SHORT COMMUNICATION

Epithelial-Specific Loss of *PTEN* Results in Colorectal Juvenile Polyp Formation and Invasive Cancer

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Cowden syndrome (CS) is a rare autosomal dominant cancer-prone disorder caused by germ-line mutation of the phosphatase and tensin homolog mutated on chromosome 10 (*PTEN*) tumor-suppressor gene. Affected patients commonly develop juvenile polyps, and show an elevated risk of developing colorectal cancers. The etiology of these peculiar polyps remains unclear, although previous work has suggested somatic *PTEN* alterations in the stroma of juvenile polyps. After a long latency period, we find epithelial-specific *PTEN* deletion to cause formation of juvenile polyps in the colorectum without stromal *PTEN* loss. More important, we find that these lesions closely recapitulate all of the characteristic histopathological features of juvenile polyps seen in patients with CS, including stromal alterations and dysplastic transformation to colorectal carcinoma. The stromal alterations we identify after epithelial-specific *PTEN* loss suggest that *PTEN* may be involved in altered epithelial-mesenchymal cross talk, which, in turn, predisposes to colorectal neoplasia and polyposis. Our transgenic model is the first to recapitulate colorectal juvenile polyposis in patients with CS. We conclude that stromal *PTEN* loss is not a prerequisite for the formation of juvenile polyps, and that colorectal juvenile polyps in CS are bona fide neoplastic precursor lesions. (*Am J Pathol* 2014, 184: 86–91; <http://dx.doi.org/10.1016/j.ajpath.2013.10.003>)

Phosphatase and tensin homolog mutated on chromosome 10 (*PTEN*) is a potent tumor-suppressor gene, originally identified through its association with sporadic malignancies of the prostate, breast, and brain.^{1–3}

Germ-line loss of one copy of *PTEN* causes a spectrum of diseases inherited in an autosomal dominant manner (alias *PTEN* hamartoma tumor syndromes), including Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome, and Lhermitte-Duclos disease. The most common of these syndromes, CS, is characterized by the development of a hamartomatous lesions within multiple tissues, including the skin and gastrointestinal tract.⁴ A recent prospective analysis of a large CS patient cohort identified gastrointestinal tract polyposis as the second most common phenotypic manifestation of the disease.⁵ Although most gastrointestinal tract polyps are nondysplastic, CS patients also show an elevated risk for the development of colorectal cancer. The etiological link between the development of colorectal polyps and frank colorectal cancer in these patients remains unclear. More important, because CS patients show loss of

one copy of *PTEN* in all cells in the body, uncertainty remains as to whether polyposis is driven by loss of *PTEN* expression in the epithelial or the stromal compartment of the intestine.⁶

Mice constitutively heterozygous for the *Pten* allele are noted to model some features of CS, including that of gastrointestinal tract polyposis.^{7–9} However, these models do not identify the tumor cell of origin nor do they progress to colorectal cancer.

Conditional deletion of *PTEN* specifically in the mouse intestinal epithelium has no immediate consequence on homeostasis or tumorigenesis in the intestine.¹⁰ In contrast, targeted deletion of *PTEN* in both intestinal epithelial and stromal cells results in expansion of the intestinal stem cell compartment and polyposis.¹¹ This suggests that *PTEN*-deficient epithelial cells can only initiate tumorigenesis in the context of stromal *PTEN* deficiency.

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Herein, we have examined the long-term consequences of epithelial-specific *PTEN* deletion to test the hypothesis that stromal loss of *PTEN* is required for the formation of colorectal polyps in human CS.

Materials and Methods

Mice

All animal procedures were conducted in accordance with local and national regulations. Mice were generated, housed, and genotyped, and Cre activity was induced as previously described.¹⁰ A total of 30 *Ah::CreER^{T/0};Pten^{F/F}* mice and 29 *Ah::CreER^{T/0};Pten^{+/+}* mice were enrolled into ageing cohorts. Tissues were harvested, fixed, and processed according to standard protocols and as previously described.¹⁰ Animals were monitored closely for symptoms of disease, and were then necropsied as previously described.¹⁰

