Cathepsin B Is the Driving Force of Esophageal Cell Invasion in a Fibroblast-Dependent Manner

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Abstract
Esophageal cancer, which frequently exhibits coordinated loss of E-cadherin (Ecad) and transforming growth factor β (TGFβ) receptor type II (TβRII), has a high mortality rate. In a three-dimensional organotypic culture model system, esophageal keratinocytes expressing dominant-negative mutant versions of both Ecad and TβRII (ECDnT) invade into the underlying matrix embedded with fibroblasts. We also find that cathepsin B induction is necessary for fibroblast-mediated invasion. Furthermore, the ECDnT cells in this physiological context activate fibroblasts through the secretion of TGFβ1, which, in turn, is activated by cathepsin B. These results suggest that the interplay between the epithelial compartment and the surrounding microenvironment is crucial to invasion into the extracellular matrix.

Introduction
Esophageal cancer carries one of the worst mortality rates of any cancer. The prognosis for patients with esophageal squamous cell cancer remains poor because of the high rate of local and distant metastases at the time of diagnosis [1]. Critical oncogenes involved in the pathogenesis of esophageal squamous cell cancer include cyclin D1 and epidermal growth factor receptor (EGFR), and key tumor suppressor genes are TP53, E-cadherin, and TβRII. We previously developed and characterized an organotypic culture model focusing on p53 mutation in concert with EGFR overexpression, thus leading to the activation of stromal fibroblasts and matrix metalloprotease–mediated degradation of the extracellular matrix [2]. To evaluate other critical genetic events that are evident in esophageal tumorigenesis, we focus here on the coordinated loss of E-cadherin (Ecad) and TGFβ receptor type II (TβRII) and elucidate their functional consequences.

Loss of Ecad expression is prominent in epithelial-mesenchymal transition; therefore, Ecad has emerged as one of the “caretakers” of the epithelial phenotype [3]. The precise mechanism that underlies suppression of cell invasion as mediated by Ecad is not well established. One possibility is that the adhesive function of Ecad prevents cells from dissociating from each other and migrating into adjacent tissues [4,5]. Alternatively, Ecad binds and sequesters β-catenin at the cell membrane, which regulates the cytoplasmic pool of β-catenin and, therefore, represents a key mediator in the Wnt signaling pathway [6]. This function of Ecad is supported by studies in Drosophila and Xenopus embryos [7–9], which demonstrate that the formation of the cadherin-catenin complex antagonizes β-catenin signaling function, for example, during axis formation.

TβRII loss in cancer has been modeled in mice through a dominant-negative approach in skin [10] and mammary glands [11] as well as conditional knock-out in the mammary glands [12]. In these models, mice develop tumors and have a high potential for metastasis, thereby supporting the tumor-suppressive function of TβRII and intact

Abbreviations: TGFβ, transforming growth factor β; Ecad, E-cadherin; EC, dominant-negative E-cadherin; TβRII, TGFβ receptor type II; dnT, dominant-negative TGFβ receptor type II

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transforming growth factor β (TGFβ) signaling. This is supported further by the restoration of wild-type TβRII in colon and breast cancer cells that lack a functional TGFBR2 allele [13] and by the overexpression in thyroid carcinomas [14], in which the expression of TβRII conferred growth inhibition, suppressed anchorage independence, and abolished tumor formation in nude mice.

In a previous study, we demonstrated the importance of Ecad and TβRII in esophageal squamous cell cancer because 70% of patient tumors had coordinated loss of both proteins [15]. To identify the biological consequences, we have established an organotypic culture model mimicking the loss of Ecad and TβRII function through the expression of dominant-negative mutants of both proteins lacking the cytoplasmic tail in each case. We demonstrate here that esophageal cell invasion is initiated by the epithelial loss of Ecad and TβRII and is mediated further through cathepsin B induction resulting in increased levels of TGFβ1.

