Extracellular Lumican Inhibits Pancreatic Cancer Cell Growth and Is Associated with Prolonged Survival after Surgery

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Abstract

Purpose: To evaluate the relevance between lumican expression patterns and the clinical course of patients with pancreatic ductal adenocarcinoma (PDAC), and to investigate the role of lumican in PDAC progression.

Experimental Design: One hundred thirty-one patient tumors were chosen for tissue microarray staining, and Cox regression analysis was used to test the associations between lumican expression and clinical, pathologic, and oncologic outcomes in all patients. Primary PDAC cells and recombinant human lumican protein were used to establish a working model to mimic the in vivo interactions between stromal lumican and PDAC cells. Using this model, we tested the effects of lumican on EGFR signaling via Akt and hypoxia-inducible factor-1α (HIF1α) and its subsequent influence on glucose consumption, lactate production, intracellular ATP, and apoptotic cell death.

Results: Lumican was present in the stroma surrounding PDAC cells in roughly one-half of primary tumors and the direct xenografts. Patients with stromal lumican were associated with a profound reduction in metastatic recurrence after surgery and 3-fold longer survival than patients without stromal lumican. In PDAC cells, extracellular lumican reduced EGFR expression and phosphorylation through enhanced dimerization and internalization of EGFR and the resultant inhibition of Akt kinase activity. Lumican also reduced HIF1α expression and activity via Akt. PDAC cells with enhanced HIF1α activity were resistant to lumican-induced inhibition of glucose consumption, lactate production, intracellular ATP, and apoptosis.

Conclusions: There is a positive association between stromal lumican in primary PDAC tumors and prolonged survival after tumor resection. Lumican plays a restrictive role in EGFR-expressing pancreatic cancer progression.

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Introduction

Lumican belongs to the class II small leucine-rich proteoglycan family (1, 2), and its overexpression has been reported in melanoma, breast, colorectal, uterine, and pancreatic cancers. The complexity and diversity of its proteoglycan structure suggest that lumican could influence cell function through a variety of mechanisms. In melanoma, decreased lumican expression correlates with increased tumor growth and progression (3, 4), and increased lumican expression impedes tumor cell migration and invasion by directly interacting with the α2β1 integrin (5) and decreasing pFAK phosphorylation (6). In neuroendocrine tumors of the colon, lumican expression in the cytoplasm is negatively correlated with tumor grade (7). In contrast, in high-grade breast cancer (8, 9) and pancreatic cancer (10), lumican is overexpressed within the stroma and is typically indicative of advanced tumors and associated with poor prognostic outcomes. It was recently discovered, however, that lumican-overexpressing pancreatic cancer cells have opposite effects on tumor growth in vitro versus in vivo. In one study (11), lumican-overexpressing cells secreted a 70-kDa lumican protein into the cell culture medium that increased proliferation in vitro; however, in vivo those same cells formed smaller tumors with reduced vascular density and enhanced Fas-mediated endothelial cell apoptosis (12). These findings suggest that lumican plays an important role in the regulation of pancreatic cancer.
growth and invasion, but the specific mechanism remains elusive.

The metabolic properties of cancer cells are different from those of normal cells. Cancer cells prefer glycolytic breakdown of glucose for energy rather than mitochondrial oxidative phosphorylation (13, 14). This process generates many key biosynthetic intermediates necessary for the synthesis of the proteins, lipids, and nucleic acids required for cell growth and proliferation (15, 16). The glycolytic shift in cancer cells is regulated by aberrant cell signaling that is itself driven by signaling via growth factor receptors, activation of oncogenes, and environmental factors. The observed overexpression of glucose transporters (Glut) and 18F-fluorodeoxyglucose accumulation on nuclear imaging studies provide evidence for preferential glucose utilization in pancreatic ductal adenocarcinoma (PDAC; refs. 17–19). No studies to date, however, have linked exposure of PDAC cells to extracellular lumican with intracellular regulation of glycolysis.

