Fibroblast-Mediated Collagen Remodeling Within the Tumor Microenvironment Facilitates Progression of Thyroid Cancers Driven by \textit{Braf}^{V600E} and Pten Loss

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Abstract

Contributions of the tumor microenvironment (TME) to progression in thyroid cancer are largely unexplored and may illuminate a basis for understanding rarer aggressive cases of this disease. In this study, we investigated the relationship between the TME and thyroid cancer progression in a mouse model where thyroid-specific expression of oncocgenic Braf and loss of Pten (\textit{Braf}^{V600E}/\textit{Pten}^−/−/\textit{TPO-Cre}) leads to papillary thyroid cancers (PTC) that rapidly progress to poorly differentiated thyroid cancer (PDTC). We found that fibroblasts were recruited to the TME of \textit{Braf}^{V600E}/\textit{Pten}^−/−/\textit{TPO-Cre} thyroid tumors. Conditioned media from cell lines established from these tumors, but not tumors driven by mutant H-ras, induced fibroblast migration and proliferation \textit{in vitro}. Notably, the extracellular matrix of \textit{Braf}^{V600E}/\textit{Pten}^−/−/\textit{TPO-Cre} tumors was enriched with stromal-derived fibrillar collagen, compared with wild-type or Hras-driven tumors. Further, type I collagen enhanced the motility of \textit{Braf}^{V600E}/\textit{Pten}^−/−/\textit{TPO-Cre} tumor cells \textit{in vitro}. In clinical specimens, we found \textit{COL1A1} and \textit{LOX} to be upregulated in PTC and expressed at highest levels in PDTC and anaplastic thyroid cancer. Additionally, increased expression levels of \textit{COL1A1} and \textit{LOX} were associated with decreased survival in thyroid cancer patients. Overall, our results identified fibroblast recruitment and remodeling of the extracellular matrix as pivotal features of the TME in promoting thyroid cancer progression, illuminating candidate therapeutic targets and biomarkers in advanced forms of this malignancy. Cancer Res; 76(7); 1804–13. ©2016 AACR.

Introduction

Thyroid cancer is the most common endocrine malignancy and is predicted to be the fourth most commonly diagnosed cancer by 2030 (1). The \textit{BRAF}^{V600E} mutation is the most common genetic alteration in thyroid cancer, in particular papillary thyroid cancer (PTC), and is associated with more aggressive disease (2). Poorly differentiated (PDTC) and anaplastic thyroid cancers (ATC) often have mutations in \textit{BRAF} as well as mutations that result in constitutive PI3K signaling, and are often unresponsive to treatments for thyroid cancer, including radiation and chemotherapy (3–5). Additionally, the success of targeted inhibition strategies for advanced thyroid cancers has been limited (6, 7). The increasing incidences of thyroid cancer, coupled with our relative lack of understanding of drivers of disease progression, underscore the need for novel therapeutics as well as identification of biomarkers that are predictive of aggressive disease.

The tumor microenvironment (TME) is comprised of multiple cellular and non-cellular components, including extracellular matrix (ECM) proteins, that converge to promote tumorigenesis in a variety of solid malignancies (8, 9). Tumor cells induce the migration of non-malignant cells, such as fibroblasts, endothelial cells, and immune cells, to the TME through direct cell–cell contact and indirect mechanisms, which collectively support the development of a primary tumor niche (10). The influence of tumor-stromal cross-talk on tumor progression is recognized in many different types of cancer. However, the mechanisms by which tumor cells establish a permissive niche that promotes thyroid cancer progression remain largely undefined.