Materials and Methods

Cell Culture

Primary esophageal epithelial cells (keratinocytes) from normal human esophagus were established as described previously [15]. Cells were cultured at 37°C and 5% CO₂ in keratinocyte serum-free medium (kSFM) supplemented with 40 μg/ml bovine pituitary extract, 1.0 ng/ml epidermal growth factor, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA). Fetal esophageal fibroblasts were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS (Hyclone; Thermo Fisher Scientific, Waltham, MA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Invitrogen). Conditioned media were collected from both esophageal keratinocytes in kSFM and DMEM and fibroblasts in DMEM with 10% FBS after 72 hours. pGIPZ-shRNAmir constructs against cathepsin B were from Openbiosystems (Huntsville, AL; available through the Vanderbilt Microarray Shared Resource): shRNA#1 corresponds to clone V2LHS151050 with the following sequence: TGCTTGTACAGTGAGCGGAGGGT-GACAATGGCTTCTTAAAAATA GTGAGGCGCACAGTGTATTTAAGAGCACCTTGCACCAGCCTCGGA, shRNA#2 is clone V2LHS151051. The sequence is TGCTTGTACAGTGAGCGGAGGGT-GACAATGGCTTCTTAAAAATA GTGAGGCGCACAGTGTATTTAAGAGCACCTTGCACCAGCCTCGGA, shRNA#2 is clone V2LHS151051. The sequence is TGCTTGTACAGTGAGCGGAGGGT-GACAATGGCTTCTTAAAAATA GTGAGGCGCACAGTGTATTTAAGAGCACCTTGCACCAGCCTCGGA, shRNA#2 is clone V2LHS151051. The sequence is TGCTTGTACAGTGAGCGGAGGGT-GACAATGGCTTCTTAAAAATA GTGAGGCGCACAGTGTATTTAAGAGCACCTTGCACCAGCCTCGGA. As a control, the sequence-scrambled, nonsilencing pGIPZ vector provided by Openbiosystems was expressed.

Tissue Microarrays

The AccuMax Array (ISU ABXIS, Co, Seoul, South Korea) contains 80 esophageal squamous cancer tissues and four normal controls and is distributed by Accurate Chemical & Scientific Corp (Westbury, NY). Immunofluorescence staining was performed using anti–cathepsin B antibody and was scored on a scale from 1 to 4, with 1 being absent and 4 being the highest signal intensity. These scores were compared with scores from a previous study [15] on a serial section of the same tissue microarray analyzing the expression of Ecad and TβRII. Scores were plotted, and linear regression was calculated using Prism4 (GraphPad Software, Inc, La Jolla, CA). For the statistical analysis of the negative correlation, we used Spearman correlation.

Organotypic Culture

Organotypic cultures were grown as described previously [16]. In brief, human esophageal epithelial cells (keratinocytes) were seeded on day 7 onto a 3:1 collagen I/Matrigel layer with 7.5 × 10⁴ human fetal esophageal fibroblasts embedded, after the matrix had contracted under the influence of the fibroblasts. Collagen I was purchased from Organogenesis (Canton, MA), and Matrigel Matrix was purchased from BD Biosciences (Franklin Lakes, NJ). On day 11, cultures were raised to an air-liquid interface to induce differentiation of the epithelium. Cultures were harvested on day 15, fixed in 10% formaldehyde (Fisher, Pittsburgh, PA), and paraffin-embedded or directly embedded into OCT for frozen sections. Conditioned media were collected at the time of harvesting 72 hours after the last medium change and were either used immediately or snap-frozen and stored at −80°C. For analysis of the invading areas, ImageJ (NIH, Bethesda, MD) was used to select areas of invasion and to measure the surface area for graphic presentation.

Coculture and Transwell Assays

For the coculture, the matrix was prepared as for organotypic cultures with and without fibroblasts and plated into four-well chamber slides (Falcon, New Brunswick, NJ). The next day, the dominant-negative mutant versions of both Ecad and TβRII (EcdnT) cells were plated onto the matrices. In transwell experiments, the bottom chamber contained fibroblasts, fibroblast-conditioned medium, and DMEM as control. The transwell insert (pore size, 0.4 μm) was coated with matrix components as previously mentioned, and EcdnT cells were plated. All of the cocultures were incubated overnight. After the methanol/acetic acid fixation, immunofluorescence staining was performed with anti–cathepsin B, and nuclear counterstain using propidium iodide was performed.

