Synergistic Function of Smad4 and PTEN in Suppressing Forestomach Squamous Cell Carcinoma in the Mouse

Yan Teng, An-Na Sun, Xiao-Chen Pan, Guan Yang, Lei-Lei Yang, Ming-Rong Wang, and Xiao Yang

Abstract

The genetic bases underlying esophageal tumorigenesis are poorly understood. Our previous studies have shown that coordinated deletion of the Smad4 and PTEN genes results in accelerated hair loss and skin tumor formation in mice. Herein, we exemplify that the concomitant inactivation of Smad4 and PTEN accelerates spontaneous forestomach carcinogenesis at complete penetrance during the first 2 months of age. All of the forestomach tumors were invasive squamous cell carcinomas (SCCs), which recapitulated the natural history and pathologic features of human esophageal SCCs. A small population of the SCC lesions was accompanied by adenocarcinomas at the adjacent submucosa region in the double mutant mice. The rapid progression of forestomach tumor formation in the Smad4 and PTEN double knockout mice corresponded to a dramatic increase in esophageal and forestomach epithelial proliferation. The decreased expression of p27, p21, and p16 together with the overexpression of cyclin D1 contributed cooperatively to the accelerated forestomach tumorigenesis in the double mutant mice. Our results point strongly to the crucial relevance of synergy between Smad4 and PTEN to suppress forestomach tumorigenesis through the cooperative induction of cell cycle inhibitors.

Introduction

Globally, esophageal cancer is one of the most frequently occurring malignancies, exhibiting considerable geographic variation, and is the sixth leading cause of cancer-related deaths (1). Esophageal carcinomas comprise two major histologic subtypes: squamous cell carcinoma (SCC), which accounts for nearly 95% of all esophageal cancer diagnoses, and adenocarcinoma (1). The esophageal epithelium is a dynamic tissue consisting of two zones: the basal zone, containing the basal layer that contains the epithelial stem cells and a variable number of transit amplifying cell layers, and the differentiated zone, containing multiple layers of progressively differentiated squames (2). The process of esophageal tumorigenesis at the cellular level is related to disorders of cell proliferation, differentiation, and programmed cell death. In addition to environmental factors involved in this process, multiple genetic alterations associated with esophageal cancer have been described. These include a loss of the genes encoding p53 or p16 and overexpression of epidermal growth factor receptor (EGFR) or cyclin D1 (1). However, other signal transduction events that occur during esophageal tumorigenesis progress are still largely unknown.

Transforming growth factor-β (TGF-β) is a multifunctional growth factor superfamily that plays important roles in maintaining epithelial homeostasis (3). Resistance to TGF-β-induced growth inhibition is widely associated with epithelial cancers, including esophageal cancer (3, 4). TGF-β signal through transmembrane receptors and intracellular mediators called Smads (3). Altered expression of the genes encoding the TGF-β receptors and Smads contributes to tumor progression and poor prognosis in esophageal SCC (ESCC; refs. 5–8). In vitro studies have shown that TGF-β inhibits epithelial cell growth through transcriptional repression of c-Myc, which in turn leads to up-regulation of the cyclin-dependent kinase (Cdk) inhibitors p21 or p15 (9, 10). We reported previously that disruption of Smad4 in keratinocytes results in the failure of hair follicle cycling, formation of skin tumors, and marked hyperkeratosis in the esophageal epithelia (11). The function of the TGF-β signaling pathway in the development of ESCCs is in need of elucidation.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor that negatively regulates cell survival and proliferation by antagonizing phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) signaling (12). Although some results suggested that PTEN mutations may not play a major role in the carcinogenesis of ESCC (13), a recent study revealed that negative nuclear PTEN expression was a significant factor indicative of poor survival (14). Activation of PI3K signaling has been observed in transgenic mice that overexpress EGFR or K-ras and display hyperplastic epithelia in the esophagus and forestomach (15, 16). The targeted disruption of PTEN in keratinocytes results in esophageal hyperkeratosis (17, 18). However, the molecular mechanisms underlying the roles of the PTEN/PI3K/Akt pathway during esophageal tumorigenesis are still poorly understood.

