween), immunoblots were incubated with antibodies overnight at 4°C in 3% bovine serum albumin/TBS-Tween using antibodies directed against Smad 2/3 (1:2000), p-Smad2 (1:1000), GAPDH (1:1000) (Cell Signaling Technology, Inc., Danvers, MA), and β-actin (1:20,000) (Millipore). Membranes were washed 6 x 10 minutes in TBS-Tween, followed by incubation in HRP-conjugated secondary antibodies (1:2000) (BioRad) in 5% non-fat dry milk/TBS-Tween at room temperature for 1 hour. Membranes were washed again 6 x 10 minutes and specific proteins were detected by chemiluminescent Western blotting substrate (ECL, Pierce, Rockford, IL). Exposed film was developed using a Konica SRX-101A medical film processor. Film was scanned using Adobe Photoshop 7.0 and images were converted to gray scale. GAPDH or β-actin were used as a loading control.

2.3.5 DNA Sequencing:

DNA was sequenced in order to identify an A→T transversion mutation in the HRas gene at codon 61. Ethanol fixed tissue was rehydrated through 3 x Histochoice, 1 x 95% ethanol, 1 x 90% ethanol, 1 x 70% ethanol and 1 x PBS each for 5 minutes and scraped into a 1.5 ml tube. DNA was extracted from tissue using the Arturus PicoPure DNA extraction kit (Molecular Devices, Sunnyvale, CA) according to manufacturer’s instructions in 20µl extraction buffer followed by incubation at 60°C for 3
hours and 95°C for 10 minutes. Samples were spun at 10,000 rpm for 1.5 minutes and supernatant was transferred to a new tube. DNA was amplified by PCR using 35 cycles for each reaction with 63.5°C annealing temperature in a 20µl reaction including 9µl 2x PCR reaction mix (Immomix, Bioline, Taunton, MA) 9µl distilled water, 1µl 10µM forward and reverse primers and 1µl DNA. First amplification primers were F:TGTGGCAGTGTCATGGTAAATG ; R:ATCTTTGACCCGCTTGATCTGCTC. A dilution of 1:40 of the first product was used for the second reaction using a similar 20µl reaction. Nested reaction primers: F:TCCATCGGGGTATGAGAGGTGCAA; R:CCTCGAAGGACTTGGGTGTGTGTA. Three nested reactions were pooled in a single well of a 2% agarose gel and elecrophoresed with a single band localizing at 270 bp. Bands were excised and purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. The final product was sequenced at the Penn State Genomics Core Facility using an ABI Hitachi 3730XL DNA Analyzer and the sequencing primer: ATTCTCTGGTGCTAGGAGAGGTGTA.

2.3.6 Statistical Analysis:

Values are expressed as the mean ± S. E. Student’s t test was used to compare the indicated groups, and the significance of the difference was described. p values of <0.05 were regarded as indicating a significant difference. Fischer’s exact test was used to determine significance of malignant conversion data.
2.4 Results

2.4.1 Topical SB431542 inhibits TGFβ signaling in the mouse epidermis

We treated primary mouse keratinocytes with SB to determine a useful dose range for inhibition of basal Smad2 phosphorylation. Figure 2-1A and 2-2A show that as expected SB significantly inhibited baseline Smad2 phosphorylation between 1µM and 10µM. Since detection of baseline p-Smad2 in the normal epidermis was difficult we analyzed expression of TGFβ1 target genes by quantitative RT-PCR following topical SB treatment, and found that expression of both Smad7, which is induced by TGFβ1 (33, 34), and latent TGFβ binding protein 1 (LTBP1) was significantly reduced within 4 hours of SB treatment (Figure 2-1B). Since TPA induces TGFβ1 expression (35) and Smad2 phosphorylation (23) in the epidermis we used indirect immunofluorescence to determine the effect of topical SB on TPA-induced TGFβ signaling. Basal levels of p-Smad2 were undetectable in acetone or SB treated skin sections (Figure 2-1C, top), but 4 and 24 hours after TPA treatment the increase in nuclear p-Smad2 localization in keratinocytes was blocked by a pre- and post-TPA treatment with SB (Figure 2-1C, middle, bottom and 2-3). Immunoblotting verified the reduced levels of TPA-induced p-Smad2 (Figure 2-1D and 2-2B), but no change was found in TPA-induced ERK phosphorylation following SB treatment (Figure 2-4). These results indicate that the effects of SB are specific for TPA-induced TGFβ signaling and sustained for at least 12 hours after dosing.
Figure 2.1. SB431542 reduces TPA-induced Smad2 phosphorylation in vivo.

(A) Reduction in Smad2 phosphorylation following treatment with SB in keratinocytes. Full length blots presented in 2-3. (B) Quantitative RT-PCR analysis of Smad7 and LTBP-1 gene expression in acetone or SB-treated whole skin. Normalized to acetone only samples, (n=4). (C) Indirect immunofluorescence showing a reduction in TPA-induced p-Smad2 by SB at 4h and 24h TPA treatment. Magnification 800x, bar represents 15µm. P-Smad2 was detected with Alexa-fluor-488 (green), and nuclei counterstained with TO-PRO3 (red), (representative of n=5). Ep, epidermis; D, dermis. Single color images presented in Supplemental Figure 2. (D) Western blot of whole skin treated 24 hours with TPA and 12 hours SB, showing reduced TPA-induced Smad2 phosphorylation with SB treatment, (representative of n=5). Full-length blots presented in Supplemental Figure S1B. C, control; A, acetone.
Figure 2-2. Full length immunoblots showing inhibition of Smad2 phosphorylation by SB.

Full length blots of Figure 2-1A (Fig. 2-2A) and Figure 2-1D (Fig. 2-2B) showing specificity of antibodies used and molecular weight of detected bands. Exposed film was developed using a Konica SRX-101A medical film processor. Film was scanned using Adobe Photoshop 7.0 and images were converted to gray scale.
Sequential scans were made using the Olympus FV300 laser scanning confocal microscope. Alexa Fluor 488 was excited using a 488nm Argon ion laser and collected through 510nm LP and 530nm SP filters. TOPRO-3 was passed through a 660nm LP filter after 633nm HeNe excitation. A 400X UplanFl objective was used with 2x zoom. Scale bar represents 15µm. Images were saved as a 24-bit TIFF file. SB, SB431542; TPA, 12-O-tetradecanoylphorbol 13-acetate.
To determine the effect of TGFβ type I receptor pharmacological inhibition on cutaneous tumor formation and progression we treated FVB/n mice with DMBA and then promoted with TPA twice a week with and without 10µM SB431542 for 25 weeks. In this protocol mice were treated with SB just prior to TPA and an additional time in between each TPA treatment to maintain suppression of TGFβ signaling. By itself SB treatment for 25 weeks did not induce tumor development in DMBA initiated skin (data not shown). Surprisingly, topical SB treatment significantly inhibited TPA-induced papilloma formation at all time points (Figure 2-5A). In mice treated with TPA alone, the peak number papilloma/mouse was 18.2 ± 8.2 at week 19 but only 11.1 ± 7.8 at week 25 in the TPA+SB treated mice. In addition the papillomas that formed with SB treatment were smaller than those that arose with TPA promotion alone between weeks 15-21 although this difference was not significant after week 23 (Figure 2-5B). Despite the reduced number of benign tumors with SB treatment the total number and frequency of SCC that formed (28 total, 1.47±0.70 SCC/mouse in the TPA and 29 total, 1.93±1.7 SCC/mouse in the SB+TPA group) as well as the latency of SCC formation (Figure 2-5C) was the same between the two treatment groups. Thus papillomas that arose with SB treatment had a 2-fold increase (15.8%) in the frequency of malignant conversion compared to TPA alone (7.4%) (Figure 2-5D). 5/5 SCCs in each group had a codon 61 mutation in *H*Ras indicating that SB was not causing outgrowth of a population of epidermal keratinocytes with a distinct initiating mutation (Figure 2-6). Since most SCC appeared after TPA+SB treatments were stopped at 25 weeks, these data suggest that the increased risk for malignant progression was established during the initial stages of promotion with SB and TPA.

**2.4.2 SB431542 inhibits papilloma formation but enhances malignant progression**

Protein extracts were made from skin of mice treated with acetone, SB, TPA or TPA+ SB for 4 hours and immunoblotted for the indicated proteins. SB, SB431542; TPA, 12-O-tetradecanoylphorbol 13-acetate.
Figure 2-5. ALK5 inhibition reduces tumor formation, but enhances malignant conversion.

(A) A significant reduction in tumor formation was observed at weeks 9-25 in mice treated with TPA+SB compared to TPA alone. (B) A significant decrease in tumor volume with SB treatment was observed at weeks 15, 17 and 21. Average volumes were determined from measurements of length x width x height using a digital micrometer. (C) Increased frequency of malignant conversion with SB treatment. Percent conversion was determined by dividing the total number of SCC that formed during the course of the study by the maximum number of papillomas. (D) No difference in latency of SCC formation determined by morphology and verified by histology. SB, SB431542; TPA, 12-O-tetradecanoylphorbol 13-acetate.
Figure 2-6. Similar A→T transversion present at codon 61 of HRas in SCCs induced with TPA and TPA+SB.

DNA was extracted from ethanol fixed tissue sections and amplified using a set of nested primers and the purified product sequenced. Image shows representative sequencing read of TPA (left) and TPA+SB (right) DNA with arrow pointing to appearance of T (red peak) at second base of codon 61.
2.4.3 Suppression of papilloma formation linked to altered epidermal homeostasis and reduced cutaneous inflammation

Since SB blocked TPA mediated activation of TGFβ signaling in the skin we tested if the reduction in papilloma formation was linked to inhibition of the tumor promoting effects of TPA on normal skin. We analyzed epidermal hyperplasia, proliferation and apoptosis following 2 weeks of repeated TPA treatment with or without SB. Figure 2-7A shows that SB treatment caused a slight inhibition of TPA-induced epidermal hyperplasia (top) associated with a 10 percent decrease in keratinocyte proliferation (middle) and an increase in TUNEL+ keratinocytes (bottom). Similar to that seen with a TGFβ type II receptor knockout (18), SB by itself caused an increase in apoptosis in the epidermis suggesting that the effects on apoptosis were due to direct inhibition of TGFβ signaling. In addition to inducing epidermal hyperplasia, TPA caused significant cutaneous inflammation which is critical for tumor promotion. Figure 2-7B shows that in mice treated topically with SB there was a sustained inhibition of both acute and chronic TPA-induced cutaneous inflammation. Twenty-four hours after treatment with TPA and SB there was a 4-fold reduction in myeloperoxidase (MPO)+ cell infiltrate into the skin compared to TPA alone, and this inhibition was maintained throughout a 2 week chronic treatment with TPA and SB (Figure 2-7B, 2-8). Based on low frequency of F4/80+ cells in the skin after TPA treatment (9%) the majority of these MPO+ cells are likely to be neutrophils (data not shown). Consistent with the inhibition of neutrophil infiltration SB suppressed acute (Figure 2-7C, top, bottom) and chronic (Figure 2-7C, middle, bottom) TPA-mediated induction of the neutrophil chemokines keratinocyte chemokine (KC) and MIP2 (macrophage-inflammatory protein 2) mRNA. Taken together these results suggest that the combined inhibition of TPA-induced changes in normal epidermal homeostasis and cutaneous inflammation underlie the significant reduction in papilloma number.
Figure 2-7. SB431542 suppresses TPA-induced inflammation.

(A) Effect of SB on chronic TPA-induced hyperplasia (top) proliferation (middle) and apoptosis (bottom) (B) Decreased cutaneous neutrophils (MPO+ cells) after acute and chronic TPA+SB treatment. (C) Decreased KC and MIP2 gene expression in acute and chronically SB and TPA-treated whole skin. Results normalized to TPA only samples. RT-PCR showing induction of these chemokines by TPA (bottom). n = 4-6 mice/treatment group. A, acetone.
Figure 2-8. SB suppresses TPA-induced inflammation.

Representative micrographs (n=4-6) of neutrophils in acute and chronic treatment (top), magnification 200x, and H&E stained sections of acetone, TPA and TPA+SB treated skin, magnification 200x (bottom). Scale bar represents 50µm for all images.
2.4.4 Early papillomas that develop following TPA and SB431542 exhibit a progressed tumor phenotype

To identify potential mechanisms underlying enhanced malignant conversion we analyzed early tumors that arose following 12 weeks of promotion with TPA alone or TPA+SB since any differences would reflect direct effects of SB rather than secondary changes due to progression. Figure 2-9A shows that tumor cell proliferation was not significantly different between early papillomas from either treatment group although at later time points after SB treatment had been discontinued tumor cell proliferation was greater in these tumors. In addition, there was no difference in tumor cell apoptosis as measured by TUNEL staining in these early papillomas (Figure 2-10). However, while the total tumor-associated neutrophils in these early premalignant lesions was not significantly different (Figure 2-9B) their localization within the tumor was distinct. In papillomas that arose during TPA promotion, neutrophils were located primarily in the tumor stroma (Figure 2-9C, left) but in tumors promoted with TPA+SB there was a significant increase in MPO+ cells within the epithelial component of the tumor (Figure 2-9C, right). In TPA promoted early tumors the number of tumor infiltrating neutrophils was less than 10/field and this increased to an average of 40 in the SCC. In contrast the average number of infiltrating neutrophils/field was 26 in early TPA+SB promoted tumors and not significantly different from that found in papillomas harvested at much later time points or in SCC that developed in either treatment protocol suggesting that SB promoted outgrowth of papillomas with a progressed inflammatory microenvironment (Figure 2-9D).

To further examine the possibility that early tumors arising with SB treatment exhibited a more progressed phenotype we analyzed expression of specific keratins that reflect tumor progression. Keratin 1 is a suprabasal differentiation marker in the epidermis that is highly expressed in early benign lesions but lost during tumor progression (36). In the majority of TPA only early papillomas keratin 1 was strongly expressed throughout all of the suprabasal layers while expression was reduced in tumors that arose during SB treatment (Figure 2-9E). In contrast TPA+SB promoted papillomas had an increase in percentage of keratin 13 positive cells compared to TPA only tumors. Since keratin 13 is not normally expressed in the epidermis or in early papillomas but is increased during tumor progression (37) these data suggest that at early time points SB treated tumors represent a more progressed stage of tumor development.
Figure 2-9. Early papillomas from SB treated mice have a progressed phenotype.

(A) Tumor cell proliferation in early (n=6) and late papillomas (n=10) and SCC (n=5) measured by BrdU incorporation counted at magnification 400x. (B) No significant change in total neutrophils in early papillomas (MPO+ cells) per field, counted at magnification 400x, (n=6). (C) Localization of neutrophils in early papillomas from TPA and SB/TPA treated mice at magnification 400x with scale bar representing 20µm. Arrows indicate neutrophils. Yellow line shows epidermal/dermal junction. (D) Effect of SB on tumor infiltrating neutrophils in early (n=6) and late (n=18) papillomas and SCC (n=5). (E) Reduced keratin 1 levels in early SB treated papillomas (left). Representative micrographs, magnification 100x, scale bar representing 50µm. Yellow line shows epidermal/dermal junction. Decreased expression of keratin 1 and increased expression of keratin 13 mRNA in early papillomas treated with SB, (n=12) (right). Percent keratin staining in the skin was scored blindly by two individuals.
2.4.5 SB rapidly alters phenotype of HRAS expressing epidermis

Since papillomas arising in SB treated skin had a phenotype distinct from that of TPA promoted papillomas and from TPA+SB treated normal skin we tested if oncogenic HRAS altered the response to ALK5 inhibition using a bitransgenic model in which human RASV12G (28) is conditionally overexpressed in the epidermis with an Involucrin tTA driver line (27). When adult bitransgenic mice are removed from 10ug/ml doxycycline (Dox) in the drinking water HRAS expression is induced within 1-2 days. Seven week old InvtTA/tetORAS mice were taken off doxycycline and treated with SB or acetone for 5 days. Overexpression of RASV12G caused epidermal hyperproliferation and this was enhanced by topical treatment with SB, although there was only a modest increase in epidermal thickness (Figure 2-11A), and no difference in apoptosis (data not shown). In contrast to normal skin, HRAS-induced neutrophil infiltration into the dermis was increased by treatment with SB from 40 MPO+ cells per field to 80 MPO+ cells per field (Figure 2-11B), and there was a 3-fold increase in expression of the proinflammatory chemokines S100A8, S100A9 and KC (Figure 2-11C). Similar to the papillomas that arose during SB treatment in the 2-stage carcinogenesis study SB treatment of HRAS expressing epidermis downregulated keratin 1 expression at both the RNA and protein level (Figure 2-11D). Although we did not detect increased expression of keratin 13, SB treatment caused a significant increase in mRNA level expression of keratin 18, a marker of simple epithelia and malignant conversion of squamous epithelia (38, 39) (Figure 2-11D, right). When InvtTA/tetORAS mice are treated with a reduced Dox concentration (250ng/ml) in drinking water, focal lesions form after 2-3 weeks. Following 5 weeks
expression of HRAS and treatment with SB, a significant increase in tumor formation was found compared to acetone alone (Figure 2-12A). SB treated tumors appeared more disorganized with an appearance that closely resembled chemically-induced SCCs (Figure 2-12B) and had significantly reduced keratin 1 expression similar to short-term SB treated InvTA/tetORAS skin and SB treated tumors from the 2-stage carcinogenesis study (Figure 2-12C).
Figure 2-11. SB rapidly induces progressed phenotype in epidermis expressing human C-Ha RASV12G oncogene.

(A) Significant increase in proliferation with SB treatment in HRAS expressing skin (left) and slight increase in epidermal thickness (right). (B) An increase in MPO+ cells in the dermis of SB treated mice expressing the InvTa x tetORASV12G transgenes. Arrows indicate neutrophils. Yellow line shows epidermal/dermal junction. Representative micrographs (right), magnification, 100x. Scale bar represents 50µm. (C) Increased mRNA expression of neutrophil chemokines s100a8, s100a9 and KC with treatment of SB. (D) SB causes significant decrease in keratin 1 staining (left) and mRNA expression (middle). Representative micrographs (left), magnification 100x. Scale bar represents 50µm. Yellow dotted line shows epidermal/dermal junction. Significant increase in keratin 18 mRNA expression by quantitative RT-PCR in RAS expressing skin following SB (right). n = 4-6 mice/treatment group.
Figure 2-12. Increased tumor formation with invasive phenotype and altered differentiation in InvlTA/tetORAS mice treated with SB.

Mice were placed on 250ng/ml doxycycline in drinking water for 5 weeks to allow focal tumor formation and treated with acetone or 10µM SB431542 (SB) three times weekly. (A) A significant increase in tumor formation following treatment with SB compared to acetone alone. Tumors were counted at 5 weeks. (B) Representative H&E staining showing heavily keratinized and benign papillomatous phenotype of acetone treated tetORAS induced tumors and disorganized invasive phenotype of SB treated tumors. Magnification 100x. Scale bar represents 50µm. N=5. (C) Representative micrographs of keratin 1 staining showing reduced expression in SB treated tumors. Magnification 100x. Scale bar represents 50µm. n=5. Dotted lines mark location of basement membrane.
2.5 Discussion

TGFβ1 can act as both a tumor suppressor for early stages of cancer and a cytokine that enhances the malignant and metastatic phenotype (25). Recent studies using small molecule inhibitors of the TGFβ type I receptor kinase show that this class of compounds can alter the growth of malignant cancers in vivo and the malignant phenotype in vitro. Thus the growth of xenotransplanted syngeneic mouse mammary carcinoma (8, 10) and mesothelioma (9) and human and mouse glioma cells (7) are suppressed in vivo by ALK5 inhibitors. Despite these promising results the finding that treatment of Eker rats with an ALK5 inhibitor enhances development of renal cell carcinoma while suppressing incidence and volume of mesenchymal tumors (40) suggests that long term treatment with these agents could provoke cancer development in epithelial tissues. Here we have tested the effect of a prototypical ALK5 inhibitor, SB431542, on tumor formation in the mouse skin chemical carcinogenesis model, a well-characterized model of multistage squamous tumor development for which TGFβ1 signaling plays an important role.

We and others have shown that treatment of mouse skin with the tumor promoter TPA causes rapid induction of TGFβ1 expression in the epidermis and phosphorylation of Smad2 (23, 35). We determined that 10µM SB431542 suppressed expression of baseline levels of two TGFβ1 target genes in the epidermis as well as the induction of Smad2 phosphorylation by topical treatment with the tumor promoter TPA, and that inhibition was apparent for up to 12 hours post-SB treatment similar to previous in vivo studies with ALK5 inhibitors like SB431542 (8, 9). Although we cannot rule out off target effects on unknown pathways the lack of effect of SB on TPA-induced MAPK pathway activation as well as previous studies on the specificity of SB431542 strongly suggest that the observed effects on tumor formation are due to inhibition of TGFβ signaling.

The carcinogen DMBA causes initiating mutations in the HRas gene in epidermal keratinocytes but repeated application of tumor promoters are required for clonal expansion and formation of premalignant lesions (36). While transgenic or knockout models with blocked TGFβ receptor signaling in cutaneous or oral keratinocytes exhibit a dependence on Ras activation for tumor formation (15, 18, 41, 42) we found that repeated application of SB431542 for 25 weeks did not cause tumor formation in DMBA initiated epidermis, suggesting either that by itself pharmacological inhibition of TGFβ signaling is not sufficient to promote squamous tumors or that inhibition of TGFβ signaling in other resident or infiltrating skin cells suppresses any promoting effects on keratinocytes with an activated HRas mutation. In support of the latter concept our results show that SB431542 suppressed the number and size of TPA-induced premalignant squamous papillomas relative to TPA alone. These results are consistent with our recent study showing that TGFβ1+/- mice developed fewer and smaller papillomas in a similar skin carcinogenesis protocol (23). This reduction in tumor number and size may be accounted for by the decrease in epidermal proliferation and increase in apoptosis observed in SB treated normal epidermis.
with chronic TPA promotion. It is significant that both pharmacological inhibition and genetic ablation of the TGFβ type I receptor (18) in the epidermis results in elevated apoptosis. However this difference appears to be lost in early papillomas and HRas expressing epidermis, supporting the idea that Ras activation suppresses pro-apoptotic effects of inactivated TGFβ1 signaling (18).