Soft Agar Assay

Soft agar assays were performed as described by Hatziapostolou et al. [17]. In brief, a 1.5-ml lower layer of 1% agar in 1× DMEM/kSFM was placed into each well of a six-well culture plate and was allowed to solidify at room temperature. EcdnT cells, fibroblasts, or cocultures of both (2.5 × 10⁴ cells total/well) were suspended in a plating layer of 0.67% agarose in 1 ml of conditioned medium from EcdnT cultures, organotypic cultures, fibroblast cultures, or control kSFM. The agar was allowed to solidify before incubating the plates at 37°C in a 5% CO₂ humidified incubator for 2 weeks. The cells were fed every 48 hours using the appropriate conditioned medium. Colonies were counted and visualized using the Oxford Optronix Gel Count with the Gel Count Software, version 1.3 (Oxford, UK), and statistical significance was determined using the Student’s t test.

Cell Invasion Assays

Invasion assays were as previously described [15] done using either 8-μm pore Biocoat Matrigel invasion chambers or Fluoroblok invasion chambers (BD Biosciences). Inserts were placed in a 24-well plate containing kSFM, including all supplements or DMEM with 10% FBS as a control for the conditioned medium in the lower chamber. Conditioned media were collected from fibroblasts growing in DMEM 10% FBS for 72 hours. Cells (5 × 10⁴ per chamber) were resuspended in starvation medium and added into each insert. After overnight incubation, the cells attached to the upper side of the membrane were removed gently, if necessary, and rinsed. Invading cells were stained with hematoxylin and eosin for quantification or with calcein AM (Molecular Probes, Invitrogen) as a fluorescent dye to count invading cells using a fluorometer (Multimode Plate Reader Synergy HT; Bio-Tek, Winooski, VT). All experiments were
done at least in triplicate, and data are presented as mean ± SD. Student’s t test was performed for statistical analysis.

**Immunohistochemistry/Immunofluorescence**

Immunohistochemistry was performed with the Vecta Elite Kit (Vector Laboratories, Burlingame, CA) following the manufacturer’s protocol. Briefly, paraffin sections were treated with xylene and then boiled for 10 minutes in 10 mM citric acid buffer. Endogenous peroxidases were quenched using hydrogen peroxide before the sections were blocked in avidin D–blocking reagent and biotin–blocking reagent. The sections were incubated with primary antibody overnight at 4°C and secondary antibody for 30 minutes at 37°C, and then the signal was developed using the DAB substrate kit for peroxidase (Vector Laboratories). For immunofluorescence staining, a biotinylated secondary antibody was detected using Texas Red—streptavidin (Vector Laboratories) or fluorescein isothiocyanate–labeled secondary antibody; stained sections were mounted with 4′,6-diamidino-2-phenylindole containing mounting medium (Vector Laboratories).

**Western Blot Analysis**

As described previously [15], subconfluent cells were harvested in lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, protease inhibitor mixture tablet [Roche Molecular Biochemicals, Indianapolis, IN]). Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The solution β-actin (1:5000 dilution) were from Sigma (St Louis, MO), and cathepsin B activity assays were from Cell Signaling Technology (Danvers, MA). The membranes were blocked in 5% nonfat milk (Bio-Rad) with the Bio-Rad protein assay (Bio-Rad, Hercules, CA), Cathepsin B activity assays were from Cell Signaling Technology (Danvers, MA). The membranes were blocked in 5% nonfat milk (Bio-Rad) with the Bio-Rad protein assay (Bio-Rad, Hercules, CA), Cathepsin B activity assays were from Cell Signaling Technology (Danvers, MA).