Recent studies have revealed that TGF-β and PI3K/Akt signals are integrated at multiple levels to regulate cell survival and proliferation (19–24). Exposure of breast cancer cells to bone morphogenic protein 2 (BMP2) results in decreased PTEN protein degradation and increased PTEN levels (19). Reciprocally, inducible PTEN expression in a glioblastoma cell line suppresses expression of TGF-β1 (20). Recent studies have reported that Akt interacts directly with Smad3 to regulate the sensitivity to TGF-β-induced apoptosis (21, 22). We showed previously that Smad4 and PTEN act synergistically to negatively regulate epidermal proliferation and tumorigenesis (11), which is consistent with the finding that inactivation of PTEN and activated Akt is associated with the development of skin SCC in Smad4 mutant mice (24).
Synergistic Role of Smad4 and PTEN in Keratinocytes

Table 1. Cancer incidence in double mutant and control mice

<table>
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<th>PTEN Co/Co</th>
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Abbreviation: NA, not applicable.

*Fisher exact test (P < 0.05) was used for comparisons with Smad4+/PTENCo/+:K5-Cre at different time points.
†Fisher exact test (P < 0.05) was used for comparisons with Smad4Co/+:PTENCo/+:K5-Cre at different time points.
‡Fisher exact test (P < 0.05) was used for comparisons with Smad4Co/+:PTENCo/+:K5-Cre at different time points.
§Fisher exact test (P < 0.05) was used for comparisons with Smad4Co/+:PTENCo/+:K5-Cre at different time points.
¶Fisher exact test (P < 0.05) was used for comparisons with Smad4Co/+:PTENCo/+:K5-Cre at different time points.

To continue exploring the synergistic function of Smad4 and PTEN in esophageal carcinogenesis, we analyzed the abnormalities in esophageal and forestomach tissues in keratinocyte-specific Smad4 and PTEN double knockout mice (Smad4Co/+:PTENCo/Co, Keratin 5-Cre), in which the deletion of Smad4 and PTEN is targeted specifically to esophageal and forestomach epithelia. We show that mice with a conditional ablation of Smad4 and PTEN develop an earlier onset of hyperplasia and dysplasia in the esophageal and forestomach epithelia and accelerated tumor formation in the forestomach. These findings suggest a cooperative effect between Smad4 and PTEN in suppressing epithelial proliferation and forestomach carcinogenesis.

Materials and Methods

Generation of tissue-specific knockout mice. By crossing the Keratin 5-Cre (K5-Cre) transgenic mice with the mice carrying conditional Smad4 alleles (Smad4Co/Co; ref. 25) and the mice bearing conditional PTEN alleles (PTENCo/Co; refs. 17, 18), we produced the Smad4Co/Co;PTENCo/Co;K5-Cre, Smad4Co/Co;PTENCo/Co;K5-Cre, Smad4Co/Co;PTENCo/Co;K5-Cre, and Smad4Co/Co;PTENCo/Co;K5-Cre mice. The offspring were genotyped by PCR analysis (11).

Histopathology. Isolated tissues from mice were directly fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm. Sections were stained with H&E. The histopathologic analysis and diagnosis of different stages of esophageal cancer progression was consistent with human tumor classification.

LacZ staining. Esophagus and forestomach tissues were fixed in 4% paraformaldehyde for 2 hours and subjected to LacZ staining according to published methods (26).

Immunohistochemistry and bromodeoxyuridine labeling. Immunohistochemistry analysis was done on formalin-fixed, paraffin-embedded sections. The primary antibodies used in these studies were as follows: cytokeratin 14 (K14), cytokeratin 6 (K6), cytokeratin 1 (K1), cytokeratin 10 (K10), and filaggrin (Covance, Berkeley, CA); Smad4, PTEN, cyclin D1, and bromodeoxyuridine (BrdUrd; Sigma, St. Louis, MO) were incubated in 10% normal human serum (NHS) supplemented with protease inhibitor cocktail (Roche, Penzburg, Germany). Proteins (50 μg) were electrophoresed on 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Immunoblotting was done using the following antibodies: Smad4, PTEN, and p16 (Santa Cruz Biotechnology); Akt, p-Akt, p-Smad2, Smad2, p21, and p27 (Cell Signaling); K6, K10, and filaggrin (Covance); actin (Sigma).