Cancer-associated fibroblasts (CAF) represent a heterogeneous population of fibroblasts that are recruited and activated to augment tumor progression in many different solid tumors (11–13). In addition to stimulating tumor cell proliferation, angiogenesis, invasion, and metastasis, CAFs also drive tumorigenesis by upregulating the production of ECM components, including type 1 collagen (Col 1) and ECM modifying enzymes (14–16). Col 1 is the most abundant ECM scaffolding protein and its increased deposition in the TME is associated with tumor progression (17–19), increased incidence of metastasis (20) and drug resistance (21) in human cancers. These observations are supported by \textit{in vivo} and \textit{in vitro} studies demonstrating that Col 1 promotes the migration, invasion, and metastasis of tumor cells (22–24).
A thorough understanding of the role of tumor derived signals in establishing an environment conducive to tumor development and the effects of stromal derived signals on tumor cell behavior in thyroid cancer is largely unexplored. To identify potential mechanisms of thyroid cancer progression in the context of the TME, a novel model of thyroid cancer progression (Braf<sup>V600E</sup>/Pten<sup>+/−</sup>/TPO-Cre) was created and the TME dissected to identify factors that influence thyroid tumorigenesis. Braf activation and Pten loss cooperate in PTC development that rapidly progresses to PDTC characterized by a fibrotic and reactive tumor stroma enriched with CAFs, fibrillar collagen deposits, and increased expression of lysyl oxidase (Lox), an ECM modifying enzyme that catalyzes collagen fiber cross-linking. We extended these findings to human disease and found that increased COL1and LOX expression is associated with more aggressive well-differentiated thyroid cancer subtypes, PDTCs, and a poorer overall survival rate. Based on these observations, we propose that a regulatory loop exists between thyroid tumor cells, CAFs, collagen, and Lox, which potentiates thyroid cancer progression. These components may serve as therapeutic targets for advanced thyroid cancers, and future studies will investigate therapeutic strategies targeting the TME and ECM in our in vivo models.

**Materials and Methods**

**Experimental animals**

All animal experiments were performed at the University of Arkansas for Medical Sciences and approved by the Institutional Animal Care and Use Committee. The LSL-Braf<sup>V600E</sup>, Pten<sup>+/−</sup>, and thyroid peroxidase promoter (TPO)-Cre strains have been previously described (25, 26). Mice were on mixed C57BL/6J/129SVJ genetic backgrounds. Genotypes were determined by PCR as previously described (25, 26).

**Histology and immunohistochemistry**

Thyroid tissues were fix ed in 10% formalin-buffered acetate and embedded in paraffin. Five-micrometer sections were prepared, and histological diagnosis was performed by a thyroid pathologist (N. Massoll). For further details, see Supplementary Materials and Methods.

**Cell lines**

The Braf, B297T, and B1180T cell lines were established from LSL-Braf<sup>V600E</sup>, Pten<sup>+/−</sup>/TPO-Cre thyroid tumors and H340T, and H245T cell lines were established from Hras<sup>LSL-C12V</sup>/Pten<sup>+/−</sup>/TPO-Cre thyroid tumors, detailed in Supplementary Materials and Methods. Cell lines were authenticated using short tandem repeat (STR) DNA profiling (DDC Medical). Independent murine mammary CAF lines (mCAF and 4F) were isolated from MMTV-PyVmT mice using the Verso cDNA synthesis kit (Thermo Scientific). Differential mRNA expression of type I collagen (Col1a1), lysyl oxidase (Lox) and 18s was measured using TaqMan Mastermix and pre-designed Taqman assays (Applied Biosystems). Four microliters (4 μL) of CAFRNA (Promega) were added to each well in a 384-well plate. Eight hours after incubation at 37°C, media from tumor lines were added to the bottom chamber of the Fluoroblok plate. Eight hours after incubation at 37°C, cells on the Fluoroblok inserts were stained with 2 μmol/L Calcein AM (Life Technologies). Fluorescent values were obtained at a wavelength of 485 ex/520 em on a Synergy H1 multimode reader (BioTek) to quantify migration. The optics position of the plate reader was set to read from the bottom of the plate in order to only image cells that had migrated through the transwell. Images were taken on the EVOS FL imaging system.

**RT-PCR analysis**

Total RNA was extracted using the RNeasy Plus Mini Kit (Qia-gen). Equal amounts of RNA template were reverse transcribed using the Verso cDNA synthesis kit (Thermo Scientific). Differential mRNA expression of type I collagen (Col1a1), lysyl oxidase (Lox) and 18s was measured using TaqMan Mastermix and pre-designed Taqman assays (Applied Biosystems). Four microliters of cDNA from tumor samples and independent passages of each cell line were run in triplicate on a Bio-Rad CFX96. Q-Gene software (28) was used to determine relative normalized expression to 18s. Data analysis was based on the C<sub>−</sub>-method.

**Migration assays**

Migration assays were performed in 24-well plates with Fluoroblok inserts (Falcon). Forty thousand mCAF or 4F fibroblasts were seeded on each Fluoroblok insert in 0.5%FBS/F12. Conditioned media from tumor lines were added to the bottom chamber of the Fluoroblok plate. Eight hours after incubation at 37°C, cells on the Fluoroblok inserts were stained with 2 μmol/L Calcein AM (Life Technologies). Fluorescent values were obtained at a wavelength of 485 ex/520 em on a Synergy H1 multimode reader (BioTek) to quantify migration. The optics position of the plate reader was set to read from the bottom of the plate in order to only image cells that had migrated through the transwell. Images were taken on the EVOS FL imaging system.