**Results**

**Coordinated Loss of Ecad and TβRII Results in Increased Invasion**

We have shown previously that 70% of esophageal squamous cell cancer tissues harbor concurrent loss of Ecad and TβRII [15]. This led us to hypothesize that abrogation of Ecad-mediated cell adhesion in concert with altered TGFβ signaling results in cell invasion during esophageal tumorigenesis. To test this hypothesis and its biological consequences, we engineered human primary esophageal keratinocytes to express dominant-negative Ecad, EC [15], and dominant-negative mutant TβRII lacking the cytoplasmic tail (ECdnT) and compared them with cells expressing full-length wild-type Ecad or dominant-negative TβRII (dnT) alone. The dominant-negative mutant version of TβRII does not contain the kinase domain [18] and has been shown to induce increased epithelial branching [19] and mammary gland hyperplasia [20,21]. Furthermore, dominant-negative TβRII alters cellular homeostasis [22] and fosters prostate cancer in a mouse model [23]. We analyzed ECdnT cells for changes in proliferation as well as for their ability to respond to exogenous TGFβ1. Whereas Ecad cells exhibited a slower proliferation rate compared with EC and ECdnT cells, which was diminished further by TGFβ1 stimulation, EC and ECdnT cells did not respond to TGFβ1 (data not shown).

When grown in organotypic culture, in which fibroblasts embedded in Matrigel/collagen form an extracellular matrix with epithelial cells seeded on top, Ecad cells developed a normal epithelium. By contrast, a lack of functional Ecad or TβRII resulted in small areas of invasion by the epithelial cells into the underlying matrix. Dual loss of Ecad and TβRII in these primary esophageal cells led to an increased invasive potential (Figure 1).

**Cathepsin B Up-regulation in ECdnT Cells Mediates Epithelial Cell Invasion**

Cathepsin B amplification has been associated with esophageal adenocarcinoma and Barrett esophagus [24,25]. Immunofluorescence staining of an organotypic section with anti–cathepsin B antibody revealed a more intense fluorescence signal for cathepsin B in organotypic cultures of invasive ECdnT cells than control Ecad and EC cells.
To evaluate the correlation between the induction of cathepsin B and the coordinated loss of Ecad and TβRII, we analyzed serial sections of a tissue microarray with normal \((n = 4)\) and squamous cell carcinoma tissues \((n = 80\) cores from 40 patients). Expression levels of cathepsin B were scored on a scale from 1 to 4, with 1 being absent and 4 having the highest signal intensity (Figure 2B) and compared with previous scoring for Ecad and TβRII expression [15]. Cathepsin B was upregulated in 50 tumor tissues \((50/80, 63\%)\) that had loss of both Ecad and TβRII. In 8 tissue cores, the signal for Ecad and TβRII was strong, but cathepsin B expression was lost, whereas the expression pattern of 22 cores demonstrated no correlation. Overall, 58 tissues of 80 or 29 of 40 patients \((72\%)\) showed a statistically significant inverse correlation \((P = .0013, \rho = -0.34)\). Representative images of immunofluorescence staining highlighting the inverse correlation for Ecad and cathepsin B are shown in Figure 2B.

**Figure 1.** ECdnT cells invade into the underlying matrix in organotypic cultures in the presence of fibroblasts. Expression of dominant-negative mutant versions of Ecad and TβRII resulted in cell invasion into the fibroblast-embedded Matrigel/collagen matrix (C, arrows). By contrast, Ecad-overexpressing cells show normal epithelium formation (A), whereas expression of mutant Ecad (B; EC) or mutant TβRII alone (D; dnT) resulted in limited invasive potential (arrows). Hematoxylin and eosin staining of a representative section. Using ImageJ, invading areas were selected, and surface area was measured and compared. Graphic representation of the measured sum of invasive areas demonstrated a significant increase in ECdnT cell invasion, \(^*P = .008, \pm SD\). The suppression of cell invasion by wild-type Ecad is statistically significant when compared with EC and dnT cells, \(^*P < .001\) and \(^{***}P = .001\), respectively.

A Fibroblast-Secreted Factor Induces Cathepsin B Expression and the Invasive Potential of ECdnT Cells

To analyze the invasive properties of these cells further, Boyden chamber invasion assays were used, revealing that ECdnT cells did
not invade (Figure 3A). Whereas cells lacking Ecad, EC, demonstrated an increased invasive potential Boyden chamber assays, ECdnT cells in contrast to their invasive behavior in organotypic cultures did not invade. This result led us to believe that the extracellular matrix or the “tumor environment” may be providing cues to the epithelial compartment for invasion into the matrix to occur.

