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Loss of TGF β Signaling Destabilizes Homeostasis and Promotes Squamous Cell Carcinomas in Stratified Epithelia

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SUMMARY

Although TGF β is a potent inhibitor of proliferation, epithelia lacking the essential receptor (T β RII) for TGF β signaling display normal tissue homeostasis. By studying asymptomatic T β RII-deficient stratified epithelia, we show that tissue homeostasis is maintained by balancing hyperproliferation with elevated apoptosis. Moreover, rectal and genital epithelia, which are naturally proliferative, develop spontaneous squamous cell carcinomas with age when T β RII is absent. This progression is associated with a reduction in apoptosis and can be accelerated in phenotypically normal epidermis by oncogenic mutations in Ras. We show that T β RII deficiency leads to enhanced keratinocyte motility and integrin-FAK-Src signaling. Together, these mechanisms provide a molecular framework to account for many of the characteristics of T β RII-deficient invasive SQCCs.

INTRODUCTION

The development of cancers depends on the ability of tumor cells to enhance growth-promoting programs and restrict growth-inhibiting mechanisms and apoptotic cell death. This endows tumors with the advantage to overcome growth limitations, which they accomplish by acquiring multiple mutations in oncogenes and tumor suppressor genes. Featured prominently in cancers are oncogenic mutations that elevate Ras-MAPK signaling to enhance the growth potential of cells. Yet the activation of growth-promoting factors alone is not sufficient to sustain tumorigenesis, which requires additional mutations to abrogate growth-inhibiting factors. Retinoblastoma (Rb), which sequesters the E2F transcription factor to arrest cell cycle progression, is also frequently mutated in cancers. Together, Ras and Rb pathways represent the core program controlling tissue homeostasis and their activities are finely tuned by a variety of signaling mechanisms.

These nodes of interaction between peripheral signaling pathways and the core machinery enable a diverse set of oncogenes and tumor suppressor genes to hijack the tissue homeostasis program and endow the mutated cells with a growth advantage which eventually promotes tumorigenesis. Understanding these complex mechanisms involving cell autonomous as well as nonautonomous effects holds the promise of therapeutic approaches to counteract tumorigenesis.

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SIGNIFICANCE: By generating a mouse model where an essential receptor (T β RII) for TGF β signaling is lost in stratified epithelia, we've discovered an unexpected and specific link between spontaneous anogenital cancers and T β RII deficiency in mice and in humans. We show that the transition from phenotypically normal homeostasis to tumor progression is associated with a reduction in apoptosis, which unmasks hyperproliferative and migratory defects also caused by T β RII deficiency. Finally, progression to carcinogenesis in T β RII-deficient stratified epithelial tissues is accelerated upon oncogenic mutations or otherwise sustained signaling of the Ras/MAPK pathway. These findings provide insights into our understanding of squamous cell carcinomas, which are among the most prevalent and life-threatening cancers in the world.

Under normal circumstances, the transforming growth factor beta (TGF β) signaling pathway restricts tumorigenesis, particularly in epithelial tissues (Bierie and Moses, 2006; Massague and Gomis, 2006). TGF β s bind to a bidimeric surface receptor complex composed of receptor types I (T β RI) and II (T β RII) to phosphorylate and activate receptor-bound Smad (Smad2/3) transcription factors enabling them to translocate into the nucleus and regulate TGF β -responsive genes. In cultured epithelial cells in vitro, TGF β s act as potent inhibitors of proliferation. They act by causing an upregulation of cyclin D kinase inhibitors p15^{Ink4B} and p21^{Cip} to prevent Rb inactivation, resulting in a concomitant downregulation of *c-Myc* expression (Iavarone and Massague, 1997; Pietenpol et al., 1990; Reynisdottir et al., 1995). Thus, TGF β signaling shows tumor suppressor characteristics, and *T β RII* is frequently mutated or transcriptionally suppressed, in human epithelial tumors.

Given the ascribed effects of TGF β signaling on growth inhibition, it is surprising that many epithelia—including mammary gland, oral mucosa, esophagus, pancreas and intestine—still develop normally upon quantitative ablation of TGF β signaling through conditional targeting of the *T β RII* gene (Biswas et al., 2004; Forrester et al., 2005; Ijichi et al., 2006; Lu et al., 2006; Munoz et al., 2006). That said, progression to cancers occurs rapidly when the *T β RII* null epithelial tissues are exposed to activated oncogenes (often oncogenic Ha-Ras) and/or loss of additional tumor suppressors, suggesting that some as yet unidentified feature of the homeostasis balancing mechanism must be compromised in the absence of TGF β signaling. Another intriguing twist is the appearance of spontaneous invasive squamous cell carcinomas (SQCCs) of the forestomach epithelium that arises non-cell autonomously from ablation of *T β RII* in stromal fibroblasts (Bhowmick et al., 2004).

Analysis of the function of TGF β signaling in surface epithelia has thus far been limited to dominant negative and overexpression strategies. Perhaps not surprisingly, results have often been conflicting, and both positive and negative effects on normal epidermal homeostasis and wound healing have been described (Amendt et al., 1998; 2002; Crowe et al., 2000; Ito et al., 2001; Wang et al., 1997b). In addition, despite TGF β 's well-documented function as a suppressor of proliferation in cultured keratinocytes, skin tumorigenesis is paradoxically promoted when carcinogenesis protocols are applied to transgenic mice that either display superactivated TGF β signaling (through overexpression of TGF β s) or suppressed TGF β signaling (through overexpression of dominant negative T β RII) (Amendt et al., 1998; Cui et al., 1996; Cui et al., 1995; Go et al., 2000; Han et al., 2005; Wang et al., 1997b; Wang et al., 1999; Weeks et al., 2001).

Some of these disparate results are likely to arise from combinatorial extrinsic and intrinsic effects, since both dermal and epidermal cells respond to TGF β s. Additionally, some studies suggest that TGF β s act as growth suppressors early but act as metastasis promoters later in tumor progression (Bierie and Moses, 2006). To this end, both gain and loss of TGF β signaling have been reported to promote invasive cell migration (Bhowmick et al., 2001; Ozdamar et al., 2005; Wang et al., 2005) as well as apoptosis (Amendt et al., 2002; Forrester et al., 2005), depending on cellular context and stage of tumorigenesis. Overall, these data suggest that the cell's signaling profile may define the functional consequences of TGF β signaling.

In the current study, we use a *keratin 14 (K14)* promoter, active in surface, oral, and anogenital stratified squamous epithelia as well as some glandular and ductal epithelia (Wang et al., 1997a) to conditionally target the loss of *T β RII* in mice. We show that mice lacking TGF β receptor signaling in K14-positive cells develop spontaneous invasive SQCCs in their anal and genital epithelia, and that TGF β signaling is diminished in human genital SQCCs. By contrast, *T β RII* null epidermis is phenotypically normal, and although wounds heal faster, oncogenic transformation with *Ha-Ras* is required to promote invasive SQCC and metastasis. Exploiting the ability to culture primary epidermal keratinocytes from our mice, we employ a combination

of in vitro and in vivo strategies to explore the intrinsic and extrinsic mechanisms underlying how asymptomatic *TβRII* null stratified epithelia are able to maintain homeostasis, how they lose it, and why this happens at a higher frequency in anogenital epithelium. We also investigate how elevated Ras-MAPK signaling accelerates an imbalance in homeostasis and progression to SQCC when TGFβ signaling is defective. Finally, we address why loss of *TβRII* leads to enhanced cell motility and provide insights as to how this may promote invasive metastatic SQCCs and accelerated wound healing in epithelial tissues.

