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Lessons learned from *SMAD4* loss in squamous cell carcinomas

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Abstract

SMAD4 is a potent tumor suppressor and the primary mediator of the TGFß signaling pathway. SMAD4 genetic loss is frequent in squamous cell carcinomas (SCCs). Reports of SMAD4 expression in SCCs vary significantly possibly due to inter-tumor heterogeneity or technical reasons. SMAD4 loss is an initiation event for SCCs. In tumor epithelial cells, SMAD4 mutant SCCs commonly present with increased proliferation, decreased apoptosis, and "Brca-like" genomic instability associated with DNA repair defects. SMAD4 loss also plays a role in expansion of cancer stem cells (CSC). Epithelial SMAD4 loss causes overexpression of TGFß which is released into the tumor microenvironment and contributes to SCC progression through pro-inflammatory and immune evasive mechanisms. SMAD4 loss, while not directly targetable, is associated with multiple targetable pathways that require further studies. Altogether, SMAD4 loss is a potential biomarker in SCCs that should be further studied to gain insight for prognosis and therapeutic approaches to potentially guide future clinical trials and improve SCC patient outcomes.

Keywords

SMAD4 loss; prevalence; tumor initiation; tumor progression; SCCs

Introduction

SMAD proteins were discovered as intracellular signaling mediators of the transforming growth factor ß (TGFß) superfamily and were named after non-mammalian homologs: *Sma* genes of *Caenorhabditis elegans* and the *Mad* gene in *Drosophila melanogaster*. SMADs are important for homeostasis during embryonic development, immune response, fibrosis, wound healing, genomic stability, and tumor development [1]. Extracellular TGFß ligands bind transmembrane receptors activating receptor kinase activity leading to phosphorylation of members of the intracellular receptor-activated SMAD family, SMAD2 and SMAD3 in the case of TGFß stimulation [2]. Phosphorylated SMAD2/3 heterotrimerize with SMAD4 and

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translocate into the nucleus to interact with SMAD binding elements (SBEs) to regulate transcription of TGFß dependent genes (Figure 1a) [2]. While the primary focus of this review is on SMAD4 as a mediator of TGFß signaling pathways, it is important to note bone morphogenic protein (BMP) and activin signaling pathways are also dependent on SMAD4 [2]. Furthermore, TGFß/BMP ligands can also signal through non-canonical pathways including ERK, p38/JNK, Rho/Rac, and PI3K/AKT dependent or independent of SMADs [3].

The role of SMAD4 as a tumor suppressor was initially identified in pancreatic cancers as Deleted in Pancreatic Cancer 4 (DPC4) [4], and SMAD4 loss has since been identified as a key driver in skin cancers and head and neck cancers as well as other cancers [5–7] TGFß signaling inhibits epithelial cell growth and promotes cellular differentiation, thus defects in TGFß signaling *via* TGFßRI, TGFßRII, and/or SMAD4 dysregulation promote tumor growth [8] Impaired TGFß signaling in epithelial cells causes additional TGFß ligand release into the tumor microenvironment (Figure 1b) that stimulates angiogenesis and inflammation in stromal cells with intact TGFß signaling (Figure 1a) that can then promotes tumor growth and progression [9]. This review will discuss the important roles of SMAD4 loss and the associated mechanisms that contribute to tumor initiation and progression of squamous cell carcinomas.

Prevalence of *SMAD4* loss in squamous cell carcinomas (SCCs)

The *SMAD4* gene is located at chromosome 18q [10] and large chromosomal deletion of 18q and loss of one or two *SMAD4* alleles is the most common reason for SMAD4 loss of function in SCCs. Overall, 56% of primary head and neck squamous cell carcinomas (HNSCCs) have SMAD4 genomic alterations [11,12]. *SMAD4* loss occurs in 35 - 68% of human HNSCCs [13–15] and occurs in up to 70% of skin SCCs [16]; however, *SMAD4* point mutations are rare (< 5%) in these types of SCCs [17]. Higher rates of genomic loss (56%) compared to point mutations (< 5%) are consistent with rates of genetic abnormalities in human cancers [18]. In contrast, *SMAD4* point mutations are more common in pancreatic cancers (~35% [19, 20]) and colon cancers (~12% [21]) than SCCs.