To address if ECdnT cells could invade in vitro in the presence of a fibroblast-secreted factor, we performed Boyden chamber invasion assays using conditioned media from fibroblasts (Figure 3A) as a chemoattractant. In the presence of conditioned media from fibroblasts, but not unconditioned DMEM (Figure 3A), ECdnT cells were able to invade through the Matrigel layer.

To analyze the effect of the fibroblast-mediated signaling on cathepsin B expression, we stimulated monolayer Ecad, EC, and ECdnT cells with fibroblast-conditioned medium and compared cathepsin B expression levels by Western blot (Figure 3B). Not only...
was the expression of cathepsin B increased, but we also observed greater activation of latent cathepsin B in ECdnT cells (Figure 3B). The smaller—molecular weight band corresponds to activated cathepsin B (around 30 kDa), and the higher—molecular weight band is recognized as the latent precursor [26].

The up-regulation of cathepsin B has been shown to be regulated by the MAPK pathway. Through analyzing the phosphorylation status of p42/44 in ECdnT cells in response to stimulation with fibroblast-conditioned media, we can show that p42/44 is phosphorylated under these conditions (Figure 3C). To demonstrate the importance of the fibroblasts in the induction of cathepsin B, we grew ECdnT cells on the same matrix as in organotypic cultures with and without embedded fibroblasts (Figure 3D). Immunofluorescence staining with anti—cathepsin B antibody shows up—regulation of cathepsin B in ECdnT keratinocytes only when fibroblasts were embedded in the matrix. These data were confirmed in transwell assays in which fibroblasts, fibroblast—conditioned media, or control DMEM was added to the bottom chamber. Again, cathepsin B expression in the ECdnT epithelial cells was only upregulated in the presence of fibroblasts or fibroblast—conditioned media (Figure 3E).

To investigate the involvement of cathepsin B in the fibroblast—dependent invasion of ECdnT cells, we infected ECdnT cells with lentivirus encoding short hairpin RNA (shRNA) against cathepsin B and showed a decrease in invasion in Boyden chamber assays (Figure 3F) in the presence of fibroblast—conditioned media. When grown in organotypic cultures, ECdnT cells with suppressed cathepsin B expression invaded less (Figure 3G). Immunofluorescence staining with anti—cathepsin B antibody confirmed the suppression of cathepsin B expression in organotypic cultures (Figure 3G). Therefore, we conclude that the increased invasive behavior of ECdnT cells is on the induction of cathepsin B through a fibroblast—secreted factor.

**Fibroblasts Are Activated through Cross Talk with Invasive Epithelial Cells**

We performed immunohistochemical staining for several markers associated with fibroblast activation, for example, vimentin, Ki-67, FSP1, and α-SMA (Figure 4A). When stained with anti—vimentin antibody, fibroblasts demonstrated increased expression of vimentin in invasive cultures (Figure 4A). Vimentin—positive fibroblasts localized closely to the areas of epithelial invasion similar to the observation made by Gaggioli et al. [27]. Interestingly, ECdnT keratinocytes also showed a stronger vimentin signal than Ecad and EC cells. Ki-67 staining demonstrated more Ki-67—positive fibroblasts in ECdnT cultures, whereas Ecad organotypic cultures did not induce proliferation of fibroblasts (Figure 4B). The number of FSP1—positive fibroblasts increased in Ecad and ECdnT organotypic cultures (Figure 4C), whereas fibroblasts in all three organotypic cultures were α-SMA—positive (Figure 4D). The increase in number of FSP1— and vimentin—positive fibroblasts, together with the observed increase in proliferation, indicates activation of these fibroblasts in an invasive environment.