RESULTS

Conditional Targeting of the TGFβ Receptor II Gene in Mice Results in Spontaneous Anal and Genital SQCCs with Age

Mice harboring the *TβRII* floxed exon 4 (Leveen et al., 2002) were bred to mice expressing Cre recombinase under the control of the human *K14* promoter, strongly active by embryonic day 15 (E15) in proliferative cells of most surface stratified squamous and glandular epithelia, as well as oral, anal, and genital stratified squamous epithelia (Vasioukhin et al., 1999) (Figure S1A in the Supplemental Data available with this article online). *K14-Cre/TβRII* (fl/fl) conditional knockout (cKO) mice were viable and appeared phenotypically normal through early adulthood. As judged by real-time PCR, in situ hybridization, and immunoblot analyses, intact *TβRII* mRNA and protein were absent by postnatal day 2 (P2) in skin epithelium and in cultured primary keratinocytes (MKs) derived from neonatal backskin epidermis (Figure 1A, and Figures S1A–S1D).

Adult *TβRII* cKO mice developed SQCCs in their anal and genital regions, starting as early as 4 months of age (Figure 1B). By 7 months, 89% of cKO mice displayed visible signs of tumor formation (Figure 1C). Histological analyses revealed SQCCs arising within the anal canal in a transitional zone between stratified squamous epithelium of anal skin and mucosal epithelium of the large intestine. Typified by keratinized cells, the SQCCs infiltrated surrounding stroma and tunica muscularis of the rectum (Figure 1D). In males, genital SQCCs arose from preputial ductal epithelium and invaded surrounding stroma (Figure S2A). In females, SQCC tumors developed from mucosal stratified squamous epithelium of vagina and rectum and from adjacent follicular/adnexal epithelium (Figure S2B).

Immunostaining of anal and genital SQCCs confirmed the loss of TβRII protein and phosphorylated (active) p-Smad2 in these keratin 14 (K14) and keratin 5 (K5)-positive tumors (Figures S3 and S4). By contrast, the TβRII-positive stromal tissue surrounding the tumors exhibited p-Smad2, indicative of TGFβ signaling, and also infiltration of smooth muscle actin-positive myofibroblasts. Similar changes were observed in the stroma surrounding spontaneous genital SQCCs.

Human Genital SQCCs Exhibit Diminished TβRII Signaling

Given that TβRII function seemed to suppress development of spontaneous anogenital tumors in mice, we wondered whether TGFβ signaling might also be affected in human genital cancers. As judged by immunohistochemistry, TβRII was reduced or absent in 73% of the 80 male genital SQCC samples tested (Figures 2A and 2B). Concomitant with this reduction was a corresponding loss of activated Smad2 within the tumor tissue, despite the presence of TGFβ1 ligand (Figures 2A and 2B). Similarly, 76% (n = 41) of female genital SQCCs displayed reduced or absent TβRII protein which correlated with reduced or absent p-Smad2 (Figures 2C and 2D). Interestingly, these features were even observed in early-stage grade I genital tumors of both sexes, suggesting that loss of TβRII signaling may be an early event in tumor progression.

In *K14-TβR11* Null Animals, Spontaneous SQCCs Develop at Transitional Zones between Two Merging Epithelial Tissue Types

It was notable that the spontaneous SQCCs arising in our *TβR11* cKO mice frequently arose within transitional zones between two merging but distinct epithelial tissue types. Tumor susceptibility was especially prominent at the juncture of K5/K14-positive mucosal stratified squamous epithelium of the anal canal and K8/K18-positive simple epithelial tissue of the large intestine. Interestingly, irrespective of the status of *TβR11* gene targeting, anal canal epithelium at this juncture displayed many features typically associated with hyperproliferative epidermis (Schäfer and Werner, 2007). This included expression of both suprabasal markers, e.g., keratin 17 (K17) and keratin 6 (K6), and basal markers, e.g., β6-integrin (β6) and Tenascin C (TnC) (Figure 3A and Figure S5). Further reflective of an atypical hyperproliferative epidermal state was the presence of a large number of macrophages (Mac1) within the underlying anogenital stroma (Figures 3Ba and 3Bb).

Overall, the natural state of anogenital transitional epithelium more closely resembled that of backskin epidermis when subjected to an imbalance in tissue homeostasis due to injury, microbial infection, inflammation, or precancerous lesions (Coulombe, 2003; Weiss et al., 1984).

Control of Epithelial Homeostasis in the Absence of *TβR11* Relies Upon the Ability to Balance Offsetting Increases in Proliferation and Apoptosis

Tissue homeostasis depends on a balance between proliferation, differentiation, and apoptosis, and aberrations in this equilibrium can result in the development of tumors. To test whether tissue homeostasis was affected in the anogenital tissue of asymptomatic cKO mice we pulsed these animals for 48 hours with BrdU and analyzed the level of proliferation. We detected a significant increase in the number of S phase cells within the epithelia of both anal canal and anal skin of 7 week cKO animals when compared to their wild-type (WT) littermates (Figure 3Cb). As mice aged, BrdU incorporation waned in anal epithelium of WT mice and in asymptomatic cKO epithelium, but it remained high in spontaneous tumors (Figure 3Cb).

Whether WT or cKO, wherever homeostasis was maintained, BrdU-positive epithelial cells were largely confined to a single epithelial layer adjacent to the basement membrane (Figure 3Ca). By contrast, proliferation often extended to suprabasal layers in the anal canal of 7-month-old cKO mice. Elevated proliferation was also noted in underlying stroma, further reflective of mesenchymal-epithelial interactions (Figures 3Ca and 3Cb). Consistent with the marked hyperproliferation, anal canal epithelium displayed signs of elevated Ras-MAPK signaling (Figures 3Da, 3Db, and 3E). Antibodies against p-MAPK (Erk1, Erk2) and pan-Ras both stained the tumor more strongly than adjacent or asymptomatic tissues. Thus, the increase in Ras-MAPK signaling correlated with tumorigenesis and a loss of homeostasis in *TβR11*-deficient anal epithelium.

Tissue homeostasis can be maintained in a hyperproliferative tissue either by increased cell death or accelerated differentiation. To test whether the hyperproliferation seen in asymptomatic anal epithelium of cKO mice might be counterbalanced by an increase in apoptosis, we conducted immunofluorescence using antibodies against activated caspase 3 (Ac-casp3). At 7 weeks of age, while apoptotic cells were rare in WT, they were frequent within the basal layer of cKO anal epithelium (Figures 3Fa and 3Fb). By 7 months of age, however, when anal tumorigenesis was common in cKO mice, the numbers of apoptotic cells were low in both WT and cKO anal epithelia.

Taken together, these findings suggest that when *TβR11* is deficient in anal epithelium of younger mice, homeostasis is still maintained by balancing enhanced proliferation with

apoptosis; however, as animals age, the spontaneous transition to tumorigenesis is accompanied by a decline in apoptosis, tipping this equilibrium between proliferation and cell death.

Malignant Conversion of *Ha-Ras*-Induced Papillomas to SQCCs in Backskin Lacking *TβRII*

Given our findings with anal epithelia, we wondered whether the counterbalancing of elevated proliferation and apoptosis might also account for why loss of *TβRII* did not overtly seem to affect backskin epidermis and its appendages. We analyzed proliferation and apoptosis levels in two different stem cell compartments of skin epithelium: the slow cycling $\alpha 6$ integrin-positive, CD34-positive cells from the hair follicle bulge (Morris et al., 2004) and the more proliferative $\alpha 6$ integrin-positive, CD34-negative cells from the basal layer of interfollicular epidermis. Following a 48 hour pulse of BrdU administered to 7-week-old mice, fluorescence-activated cell sorting (FACS) was used to analyze the two populations from skin.

Despite signs of elevated TGF β signaling normally displayed by bulge stem cells (Figure S7A) (Tumbar et al., 2004), the loss of *TβRII* did not appear to alter their quiescent state nor affect apoptosis (Figure 4A). By contrast, basal interfollicular epidermal cells exhibited an $\sim 2\times$ increase in BrdU incorporation within 48 hours, which was counterbalanced by elevated apoptosis (Figure 4A). Although perturbations in proliferation and apoptosis were lower in cKO backskin epidermis than in cKO anogenital epithelium, the outcome was similar, namely, a morphologically normal epithelium. In contrast to anogenital epithelium, however, epidermal homeostasis was maintained as animals aged.