Reduced SMAD4 protein staining is associated with aggressive SCC tumor progression [14, 15, 22]. However, reports of reduced SMAD4 expression vary widely at 12 – 86% [6, 23]. This wide range of reported SMAD4 reduction may be explained by the criteria used to define "reduced SMAD4 expression" and the tissue samples used as SMAD4 positive controls such as adjacent non-malignant tissue versus unrelated normal tissue. For example, reduced *SMAD4* expression, defined as > 50% reduction of mRNA expression per specimen, was observed in 86% of human HNSCCs compared to 67% of the non-malignant adjacent mucosal specimens [6]. By this criteria, "SMAD4 loss" would be under-reported in studies that compared SCCs to non-malignant adjacent mucosal tissue where SMAD4 reduction may also have occurred. Furthermore, multiple reports support that single copy loss of *SMAD4* occurs in 30 – 50% of HNSCCs [6, 10, 12, 13, 24]; however, other reports suggest reduced SMAD4 immunostaining in < 30% of HNSCC cases which maybe be due to control tissues or poor antibody specificity. Additionally, intra-tumor heterogeneity of genomic *SMAD4* loss as well as aneuploidy of chromosome 18 may also contribute to variations in reported *SMAD4* loss [13]. While genomic loss of *SMAD4* is evident in ~ 50%

of SCCs, its detection by immunostaining or RNA expression analyses are not standardized and ideal expression standards for SMAD4 are lacking. With a central role in tumor development and potential therapeutic response marker as discussed below, a standard for "SMAD4 loss" is a critical and logical need in future studies.

SMAD4 loss initiates SCCs

We have shown that SMAD4 downregulation occurs in preneoplastic oral mucosa and actinic keratosis (AKs), suggesting SMAD4 downregulation is an early event in human SCC development [6, 25]. In genetically engineered mouse models, keratinocyte-specific Smad4 deletion in the oral cavity or skin spontaneously induces SCCs [5, 6, 25, 26] demonstrating that SMAD4 loss, as a single event, can initiate SCCs. Therefore early stage keratinocyte SMAD4 loss in patients may have a significant impact on SCC initiation in patients. This is quite different from pancreatic and colon cancers where SMAD4 loss occurs at later stages of cancer development and is more associated with metastatic progression [27–29]. Interestingly, single copy deletion of Smad4 did not initiate HNSCC formation, but it accelerated HNSCC development initiated by oncogenic Kras^{G12D} [6], which suggests that Smad4 haploid insufficiency can promote oncogene-driven HNSCC development. Keratinocytes-specific Smad4 deletion caused interruption of hair follicle cycling, hyperproliferative hair follicles, progressive hair loss, and well-differentiated skin SCCs [5, 26]. SCCs with SMAD4 loss activate survival factors including increased AKT, cyclin D1, and c-myc expression and promotes growth of Smad4$^{-/-}$ skin stem cells to promote development of sebaceous adenomas and basal cell carcinomas as well as other cancer types [5, 26]. Smad4 deletion in mammary epithelial cells of mice also caused mammary tumors with transdifferentiation to squamous histology [30]. Furthermore, Smad4 deletion promotes PTEN$^{-/-}$ skin tumor formation [5]. Collectively, these reports demonstrate that Smad4 loss promotes SCC initiation and accelerates oncogene-driven SCC development. Thus, early loss of Smad4 appears uniquely pathogenic in SCCs, further emphasizing the need for SMAD4 detection and evaluation in human SCCs.

Survival and invasive mechanisms of SMAD4 mutant SCCs

Several reports have shown that TGFβ/SMAD4 signaling induces growth arrest and/or apoptosis in normal keratinocytes [1, 6, 31]. TGFß-induced growth arrest requires SMAD-2, -3, and -4 [32]. Spontaneous HNSCCs development driven by Smad4 deletion was associated with increased proliferation in the Smad4$^{-/-}$ malignant and adjacent-premalignant mucosa compared to Smad4$^{+/+}$ normal mucosa [6]. Inversely, apoptotic cells were less common in Smad4$^{-/-}$ malignant and adjacent-premalignant mucosa compared to normal mucosa [6]. Therefore, Smad4 loss allows for epithelial derived SCCs to escape TGFβ-mediated growth inhibition and survive into tumor development. Mechanistically, Smad4 loss is associated with inactivation of tumor suppressors PTEN and p21 to bypass cell cycle arrest and promote cell proliferation and survival in SCCs [26]. Furthermore, Smad4 mutant mouse models developed hyperplasia and hyperproliferative hair follicles associated with upregulated c-Myc and cyclin D1 to promote proliferation and downregulated p27 to promote survival [5]. Taken together, SMAD4 loss leads to increased proliferation and

decreased apoptosis to promote tumor development of SCCs; however, additional survival mechanisms are at play.