Histological and IHC Data

Formalin-fixed, paraffin-embedded tissues were divided into sections and stained according to standard protocols and as previously described.¹⁰ Antibodies used for immunohistochemistry (IHC) were as follows: rabbit anti-PTEN (1:100; Cell Signaling Technologies, Danvers, MA), mouse anti- β -catenin (1:100; BD Transduction Laboratories, Oxford, UK), rabbit anti-p53 (CM5, 1:1000; Novocastra/Leica Biosystems, Wetzlar, Germany), rabbit anti-phospho-AKT (S473) (1:50; Cell Signaling Technologies), and mouse anti-Ki-67 (1:100; Novocastra). All sections were subject to heat-mediated antigen retrieval by boiling in citrate buffer (LabVision Corp./Thermo Fisher Scientific Inc., Kalamazoo, MI) before incubation with primary antibody. Primary antibody binding was detected by incubation with appropriate secondary antibody (goat anti-rabbit or goat anti-mouse, both from Dako; Agilent Technologies, Cambridge, UK), followed by signal amplification using the avidin-biotin complex method (Vector Labs, Burlingame, CA) and chromogenic signal detection using 3,3'-diaminobenzidine reagents (Dako).

Histopathological Data

Representative H&E-stained sections of juvenile polyps from *Ah::CreER^T;Pten^{F/F}* mice were presented and jointly reviewed by the mouse models' histopathological consensus committee at the Colon Cancer in Murine Models and Humans III meeting (Bar Harbor, ME), October 18 to 22, 2010.

Statistical Analysis

Kaplan-Meier survival analysis was performed using SPSS software version 20 (IBM, Inc., Armonk, NY).

Results

Intestinal Epithelial *Pten* Deletion Causes Colorectal Juvenile Polyposis

Previously,¹⁰ we used an inducible Cre-LoxP system to delete *PTEN* specifically within adult mouse intestinal epithelium. We described three independent Cre-expressing transgenes to drive epithelial-specific deletion of both copies of the *Pten* gene. In each system, induction of *PTEN* loss did not result in any detectable tumorigenic phenotype within the intestine up to 200 days after induction. Herein, we have further aged one group of animals (*Ah::CreER^{T/0};Pten^{F/F}*, herein referred to as *Pten^{F/F}*) and appropriate controls [*Ah::CreER^{T/0};Pten^{+/+}*, referred to as wild type (WT)].

Pten^{F/F} animals had significantly reduced longevity compared with WT controls. Kaplan-Meier analysis (Figure 1A) revealed the median survival of *Pten^{F/F}* animals to be approximately half that of WT animals (median, 416 and 726 days, respectively; $P < 0.001$, $\chi^2 = 46.47$, $df = 1$, numbers at risk are shown) (Supplemental Table S1). At necropsy, *Pten^{F/F}* animals bore a range of pathological conditions, including prostate neoplasia¹² and liver steatosis,¹³ which have been reported elsewhere. In addition, 13 (57%) of 23 *Pten^{F/F}* animals presented polyp-like lesions of the intestine, which occurred most commonly in the large intestine (31 of 34 total intestinal lesions) (Table 1). The median time point of intestinal tumor incidence was 422 days (Figure 1B). Evidence of intestinal lesions was not found in any WT control animals analyzed ($n = 9$).

Histopathological assessment of intestines from *Pten^{F/F}* mice showed large polypoid lesions throughout the colorectum of *Pten^{F/F}* animals, often pedunculated with a spherical head (Figure 1C). The mucosa separating the lesions bore no gross abnormalities and was indistinguishable from WT mucosa (Supplemental Figure S1). Polyps displayed expansion of the mesenchymal compartment relative to the crypt epithelium (Figure 1C and Figure 2A). Many crypts within polyps showed distended architecture and focal cystic change with inspissated mucin. The crypts were lined by normal goblet cell-bearing colonic epithelium. The mesenchyme was edematous and characterized by increased inflammatory infiltrate consisting of plasma cells, lymphocytes, and eosinophils. Many polyps contained prominent lymphoid aggregates within the stroma. Based on this constellation of features, we classified the most common lesions we observed as juvenile polyps. Of 31 colonic lesions, 28 identified across 23 *Pten^{F/F}* mice were found to be of juvenile histopathological features (Table 1), with a further two juvenile polyps identified, which also showed evidence of dysplasia. One frank colonic adenocarcinoma was also identified. We observed no hyperplastic or adenomatous polyps (without co-existent juvenile features) in the large intestine. Lesions in the small intestines were rarely identified (average, 0.13 lesions per mouse), and were tubular adenomas (one of three) or adenocarcinomas (two of three).