Hypoxia-inducible factor-1α (HIF1α) plays a central role in reprogramming cell metabolism from oxidative phosphorylation to aerobic glycolysis. HIF1α increases the expression of many metabolic enzymes, including PFKFB3 (an isoform of the glycolytic enzyme PFK2; ref. 20), pyruvate dehydrogenase kinase (21), LDHA (22), MCT4 (a lactate transporter; ref. 23), and Glut1 (24). HIF1α also promotes cell survival through induction of antiapoptotic proteins, such as Survivin, Bcl-Xl, Mcl-1, BNIP3, and BNIP2L. Previous work has demonstrated that HIF1α lies downstream of EGFR, and anti-EGFR treatment using cetuximab (an EGFR-blocking monoclonal antibody) can down-regulate HIF1α protein by inhibiting the PI3K/Akt and MEK/Erk pathways. In fact, downregulation of HIF1α is required for cetuximab to inhibit cell glycolysis and induce cell apoptosis (25, 26).

EGFR and its downstream signaling partners, Akt and HIF1α, are frequently overexpressed in human PDAC and play important roles in its development and progression (27–31). Negative feedback mechanisms, both extracellular and intracellular, have evolved to prevent the dire consequences of uncontrolled activation of EGFR. Recently, some studies reported that decorin, another member of the small leucine-rich proteoglycan family, binds EGFR and causes its internalization via caveolae-mediated endocytosis, which results in EGFR degradation and attenuation of its signaling pathway (32–34). On the basis of these previous findings, we hypothesized that lumican inhibits HIF1α activity via downregulation of the EGFR/Akt signaling pathway, which leads to inhibition of glycolysis and enhancement of apoptosis in PDAC cells.

In this study, we evaluated the expression patterns of lumican in untreated human PDAC tumors as well as direct xenograft tumors and low-passage cell lines derived from the primary tumors. Cancer-related outcomes for patients based upon lumican expression patterns were measured. The derived PDAC cell lines were exposed to recombinant human lumican protein to mimic extracellular lumican in the PDAC tumor microenvironment. Using this model, we tested the effect of lumican on EGFR signaling via Akt and HIF1α and its subsequent influence on glucose consumption, lactate production, intracellular ATP, and apoptotic cell death. The findings conveyed herein confirm our hypothesis and provide novel insights into the mechanisms underlying the observed prolonged survival after surgical resection of PDAC tumors in patients with lumican present within the tumor microenvironment.

Materials and Methods

Antibodies and reagents

The following antibodies and reagents were purchased: anti-lumican (Western blot) and anti-VEGF from Abcam; goat anti-human lumican (IHC) from R&D Systems; recombinant human lumican and anti-HIF1α from BD Biosciences; anti-Akt, anti–phospho-Akt (S473), anti–phospho-EGFR (Y1068), anti–phospho-GSK-3β (S21/9), anti–GSK3β, anti–PARP, anti–Bcl-2, anti–Bcl-XL, IAP family antibody sampler kit, and Akt kinase assay kit (nonradioactive) from Cell Signaling Technology; Human Lumican ELISA kit from Mybiosource; anti-EGFR from Santa Cruz Biotechnology; glucose assay kit, ATP bioluminescent somatic cell assay kit, and anti–β-actin from Sigma-Aldrich; cross-linking reagent BS3 from Thermo Scientific Pierce; VEGF human enzyme-linked immunosorbent assay (ELISA) kit from Invitrogen; luciferase assay system from Promega; lactate assay kit from BioVision; live/dead viability kit from Life Technologies; Cell Death Detection ELISAPLUS from Roche. All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich.
Cell lines and cDNA constructs

We generated the stable PDAC cell lines MDA-PATC50, MDA-PATC53, MDA-PATC43, and MDA-PATC66 from direct xenograft tumors. All of them were validated to be new authentic human pancreatic cancer cells by STR/DNA-fingerprint in The University of Texas MD Anderson Cancer Center. Human pancreatic stellate cells (HPSCs) were kindly gifted by Dr. Rosa F. Hwang (UT MD Anderson Cancer Center). cDNA constructs expressing HIF1α-ΔODD, Myr-Akt, and pB-L-GL-V6L (VEGF hypoxia response element) were kindly provided by Dr. Zhen Fan (UT MD Anderson Cancer Center).