Cancer stem cells (CSCs) with Smad4 deletion also contribute to tumor development and progression. Mouse models with keratinocyte-specific Smad4 deletion developed tumors in 6-8 months [5, 26], but when Smad4 was deleted from K15 positive (K15.Smad4$^{-/-}$) bulge stem cells, spontaneous skin tumors required 1 year to form [33]. The slowed tumor development was likely due to the quiescent nature of bulge stem cells, suggesting that Smad4$^{-/-}$ driven tumor development requires more rapid proliferation. When oncogenic Kras^{G12D} was co-expressed in K15.Smad4$^{-/-}$ bulge stem cells (K15.Kras^{G12D}.Smad4$^{-/-}$) tumor latency was accelerated to 1 month [33]. However, tumor development required Smad4 deletion as mice solely expressing the K15.Kras^{G12D} mutation only developed benign papillomas [33]. In contrast, K15.Kras^{G12D}.Smad4$^{-/-}$ bulge stem cells led to the development of multi-lineage skin tumors (SCCs, basal cell carcinomas, trichoepitheliomas, and sebaceous adenomas) likely from uncommitted multipotent stem cells or immediate progenitor cells [33].

Smad4 deletion in stem cells also contributed to the metastatic progression of SCCs. Serial passage of K15.Kras^{G12D}.Smad4$^{-/-}$ tumors presented with more aggressive outgrowth and epithelial-mesenchymal transition (EMT)-mediated metastatic progression which correlated with increased side population (SP) cells, a metastasis-associated CSC population [33]. Expansion of metastatic CSCs was associated with miR-9 overexpression [33], and increased miR-9 correlated with a loss of E-cadherin and α-catenin, previously reported potential miR-9 targets associated with EMT and metastasis [34, 35]. In human SCC metastases, the loss of α-catenin and E-cadherin was more commonly observed in miR-9 positive tumors than in miR-9 negative tumors [33], which suggest that miR-9 is an important driver of metastasis. These Smad4 loss-associated mechanistic findings are consistent with reports that EMT promotes an increase in CSCs and metastasis in other cancer types [36, 37].

While it has been well documented that Smad4 is required for TGFß-induced EMT to promote metastasis [29, 38], EMT is not always required for metastasis. Early stage EMT is not observed in Smad4$^{-/-}$ SCCs [16] that retain the ability to metastasize [6, 33]. However, one report suggest that Smad4 downregulation is important for EMT induction by HNSCC cell lines that are already resistant to cetuximab, an EGFR inhibitor [39]. One may speculate that these cetuximab-resistant, Smad4-deficient HNSCC cell lines unlocked EMT induction through non-TGFß-dependent mechanisms. In addition to EMT, SCCs with Smad4 deficiency may metastasize through other mechanisms induced by pro-inflammatory cytokines and angiogenic response within the tumor-associated stroma [6]. Together, these findings suggest further studies are required to understand how SMAD4 deficient SCCs survive and metastasize through non-EMT pathways such as increased pro-tumorigenic inflammation.

"Brca-like" genomic instability in SMAD4 mutant SCCs

Deletion of Smad4 in the mouse oral epithelia caused spontaneous HNSCC formation associated with increased genomic instability [6]. In particular, Smad4 deletion down-

regulated Fanc/Brca (Fanconi anemia and breast cancer associated) family genes that maintain DNA integrity and are therefore necessary for sustained genomic stability [6]. Fanc family genes were functionally identified as germline mutations in Fanconi anemia (FA) patients who typically died at a young age without bone marrow transplantation due to chromosome instability-associated bone marrow failure [40]. FA patients that survive to adulthood have an HNSCC-risk 500-fold greater than the general population [41]. Furthermore, *BRCA1* and *BRCA2* mutations increase genomic instability and are associated with the development of breast and ovarian cancers [42–44]. Therefore, early SMAD4 loss associated-downregulation of FANC/BRCA expression and the associated genomic instability may lead to the accumulation of genetic defects required to initiate HNSCC development.