An additional mechanism for suppression of epidermal proliferation, papilloma number and size is the inhibition of TPA-induced cutaneous inflammation which has repeatedly been shown to be a critical component of tumor promotion in the skin (43, 44). Our previous study showed that TPA-induced cutaneous inflammation was exaggerated in TGFβ1+/- mice (23) suggesting an important role for TPA-induced TGFβ1 in limiting the inflammatory response. In contrast SB431542 significantly suppressed acute or chronic TPA-induced infiltration of MPO+ cells into the skin, and this was associated with reduced expression of two proinflammatory cytokines KC and MIP2. However we could not detect an effect of SB431542 on TPA-induced expression of these or other proinflammatory molecules in primary mouse keratinocytes or fibroblasts (data not shown), indicating that SB431542 is not directly blocking TPA-induced signaling pathways that activate these genes. Interestingly, it has been shown that TGFβ1 is a potent chemoattractant for neutrophils (45) and the TGFβ type I receptor on neutrophils mediates this response (46), suggesting either that inhibition of TGFβ signaling in a skin resident immune cell was critical for blocking the inflammatory response, or that inhibition occurred in neutrophils directly. Taken together these results suggest that the response to TGFβ in an as yet unidentified cutaneous cell type is critical for amplification of TPA-induced inflammation and enhanced outgrowth of benign lesions.

Similar to transgenic and knockout models with blocked TGFβ receptor signaling (16-18, 47, 48) our results show that chronic treatment with SB431542 increases the conversion to SCC. However our data reveal new insights into the role of TGFβ signaling in malignant progression of squamous tumors. We have previously shown that in the 2-stage chemical carcinogenesis model SCC arise from a small subpopulation of benign lesions with biochemically distinct properties at an early stage of tumor development, while the majority of papillomas do not have the capacity to progress to SCC or do so at a much slower rate (36). Microarray analysis of these different premalignant tumor subclasses showed that early non progressing papillomas or low-risk lesions had an elevated inflammatory gene signature (49) suggesting these tumors are dependent on inflammation for outgrowth. Our results suggest that SB431542 blocks a TGFβ dependent pathway of cutaneous inflammation that is critical for outgrowth of low-risk benign lesions but at the same time promotes outgrowth of lesions with a rapid progression phenotype. Thus unlike TPA only treated papillomas, early lesions in SB treated mice have an inflammatory microenvironment similar to SCC, with intraepithelial infiltration of MPO+ cells, reduced expression of normal squamous differentiation markers, and expression of keratin markers of progression. The rapid induction of this phenotype in HRAS expressing transgenic epidermis and tumors provides strong
evidence that pharmacological inhibition of TGFβ signaling during DMBA-TPA induced tumor formation directly provokes this response. The observation that keratin 8, a marker of simple epithelium and malignant conversion in squamous epithelium is also induced in pancreas expressing a dominant negative TβRII transgene (50) and keratinocytes by a dominant negative TβRII adenovirus (51) indicates that the induction of these simple epithelial markers in the transgenic HRAS epidermis by SB431542 is the result of blocked TGFβ signaling rather than off target effects and directly implicates TGFβ signaling in the maintenance of the squamous differentiation phenotype in the epidermis. It is clear that subsets of keratinocytes with an activated Hras oncogene respond differently to inhibition of ALK5 in the tissue microenvironment. Our results show that a large number of initiated keratinocytes are dependent on ALK5 signaling for inflammatory pathways that enhance tumor outgrowth. A second subset can form tumors despite inhibition of cutaneous inflammation, in part through generation of an altered inflammatory microenvironment and through altered squamous differentiation induced by inhibition of ALK5. This latter group has a higher frequency of malignant conversion. The basis of this differential response to ALK5 inhibition is unknown but could reflect different roles of TGFβ signaling in epidermal keratinocytes with distinct developmental fates or capacity.

Previous in vivo studies with different small molecule inhibitors of the TGFβ type I receptor kinase suggest that these compounds could have potential use as anti-cancer and anti-fibrotic therapeutics and point to immune mechanisms as key to their ability to block growth of syngeneic tumors in mice. Thus in keeping with the well-documented role of TGFβ1 as an immunosuppressive cytokine in the tumor microenvironment, this class of inhibitors has been shown to increase the T cell antitumor response (8-10), and natural killer cells and macrophages (7). In a model of squamous cancer driven by chronic inflammation and tissue remodeling our results show that pharmacological inhibition of TGFβ signaling also blocks tumor outgrowth in part through inhibition of TGFβ-dependent epidermal inflammation. However, the differential outgrowth of premalignant lesions with an inflammatory phenotype similar to SCC following long-term treatment with SB431542 and at high risk for malignant conversion suggests that effects of these inhibitors on the tumor immune microenvironment during tumor formation and premalignant progression may be different than on malignant tumors. Thus these data open the possibility for use of these inhibitors in settings where TGFβ signaling is a component of disease processes associated with chronic inflammation but also suggests that long-term use of this inhibitor class should be approached with caution.
2.6 Bibliography


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Chapter 3

Terminal Differentiation is Regulated by TGFβ Signaling in Preneoplastic Keratinocytes

3.1 Abstract

We have shown that pharmacological transforming growth factor β (TGFβ) type I receptor (ALK5) inhibition suppressed tumor formation and enhanced progression in a mouse two-stage skin chemical carcinogenesis assay in part through altered squamous differentiation. Here we investigate the regulation of squamous differentiation in preneoplastic keratinocytes by treating oncogenic HRAS-expressing keratinocytes with the ALK5 inhibitor SB431542 (SB). SB significantly enhanced HRAS-induced cornification which correlated with the increased expression of terminal differentiation genes transglutaminase 1 (TGM1) and 3 (TGM3) and small proline-rich protein 1A (SPR1A) and 2H (SPR2H) which cross-link structural proteins that make up the cornified envelope. There was a similar increase in cornified layers and TGM1 and SPR1A following SB treatment of mice expressing an inducible epidermal HRAS transgene. Alternatively, treatment of HRAS-expressing keratinocytes with TGFβ1 or overexpression of TGFβ1 and HRAS in an inducible epidermal transgenic model resulted in reduced expression of TGM1 and TGM3 and cornification in vitro. Papilloma (SP1) and squamous cell carcinoma (PAM2.12) cell lines were less responsive to TGFβ1 suppression of markers and marker induction by SB. Interestingly, a subpopulation of HRAS-expressing keratinocytes were resistant to induction of terminal differentiation by ALK5 inhibition. These cells were also resistant to senescence and rapidly immortalized, suggesting that they represent a progressed phenotype. However, these cells were unable to grow in elevated calcium or produce tumors after grafting nude mice indicating that they were not fully malignant. Thus, selective responsiveness to differentiation may represent a mechanism by which blocked TGFβ signaling can inhibit the outgrowth of preneoplastic lesions but may cause a more progressed phenotype in a separate keratinocyte population.

3.2 Introduction

The cornified envelope (CE) is the end product of keratinocyte differentiation which occurs as keratinocytes leave the basement membrane and express specific proteins that are involved in formation of the CE. Biologically, the CE is highly insoluble and essential for barrier function of the epidermis. During terminal differentiation, keratinocytes undergo morphological and cytostructural changes that are regulated by structural and catalytic proteins including involucrin, filaggrin, keratins, transglutaminase and small proline-rich proteins (SPRs) (1, 2). Transglutaminase 1 (TGM1) and transglutaminase 3 (TGM3) are expressed in the spinous and granular layers of the epidermis and catalyze the formation of isopeptide bonds resulting in the formation of covalent intra- and interprotein cross-linking that are
responsible for cornification. TGM3 first cross-links loricrin and SPRs to form small interchain oligomers, and the TGM1 enzyme causes further cross-linking which permanently fixes the developing CE just below the plasma membrane (3-5).

Epidermal homeostasis depends on the modulation of keratinocyte proliferation, differentiation, and cell death and deregulation of these processes can result in a number of skin diseases including cancer. HRas-expressing keratinocytes exhibit suppressed induction of early differentiation markers keratin 1 and 10, and upregulate expression of late differentiation markers loricrin, filaggrin and transglutaminase (6). Previous studies have shown that keratinocytes expressing the Ras oncogene undergo cell cycle arrest that shows features of terminal differentiation and may function as a counter to uncontrolled mitogenic signaling (7, 8). These results are supported by the two-stage chemical carcinogenesis assay in which DMBA initiates an HRas mutation, and repeated treatment with the tumor promoter TPA causes the formation of benign papillomas that rarely progress to malignancy (9). In agreement, keratinocytes that resist terminal differentiation show characteristics of malignant conversion through evasion of this tissue-specific mechanism for limiting growth (10-12).

Transforming growth factor β1 (TGFβ1) is a member of a large family of regulatory molecules that play both positive and negative roles in epithelial cancers. Reports on a role for TGFβ in terminal differentiation have been variable. While some studies have shown that TGFβ1 does not induce terminal differentiation in normal human foreskin epidermal keratinocytes (HFEC) (13, 14) or mouse keratinocytes (15), others report that TGFβ1 inhibits CE formation in HFEC (12) and reduces the expression of IFN-γ (16) and TPA (17) induced TGM1 expression in both HFEC and the head and neck squamous cell carcinoma (SCC) cell line SCC25. Further, this response can be reversed by the overexpression of Smad7 indicating that TGFβ1-mediated suppression of TGM1 is Smad7-dependent, although it is unclear whether this is a direct effect or a result of downstream signaling (17).

Here we show that TGFβ1 suppresses terminal differentiation of HRAS-expressing keratinocytes while inhibition of TGFβ signaling enhances terminal differentiation. Using the doxycycline (Dox) inducible keratin 5/tetORAS (K5/tetORAS) model of preneoplastic epithelial cancer to induce HRAS expression, we show that the pharmacological TGFβ type I receptor (ALK5) inhibitor SB431542 (SB) enhances HRAS-induced cornification through increased cornified envelope formation and gene expression of transglutaminases and small proline-rich proteins in vitro and in vivo, and this response is inhibited by TGFβ1. Similar regulation of differentiation gene expression is observed in the papilloma and SCC cell lines SP1 and PAM2.12, respectively. Surprisingly, we find that following long-term ALK5 inhibition, a population of HRAS-expressing keratinocytes emerges that is resistant to induction of cornification. We hypothesize that these results show a mechanism by which blocked TGFβ signaling can inhibit the outgrowth of preneoplastic keratinocytes but may cause a more progressed phenotype in a
separate keratinocyte population, leading to reduced benign tumor formation but enhanced malignant conversion in a distinct population of initiated epidermal cells.

3.3 Materials and Methods

3.3.1 Cell culture: 
Primary mouse keratinocytes were isolated according to a standard protocol (18) from newborn transgenic littermates. tetORASV12G (19) mice that express HRAS when induced were obtained from the NCI mouse repository and newborn mice of each genotype identified by PCR were obtained from crosses of heterozygous K5rtTA (K5) and homozygous tetORASV12G (tetORAS) mice. Keratinocytes were plated in minimum essential media (Formula # 98-0216DJ, Gibco Invitrogen, Carlsbad, CA) containing 8% chelex-treated fetal bovine serum, 18.3 IU/ml penicillin, 18.3 µg/ml streptomycin (Cellgro, Manassas, VA) and 0.2 mM Ca\(^{2+}\). The following day, media was changed to 0.05 mM Ca\(^{2+}\). Conditional expression of the tetORASV12G transgene in mouse keratinocytes was achieved using keratinocytes double transgenic for the K5 and tetORAS transgene. HRAS expression was induced by addition of 1µg/ml doxycycline (Dox) to culture media. Pharmacological inhibition of ALK5 was achieved with 0.5µM SB431542 (Sigma, St. Louis, MO). The papilloma cell line SP1 (20) and SCC cell line PAM2.12 (21) were plated in minimum essential media (Formula # 98-0216DJ, Gibco Invitrogen) containing 8% chelex-treated fetal bovine serum, 18.3 IU/ml penicillin, 18.3 µg/ml streptomycin (Cellgro) and 0.05 mM Ca\(^{2+}\). The immortalized cell line was generated from K5/tetORAS keratinocytes treated with 50 ng/ml doxycycline and 0.5 µM SB for 11 days and subsequently passaged more than 15 times suggesting that they are immortalized. 300,000 cells were plated in p60 dishes for protein and RNA analysis. For all long-term experiments media was changed every other day.

3.3.2 Virus production and infection: 
The v-Ras\(^{HA}\) retrovirus was generated from ψ2 producer cells as described previously. Virus titer was determined using a NIH3T3 focus-forming assay. FVB/n keratinocytes were infected with v-Ras\(^{HA}\) retrovirus on day 3 of culture for 3 days at a dilution of 1:2 to ensure sufficient infection of cells.

3.3.3 Cell counting: 
For cell death curves, K5/tetORAS keratinocytes were plated in high calcium (1.4mM) for 2 hours to remove contaminating fibroblasts which attach faster than keratinocytes. Keratinocytes in the supernatant were pelleted, resuspended in 0.2mM calcium media, and seeded on 24-well tissue culture plates. The following day, media was changed to low calcium (0.05mM). On day 2 post-plating, keratinocytes were treated with doxycycline and/or SB 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, Hialeah, FL).

### 3.3.4 Cornified envelope assay:

A protocol adapted from published methods (22) was used to quantify cornified envelope formation. Fibroblasts were removed from primary keratinocyte preparations as described and keratinocytes seeded on p60 dishes in 0.2mM calcium media. The following day, media was changed to low calcium (0.05mM). Keratinocytes were treated with 1µl/ml Dox and/or 0.5µM SB on day 3 post-plating. To assay CE formation, media was collected and dishes were washed twice with PBS and collected in a 15 ml tube. Media was spun down at 800 rpm for 5 minutes to pellet floating/cornified cells. 100µl of 2% SDS/20mM DTT solution was added to each pellet and transferred to an eppendorf tube. Cells were boiled at 90°C for 10 minutes as cornified cells are resistant to the SDS/DTT treatment. The cornified envelopes appear as ghost cells (23). Phase contrast microscopy was used to view and count them with a hemocytometer as shown below (Figure 3-1). The attached cells on the washed p60 dishes were trypsinized in 500 µl trypsin and counted using a Z1 Coulter particle counter (Beckman Coulter). Triplicate samples were analyzed for each treatment group. The result was expressed as a percentage of floating, cornified cells per attached cell.

Figure 3-1 Cornified cells resistant to SDS/DTT treatment

Following boiling with 2% SDS/ 20mM DTT solution, cornified cells appear as irregular shaped “ghost cells” (white arrows). Perfectly circular objects are likely cornified cells but are probably not linked to other cells by desmosomes which gives them a different shape during cornification in the stratum corneum (red arrow). Four quadrants were counted under a hemocytometer for each sample.

### 3.3.5 Luciferase assay:

PAM2.12 and SP1 cell lines were transfected at 60% confluence with 1.0 µg of SBE4-luciferase reporter plasmid containing the synthetic sequence with four copies of connective Smad binding elements (SBEs) and 9 ng of a renilla-luciferase control plasmid pRL-CMV using Lipofectamine 2000 transfection
reagent (Invitrogen). Cells were pre-treated with 0.5 µM SB for 30 minutes prior to treatment with 1 ng/ml TGFβ1 for 24 hours. Luciferase activity was determined using the dual glow luciferase assay kit (Promega) according to protocol and a Promega 20/20 luminometer.

3.3.6 Calcium responsiveness:

The immortalized K5/tetORAS keratinocytes treated with 50 ng/ml doxycycline and 0.5 µM SB for 11 days were maintained as a cell line. To determine responsiveness to increased calcium concentrations, 5000 cells/plate were seeded in 0.05 mM Ca²⁺ media with 1 µg/ml dox and 0.5 µM SB. After 48 hours the calcium concentration was increased to 0.5 mM and treatment continued for 9 days. Calcium resistant colonies were counted after the dishes were fixed and stained in hematoxylin. The experiment was repeated. Immortalized cells were also treated with either 0.05 mM or 0.12 mM Ca²⁺ and protein harvested using (0.25M Tris, 5% SDS, 20% BME) followed by sonication and boiling at 99°C. SDS-PAGE was performed using equal volumes of lysate.

3.3.7 β-galactosidase senescence assay:

Keratinocytes were plated in 12-well tissue culture plates and on day 3 post-plating treated with doxycycline and/or SB for 11 days. For staining, cells were washed 2 times with PBS and fixed for 5 minutes in 0.5% glutaraldehyde in PBS (pH 7.2). The fixative was aspirated and cells were washed twice with PBS (pH 7.2)/1mM MgCl₂. The PBS/MgCl₂ solution was removed and 500 µl of staining solution (0.12 mM K₃Fe[CN]₅, 0.12 mM K₄Fe[CN]₆, 1 mM MgCl₂, 10 mg X-gal (V394A, Promega, Madison, WI) in PBS, pH 6.0) was added to each well. Cells were incubated with staining solution for 24-48 hours at 37°C in a non-CO₂ incubator. Positive cells stained blue. The percentage of β-galactosidase positive cells was quantified using a Nikon inverted microscope (20x microscope frame) and 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.

3.3.8 Cell cycle analysis:

Keratinocytes were analyzed for cell cycle progression by 5-bromo-2-deoxyuridine/ propidium iodide (BrdU/PI) staining. One hour before the time point 40 µM BrdU in DMSO was added to each plate with the last media change 24 hours prior to fixation. Cells were washed with PBS and trypsinized for 15 minutes with trypsin. Media was added to trypsinized cells and transferred to 15 ml conical tubes. Cells were centrifuged (300 x g for 6 minutes). Cells were washed with PBS and centrifuged again. The supernatant was discarded and the pellet loosened by tapping the bottom of tube on bench top. While vortexing on a low speed, the pellet was resuspended in ice cold 70% Ethanol in a drop-wise manner to
avoid cells sticking together. Cells were incubated at room temperature for 20 minutes before storing at 
-20°C at minimum overnight before proceeding with BrdU and PI staining.

For staining, 1ml of fixed cells were transferred to flow tubes and wash buffer added (0.5 % 
Tween-20/PBS). The tubes were centrifuged and supernatant removed. A pellet should be visible as a 
smear on the side of the microfuge tube with about 50 µL of the remaining supernatant. This was 
followed by a second wash with wash buffer. Cells were resuspended in freshly made denaturing solution 
(2M HCl/0.5 % Triton-X), mixed well, and incubated for 20 minutes at room temperature. Wash buffer 
was added to suspension, mixed and centrifuged, followed by a second wash with wash buffer. The 
supernatant was discarded and acid neutralizing solution added (0.1 M Sodium Borate pH 8.5), mixed and 
incubated at room temperature for 2 minutes. The cells were centrifuged, followed by a second wash with 
wash buffer. BrdU-FITC antibody (ABFM-18, Phoenix Flow Systems, San Diego, CA) was made in 
dilution buffer (0.5 % Tween 20-PBS/0.5 % BSA PBS). Cells were mixed well and incubated at room 
temperature in dark for 45 min to 1 hr. Cells were then washed twice and resuspended in PI solution 
(0.00625 % RNase/PBS with PI at 5 µg/ml) and incubated at room temperature for 10 minutes protected 
from light.

Approximately 10,000 cells/sample were analyzed using an EPICS-XL-MCL flow cytometer 
(Beckman Coulter Electronics, Hileah, FL). The percentage of cells at each phase of the cell cycle was 
determined with FlowJo Flow Cytometry analysis software.

3.3.9 Animal studies

Involucrin tTA (InvtTA) x tetORAS mice were maintained on 10 µg/ml doxycycline water, which 
was switched to water alone to induce HRAS expression. Seven-week-old mice were treated with 200 µL 
of acetone and/or 200 µL of 10.0µM SB431542 every other day for 5 days, and skin was harvested 24h 
after the last SB treatment. Non-transgenic, K5 x tetOTGFβ1, K5 x tetORAS, and K5 x tetORAS 
tetOTGFβ1 mice were treated with doxycycline for 2 days in chow (1gm/kg) to induce expression of 
RAS and/or TGFβ1.

Skin from each mouse was fixed in 10% neutral buffered formalin or 70% ethanol and then 
paraffin embedded. Sectioned tissue was cut at 5µm and placed on charged slides to ensure adhesion 
during the staining process. All animals were injected intraperitoneally with 5-bromo-2-deoxyuridine 
(BrdU) 1h before sacrifice (6 mg in 300µl PBS per mouse). All animals were kept under a controlled 
environment of temperature and humidity and a 12h light/dark cycle. Animal studies were conducted 
under approved IACUC protocols.

Skin grafts were performed according to an approved IACUC protocol. All instruments, reagents 
and surfaces were sterile/clean. Clean recovery cages were prepared under the hood for mouse recovery
and placed on a slide warmer at 40°C. Mice were injected with 0.4 cc/mouse of 2.5% avertin and given approximately 10 minutes to ensure sufficiently anesthetized. The area to be grafted was wiped with betadine using sterile gauze pads followed by wiping with 70% ethanol. Using forceps, the back of each mouse was pinched, lifted straight up and 1cm in length cut using curved scissors. This gave an area sufficient for inserting the grafting domes. Sterilized domes were then placed in a Petri dish with 1x PBS. Forceps were dipped in PBS and gently wiped around edges of incision, making it easier to insert domes. Domes were inserted, secured with a surgical staple if necessary, and cell suspension for graft was injected into dome directly onto exposed flesh. Three million keratinocytes treated with 1µg/ml Dox and 0.5µM SB for 11 days and six million FVB/n fibroblasts were used for each graft. Mice were then placed in warmed clean cages. Ketoprofen at a dose of 0.25cc/mouse was administered subcutaneously immediately following surgery as an analgesic. Mice were observed until they woke up (about 2 hours) and were then observed for an additional hour. Domes were removed 1 week later by gently squeezing dome and pulling up. Mice were observed daily and records maintained in a surgical log until the wounds healed. Mice were maintained on doxycycline water before and after the procedure in order to ensure growth of grafts based on in vitro treatment media.