Immunohistochemical and immunocytochemical staining

Both of them were carried out as described in our previous work (35). In immunohistochemical (IHC) staining, the primary antibody was goat anti-human lumican (R&D Systems). The sections were counterstained with Nuclear Fast Red (Vector Laboratories). IHC scoring was determined by staining intensity (0–3) and proportion (%) of positively stained cells (10). Positivity by IHC was defined as positive as signal intensity equal to or greater than 2 and more than 30% of cells with positive staining. Scoring was performed by two independent pathologists, and any scoring disagreement was resolved by joint pathologic review of the slides using a multi-head microscope.

Engraftment and expansion of direct xenograft tumors

We previously published a protocol detailing heterotopic engraftment of patient tumors into immunodeficient mice (36) and expansion of direct xenograft tumors (35). Briefly, excised patient tumor tissue was mechanically minced into fragments (<1 mm), and five tumor fragments were individually placed into formed tissue pockets. Once tumors reached 1.2 cm in greatest diameter, the mice were sacrificed and the tumors dissected from mouse subcutaneous tissue under sterile conditions. Approximately one third of the tumor was prepared for paraffin embedment, one third was cut into quadrants and placed in liquid nitrogen for future study (F1), and other one third was injected into NOD/SCID mice again to generate additional generations (F2, F3) of direct xenograft tumors.

Western blotting

Cell pellets were lysed in RIPA buffer. Patient tumor tissues were cut into very small pieces and disrupted with a Dounce homogenizer in RIPA buffer at 4°C for use in all procedures. The lysates were then separated by SDS-PAGE and subjected to Western blotting with the primary antibodies.

Measurement of lumican levels in conditioned medium

Lumican levels in the conditioned medium were measured using the lumican ELISA Kit according to the manufacturers’ instructions. The results were normalized by the number of cells in each sample in the culture plates.

Chemical cross-linking

Chemical cross-linking was carried out as described previously, with minor modifications (37). Briefly, the cells were washed twice with cold PBS to remove serum proteins and incubated with 3 mmol/LBS3 in PBS for 30 minutes at room temperature with gentle shaking. The reaction was quenched at room temperature with 90 mmol/L glycine, 9 mmol/L Tris-HCl (final concentrations), pH 8.5, for 15 minutes. Cell lysates were subjected to Western immunoblotting for EGFR and β-actin.

Quantitative RT-PCR

Total RNA was extracted from lumican-treated cells with TRIzol (Invitrogen) and then reverse-transcribed to cDNA using TaqMan Reverse Transcription Reagents (Life Technologies). Real-time monitoring of PCR products was performed by measuring the fluorescence (SYBR Green) of PCR products with the LightCycler (Roche Molecular Biochemicals). Normalization and quantification of RT-PCR were performed using Light Cycler software (Roche Molecular Biochemicals). EGFR forward primer: 5′-GGGTTTTCCTTCTGTCGGG-3′, reverse primer: 5′-TTTCCATGCATGCTGCTAAAAGG-3′.

Live/dead double staining assay

Live/dead double staining was measured using a live/dead viability kit. Live cells and dead cells were identified by a green and red fluorescence, respectively.

Apoptosis assay

After various treatments, cell apoptosis was measured by Cell Death Detection ELISAPLUS and by Western blotting with an antibody that recognizes both uncleaved and cleaved PARP.

Akt kinase assay

Akt kinase activity was detected by an Akt kinase assay kit (nonradioactive) according to the manufacturers’ instructions.