Since Ras-MAPK activity was strongly enhanced in *TβRII*-deficient anogenital tumors, we wondered whether oncogenic mutations in this pathway might tilt tissue homeostasis in *TβRII*-deficient epidermis to promote SQCC formation. To test this hypothesis, we briefly cultured freshly isolated epidermal cells from WT and cKO mice, infected them with a retroviral vector expressing an oncogenic mutation in the GTPase domain of Ha-Ras (Ha-RasV12), and grafted these cells onto backs of athymic *Nude* mice (Figure 4B; Figures S6A and S6B). By 4 weeks, control grafts from *Ha-Ras* infected WT epidermal cells displayed visible and morphological signs of benign papilloma expected from *Ha-Ras* transformation of skin (Baillou et al., 1990) (Figures 4C and 4D, and Figure S6C). Notably, T β RII expression was maintained in these papillomas, as judged by immunohistochemistry (Figure S6E).

In striking contrast, grafts from *Ha-Ras*-infected KO MKs displayed overt aberrations soon after grafting (Figure S6D). By 4 weeks, 100% of *cKO-Ras* grafts had developed large, aggressive tumors, which often developed signs of ulcerations and necroses in the tumor center (Figure 4C). Ethical concerns mandated sacrificing mice within 4 weeks, at which time the *cKO-Ras* engrafted skin displayed signs of poorly differentiated SQCCs, including cellular atypia, mitoses, cellular disorganization, and invasion (Figure 4D). At the ultrastructural level, an intact basal lamina demarcated the dermal-epidermal boundary in normal skin of the *Nude* mouse (Figure 4E, left frame), while in invasive, *Ha-Ras* transformed *TβRII null* SQCCs, it was discontinuous and often absent (Figure 4E, right frame).

Both *Ha-Ras* papillomas and *TβRII null/Ha-Ras* SQCCs displayed elevated BrdU incorporation relative to WT backskin (compare Figures 4F and 4A). While BrdU-positive cells were confined to the basal and first few supra-basal layers of papillomas, they were dispersed throughout the *TβRII null/Ha-Ras* SQCCs (Figure S6G). Outside necrotic regions, $\sim 2.5\times$ more BrdU-positive cells were found in the *TβRII null/Ha-Ras* SQCCs when compared to *Ha-Ras* papillomas (Figure 4F), while apoptoses were comparable and low compared to *Ha-Ras* papillomas (Figures S6H, Figure 4F).

To determine whether alterations in epithelial proliferation in *TβRII* null cells are cell-intrinsic or dependent upon their environment, we examined KO MKs in vitro. In contrast to their WT counterparts, these cells were refractory to TGFβ signaling and displayed no signs of activated phospho-Smad2 when exposed to recombinant active TGFβ1 or TGFβ2 (Figure 4G). KO MKs also failed to undergo TGFβ-mediated growth arrest (Figure 4H), and did not accumulate in the G0/G1 phase of the cell cycle at the expense of S phase cells (Figure 4I and Figure S7B). Similarly, only WT MKs responded to TGFβ1 by enhanced expression of cyclin-dependent kinase inhibitors p15INK4b and p27Kip1 (Figure S7C). Thus, although KO and WT MK were comparably hyperproliferative in the absence of TGFβ signaling, marked differences were noted when TGFβs were present. Notably, p-Smad2 and TGFβ1 were both upregulated in the stroma surrounding *Ha-Ras* cKO SQCCs (Figure S6F).

To test whether differences in apoptosis are intrinsic to the loss of *TβRII* function, we treated MKs with TGFβ1 (test) or the potent apoptosis-inducer TNFα (control), and used FACS to quantify expression of the apoptosis marker Annexin V. Even after treating with TGFβ1 for 72 hours, apoptosis levels remained low, and no significant differences were noted between WT and KO MKs (Figure 4J, blue and red bars, respectively). We next examined the ability of *Ha-Ras* transformation to impact apoptosis. Even without exogenously added TGFβ1, *Ha-Ras* MKs exhibited an ~2.5× increase in apoptosis, and when TGFβ1 was added, this level rose another 2×. In striking contrast, apoptosis levels remained low in the *TβRII* null/*Ha-Ras* MKs (Figure 4J). Thus, the loss of *TβRII* appeared to render *Ha-Ras* transformed MKs refractory to TGFβ1-mediated apoptosis.

The similarities in *Ha-Ras* transformed epidermis and WT anal epithelium to loss of *TβRII* prompted us to examine the status of the molecular markers that we previously found to distinguish the anal canal from backskin of WT mice. *Ha-Ras*-induced papillomas expressed K17, K6, and TnC, and caused a marked macrophage infiltration in underlying dermis (Figure 4K, left). These alterations were as or more pronounced in *TβRII* null/*Ha-Ras*-induced SQCCs, and in addition, β6-integrin was selectively found at invasive fronts (Figure 4K, right).

Taken together, these data suggest that loss of *TβRII* in epidermal keratinocytes cooperates with oncogenic mutations in *Ha-Ras* to promote hyperproliferation and maintain low levels of apoptosis, resulting in a gross imbalance in tissue homeostasis, which progresses to SQCC. *Ha-Ras* transformation of epidermis also induced features of WT anogenital epithelium, and although the initiating event remains to be uncovered, Ras-MAPK signaling was elevated in spontaneous anogenital tumors that formed in older *TβRII* cKO mice. These findings may account for differences in susceptibility of *TβRII*-deficient anogenital and backskin epithelium to homeostatic imbalancing and tumor progression.

Elevated Integrin and FAK Activity Is Intrinsic to the Loss of *TβRII* in Stratified Epithelia

The increased susceptibility of *TβRII* null epidermis to *Ha-Ras* induced SQCC formation was further tested by treating mice with the chemical mutagen 7,12-dimethyl-benz [a] anthracene (DMBA), which typically induces mutations in *Ha-Ras* (Balmain et al., 1984) but requires additional promoting agents to generate SQCCs (Yuspa et al., 1995). In contrast to WT mice, cKO mice developed visible backskin tumors within 5–6 weeks following DMBA treatments (Figure 5A). After 15–20 weeks, cKO mice had an average of 7 tumors each, and pathological analyses revealed signs of K14-positive SQCCs in the lungs, suggestive of metastases (Figure 5B).

Elevated focal adhesion kinase (FAK) has been detected in head and neck SQCCs (Canel et al., 2006), and mice lacking epidermal *FAK* are more resistant to DMBA/TPA-induced SQCCs (McLean et al., 2004). To determine whether *FAK* expression and/or activation might be involved in SQCCs formed in *TβRII* cKO animals, we first conducted immunohistochemistry.

Whereas antibodies against FAK only weakly labeled WT tissue, staining was readily detected in cKO anal epithelium (Figures 5C and 5D). Signs of activated (pY397) FAK were even more pronounced in the SQCCs that formed (Figures 5E and 5F). Elevated FAK activity was observed in both basal and suprabasal layers, consistent with the atypical supra-basal expression of integrins known to occur in SQCCs (Owens and Watt, 2001).

To better assess the relationship between loss of *TβRII* and elevated FAK activity, we next plated MKs on fibronectin (FN)-coated dishes and cultured them in absence or presence of serum. Immunoblot analyses of protein extracts revealed that in vitro as in vivo, KO MKs displayed elevated FAK activity (Figure 5G). Using analogous phosphospecific antibodies, we found that Src and MAPK activities, but not the cell survival kinase AKT, were elevated in *TβRII* null MKs compared to WT MKs (Figures 5H and 5I and data not shown). FAK, Src, and MAPK activities were enhanced even in serum-free media (Figures 5G–5I), suggesting that signaling through integrins and cell matrix interactions might be responsible for these differences. We tested this hypothesis by analyzing the in vivo expression and activity of β1-integrin, a key activator of FAK. In adult cKO backskin, anti-β1-integrin labeled the basal epidermal layer as well as the dermis, while antibodies against the activated form of β1-integrin only showed strong labeling in the dermis (Figure 5J). By contrast, both antibodies labeled the cKO anal canal prominently, in stroma and in SQCC cells (Figures 5K and 5L). *TβRII* null/*Ha-Ras* SQCC cells also exhibited enhanced β1-integrin signaling (Figure 5M). The association of increased β1-integrin activation with *TβRII*-ablation was also observed in vitro, where we could use FACS analysis to quantify surface integrin levels (Figure 5N).