We have shown that pre-malignant Smad4$^{-/-}$ oral epithelium with downregulation of Fanc/Brca genes occurred prior to the development of malignancy in mouse models [6]. Similar to FA cells in which DNA crosslinking agents, such as mitomycin C, induces chromosomal breaks and radial structures (Figure 2a) [45], Smad4$^{-/-}$ keratinocytes exhibit the same characteristics (Figure 2b) [6]. Along with cancer progression, Smad4$^{-/-}$ SCCs had increased genomic instability as demonstrated by abnormal centrosome number and genomic aberrations [6]. SMAD4 restoration in SMAD4 deficient human HNSCC cell lines increased Brca1 and Rad51 expression as well as increased Brca1 and Rad51 DNA repair foci found in the nucleus of cells treated with mitomycin C [6]. Conversely, reduced SMAD4 immunostaining correlated with reduced immunostaining of BRCA1 and RAD51 in human HNSCCs and the adjacent mucosal. Taken together, these data suggest that reduced SMAD4 leads to downregulation of BRCA1 and RAD51 in human HNSCCs [6]. This SMAD4 loss associated-reduction of Brca1 and Rad51 as well as the FA phenotype causes "Brca-like" phenotype:, defined as reduced expression or loss of other DNA repair genes-associated functional defect of homologous recombination rather than BRCA mutations *per se*, in SMAD4 deficient SCCs [46]. While single gene deletions of the Fanc/Brca family do not develop HNSCCs [47], Smad4 loss in keratinocytes disrupts multiple functionally redundant Fanc/Brca family genes to initiate self-perpetuating DNA damage leading to genomic instability and HNSCC development. This is rather unique to keratinocytes, as Smad4 deletion does not predict genomic instability in pancreatic cancers [48], nor do FA patients have increased incidence to colon or pancreatic cancers[40].

Skin SCC mouse models driven by Smad4 loss was found to increase susceptibility to ultraviolet (UV)-induced carcinogenesis and subsequent genomic instability[25]. Smad4 deletion in epidermal keratinocytes accelerated UV-induced skin SCCs [25]. Multiple DNA repair genes were down regulated by UV-radiated Smad4-null tumors, most notably reduced was Ercc1 [25]. Ercc1 is an essential protein for DNA repair of nucleotide mismatches, double-strand breaks, and crosslinks [49]. Furthermore, most human skin SCCs cases reported co-expressed (86%) or co-repressed (60%) SMAD4 and ERCC1 [25]. However, Ercc1 is not a direct target of Smad4 [50], but instead Ercc1 was shown to be directly regulated by Snail [25]. Snail is a direct target of Smad4 [16, 51] that was also down regulated in the UV-radiated Smad4-null specimens [25]. This suggests that Smad4 protects keratinocytes from UV-induced DNA damage by upregulating Snail-dependent Ercc1 to maintain functional DNA repair. Similar expression patterns of SNAIL and ERCC1 have been reported in HNSCCs and

bladder cancer [52, 53], therefore, SNAIL-regulated ERCC1 expression may occur broadly in human cancers. Future studies are necessary to identify the stage-specific effects of Smad4, Snail, and Ercc1-associated DNA repair in SCC initiation and progression. Additionally, Smad4 loss was associated with reduced expression of other DNA repair pathway genes (Exo1, Chaf1a, and Mre11 [25]) although it remains unknown how reduced expression of these genes affects UV-induced SCC development. Thus, SMAD4 regulates multiple nodes of DNA damage response and loss of SMAD4 causes genomic instability to promote SCC progression, placing SMAD4 as a central mediator of genomic stability.

Tumor microenvironment and progression of SMAD4 deficient SCCs

Loss of SMAD4 led to spontaneous SCC development associated with increased inflammatory infiltration of the tumor microenvironment [6] likely exasperated by neoantigens associated with genomic instability. TGFß1 ligand expression is increased in the tumor epithelial cells of Smad4$^{-/-}$ SCCs, and TGFß1 overexpression was associated with increased infiltration of neutrophils, T cells, and Th17 cells in the tumor microenvironment [6]. Smad4 loss-associated inflammation was abrogated in a Smad3+/− background, suggesting that inflammation in the microenvironment of Smad4$^{-/-}$ SCCs requires Smad3-dependent TGFß signaling [6]. Smad4$^{-/-}$ SCCs show elevated cytokines including the chemoattractant proteins MCP-1, MCP-2, and MIP-2, compared Smad4$^{+/+}$ mucosa [6]. These cytokines have also been shown to be upregulated in skin keratinocytes with overexpressed TGFß1 [54]. It is well known that tumor cells can influence the local immune response within the tumor microenvironment [55] by producing inflammatory or immunosuppressive cytokines, recruiting immune suppressive cells, restraining T cell response via modulating the expression of checkpoint pathway components, and reprograming T regulatory cells (Tregs) to be more suppressive [56–59]. Therefore, SMAD4 deficient SCCs induce a pro-tumorigenic immune response to promote SCC development.