3.3.10 RNA analysis

Whole skin was homogenized using a Qiagen Tissuelyzer (Qiagen, Valencia, CA) in Trizol (Invitrogen). RNA was quantitated using a Beckman Coulter DU-800 Spectrophotometer at wavelengths 260nm for quantitation and 280nm for detection of protein contamination. DNA was removed from samples using Turbo DNA-free (AM-1907, Applied Biosystems/Ambion, Austin, TX) according to manufacturer’s recommended procedures, and cDNA was made from RNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems/Ambion). Quantitative RT-PCR (qPCR) was done for the indicated genes using the MyIQ system (BioRad Laboratories, Hercules, CA) and PerfeCTa SYBR Green SuperMix for iQ (#95053-500, Quanta Biosciences, Gaithersburg, MD). The following conditions were used for qPCR: 50°C, 2min.; 95°C, 10min.; (95°C, 15sec.; 60°C, 1min. x 40 cycles). All qPCR values were normalized to 18s rRNA. For RT-PCR, 94°C, 10min.; (95°C, 15sec.; 60°C, 15 sec.; 72°C, 30 sec. x 40 cycles), 72°C, 10 min. All RT-PCR samples were normalized to GAPDH and 18s rRNA. Primer sequences were obtained from published studies or using Primer 3 (24) software with Genebank sequence information.
Protein analysis:

Following treatment, keratinocytes were washed twice with PBS, scraped off dishes, and lysed with 200µl of 0.5% NP-40 lysis buffer (0.5% IGEPAL CA-630, 250mM NaCl, 50mM Tris HCl, pH 7.4) with 1µl/ml each protease and phosphatase inhibitors including 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). Cells were rotated at 4°C for 1 hour, spun at 14,000 rpm for 15 minutes at 4°C, and the supernatant was transferred to new tubes. Protein was quantitated using the Bio-Rad protein assay (#500-0006, Bio-Rad) based on the Bradford Method and measured at 595nm wavelength. SDS-PAGE was performed using 20µl protein per sample and transferred to a nitrocellulose membrane. Transfer efficiency and equal loading was verified by Ponceau-S staining, and GAPDH or β-actin immunoblotting. Following blocking in 5% non-fat dry milk in 0.1% Tween-20/Tris-buffered saline (TBS-Tween), immunoblots were incubated with antibodies overnight at 4°C in 3% bovine serum albumin/TBS-Tween using antibodies directed against Smad 2/3 (1:2000) (Cell Signaling Technology, Inc., Danvers, MA); p-Smad2 (1:1000) (Cell Signaling); HRAS, (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); Keratin 1 (1:1000); p-ERK (1:1000) (Cell Signaling); ERK (1:1000) (Cell Signaling); p-AKT (1:1000) (Cell Signaling); p-p38 (1:1000) (New England Biolabs, Ipswich, MA); p-38 (1:500) (Santa Cruz); GAPDH (1:1000) (Cell Signaling) and β-actin (1:20,000) (Millipore). Membranes were washed 6 x 10 minutes in TBS-Tween, followed by incubation in HRP-conjugated secondary antibodies (1:2000) (BioRad) in 5% non-fat dry milk/TBS-Tween at room temperature for 1 hour. Membranes were washed again 6 x 10 minutes and specific proteins were detected by chemiluminescent Western blotting substrate (ECL, Pierce, Rockford, IL). Exposed film was developed using a Konica SRX-101A medical film processor. Film was scanned using Adobe Photoshop 7.0 and images were converted to gray scale.

Table 3-1. Primer sequences
Changes in gene expression of differentiation markers were investigated using the following primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<tbody>
<tr>
<td>18srRNA</td>
<td>TCGATGCTCTTAGCTGAGTGATGTC</td>
<td>TATTTCAAGCTACGGTATCAGGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAATGGTGAGGTGTTGAGTGAACG</td>
<td>TGGCAACATCTTCACCTTTGCAC</td>
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<tr>
<td>Involucrin</td>
<td>CCCTCTGCTTGATTTGTTGGCT</td>
<td>ACCTTGCATTTGTAGGTAG</td>
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<tr>
<td>SPRR1A</td>
<td>CCATTGCCCTTGTCTACCAA</td>
<td>TCAGAGCCCTTGAAGATGAG</td>
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<tr>
<td>SPRR2H</td>
<td>CTTCCTCCAAAGCCATTCA</td>
<td>TGAAGCGCTGAGGAGGACTA</td>
</tr>
<tr>
<td>RAS</td>
<td>CCAAGCTGATCCAGAAACCCT</td>
<td>GCGGCGGTATCAGGATGTC</td>
</tr>
<tr>
<td>Transglutaminase 1</td>
<td>ACACAACCTAAACCTACCGTCCT</td>
<td>ACATTCTGTGCCCCAGTCCTC</td>
</tr>
<tr>
<td>Transglutaminase 3</td>
<td>CGCAACACATCTTTGAGGAAATC</td>
<td>TCCTTCACACTTTCGAGGACAA</td>
</tr>
</tbody>
</table>

3.3.11 Protein analysis:

Following treatment, keratinocytes were washed twice with PBS, scraped off dishes, and lysed with 200µl of 0.5% NP-40 lysis buffer (0.5% IGEPAL CA-630, 250mM NaCl, 50mM Tris HCl, pH 7.4) with 1µl/ml each protease and phosphatase inhibitors including 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). Cells were rotated at 4°C for 1 hour, spun at 14,000 rpm for 15 minutes at 4°C, and the supernatant was transferred to new tubes. Protein was quantitated using the Bio-Rad protein assay (#500-0006, Bio-Rad) based on the Bradford Method and measured at 595nm wavelength. SDS-PAGE was performed using 20µl protein per sample and transferred to a nitrocellulose membrane. Transfer efficiency and equal loading was verified by Ponceau-S staining, and GAPDH or β-actin immunoblotting. Following blocking in 5% non-fat dry milk in 0.1% Tween-20/Tris-buffered saline (TBS-Tween), immunoblots were incubated with antibodies overnight at 4°C in 3% bovine serum albumin/TBS-Tween using antibodies directed against Smad 2/3 (1:2000) (Cell Signaling Technology, Inc., Danvers, MA); p-Smad2 (1:1000) (Cell Signaling); HRAS, (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); Keratin 1 (1:1000); p-ERK (1:1000) (Cell Signaling); ERK (1:1000) (Cell Signaling); p-AKT (1:1000) (Cell Signaling); p-p38 (1:1000) (New England Biolabs, Ipswich, MA); p-38 (1:500) (Santa Cruz); GAPDH (1:1000) (Cell Signaling) and β-actin (1:20,000) (Millipore). Membranes were washed 6 x 10 minutes in TBS-Tween, followed by incubation in HRP-conjugated secondary antibodies (1:2000) (BioRad) in 5% non-fat dry milk/TBS-Tween at room temperature for 1 hour. Membranes were washed again 6 x 10 minutes and specific proteins were detected by chemiluminescent Western blotting substrate (ECL, Pierce, Rockford, IL). Exposed film was developed using a Konica SRX-101A medical film processor. Film was scanned using Adobe Photoshop 7.0 and images were converted to gray scale.
3.3.12 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. P-values of <0.05 were regarded as a significant difference as indicated by an asterisk. Error bars represent standard error of the mean.
3.4 Results

3.4.1 SB431542 inhibits Smad2 phosphorylation in keratinocytes

FVB/n keratinocytes were treated with SB to determine the duration of inhibition of endogenous TGFβ signaling in vitro for long-term experiments. Figure 3-2A shows that 24 hour treatment with TGFβ1 (T) activated Smad2 phosphorylation compared to baseline levels. At 0.5 hours, 0.5µM SB inhibited baseline Smad2 phosphorylation and this inhibition was maintained through 48 hours. In a dose response of ALK5 inhibition in FVB/n keratinocytes, SB blocked Smad2 phosphorylation, but had no effect on off-target kinases including ERK and p38 MAPK (Figure 3-2B). In order to create a proneoplastic keratinocyte mouse model, a bitransgenic line in which human RASV12G (19) is conditionally overexpressed in the epidermis with a keratin 5 rtTA driver line by treatment with Dox was used (25). After treatment of bitransgenic keratinocytes for 4 days with Dox to induce HRAS expression, a dose-dependent induction of HRAS protein was evident (Figure 3-2C). Figure 3-2D shows that SB blocked Smad2 phosphorylation in bitransgenic keratinocytes in which HRAS was expressed for either 1 or 4 days. These data demonstrate that SB can inhibit TGFβ signaling for at least 48 hours and that it is effective in blocking TGFβ signaling in cells expressing an activated HRAS oncogene.
Figure 3-2. SB inhibits Smad2 phosphorylation in vitro.

(A) Reduction in Smad2 phosphorylation maintained up to 48 hours following treatment with SB in FVB/n keratinocytes. (B) SB does not inhibit phosphorylation of ERK and p38. (C) Dose-dependent increase in HRAS protein with specified Dox treatment for 48 hours in double transgenic K5/tetORAS compared to single transgenic tetORAS keratinocytes. Experiment repeated. (D) Reduced Smad2 phosphorylation in K5/tetORAS keratinocytes treated for 1 or 4 days with Dox+SB. Protein analysis and isolation as described in Materials and Methods. Experiment repeated. C, control; SB, SB435142; D or Dox, Doxycycline; T, TGFβ1.
3.4.2 ALK5 inhibition causes terminal differentiation in HRAS-expressing keratinocytes

TGFβ1 is a potent growth inhibitor and inhibits the formation of benign lesions, but promotes malignant conversion at later stages (26). To determine the impact of ALK5 inhibition on the growth of HRAS-expressing keratinocytes, we investigated changes in cell cycle phase. While Figure 3-3A shows that we did not see a difference in G1, S or G2 phases between activated HRAS and HRAS+SB groups, Figure 3-3B shows that following 5 days of treatment, the cell number in HRAS-expressing keratinocytes increased 45% above control and SB treated keratinocytes, while HRAS+SB treatment caused a 50% decrease in cell number compared to HRAS alone to levels below that of SB and control cells. Photomicrographs show reduced confluence and cells with a more spindle phenotype (Figure 3-3C). In order to determine whether there was a difference in cell death between HRAS and HRAS +SB, a cornified envelope assay was performed which detects differences in keratinocytes that have undergone terminal differentiation. As expected based on previous studies, HRAS expression by Dox caused a significant increase in CE formation. However, while SB had no effect on CE formation in normal keratinocytes, it more than doubled the number of CE cells in the HRAS-expressing epidermal cells (Figure 3-3D). To identify a potential mechanism for this change, we investigated the mRNA levels of transglutaminase 1 (TGM1), transglutaminase 3 (TGM3), and small proline-rich proteins SPR1A and SPR2H, all of which encode for proteins that cause extensive cross-linking and are significant in the formation of the cornified envelope. We show that ALK5 inhibition increased expression of TGM1 and TGM3 and significantly increased the expression of SPR1A and SPR2H (Figure 3-3D), indicating that the expression of genes responsible for protein cross-linking of the cornified envelope are responsible for enhanced terminal differentiation.
Figure 3-3. ALK5 inhibition causes terminal differentiation in HRAS-expressing keratinocytes.

(A) Keratinocytes attached to culture dishes were pulsed with BrdU and FLOW analysis of BrdU/PI stained cells analyzed according to Materials and Methods following specified treatments for 4 days. No significant difference between HRAS and HRAS+SB keratinocytes with all phases of cell cycle. n=4. (B) Inhibition of cell confluence in HRAS-expressing keratinocytes treated with SB. Cells were trypsinized in wells and counted in triplicate using a Coulter Counter on day 5. Experiment repeated. (C) Images of cell confluence of K5/tetORAS keratinocytes treated with Dox and/or SB on day 5. (D) Enhanced formation of cornified envelopes in keratinocytes expressing HRAS treated with SB. Floating cells counted by cornified envelope assay and compared to attached cells on day 5. n=3 and performed 3 times. Image captured at 200x magnification, scale bar represents 50µm (right). (D) Increased transcription of genes involved in terminal differentiation and cross-linking following 4 days treatment that correlates with increased cornification. n=4 and repeated. C, control; SB, SB435142; D, Doxycycline; SPR1A, small proline rich protein 1A; SPR2H, small proline rich protein 2H; TGM1, transglutaminase 1; TGM3, transglutaminase 3.
3.4.3 Increased expression of transglutaminases, but not SPRs in vivo by ALK5 inhibition

Since SB caused an upregulation in transglutaminases and SPRs in keratinocytes, we tested if similar regulation of HRAS-induced differentiation could be detected in vivo. We used a bitransgenic model in which human RASV12G (19) is conditionally overexpressed in the epidermis with an InvtTA driver line to test this hypothesis (27). When adult bitransgenic mice are removed from 10µg/ml doxycycline in the drinking water HRAS expression is induced within 1-2 days. Seven week old InvtTA/tetORAS mice were taken off Dox and treated with SB or acetone for 5 days. In Figure 3-4A we show that treatment with SB resulted in a significant increase in TGM1 and SPR1A in mouse skin expressing HRAS, with a similar trend observed with TGM3 and SPR2H expression. This result was also associated with an increase in proliferation (Figure 2-11A). In agreement, an increase in the thickness of cornified layers was also detected with SB treatment compared to acetone (Figure 3-4B, C). These results show that ALK5 inhibition can induce the expression of specific markers of late differentiation in preneoplastic skin even though we also observe inhibition of keratin 1 and induction of keratin 18 as shown in Figure 2-11A.
Table 3-4. Terminal differentiation markers regulated *in vivo* by ALK5.

(A) Significantly increased gene expression of TGM1 and SPR1A, with similar trends in TGM3 and SPR2H expression was detected in InvtTA/tetORAS mice treated with SB and removed from Dox to induce HRAS expression. n=5. (B) Correlated with increased cornified layers. Magnification 200x, scale bar represents 50 µm. (C) Cornified layer thickness measured at magnification 200x, 30 measurements/slide. n=5.

A, Acetone; SB, SB431542; Dox, Doxycycline; SPR1A, small proline rich protein 1A; SPR2H, small proline rich protein 2H; TGM1, transglutaminase 1; TGM3, transglutaminase 3; C, Cornified layer; E, Epidermis.
3.4.4 TGFβ1 overexpression inhibits expression of transglutaminases; response not limited to ALK5

The enhancement of CE formation and terminal differentiation gene expression by inhibition of ALK5 suggests that TGFβ should suppress terminal differentiation. To test this we treated K5/tetORAS keratinocytes with exogenous TGFβ1. Figure 3-5A shows that TGFβ1 inhibited TGM1 and TGM3 compared to HRAS-expression alone, although no change was detected in SPR genes following 24 hours pretreatment with Dox and 12 hours treatment with SB (data not shown). A cornified envelope assay was then performed to determine if these alterations resulted in the biological endpoint of terminal differentiation. In Figure 3-5B, exposure of keratinocytes to TGFβ1 for 48 hours following 3 days of HRAS expression by Dox resulted in a significant decrease in HRAS-induced cornification. These results correlated well with results obtained in the bitransgenic K5/tetORAS, K5/tetOTGFβ1 and triple transgenic K5/tetORAS/tetOTGFβ1 mice. Following treatment with Dox in chow for 2 days, HRAS and TGFβ1 are overexpressed in the basal layers of the skin. We show that mice induced to express HRAS had significantly lower levels of TGM3 and a similar trend with TGM1 expression when TGFβ1 was coexpressed (Figure 3-5C). Although there was an induction of SPR genes with HRAS expression, TGFβ1 did not alter this response and no change was detected with TGFβ1 expression alone compared to control (data not shown). Additionally, we found that TGFβ1 alone inhibited baseline TGM1 expression in vivo (Figure 3-5C) and significantly in vitro (Figure 3-5A).
Figure 3-5. TGFβ1 inhibits terminal differentiation in vitro and in vivo.

(A) In contrast to ALK5 inhibition, K5/tetORAS keratinocytes treated with TGFβ1 have reduced expression of RAS-induced TGM1 and similar trend in reduced TGM3 expression with 24h pretreatment with Dox and 12h treatment with Dox±SB. n=4. Experiment repeated. (B) Reduced expression of TGM1 and TGM3 correlated with reduced cornification. Floating cells counted by cornified envelope assay and compared to attached cells with 3 days Dox pretreatment followed by 2 days Dox±TGFβ1. n=3. Experiment repeated. C, Control; SB, SB431542; TGFβ1, Transforming Growth Factor β1; Dox, Doxycycline. (C) Reduced TGM1 and TGM3 following overexpression of TGFβ1 and HRAS compared to HRAS alone in transgenic mice. n=5. TGFβ, K5/tetOTGFβ1 mice; Ras, K5/tetORAS mice; Ras+TGFβ, K5/tetORAS/tetOTGFβ mice; TGM1, transglutaminase 1; TGM3, transglutaminase 3.
3.4.5 Altered TGFβ signaling regulates differentiation in papilloma and SCC cell lines

Since we demonstrated that ALK5 inhibition and activation of TGFβ1 signaling alters regulators of terminal differentiation in HRAS-expressing keratinocytes, we tested whether similar regulation occurred in other models of tumor progression. The papilloma cell line SP1 was generated from tumors isolated from a skin chemical carcinogenesis assay and has a mutation in the HRas gene at codon 61 (20). The SCC cell line PAM2.12 was generated from primary BALB/c keratinocytes resistant to terminal differentiation and mild trypsin treatment. Tumors derived from PAM2.12 cells were well-differentiated, occurred at 100% incidence and grew rapidly (21). As a number of carcinoma cell lines have been shown to have defects in TGFβ1 signaling (28, 29), we show that both SP1 (Figure 3-6A) and PAM2.12 (Figure 3-6B) cell lines have an intact receptor/Smad signaling pathway as demonstrated by increased SBE-luciferase activity following treatment with TGFβ1, and this was suppressed when also treated with SB. Similar to results found in the K5/tetORAS keratinocyte model, we found that SB induced a slight increase in expression of the differentiation genes TGM1, TGM3, SPR1A and SPR2H in both the papilloma and SCC cell lines (Figure 3-6C, D, E, F). In contrast, TGFβ1 significantly reduced the expression of TGM1 and SPR1A in both cell lines (Figure 3-6C, E), with a similar trend for SPR2H expression (Figure 3-6F). Interestingly, the changes in TGM3 (Figure 3-6D) were not as pronounced as was found in skin (Figure 3-4, 3-5). These data indicate that TGFβ1 treatment is sufficient for downregulating the expression of epidermal differentiation genes. However, the muted response to ALK5 inhibition suggests that targeting this receptor alone may not completely regulate this response in these cell lines and may reflect a more subtle and altered response of TGFβ signaling on squamous differentiation at varying stages of tumor progression.
Figure 3-6. Papilloma (SP1) and SCC (PAM2.12) cell lines responsive to regulation of cross-linking genes.

(A) SB reduces TGFβ1-induced luciferase activity in SP1 cell line. n=3. (B) SB reduces TGFβ1-induced luciferase activity in PAM2.12 cell line. n=3. (C) Transglutaminase 1 gene expression significantly regulated by TGFβ1 in SP1 and PAM2.12 cells. (D) Transglutaminase 3 gene expression does not significantly change with SB or TGFβ1 treatment. (E) SPR1A gene expression regulated by TGFβ1 in SP1 and PAM2.12 cells. (F) SPR2H gene expression only significantly downregulated in PAM2.12 cells by TGFβ1. Trends toward increased gene expression by SB observed with TGM1, SPR1A and SPR2H genes. (C-F) n=4. C, Control; SB, SB431542; TGFβ1, Transforming Growth Factor β1; SPR1A, small proline rich protein 1A, SPR2H, small proline rich protein 2H; TGM1, transglutaminase 1; TGM3, transglutaminase 3.
3.4.6 Subpopulation of immortalized keratinocytes is less responsive to terminal differentiation

We observed that following long-term exposure of K5/tetORAS keratinocytes to Dox+SB, a population resistant to the SB-induced terminal differentiation expands in culture by day 11 as shown by cell number and images of confluence (Figure 3-7A,B). To test whether these keratinocytes were resistant to HRAS- or HRAS+SB- induced cornification, K5/tetORAS keratinocytes were treated with Dox+SB for 11 days, removed from Dox+SB for 48 hours, and then treated for an additional 5 days. Figure 3-7C shows that these keratinocytes terminally differentiate in control or SB media but do not respond as robustly to HRAS-induced cornification as was seen at day 5. There was a significant increase in cornification induced by ALK5 inhibition in HRAS-expressing cells compared to HRAS alone, although these levels of cornification were reduced 5-fold from that observed at day 4. These data demonstrate that this population of keratinocytes has reduced responsiveness to cornification by ALK5 inhibition. Figure 3-7D shows that HRAS+SB treated keratinocytes also have reduced senescence at day 11 compared to RAS expression alone and this was also observed in v-RasHA retrovirus infected FVB/n keratinocytes treated with SB for 11 days (Figure 3-7E). As expected, about 75% of keratinocytes treated with media alone or SB were arrested in G1 of the cell cycle with low levels of proliferation. While keratinocytes expressing HRAS had higher levels of proliferation, there was no difference between HRAS and HRAS+SB treated cells (Figure 3-7F).
Figure 3-7. Immortalized keratinocytes are resistant to terminal differentiation and senescence, but no change in proliferation.