Measurement of VEGF levels in conditioned medium

VEGF levels in the conditioned medium were measured using the VEGF ELISA Kit according to the manufacturers’ instructions. Relative VEGF levels were expressed as the optical density value of the conditioned medium normalized by the number of cells in each sample in the culture plates. Experiments were repeated twice.

Glucose detection assay, lactate production assay, and intracellular ATP assay

Cells were treated with lumican in phenol red-free, low-glucose (1 g/L), 0.5% FBS cell culture medium for the indicated time periods. Glucose, lactate production, and intracellular ATP were measured using glucose and lactate assay kits and an ATP bioluminescent assay according to the manufacturers’ instructions and a previous report (26).
Results

Protein expression and localization of lumican in human PDAC

We first identified that lumican is expressed within the cancer cell and stromal compartments of human PDAC tumors. To evaluate the expression patterns and clinical importance of lumican, we assessed the expression and localization of lumican using a tissue microarray of 131 untreated PDAC tumors surgically removed from patients at MD Anderson Cancer Center (Supplementary Methods). Of the 131 patients chosen for tissue microarray staining, pathologically scorable results were evident in 123 patient tumors, and a relatively equal distribution of lumican staining was observed (Fig. 1A): stromal (−)/tumor (−) = 25%; stromal (+)/tumor (−) = 24%; stromal (−)/tumor (+) = 28%; and stromal (+)/tumor (+) = 23%.

Stromal-only lumican expression and clinical outcome

To determine whether lumican expression patterns are associated with the clinical course of patients with PDAC, we used Cox regression analysis to test the associations between lumican expression and clinical, pathologic, and oncologic outcomes in all 123 patients. No demographic or fundamental clinical data were statistically associated with any pattern of lumican expression; however, better pathologic and cancer outcomes were associated with lumican staining within the stroma of the primary PDAC tumor (Supplementary Table S1). For example, patients with stromal-only lumican staining were less likely to have lymph node metastasis within the surgical specimen compared with all other expression subgroups (P = 0.006; Supplementary Table S1). Stromal expression within the primary tumor was markedly associated with longer time from surgical resection to recurrence in the liver or any other site, and this translated into prolonged survival: overall survival (OS) and recurrence-free survival (RFS), durations were 66.1 and 43.2 months in the stromal-only lumican subgroup versus 18.8, and 10.5 months, respectively, in all other patients (P = 0.0006, P = 0.003; Fig. 1B–D). This observation persisted after performing a univariate Cox proportional hazards model of risk factors (Supplementary Table S1).
and after adjusting for the effects of patient age, tumor size, regional lymph node metastases, margin status, adjuvant therapy, and lumican expression subgroups, only stromal-only lumican expression ($P = 0.001$) and the use of adjuvant therapy ($P = 0.007$) were independently associated with improved OS and RFS in the multivariate Cox proportional hazards model (Supplementary Table S3).

### Lumican expression in patient-derived reagents

Previous studies exploring the mechanisms of response to pancreatic cancer treatment have traditionally used established cell lines or xenograft tumors arising from indirect xenografts (cell line intermediary between patient and mouse). These in vitro and in vivo models based on cultured cell lines may not reflect inherent properties of patient tumors that contribute to clinical response and may be the main reason that some novel therapeutic agents have failed to demonstrate clinical efficacy. To avoid this limitation of established cell lines, we directly implanted patient tumors (F0) into immunodeficient mice, without a cell line intermediary, to obtain direct xenograft tumors (F1, F2, F3) and low-passage (<20 generations) primary PDAC cell lines (Fig. 2A). IHC examination of lumican staining in our patient-derived xenografts demonstrated the same four distinct expression patterns in the same distribution of frequency as the patient tumor samples (Fig. 2B). In addition, immunoblotting of tumor protein lysates from a large panel of xenografts detected lumican in all generations (F1–F3), without marked changes in expression from F1 to F3 in most tumors (Fig. 2C). In four primary cell lines, lumican was expressed as well (Fig. 2D, left). In Fig. 2C and D (left), we also detected the protein expressions of EGFR, Akt, and HIF1α, which have been determined to play important roles in the regulation of growth in pancreatic cancer cells. Compared with other three primary cell lines, MDA-PATC66 has very low EGFR and HIF1α expression. Figure. 2D (right) showed that the secreted lumican from human pancreatic stellate cells (HPSCT) is much more than that from the four primary cell lines. These findings suggest that lumican and EGFR, Akt, and HIF1α are widely expressed in pancreatic tumor tissues and primary cells.