SMAD4 loss may also be necessary for tumor evasion of the immune system and subsequent progression of SCCs. K15.Kras^{G12D}.Smad4$^{-/-}$ SCC cells are metastatic in mouse models [33]. K15.Kras^{G12D}.Smad4$^{-/-}$ SCC tumor transplants develop secondary lesions, despite infiltration of active CD8+ T cell into the primary tumor microenvironment [60]. However, K15.Kras^{G12D}.Smad4$^{-/-}$ SCC primary tumor growth was unaffected by the presence of CD8+ cells, suggesting that immune evasion or T cell exhaustion. Several immune checkpoints have been implicated in preventing cytotoxic T cell killing of tumor cells including programmed cell death 1 (PD-1), PD-ligand 1 (PD-L1) [61] and lymphocyte activation gene-3 (LAG-3) [62]. The K15.Kras^{G12D}.Smad4$^{-/-}$ SCC tumors were infiltrated by active CD8+ T cells that were PD-1+ and LAG-3+ to indicate immune evasion by the tumors [60]. Furthermore, a large percent of the CD8+ T cells downregulated T cell antigen receptor (TCR) ß chain, an indication of suppressed CD8+ T cells [60]. Isolated K15.Kras^{G12D}.Smad4$^{-/-}$ SCC tumor cells also expressed higher PD-L1 in response to infiltrating CD8+ T cells, and K15.Kras^{G12D}.Smad4$^{-/-}$ SCC tumor cells isolated from CD8-null mice had reduced PD-L1 expression [60] to further support an immune-evasion phenotype by these Smad4-null tumors. Therefore, SMAD4 loss may predict evasion of active T cells by PD-1/PD-L1 and LAG3 in SCCs, but testing of this hypothesis in other models is necessary.

Potential therapeutic approaches against Smad4 deficient SCCs

SMAD4 status may function as a biomarker for drug selection in SCCs as *BRCA* mutations have in breast and ovarian cancers. For example, *BRCA* mutant breast and ovarian cancer patients are being treated with the FDA approved PARP inhibitor, olaparib, which causes synthetic lethality due to the defective DNA damage repair mechanisms and increased genomic instability inherent with *BRCA* mutation and exacerbated by DNA damage repair inhibition [63, 64]. SMAD4 deficient SCCs present a BRCA-like phenotype with decreased Fanc/Brca expression and increased sensitivity to genotoxic agents [6]. Phase I clinical trials have reported olaparib with standard care (cetuximab and radiation) was well tolerated by patients with advanced head and neck cancer [65]. Therefore, it is worthwhile to assess if SMAD4 deficient SCCs are more sensitive to PARP inhibition. Additionally, numerous DNA repair inhibitors or modulators are being studied as anti-cancer agents [66], such as DNA topoisomerase inhibitors, which suppressed Smad4 deficient lung cancers [67, 68]. Clinical data from SCC patients treated with these drugs could also reveal if SMAD4 status predicts their therapeutic responses.

The increased TGFß-mediated signaling in the tumor microenvironment of SMAD4 mutant SCCs [6, 69] suggests therapeutic strategies inhibiting stromal TGFß. Alternatively, immunotherapies may be effective against SMAD4 deficient SCCs as there are several checkpoint inhibitors FDA approved that target PD-1 or PD-L1. For instance, K15.Kras^{G12D}.Smad4$^{-/-}$ SCC tumor growth was suppressed by dual-blockade of immune checkpoint inhibitors, anti-PD-1 and anti-LAG3 [60]. Immunotherapies also have the potential to prime the host immune system to recognize tumor neoantigens associated with genomic instability and help prevent future tumor reoccurrence. Because single therapeutic approaches are often met with resistance, it is more likely that a combination targeting multiple SMAD4 deficient-associated mechanisms in addition to standard care approaches, such as olaparib with PD-1/PD-L1 blockade and radiation, would be more effective at reducing SMAD4 deficient SCC tumor burden.