***TβRII*-Deficient Epidermal Keratinocytes Possess a Cell-Autonomous, Enhanced Ability to Migrate, Degrade Extracellular Matrix, and Invade: In Vivo and In Vitro Studies**

The data presented in Figure 5 suggest that loss of *TβRII* might enhance keratinocyte migration through activation of integrin-FAK-Src signaling. To explore this possibility further, we next monitored the response of our mice to 4 mm punch biopsy wounds. Interestingly, cKO wounds healed faster (Figure 6A). FAK and MAPK were hyperactivated at the wound edge in both WT and cKO skins, although the wound edge was noticeably thicker in cKO skin (Figures 6B and 6C). BrdU labeling substantiated the hyperproliferative status of cKO epidermis in the wound area, and anti-Ac-casp 3 immunofluorescence suggested that apoptosis was selectively reduced at the wound site, thereby accounting for the corresponding thickened tissue (Figures 6D and 6E). When tissue explants were transferred from backskin to FN-coated culture dishes, epidermal outgrowth was also accelerated when *TβRII* was absent (frames in Figure 6F). These differences were striking when 5 ng/ml active TGFβ1 was added to the culture medium, a feature which abruptly inhibited outgrowth from WT explants while leaving cKO explants unaffected (graph in Figure 6F).

A priori, the enhanced wound healing and explant outgrowth could be due solely to the imbalance in tissue homeostasis caused by loss of *TβRII*. Alternatively, it might reflect an additional migratory and/or invasive advantage. To distinguish between these possibilities, we conducted transwell migration assays in which primary MKs were placed in the upper compartment of a Boyden chamber, while fibroblast-conditioned medium was placed as a stimulus in the bottom chamber. In these assays, KO MKs exhibited a migratory advantage over WT cells with and without additional expression of *Ha-Ras*, but *Ha-Ras* enhanced the effects (Figure 7A). When the assay was repeated, this time after coating the chamber filter with Matrigel (extracellular matrix), KO MKs exhibited an increased ability to invade the matrix and traverse the filter (Figure 7B). Again, the presence of oncogenic *Ha-Ras* enhanced the invasive behavior both in KO and WT cells. Moreover, since the stimulus used in the bottom chamber was always the same, the observed differences in migration and invasion must initiate

from keratinocyte-autonomous changes resulting from *TβRII*-deficiency and/or elevated *Ha-Ras* activity, which appeared to act synergistically.

We quantified cell velocities by imaging the migrations of MKs on FN-coated slides (n = 30 over 12 hr). By video-microscopy, the significantly faster velocities of KO MKs were readily visualized (Figure 7C). Moreover, the accelerated movement was directly due to the loss of *TβRII*, as re-expression of *TβRII* restored p-Smad2 activity and rescued the cell motility advantage (Figures 7C and 7D). Furthermore, although treatment of WT MKs with TGFβ1 slightly accelerated their motility, they did not reach the pace of KO cells, which were insensitive to TGFβ1 treatment (Figure 7E). Taken together, these findings underscore the intrinsic importance of *TβRII*-deficiency in the enhanced migratory behavior of MKs.

Finally, we treated MKs with the MEK1 inhibitor PD98059 and the Src/FAK inhibitor PP2 to test the degree to which downstream elevation of MAPK or FAK/Src activities might account for the differences in cell motility. Although inhibition of MEK1 only modestly reduced motilities, inhibition of Src/FAK had a more robust effect (Figures 7F and 7G). These findings suggest that the elevated Src/FAK activities contribute to the enhanced migratory behavior of *TβRII* cKO epithelia, which could be important in their increased susceptibility to homeostatic imbalance and tumorigenesis.

DISCUSSION

Balancing Hyperproliferation and Apoptosis to Maintain Homeostasis

The development of spontaneous anogenital SQCC in our *TβRII* cKO mice was unexpected. No significant pathological changes have been identified in internal stratified or simple epithelia lacking *TβRII* expression (Biswas et al., 2004; Ijichi et al., 2006; Lu et al., 2006), and while *TβRII* null mammary epithelia exhibited a transient hyperplasia in neonatal mice, this regressed during adulthood, and tissue homeostasis was maintained thereafter (Forrester et al., 2005). Similarly in our own studies, tissue homeostasis was maintained in the absence of *TβRII*, and both in backskin epidermis and in juvenile anogenital epithelium, enhanced hyperproliferation was counterbalanced by an increased apoptosis rate.

As cKO mice aged, apoptosis decreased significantly in the anogenital epithelium, while cells remained hyperproliferative and sustained elevated Ras-MAPK signaling. This age-related imbalance in *TβRII*-deficient tissue homeostasis correlated with the appearance of spontaneous anogenital SQCCs. Moreover, even though we did not detect spontaneous SQCCs in cKO backskin, invasive metastatic SQCCs were readily induced by oncogenic mutations in *Ha-Ras* or treatment with the mutagen DMBA.

Like the spontaneous anogenital tumors which formed, *Ha-Ras* transformed cKO backskin tumors exhibited significantly reduced apoptosis rates while simultaneously remaining hyperproliferative. Our data on cultured MKs revealed that TGFβ signaling in cells transformed by oncogenic *Ha-Ras* induces apoptosis in WT cells, and this effect is quantitatively lost when *TβRII* is mutated. Taken together, these findings suggest that loss of *TβRII* signaling stresses the homeostatic circuitry of stratified squamous epithelia by uncoupling the ability of keratinocytes to execute apoptosis in the face of oncogenic mutations that promote a sustained hyperproliferative state.

Hyperactivation of Regulatory Circuitries Involving Integrin-FAK-Src-MAPK Signaling and Cell Migration: Links between Loss of *TβRII* and Enhanced Wound Healing, SQCC Progression, and Metastasis

In exploring the underlying basis for why the junction between simple epithelium of the intestine and squamous epithelium of the anal canal might be particularly sensitive to SQCC

progression, we began to realize that this transitional epithelium naturally possesses many of the same features that are displayed by the epidermis when it either responds to wounding in normal individuals or exists in patients with hyperproliferative skin disorders (Coulombe, 2003; Weiss et al., 1984). Thus, WT anogenital epithelium is not only substantially thicker and more proliferative than backskin epidermis, but in addition it expresses hyperproliferative-associated suprabasal keratins as well as migration-associated integrins and ECM ligands, and even displays a high number of inflammatory cells in the underlying stroma. These findings suggest that this zone of stratified epithelium may be naturally subjected to a chronic hyperproliferative state, a trait which renders the epithelium more susceptible to loss of tissue homeostasis when TGF β signaling is compromised.

It is particularly intriguing that TnC and β 6-integrin are expressed at this site as their expression is known to correlate with invasive growth during wound healing and tumorigenesis (Bates et al., 2005; Munger et al., 1999). Furthermore, α v β 6 can activate latent TGF β 1 and TGF β 3 leading to TGF β stimulation in mice (Annes et al., 2004). In this way, the activation of α v β 6/TnC may provide a mechanism for hyperproliferative epithelial tissues to keep homeostasis in check by stimulating and responding to TGF β signaling, a process interrupted by conditionally targeting *T β RII*.

Another link between loss of *T β RII* and altered integrin activation came from our studies in cultured MKs, which like anal epithelia, naturally display elevated α v β 6 signaling (Schober et al., 2007). In the absence of T β RII, however, cultured MKs additionally displayed higher β 1-integrin, FAK, Src, and MAPK activities, and enhanced migration and invasion. This was the case even when cells were cultured in the absence of exogenous growth factors, suggesting that the mechanism is intrinsic to the keratinocyte, and is mediated through integrin signaling. It is also dependent upon T β RII deficiency, as reintroduction of T β RII expression into *T β RII* null cells reduced their migratory behavior to WT levels.