**Proliferation assays**

Proliferation assays were performed in 96-well plates. One thousand fibroblasts were seeded in quadruplicate per treatment condition in complete media for attachment. Following overnight attachment, the media were replaced with medium containing 0.5% FBS, 10% FBS, or conditioned media from tumor cell lines. Proliferation was assessed using the CellTiter-Glo luminescent cell viability assay (Promega). One well per treatment was incubated with a 1 μmol/L Calcein AM for 30 minutes at 37°C and imaged using an EVOS FL imaging system.

**Live-cell microscopy**

Tumor cells were plated in 35-mm cell culture dishes either tissue culture treated or coated with 100 μg/mL rat tail collagen I (Sigma). Following attachment in complete medium, cells were serum starved overnight, then stimulated with 10% FBS and immediately imaged under phase contrast on an Axiovert 200 M microscope fitted with a Zeiss AxioCam ICM1 camera and maintained at 37°C and 5% CO$_2$ using a Live Cell Pathology incubator. Images were collected every minute for 4 hours. The images were analyzed using NIH ImageJ software (Version 1.50e) with the MTrack Plugin to determine distance travelled by cells. The center of each cell nucleus was used as the point of tracking, and cells undergoing mitosis were excluded from analysis. Track length was measured for 10 individual cells per treatment and repeated at least thrice.

**Human thyroid cancer database analysis**

The Oncomine platform (www.oncomine.org; ref. 29) was used to compare the expression levels of COL1A1 and LOX mRNA between thyroid cancer subtypes (GSE27155; ref. 30), which included 4 normal thyroid samples, 15 follicular variant papillary thyroid cancer samples (FVPTC), 10 tall cell PTCs, 10 follicular adenomas, 13 follicular thyroid cancers (FTC), 2 medullary thyroid cancer samples, 7 oncocytic adenomas, 8 oncocyctic FTCs, 26 PTCs, and 4 undifferentiated/anaplastic thyroid cancer samples. The log, median-centered intensity values for COL1A1 and LOX were extracted for the analysis using all samples. eBioPortal, the web-based open platform for analyzing multidimensional cancer genomics data (31, 32), was used to obtain summary statistics on co-occurrence of genomic alterations in Braf, Nras, Hras, Kras, Col1a1, and Lox in thyroid carcinomas in 397 thyroid cancer cases. Odds ratios to indicate the likelihood of mutual exclusivity or co-occurrence of each pair of genes were calculated. P values were determined by the Fisher exact test.
BrafV600E and PI3K signaling cooperate in the development of PTCs that rapidly progress to PDTC in vivo

MAPK signaling plays a critical role in thyroid cancer initiation, as evidenced by our previous studies demonstrating that endogenous expression of BrafV600E is sufficient to induce murine PTCs that recapitulate human disease (25). Braf mutations are associated with more aggressive PTC and are often found in conjunction with mutations that result in constitutive PI3K/AKT signaling, including PIK3CA and PTEN mutations, in PTCs (33). This led to the hypothesis that simultaneous MAPK activation via BrafV600E and PI3K activation via Pten loss would cooperate in thyroid cancer initiation and progression to advanced disease. To determine whether BrafV600E and PI3K signaling could cooperate in thyroid cancer progression in vivo, LSL-BrafV600E/Ptenfl/fl mice were crossed with Ptenfl/fl/TPO-Cre mice to generate mice in which BrafV600E is conditionally activated and Pten is homozygously inactivated through thyroid-specific Cre recombinase activation (BrafV600E/Ptenfl/fl/TPO-Cre). BrafV600E/Ptenfl/fl/TPO-Cre mice developed PTCs that rapidly progressed to PDTCs with 100% penetrance and lethality by weaning (Fig. 1A). In stark contrast to wild-type (WT) thyroid glands with normal follicular architecture (Fig. 1B), BrafV600E/Ptenfl/fl/TPO-Cre tumors encompass the entire thyroid gland and display many of the classical hallmarks of high-grade human PTC, including formation of papillae, fine chromatin, and nuclear grooves (Fig. 1 C–E), as well as features of PDTC, including central necrosis (Fig. 1 F and G) and invasion into surrounding tissue (Fig. 1H). The very early lethality of BrafV600E/Ptenfl/fl/TPO-Cre mice precludes long-term studies to determine factors involved in disease progression. However, the rapid tumor development and pathological features of PDTC that are recapitulated in BrafV600E/Ptenfl/fl/TPO-Cre mice provide a model by which to investigate factors within the TME that may contribute to disease progression.