Statistical analysis. All statistical analysis was done using SPSS software. Statistical differences were determined by Student’s t test in Fig. 2Q and Fisher exact test in Table 1. P < 0.05 was considered significant.

Results

Conditional deletion of Smad4 and PTEN in esophageal and forestomach epithelia. We first examined the efficiency and tissue specificity of Cre-mediated recombination in esophageal and forestomach tissues by breeding the K5-Cre mice (11) with the ROSA26 reporter mice (27). Analysis of the tissues from the double transgenic offspring at postnatal day 15 (P15) for the LacZ staining revealed specific Cre-mediated recombination in all layers of the esophageal (Fig. 1A) and forestomach stratified squamous epithelium (Fig. 1B). The expression of Smad4 and PTEN was detected in the basal cell layer and the suprabasal layers in the Smad4Co/Co;PTENCo/Co; K5-Cre esophageal (Fig. 1C and E) and forestomach epithelia (data not shown) but was dramatically decreased in the Smad4Co/Co; PTENCo/Co;K5-Cre double mutant epithelia (Fig. 1D and F). Western blot analysis confirmed that both the expression of Smad4 and PTEN was markedly reduced in the double mutant esophageal epithelia (Fig. 1G). As expected, p-Akt expression was elevated in the double mutant esophageal epithelia (Fig. 1G). We also checked the expression of p-Smad2 by immunohistochemical analysis. Less nuclear p-Smad2 staining was found in double mutant esophageal epithelia (Fig. 1I) compared with that in the Smad4Co/Co;PTENCo/Co; K5-Cre esophageal epithelia (Fig. 1H). These data indicated that both Smad4 and PTEN were efficiently and simultaneously disrupted in the esophageal and forestomach epithelia by Cre-mediated recombination.

Shortened life span and forestomach tumorigenesis in the Smad4 and PTEN double mutant mice. The Smad4 and PTEN compound mutant newborn mice presented no genotype dependent differences in body weight, size, or suckling ability. However, all Smad4Co/Co;PTENCo/Co;K5-Cre mice (40 of 40) exhibited progressive growth retardation and died between P10 and P100 (Fig. 1J). At P10 and P100, all of the Smad4Co/Co;PTENCo/Co;K5-Cre mice (40 of 40) were alive, whereas 25% of the Smad4Co/Co;PTENCo/Co;K5-Cre mice (10 of 40) died between
P20 and P60 (Fig. 1J). At P90, the double mutant mice exhibited lower serum blood sugar levels and body weights than the heterozygous controls (4.09 ± 1.22 mmol/L and 23.3 ± 2.40 g in the double mutants versus 10.57 ± 1.82 mmol/L and 34.9 ± 1.91 g in the double heterozygous controls, respectively; n = 5; P < 0.01), indicating that the double knockout mice might have died of starvation. In all cases, postmortem analysis of the Smad4Co/Co;PTENCo/Co;K5-Cre mice revealed dramatically thickened esophagi and forestomachs, with large, fused forestomach tumors at unapparent squamocolumnar junctions (SCJs; Fig. 1K). These were the likely causes of death. In contrast, the littermate Smad4Co/+;PTENCo/+;K5-Cre mice showed thin and transparent forestomachs with distinct SCJs (Fig. 1K).

Enhanced cell proliferation in the Smad4 and PTEN double deficient esophagus and forestomach. Histologic examination of the esophageal epithelia at P60 revealed a very clearly defined epithelium with a thin cornified layer in the Smad4Co/+;PTENCo/+;K5-Cre mice (Fig. 2A). In contrast, the double mutant mice presented marked hyperplastic epithelia, which were characteristic of more layers of small basophilic cells in the basal zone and more layers of flattened, differentiated cells in the differentiated zone (Fig. 2D). The esophageal epithelia from the Smad4Co/+;PTENCo/+;K5-Cre and the Smad4Co/+;PTENCo/+;K5-Cre mice (Fig. 2B and C) did not show obvious hyperplasia but instead exhibited thicker cornified layers than those in the Smad4Co/+;PTENCo/+;K5-Cre mice. Similar to the results we documented for the esophageal epithelia, we found severe hyperplastic lesions in the forestomach epithelia of the double mutant mice (Fig. 2E-H).