### Lumican downregulates EGFR

The growth of pancreatic cancer cells is dependent on or facilitated by EGFR. Previous studies showed that decorin, another small leucine-rich proteoglycan, is capable of suppressing EGFR tyrosine kinase by directly interacting with

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**Table S2.**

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**Figure 2.** Lumican, EGFR, Akt, and HIF1α are expressed in direct xenograft tumors and related primary cells. A, flowchart depicts pancreatic tumor implantation, expansion, and primary cell isolation. B, IHC images of lumican in direct xenograft tumors. C, Western blot analysis of lumican, EGFR, Akt, and HIF1α levels in first-, second-, and third-generation direct xenograft tumors. D, the indicated cells were subjected to Western blot analysis (left), and secreted lumican was detected using Human Lumican ELISA Kit (right).
EGFR (32). Here, we explore whether lumican also regulates EGFR and related signalings. Compared with low EGFR expression cell line MDA-PATC66, EGFR overexpression cell lines MDA-PATC43, MDA-PATC50, and MDA-PATC53 (shown in Fig. 2D, left) showed better response to lumican treatment in cell MTT assay (Fig. 3A, top). This suggested that EGFR may be involved in lumican-induced cell inhibition. MDA-PATC53 and MDA-PATC50 cells exposed to extracellular lumican exhibited marked down-regulation of EGFR protein and phosphorylation levels; after 120 minutes of lumican exposure, EGFR levels were reduced by 71% in MDA-PATC53 and 43% in MDA-PATC50 cells (Fig. 3A, bottom). To elucidate the mechanism for EGFR reduction, immunofluorescent staining of EGFR demonstrated a reduction in membranous, with a concomitant increase in cytoplasmic, after 30 and 120 minutes of exposure to lumican (Fig. 3B). This result suggests that lumican enhances the internalization of EGFR from the cell membrane into the cytoplasm. Previous studies have demonstrated that the formation of dimers of EGFR results in autophosphorylation and subsequent internalization (38), so we investigated whether lumican affected EGFR dimerization. Cells were exposed to lumican for various time intervals and then treated with the membrane-impermeable chemical cross-linker BS3, which allowed resolution of monomeric and dimeric components of EGFR. Exposure to extracellular lumican produced a rapid (15 minutes) and substantial increase in EGFR dimers and a significant decline in EGFR monomers, but both of which were weaker than that induced by EGF stimulation (Fig. 3C). To test whether lumican also decreases EGFR level through inhibiting EGFR protein synthesis, EGFR mRNA levels were measured. Figure 3D showed EGFR mRNA levels were not decreased, on the contrary, they were enhanced especially on 48H (P < 0.01) in MDA-PATC53 cells, after lumican treatment. These data suggest that lumican stimulates the dimerization, internalization, and subsequent degradation of EGFR.