The TME of BrafV600E/Ptenfl/fl/TPO-Cre tumors is enriched with tumor-associated fibroblasts

The TME is composed of many different cell types that influence tumor progression, including CAFs. CAFs promote...
tumorigenesis in human cancers and in vivo model systems (11). Interestingly, fibroblast growth factors (FGF) and their receptors (FGFR) are overexpressed in thyroid cancer (34, 35) and correlate with thyroid cancer progression (36). Hematoxylin and eosin (H&E) staining of tumor sections revealed areas of fibrosis along the tumor periphery and cells with fibroblast morphology (Fig. 2A and B, top, arrows). In contrast, no areas of fibrosis were observed in WT thyroid tissue. These cells were confirmed as fibroblasts via immunostaining with α-SMA. In contrast to WT thyroid, in which no α-SMA staining was observed, BrafV600E/Pten−/−/TPO-Cre tumors displayed robust peripheral and intratumoral α-SMA staining, indicating fibroblast recruitment and infiltration (Fig. 2A and B, bottom).

Tumor cells isolated from BrafV600E/Pten−/−/TPO-Cre mice stimulate fibroblast proliferation and migration in vitro

Given the increased fibroblast infiltrate observed in BrafV600E/Pten−/−/TPO-Cre tumors, we asked whether Braf-driven thyroid tumor cells could stimulate the proliferation and/or migration of fibroblasts. We generated multiple stable tumor cell lines from BrafV600E/Pten−/−/TPO-Cre mice (Braf-1, B1180T, and B297T) and tested the ability of these cell lines to drive the proliferation and migration of two independent CAF lines in vitro (27). Conditioned media collected from Braf and B297T cells significantly increased mCAF and 4F fibroblast proliferation in comparison with serum-free controls after 48 hours of incubation (Fig. 3A and Supplementary Fig. S1A). To determine whether increased proliferation was specific to factors secreted by Braf-driven thyroid tumor cells, the experiments were repeated with the Hras-driven thyroid tumor cell line H340T. Conditioned medium isolated from H340T cells had no effect on the proliferation of mCAF or 4F after 48 hours compared with the serum-free control (Fig. 3A and Supplementary Fig. S1A). Additionally, conditioned media from Braf, B1180T, and B297T cells significantly increased the migration of mCAF and 4F fibroblasts in Transwell assays compared with serum-free controls (Fig. 3B and C; Supplementary Fig. S1B and S1C), demonstrating that tumor cells from BrafV600E/Pten−/−/TPO-Cre tumors secrete factors that induce fibroblast migration and likely drives fibroblast recruitment to BrafV600E/Pten−/−/TPO-Cre tumors in vivo. Consistent with the proliferation studies, conditioned media from HrasG12V-driven murine thyroid tumor cell lines did not stimulate mCAF or 4F migration compared with serum-free controls (Fig. 3B and C; Supplementary Fig. S1B and S1C). Together, these results suggest that Braf, but not Hras, activation results in secretion of tumor-derived factors that induce the proliferation and recruitment of fibroblasts in murine thyroid cancer. TGFβ is a key mediator of fibroblast activation during wound healing and exerts promitogenic and chemotactic effects on fibroblasts (reviewed in 12). To determine whether the induction of fibroblast migration and proliferation in response to conditioned medium from Braf-driven tumor cells is TGFβ dependent, proliferation and migration experiments were repeated with TGFβRII knockout fibroblasts (27). Treatment with conditioned medium from Braf, B1180T, B297T, and H340T inhibited the proliferation of TGFβRII knockout fibroblasts compared with 0.5% FBS (Supplementary Fig. S2A). No migration through transwells was observed in any treatment group, even after 24 hours of exposure to 10% FBS (Supplementary Fig. S2B), indicating that intact TGFβRII signaling is required for the migration of fibroblasts. To determine whether Braf-driven tumor cells...
could induce activation of TGFβ signaling in fibroblasts, 4F fibroblasts were treated with conditioned medium from Braf, B1180T, and B297T cells and Western blot analysis for phosphorylated SMAD 2 or 3 performed. Treatment with conditioned medium from Braf-driven thyroid tumor cell lines did not induce the phosphorylation of SMAD 2 or 3 in fibroblasts (Supplementary Fig. S2C). Collectively, these data suggest that while TGFβ signaling is permissive for the induction of fibroblast migration in response to Braf-driven tumor cell–derived signals, alternative pathways are likely being activated by tumor-derived factors to induce fibroblast proliferation and migration.