To evaluate the premise that fibroblasts may be activated through a factor secreted by the epithelial cells, we performed experiments using conditioned media. Treatment with conditioned media from noninvasive cells, for example, Ecad—overexpressing cells versus invasive ECdnT cells, demonstrated the induction of vimentin, α-SMA, and FSP1 in fibroblasts only in the invasive microenvironment (Figure 5A). The suppression of ECdnT cell invasion through infection with shRNA against cathepsin B results in FSP1—negative fibroblasts that are less proliferative (Figure 5B, a and b) and express low levels of vimentin (Figure 5B, c); this is similar to the Ecad control cells in Figure 4A. As in Figure 4A, fibroblasts in the noninvasive environment as well as in the invasive environment are α-SMA—positive (Figure 5B, d). Furthermore, we analyzed fibroblast expression of
vimentin and α-SMA grown in monolayer in response to stimulation with TGFβ1, ECdnt-conditioned media, ECdnt-shRNA cathepsin B–conditioned media, and control media to demonstrate the link between fibroblast activation and invasive ECdnt cells. Fibroblasts are vimentin-positive in the presence of TGFβ1 and ECdnt-conditioned media but not in the presence of ECdnt-conditioned media from cells expressing shRNA against cathepsin B or control media. There were no differences in α-SMA expression other than slightly lower levels in fibroblasts stimulated with ECdnt-conditioned medium (Figure 5C).

Furthermore, we could show that, when grown in soft agar, ECdnt cells were unable to grow in an anchorage-independent fashion.
Figure 3. (continued).
(Figure 5D). By contrast, fibroblasts alone, in addition to cocultures of fibroblasts and ECdnT cells, were able to grow in soft agar when stimulated with conditioned media from ECdnT cells or organotypic cultures (Figure 5D).

**Cathepsin B and TGFβ1 Are Activated Interdependently**

TGFβ1 not only is a key factor in the activation of fibroblasts and known to promote squamous cancer cell invasion [28] but also has been linked to cathepsin B because TGFβ1 activity can be regulated by cathepsin B [29,30]. To investigate the link between the up-regulation of cathepsin B and the secretion of TGFβ1 in ECdnT cells, we performed ELISA with conditioned media collected from noninvasive and invasive organotypic cultures. This analysis demonstrates increased levels of TGFβ1 in ECdnT cells, potentially induced to compensate for the disruption of TGFβ signaling through the expression of dominant-negative TβRII (Figure 6A). The levels of TGFβ1 secretion were elevated in monolayer ECdnT cells when grown on collagen (Figure 6B) or after treatment with conditioned media from fibroblast cultures (Figure 6C). This increase correlated with an increase in cathepsin B activity in response to collagen extracellular matrix (Figure 6B, lower panel) or treatment of monolayer ECdnT cells with fibroblast-conditioned medium (Figure 6C, lower panel).

**Discussion**

Esophageal squamous cell cancer patients harbor high mortality rates because of the invasive and metastatic nature of this disease. We demonstrate the importance of the tumor microenvironment in

![Figure 4.](image-url) **Figure 4.** Fibroblasts in the presence of invasive ECdnT cells express markers of activated fibroblasts. A panel of markers detecting activated fibroblasts identifies (A) vimentin-positive, proliferating (B), and FSP1-positive (C) cells in ECdnT organotypic cultures, whereas fibroblasts in all cultures were α-SMA-positive (D). Gray demarcations (C) show basal membrane of the epithelium and invasive areas.
Figure 5. Conditioned medium from ECdnT cultures induces activation and anchorage-independent growth of fibroblasts. (A) Fibroblasts show upregulated levels of vimentin, α-SMA, and FSP1/S100 when stimulated with ECdnT-conditioned medium compared with conditioned medium from Ecad and EC cells as shown by Western blot. Cathepsin B shRNA expression results in the suppression of vimentin and FSP1 in fibroblasts in organotypic cultures (B) and monolayer (C). (B) Ki-67 (a, arrows), FSP1 (b, arrows), and vimentin (c) were not detected in fibroblasts in the presence of cathepsin B suppression in noninvasive cultures, whereas α-SMA was expressed in fibroblasts from all cultures. (C) Western blot analysis of fibroblasts grown in the presence of cathepsin B–suppression in noninvasive cultures, whereas α-SMA is present under all conditions (bottom arrow), although lower levels are detected in ECdnT-conditioned media. (D) Fibroblasts (black bars) and fibroblast/ECdnT cocultures (gray bars) grown in soft agar in the presence of ECdnT-conditioned media (**P = .05) and organotypic (OTC)-conditioned media (***P = .05 and ****P = .17, not statistically significant) formed a higher number of colonies compared with those grown in kSFM. ECdnT cells alone lack the ability to grow anchorage-independent (white bars). Fibroblast-conditioned medium alone did not induce the ability for anchorage-independent growth in any of the cell lines.
of pancreatic cancer [41]. A model of the tumor microenvironment analyzed the effects of nitric oxide on cathepsin B expression [42], with the finding that cathepsin B is downregulated in response to endothelial nitric oxide synthase and cell invasion is suppressed. Increased levels of cathepsin B have been attributed to changes in TGFβ/BMP signaling, mainly stimulation with TGFβ1 in the presence of Smad1 reduction as has been reported in myeloid tumors [43].