We also investigated whether the increased number of keratinocyte layers in the esophageal and forestomach epithelia from the double mutant mice was due to enhanced cellular proliferation. Results of BrdUrd labeling showed that the replicating cells were localized sparsely in the basal layer of the control mice (Fig. 2I and M) and significantly increased in the Smad4Co/+;PTENCo/+;K5-Cre and Smad4Co/+;PTENCo/+;K5-Cre mice (Fig. 2I, N, K, O, and Q). The BrdUrd-labeled cells in the basal layer were even more dramatically increased in the esophageal and forestomach epithelia of the double mutant mice (Fig. 2I, P, and Q). Notably, the double mutant mice also showed extensive BrdUrd-positive staining in the cells of layers of flattened, differentiated cells in the differentiated zone (Fig. 2D). The esophageal epithelia from the Smad4Co/+;PTENCo/+;K5-Cre and the Smad4Co/+;PTENCo/+;K5-Cre mice (Fig. 2B and C) did not show obvious hyperplasia but instead exhibited thicker cornified layers than those in the Smad4Co/+;PTENCo/+;K5-Cre mice. Similar to the results we documented for the esophageal epithelia, we found severe hyperplastic lesions in the forestomach epithelia of the double mutant mice (Fig. 2E-H).

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the suprabasal layers of the esophageal and forestomach epithelia (Fig. 2L and P). These data indicated that the combined Smad4 and PTEN deficiencies increased cell proliferation in the esophagus and forestomach of the double mutant mice much more than either single gene ablation.

**Altered expression of keratins in Smad4 and PTEN double knockout mice.** Next, we examined whether abnormal epithelial proliferation correlated with terminal differentiation defects in the Smad4 and PTEN double mutants. We determined that the expression of K14 was restricted mainly to the proliferating basal layer of the forestomach epithelium in the control mice (Fig. 3A). In contrast, we detected K14 expression in the basal and suprabasal cell layers of the hyperplastic epithelium in the double mutant mice (Fig. 3B). The expression of K6, an early differentiation marker, was enhanced in the compound mutant forestomachs (Fig. 3C and D). The absence of Smad4 and PTEN in the epithelium resulted in an expanded area of suprabasal K1 and K10 expression (Fig. 3E-H). Filaggrin, which is a terminal differentiation marker in mature corneal cell layers, was expressed at comparable levels in the double mutant and control mice (Fig. 3I and J), indicating that the epithelial terminal differentiation program was not blocked completely in the compound mutant mice.

**Accelerated tumor formation in the forestomachs of the Smad4 and PTEN double mutant mice.** The histologic analyses revealed a more rapid progression of tumors in the double mutant forestomachs (Table 1). The double knockout mice began to develop SCCs as early as 1 month of age and exhibited invasive SCCs with 100% penetrance by 2 months (Table 1). None of the Smad4Co/Co; PTENCo/Co; K5-Cre and Smad4Co/Co; PTENCo/Co; K5-Cre mice developed SCCs during the same time period. In contrast, 53% of the Smad4Co/Co; PTENCo/Co; K5-Cre mice developed SCCs by 4 months of age (Table 1). At 8 months, the Smad4Co/Co; PTENCo/Co; K5-Cre mice exhibited the highest cancer frequency followed by the Smad4Co/Co; PTENCo/Co; K5-Cre mice and then the Smad4Co/Co; PTENCo/Co; K5-Cre mice (Table 1).