(A) Recovery of cell confluence as measured by number of attached cells in HRAS-expressing keratinocytes treated with SB at day 11 following cell death. n=3, experiment repeated. (B) Images of cell confluence of K5/tetORAS keratinocytes treated with Dox+SB on day 5 and regrowth by day 11. (C) Cornification still occurs in resistant keratinocytes following Dox and Dox+SB treatment, but levels are 5 times lower than day 5. n=3, experiment repeated. (D) Dox-induced K5/tetORAS keratinocytes treated with SB resist senescence compared to HRAS expression alone. n=3, experiment repeated. (E) v-RasHA infected keratinocytes treated with SB for 11 days resist oncogene-induced senescence. n=3, experiment repeated. (F) No change in proliferation between HRAS and HRAS+SB keratinocytes at day 11 of continuous treatment. n=4, experiment repeated. C, Control; SB, SB431542; TGFβ1, Transforming Growth Factor β1; Dox, Doxycycline.
3.4.7 Characterization of immortalized K5/tetORAS differentiation resistant keratinocytes

Resistance to terminal differentiation is a characteristic of malignant conversion (10-12). Typical of cells that have undergone transformation, these keratinocytes were immortalized and were passaged more than 15 times. Therefore, a variety of assays were performed in order to determine if these immortalized keratinocytes possessed properties of a more malignant cell type. In contrast to keratinocytes at day 5 that respond to Dox+SB by terminally differentiating (Figure 3-2B), the immortalized cells grew more vigorously with HRAS expression and ALK5 inhibition compared to HRAS-expression and ALK5 inhibition alone (Figure 3-8A), suggesting that there may be a switch to a growth inhibitory function of ALK5. To test the hypothesis that the immortalized cells were transformed, K5/tetORAS keratinocytes treated continuous with Dox+SB for 11 days were grafted onto athymic nude mice to determine whether tumor formation occurred. Although small tumors did form in control K5/tetORAS keratinocyte grafts, no tumors formed from the immortalized cell grafts (data not shown).

Keratinocytes that possess a more malignant phenotype have been shown to grow in elevated calcium (0.5 mM) compared to normal growth conditions (0.05 mM) (21, 30). Corresponding with the inability to form grafted tumors, these cells did not grow in media with an elevated calcium concentration (Figure 3-8B). These results correlated with continued responsiveness to calcium-induced expression of keratin 1 shown in Figure 3-8C (21). Protein analysis shows that the cells are still responsive to activation by TGFβ1 and inhibition of ALK5 by SB of Smad2 phosphorylation (Figure 3-8D). However, although still HRAS-inducible by Dox at the RNA level (Figure 3-8E), HRAS protein expression is far less than that of primary keratinocytes with 48h Dox treatment (Figure 3-8F). Similarly, primary K5/tetORAS keratinocyte still responded to transgene induction by Dox through day 11 (Figure 3-9A). However, expression of HRAS protein was reduced after being in culture long-term in both the Dox inducible model (Figure 3-9B) and in v-RasHA infected keratinocytes (Figure 3-9C), and this is further reduced by SB (Figure 3-9B,C). Yet, ERK activation was still present despite the lack of HRAS protein (Figure 3-9B).
Figure 3-8. Characterization of immortalized keratinocytes.

(A) Dox+SB treatment enhances confluence in resistant cells. Attached cells were trypsinized in wells and counted using a Coulter Counter. Significant increase with Dox+SB compared to control. n=3. Experiment repeated. (B) Immortalized cells do not grow in 0.5 mM Ca\(^{2+}\) media (right) compared to normal 0.05 mM Ca\(^{2+}\) conditions (left). Experiment repeated. (C) Keratinocytes that escape cell death express keratin 1 following Ca\(^{2+}\) elevation. Cells were treated with standard (0.05mM) and elevated (0.12) Ca\(^{2+}\) media for 24 hours. Protein lysates prepared in 0.25M Tris HCl, 5% SDS, 20% BME lysis buffer. Equal volumes of lysates were loaded. (D) Immortalized keratinocytes are still responsive to SB and TGF\(\beta\) treatment as measured by expression of p-Smad2. Cells treated with specified media for 48 hours and harvested in 0.1% NP-40 lysis buffer. Experiment repeated. (E) Immortalized cells still responsive to Dox-induced transcription of tetORAS transgene at varying doses Dox at measured by RT-PCR. (F) However, lower protein levels of HRAS detected compared to primary K5/tetORAS keratinocytes following 48 hours Dox. Experiment repeated. All cells were removed from SB+Dox maintenance media for 48 hours prior to treatments. C, Control; SB, SB431542; TGF\(\beta\)1, Transforming Growth Factor \(\beta\)1; Dox, Doxycycline.
Figure 3-9. HRAS induced at mRNA level, but protein expression reduced over time.

(A) The tetORAS transgene is expressed evenly following treatment with 1µg/ml Dox and Dox+SB. (B) HRAS protein expression was reduced at day 11, and further reduced by SB. ERK and AKT phosphorylation remain high throughout. Experiment repeated. (C) HRas protein reduced in v-ras<sup>HA</sup> transduced keratinocytes at day 11, and further reduced by SB. Experiment repeated. C, Control; SB, SB431542; Dox, Doxycycline.
3.5 Discussion

As described in Chapter 2, treatment of the mouse epidermis during the promotion phase of a two-stage skin chemical carcinogenesis assay with the ALK5 inhibitor SB suppressed the number and size of TPA-induced papillomas compared to TPA alone. We showed that this may be a result of both an SB-associated decrease in epidermal proliferation and increase in apoptosis, as well as reduced TPA-induced inflammation. However, chronic SB treatment increased the conversion rate of remaining papillomas to SCC and this was associated with increased neutrophil infiltration into SB treated tumors. In an inducible HRAS model SB treatment caused increased inflammation in skin and formation of tumors with a more malignant phenotype than HRAS expression alone. Taken together these results suggest that inhibition of papilloma formation and increased conversion to SCC was due to separate keratinocyte populations, one that requires ALK5 signaling for tumor outgrowth and another that forms tumors despite ALK5 inhibition through a differential response to the altered inflammatory microenvironment.

Here we used an in vitro model to further investigate the differential effects of ALK5 inhibition and TGFβ1 signaling on HRAS-expressing keratinocytes. Similar to the two-stage chemical carcinogenesis model in which DMBA causes an initiating mutation in codon 61 of the HRas oncogene, we used a K5/tetORAS transgenic model which has a HRAS mutation in codon 12 and overexpresses this gene when induced. We show that these keratinocytes can be induced to express HRAS protein in a dose-dependent manner which is absent in cells lacking the K5 transgene. As shown in our and other studies, the specificity of ALK5 inhibition (31, 32) and the ability to maintain inhibition of endogenous and HRAS-induced TGFβ signaling by SB long-term verified that the observed effects on terminal differentiation are likely due to inhibition of TGFβ1 signaling.

In agreement with previous studies that show pharmacological ALK5 inhibitors have anti-cancer properties (33-36), we showed that blocking TGFβ signaling by SB inhibited cell growth and caused an induction in HRAS-induced cornified envelope formation. The increased expression of TGM and SPR mRNA in vitro by SB suggests that TGFβ plays a specific role in terminal differentiation, causing enhanced protein cross-linking as no differences were detected in expression of involucrin (data not shown). There was a similar response in vivo, since the expression of TGM1 and SPR1A was significantly induced in HRAS-expressing skin by SB and this correlated with increased thickness of the cornified layers.

We demonstrated that the regulation of genes that control protein cross-linking and CE formation is a more widespread response to the regulation of the TGFβ signaling pathway rather than TGFβ type I receptor inhibition. A significant decrease in TGM1 and TGM3 by the addition of TGFβ1 to HRAS-expressing keratinocytes and genetic overexpression of TGFβ1 in vivo was detected. Furthermore, we correlated the response in vitro to reduced cornification. Although the role of ALK5 in terminal
differentiation has not been investigated, previous studies have shown that TGFβ1 suppresses the expression of IFN-γ and TPA-induced TGM1 (16, 17), although one study showed that TGFβ1 increased TPA-induced cornified envelope formation (37). Calcium-induced terminal differentiation was also inhibited by TGFβ1 (38) suggesting that modulation by TGFβ1 requires the induction of differentiation by a variety of stimuli to yield a response. Others treating normal human epidermal keratinocytes with nearly 6 times more cytokine did not see a difference in TGM1 gene expression by TGFβ1 alone (14, 16, 17).

However, we showed significant TGM1 downregulation by TGFβ1 in vitro and a similar trend in vivo which may reflect a difference in sustainability of inhibition, origin of tissue and interaction with the external environment. We show here for the first time that alteration to TGFβ signaling not only regulates the expression of HRA-induced markers of protein cross-linking, but also results in keratinocytes that show characteristics of cornification through resistance to solubilization in boiling SDS-βME (39).

Whereas TGFβ1 inhibits cornification, other members of the TGFβ superfamily induce terminal differentiation. Expressed in the suprabasal layer of the skin (40), bone morphogenic protein (BMP) induced the expression of differentiation markers keratin 10 and involucrin in normal keratinocytes (41) and keratin 1 and 10 in keratinocytes induced to differentiate by suspension culture (42, 43). TGFβ1 and adenoviral expression of Smad6 and Mitogen-activated protein kinase kinase 4 (M KK4), but not p38αMAPK, inhibited BMP-6 induced differentiation (43). Since others have shown that Smad7 induced expression of differentiation markers and genes in differentiated keratinocytes (17, 44), it will be important to investigate whether HRA-induced differentiation is modulated by particular members of the TGFβ signaling pathway.

Despite the increase in cornification, we found that a subpopulation of keratinocytes were resistant to terminal differentiation and rapidly immortalized. Previous studies showed that growth arrest and senescence is suppressed in TGFβ1(-/-), DN-TGFβ type II receptor and Smad3(-/-) keratinocytes transduced with a v-RasHa retrovirus (45, 46). Similarly, oncogene-induced senescence is suppressed following long-term HRAS+SB treatment, which is supported by evidence that the expression of genes associated with squamous differentiation may also be linked to senescence (47). In contrast to earlier time points where keratinocytes were induced to growth arrest, the immortalized subpopulation of HRAS-expressing keratinocytes responded to SB by increased growth. Similarly, others have shown that a subpopulation of non-transformed cells under the control of the endogenous KRas2 promoter fail to undergo senescence in vivo and are immortalized in vitro (48-50). As an in vivo correlate, SA-β-galactosidase mosaicism within tumors suggests that a non-senescent tumor cell population emerge from senescent regions indicating that cells are capable of evading tumor suppressor checkpoints, potentially by spontaneous upregulation of Ras which is required for tumorigenesis (51). These results collectively suggest that this population of keratinocytes may have a more malignant phenotype as has been shown in
a number of models where inhibition of ALK5 resulted in increased SCC formation (52-54). However, the terminal differentiation resistant cells did not show other characteristics of malignancy, as they did not form tumors in skin grafts and were not capable of growth in 0.5mM calcium (21). Although this does not fully support our finding that SB treated papillomas have a higher rate of malignant conversion as shown in Chapter 2, the switch in responsiveness to HRAS expression and ALK5 inhibition, immortalization and resistance to senescence suggests that the differential response of this keratinocyte subpopulation is responsible for an altered tumor phenotype. This interpretation is strengthened by data from Chapter 2 in which SCC from the two-stage chemical carcinogenesis model arise from a subpopulation of benign lesions with biochemically distinct properties at an early stage of tumor development, while the majority of papillomas do not have the capacity to progress to malignancy (55, 56).

While we show that terminal differentiation is induced in HRAS-expressing keratinocytes treated with SB compared to HRAS alone, we did not detect differences in proliferation or cell cycle phase between these groups at day 4. We speculate that differences in the heterogeneous keratinocyte population composed of both immortalized cells that are retained and keratinocytes destined for terminal differentiation that become detached from culture plates cannot be detected. Although immortalized cells appear to respond positively to growth with ALK5 inhibition and HRAS expression, at day 11 it appears that both immortalized keratinocytes and those expressing HRAS alone have similar cell cycle profiles. In chapter 2, we also showed that SB treatment had no effect on proliferation in SB and TPA+SB treated skin following 24 hours treatment, and only a mild difference (4%-1% proliferation in control v. SB; 60% to 50% proliferation in TPA v. TPA+SB) following 2 weeks treatment.

As a growth inhibitor, it is conceivable that ALK5 inhibition by SB treatment alone would cause enhanced growth in keratinocytes. However, we did not see a difference with SB treatment alone at both day 4 and 11. Similar to these results, others have shown that SB and other ALK5 inhibitors do not modulate proliferation to the extent that TGFβ1 classically does. At concentrations much higher than what we have used, SB caused a 50% reversal of TGFβ1-induced proliferation inhibition after 28 hours in FET cells (non-tumorigenic colon cancer cells) and no change in cell number four days after TGFβ1+SB treatment compared to control and TGFβ1 alone. In FET, Mv1Lu (mink lung epithelial cells), and RIE (rat intestinal epithelial cells) 10.0µM SB only resulted in a 10% increase in keratinocytes in TGFβ1-induced S-phase and 8% decrease in G0-G1 phase following 35 hours treatment (35). At a concentration similar to that used in our work, SB431542 mildly recovered TGFβ1-induced growth inhibition and with reduced potency compared to another ALK5 inhibitor (A-83-01) in MvLu1 cells (57). The ALK5 inhibitor SD-208 had no effect on endogenous and exogenous TGFβ1-induced proliferation in MvLu1 cells (36), no effect on cell proliferation in mouse mammary carcinoma (R3T) tumors, and in the absence of exogenous TGFβ1, SD-093 did not reduce cell number following 72 hours treatment of the 4T1 murine
mammary carcinoma cell line (33). Collectively, these results may indicate the importance of Smad and ALK5-independent signaling in regulation of TGFβ1-mediated growth inhibition.

Since terminal differentiation and the expression of differentiation genes have been shown to be altered in transformed epidermal cells (10, 58), we tested the response of SP1 and PAM2.12 cells to ALK5 inhibition. It has been shown that TGFβ1 inhibited TGM1 expression in SCC25 cells alone and following treatment with either TPA or IFN-γ (17). We show here that both papilloma and SCC cell lines were responsive to downregulation of TGM and SPR genes by TGFβ1, and the differences in TGM1 and SPR1A were significant. Luciferase data (Figure 3-7A,B) indicate that TGFβ signaling via ALK5 was intact. Interestingly, ALK5 inhibition did not cause a dramatic increase in any of the genes although trends similar to those observed in HRAS-expressing keratinocytes were found, indicating that regulation in these cell types may depend on a difference in level of Ras-expression or state of progression. Whether the expression of these genes results in cornified envelope formation by long-term ALK5 inhibition occurs is yet to be determined.

Due to mechanisms that are still unclear, we observed that cells which continued to proliferate following long-term induction of HRAS had reduced HRAS protein levels despite no change in expression of the tetORAS transgene. Similar results were obtained in keratinocytes transduced with a v-RasHA retrovirus. HRAS protein expression is further decreased by ALK5 inhibition at a number of time points. It may seem plausible that reduced expression of HRAS due to inhibition of TGFβ signaling may lead to a reduction in the endpoints seen in our immortalized population including HRAS-induced terminal differentiation and senescence. However, others have shown that consistent low levels of Ras expression in mammary glands (51), lung bronchio-alveolar cells (49) and fibroblasts (50) stimulate proliferation and hyperplasia but not senescence and high levels of Ras protein expression results in Ink4a-ARF dependent cellular senescence (51). Our data show that downstream pathways including ERK are still activated, potentially via alternative signaling. In agreement, others have shown that impaired terminal differentiation through mutation of β1 integrin resulted in sustained ERK/MAPK phosphorylation (59), and the switch in keratinocyte phenotype including resistance to terminal differentiation may contribute to this activation. As a correlate to enhanced malignant conversion in the two-stage chemical carcinogenesis model, accumulation of additional mutations may be required in vivo to develop a malignant phenotype beyond that produced here with HRAS-expression.

Using an in vitro model of initiated keratinocytes, we have built upon previous studies showing that the TGFβ signaling pathway regulates terminal differentiation and have expanded our understanding of the mechanisms by which this occurs through the use of a pharmacological ALK5 inhibitor. Considering that the TGFβ signaling pathway is notorious for its dual role in carcinogenesis as both a tumor suppressor and an oncogene at varying stages in progression (60, 61), we have provided new
insight into the potential for subpopulations of keratinocytes with differential responses to alterations in TGFβ signaling over time. The presence of HRAS-expressing cells that are induced to terminally differentiate by pharmacological ALK5 inhibition among others that are resistant to this growth arrest suggests a previously unidentified mechanism by which ALK5 inhibition can inhibit tumor formation and enhance progression in vivo.
3.6 Bibliography

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Chapter 4
Discussion

Purpose

Transforming growth factor β1 (TGFβ1) is a cytokine that regulates a number of cellular processes including proliferation, differentiation, immune regulation and carcinogenesis. Pharmaceutical companies have identified that TGFβ type I receptor (ALK5) inhibitors have potential as anti-fibrotic and anti-cancer therapeutics. For example, scleroderma is a fibrotic skin disease that occurs due to a failure to terminate the wound-healing response and is characterized by early inflammation and vascular injury, followed by fibrosis of skin and other organs (1). The fibroblasts of this diseased tissue secrete TGFβ1 (2) which causes increased signaling via phosphorylation of Smad2 and Smad3 (3). ALK5 inhibition has been successful in reducing the expression of fibrotic markers in patients with diffuse cutaneous systemic sclerosis (4-6) and an anti-TGFβ antibody reduced fibrosis in the lungs and skin in a bleomycin-induced model of scleroderma (7, 8). Linked to the overexpression of TGFβ1 signaling, chronic renal diseases such as diabetic nephropathy, hypertension and glomerulonephritis are caused by fibrosis and reduced kidney filtration function (9). Small molecule ALK5 inhibitors cause reduced expression of genes involved in these diseases as well as improvement in renal function in vivo (10, 11).

Alterations and mutations to the TGFβ signaling pathway are also present in many human cancer types and there is a significant correlation between TGFβ1 expression in tumors and poor prognosis for cancer patients (12). TGFβ1 classically plays a dual role in carcinogenesis, acting as a growth inhibitor early in tumor development and as an oncogene during tumor progression (13-15). Investigation of the role of ALK5 in skin carcinogenesis suggests that this receptor is important for blocking epithelial tumor progression through mechanisms that regulate the immune and proliferative response (16-19). In vitro studies have shown that pharmacological inhibition of ALK5 inhibited epithelial to mesenchymal transition, cell motility, migration and invasion in human cell lines (20, 21). Short term in vivo treatment with ALK5 inhibitors inhibited growth and metastasis in mouse mammary carcinoma (22, 23). Pharmacological ALK5 inhibition also resulted in an anti-cancer response through affects on CD8+ cells (24), cytotoxic T lymphocytes (22-24), and natural killer cells and macrophages (25), but also promoted epithelial cell carcinoma in Eker rats (26).

Skin cancer affects one in five Americans in their lifetime (27) and more than 2 million people are diagnosed with skin cancer each year in the United States (28). While clinical trials have shown there is promise for the use of small molecule ALK5 inhibitors in treating fibrotic diseases, initial studies showing ALK5 as a potential anti-cancer target have overwhelmingly utilized in vitro methodologies and have not addressed the impact of cancer stage and long-term ALK5 inhibition in vivo. Furthermore, the role of ALK5 in skin carcinogenesis has not been fully explored and pharmacological ALK5 inhibition
may actually have unfavorable and off-target effects. Therefore, these preclinical studies are important in identifying risks involved in targeting the TGFβ signaling pathway in skin therapeutics.

**Current Studies**

In Chapter 2, we identified how pharmacological ALK5 inhibition alters tumor formation and progression *in vivo* using the two-stage skin chemical carcinogenesis assay. For the first time, we show that SB431542 (SB) is effective in blocking the TGFβ signaling pathway *in vivo* through topical treatment of mouse skin. SB inhibited the endogenous expression of TGFβ1 target genes *LTBP-1* and *Smad7* as well as tumor promoter-induced phosphorylation of Smad2 detected by indirect immunofluorescence and western blot. These data established the effectiveness of this ALK5 inhibitor in blocking TGFβ signaling in mouse skin. Using the two-stage skin chemical carcinogenesis model, we demonstrated that treatment with SB significantly reduced papilloma formation compared to mice treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) alone. This correlated with our and other chemical carcinogenesis studies that showed TGFβ1 heterozygosity (29) and Smad3 knockout (30) inhibited tumor formation. We also found reduced tumor volumes with SB treatment at early time points, although there was no significant difference in tumor volume beyond 21 weeks. Investigation of the role of ALK5 in tumor promotion by TPA showed that ALK5 inhibition reduced proliferation and slightly increased apoptosis, with the net results being no change in epidermal thickness. This is contrary to the current paradigm of TGFβ1 as a growth suppressor during the early stages of tumorigenesis (15). Remarkably, the impact on cutaneous inflammation appears to be a more essential mechanism by which TGFβ1 signaling regulates tumor formation. We showed that treatment with SB during tumor promotion dramatically reduced the number of neutrophils infiltrating into the skin, correlating with a decrease in the neutrophil chemokines KC and MIP2. These results show that ALK5 inhibition may have an anti-tumoral effect and support an association between a TGFβ-induced inflammatory response and tumor promotion.

Although there was a significant reduction in papilloma formation due to pharmacological ALK5 inhibition, of the papillomas that did form, there was a two-fold higher rate of conversion to squamous cell carcinoma (SCC) showing that ALK5 inhibition enhanced malignant tumor progression and that there is also a risk associated with the use of these pharmaceutical agents. In agreement with our studies, transgenic and knockout models with blocked TGFβ receptor signaling (17, 19, 31-33) showed increased incidence of malignant conversion. ALK5 knockout resulted in a 45% increase in development of head and neck squamous cell carcinoma (16) and a similar result was observed in SCC development of the periorbital and perianal regions (17) as well as with colorectal cancer development (18). These data support a surprising role for the TGFβ type I receptor in tumor development and progression.
contradictory to the classical role of TGFβ1 as a tumor suppressor during initiation and oncogene during tumor progression.