Figure 1 Epithelial-specific deletion of PTEN causes decreased survival and causes juvenile polyposis of the colon. **A:** Kaplan-Meier survival analysis shows a significant reduction in survival of *Pten*^{F/F} animals [median survival = 416 days (solid line)] compared with WT controls [median survival = 726 days (dashed line)] ($P < 0.001$, $\chi^2 = 46.47$, $df = 1$). **B:** Kaplan-Meier analysis to visualize the time course of development of intestinal lesions after induction of recombination in *Pten*^{F/F} animals [$n = 23$ (solid line)]. None of the animals developed intestinal tumors at <260 days after induction; intestinal tumor incidence in *Pten*^{F/F} animals steadily increased, with a median time of 422 days after induction. In contrast, WT controls (dashed line) remained free of intestinal tumors. **C:** Overview of an H&E-stained Swiss roll section of a representative *Pten*^{F/F} mouse colon shows a series of polyps varying in size along the length of the colorectum. This animal was necropsied at 60 weeks after induction. The boxed area of the left panel is magnified in the right panel, showing a low-power light micrograph of a representative larger polyp observed in the colorectum of *Pten*^{F/F} animals. It shows the haphazard crypt arrangement, focal cystic dilatation, and inflammatory stroma. The boxed areas of this panel are magnified in Figure 3A (left boxed area) and Figure 2A (right boxed area). **D:** Anti-Pten IHC staining on normal colon mucosa of an aged *Pten*^{F/F} animal (left panel) and on the juvenile polyp shown in C (right panel), showing depletion of PTEN specifically in the epithelium. In both cases, PTEN expression in the stroma is retained. This demonstrates that mesenchymal PTEN loss is not required for juvenile polyp formation. The boxed area indicates a region magnified in Figure 3C. **E:** Anti-pAKT (S473) IHC on colon tissue from aged *Pten*^{F/F} animals reveals elevation of pAKT staining in epithelial lesions. Small lesions tend to be more homogeneous for pAKT expression (left panel) and may represent incipient polyps (right panel, H&E-stained adjacent section). **F:** Anti-pAKT (S473) staining of larger polyps from *Pten*^{F/F} animals reveals strong focal increases in staining for pAKT (left panel). The localization of this staining does not correspond to areas of focal dysplasia (right panel, H&E-stained adjacent section). solid line, *Pten*^{F/F} animals; dashed line, WT controls.

To confirm that colorectal polyps were PTEN deficient, we performed anti-PTEN IHC. PTEN expression was readily detected in WT controls (Supplemental Figure S2). In contrast, the epithelium of unaffected mucosa and that covering juvenile polyps in *Pten*^{F/F} mice was deficient for anti-PTEN staining (Figure 1D and Supplemental Figure S2). The underlying stroma retained strong positivity for PTEN, consistent with the pattern of recombination we have previously observed.¹⁰ This indicates that PTEN loss from the underlying stroma is not required for juvenile polyp formation.

To further analyze the downstream consequences of epithelial PTEN deletion, we next performed IHC against phosphorylated (p) AKT, a key effector of the phosphatidylinositol 3'-kinase pathway. We did not observe pAKT

staining in the intestinal epithelium of WT animals or in histologically normal *PTEN*-deficient epithelium derived from *Pten*^{F/F} animals (data not shown). Clearly delineable regions of increased pAKT labeling were, however, evident in lesions of *Pten*^{F/F} animals. Smaller lesions, which might represent incipient juvenile polyps, tended to show more homogeneous labeling for pAKT across the whole tumor (Figure 1E). Larger, manifest polyps were also noted to show increased pAKT labeling (Figure 1F), although this was not homogeneous across the whole tumor but was restricted to clusters of epithelial cells. These regions of elevated pAKT expression were not found to be related to foci of dysplasia.