**Increased total and fibrillar collagen deposition and Lox expression in BrafV600E/Pten-/-/TPO-Cre tumors**

Collagens, in particular collagen 1 (Col 1), are primarily derived from fibroblasts and augment tumor cell invasion and migration in vivo and in vitro (13–20). We hypothesized that the recruitment of fibroblasts to BrafV600E/Pten-/-/TPO-Cre tumors would result in increased collagen deposition in the thyroid TME. Col1a1, which encodes the α1 chain of Col 1, expression levels were consistently upregulated in BrafV600E/Pten-/-/TPO-Cre tumors compared with WT thyroid (Fig. 4A). By contrast, Col1a1 expression in tumor cell lines derived from BrafV600E/Pten-/-/TPO-Cre tumors (B1180T and B297T) was no different than WT controls (Fig. 4B), suggesting that Col1a1 expression in BrafV600E/Pten-/-/TPO-Cre tumors is not derived by tumor cells. Col1a1 expression was significantly upregulated in the parent tumors (B1180 and B297) from which these cell lines were derived (Fig. 4B), suggesting that the increased Col1a1 expression in whole BrafV600E/Pten-/-/TPO-Cre tumors occurs primarily in stromal cells, likely fibroblasts, rather than tumor cells. Immunostaining revealed increased Col 1 in BrafV600E/Pten-/-/TPO-Cre tumors compared with WT thyroid (Fig. 4D). Additionally, Col 1 was undetectable in ECM derived from BrafV600E-driven tumor cells in vitro (data not shown). To test the hypothesis that fibroblasts are the predominant source of Col 1 within the TME of BrafV600E/Pten-/-/TPO-Cre tumors, tumor sections were immunostained with Col 1 and αSMA to determine if they colocalized. Both Col 1 and αSMA staining localized to the tumor-stromal interface (Fig. 4D), supporting the hypothesis that fibroblasts are the predominant source of Col 1 within the TME of BrafV600E/Pten-/-/TPO-Cre tumors.

The biomechanical properties and deposition of ECM proteins are altered during tumorigenesis. Further, the activity of tumor and stromal derived matrix metalloproteinases (MMP) and collagen-cross-linking enzymes, which modulate the structural stability of ECM proteins, is increased in different cancers (37). Lysoxydase (Lox) is an ECM-modifying enzyme that catalyzes the cross-linking of collagen fibers, resulting in increased collagen fiber stability and ECM stiffness, which can enhance the invasive capacity of tumor cells in vivo (38). Upregulation of Lox is observed in a variety of solid tumors (39–41) and correlates with reduced metastasis-free survival in breast and head and neck cancers (39). Lox has recently been found to be upregulated in thyroid cancer and potentiates metastasis and invasion of anaplastic thyroid cancer cell lines in vivo (40). Lox expression was significantly upregulated in BrafV600E/Pten-/-/TPO-Cre tumors compared with WT thyroid controls (Fig. 4C). Lox expression was also increased in B1180T and B297T cell lines in comparison with WT controls (Supplementary Fig. S3). Picrosirius red staining of tumor sections revealed increased polarized intensity in BrafV600E/Pten-/-/TPO-Cre tumors, demonstrating higher content of mature and cross-linked collagen fibers (Fig. 4E). In WT thyroid, only tracheal cartilage contained collagen. These results indicate that BrafV600E/Pten-/-/TPO-Cre tumors promote increased collagen synthesis and cross-linking through upregulation of Col1a1 and Lox, resulting in increased collagen deposition and stability in the TME of BrafV600E/Pten-/-/TPO-Cre tumors. To determine whether Col 1 modulates tumor cell phenotype, cell motility was measured on tissue culture plates and Col 1 coated TPO-Cre. Live-cell microscopy and tracking analysis demonstrated that BrafV600E/Pten-/-/TPO-Cre tumor cell lines Braf and B297T displayed significantly increased motility when plated on Col 1 versus tissue culture plastic (Fig. 4F and G; Supplementary S4). No increase in motility was observed when BrafV600E/Pten-/-/TPO-Cre tumor cell line B1180T was plated on Col 1. However, B1180T cells plated on Col 1 exhibited an increase in the mitotic index; therefore, less total cells were included in the final analysis. Further, Col 1 had no effect on the motility HrasG12V/Pten-/-/TPO-Cre tumor cell lines.
H340T and Hras1, suggesting that the increased motility response to Col 1 is specific to Braf and not Hras-driven thyroid tumor cells.