In Chapter 2, we used the skin chemical carcinogenesis model which utilizes the carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA) to predominantly cause an A to T transversion mutation in codon 61 of the Hras gene (34). In chapter 3 we used a similar model in which the keratin 5 promoter drives HRAS expression in keratinocytes (K5/tetORAS) in order to understand additional mechanisms by which the interaction of Ras and TGFβ signaling in epidermal cells may result in changes in tumor formation and progression. We discovered an interesting biological endpoint associated with ALK5 inhibition of HRAS-expressing keratinocytes. Keratinocytes that express the HRAS oncogene underwent terminal differentiation and cell death that was enhanced by ALK5 inhibition. This biological endpoint may be another mechanism by which inhibition of TGFβ signaling results in reduced tumor formation as shown in tumor studies performed in chapter 2. We showed that TGFβ signaling is responsible for selectively altering the expression of differentiation markers responsible for protein cross-linking of the cornified envelope. The mRNA levels of TGM1 and TGM3 as well as SPR1A and SPR2H are increased following treatment with SB in mouse HRAS-expressing keratinocytes. Transglutaminases catalyze the cross-linking of SPRs to loricrin and this permanently fixes the cornified envelope and results in terminally differentiated keratinocytes. Supporting a role for TGFβ1 as a negative regulator of cornification, treatment with TGFβ1 inhibited expression of TGM1 and TGM3 both in vivo and in vitro, and inhibited HRAS-induced cornified envelope formation. Interestingly, expression of other markers of terminal differentiation such as involucrin was not altered indicating that ALK5 specifically alters the expression of genes involved in cross-linking of the cornified envelope. This is also the first time that changes in these genes have been identified in vivo as a response to altered TGFβ signaling following a differentiation stimuli. However, in vivo data showed differences in transglutaminases, but not SPRs. Since SPRs only account for 5% of the cornified envelope (35), it may be difficult to detect changes in expression in whole skin, expression may not be altered, or altered expression may occur at a different time during cornification than the experiments we have performed. Considering that TGFβ1 inhibits expression of differentiation markers in other models where keratinocytes are induced to differentiate by TPA and calcium (36-38), these results imply that TGFβ signaling may be important in modulating the signaling associated with differentiation induced by exogenous factors. More broadly, these data indicate that TGFβ may have tumor formation stimulatory properties as has been supported in chapter 2.

Following terminal differentiation in ALK5 inhibited keratinocytes expressing HRAS, we observed a subpopulation of keratinocytes with characteristics very different from the primary K5/tetORAS keratinocytes. These cells had increased growth when ALK5-inhibited and induced to express HRAS, resisted terminal differentiation and senescence, and were immortalized as cells could be
passed more than 15 times. Others have also shown that a subpopulation of non-transformed cells under the control of the endogenous KRas2 promoter fail to undergo senescence in vivo and are immortalized in vitro (39-41). Although these keratinocytes still responded to exogenous TGFβ1 and induced expression of keratin 1 by increased calcium, they did not possess a tumorigenic phenotype, as they did not form tumors when keratinocytes were grafted onto athymic nude mice and were not capable of growing in elevated calcium media. In contrast to these data, others have shown that keratinocytes resistant to calcium-induced terminal differentiation show characteristics of malignant conversion (42-44). We proposed that this population of resistant keratinocytes is no longer responsive to terminal differentiation by SB, and a parallel population may exist in vivo where there is a correlation between the pro-inflammatory effects of ALK5 inhibition and tumor progression. This causes a switch in the role of TGFβ signaling from tumor promoter to suppressor of progression, potentially resulting in malignant conversion by ALK5 inhibition through not only a pro-inflammatory microenvironment but also altered squamous differentiation. It is also possible that the keratinocytes with altered responsiveness to TGFβ signaling are a specific subpopulation in the epidermis, such as epidermal stem cells. However, we did not find an increase in expression of epidermal stem cell markers CD34 and keratin 15 in vitro, and the frequency of colony formation (0.9%) was less than what others have found in culture (6%) (Personal communication, Rebecca Morris, Hormel Institute). Therefore, it is unlikely that this is the source in vitro. Overall, these data show that ALK5 regulates the suppression of the TPA-induced inflammatory response and terminal differentiation, and this is required for anti-tumoral effects. Yet, the data from chapters 2 and 3 are novel in that we identify the presence of subpopulations of epidermal cells that are dependent on HRAS and inhibition of TGFβ signaling for tumor progression.

Observations from the InvTA/tetORAS model further support the interpretation of multiple epidermal cell populations that respond differently to altered TGFβ signaling. We showed that papilloma formation following SB treatment was inhibited due to reduced cutaneous inflammation, yet progression of these tumors is enhanced through neutrophil infiltration and an altered inflammatory microenvironment. These data were supported by the InvTA/tetORAS model (Figure 2-11) where ALK5 inhibition enhanced neutrophil infiltration. We also showed in Figure 3-5 that in vivo treatment of InvTA/tetORAS skin with SB resulted in increased cornified layers. Interestingly, others have shown a similar connection between inflammatory diseases and altered squamous differentiation. In a model similar to InvTA/tetORAS, abnormal epidermal thickening and accumulation of cornified layers is observed in mice that constitutively express mitogen-activated protein kinase kinase (MEK1) through an involucrin driven promoter. This was associated with a considerable inflammatory infiltrate, linking these two seemingly dissimilar biological endpoints potentially via IL-1 expression. Although constitutive expression of MEK1 did not result in spontaneous papilloma formation, papillomas were observed at sites
of skin injury (45). The expression of differentiation genes SPR1 and SPR2 also correlated with inflammatory diseases and expression was increased in benign papilloma and SCC compared to epidermis alone (46). We hypothesized that increased inflammation seen in HRAS-expressing skin treated with SB enhances malignant progression, yet we also postulate that enhanced terminal differentiation and cornification are a block for tumor formation. The regulation by TGFβ in these responses may be multifaceted. As shown in chapter 3 there may be epidermal cells resistant to terminal differentiation that remain following the upregulation of differentiation genes and cornification. This subset of resistant cells may respond differently to the inflammatory microenvironment induced by ALK5 inhibition and HRAS with a higher frequency of malignant conversion, as shown in chapter 2 by increased expression of keratin 18, a marker of simple epithelia and malignant conversion of squamous epithelia (47, 48). Since SB treated papillomas had a higher rate of progression and we observed a poorly differentiated and more invasive phenotype in InvTATetORAS tumors treated for 5 weeks with SB, this supports the important role for HRas expression in papilloma formation and the differential role of TGFβ signaling in altering the epidermal cell populations.

**Pharmacological vs. Genetic Inhibition**

The two stage chemical skin carcinogenesis assay has been successful in dissecting the role of TGFβ signaling in tumor formation and progression. However, variable results have been obtained in different model systems. Overexpression of TGFβ1 in the suprabasal layer of the epidermis caused reduced papilloma formation (49) but this result was also observed in TGFβ1(+/-) mice with a loss of function mutation in the TGFβ1 gene of all tissues (29) and Smad3 knockout mice with targeted disruption of the Smad3 gene in all tissues (30). Both TGFβ1 overexpression (49) and TGFβ1(+/-) mice (29) showed increased conversion to SCC, although in Smad3 knockout mice this response was reduced (30). Opposingly, dominant negative TGFβ type II receptor targeted to the suprabasal (33) and basal layers (32) showed increased tumor formation and malignant conversion. Conditional ALK5 deletion driven by the K14 promoter in the oral cavity of mice resulted in increased SCC incidence following initiation by DMBA (16) and neuronal conditional knockout resulted in increased spontaneous SCC in the periorbital and perianal regions after 6 months (17). The variability exemplified in these results may be contributed to model systems in which different cell types have altered TGFβ signaling. In TGFβ1 and Smad3 knockout models, it is difficult to ascertain the differences between epidermal, dermal and inflammatory cell contributions to skin tumor formation and progression. Furthermore, blocking different mediators (ligand, receptors, transcription factors) in the TGFβ signaling pathway may yield inconsistent results. Reduced levels of TGFβ1 cytokine may affect other signaling pathways such as MAPK, NFkB and AP1. It may also alter the responses of inflammatory cells that require TGFβ1 for chemotaxis.
Blocking the TGFβ type II receptor may alter interactions with not only ALK5, but alternative type I receptors such as ALK1 and ALK2 that signal through Smad1 and Smad5 (50). Others have shown that conditional ALK5 deletion in the oral cavity results in activation of the PI3K-AKT pathway (16), showing the importance of Smad-independent signaling. Therefore, it is difficult to fully predict the result of blocking specific aspects of TGFβ signaling since it is so heavily involved in activating numerous pathways and can have varying effects on different stages of disease pathologies as well as cell types.

Previous studies have not addressed how pharmacological inhibition of ALK5 alters tumor formation and progression. Yet, topical treatment of a drug is more applicable to clinical studies. One of the main drawbacks of pharmacological inhibition is off-target effects. Most ALK5 inhibitors including SB are specific for ALK5, but inhibit the activin receptor ALK4 and nodal receptor ALK7 to a lesser extent. Protein kinase domains such as p38α MAPK, ERK and JNK are also structurally similar to the ALK5 kinase. Although it has been demonstrated that SB has no direct effect on these Smad-independent signaling pathways, nor the Activin and BMP receptors ALK2 and ALK6, respectively (51), we cannot completely rule out off-target-signaling. This potentially makes it difficult to determine the origin of adverse effects unless a high level of specificity exists. While genetic models may target TGFβ1 signaling disruption to certain tissues, pharmacological inhibition may affect a number of cell types following topical application. We showed that epidermal cells are primarily affected by the topical treatment of SB431542 through visualization of Smad2 phosphorylation by immunofluorescence, and whole skin extracts also show reduced Smad2 phosphorylation by protein analysis, but we have not determined the depth of penetration of SB following topical treatment. Therefore, epidermal, dermal, and inflammatory cells may be affected by treatment with the potential for systemic exposure. Additionally, the level to which TGFβ signaling is blocked may be dramatically different in genetic models, which is another reason why differing results could be attained.

We originally hypothesized that reduced TPA-induced neutrophil infiltration following SB was due to reduced secretion of neutrophil chemokines MIP2 and KC by keratinocytes. However, we did not detect a decrease in mRNA levels of these chemokines in vitro in either TPA+SB treated keratinocytes or fibroblasts compared to TPA alone (data not shown). This discrepancy may originate from the fact that in vivo results may not recapitulate in vitro and the crosstalk between the dermis with the epidermis may be required. Since ALK5 knockout mice are not viable (52), generation of a tissue specific ALK5 knockout mouse model would greatly enhance the understanding of the role that this receptor plays in specific cell types and in carcinogenesis. It would be advantageous to target ALK5 deletion to the epidermis and dermis separately during a two-stage skin carcinogenesis assay and with tumor promotion studies in order to determine the contributions of each cell type to tumor formation and progression. These data would also further support the use of pharmacological inhibitors that are currently not specific for ALK5 and
may also non-specifically inhibit ALK4 and ALK7 due to structure similarities. An ALK5 flox/flox mouse has been generated, although the studies performed were targeted to hematopoietic stem cells (53) and neuronal cells (17). A keratin 14 or keratin 5 regulated Cre mouse that causes conditional tissue-specific knockout of ALK5 in the epidermis or a type I collagen α2 Cre mouse that drives expression in the dermis could be used in conjunction with the ALK5 flox/flox mice to address these issues.

Since neither the dermis nor epidermis appeared to be responsible for altered chemokine secretion in vitro, there may also be a difference in the immune cell response to ALK5 inhibition. TGFβ1 is a strong chemoattractant for neutrophils (54, 55) and neutrophils express TGFβ type I receptors which exhibit a high affinity for the TGFβ1 ligand, demonstrating an important role for TGFβ1 in mediating the pro-inflammatory and chemotactic effects of neutrophils (55). Studies showing that SB can inhibit neutrophil chemotaxis during tumor promotion and that the opposite response occurs with targeted HRas-expression would enhance the hypothesis that inhibition of TGFβ signaling is directly responsible for the change in neutrophil infiltration.

Additional characterization of these neutrophils would aid in the interpretation of cell specific effects. We show in chapter 2 that SB resulted in reduced neutrophil infiltration in tumor promotion studies, but early papillomas treated with SB had increased intratumoral neutrophils and decreased keratin 1 staining that suggested a more progressed phenotype, and a similar result was observed in HRAS-expressing skin following SB treatment. However, the characteristics of these immune cells have not been established. Tumor-associated macrophages (TAMs) can be classified as M1 or M2 phenotype and are characterized by their response to the cytokine microenvironment. M1 macrophages are classically activated, which means that they respond to endogenous inflammatory insults. They are anti-tumorigenic and proinflammatory. M2 macrophages and the related myeloid derived suppressor cells are alternatively activated, inhibiting the M1 phenotype, and are protumorigenic and anti-inflammatory based on the cytokines that they secrete (56). Overexpression of the DN-TGFβ type II receptor in a murine macrophage cell line showed that TGFβ resistance induces an M1 phenotype in vitro (57). Conversely, TGFβ promotes the M2 phenotype (58). TGFβ1 can block neutrophil degranulation and activation (59) and migration of neutrophils (54, 55). Recent work shows that the change in phenotype and function that occurs with macrophages may also occur with neutrophils. Tumor associated neutrophils (TANs) can acquire a protumorigenic phenotype (N2) that appears to be driven by TGFβ, while inhibition of TGFβ signaling results in the antitumor phenotype (N1). Similar to results in chapter 2, pharmacological ALK5 inhibition of xenotransplanted AB12 cells (murine malignant mesothelioma cell line) into syngeneic mice resulted in an increase in intratumoral CD11b+, Ly6G+ cells that were overwhelmingly neutrophils, and this was also associated with an increased in neutrophil chemokines. However, in this study with a xenotransplanted tumor, treatment with the ALK5 inhibitor SM16 resulted in an anti-tumoral N1
neutrophil phenotype characterized by reduced expression of arginase, CCL2 and CCL5, increased TNFα and a more lobulated and hypersegmented neutrophil morphology. Similar results were achieved with whole lungs containing KRas derived tumors and xenotransplanted murine lung cancer cell derived tumors. The authors concluded that suppression of TGFβ signaling shifted neutrophils to an N1 phenotype that caused a reduction in tumor growth (60). In contrast, we showed an association between intratumoral neutrophils and increased progression with pharmacological ALK5 inhibition. However, ALK5 inhibition by SM16 was tested using preexisting tumors with short-term treatments (1 week). In addition, SM16 was administered orally compared to topical SB treatment. This likely alters the cell types that are subjected to TGFβ signaling inhibition, and may cause a differential response of neutrophils to environmental stimuli. In order to determine the precise role of neutrophil influx on tumor progression, classification of N1 or N2 status should be performed in papillomas and SCCs as there may be a differential role for these neutrophil types in different cancer stages. Moreover, it may also be important to determine the phenotype of neutrophils following tumor promotion, as this may contribute to our interpretation of a mechanism for reduced tumor formation with ALK5 inhibition.

Preclinical and Clinical Relevance:

More recently, the inhibitor 4-(4-[3-(Pyridin-2-yl)-1H-pyrazol-4-yl]pyridine-2-yl)-N-(tetrahydro-2Hpyran-4-yl) benzamide or GW788388 (GW) has been used as a treatment for fibrosis. Like SB, this inhibitor blocks ALK4, ALK5 and ALK7 but has no effect on ALK2, ALK3 and ALK6 as well as p38α MAPK signaling. It also inhibits TGFβ1-induced growth inhibition and epithelial to mesenchymal transition. Unlike SB431542, it is orally bioavailable and has better in vivo exposure as its half-life is more than 8 times that of SB (4.1±1.8h for GW v. 28.5±16.1min for SB). Oral administration of GW at 2mg/kg/day for 5 weeks resulted in decreased glomerulopathy in a model of spontaneous diabetic nephropathy. This was supported by reduced levels of fibrotic markers PAI-1, type I collagen and type III collagen to nearly the same levels as non-diabetic littersmates. Although GW showed specificity in inhibiting kinase activity of both the TGFβ type I and II receptors, but not the BMP type II receptor, these results demonstrate that this inhibitor has potential as an anti-fibrotic therapeutic (10). Another orally bioavailable ALK5 inhibitor 3-[6-(2-Morpholin-4-yl-ethoxy)-naphthalen-1-yl]-2-pyridin-2-yl-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole or LY2109761 (LY) has been optimized. It was the most potent of the series as tested with an in vivo target inhibition assay of Smad2 phosphorylation and was specific for the TGFβ type I receptor. As expected, in a rat exposure study good exposure and bioavailability was achieved and long duration was observed with 50% inhibition after 4 hours. In vivo evaluation of efficacy showed that this inhibitor, administered by oral gavage in saline solution, significantly delayed tumor growth in xenografted mice with human MX-1 breast carcinoma cells which had previously been shown
to be sensitive to TGFβ1 inhibition (61). Use of these inhibitors could provide longer and more efficacious blockade of TGFβ signaling and provide a route of administration that is more applicable to pharmaceutical use. It may be important to investigate whether this mode of drug delivery yields similar inhibition of tumor formation.

As a potential anti-tumor agent, it can be anticipated that ALK5 inhibitors may be used in order to reduce the growth of skin tumors that are already present. Therefore, future studies should investigate the effect of pharmacological ALK5 inhibition in preexisting papillomas in order to determine if an outcome similar to treatment before the appearance of papillomas is achieved. A two-stage skin carcinogenesis assay should be performed, forming papillomas with the standard DMBA/TPA protocol. By assessing changes in papilloma progression or regression, as well as mechanisms including neutrophil infiltration, apoptosis, and proliferation, we will have a better understanding of how this class of small molecule inhibitors can be used in a more realistic scenario.

It is estimated that 90% of non-melanoma skin cancers are associated with exposure to UV light (62) rather than exposure to environmental/industrial toxicants, viruses, genetics and lifestyle choices such as smoking, drinking and diet. Sun-exposure related skin cancers are a result of ultraviolet radiation: UVA (320-400nm) and UVB (280-310nm). The p53 tumor suppressor gene is the primary target for DNA mutations by UV, which is known to cause C to T transitions or CC to TT tandem mutations in the p53 gene (63). Clusters of epidermal cells with mutant p53 have been found in hairless mice long before the appearance of malignant tumors (64), and this has also been demonstrated in humans (65). Understanding how ALK5 inhibition alters tumor formation and progression in this mode of skin carcinogenesis will be an important aspect of understanding the efficacies and risks involved with using these pharmaceutical agents. Current studies being performed in our lab are designed to address this question. Preliminary analysis suggests that ALK5 inhibition by SB reduced the number of papillomas formed following exposure to UVB, yet the conversion rate to SCC may be higher in the SB treatment group. These results are strikingly similar to the data presented in chapter 3 using the chemical carcinogenesis mouse model and are yet another example of the risks involved with long-term use of ALK5 inhibitors in the skin. However, the mechanisms by which this occurs may be different and should be developed in order to potentially optimize the effects of ALK5 inhibition in skin carcinogenesis. Additionally, the ex vivo human skin carcinogenesis HaCaT cell line (human keratinocyte spontaneously immortalized cell line) which harbors a p53 activating mutation (66) should be used to correlate changes in markers of epidermal homeostasis.

We also demonstrated that early papillomas have a lower level of expression of keratin 1 and a slight increase in keratin 13 and InvTA/tetORAS transgenic mice have a greater expression of keratin 18 following treatment with SB. These markers suggest a progressed phenotype associated with a higher risk
for malignant conversion. A high degree of variability in potential for progression has also been observed in human premalignant lesions such as oral leukoplakia, actinic keratosis and cervical dysplasia (67). The identification of a correlation between a specific phenotype/ biomarkers and risk may aid in differentiating this potential. As explained above, there may be a difference in the functionality of intratumoral neutrophils with a protumorigenic N2 phenotype that may contribute to enhanced progression. While tumor-associated CD3+ cells have been associated with low-risk for malignant conversion (68), further establishment of an immune cell phenotype for high-risk and low-risk papillomas using the two-stage carcinogenesis model may translate to human cancers and a clinician’s ability to determine an optimal course of treatment for patients.

While changes in terminal differentiation and senescence have been observed in mouse epidermal keratinocytes in vitro as shown in chapter 3, we are ultimately interested in whether these result translate to human keratinocyte models. In order to investigate this aim, human foreskin epidermal cells should be transduced with pBabe-puro H-RasG12V retroviral construct (69) or retroviral vector pLXSN containing the untagged cDNA for V12Ras. Transient transfection should be attained via plasmid vector pcDNA3 containing untagged variants of V12Ras that harbor point mutations in the effector domain (70). Cells would be treated with SB431542 over time and terminal differentiation, gene expression and senescence investigated. Additionally, these keratinocytes could be infected with dominant negative ALK5-K232R (71) to demonstrate similar results through genetic TGFβ1 pathway inhibition. Although HaCaT cells would be readily available, these cells possess a p53 mutation and do not undergo senescence (72), both of which would inhibit analysis of our hypotheses. These results would provide substantial support to our experiments performed in mouse models, validate the use of pharmacological ALK5 inhibitors as anti-cancer therapeutics in certain clinical scenarios and aid in the risk-assessment of these pharmaceuticals for cancer and other diseases such as fibrosis.