TGF β signaling has been attributed to wound healing and cell motility, although a variety of Smad-dependent and independent mechanisms have been implicated, and both positive and negative effects have been described (Arany et al., 2006; Ashcroft et al., 1999; Crowe et al., 2000; Koch et al., 2000; Oft et al., 1998; Zhu et al., 1998). Our studies provide compelling evidence that loss of TGF β signaling in keratinocytes promotes wound closure in vivo and leads to enhanced epidermal outgrowth from explants in vitro. The advantage of *T β RII* null epidermis in wound healing does not appear to be attributable solely to the alterations in proliferation and apoptosis, as the average velocities of migrating *T β RII* null MKs were significantly higher than WT MKs even in vitro and in the absence of active TGF β s, i.e., conditions where proliferative and apoptotic levels were comparable.

At first glance, our finding might seem paradoxical in light of those studies where TGF β stimulates cell motility in MKs or in other cell types. That said, a biphasic concentration-dependent response is often observed in migrating cells where suboptimal cell velocities have resulted from either elevated or abrogated chemotactic signaling or elevated or decreased adhesiveness to the underlying substratum (Lauffenburger and Horwitz, 1996). In this regard, hyperactivation of β 1-integrin-FAK-Src-MAPK signaling can profoundly influence cell motility and conversely pharmacological inhibition of FAK and Src activities strongly suppressed the migratory advantage of *T β RII* null MKs. These data argue for a role for FAK and Src downstream of T β RII in controlling cell motility in keratinocytes.

The increased activity of β 1, FAK, Src, and MAPK activities coupled with enhanced migratory activity could also explain why cKO mice repaired their wounds faster than their WT counterparts. Not only is FAK hyperactive in normal wound repair, but conversely, conditional ablation of *FAK* in keratinocytes impairs epidermal outgrowth from skin explants in vivo and

causes defects in hair follicle downgrowth in vivo (Mitra et al., 2006; Schober et al., 2007). Interestingly, *FAK* deficiency also renders stratified squamous epithelia resistant to *Ha-Ras*-induced SQCC formation (McLean et al., 2004), further strengthening the importance of the connection between loss of *TβRII*, elevated integrin-FAK signaling, and enhanced cell migration that we've uncovered here.

In closing, although future studies will be necessary to illuminate more of the details underlying the circuitry we've described, our data provide a mechanistic framework for understanding the role of TGFβ signaling in regulating homeostasis, injury response, and carcinogenesis in epithelial tissues, and offer a molecular explanation for the surface epithelial tumorigenesis that occurs when TGFβ signaling is compromised.

EXPERIMENTAL PROCEDURES

Generation of cKO Mice, Carcinogenesis Protocols, and Analysis of Tumors

TβRII floxed mice and *K14-Cre* transgenic mice were generated as described (Leveen et al., 2002; Vasioukhin et al., 1999). Genotyping was conducted by PCR of tail skin DNAs. Complete carcinogenesis protocols were performed on cohorts of 6 WT and 5 cKO mice at 6–8 weeks of age as described (Harper et al., 1987). Tumor pathologies were analyzed by at least two independent pathologists in the Laboratory of Comparative Pathology at Cornell University Medical College. All studies were approved by the IACUC committee and follow the NIH guidelines.

Immunofluorescence, Antibodies, Inhibitors, and In Situ Hybridization

Primary antibodies are described in the Supplemental Experimental Procedures. For detection of apoptosis Annexin V (Molecular Probes) was used. Chemical inhibitors used: PD98059 (MEK, Calbiochem, 50 μM for 6 hr), and PP2 (Src, Calbiochem, 5 μM).

Human Tumor Studies and Anatomic Pathology

Tissue microarray from 118 human skin SQCCs (SK241, SK242, SK801, SK802) and from 80 male and 41 female genital SQCCs (UV241, UV801, PE241, PE801) were obtained from US Biomax, Rockville, MD. Paraffin slides were deparaffinized and stained with either TβRII or p-Smad2 antibodies, followed by the DAB substrate kit for peroxidase visualization of secondary antibodies (Vector Laboratories).

Wound, Explant, and Migration/Invasion Assays

In vivo wounds on 7-week-old animals were performed using a 4 mm dermal biopsy punch (Miltex). The size of the closing wound was monitored daily until day 8. Skin explants and migration assays were performed as described (Schober et al., 2007). Invasion assays were performed in precoated Matrigel invasion chamber (BD Biosciences). Individual MKs were imaged with an Olympus phase-contrast microscope (20X) for 12 hr at 1 frame/min and manually tracked in Metamorph (Universal Imaging).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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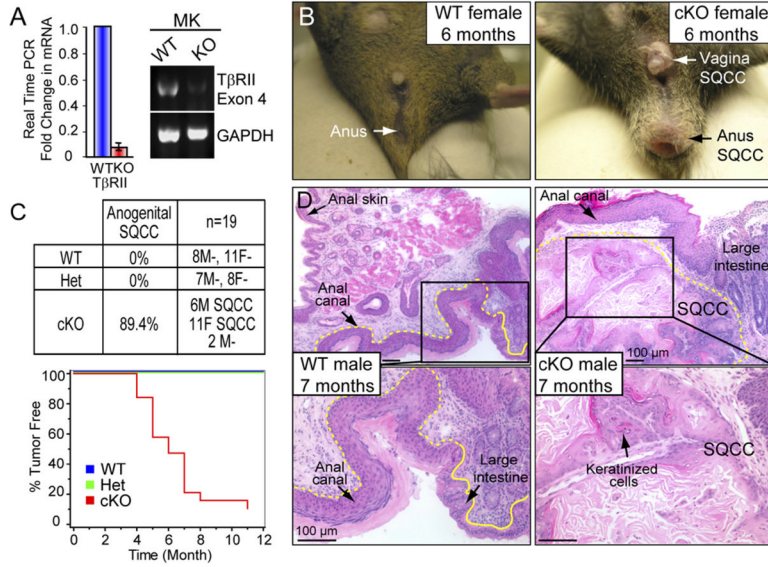


Figure 1. Adult TβRII cKO Mice Develop Spontaneous Anogenital SQCCs

(A) Efficient targeting and loss of *TβRII* mRNA was assessed by real-time PCR using primer pairs corresponding to the floxed exon 4 of *TβRII*. MK, primary keratinocytes. Mean ± SD.

(B) Anal and vaginal tumors were visible in 6-month-old cKO, but not WT mice.

(C) Kaplan-Meier curves depicting the probability of tumor-free survival with age. M, male; F, female. Note that >89% of cKO mice developed anogenital tumors within 7 months, while WT and heterozygous (Het) animals were tumor-free.

(D) Hematoxylin- and eosinstained longitudinal sections of the distal colon, rectal, and anal/perianal tissues revealed classical SQCC pathology in 7-month-old cKO, but not in WT mice. SQCCs displayed anastomosing trabeculae and nests of keratinized squamous cells organized around central cavities, and were associated with moderate, multifocal, and chronic lymphoplasmacytic inflammation around the tumor masses.