We observed obvious hyperplasia in the double mutant forestomachs as early as P15 (Fig. 4A). By 30 postnatal days, severe...
dysplasia with a loss of epithelial organization was obvious in all double knockout mice (Fig. 4B). Carcinoma in situ (Fig. 4C), which is characterized by dysplasia involving the entire thickness of epithelium, was present in the double knockout mice for the duration. By 60 postnatal days, all of the double mutant mice displayed invasive SCCs at SCJs, which showed that the neoplastic epithelial cells invaded the subepithelial tissues through the basement membrane (Fig. 4D) and replaced the muscle fibers of destructive infiltrative growth (Fig. 4E). In addition, the invasive well-differentiated adenocarcinomas were found to accompany the SCCs in 15% (3 of 20) of the double mutant mice (Fig. 4F). Approximately 10% (2 of 20) of the double mutant mice displayed metastatic SCC lesions below the esophageal adventitia (Fig. 4G), which were confirmed by K14 staining (Fig. 4H). We found an abundance of BrdUrd-positive cells in the SCCs (Fig. 4I) and the adenocarcinomas (Fig. 4J).

To determine the nature of neoplastic cells in the double deletion murine forestomachs, we investigated the expression of differentiation markers using immunohistochemistry. Previous studies showed that high levels of K14 expression is an important biomarker in human esophageal carcinogenesis (28) and is found throughout the epithelium in the majority of cases of human SCC, regardless of tumor origin or degree of differentiation (29). We found abundant, strong K14-staining in all squamous cells (Fig. 5A) but negligible amounts in well-differentiated adenocarcinoma cells (Fig. 5B). K6, a putative marker of proliferation, was strongly expressed in squamous cells of tumor tissues from the double mutant forestomachs (Fig. 5C). In contrast, K1, K10, and filaggrin,
as terminal differentiation markers, were abundant in hyperplastic epithelia but scarcely detectable in invasive lesions (Fig. 5D-F). It has been reported that SPRR3, a marker for terminally differentiating squamous cells, was decreased dramatically in primary esophageal carcinomas (30). In the double knockout forestomachs, SPRR3 staining was strong in the hyperplastic epithelia but was undetectable in the invasive SCCs (Fig. 5G). Western blot analysis confirmed that K6 was markedly up-regulated, and filaggrin and SPRR3 were significantly diminished in the double mutant invasive tumors compared with those in the esophageal epithelia (Fig. 5H). These findings indicate that the invasive tumor cells originating from the basal layer failed to undergo terminal differentiation.

**Absence of Smad4 and PTEN results in down-regulation of cell cycle inhibitors.** Previous studies have shown that Akt activation and overexpression of cyclin D1 are key processes during SCC development in humans and mice (24, 31–34). Therefore, we examined the expression levels of p-Akt and cyclin D1 in Smad4 and PTEN double knockout tumors with immunohistochemical staining. The expression of p-Akt was barely detectable in the control forestomach epithelia but was elevated significantly in the double mutant tumors (Fig. 6A and B). The nuclear expression of cyclin D1 was detected in a patchy pattern in the double knockout tumor tissues but not in the forestomach epithelia of the control mice (Fig. 6C and D). We also checked the expression of p-Smad2 in...
double knockout tumor tissues. Although the expression of p-Smad2 was decreased in the Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre, Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre and double mutant esophageal epithelia, it was dramatically up-regulated in tumors of double knockout mice (Fig. 6E).

Previous studies indicate that the Cdk inhibitors are downstream mediators of the biological effects of PTEN and TGF-\(\beta\) signals (3, 35–39). To examine whether Smad4 and PTEN could cooperate to regulate the Cdk inhibitors and thereby repress esophageal epithelial proliferation, we measured the protein levels of p27, p21, and p16 in the esophageal epithelia of different genotypes and tumor tissues from the double knockout mice using Western blot analysis (Fig. 6E). The expression of p27 was decreased slightly in the Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre and double mutant esophageal epithelia but was down-regulated significantly in the double knockout epithelia and tumors. The expression of p21 was decreased dramatically in the Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre, double knockout epithelia and tumors, compared with that in wild-type, Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre, and Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre mice. The expression level of p16 in the Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre epithelia was comparable with that in the Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre epithelia but was barely detectable in the Smad\(^4^{+/+}\);PTEN\(^{+/+}\);K5-Cre, double mutant epithelia and tumor tissues. In addition, the expression of Cdk4, which is the catalytic partner of cyclin D1, was not altered in the double mutant epithelia (Fig. 6E). These results suggest that Smad4 and PTEN can cooperatively inhibit the proliferation of esophageal and forestomach epithelia by up-regulating the Cdk inhibitors.