Preclinical data has been used as proof-of-concept for clinical trials using drugs that block TGFβ signaling as therapeutics for fibrosis and cancer as documented by the U.S. National Institute of Health. Peptide 144 (P144) is the acetic salt of a 14mer peptide from human TGFβ type III receptor and has been shown to significantly decrease fibrosis in a bleomycin-induced mouse model of scleroderma (73). It blocks the interaction between TGFβ and the TGFβ type III receptor (betaglycan) and thus downstream TGFβ and Smad signaling. In phase I clinical trials the bioavailability in serum and tolerability, as tested using the visual scale of Frosch and Kligman, of topical P144 was tested over 21 days with positive results for use at the highest dose (0.03%) (NCT00656825). A phase II clinical trial for 3 months (NCT00574613) and an open label extension phase II clinical trial for 6 months (NCT00781053) with daily topical treatment of 0.03% P144 are currently recruiting patients and will investigate changes in soluble collagen content and skin hardness as indicators of disease improvement. This peptide has also
been tested as an anti-tumor immunotherapy in mouse models. As an adjuvant therapy, p144 enhanced tumor rejection from 15 to 70% due to the inhibition of TGFβ1 produced by regulatory CD4+CD25+ T cells rather than tumor cells. Higher activity of dendritic cells, natural killer cells and tumor antigen-specific T cells and decreased myeloid-derived suppressor cells was also observed (74). Another phase I clinical trial is investigating the effects of the pan-neutralizing IgG4 anti-TGFβ monoclonal antibody GC1008 that is directed against all 3 TGFβ isoforms (NCT00899444) in unresectable advanced or metastatic kidney cancer development or malignant melanoma in order to determine whether treatment results in a decrease in Smad2 phosphorylation as a marker for inhibition of TGFβ signaling. Preliminary analysis using GC1008 from another clinical trial showed that 5/22 patients treated with GC1008 had shrinking of liver metastasis and other sites. One significant adverse affect possibly related to treatment with GC1008 was a well-differentiated SCC in a patient with a history of this cancer (75). Since ALK5 inhibition has shown similar promise in preclinical models of both fibrosis and cancer, these clinical trials should consider the data presented in chapters 2 and 3 for potential off-target or adverse effects associated with long-term TGFβ signaling blockade. It is specifically interesting that the adverse effect of SCC formation was noted with GC1008 use, which parallels the studies presented in chapters 2 since we also see a risk of malignant conversion associated with use of SB431542. Overall, the use of TGFβ inhibitors in clinical studies demonstrates a need for further pre-clinical analysis of long-term effects following treatment as has been developed here.

**Cellular and Molecular Mechanisms:**

Oncogenic Ras is known to alter keratinocyte terminal differentiation through the protein kinase C (PKC) pathway (76). We have shown that PKCα phosphorylation is not altered following long-term SB treatment of RAS-induced keratinocytes (data not shown). However, data show that phosphorylation of PKCδ causes keratinocytes expressing Ras to resist terminal differentiation after exposure to differentiation signals such as elevated Ca\(^{2+}\) or phorbol esters (77). To test this, future studies could use immunoprecipitation to detect PKCδ tyrosine phosphorylation both short and long-term following doxycycline and/or SB treatment to determine changes in this posttranslational modification. Other PKC isoforms as well as PKC activity could be inspected for changes in overall expression by western blot. Bryostatin, a PKC activator, could be used to determine if markers of terminal differentiation can be inhibited in ALK5-inhibited, RAS-expressing keratinocytes. Ras-mediated signaling can also act through the PI3K (phosphatidylinositol-3,4,5-triphosphate) -AKT pathway. PI3K-AKT signaling is activated when keratinocytes are induced to differentiate, and inhibition of this pathway correlated with decreased protein expression of late differentiation markers loricrin and filaggrin (78). However, we show in chapter
that the level of p-AKT does not change between day 4 and 11 keratinocytes, indicating that this signaling pathway is likely not responsible for resistance to terminal differentiation.

Many of the genes involved in differentiation are encoded on the epidermal differentiation complex (EDC) which is located within a 2.05-Mb region on human chromosome 1q21 (79) and mouse chromosome 3 (80) which can cause coordinated expression as we have seen here with SPR and TGM genes. There is also evidence that TGM and SPR among other differentiation genes are regulated by c-Jun. The TGM1 promoter contains an AP-1 binding element and supershift analysis identified AP-1 members in protein complexes binding to these sites in differentiated epithelial cells (81). During human keratinocyte differentiation, c-Jun inhibited SPR2 promoter activity and involucrin expression (82). Since we have shown that TGFβ(+/−) skin and keratinocytes have reduced TPA-induced c-Jun phosphorylation (29), we hypothesize that downregulation of AP1 by ALK5 inhibition may contribute to increased RAS-induced terminal differentiation through the expression of this battery of genes. Regulation of AP1 may also contribute to the changes in inflammation observed in chapter 2. As a chemoattractant for all leukocytes, TGFβ1 is known to induce inflammation (83), and epidermal deletion of AP1 proteins JunB and c-Jun causes a psoriasis-like disease and arthritis in part through increased expression of s100a8 and s100a9 proteins (84, 85) and TNFα expression (86). Therefore, downregulation of AP-1 by inhibition of TGFβ signaling may also contribute to the anti-inflammatory effects of SB on tumor promotion. Investigating of AP1 signaling will provide more insight into the molecular mechanisms of altered tumor formation and progression.

Like terminal differentiation, senescence is a biological response to terminate the propagation of cells that have undergone damage. In chapter 3, we observed that long-term treatment of RAS-expressing keratinocytes with SB caused the outgrowth of a population of cells that are resistant to senescence. We have also shown reduced senescence in RAS-infected keratinocytes treated with SB long-term. This indicates that these cells may have a growth advantage as demonstrated by their immortalization and may reflect the ability of these cells to cause a more progressed phenotype as was observed with the two-stage carcinogenesis assay in chapter 2. In support of this, v-rasH1a retrovirus infected TGFβ1-null, dominant negative TGFβ type II receptor and TGFβ1 neutralizing antibody treated keratinocytes resist senescence and this is associated with the formation of SCCs following skin grafting (87). Exploring the presence of senescent cells in not only the papillomas and SCCs formed during this assay, but also following TPA/SB treatment in the skin would provide insight into whether ALK5 inhibition is altering this permanent cell cycle arrest in vivo. Frozen sections of normal skin, papillomas and carcinomas should be used to identify differences in the senescence response. We would predict that SB431542 treated mice would exhibit reduced senescence which would explain an increase in carcinoma incidence. While K5rtTA/tetORAS adult mice do not survive more than a week post doxycycline exposure, InvrtTA/tetORAS mice removed
from doxycycline for 2 weeks generate focal tumors as shown in chapter 2. Tissue from this experiment would also be useful for further analysis. To determine a mechanism for this response, changes in the expression of cell cycle regulators such as p16\textsuperscript{ink4A} and p21 should be investigated.

TGFβ1 is classically a growth inhibitor of keratinocytes, so we hypothesize that inhibiting ALK5 would enhance growth. However, we found in chapter 2 that proliferation was slightly reduced following 2 weeks treatment of skin with TPA and SB, and no change was detected in early tumors. Reduced proliferation measured by 5-bromo-2-deoxyuridine (BrdU) incorporation was also observed in TGFβ1 (+/-) mice following short-term and chronic treatment with TPA and in papillomas isolated at 10 weeks (29), and this was consistent with reduced tumor formation observed in Smad3 null mice (30).

Furthermore, senescence and terminal differentiation are often associated with a change in proliferation, but we did not detect a difference in cell cycle phases or proliferation following SB treatment in vitro by BrdU/propidium iodide Flow analysis. Considering there is evidence for multiple epidermal cell populations both in vitro and in vivo, it may be difficult to distinguish the characteristics and responses of each to ALK5 inhibition. An increase in proliferation may be diluted out by reduced proliferation in keratinocytes terminally differentiating. We also speculate that overexpression of TGFβ1 versus reduced expression of physiological levels may result in different proliferative responses to tumor promoters. This is further supported by differences in the regulation of proliferation in vitro by ALK5 inhibitors with and without exogenous TGFβ1. In the absence of exogenous TGFβ1, SD-093 did not reduce cell number following 72 hours treatment of the 4T1 murine mammary carcinoma cell line (22). Also, in FET (non-tumorigenic colon cancer cells), Mv1Lu (mink lung epithelial cells), and RIE (rat intestinal epithelial cells) 10.0µM SB resulted in a 10% increase in keratinocytes in TGFβ1-induced S-phase and 8% decrease in G0-G1 phase following 35 hours treatment (20). Although the effects of ALK5 inhibition on proliferation in tumor promoter experiments were statistically significant, tumor formation and progression may depend more heavily on changes in the inflammatory microenvironment.

Over 90% of all human malignancies arise from epithelial cells. Therefore, it is important to understand the characteristics of cells that are able to escape tumor suppressive checkpoints such as terminal differentiation and senescence to malignancy. In chapter 2, we observed that papillomas and RAS expressing skin treated with SB both had reduced expression of keratin 1 and an increase in the expression of markers such as keratin 13 and keratin 18 which are indicative of a more progressed phenotype (68). Markers of malignant conversion, such as keratin 8/18 (47, 88) and keratin 13 should be investigated in the immortalized keratinocyte population compared to earlier time points. Studies in primary mouse keratinocytes show that treatment with SB431542 induces expression of K8, which was also achieved by dominant negative TβRII adenoviral infection (89). Investigation of markers of progression should also be extended to the papilloma and SCC cell lines SP1 and PAM2.12, respectively,
in order to determine the potential for enhanced progression of ALK5 inhibition on cells that are at different stages in tumor progression.

In chapter 3 we proposed that a subpopulation of RAS-expressing keratinocytes respond to ALK5 inhibition by terminally differentiating, and this cell death may be a mechanism by which there is a reduction in tumor formation following a chemically-induced Ras mutation in epidermal cells. Because we saw a difference in the expression of differentiation genes in papilloma and SCC cell lines in chapter 3, we were interested in whether expression is altered in tumors collected in the two-stage chemical carcinogenesis assay performed in chapter 2. We did not see a difference in expression of transglutaminases in either the papillomas or SCC that were collected at later time points (data not shown). The biological changes that occur due to altered terminal differentiation by ALK5 inhibition may be more apparent at earlier time points. Since similar results are seen in both RAS and TPA-induced terminal differentiation following TGFβ1 treatment (37), future experiments should determine whether alterations in gene expression can be detected in vivo in tumor promotion studies performed in chapter 2. Investigating the alteration of differentiation at the level of tumor promotion may provide more insight into the mechanism of reduced tumor formation.

One finding from the studies that is not completely understood is related the interaction between Ras and TGFβ signaling. We show in chapter 3 that although the RAS transgene is activated by doxycycline and transgene expression does not change, there is a decrease in HRAS protein levels and this expression is further reduced with ALK5 inhibition (Figure 3-10A,B). Similar results were observed in v-RasHA infected keratinocytes as shown in figure 3-10C, with decreased HRAS expression following 11 days in culture and an even greater reduction with SB. Unpublished work from our lab has also shown a similar decrease in HRAS protein levels in v-rasHA infected Smad3 knockout keratinocytes. Therefore, it seems plausible that TGFβ may regulate the expression of the HRAS protein. Extensive work from the Mulder Lab at the Pennsylvania State University College of Medicine show that TGFβ1 in the absence of serum or exogenous factors can rapidly activate (3-6 minutes) Ras, ERKs and SAPKs (90, 91). The quick response suggests that the activation of Ras is a direct effect of TGFβ1 signaling. Our data suggest that ALK5 inhibition downregulates HRAS protein expression long-term, which suggests that it may not be a direct effect. It is possible that the difference in HRAS expression is due to selection of keratinocytes expressing low levels of RAS that do not require TGFβ signaling via ALK5 for survival. As others have shown in mammary tumorigenesis (92), low levels of RAS expression correlates with reduced senescence and tumor formation, while epithelial cells that express high levels of RAS undergo senescence and do not proliferate. We have emphasized the possibility for multiple cell populations in early papillomas where ALK5 inhibition caused a phenotype of high-risk for malignant conversion and in chapter 3 where ALK5 inhibition caused the outgrowth of an immortalized, terminal differentiation-resistant cell type. We have
also hypothesized that TGFβ enhances HRAS protein stability, and ALK5 inhibition may results in increased protein turnover. Yet, we show in repeated experiments that TGFβ1 treatment does not inhibit the protein stability of HRAS between 1-6 hours (data not shown). However, the half-life of HRAS may be much longer and warrant a change in experimental design. This should be further developed in the keratinocyte models described above. If a connection between Ras and TGFβ is not identified based on protein turnover, the potential for a cell selection process is strengthened.

Overall, the experiments performed in this dissertation reveal a dual role of TGFβ in tumor formation and progression in the skin through context-dependent homeostatic and inflammatory mechanisms, emphasizing the necessity of testing pharmacological inhibitors in in vivo, multi-stage models of skin carcinogenesis.
4.2 Bibliography


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Appendix A

Transforming growth factor β1 enhances tumor promotion in mouse skin carcinogenesis

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Transforming growth factor β1 (TGFβ1) expression is elevated by tumor promoters in the mouse skin, but its role in tumor promotion has not been well defined. To investigate this, we have compared TGFβ1+/+ and +/- mice in a two-stage skin chemical carcinogenesis protocol. Surprisingly, TGFβ1+/+ mice had fewer number and incidence of benign papillomas, reduced epidermal and tumor cell proliferation and reduced epidermal TGFβ1 and nuclear p-Smad2 localization in response to the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) compared with TGFβ1+/+ mice. Maximal TPA activation of protein kinase C (PKCα) as measured by activity assays and activation of target genes and induction of cornified envelopes correlated with TGFβ1 gene dosage in keratinocytes and addition of exogenous TGFβ1 restored the cornification defect in TGFβ1+/+ keratinocytes. Similarly, inhibition of ALK5-suppressed TPA-mediated PKCα activation suggesting that physiologic levels of TGFβ1 are required for maximal activation of PKC-dependent mitogenic responses. Paradoxically, the TPA-induced inflammatory response was greater in TGFβ1+/+ skin, but TGFβ1+/+ papillomas had more infiltrating myeloperoxidase-positive cells and pro-inflammatory gene expression was elevated in v-ras-transduced TGFβ1+/+ but not TGFβ1+/+ keratinocytes. Thus, TGFβ1+/+ keratinocytes were more pro-inflammatory and pro-inflammatory cytokines were more abundant in response to TPA and enhanced papilloma formation in the TGFβ1+/+ mice, the frequency of malignant conversion was reduced compared with TGFβ1+/+ mice. Therefore, TGFβ1 promotes benign tumors by modifying tumor promoter-induced cell proliferation and inflammation but retains a suppressive function for malignant conversion.

Introduction

Transforming growth factor β1 (TGFβ1) is a regulatory cytokine that has stage-specific stimulatory and suppressive actions in cancer development (1). In the two-stage mouse skin carcinogenesis model, benign epidermal papillomas are caused by topical application of the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) and repeated promotion with 12-O-tetradecanoylphorbol 13-acetate (TPA). Inhibition of TGFβ1 signaling in this model accelerates malignancy and overexpression of TGFβ1 suppresses benign tumor formation (1) but causes outgrowth of highly malignant spindle cell cancers (2) with increased tumor cell metastases (3). TPA and other tumor promoters cause epidermal hyperplasia, dermal inflammation (4) and induce TGFβ1 expression in the epidermis (5). Although in mammary carcinogenesis, reduced TGFβ1 levels in the TGFβ1+/+ mouse enhance tumor growth (6), it is not known what role TGFβ1 has in tumor promotion. The surprising resistance of Smaβ-null mice to skin tumor formation (7) suggests that in the epidermis, TGFβ1 signaling may not simply act as a negative feedback pathway. To examine the role of TGFβ1 in skin tumor promotion, we compared the response of TGFβ1+/+ and +/- mice to acute and chronic treatment with TPA and evaluated tumor development in a two-stage skin carcinogenesis assay. Our studies show that TGFβ1 enhances tumor promotion through effects on PKC, but the benign tumors that form have a low frequency of premalignant progression to squamous cell carcinoma (SCC).

Materials and methods

Animal studies

Seven to eight-week-old TGFβ1+/+ and TGFβ1+/+ mice (8) backcrossed onto a Balb/c background were used for all in vivo studies. Adult TGFβ1+/+ mice were not used due to post-natal lethality (8). TGFβ1+/+ and TGFβ1+/+ mice were given topically with a single dose of 50 μg of DMBA (Sigma, St. Louis, MO) and 5 or 10 μg of TPA (Cayman, La Jolla, CA) in 200 μl of acetone twice a week for 25 weeks and tumours >2 mm recorded weekly. Skin tumour induction was done similarly for 10 weeks. For acute TPA, mice were treated once with 5 μg of TPA/200 μl acetone or acetone alone and dorsal skins were harvested as indicated. For chronic promotion, mice were treated with TPA (5 μg) twice weekly for 5 weeks, and tissues were isolated after 72 h. Double transgenic mice with conditional expression of active TGFβ1 (9) were given doxycycline (2 mg) intraperitoneally 24 h prior to TPA treatment. All animals were housed and treated according to approved Institutional animal protocols.

Tissue analysis

Analysis of tumors and measurement of epidermal thickness was done on hematoxylin- and eosin-stained sections of neutral buffered formalin-fixed tissues. Epidermal layers were quantitated at every 20 basal cell for each section, and five sections averaged per treatment group. Specific antibodies to Smaβ2 (Santa Cruz Biotechnology, Santa Cruz, CA) and p38α-Smaβ2 (Millipore, Billerica, MA) (10–12) were used to detect these proteins in frozen and ethanol-fixed sections, respectively, by indirect immunofluorescence. Stained sections were imaged using an Olympus V300 Laser Scanning Confocal Microscope. A Smaβ2-specific blocking peptide (Santa Cruz Biotechnology) was used to demonstrate specificity of Smaβ2 cytoplasmic and nuclear staining. Cell proliferation was measured using anti-bromodeoxyuridine immunohistochemistry as described previously (9) and p20 cells identified using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick and labeling assay (TUNEL) and expressed as a percentage of total basal cell per section. Immunofluorescent cells were detected using anti-myeloperoxidase (MPO) antibodies (Dako, Carpinteria, CA) and the average of positive cells per section was scored. Quantification of epidermal layers and immunostaining was done blindly. Photomicrographs of tissue sections were made using an Olympus BX61/Epi-Fluorescence Microscope.

Cell culture

Primary keratinocytes or dermal fibroblasts obtained from crosses of TGFβ1+/+ adult mice were isolated and cultured as described previously (13) and treated with TGFβ1 (R&D Systems, Minneapolis, MN) and TPA as indicated. Where indicated, SB431542 (Sigma, St. Louis, MO) was added 15 min before TPA. Keratinocytes were infected with the v-ras-transduced retrovirus as described (13). Keratinocytes were transduced with 0.25 μg activator protein 1 (AP-1) luciferase reporter (Stratagene, Cedar Creek, TX) and a renilla-luciferase control plasmid were treated with 5 ng/ml TPA, and luciferase activity was determined using a Promega Renata luminometer. PKC activity was measured in 0.3% Triton X-100 extracts of TPA-treated (25 ng/ml) TGFβ1+/+ and +/- primary keratinocytes as described (13–14) with PKC substrate (Ser)3 (19-31) peptide (AnaSpec, Fremont, CA). Cornified envelopes were measured 36 h after TPA treatment as described elsewhere (14,15).

Analysis of protein and RNA

Keratinocytes or whole skin were homogenized in a 1% Triton X-100 lysis buffer with protease and phosphatase inhibitors, and specific proteins were detected by immunoblotting and enhanced chemiluminescence (Pierce, Illinois, USA).

Abbreviations: AP-1, Activator protein 1; DMBA, 7,12-dimethylbenz[a]anthracene; MPO, myeloperoxidase; mRNA, messenger RNA; PKC, protein kinase C, SCC, squamous cell carcinoma; TGFβ1, transforming growth factor β1; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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Rockford, IL) using antibodies directed against Smad 2/3, p-Smad2, p-Erk, Erk, p-stress-activated protein kinase/N-terminal kinase, p-c-jun or c-jun (Cell Signaling Technology, Danvers, MA). Densitometric analysis of c-jun and p-c-jun blots was done using the Kodak Gel Logic Imaging System and Molecular Imaging Software. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), and quantitative reverse transcription–polymerase chain reaction done for the indicated genes using the MxIQ system (BioRad Laboratories, Hercules, CA). All values were normalized to β-actin. Primer sequences were obtained from published studies or using Primer 3 (16) software with GenBank sequence information.

Statistical analysis

Values are expressed as the mean ± SE. Student’s t-test was used to compare the indicated groups, and the significance of the difference was described. P values of <0.05 were regarded as indicating a significant difference. Difference in papilloma per mouse for each genotype was measured using an unpaired t-test.

Results

Papilloma formation but not malignant conversion is suppressed in TGFβ1 +/− mice

To determine if TGFβ1+/+ and TGFβ1+/− mice differ in the induction of epidermal squamous tumors, 7- to 8-week-old TGFβ1+/+ and TGFβ1+/− mice were treated topically with DMBA and promoted with 5 or 10 μg TPA twice a week for 25 weeks. At both doses of TPA, the papilloma frequency was significantly reduced in TGFβ1+/− mice compared with TGFβ1+/+ mice, with maximum frequency of 1.8 and 2.6 (5 μg) papillomas per mouse and 3.6 and 4.7 (10 μg) papillomas per mouse, respectively (Figure 1A). As expected the papillomas frequency declined after cessation of TPA promotion due to regression and removal of mice with malignancies. The percentage of mice developing tumors was lower in the TGFβ1+/− mice at 5 μg TPA but there was little difference between genotypes at the higher dose (Figure 1A). Similar results were obtained in a pilot study with fewer mice per group. Additionally, at early time points measured between weeks 20–26, papillomas in the TGFβ1+/+ mice were significantly larger than in the TGFβ1+/− animals, although this was not significant at later time points (Figure 1B). Despite larger numbers of papillomas in the TGFβ1+/+ mice at both TPA doses, similar numbers of SCC formed in both genotypes (Figure 1C), indicating a 2-fold increase in frequency of malignant conversion in the TGFβ1+/− mice. Thus, the additional TGFβ1+/+ papillomas are not at high risk for malignant conversion, suggesting that TGFβ1 acts as a suppressor of malignant conversion but enhances benign tumor formation.