Juvenile Polyps Are Characterized by Ectopic Crypt Formation

We next compared the proliferation status and pattern of tumors arising in *Pten*^{F/F} animals with bona fide human CS polypectomy samples. We performed IHC against Ki-67 antigen, a marker of proliferation. Anti-Ki-67 staining revealed the presence of proliferative, ectopic crypt units that were seen to be haphazardly budding off parent crypts in the polyps of *Pten*^{F/F} animals (Figure 2B). A similar organization of crypt units was seen on Ki-67 staining of the CS patient material (Figure 2D). The unusual pattern of proliferation we see in *Pten*^{F/F} polyps is highly reminiscent of a mouse model of juvenile polyposis caused by deregulation of epithelial-mesenchymal interaction, which has previously been described.¹⁴

Clinically, CS patients develop juvenile polyps, which can be histopathologically indistinguishable from the polyps observed in patients with juvenile polyposis.¹⁵ The lesions described herein were found to faithfully recapitulate the polyps observed in CS, as demonstrated in a polypectomy specimen from a CS patient (Figure 2C).

Colorectal Juvenile Polyps Progress to Carcinoma

In addition to the formation of benign intestinal polyps, CS patients also show increased colorectal cancer risk. In our

Table 1 Overview of WT and *PTEN*^{F/F} Intestinal Histopathological Characteristics

Characteristics	WT data (n = 9)	<i>PTEN</i> ^{F/F} data (n = 23)
Animals with polyps	0	13
Total lesions, colon and SI	0	34
Small intestine, total	0	3
Tubular adenoma, LGD	0	1
Adenocarcinoma	0	2
Large intestine, total	0	31
Juvenile polyp	0	28
Juvenile polyp with dysplasia	0	2
Adenocarcinoma	0	1
Tubular adenoma	0	0
Hyperplastic polyp	0	0

LGD, low-grade dysplasia; SI, small intestine.

Figure 2 Juvenile polyps in *Pten*^{F/F} animals accurately model juvenile polyps in CS. **A:** Details of the boxed area in Figure 1C, demonstrating ectopic crypt formation along the length of a parent crypt in the juvenile polyp. **B:** IHC against Ki-67 on a consecutive section highlights proliferation zones in ectopic crypt units (arrows). **C:** Overview of an H&E-stained section of a polypectomy specimen of a CS patient carrying a germ-line point mutation in exon 8 (C.940del) of *PTEN* for comparison. Note the bizarre Maltese cross-like crypt architecture and the inflammatory stroma, features recapitulated in the *Pten*^{F/F} mice colorectal polyps. **D:** IHC against Ki-67 in the CS patient polypectomy specimen highlights ectopic crypt units (arrows), which is highly reminiscent of the pattern observed in polyps of *Pten*^{F/F} mice.

model, we found juvenile polyps to bear focal dysplasia (intraepithelial neoplasia) characterized by crowding of glands, cytonuclear pleomorphism, and loss of normal epithelial maturation (Figure 3, A and B). More important, we noted *PTEN* expression to be retained in the stroma, even in regions of focal dysplasia (Figure 3C).

Furthermore, we identified at least one lesion that had progressed to a frankly invasive carcinoma (Figure 3, D and E), recapitulating the pattern noted in CS patients. Similar to the dysplastic foci, *PTEN* expression was retained in the stroma surrounding invasive glands (data not shown). Similar findings regarding the preservation of *PTEN* expression in the mesenchymal compartment have recently been reported for two cases of colorectal carcinoma in CS patients,¹⁶ corroborating our findings in this model.

Given that disruptions in epithelial-mesenchymal interactions have previously been shown to cause dysregulation of key signaling pathways governing intestinal epithelial homeostasis,¹⁷ we next assessed the activation status of the Wnt pathway by IHC β -catenin staining. We did not observe nuclear β -catenin accumulation in either WT colon or histologically normal colonic epithelium from *Pten*^{F/F} mice, except for cells at the base of the crypt, as expected (Supplemental Figure S1, E and F). Furthermore, we did not observe nuclear β -catenin accumulation in epithelial cells of the (dysplastic) juvenile polyps (data not shown) or in the invasive colorectal carcinoma (Figure 3E). Likewise, p53 IHC did not reveal increased labeling in any of the juvenile polyps or carcinoma (Figure 3F).

Discussion

The findings we report herein are significant for two reasons. First, the lesions we describe faithfully recapitulate the gastrointestinal tract features of CS, providing a new model of this disease. Although other mouse models of *PTEN*-deficient gastrointestinal tract tumors have been described,^{11,18} these do not model the key features of CS that we describe herein. Second, these data provide further insight into the role of *PTEN* in epithelial-stromal interactions and initiation of intestinal tumorigenesis.