**Discussion**

Our previous studies have suggested that Smad4 and PTEN may act synergistically to regulate epidermal proliferation and inhibit skin tumorigenesis. Herein, we showed that simultaneous
inactivation of Smad4 and PTEN in mouse esophagi and forestomachs resulted in accelerated cellular hyperproliferation and earlier onset of forestomach tumors. Importantly, we revealed that Smad4 cooperated with PTEN to inhibit esophageal epithelial proliferation through collaborative regulation of cell cycle inhibitors.

Our previous results showed that knocking out Smad4 blocked the response of the keratinocytes to the growth inhibition of TGF-β1 (11). In this study, we showed that deletion of Smad4 led to the decreased expression of p-Smad2 (Fig. 6E) and less nuclear accumulation in the esophageal epithelia (Fig. 1H and I). This confirms our previous finding in Smad4 mutant cardiomyocyte (40) but conflicts some previous studies. There are several articles indicating that the TGF-β-induced phosphorylation and nuclear localization of Smad2/Smad3 are not affected by the absence of Smad4 (41, 42). The discrepancy is probably because of the fact that we studied the consequence of Smad4 deficiency in a physiologic setting, whereas most of the previous studies in cancer cell lines were in vitro. Our results suggested that p-Smad2 could be downregulated upon lack of Smad4 via a feedback mechanism in vivo.

However, we also found that the expression of p-Smad2 was significantly elevated in tumors of double knockout mice (Fig. 6E), which is consistent with the previous point that Smad4 is not necessary for activation of R-Smads (41, 42). Further work is required to define the importance of Smad4-independent activation of R-Smads in tumorigenesis.

In this analysis, we present a novel genetic mouse model of esophageal and forestomach SCC and provide new insights into the molecular relationship between TGF-β and PI3K signaling pathways. Keratinocyte-specific knockout of Smad4 led to the development of spontaneous forestomach SCCs in roughly 42% of the 8-month-old mice (Table 1), whereas deletion of PTEN only led to a moderate to severe dysplasia by 8 months (data not shown). Strikingly, all (100%) of the double mutant mice exhibited destructive invasive SCCs by 60 days of age. These findings show that concurrent inactivation of Smad4 and PTEN is sufficient for initiation and promotion and malignant conversion of forestomach SCCs in mice. Notably, the occurrence of forestomach cancer in the Smad4Co/Co;PTENCo/+;K5-Cre mice at 8 months of age was statistically significant, relative to that in the Smad4Co/Co;PTENCo/Co;K5-Cre mice at 8 months of age.
K5-Cre mice (83% versus 42%, P < 0.05; Table 1), suggesting that PTEN haploinsufficiency played a role in mouse forestomach carcinogenesis in the Smad4 mutant mice. Similar to the effect of PTEN haploinsufficiency, combining the PTEN deficiency with the inactivation of one of the Smad4 alleles also resulted in a higher incidence of forestomach SCCs (Table 1). It is noteworthy that the survival rates were significantly lower in ESCC patients with reduced Smad4 or nuclear PTEN expression (6, 14). Our data provided the first direct evidence of synergistic function of Smad4 and PTEN in suppression of esophageal and forestomach tumorigenesis.