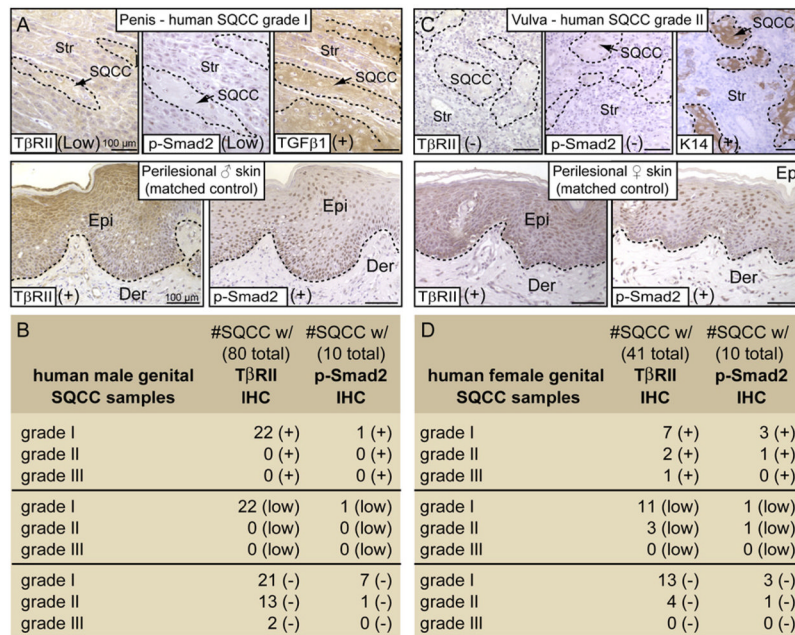


Figure 2. Diminished TGFβ Signaling in Human Genital SQCCs

Male (B) and female (D) human genital SQCCs were scored according to their grade of severity (I-lowest to III-highest) and analyzed for TβRII, phosphorylated (activated) Smad2, TGFβ1 and/or K14 by immunohistochemistry (IHC). Examples of IHC staining from a male genital SQCC grade I (A) and from a female SQCC grade II (C) show reduced or no anti-TβRII, in contrast to perilesional, matched control skins. TβRII loss correlated with a lack of p-Smad2 staining in 10 out of 10 SQCC samples. TGFβ1 and K14 served as internal quality controls and were detected in TβRII-deficient human samples. Scoring: (+), positive staining; (-), negative staining; (low), reduced expression. Epi, epidermis; Der, dermis; Str, stroma. Lines encircle SQCCs in the top panels.

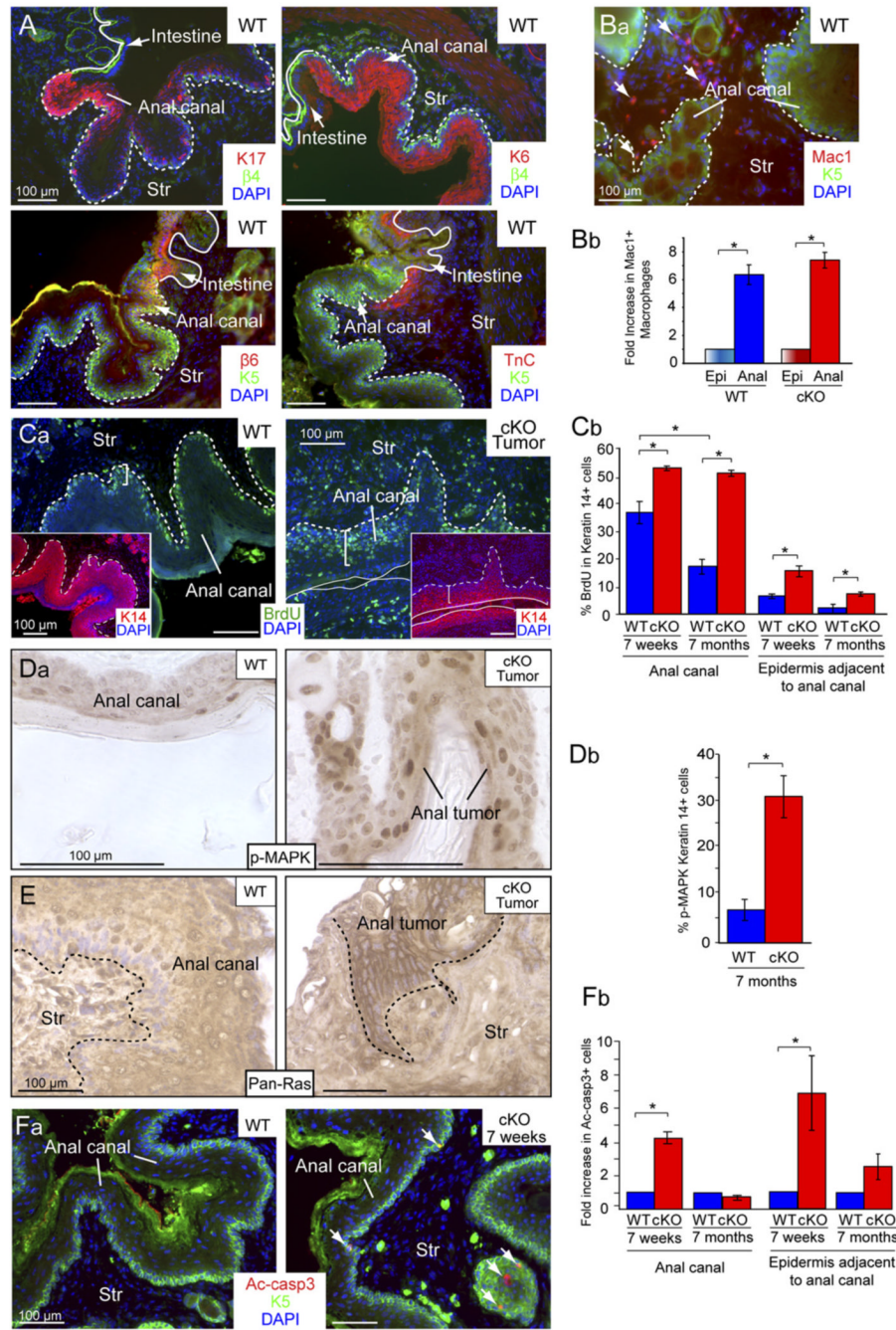


Figure 3. Tissue Homeostasis Is Impaired in the Anal Canal of Older *TβRII* cKO Mice
 (A) The anal canal epithelium is a transitional epithelium bordering stratified squamous epithelium of anal skin and simple epithelium of large intestine. Immunofluorescence microscopy of frozen tissue sections revealed that this epithelium naturally expresses many markers of a hyperproliferative state, including K17, K6, β6-integrin (β6), and tenascin C (TnC). β4-integrin (β4) or K5 label the basal-like cells of the anal canal. Epithelial-stromal border is denoted by white lines. DAPI (blue) labels nuclear chromatin. Str, stroma.
 (B) (Ba) Inflammatory Macrophages (Mac1) are prevalent in the stroma underlying the anal epithelium. (Bb) Mac1-positive cells are much more prevalent in the anal region (Anal) than in backskin epidermis (Epi) in both WT and cKO mice.

(C–F) *TβRII* null anal epithelium exhibits sustained hyperproliferation but maintains homeostasis through enhanced apoptosis, which is lost in spontaneous tumors. (Ca–E) Proliferation was assayed by BrdU incorporation and correlates with pronounced Ras/p-MAPK staining. (Fa) Apoptosis was assayed by immunostaining for activated caspase 3 (Ac-casp3) (arrows). (Cb, Db, and Fb) Quantification of proliferation and apoptosis in WT and cKO anal canal and adjacent anal epidermis at 7 weeks when the cKO anal canal is histologically normal, and 7 months when it appears hyperplastic. Accelerated proliferation is balanced by increased apoptosis when homeostasis is maintained, while alleviated apoptosis correlated with tumor formation. Graphs represent the mean (\pm SD) of 3 different WT and cKO mice. * $p < 0.05$.