Posttranslational regulation of cathepsin B has centered on the conversion of latent to active cathepsin B protein. A study in glioblastoma showed an increase in cathepsin B activity to be a key regulator of cell invasion using synthetic inhibitors and genetic knock-down approaches [44]. Another mechanism involved in the processing of mature forms of cathepsin B is in the presence of tumor cells in contact with collagen I [45]. In contrast, collagen type II enhances messenger RNA expression of cathepsin B in chondrocytes after induction of protein kinase C and p38 MAPK [46]. Taken together, the mechanisms underlying cathepsin B activation highlight the role of cathepsin B in cancer cell invasion.

**Link between Cathepsin B and TGFβ1**

We show that the up-regulation of cathepsin B activity correlates with an increase in TGFβ1 concentration in conditioned media of ECdNT cells (Figure 6). The increase in cathepsin B secretion and activity stimulated by collagen has been observed previously [47]. We conclude that the extracellular matrix might aid in the up-regulation of cathepsin B, and cathepsin B, in turn, might increase the levels of activated TGFβ1. Furthermore, the up-regulation of cathepsin B expression and activity induced by fibroblast-conditioned media confirmed the association of cathepsin B and TGFβ1. TGFβ1 is secreted in a latent form and activated through cleavage after secretion, and exocytosis of cathepsin B activates TGFβ1 [30]. In another model system using *Leishmania chagasi*, TGFβ1 release from the latent complex was dependent on cathepsin B activity [29]. Activated TGFβ1 will then signal to fibroblasts resulting in their activation and the observed epithelial cell invasion.

**Fibroblast Activation through ECdNT Cells**

The tumor stroma is an important regulator of cancer progression [48,49]. Malignant transformation can be induced in nontumorigenic epithelia through cancer-associated fibroblasts [50,51]. In our study, fibroblasts in organotypic cultures, whether with invasive or noninvasive phenotypes, stained positive for α-SMA. However, the expression of FSP1 and vimentin was increased only in organotypic cultures with more invasive epithelial cells. FSP1 and α-SMA are markers for activated fibroblasts [49]. Furthermore, the expression of FSP1 together with an increase in proliferation as measured by Ki-67 demonstrates that the fibroblasts in coculture with invasive epithelial cells are activated. FSP1 is a lineage marker for fibroblasts and a potential marker for cells undergoing epithelial-mesenchymal transition and acquiring the capacity for increased motility [52,53]. In addition, metastatic carcinoma cells are less likely to form tumors and to metastasize when injected into FSP1−/− mice [54]. To restore tumor formation and metastasis, Grum-Schwensen et al. [54] co-injected the carcinoma cells with FSP1+/− and FSP1−/− fibroblasts, supporting the notion that FSP1-positive fibroblasts are necessary for invasive tumor growth. TGFβ1 can induce the transition from fibroblasts to myofibroblasts and the activation of cancer-associated fibroblasts [49].

**Mechanisms of Cathepsin B Regulation**

The mechanisms of cathepsin B up-regulation are twofold: 1) increased cathepsin B expression as observed in the organotypic model and in the tumor tissues and 2) augmented cathepsin B activity based on posttranslational processing as seen in Western blot analysis after conditioned media transfer. Cathepsin B has been noted to be a target of Hedgehog signaling and is associated with enhanced invasiveness of pancreatic cancer [41].
Overall, we show here a model of cathepsin B–mediated invasion of epithelial cells induced by cues from activated fibroblasts. This study demonstrates the importance of epithelial-mesenchymal communication and point to secreted factors as the key factors orchestrating the delicate balance between normal and invasive cell fate.

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