**COL 1 and LOX** are upregulated in human PTC and are associated with aggressive histologic variants of PTC and PDTC

To determine whether these murine models recapitulated human disease and reflected changes observed in patients, the Oncomine database (29) was used to investigate COL1A1 and LOX expression in thyroid tumors from the Giordano cohort (30). COL1A1 and LOX expression were increased in PTC (Fig. 5A) compared with normal thyroid, FTC, and FVPTC, which is associated almost exclusively with RAS mutations and displays many pathologic features similar to FTC (2). COL1A1 and LOX expression levels were further increased in tall-cell variant PTC, a more aggressive form of PTC, and highest in undifferentiated thyroid cancers (Fig. 5A). The CibioPortal was used to analyze thyroid cancer data in The Cancer Genome Atlas (TCGA) dataset in order to correlate COL1A1 and LOX upregulation with mutational status. COL1A1 and LOX upregulation occurred in 8% and 10%, respectively, of all thyroid tumors analyzed (397 cases), and occur exclusively in thyroid tumors harboring BRAF, but not RAS, mutations (Fig. 5B). Strong tendencies in the rate of cooccurrence between BRAF mutations and COL1A1 upregulation, BRAF mutations and LOX upregulation, and COL1A1 and LOX upregulation in thyroid cancers were found (Table 1). Together, these results suggest that COL1A1 and LOX cooperate in thyroid cancer progression and that upregulation of COL1A1 and LOX occurs in response to BRAF, but not RAS, activation in thyroid cancer.

Upregulation of COL1A1 and LOX is associated with decreased overall survival in thyroid cancer patients

Mutations in BRAF correlate with decreased overall survival in thyroid cancer patients (41). Given that upregulation of COL1A1 and LOX is
and LOX occurs predominantly in thyroid tumors harboring BRAF mutations, we sought to determine whether COL1A1 and LOX overexpression was associated with reduction of overall survival in thyroid cancer. Co-upregulation of COL1A1 and LOX in thyroid cancer (N = 41 cases) results in a significant decrease in overall survival in thyroid cancer patients (Fig. 5D) compared with patients with tumors without COL1A1 and LOX upregulation. These results suggest that overexpression of COL1A1 and LOX contributes to disease progression in thyroid cancer and may contribute to thyroid cancer–related mortality.

Discussion

Each of the components that make up the TME, including tumor cells, non-malignant infiltrating stromal cells, and ECM proteins, work in concert to establish a permissive niche that is essential for tumorigenesis (42). While many studies have addressed the involvement of a singular cell type, such as fibroblasts or immune cells, or ECM component in tumor development, few studies have investigated the cross-talk between multiple components within the TME and how these complex relationships function together to promote tumor development. In this study, we dissected the cellular and non-cellular components within the TME of thyroid cancer in order to understand how interactions between these components contribute to thyroid cancer progression.

The BRAFV600E mutation is associated with a more aggressive tumor phenotype in thyroid cancer patients and has recently been implicated in the modulation of the TME through the regulation of ECM components (43). Genes associated with ECM remodeling, including integrins, TGFβ1, and fibronectin, are upregulated

![Table 1. BRAF mutations are associated with LOX and COL1A1 upregulation.](image)
in PTCs with BRAFV600E mutations when compared with PTCs without the mutation (44), suggesting activation of BRAF is critical for the development of a fibrotic tumor stroma. In agreement with these findings, our data demonstrate that activation of Braf and PI3K signaling in thyrocytes results in the development of a fibrotic and reactive tumor stroma in BravV600E/Pten−/− /TPO-Cre tumors, characterized by increased fibroblast recruitment and stromal deposition of Col 1 (Fig. 6). In this model, we propose that fibroblasts are recruited to the thyroid TME by BravV600E/Pten−/− /TPO-Cre tumor cells, which activate fibroblasts to produce and deposit Col 1. In turn, tumor cells cross-link the fibroblast derived Col 1 fibers in the TME via upregulation of Lox, resulting in a stiffer Col 1 matrix that augments tumor cell motility and promotes tumor progression.