Previously, we reported that epithelial *PTEN* loss had no immediate effect on intestinal homeostasis or tumorigenesis,¹⁰ despite contrasting data from a different laboratory indicating that intestinal *PTEN* deletion causes rapid tumorigenesis.¹⁹ The main experimental disparity between these two studies lies in the construct used to express Cre recombinase. He et al¹⁹ used *Mx1::Cre*, which drives

Figure 3 Juvenile polyps in *Pten*^{F/F} animals develop focal dysplasia and progress to invasive adenocarcinoma. **A:** Details of the boxed area of Figure 1C, demonstrating back-to-back arrangement of crypts lined by mildly atypical cells with loss of goblet cell maturation. This corresponds to a focus of low-grade dysplasia arising in a pre-existent juvenile polyp. **B:** Anti-Ki-67 IHC outlines the dysplastic focus, showing loss of normal compartmentalization of proliferating cells. **C:** Details of the boxed area in Figure 1D reveal that the mesenchyme underlying *PTEN*-deficient epithelium in juvenile polyps of *Pten*^{F/F} animals retains strong positivity for anti-*PTEN* staining, even in dysplastic foci, as shown herein. **D:** H&E-stained section of an invasive colorectal adenocarcinoma arising in the distal colon of a *Pten*^{F/F} animal necropsied at 79 weeks after induction. The invasive carcinoma presumably developed from a dysplastic region in a preexistent juvenile polyp (left panel). The boxed area indicates a region shown at higher magnification in the right panel. **E:** IHC against β -catenin shows strong subcellular localization of β -catenin at the cell membrane, with no localization to the nucleus. Progression of disease to invasive carcinoma is not associated with activation of Wnt signaling in these lesions. **F:** IHC against p53 reveals no accumulation of stabilized p53 in invasive lesions.

recombination in both epithelial and stromal cells within the intestine in response to interferon induction.²⁰ In contrast, we have used *AhCreER^T*,²¹ an inducible construct that is expressed specifically in the epithelial cell layer of the intestine. This difference prompted us to hypothesize that the interaction between PTEN-deficient epithelial cells and the underlying stromal cells of the intestine is crucial for the initiation of tumorigenesis.¹⁰

Our current work demonstrates that, after a long latency, *Ah::CreER^T;Pten^{F/F}* mice develop intestinal tumors that are histologically consistent with juvenile polyps. The histological characteristics of these tumors interested us because they are remarkably similar to polyps seen in the CS patients, characterized by the presence of bizarre, tortuous crypt architecture. Juvenile polyps are known to arise as the result of defects in epithelial-stromal interactions, as previously demonstrated in a mouse model.²² Thus, our data suggest that defective mesenchymal-epithelial cross talk may govern juvenile polyp formation in both this model and in CS patients.

Herein, we find that stromal PTEN loss is not associated with juvenile polyp formation. This is in contrast to the situation in CS patients, however, in whom all cells in the body are heterozygous for *PTEN*. For CS patients, it remains unclear whether intestinal tumors are initiated by loss of the WT copy of *PTEN* in the stromal compartment or in the epithelial compartment, or whether loss of both copies of *PTEN* is required at all. By using the experimental system we present herein, we are unable to completely resolve this conundrum. Nonetheless, we are able to show that deletion of PTEN, when tightly restricted to the epithelial cell compartment, results in polyps that phenocopy all of the stromal changes (eg, the expanded mesenchymal compartment and inflammatory infiltrate) observed in CS polyps. Thus, epithelial PTEN loss provokes reactive changes in the surrounding PTEN-proficient stroma.

Finally, we have shown that juvenile polyps can progress to invasive epithelial neoplasia in our mouse model. This indicates that juvenile polyps in patients with CS are likely to be genuine preneoplastic lesions.

CS patients also develop benign nonepithelial colorectal polypoid lesions, such as ganglioneuromas. Previous studies have shown that PTEN deletion from the enteric nervous system elicits ganglioneuromatosis.²³ Although we note reactive stromal changes after epithelial PTEN deletion, we did not observe mesenchymal lesions, such as ganglioneuromas, even in aged mice. This suggests that, although we accurately model juvenile polyp formation and colon cancer formation in CS patients, the full spectrum of clinicopathological manifestations in CS requires either an epithelial or a stromal PTEN mutation, depending on the context.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.10.003>.

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