**Lumican reduces Akt activity via inhibition of EGFR**

The PI3K/Akt-mediated signaling pathway is a major downstream pathway of EGFR. Given our observations that lumican downregulates EGFR levels in MDA-PATC53 and MDA-PATC50 pancreatic cancer cells, experiments were designed to determine whether downregulation of EGFR after lumican exposure also decreases Akt activity. We first tested the response of Akt to lumican exposure and observed a brief increase in Akt phosphorylation at Ser473 after 10 minutes, followed by a progressive decline
Because Akt phosphorylation at Ser473 promotes kinase function, we examined Akt kinase activity using a direct Akt kinase assay and observed that cell exposure to lumican significantly decreased phosphorylation levels of Gsk, a direct substrate of Akt, demonstrating that lumican exposure at 30 and 120 minutes (Fig. 4A, top).
abolishes Akt activity (Fig. 4A, bottom). We next investigated whether lumican reduces Akt activity via EGFR. As demonstrated in previous studies (39), the proteasome inhibitor MG132 blocks ligand-induced internalization of EGFR through altered receptor ubiquitination and phosphorylation. When cells were pretreated with MG132 and subsequently exposed to lumican, the previously observed EGFR degradation was blocked as was the resultant Akt phosphorylation (Fig. 4B). Taken together, these results indicate that lumican decreases Akt activity through its inhibition of EGFR.

**Lumican downregulates HIF1α activity through inhibition of the Akt pathway**

HIF1α is a major effector molecule of EGFR-mediated cell signaling and is regulated through the PI3K/Akt pathway (25, 40). Immunoblotting and ELISA of cell protein lysates harvested from MDA-PATC53 and MDA-PATC50 cells after exposure to lumican identified a marked reduction in expression of both HIF1α and VEGF (Fig. 4C, left) and secretion of VEGF in the conditioned medium (Fig. 4C, right), the protein product of a major HIF1α target gene. To determine whether inhibition of Akt mediates the observed downregulation of HIF1α, the effect of lumican on HIF1α levels within PDAC cells possessing constitutively active Akt was explored. Expression of a myristoylated Akt resulted in an overall increase in the basal level of HIF1α and resistance to the decrease in HIF1α and VEGF levels previously observed after exposure to lumican (Fig. 4D, top). Levels of secreted VEGF (Fig. 4D, lower-left) and HIF1α activity (Fig. 4D, lower-right) were similarly affected, indicating a critical role of Akt inhibition in the observed lumican-mediated decrease in HIF1α. In addition, we also tested the effect of lumican on downstream survival markers of Akt/HIF1α, and found lumican decreased the expression levels of IAPs, such as c-IAP1, XIAP, Livin, and Survivin, but had no effect on regulation of Bcl-2 and Bcl-XL (Fig. 4C, left). These data not only confirmed the fact that lumican decreases HIF1α activity through inhibition of the Akt pathway but also suggests a relationship between lumican and cell survival.

**Lumican reprograms cell metabolism and promotes apoptosis via HIF1α downregulation**

HIF1α plays a key role in reprogramming cell metabolism from oxidative phosphorylation to aerobic glycolysis. To assess the ability of lumican to inhibit aerobic glycolysis and to determine whether this effect correlates with lumican-induced HIF1α inhibition, we measured glucose consumption, lactate production, and intracellular ATP levels after lumican exposure in MDA-PATC50 and MDA-PATC53 cells that did or did not overexpress an HIF1α mutant (HIF1α/ΔODD). This mutant retains the majority of the transcriptional activity of full-length HIF1α and can be stably overexpressed in normoxic culture (25, 26). We found that lumican inhibited glucose consumption and lactate production in a time-dependent manner (Fig. 5A), and significantly (P < 0.01) reduced ATP levels in MDA-PATC50 cells (Fig. 5B). Transfection with HIF1α/ΔODD increased glucose consumption and lactate production in MDA-PATC53 cells, which is consistent with established knowledge that HIF1α upregulates glycolysis. Importantly, transfection with HIF-1α/ΔODD conferred resistance to lumican-induced inhibition of glucose consumption and lactate production (Fig. 5C). The ATP level of MDA-PATC53–HIF1α/ΔODD cells treated with lumican was only modestly lower (P > 0.05) when compared with untreated cells (Fig. 5D).