Summary and future perspectives

Here, we review the unique functional impacts of SMAD4 loss in SCCs through its direct effects on stratified epithelial cells and indirect effects on stromal microenvironment (Figure 3), and suggest various targetable mechanisms important for tumor initiation and progression of SMAD4 deficient SCCs. Therefore, rational clinical trials that include measurement of SMAD4 loss as a relevant biomarker of response will be required to determine which biological observations are relevant for therapeutic approaches against human SCCs.

Acknowledgements

Abbreviations:

SCCs	squamous cell carcinomas
LOH	Loss of heterozygosity
CSC	cancer stem cells
SBEs	SMAD binding elements
EMT	epithelial-mesenchymal transition
FA	Fanconi anemia

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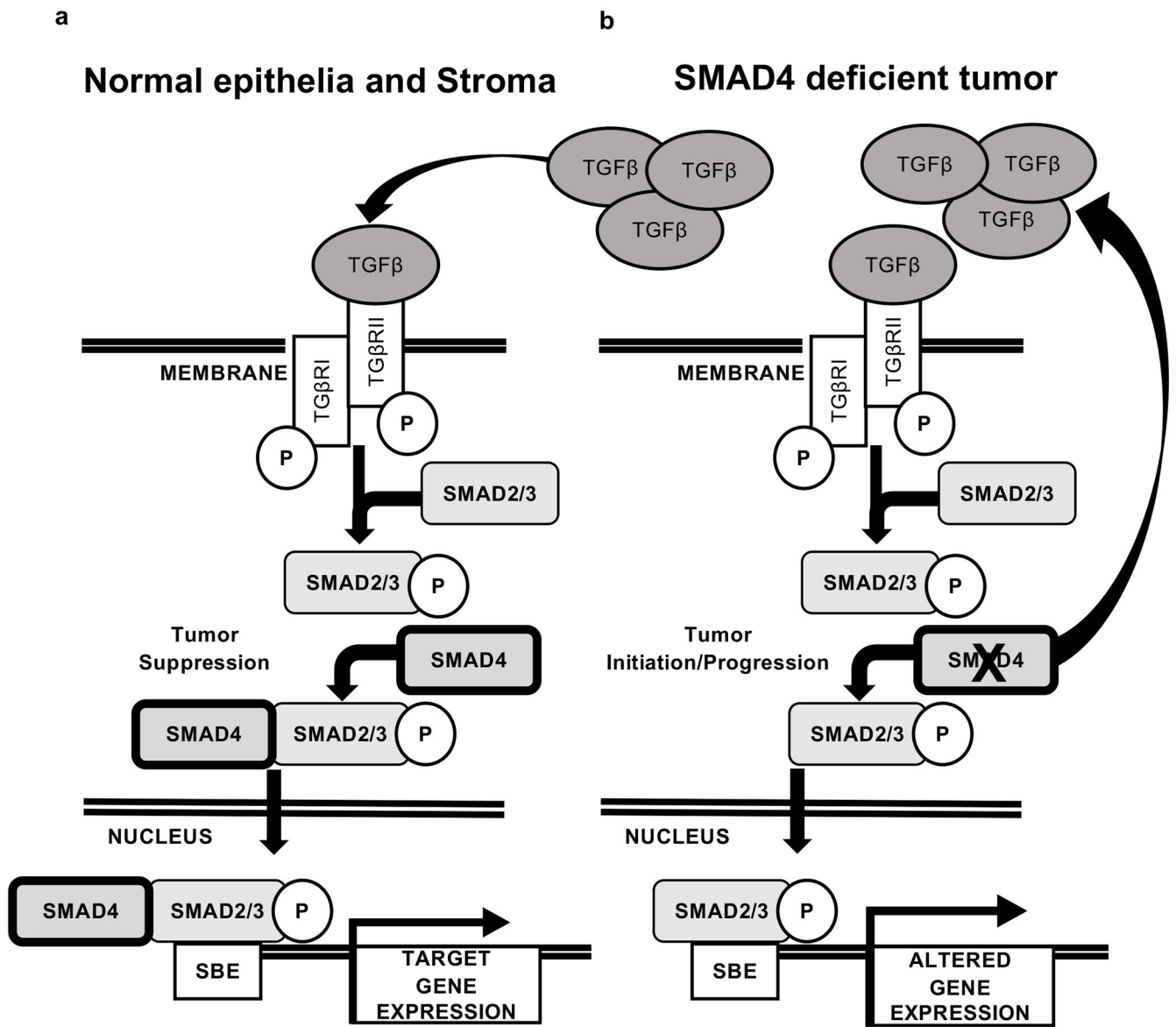
Figure 1. Normal versus deficient SMAD4 signaling.
(a) SMAD4 dependent TGFβ signaling in normal epithelial and stromal cells. (b) SMAD4 loss results in increased TGFβ production and release TGFβ into the extracellular matrix that acts on stromal cells, inducing a pro-tumorigenic microenvironment.
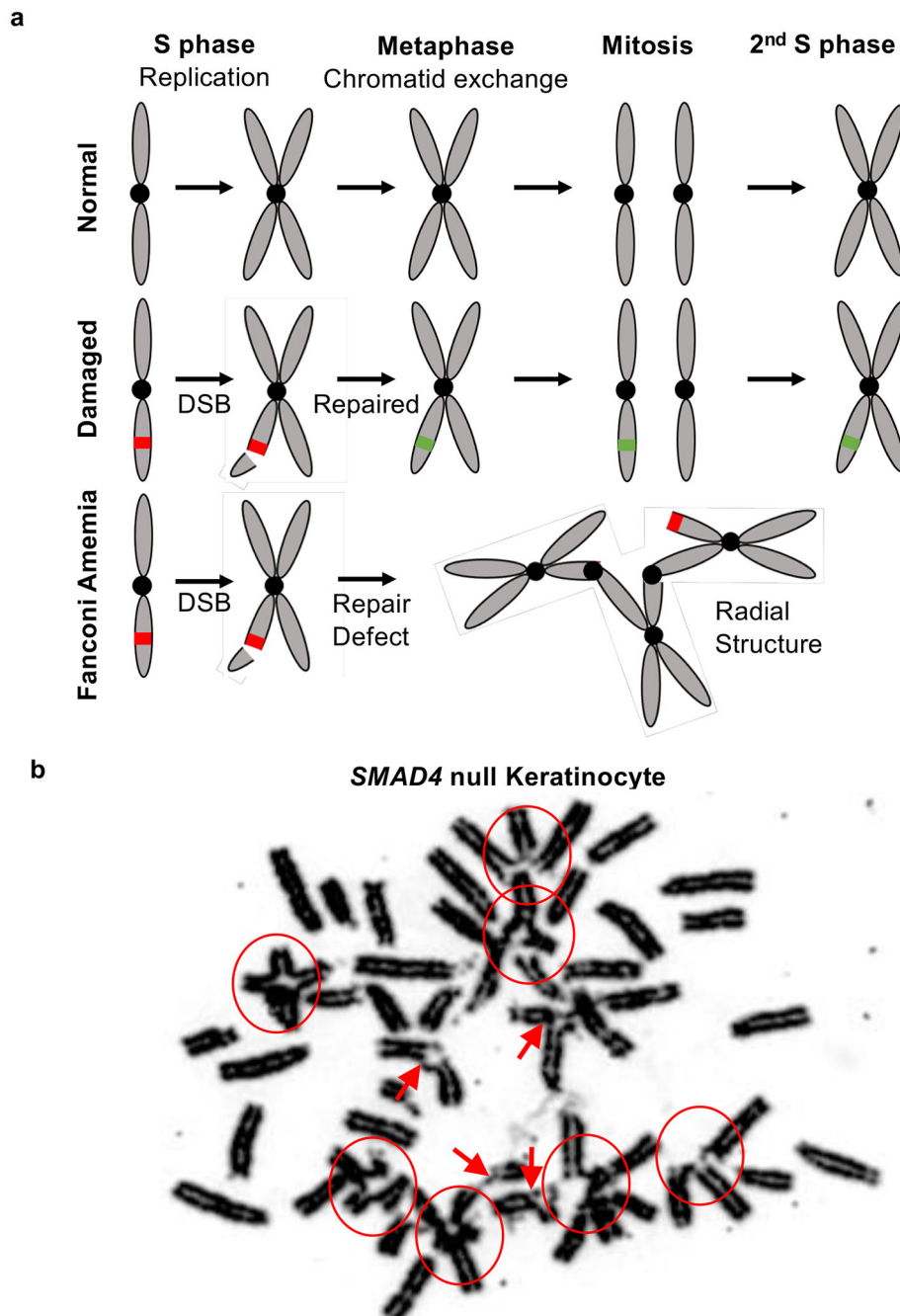
Figure 2. "Brca-like" phenotype in SMAD4 deficient keratinocytes.