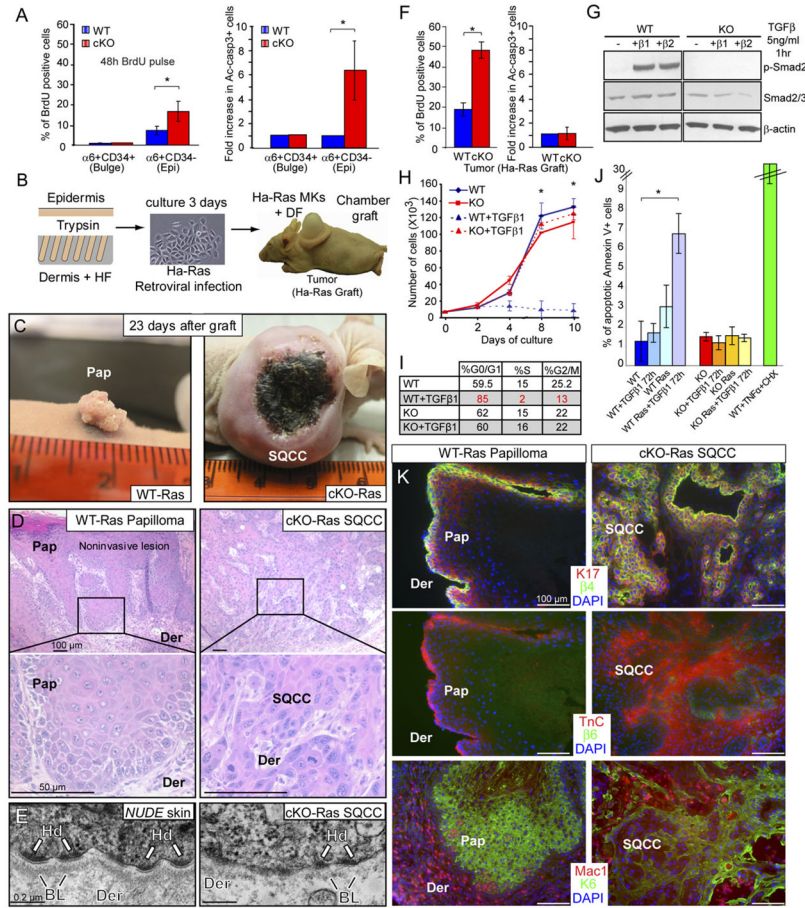


Figure 4. TGFβ and Ras-MAPK Signaling Also Cooperate to Control Tissue Homeostasis in the Epidermis

(A) Proliferation and apoptosis rates are increased in the stem cell compartment (basal layer) of *TβRII* null epidermis (Epi) ($\alpha6+CD34+$), but not in the stem cell compartment (bulge) of the hair follicle ($\alpha6+CD34+$). Analyses were performed on adult mice (7 wk). Graphs represent the mean (\pm SD) of 3 different WT and cKO mice. * $p < 0.05$.

(B) Schematic of tumor susceptibility assay where primary keratinocytes (MKs) from WT or cKO backskins were isolated, infected with a retrovirus expressing constitutively active (oncogenic) Ha-Ras, and grafted together with WT dermal fibroblasts (DF) onto the backs of *Nude* mice. HF, Hair follicle.

(C) At 23 d, papillomas formed from *Ha-Ras* MKs, while invasive SQCCs formed from *TβRII* null/*Ha-Ras* MKs. Note necrosis in center of large tumor.

(D) Hematoxylin- and eosin-stained tissue sections revealed papilloma (Pap) pathology in *Ha-Ras* graft and SQCC pathology in *TβRII* null/*Ha-Ras* graft.

(E) Ultrastructural analysis indicated a compromised basal lamina underlying *Ha-Ras* transformed, *TβRII* null SQCCs (right frame). Scarcity of hemidesmosomes (Hd) and perturbed basal lamina (BL) is typical of invasive SQCCs. The basal lamina appeared uncompromised at the boundary between Epi and dermis (Der) in adjacent nude skin (left frame).

(F) *TβRII* null/*Ha-Ras*-induced SQCCs exhibited sustained hyperproliferation without enhanced apoptosis. Graphs represent the mean (\pm SD) of 3 different WT and cKO mice. * $p < 0.05$.

(G) *TβRII* null MKs fail to activate Smad2 and remain resistant to a G1 growth arrest when stimulated with TGFβs as judged by growth curves and cell cycle profiles.

(H and I) MKs were cultured in low Ca^{2+} medium in the presence or absence of 5 ng/ml TGF β 1, added at day 2. Growth curves (H) indicate the average value of 3 independent experiments performed in triplicate (\pm SD). (Note: by this assay and Elisas, active TGF β s are low/absent in epidermal culture medium.) (I) Cell cycle analyses with propidium iodide staining.

(J) FACS analysis for the presence of the apoptotic marker annexin-V revealed that Ha-Ras and TGF β signaling cooperate to induce apoptosis in WT MKs, while apoptosis rates remain low and unaltered in KO MKs under these conditions. TNF α (100 ng/ml) and cycloheximide (5 μ g/ml) induced apoptosis in WT MKs served as a positive control. Bar graphs indicate mean value (\pm SD) on duplicate experiments.

(K) Keratin 17 (K17), Tenascin C (Tnc), and β 6-integrin are expressed in both *Ha-Ras* papillomas and *T β RII* null/*Ha-Ras* SQCCs. β 4-integrin marks the basal cell layer and Macrophages (Mac1) are recruited to the tumor stroma. Note similarities between the expression patterns in *Ha-Ras* papillomas (here) and WT anogenital epithelia (Figure 3).

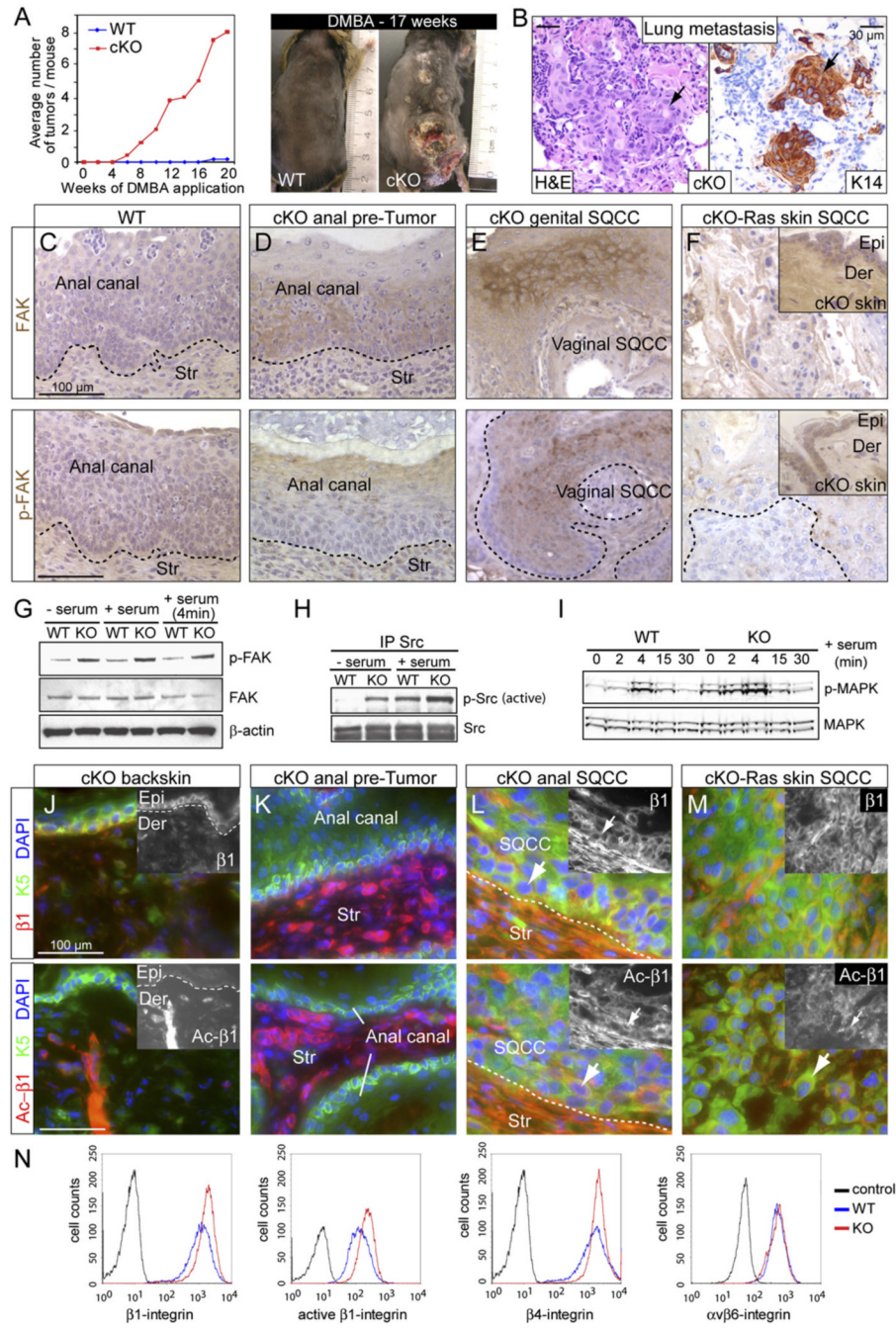


Figure 5. Invasive Metastatic Skin Tumors Correlate with Increased Integrin, FAK, Src, and MAPK Activities

(A) DMBA treatment of mice revealed a marked increase in DMBA-induced tumor formation in the absence of *TβRII*.