To determine whether these effects on HIF1α levels could mediate the antitumor activity of lumican, we examined whether lumican exposure induces apoptotic cell death and whether experimental elevation of the HIF1α level would mitigate the proapoptotic effects of lumican. Using three complementary methods to detect apoptotic death, we found that lumican induced marked apoptosis in MDA-PATC50 and MDA-PAT-53 cells (Fig. 6A), which is in consistent with lumican-induced downregulation of EGFR and EGFR phosphorylation at the same time points (Fig. 6A, middle-lower). However, overexpression of HIF1α/ΔODD counteracted the apoptotic effects of lumican in MDA-PATC53 cells (Fig. 6B). These results demonstrate that lumican decreases glucose consumption, lactate production, and intracellular ATP level and induces apoptosis through downregulation of HIF1α.

**Discussion**

The results of our study highlight two complementary findings: (i) a positive association between stromal lumican in primary PDAC tumors and prolonged survival after tumor resection; and (ii) reduced proliferation of EGFR-expressing pancreatic cancer cells when exposed to extracellular lumican. These data suggest a new mechanism, depicted in Fig. 6C, in which lumican stimulates EGFR dimerization and internalization, resulting in decreased EGFR kinase activity and attenuation of its downstream activators Akt and HIF1α. Reduced HIF1α inhibits glycolytic metabolism and triggers apoptotic cell death. Together, these observations offer a unifying mechanism for the clinical observations linking high levels of stromal lumican with reduced cancer recurrence and death after tumor resection in patients with early-stage PDAC tumors.

To heighten their relevance to human PDAC, all experiments were performed using patient data and the unique reagents derived from PDAC tumors surgically removed at MD Anderson Cancer Center. IHC examination of human PDAC tumors identified that the presence of lumican in the extracellular space surrounding PDAC cells was associated with a profound reduction in metastatic recurrence after surgery and 3-fold longer survival. This marked association drove us to investigate how extracellular lumican affects PDAC cells. We used primary PDAC cells and recombinant human lumican protein within the
media to establish a working model designed to mimic the in vivo interactions between stromal lumican and PDAC cells. We chose this approach for two reasons. First, the primary cell source of lumican in the PDAC tumor microenvironment has not been clarified. Second, recent reports suggest that lumican expression in the cytoplasm of pancreatic cancer cells does not correlate with clinicopathologic factors, whereas lumican expression in stromal tissues has been more closely linked to such factors (10).

Our experimental approach has yielded novel findings demonstrating that a soluble proteoglycan, lumican, can control EGFR, an important receptor tyrosine kinase widely implicated in the pathogenesis and progression of pancreatic cancer. Our results indicate that lumican exposure induces rapid EGFR dimerization (15 minutes) and internalization (30 minutes), which results in EGFR degradation. This process is similar to EGF-induced EGFR endocytosis that depends upon the clathrin pathway (41, 42), but it is different from that induced by decorin, another member of the small leucine-rich proteoglycan family. Decorin directly binds to EGFR, decreases EGFR dimerization, and causes a slow and sustained internalization (2–3 hours) of EGFR via caveolae-mediated endocytosis (32, 33).

The results of our analysis of lumican expression and cancer-related outcomes differ from previously reported data (10). Our examination of 123 early primary PDAC tumors demonstrated an association between stromal lumican and improved clinical outcome, but the previous study of 53 PDAC tumors linked high levels of stromal lumican to local tumor invasion (10). Close comparison of the two studies, however, detects important differences between the primary tumors in the two groups: the majority (60%) of the previously reported cases were T4 (stage III; ref. 43) tumors with cancer cells metastasizing beyond the pancreas into adjacent anatomic structures. Conversely, all of the 123 tumors in our group were confined to the pancreas, with none larger than T3 (stage II; Supplementary Table S1). It is plausible that the different associations between lumican expression within the tumor, metastasis, and outcome in these two reports reflect a difference in the PDAC cells populating the tumor microenvironment in primary versus metastatic tumors. Lumican-sensitive cells fail to propagate and undergo apoptosis, whereas lumican-resistant cells survive and metastasize. This is supported by additional studies we have performed (data not shown) in which MDA-PATC50 cells (derived from primary PDAC) were eliminated through apoptosis, whereas MDA-PATC53