The early onset and full penetrance of forestomach SCCs in double knockout mice suggests that Smad4 and PTEN act synergistically to regulate tissue homeostasis, possibly by regulating stem cell self-renewal in esophageal and forestomach epithelia. Our BrdUrd labeling results revealed that the double knockout epithelia possessed more proliferating layers (Fig. 2L and P) than the control epithelia, indicating that the number of stem cells or the rate of stem cell self-renewal could be affected by the double gene deficiency. The invasive SCCs from the double mutant forestomach displayed strong and extensive expression of Ki4, showing that tumor cells originate from the basal layer that contains epithelial stem cells. It is intriguing to note that 15% of the double mutant mice displayed invasive adenocarcinomas in addition to SCCs. This finding supports the viewpoint that esophageal adenocarcinomas may have arisen directly by metastatic changes from stem cells (43). Recent studies have revealed that BMP signaling inhibits intestinal stem cell self-renewal by regulating PTEN activity (44). The molecular mechanism of synergistic interaction between Smad4 and PTEN to regulate esophageal stem cell self-renewal and differentiation needs further clarification.

In the present study, we show that Smad4 and PTEN cooperatively control the proliferative rate of the esophageal and forestomach epithelia. This is substantiated by thickened epithelium (Fig. 2A–H) and significantly increased BrdUrd incorporation (Fig. 2F–Q) in double mutants. These results suggest that the impaired forestomach proliferation process may cause the observed accelerated forestomach cancer formation in the double mutants. Several lines of evidence suggest that TGF-β and PI3K signals interact to modulate cell proliferation and apoptosis (21, 22, 44, 45). A recent study has shown that ectopic activation of TGF-β signaling by a constitutively active TGF-β type I receptor (TGF-βRI) can restrain anchorage-independent proliferation elicited by PTEN knockdown (45). Previous studies have also revealed a mechanism whereby PI3K induces an interaction between PKB/Akt and Smad3 to reduce the pool of Smad3 available for TGF-β signaling (21, 22).

In the current work, we provide additional supportive evidence that Smad4 and PTEN synergistically inhibit the proliferation of esophageal epithelial keratinocytes and tumor formation by regulating the Cdk inhibitors collaboratively. Two families of mammalian Cdk inhibitors have been identified. One comprises the CIP/KIP family (p21, p27, and p57), and the other includes the INK4 family proteins (p16, p15, p18, and p19; ref. 46). Loss of function in either family plays a principle role in the development of most human cancers, including ESCC (1). In vitro studies have shown that an activated PI3K/AKT pathway can down-regulate the expression of p27 (35–38). Lower expression of p27 was found from forestomach tissues of fibroblast-specific inactivation of TGF-βRII mice (39). We observed previously the reduced expression of p27 in conditional Smad4 mutant epidermis (11). In this study, the expression of p27 was reduced dramatically in the double mutant epithelia and in the SCCs relative to that in the mutants with a single gene deficiency, showing that Smad4 collaborates with PTEN to up-regulate p27. Previous studies have shown that the process of malignant transformation of the esophageal mucosa in mice by gastroesophageal-reflux together with N-methyl-N-nitrosourea is enhanced in a p27 null background (47). Concomitant inactivation of one PTEN allele and one or both Cdkn 1b alleles (encoding p27) accelerates spontaneous neoplastic transformation and the incidence of tumors of various histologic origins (48), suggesting a role for p27 in the prevention of malignant progression. The dramatically decreased expression of p27 may, in part, explain the phenotype in which tumor onset and the malignant degree were significantly accelerated in the double mutant mice.

In vitro studies have shown that TGF-β inhibits cell growth by up-regulating the Cdk inhibitors, including p21 (3). We showed herein that the expression of p21 was significantly decreased in the Smad4+/−;PTEN+/−;K5-Cre and double mutant epithelia and in the SCCs, which is in line with our previous observations (11). Inactivation of the p16 gene caused by homozygous deletion and de novo methylation is common among ESCCs (49). Moreover, a loss of p16 expression together with cyclin D1 overexpression is associated with the poor prognosis of ESCCs (50). Notably, the expression of p16 was dramatically decreased in the Smad4+/−;PTEN+/−;K5-Cre and double mutant epithelia. All of these results indicate that the loss of both Smad4 and PTEN led to a down-regulation of the CIP/KIP and INK4 Cdk inhibitors. Cooperativity among p27, p21, and p16 underexpression and cyclin D1 overexpression contributed to the forestomach tumorigenesis acceleration in the double mutant mice.

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