(a) DNA repair and replication in normal, DNA damaged, and Fanconi anemia cells. (b) Karyotype of SMAD4 null keratinocytes treated with mitomycin C (40 ng/mL) leads to chromatin with radial structures (red circles) and chromosome breaks (red arrows), hallmarks observed in Fanconi anemia patients.
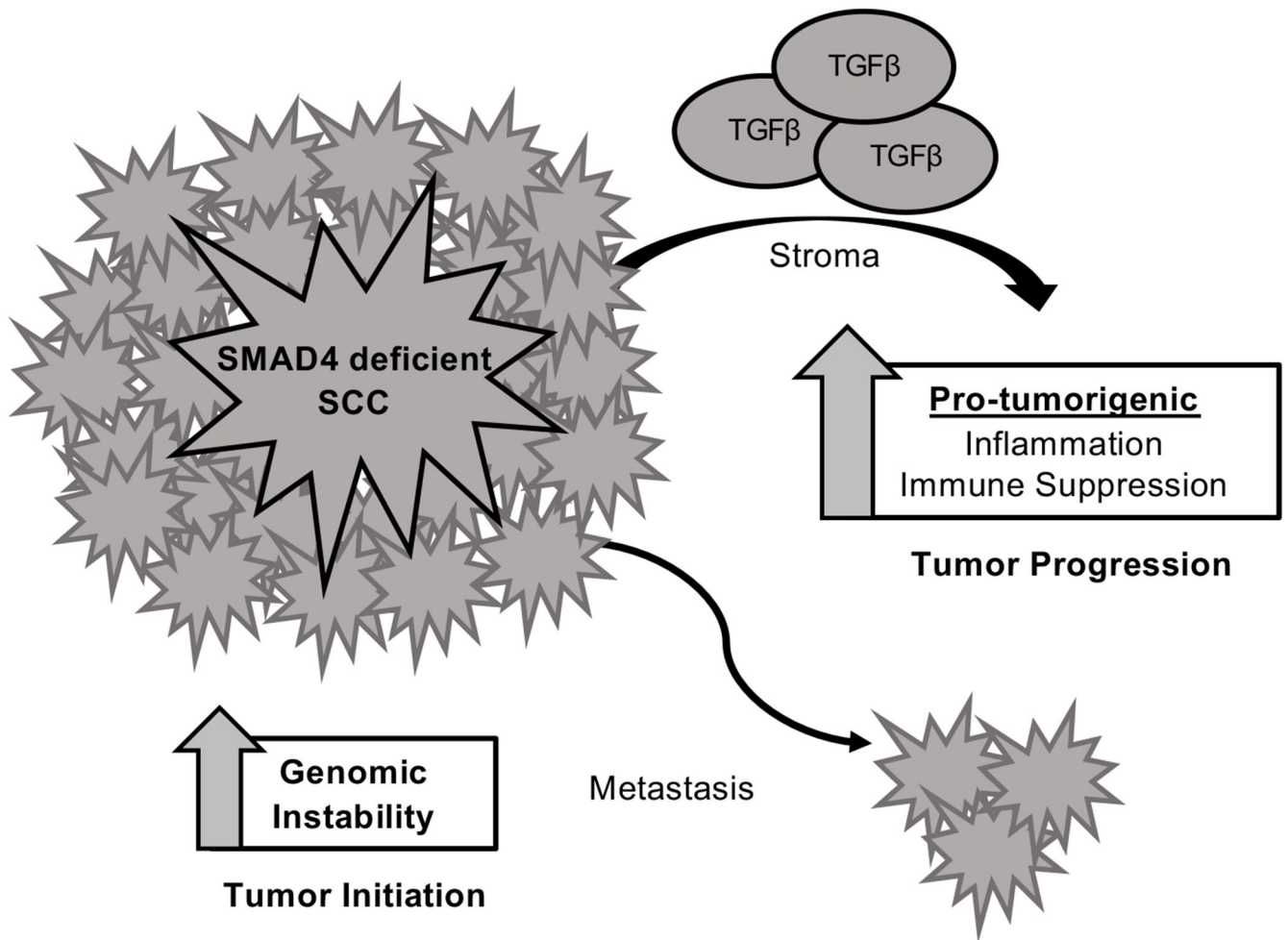
Figure 3. SMAD4 deficient SCC initiation and progression.
Loss of SMAD4 causes increased genomic instability to initiate SCC formation and
progression. A pro-tumorigenic microenvironment is induced by TGFβ-associated
inflammation and immune suppression to promote tumor growth and metastatic progression.