TPA-induced proliferation is reduced in epidermis and papillomas of TGFβ1+/− mice

To test if TGFβ1 levels altered the epidermal response to TPA, TGFβ1+/+ and TGFβ1+/− mice were treated with TPA alone once

Fig. 1. Papilloma frequency and size are greater in TGFβ1+/+ mice, but conversion to SCC is reduced. TGFβ1+/+ and +/− mice were initiated with DMBA and promoted with TPA twice per week for 23 weeks. The number of papillomas ≥2 mm was determined on a weekly basis. (A) Papillomas per mouse from mice promoted with 5 μg (top) and 10 μg (middle) TPA and percent of mice with papillomas (bottom). The papilloma frequency was significantly higher in TGFβ1+/− mice promoted with 5 μg TPA (P = 0.0013) and 10 μg TPA (P = 0.01). At each dose, 21/28 and 20/24 TGFβ1+/+ and 13/20 and 19/20 TGFβ1+/− mice developed tumors. (B) Tumor volumes between weeks 20–30 were measured using a digital micrometer. Average volumes were determined from measurements of length × width × height, and statistical significance determined using a t-test to compare genotypes at each time point. (C) The total papilloma and SCC yield and percent conversion for each genotype. Percent conversion at each TPA dose was determined by dividing the total number of SCC that formed during the course of the study by the maximum number of papillomas; P, papilloma; C, carcinoma.
or biweekly for 5 weeks. TPA-induced epidermal hyperplasia was significantly greater in the TGFβ1−/− mice after 72 h of treatment (4.03 ± 0.17 versus 3.40 ± 0.08) (Figure 2A and B), and although the initial increase in epidermal proliferation was similar between the two genotypes, by 72 h, post-TPA epidermal proliferation had decreased significantly in the TGFβ1−/− mice (Figure 2B, bottom). Similarly, after chronic TPA treatment, there was a greater number of epidermal layers (Figure 2A) and higher epidermal labeling index (Figure 2C) in the TGFβ1−/− mice compared with TGFβ1+/+ mice, but no significant difference in TPA-induced hyperkeratinization. Papillomas generated in TGFβ1−/− mice after DMBA and 10 week TPA promotion also had a significantly higher percentage of bromodeoxyuridine-positive tumor cells (29.1 ± 6.6%) compared with TGFβ1+/+ (15.4 ± 3.4%) (Figure 2A and D). There was no difference in expression of the known TGFβ1 target gene p21(WAF1)/Cip1 in response to TPA (data not shown). There was also no significant difference in TUNEL-positive epidermal keratinocytes between genotypes after acute (TGFβ1−/− 2.2 ± 0.3% versus TGFβ1+/+ 1.73 ± 0.3% at 72 h; P = 0.1) or chronic TPA treatment (TGFβ1−/− 3.04 ± 0.16% versus TGFβ1+/+ 2.25 ± 0.3%; P = 0.1).

**Fig. 2.** TGFβ1 enhances TPA-induced proliferation in normal epidermis and tumors. (A) Tgfr2−/− representative hematoxylin- and eosin-stained sections of acetone or TPA-treated skin at 72 h post-treatment and after 5 weeks chronic TPA treatment, magnification ×200. Bottom: detection of proliferating tumor cells (arrows) with anti-bromodeoxyuridine (BrDU) immunohistochemistry in TGFβ1−/− and TGFβ1+/+ 10-week papillomas. Magnification ×400. Tumor basement membrane indicated by dashed line. Scale bars represent 20 μm for all images. (B) Quantiﬁcation of epidermal hyperplasia (top) and proliferation (bottom) in TPA-treated TGFβ1−/− mice compared with TGFβ1+/+ mice. The number of cell layers in hematoxylin- and eosin-stained sections was determined every 20 basal cells along a section and averaged from 5 mice per group. BrDU-positive cells were quantified from anti-BrDU-stained sections and averaged from 5 mice per time point for each genotype. (C) Quantiﬁcation of epidermal proliferation in TGFβ1−/− and TGFβ1+/+ skin after chronic TPA treatment. BrDU-positive epidermal keratinocytes were quantiﬁed from mice treated twice per week with TPA or acetone (c) for 5 weeks (N = 5 mice per group). (D) Quantiﬁcation of tumor cell proliferation. BrDU-positive tumor cells were quantiﬁed from papillomas generated in each genotype with DMBA and 10-week TPA promotion. (N = 5 tumors per group). Papillomas were isolated 72 h after last TPA treatment. Asterisk represents signiﬁcantly different from indicated group.

**TGFβ1 response to TPA is reduced in TGFβ1−/− skin and keratinocytes**

Since, surprisingly, a wild-type TGFβ1 genotype was associated with enhanced proliferative responses to TPA, we examined TGFβ1 induction, a well-characterized response to tumor promoters in the mouse epidermis (5,18). Twelve hours after TPA treatment, TGFβ1 protein levels were increased in all the layers of the TGFβ1−/− epidermis consistent with published reports (19), but there was little detectable change in the TGFβ1+/− epidermis (Figure 3A). Similarly, levels of nuclear phospho-Smad2 and total Smad2 as detected by indirect immunofluorescence with two well-characterized antibodies (10–12) were reduced in TPA-treated TGFβ1+/+ epidermal keratinocytes compared with TGFβ1−/− keratinocytes (Figure 3A, bottom and Figure S1 is available at CancerResearchOnline). In agreement, immunoblot analysis of total skin protein extracts in TGFβ1+/− skin showed an increase in total Smad2 24 h post-TPA that was sustained through 96 h, whereas in the TGFβ1+/− skin, the increase in total Smad2 was reduced and delayed until 72 h (Figure 3B). Phosphorylation of Smad2 was seen 12 h after topical TPA treatment in both genotypes. However, p-Smad2 levels in the TGFβ1+/− skin after 24 h and throughout 96 h were consistently higher than in the TGFβ1+/− skin, particularly at the later time points (Figure 3B). Although there was an induction of p-Smad2 from 12 to 96 h in the TPA-treated TGFβ1+/− skin (Figure 3B), it is possible that this is due to TGFβ1 pathway activation in non-epidermal cells since phospho-Smad2-positive cells were detected in the dermis of TPA-treated skin (Figures S1A and S2A are available at CancerResearchOnline). Figure 3C shows that as expected for a hemizygous state, TGFβ1+/− keratinocytes had roughly 50% basal expression of TGFβ1 messenger RNA (mRNA) compared with TGFβ1+/+ keratinocytes. However, TPA treatment caused a rapid 9-fold increase in TGFβ1 mRNA in the TGFβ1+/− keratinocytes but a slower 5-fold induction by 8 h in the TGFβ1+/− keratinocytes. In contrast, TPA caused a similar fold induction of TGFβ1 mRNA in primary dermal fibroblasts even though the absolute level was half in the heterozygote fibroblasts (Figure 3C). Keratinocytes from both genotypes responded similarly to exogenous TGFβ1 indicating that TGFβ1+/− keratinocytes do not have a general defect in ability to activate the TGFβ1 pathway (data not shown).

**Activation of PKC and AP-1 pathway is reduced in TGFβ1+/− keratinocytes**

Since TPA effects are mediated primarily through PKC, we investigated if TGFβ1 levels influence PKC activation in primary keratinocytes from each genotype. Within 30 min after TPA treatment, there was a 5.5-fold increase in PKC activity in the TGFβ1+/+ keratinocytes relative to basal levels that was sustained through 2 h post treatment but declined to near baseline by 4 h (Figure 4). In contrast, the maximal level of TPA-induced PKC activity was less and was not sustained in the TGFβ1−/− keratinocytes, suggesting more rapid downregulation of enzyme activity. Furthermore, pretreatment of keratinocytes of either genotype with the TGFβ1 type 1 receptor (ALK5) inhibitor SB431542 reduced PKC activity (Figure 4), supporting the idea that maximal PKC activity in keratinocytes is dependent on activation of TGFβ1 signaling. As expected, inclusion of the PKC inhibitor bisindolymaleimide 1 (25 mM) and 2.5 mM
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Fig. 3. TPA induction of TGFβ1 and p-Smad2 nuclear localization is reduced in TGFβ1+/− epidermis. (A) Top and middle: immunohistochemical detection of TGFβ1 protein in skin 12 h after acetone or TPA treatment, (magnification ×400). Bottom: detection of phospho-Smad2 by indirect immunofluorescence with an anti-p-Smad2 ser 465/467 antibody in tissue 24 h after TPA treatment. Magnification ×1000 and scale bars represent 20 μm. Exposure times for TGFβ1+/+ and TGFβ1+/− skins were identical. Figure S1A available at CurrGene Online shows individual and merged images with TO-PRO 3 nuclear counterstaining. Location of the basement membrane is indicated by a dashed line. (B) Immunodetection of total Smad2 and p-Smad2 in whole skin protein extracts isolated at the indicated time points after TPA treatment using specific anti-Smad2/3 and p-Smad2 antibodies and β-actin as a loading control. (C) Quantitative reverse transcription-polymerase chain reaction analysis of TGFβ1 mRNA induction by TPA (25 ng/ml) in primary keratinocytes (top) and fibroblasts (bottom) of the indicated genotype. Results are the average of three independent experiments. Asterisk represents significantly different from TGFβ1+/+ (P < 0.05).

Fig. 4. TGFβ1 modulates PKC activation by TPA. PKC enzyme activity was measured at the indicated times after TPA treatment (25 ng/ml) in triplicate cultures of primary keratinocytes of each genotype and expressed as fold increase over untreated control (1751.5 ± 536 c.p.m. for TGFβ1+/+ and 1642.2 ± 171.19 c.p.m. for TGFβ1+/− cells). TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ keratinocytes were also pretreated for 15 min with the small molecule ALK5 inhibitor SB431542 (0.5 μM) for TGFβ1+/− cells. TGFβ1+/+ kinase was significantly reduced in TGFβ1+/− keratinocytes compared with TGFβ1+/− keratinocytes (Figure 5C). No significant differences were found between treatments within the TGFβ1+/− cells or between untreated control cells (P > 0.5). This response was blocked by SB431542 (Figure 5C), supporting the involvement of the TGFβ1 signaling pathway. Interestingly, this difference between genotypes was not observed at higher TPA doses (data not shown), correlating with diminution of TGFβ1 genotype affects on tumor development at higher TPA doses. Finally, the induction of cornified envelopes, the end product of epidermal differentiation and a well-characterized response of keratinocytes to...
Fig. 5. TGFβ1 regulates AP-1-mediated effects of TPA. (A) Reduced induction of c-jun phosphorylation in TGFβ1+/− keratinocytes. Primary keratinocytes of each genotype were treated with 25 ng/ml TPA and cell lysates immunoblotted for the indicated proteins using total and phospho-specific antibodies, and β-actin as a loading control. (B) Reduced induction of c-jun phosphorylation in TPA-treated TGFβ1+/+ skin. TGFβ1+/+ and −/− mice were treated with 5 μg TPA or acetone and whole skin protein extracts were immunoblotted with anti-c-jun and total c-jun antibodies, and β-actin as a loading control. Detection of c-jun was confirmed using a positive control from keratinocytes (data not shown). The fold increase in c-jun phosphorylation was averaged from densitometric measurement of band intensity from four different samples at each time point. Asterisk represents significantly different from TGFβ1+/+ at corresponding time points P < 0.05. (C) Reduced AP-1 transactivation in TGFβ1+/− keratinocytes. Keratinocytes of each genotype were transfected with an AP-1 luciferase-reporter, treated with TPA (5 μg/ml) and 0.5 μM SB431542 (SB) were indicated and finally luciferase activity normalized to that of empty luciferase control. Data are representative of three independent experiments and each histogram is the average of five replicate transfections. Asterisk represents significantly different from other genotype. (D) Top: reduced TPA-induced terminal differentiation in TGFβ1+/− keratinocytes. Keratinocytes of each genotype were treated with TPA and cornified envelopes counted with a hemocytometer. Bottom, TGFβ1+/− were treated with TPA with or without 0.05 ng/ml TGFβ1 and cornified envelopes were quantitated. Average of three experiments is shown.

TPA dependent on AP-1 activity (23,24), was significantly higher in the TGFβ1+/+ keratinocytes (Figure 5D). In TGFβ1+/− keratinocytes, addition of exogenous TGFβ1 enhanced the induction of cornified envelopes by TPA (Figure 5D, P < 0.01) indicating a direct relationship between reduced TGFβ1 induction and altered differentiation in response to TPA in this genotype. Taken together these results indicate that maximal biological and biochemical responses to TPA in keratinocytes require physiological levels of TGFβ1.

TGFβ1 reduces the TPA-induced skin inflammatory response

In addition to proliferation, inflammation is a critical component of the response to TPA. Dermal inflammation was detected as early as 12 h post-TPA in both TGFβ1+/+ and TGFβ1+/− mice and was mainly composed of MPO-positive cells (Figure 6A). In contrast to the proliferative response, there was a decrease in MPO+ cells by 72 h post-TPA in the TGFβ1+/+ skin that did not occur in the TGFβ1+/− mice (Figure 6A and B top), and there were twice as many dermal inflammatory cells in chronically TPA-treated TGFβ1+/− skin compared with TGFβ1+/+ skin (Figure 6B top). Additionally, to test if TGFβ1 expression could block TPA-induced cutaneous inflammation, we used a transgenic K5/Tα X tetOTGFβ1 mouse line in which the expression of a constitutively active form of TGFβ1 can be regulated by doxycycline with an epidermally targeted reverse tetr transactivator (9). When these transgenic mice were dosed with doxycycline to induce TGFβ1 expression in the epidermis (2 mg intraperitoneally 24 h prior to topical TPA treatment), the number of skin infiltrating MPO+ cells was decreased by ~50% compared with mice treated with TPA alone (Figure 6B middle) indicating that TGFβ1 could directly inhibit TPA-induced inflammation.

The difference in cutaneous inflammation between genotypes was mirrored by expression of pro-inflammatory genes. We analyzed the expression of pro-inflammatory and neutrophil chemotactic molecules such as S100a8, S100a9 and KC (Cxc11) (25,26) and COX-2 that regulates inflammatory responses through prostaglandin and thromboxane metabolism (27). Although transcripts for the KC, S100a8 and S100a9 were induced in both genotypes, the levels were sustained and significantly higher in the TGFβ1+/+ skin for at least 48 h post-TPA, but these decayed by 24 h in the TGFβ1+/− skin (Figure 6C). No change in another S100 family member, S100a1 was seen in either genotype after TPA treatment (data not shown). With chronic TPA treatment, the expression of COX-2 and S100a8 was significantly higher in the TGFβ1+/− skin but there was no significant difference in KC or S100a9 between genotypes (Figure 6C). Similarly, in primary TGFβ1+/− keratinocytes, the absolute level and fold induction of COX-2, S100a8 and S100a9 was higher compared with TGFβ1+/− keratinocytes (Figure 6C). Thus, reduced levels of TGFβ1 directly enhanced TPA-induced pro-inflammatory gene expression in keratinocytes. Although the absolute expression levels were lower in isolated fibroblasts, the TPA-driven induction of COX-2, S100a8 and S100a9 expression was transient in TGFβ1+/+ fibroblasts but sustained in TGFβ1+/− fibroblasts, resembling the difference in expression pattern between keratinocytes of each genotype (Figure S2B).
is available at Carcinogenesis Online). Although TPA induced KC expression in TGFβ1+/− keratinocytes, the pattern of expression between the genotypes did not match that observed in whole TPA-treated skin (Figure 5C). However, the expression pattern of KC in TPA-treated fibroblasts was similar to that in whole skin (Figure S2B is available at Carcinogenesis Online), suggesting that responses from fibroblasts could be responsible for the higher induction of this chemokine in whole TGFβ1+/− skin.

**Elevated inflammatory response in TGFβ1+/+ tumors**

In contrast to TPA-treated normal skin, papillomas that developed after 10 weeks of promotion had approximately four times as many tumor infiltrating MPO+ cells in the TGFβ1+/+ papillomas (3.7 ± 1 MPO+ cells per 100 tumor cells) compared to the TGFβ1+/− papillomas (0.86 ± 0.13 MPO+ cells per 100 tumor cells) (Figure 6A and B, bottom). In the two-stage skin chemical carcinogenesis model with DMBA as the initiating carcinogen, mutations at codon 61 in the c-ras<sub>Ha</sub> gene occur at an extremely high frequency in papillomas and carcinomas (28,29). Similarly, introduction of oncogenic v-ras into primary keratinocytes with a replication-defective retrovirus generates a benign tumor cell phenotype in vitro and in vivo following skin grafting of transduced keratinocytes (30,31). Thus, to determine if ras activation altered effects of TGFβ1 on the inflammatory response, we examined pro-inflammatory cytokine gene expression in primary keratinocytes of both genotypes transduced with the v-ras<sub>Ha</sub> retrovirus. As expected TGFβ1 expression is higher in normal and v-ras<sub>Ha</sub> expressing TGFβ1+/+ keratinocytes (Figure 6D). While there was little difference in expression of S100a8 or KC between primary keratinocytes of each genotype, there were 5-fold higher level of S100a8 and 3-fold higher level of KC in v-ras<sub>Ha</sub> expressing TGFβ1+/+ keratinocytes. There was no effect of v-ras<sub>Ha</sub> on the expression of S100a8 and COX-2 between genotypes (Figure 6D). Thus, in the context of ras oncogene activation and developing papillomas, TGFβ1 acts as a pro-inflammatory cytokine.
Discussion

In the two-stage skin carcinogenesis model, repeated application of the phorbol ester tumor promoter TPA drives expansion of initiated clones of keratinocytes by enhancing proliferation and creating a chronic inflammatory environment (32). Many studies detailing the growth inhibitory and tumor suppressive actions of TGFβ1 support the idea that the induction of TGFβ1 by tumor promoters in keratinocytes is a negative feedback pathway to re-establish tissue homeostasis (13,33). Indeed, several skin-targeted TGFβ1 transgenic mouse models show that overexpression of this growth factor can inhibit tumor promotion and epidermal proliferation (2.34) as well as enhance cutaneous inflammation (7,9). However, using a model of TGFβ1 haploinsufficiency, we find that TGFβ1+/− mice developed fewer benign tumors with reduced incidence and size compared with TGFβ1+/− mice. Furthermore, we observed that the proliferative response to the tumor promoter TPA was less in the epidermis and papillomas that formed in TGFβ1+/− mice, consistent with the resistance of Smad3 null mice to skin carcinogenesis (7). In contrast, TPA-induced inflammation was reduced in TGFβ1+/+ skin but enhanced in TGFβ1+/− papillomas. Taken together these results suggest that physiological levels of TGFβ play an important positive role in tumor promotion by paradoxically enhancing epidermal proliferation and limiting cutaneous inflammatory responses to a tumor promoting stimulus in normal skin but stimulating inflammation within a developing tumor. Since the differences in tumor number and incidence between genotypes were reduced at higher TPA doses, tissue levels of TGFβ are probably to play a determinative role in tumor development at suboptimal promoter doses and could positively impact human tumor development under conditions of weak or intermittent chronic promoting stimuli.

In keratinocytes, PKCs is one of the major targets for TPA during tumor promotion (30,35). Our data showing transient activation of PKC, transient phosphorylation of downstream targets such as c-jun, reduced induction of TGFβ1 an AP-1 target gene (21,36) and AP-1 luciferase activity in TGFβ1+/− keratinocytes as well as suppression of PKC activation and AP-1 luciferase activity by the ALK5 inhibitor SB431542 in wild-type keratinocytes suggest that TGFβ1 directly influences the extent of PKC activation in response to TPA. These data also show that TGFβ1 does not influence initial activation of PKC but rather pathways regulating sustained activity or down-regulation, such that the signal strength from PKC activation is diminished. Previous reports have linked PKCα and PKCδ activation by TGFβ1 to p21induction, collagen I expression and phosphatase and tensin homolog transcriptional down-regulation (37-39) but we did not observe altered expression of p21 between genotypes. Our results suggest that regulation of AP-1 activity is a key PKC target that is modulated by TGFβ levels. Previous studies have shown that induction of TGFβ1 mRNA and protein is a rapid response of the epidermis to TPA (5,19). Our results suggest that the induction of TGFβ1 in response to TPA may be important for the comitogenic response as this was reduced in TGFβ1+/− keratinocytes and enhanced in these cells with addition of exogenous TGFβ1. Although we did not observe a significant difference in hyperkeratinosis in response to TPA between the genotypes, the reduced formation of cornified envelopes in vitro in the TPA-treated TGFβ1+/− keratinocytes suggests that the observed differences in proliferation and hyperplasia may be indirectly linked to TPA-induced terminal differentiation and epidermal turnover, which is mediated in part in keratinocytes by AP-1 (40,41). Further studies will be required to explore this possibility in more detail.

TGFβ1 is known to have both pro-inflammatory and anti-inflammatory properties in the skin and other tissues (42). In contrast to the reduced proliferative response in the TGFβ1+/− papillomas and epidermis, the TPA-induced inflammatory response in normal skin, as measured by total inflammatory infiltrate, MPO+ cells number and expression of pro-inflammatory cytokines in vitro, and in primary keratinocytes in vitro was greater and sustained over a longer time period in TGFβ1+/− mice compared with TGFβ1+/+ mice. Thus, in the normal epidermis, TGFβ1 acts to suppress inflammation and this is probably to be a direct effect since overexpression of TGFβ1 reduced TPA-associated inflammation. Since AP-1 can repress transcription of S100a8 and S100a9 genes in keratinocytes (43), it is possible that the rapid and increased induction of these pro-inflammatory mediators by TPA in TGFβ1+/− keratinocytes is due to reduced AP-1 activity. However, we observed that TGFβ1+/− early papillomas had abundant intraepithelial MPO+ cells, whereas there were virtually none in the TGFβ1+/− lesions. Thus, in the context of a developing papilloma with profound nuclear oncogene activation TGFβ1 production by keratinocytes contributes to a pro-inflammatory environment. Support this pro-inflammatory switch, we found that v-raf-transduced TGFβ1+/− keratinocytes expressed elevated levels of the pro-inflammatory genes S100a9 and KC (25) as well as TGFβ1, relative to v-Raf-transduced TGFβ1+− keratinocytes. The neutral chemotactic properties of TGFβ1 (44,45) as well as these additional pro-inflammatory cytokines could contribute to the pro-inflammatory switch in developing tumors, although additional factors must also allow infiltration of inflammatory cells into the epithelial component of the papillomas, as this was never observed in TPA treated normal skin of either genotype. While the data presented here provide strong evidence that the observed differential proliferative and inflammatory responses are due in part to reduced TGFβ1 expression in keratinocytes, we cannot rule out the influence of reduced TGFβ1 levels by fibroblasts and inflammatory cells as contributing to the observed responses in the intact animal. Tissue specific knockout of TGFβ1 in keratinocytes and other cutaneous cell types will be needed to directly test their role in proliferative and inflammatory responses associated with tumor promotion. Nevertheless, these data point to TGFβ1-mediated compartmentalization of inflammatory responses between normal skin and expanding clones of initiated keratinocytes as important factors for tumor promoting.