![Figure 5. Lumican inhibits glucose consumption, lactate production, and intracellular ATP levels in a HIF1α inhibition–dependent manner. A, MDA-PATC50 cells were treated with lumican (2 μg/mL) for the indicated times in low glucose (1 g/L), 0.5% FBS DMEM medium. Left, the level of glucose remaining. Right, the level of lactate produced in the conditioned medium. B, intracellular levels of ATP were measured using a luciferase-based ATP determination assay. The relative values of ATP in the treated groups were expressed as percentage of the value of ATP in the corresponding untreated groups. C, MDA-PATC53-vector and MDA-PATC53–HIF1α/DODD pool cells were treated with lumican (2 μg/mL) for the indicated times. Left, the levels of glucose. Right, lactate produced. D, intracellular ATP was detected using the same methods as described in B.](https://www.aacrjournals.org/doi/fig/10.1158/1078-0432.CCR-14-0970)
cells (derived from a metastatic PDAC) survived after long-term exposure (27 days) to lumican. The surviving cells, although proliferating slowly, demonstrated increased Src phosphorylation and decreased LKB1 phosphorylation, which synergistically promote cancer cell survival, tumor formation, angiogenesis, and metastasis (44–49) and are possible mechanisms by which PDAC cells acquire resistance to lumican and survive in metastatic sites.

This work unambiguously links lumican with PDAC cell metabolism and identifies the lumican/EGFR/Akt/HIF1α signaling pathway as a mechanism by which lumican inhibits pancreatic cancer cell survival and proliferation. These studies are further supported by related histopathologic observations in carefully annotated human PDAC samples. Together, these observations provide strong evidence that lumican is a critical host modifier of PDAC cell growth and survival. Because HIF1α/VEGF plays a pivotal role in progression of pancreatic cancer, in further studies we need to evaluate whether lumican is correlated with angiogenesis and metastasis, and whether lumican sensitizes PDAC cells to chemotherapy. Increasing our understanding of these areas will help delineate the clinical utility of lumican in PDAC.

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

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Conception and design: X. Li, M.A. Truty, H. Wang, J.B. Fleming
Development of methodology: X. Li, M.A. Truty, J.B. Fleming
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Li, M.A. Truty, X. Chopin-Laly, R. Zhang, R.M. Thomas, H. Wang, M.H. Katz, J.B. Fleming
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Li, M.A. Truty, Y. Kang, D.J. Roife, D. Chatterjee, E. Lin, R.M. Thomas, J.B. Fleming
Writing, review, and/or revision of the manuscript: X. Li, M.A. Truty, X. Chopin-Laly, D.J. Roife, R.M. Thomas, H. Wang, M.H. Katz, J.B. Fleming
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Li, R. Zhang, J.B. Fleming
Study supervision: J.B. Fleming

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Figure 6. Lumican-induced cell apoptosis can be overcome by overexpression of HIF1α/ΔODD. A, left, MDA-PATC50 and MDA-PATC53 cells were treated with lumican (2 μg/mL) for 24 hours in low-glucose, 0.5% FBS DMEM medium, and the live and dead cells were measured using a live/dead assay. The results shown are the average from three independent experiments. Middle, cell lysates were subjected to a cell death detection ELISA kit (top) and Western blotting with the indicated antibodies (bottom). Right, Western blotting analysis of PARP and cleaved PARP. B, MDA-PATC53 cells were transfected with HIF1α/ΔODD for 24 hours and treated with lumican (2 μg/mL) for another 24 hours. Left, live/dead cell assay was performed. Middle, cell lysates were subjected to cell death detection ELISA assay. Right, Western blotting with the indicated antibodies. C, schematic model depicts how lumican inhibits glycolysis and induces apoptosis through the EGFR/Akt/HIF1α signaling pathway.
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