BrafV600E/Pten−/− /TPO-Cre tumors contained higher levels of total and fibrillar collagen and increased expression of Lox. Col 1 augmented the motility of BravV600E/Ptn−/− /TPO-Cre tumor cells in vitro. Interestingly, no fibroblast recruitment or collagen deposition was observed in the TME in response to Hras activation in HrasG12V/Pten−/− /TPO-Cre mice, a closely related murine model of thyroid cancer in which mice develop follicular carcinomas that progress to PDTC (A. Franco; manuscript in preparation). In addition, Col 1 had no effect on the motility of Hras-driven tumor cell lines in vitro. These data indicate that in the context of Pten loss, activation of Braf, but not Hras, results in a fibroblastic response in the TME of thyroid cancer that promotes tumor progression and potentially invasion. Further supporting fibroblast recruitment to the thyroid TME is BrafV600E specific, only conditioned media from BrafV600E/Pten−/− /TPO-Cre cells was able to induce proliferation and migration of fibroblasts in vitro. These results suggest that activation of Braf, but not Hras, induces secretion of factors that promote fibroblast migration and likely leads to the increased fibroblast recruitment observed in vivo. Interestingly, RAS activation is associated with increased inflammation and tumor immune cell infiltration in murine models of lung and pancreatic adenocarcinoma (45, 46), and mutant BRAFV600E induces fibroblast activation in melanoma cell lines (47). Future studies are needed to unravel the molecular mechanisms by which the activation of closely related MAPK effectors, like RAS and RAF, lead to the development of distinct TMEs through the differential recruitment of various cell types or ECM remodeling.

The biomechanical properties of a tumor-associated matrix can have a strong influence on cellular behavior (48). Lox is a known driver of ECM stiffness within the TME due to its ability to cross-link collagen fibers, and inhibition of Lox attenuates metastasis in mouse models of breast cancer and, more recently, thyroid cancer (39, 40, 49). Increased matrix stiffness also induces the activation of integrin signaling and downstream ERK activation and promotes the stabilization of focal adhesion complexes that can drive malignancy (50). BravV600E/Pten−/− /TPO-Cre tumors contained abundant total and fibrillar collagen. Future studies will investigate whether inhibition of Lox decreases matrix stiffness and can attenuate thyroid cancer progression in BravV600E/Pten−/− /TPO-Cre tumors.

Advanced forms of thyroid cancer are associated with mutations in the MAPK pathway and additional mutations that result in constitutive PI3K activation (33). These data demonstrate that activation of BravV600E and PI3K leads to the development of PTCs that rapidly progress to PDTCs. These tumors are associated with a fibrotic TME characterized by increased stromal collagen deposition and Lox upregulation. These murine models faithfully recapitulate patient tumors by which increased COL1A1 and Lox expression is associated with PTC compared with FTC and normal thyroid, and that COL1A1 and Lox are expressed at highest levels in PDTC and ATC. Together, these data support the critical role of these ECM components in
promoting thyroid cancer progression. COL1A1 and LOX expression in human PTCs is strongly correlated with BRAF, but not RAS mutations. RAS mutations are very common in FTC and FVPTC, while BRAF mutations are associated with classical PTC, suggesting that COL1A1 and LOX upregulation in thyroid cancer occurs in response to BRAF activation and may drive PTC versus FTC development. Finally, COL1A1 and LOX upregulation is associated with decreased overall survival in thyroid cancer, implicating COL1A1 and LOX as mediators of cancer progression and may serve as a prognostic indicator of disease status in addition to the BRAFV600E mutation in thyroid cancer.

While it is now widely accepted that the TME is essential for tumorigenesis, most studies only address the contribution of singular TME component to cancer progression. Considering that the TME is composed of multiple components (both cellular and non-cellular), studies that aim to investigate how these components work together to establish a niche permissive for tumorigenesis are needed to fully understand the mechanisms of tumor development and therapeutic resistance. This study is the first to identify and describe the interaction between tumor cells, fibroblasts, collagen, and Lox in the TME of thyroid tumors, providing a model by which this dynamic interaction may drive thyroid tumor progression (Fig. 6). We hope that these results will lead to the development of more effective therapeutic strategies for thyroid cancer that account for the complexity of the TME in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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