Despite the increased number of benign tumors, there was no increase in SCC in the TGFβ1+/− compared with the TGFβ1+/+ mice at either promoter dose. Thus, the additional papillomas that developed in the TGFβ1+/− mice are at low risk for malignant conversion. Studies in SenCarc mice show that the majority of papillomas do not progress to SCC, whereas a much smaller subpopulation is the precursors to SCC (46). These high-risk papillomas are characterized by reduced or absent expression of TGFβ1 relative to low-risk papillomas (47) and a reduced inflammatory gene expression signature similar to SCC (48). While the TGFβ1-associated increase in tumor cell proliferation and inflammation is linked to tumor outgrowth, the latter may suppress premalignant progression. In contrast, reduced levels of tumor-associated TGFβ1 may prevent inflammatory responses but enhance genetic instability (47,49,50) leading to more rapid malignant progression. Therefore, even at the earliest stages, physiological levels of TGFβ1 play a paradoxical role in cancer by enhancing tumor promotion and tumor outgrowth, but inhibiting premalignant progression.

Supplementary material

Supplementary Figures S1 and S2 can be found at http://earcin.oxfordjournals.org/

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Figure S1. Reduced levels of nuclear Smad2 and p-Smad2 in TPA treated TGFβ1+/− epidermis.

(A) Detection of p-Smad2 by indirect immunofluorescence in ethanol fixed sections from 24h TPA treated TGFβ1+/+ and TGFβ1+/− skin. Sections were stained with rabbit anti-pSmad2 (Millipore) and visualized with streptavidin-Alexa-Fluor-488. Nuclei were identified using TO-PRO 3 staining (middle panel) and merged images showing nuclear localization of p-Smad2 in TPA treated TGFβ1+/+ epidermis (right panel). Location of basement membrane is indicated by dashed lines. Arrows show p-Smad2 positive cells in the dermis, magnification 1000X. (B) Detection of Smad2 by indirect immunofluorescence in frozen sections from 24h TPA treated TGFβ1+/+ and TGFβ1+/− skin using anti-Smad2 antibody (Santa Cruz Biotechnology), and streptavidin-Alexa-Fluor-488 and TO-PRO 3 to localize nuclei. Arrows indicate representative nuclei with Smad2 localization. (C) Smad2 peptide blocks detection of Smad2. Anti-Smad2 antibody was preincubated with a Smad2 blocking peptide (Santa Cruz Biotechnology) prior to incubation with tissue sections, and detection with streptavidin-Alexa Fluor-488, Magnification 600X.
Figure S2. Response of TGFβ1+/+ and TGFβ1+- fibroblasts to TPA.

(A) Detection of p-Smad2 in dermal cells of TPA-treated TGFβ1+/+ and +/- skin. Skin sections were immunostained with an anti-pSmad2. Arrows show positive cells in the dermis of both genotypes. Magnification 400X. (E: epidermis; D: dermis) (B) Quantitative rt-PCR analysis of KC, S100a8, S100a9 and COX-2 gene expression in TPA-treated primary dermal fibroblasts. Fibroblasts were isolated from trypsin separated TGFβ1+/+ and +/- newborn skin and cultured in DMEM/10% FBS. Cells were treated with TPA (25 ng/mL) and RNA isolated at the indicated times. Histograms represent the average of 2 experiments with expression of each gene normalized to GAPDH.
APPENDIX B

H&E stain
Formalin fixed section

1. Deparaffinize and hydrate to PBS (five minutes each).
2. 30 min Gill’s Hematoxylin
3. 10 min tap water
4. 30 seconds Eosin
5. 2x5min 95% EtOH
6. 3x5min Histochoice
7. Mount

Anti-BrdU Immunohistochemistry
[Becton Dickinson 347580]

Ethanol fixed sections

1. Deparaffinize and hydrate (five minutes each). Last step PBS.
2. Incubate in 4N HCl seven minutes. Wash 3X5 in PBS.
3. Quenching endogenous peroxidase with 3% H$_2$O$_2$ in dH$_2$O for 5 minutes at RT. Wash 5 min in PBS.
4. 10% Normal Goat Serum in 3% BSA-PBS, 20 minutes at RT.
5. Primary antibody (Mouse anti-BrdU; BD): 1:50 in 10% Normal Goat Serum in 3% BSA-PBS. ON at 4°C. Wash 3X5 in PBS.
6. Secondary antibody: 1:1000 in PBS, 40 min at RT. Wash 3X5 in PBS.
   a. Biotin-SP-conjugated AffiniPure F(ab’)$_2$ Fragment Goat anti-Mouse IgG, F(ab’)$_2$ Fragment specific (115-066-072, Jackson ImmunoResearch, West Grove, PA)
7. Vectastain ABC Elite (PK-6200, Vector). 30 minutes at RT. Wash 3X5 in PBS. ABC MIX MUST STAND FOR 30 MINUTES AT RT BEFORE USE.
8. DAB-Substrate (Vector, SK-4100) 6 min at RT (Protected from direct light). Wash 3X2 with distilled water.
9. Counterstain nuclei with Gill’s hematoxylin for 30s and rinse with running tap water for five minutes.
10. Dehydrate and mount.

Immunohistochemistry
TUNEL ASSAY- using homemade buffers
Ethanol fixed sections

1. Deparaffinize and hydrate (five minutes each). Last step PBS.
2. Incubate in 20 μg/mL Proteinase K (1:1000 dilution of 20μg/μL Proteinase K stock in PBS) at RT for 15 min. Wash 3x5 min in PBS.
3. Incubate in 3% H$_2$O$_2$ (1:10 of 30% H$_2$O$_2$ in dH$_2$O) for 5 min at RT. Wash 3x5 min in PBS.
4. Make up appropriate amount of terminal deoxyribonucleotidyltransferase (TdT) Reaction Buffer. Keep reagents on ice.
   - account for steps 5 and 6 (50 μL per section for step 6).
   
   TdT Reaction Buffer
   (25 mM Tris-HCl, 200mM Sodium Cacodylate, 0.25 mg/mL BSA, 1mM CoCl$_2$)
   TdT Buffer Stock ........... 10 μL
   CoCl$_2$ (25 mM stock) ........ 2 μL
   Distilled H$_2$O ............... 38 μL
5. Incubate 5 min in 1x Trevigen TdT buffer.
6. Make up 1:20 dilution of TdT enzyme (New England Biolabs #M0252) in TdT Reaction Buffer. You will need 1μL of this for each reaction.
7. **TdT Reaction Mix*** Make extra reaction mix for 1 additional reaction ***
   **Per reaction:**
   - TdT Reaction Buffer……………………………………………... 50.0 μL
   - TdT enzyme dilution (from Step 6)………………………………… 1.0 μL
digoxigenin-11-uridine-5'-triphosphate (10 μM, 1:10 dilution)….0.5 μL
   (Roche #11558706910)

8. Add 50 μL TdT Reaction Mix (from Step 7) to each section. Incubate sections in a humidified box at 37º C for 1 hr.
9. Rinse off reaction mix in PBS. Stop reaction by adding 50-100 μL 1X Trevigen TdT Stop Wash Buffer to each section and incubating for 5 min at RT. Wash 3x5 min in PBS.
10. Block using 10% normal rabbit serum in 3% BSA for 20 min at RT.
11. Incubate sections in sheep anti-digoxigenin (cat#11333089001, Roche, Basel, Switzerland ) diluted 1:800 (400μg/ml ) in 3% BSA for 30 min at RT. Wash 3x5 min in PBS.
12. Incubate sections in biotinylated rabbit anti-sheep IgG (BA-6000, Vector) diluted 1:2000 in 3% BSA for 20 min at RT.
13. Vectastain ABC Elite (PK-6200, Vector). 20 minutes at RT. Wash 3X5 in PBS. ABC MIX MUST STAND FOR 30 MINUTES AT RT BEFORE USE.
14. DAB-Substrate (Vector, SK-4100). This is a fast reaction and you will need to monitor using microscope in culture room. Pull slider all the way to left to view sections with scope. Do one or two at a time to make sure that all sections are incubated for the same amount of time in DAB. It is likely that the reaction will need to be stopped within 25-30 seconds or you will get a lot of background labeled nuclei.
15. Stop reaction by placing section immediately in Hematoxylin for 1 minute, then wash in 2 changes of water.
16. Dehydrate and mount.

**TUNEL ASSAY**

**Buffer and reagent recipes**

**TdT Buffer Stock Solution (125 mM Tris-HCl, 1 M Sodium cacodylate, 1.25 mg/mL BSA, pH 6.6)**

- Tris.................................................................1.51 g
- Sodium cacodylate, Trihydrate ............................21.4 g
- BSA.................................................................0.125 g
- Distilled water..................................................100 mL

Fill halfway with water, adjust pH to 6.6 using HCl, top off to 100 mL. Make aliquots, store at -20º C.

**TdT Reaction Buffer (25 mM Tris-HCl, 200 mM Sodium cacodylate, 0.25 mg/mL BSA, 1 mM CoCl₂)**

- TdT Buffer Stock Solution.................................10 μL
- CoCl₂ (25 mM) .....................................................2 μL
- Distilled water..................................................38 μL

**Stop Wash Buffer (300 mM NaCl, 30 mM Sodium Citrate)**

- NaCl.................................................................1.75 g
- Sodium citrate, Trihydrate .................................0.88 g
- Distilled water..................................................100 mL

Mix until fully dissolved. Store at room temperature.
Anti-MPO Immunohistochemistry
[Rabbit anti-human MPO, DAKO A0398]
Ethanol fixed sections

1. Deparaffinize and hydrate to PBS (five minutes each).
2. Quenching endogenous peroxidase with 3% H₂O₂ in methanol for 20 minutes at RT. Wash 3X5 in PBS
3. 5% Normal Goat serum, 30 minutes at RT.
4. Primary antibody: 1:500 in 5% BSA in PBS. 30 min at RT. Wash 3X5 in PBS.
5. Secondary antibody: 1:2000 in PBS, 30 minutes at RT. Wash 3X5 in PBS.
   a. Biotin-conjugated Goat anti-Rabbit IgG (Vector BA-1000)
6. Vectastain ABC Elite (PK-6200, Vector). 30 minutes at RT. Wash 3X5 in PBS.
   a. ABC MIX MUST STAND FOR 30 MINUTES AT RT BEFORE USE.
7. DAB-Substrate (Vector, SK-4100) 5 min at RT (Protected from direct light). Wash 3X2 with distilled water.
8. Counterstain nuclei with Gill’s hematoxylin for 2 min and rinse with running tap water for five minutes.
9. Dehydrate, clear and mount.

Anti-F4/80 Immunofluorescence
[Clone C1:A3-1, Serotec MCAP947]
Ethanol fixed sections

1. Deparaffinize and hydrate (five minutes each). Last step PBS.
2. Microwave antigen retrieval (Vector Antigen Unmasking Solution 3.75ml + 400ml dH₂O)
   Warm up solution. Heat slides 2x5 min at 20% power. Add solution after 5 min. Cool down 10 min at RT. Wash 3 min PBS.
3. Block 30 min 10% Normal Goat Serum in 3% BSA/PBS.
4. Block 1h. in working solution of M.O.M. Mouse IgG Blocking reagent (Vector kit, BMK-2202).
   (Add 1 drop/45µL of stock solution to 1.25 ml PBS)
5. Wash 2x2 min in PBS
6. Incubate sections for 5 min in working solution of MOM Diluent- Protein concentrate.
   (Add 120µl of stock solution to 1.5 ml PBS)
7. Dilute primary antibody in MOM diluent to appropriate concentration. 1:100. 30 min at RT.
8. Wash sections 2 x 2min in PBS.
9. Apply secondary Ab, F(ab)₂ goat anti-rat (112-066-003, Jackson ImmunoResearch) 1:250 in PBS.
   Incubate 30 min at RT.
10. Wash 2x2min in PBS
11. AlexaFluor 488-SA- 1:200 in PBS, 30 min at 4°C protected from light.
12. Wash 2x2 min in PBS.
13. Counterstain- TO-PRO3- 1:5000 in PBS, 20 min at 4°C protected from light.
14. Wash 1x2 min in PBS.
15. Coverslip with Vectashield (Vector, H-1400) mounting medium with fluorescence, hard set. Keep at 4°C protected from light until ready to go to the microscope.
Anti-Keratin 1, Keratin 8, Keratin 10, Keratin 13, Loricrin Immunohistochemistry
[From NCI]
Ethanol fixed sections

1. Deparaffinize and hydrate (five minutes each). Last step PBS.
2. Quenching endogenous peroxidase with 3% H₂O₂ in dH₂O for 5 minutes at RT. Wash 5 min in PBS.
3. 10% Normal Goat Serum in 3% BSA-PBS, 20 minutes at RT.
4. Primary antibody in 10% Normal Goat Serum in 3% BSA-PBS. ON at 4°C. Wash 3X5 in PBS.
   - Keratin 1- 1:2000
   - Keratin 10- 1:500
   - Keratin 8- 1:5
   - Keratin 13- 1:1500
   - Loricrin- 1:1000
5. Secondary antibody: 1:1000 in PBS, 40 min at RT. Wash 3X5 in PBS.
   a. Biotin-SP-conjugated AffiniPure F(ab’)_2 Fragment Goat anti-Rabbit IgG, F(ab’)_2 Fragment specific (BA-1000)
6. Vectastain ABC Elite (PK-6200, Vector). 40 minutes at RT. Wash 3X5 in PBS. ABC MIX MUST STAND FOR 30 MINUTES AT RT BEFORE USE.
7. DAB-Substrate (Vector, SK-4100) 1 min at RT (Protected from direct light). Wash
8. 3X2 with distilled water.
9. Counterstain nuclei with Gill’s hematoxylin for 30s and rinse with running tap water for five minutes.
10. Dehydrate and mount.

Anti-Smad2, phospho-specific (Ser 465/467) Immunofluorescence
[Millipore/Upstate, Ab AB3849]
Ethanol Fixed Sections

1. Deparaffinize and hydrate (five minutes each). Last step PBS.
2. Microwave antigen retrieval (Vector Antigen Unmasking Solution 3.75ml + 400ml dH₂O)
   Warm up solution. Heat slides 2x5 min at 20% power. Add solution after 5 min. Cool down 10 min at RT. Wash 3 min PBS.
3. Blocking- 5% normal goat serum in PBS, 30 min at RT.
4. Primary Ab- 1:50 in PBS, 1h at RT. Wash 3x2 min in PBS.
5. Secondary Ab- biotinylated goat anti-rabbit (Vector, BA-1000)
   1:200 in PBS, 30 min at RT. Wash 3x2 min in PBS.
6. AlexaFluor 488-SA- 1:200 in PBS, 30 min at 4°C protected from light. Wash 3x2 min.
7. Counterstain- TO-PRO3- 1:5000 in PBS, 20 min at 4°C protected from light.
   Wash 1x2 min in PBS.
8. Coverslip with Vectashield (Vector, H-1400) mounting medium with fluorescence, hard set. Keep at 4°C protected from light until ready to go to the microscope.
Anti-TGFβ1 Immunohistochemistry
[Santa Cruz, SC-146]
NBF/ Ethanol fixed sections

1. Deparaffinize and hydrate to PBS (five minutes each).
2. 3% H₂O₂ in methanol for 20 minutes at RT. Wash 3X2 in PBS.
3. Heat antigen retrieval.
   a. Antigen unmasking solution (Vector laboratories H-3300)
      i. Prepare as indicated (400 mL distilled water + 3.75 mL concentrated stock) and bring it to boil.
   b. Treatment of the slides
      i. Put the slides into the pre-heated unmasking solution.
      ii. Set the microwave at power 20 and heat the slides for five minutes 2 times. Replenish with unmasking solution to the original volume between sets.
      iii. Cool down at RT for 10 minutes.
      iv. Wash for 5 more minutes in PBS
4. 10% Normal Goat serum in 3% BSA-PBS, 30 minutes at RT.
5. Primary antibody (Rabbit polyclonal IgG, SC-146): 1:100 in 10% Normal Goat serum in 3% BSA-PBS. Incubate overnight at 4°C. Wash 3X2 in PBS.
   a. Biotin-conjugated goat anti-Rabbit IgG (Vector, BA-1000)
7. Vectastain ABC Elite (PK-6200, Vector). 30 minutes at RT. Wash 3X2 in PBS. ABC MIX MUST STAND FOR 30 MINUTES AT RT BEFORE USE.
8. DAB-Substrate (Vector laboratories SK-4100) 5 min at RT (Protected from direct light). Wash 3X2 with distilled water.
9. Counterstain nuclei with Gill’s hematoxylin for 2 min and rinse with running tap water for five minutes.

BrdU/PI staining

Reagents needed: Fixative : 70 % ice cold ethanol
Wash Buffer : 0.5 % Tween-20/PBS
Denaturing Solution : 2 M HCl/0.5 % Triton-X (always prepared fresh)
Acid Neutralizing Solution : 0.1 M Sodium Borate pH 8.5
Dilution Buffer : 0.5 % Tween 20-PBS/0.5 % BSA PBS
PI Solution : 0.00625 % RNase/PBS with PI-5 µg/ml

Controls needed: Cells only, PI only, BrdU only, No BrdU pulse + BrdU

BrdU Pulse:
1. Deparaffinize and hydrate to PBS (five minutes each).
2. One hour before the time-point, add 40µM BrdU (in DMSO) to each of the plates. (last media change should be 24 hours prior to fixation)
3. Wash the cells with PBS and trypsinize about 15 min. with 1 ml Trypsin (for a p60 dish).
4. Add 1 ml media with SERUM (Lo Ca²⁺) to trypsinized cells and transfer this to 15 ml conical tubes.
5. Centrifuge cells at 300xg for 6min. Wash cells with 3-4 ml PBS and centrifuge again at 300xg for 6 min. Discard the supernatant and loosen the pellet by tapping bottom of tube on bench top.
6. While vortexing on a low speed, resuspend pellet in 2.5-3 ml ice cold 70 % Ethanol in a dropwise manner (to avoid precipitation/sticking of cells).
7. Incubate at room temperature for 20 min before storing at -20°C until later. The samples must be stored at -20°C at least overnight before proceeding for the Flow Staining.
Flow Staining:
1. Transfer the fixed cells to flow tubes (typically 1 ml) and add 500 µL of wash buffer.
2. Centrifuge at 300xg for 6 min. (Flick the tubes to get rid of the supernatant; you should be able to see a visible pellet, as a smear with about 50 µL of the remaining supernatant).
3. Repeat the wash step with 1 ml Wash Buffer.
4. Resuspend pellet in 1 ml of fresh denaturing solution. Mix well and incubate for 20 min at room temperature.
5. Add 0.5 ml wash buffer to this, mix and centrifuge at 300xg for 6 min.
6. Repeat the wash step with 1 ml Wash Buffer.
7. Discard supernatant and add 1 ml NaBorate to neutralize; mix well and incubate at room temperature for 2 min.
8. Centrifuge again at 300xg for 6 min.
9. Add 1 ml of wash buffer and mix well; Centrifuge at 300xg for 6 min. 1 hr 15 mins before the Flow Slot and all subsequent steps in DARK.
10. Make BrdU-FITC antibody master-mix. Dilute such that each cell pellet gets 0.8 µL of the BrdU-FITC (Phoenix ABFM-18) in 60 µL of the Dilution Buffer. Mix well and incubate at room temperature in dark for 45 min to 1 hr.
11. Add 1 ml wash buffer and mix well. Centrifuge at 300xg for 6 min at 4°C to pellet and aspirate supernatant.
12. Repeat the wash step with 1 ml wash buffer.
13. Resuspend the pellet in 500 µL PI solution and incubate at room temperature for 10° in dark.

β-galactosidase senescence assay
1. Wash cells 2 times with PBS.
2. Fix for 5 minutes in 0.5% glutaraldehyde in PBS (pH 7.2).
3. Aspirate fixative and wash cells twice with PBS (pH 7.2)/1mM MgCl₂.
4. Aspirate PBS/MgCl₂ solution and add 500 µl of staining solution (0.12 mM K₃Fe(CN)₅, 0.12mM K₄Fe(CN)₆, 1mM MgCl₂, 10mg X-gal (V394A, Promega, Madison, WI) in PBS, pH 6.0).
5. Incubate cells with staining solution for 24-48 hours at 37°C in a non-CO₂ incubator.
6. Positive cells stained blue.

Staining solution:
- 2.4 µl 500 mM K₃Fe(CN)₅
- 2.4 µl 500 mM K₄Fe(CN)₆
- 10 µl 1M MgCl₂
- 200 µl 50mg/ml X-gal (V394A, Promega, Madison, WI)
- 9.8 ml PBS pH 6.0
VITA

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PUBLICATIONS

Mordasky Markell, L., Pérez-Lorenzo, R., Masiuk, K., Kennett, MJ., Glick, AB., “Use of a TGFβ1 Type I Receptor Inhibitor in Mouse Skin Carcinogenesis Reveals a Dual Role for TGFβ1 Signaling in Tumor Promotion and Progression” Accepted to Carcinogenesis.

Pérez-Lorenzo, R., Mordasky Markell, L., Hogan, KA., Yuspa, S., Glick, AB., “Transforming Growth Factor β1 Enhances Tumor Promotion in Mouse Skin Carcinogenesis” Carcinogenesis 2010;31:1116-23.

PRESENTATIONS

Abstract: Mordasky Markell, L., Pérez-Lorenzo, R., Masiuk, KE., Glick AB. (2010) Use of a TGFβ1 Type I Receptor Inhibitor in Mouse Skin Carcinogenesis Reveals a Dual Role for TGFβ1 Signaling in Tumor Promotion and Progression. International Skin Carcinogenesis Conference. State College, PA.