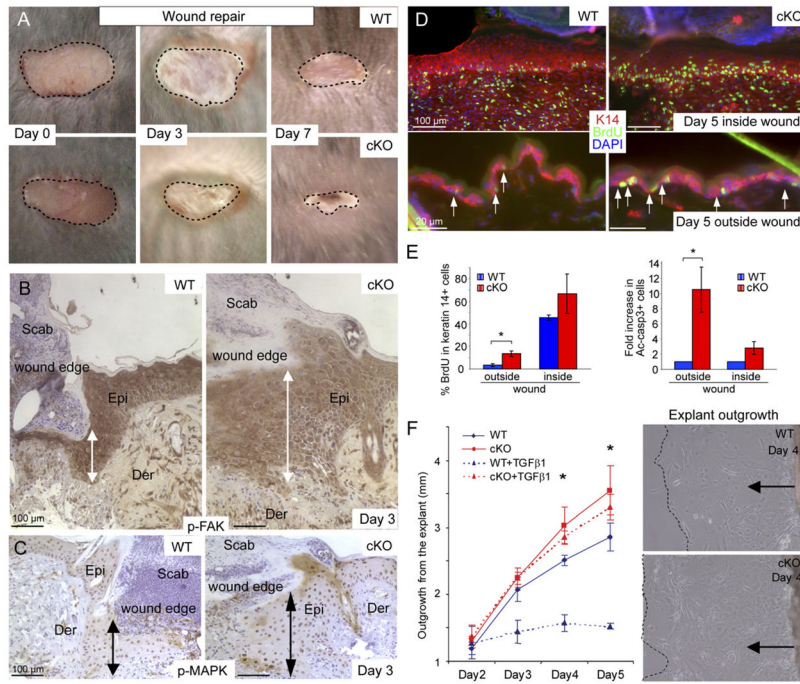
(B) Lung metastasis from *TβRII*-cKO mouse topically treated with DMBA to induce backskin SQCCs. Note typical keratinized SQCC histology as revealed by hematoxylin and eosin staining and K14 immunohistochemistry.

(C–F) Anti-FAK and p-FAK immunohistochemistry on sections from WT anal canal (C), cKO pretumor and tumor stage of anogenital skin (D and E), and cKO-Ras backskin SQCC (F). Str, stroma; Epi, epidermis; Der, dermis.

(G–I) Immunoblots of protein lysates from MKs grown in the presence or absence of serum, ± 4 min serum stimulation where indicated. Note: Src activity was assessed by immunoprecipitation/immunoblot analysis using Src and pY417-Src antibodies.

(J–M) Immunofluorescence with antibodies against $\beta 1$ - and active $\beta 1$ -integrin (Ac- $\beta 1$) performed on tissue sections as indicated.

(N) Quantitative FACS analysis of KO (red) versus WT (blue) cultured MKs with antibodies against the cell surface integrins indicated. Gray lines depict secondary antibody-only control.



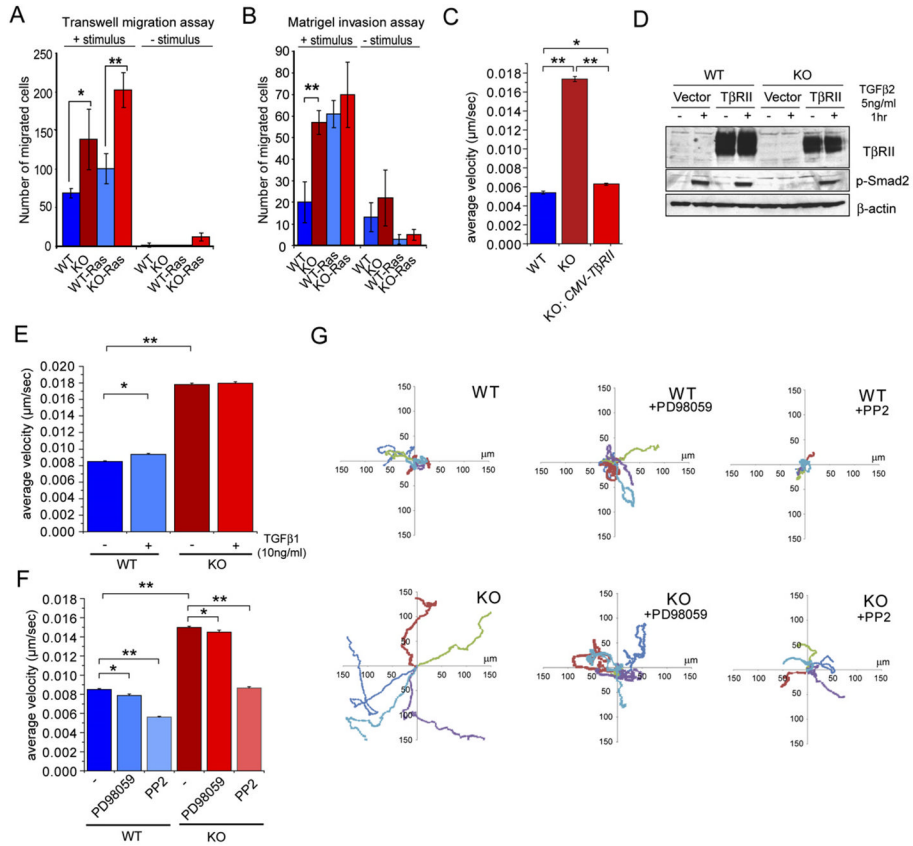


Figure 7. Elevated Cell Motility in the Absence of *TβRII*
 (A and B) Elevated cell motility in the absence of *TβRII*. Transwell migration assays were carried out on Boyden chambers ± coating with Matrigel ECM on the top chamber and in the absence and presence of fibroblast-conditioned medium, used as stimulus in the bottom chamber. Bar graphs depict means of 3 independent experiments ± SD. **p* < 0.03. ***p* < 0.004. Note that KO MKs showed elevated migration and invasion over WT MKs, and Ha-Ras transformation enhanced these effects.
 (C and D) Rescue experiment to show that the enhanced migratory ability of KO MKs is specifically attributable to *TβRII* loss. The *CMV* promoter was used to drive *TβRII* cDNA, which restored cell velocities to WT levels (C). Immunoblot shows that re-expression of *TβRII* in KO MKs restores TGFβ2 responsiveness (D).
 (E) TGFβ1 treatment has only a slight effect on the velocity of WT MKs, which are still significantly less motile than KO MKs.
 (F and G) Enhanced MAPK (Erk) and Src/FAK activities promote cell motility in KO MKs. (F) Cell velocities were measured ± 50 μM of the MEK1 inhibitor PD98059 and ± 5 μM of the Src inhibitor PP2, which also inhibits FAK activity. (C, E, and F) Thirty cells were tracked by phase-contrast microscopy with an acquisition rate of 1 frame/min over 12 hr. Bar graphs indicate average velocity ± SEM. **p* < 0.05. ***p* < 0.0001. (G) Scatter plots depict the migration tracks of 5 representative cells for each condition.