Extracellular lumican inhibits pancreatic cancer cell growth and is associated with prolonged survival after surgery

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Lumican is widely expressed in PDAC and its extracellular matrix, but the biologic roles of lumican are quite variable. In this study, 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 and analyzed the associations between lumican expression and clinical, pathologic, and oncologic outcomes in these patients. Our findings indicate that patients with stromal lumican lived three times longer than patients without stromal lumican. This marked association was further verified by our in vitro studies, in which extracellular lumican inhibits glycolytic metabolism and triggers apoptotic cell death in PDAC cells through EGFR/Akt/HIF-1α signal pathway. These observations suggest that high expression of lumican in the stroma surrounding tumors may represent an effective marker to predict reduced cancer recurrence and death after tumor resection in patients with early stage PDAC tumors.
Abstract

**Purpose:** To evaluate the relevance between lumican expression patterns and the clinical course of PDAC patients, and to investigate the role of lumican in PDAC progression.

**Experimental Design:** 131 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 HIF-1α 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 three-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 HIF-1α expression and activity via Akt. PDAC cells with enhanced HIF-1α 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.
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 $^{18}$F-fluorodeoxyglucose accumulation on nuclear imaging studies provide evidence for preferential glucose utilization in pancreatic ductal adenocarcinoma (PDAC) (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 (HIF-1α) plays a central role in reprogramming cell metabolism from oxidative phosphorylation to aerobic glycolysis. HIF-1α increases the expression of many metabolic enzymes, including PFKFB3 (an isoform of the glycolytic enzyme PFK2) (20), pyruvate dehydrogenase kinase (21), LDHA (22), MCT4 (a lactate transporter) (23), and GLUT1 (24). HIF-1α also promotes cell survival through induction of anti-apoptotic proteins, such as Survivin, Bcl-Xl, Mcl-1, BNIP3, and BNIP2L. Previous work has demonstrated that HIF-1α lies downstream of epidermal growth factor receptor (EGFR), and anti-EGFR treatment using cetuximab (an EGFR-blocking monoclonal antibody) can downregulate HIF-1α protein by inhibiting the PI3K/Akt and MEK/Erk pathways. In fact, downregulation of HIF-1α is required for cetuximab to inhibit cell glycolysis and induce cell apoptosis (25, 26).

EGFR and its downstream signaling partners, Akt and HIF-1α, 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 caveolar-mediated endocytosis, which results in EGFR degradation and attenuation of its signaling pathway (32-34). Based on these previous findings, we hypothesized
that lumican inhibits HIF-1α 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 in order to mimic extracellular lumican in the PDAC tumor microenvironment. Using this model, we tested the effect of lumican on EGFR signaling via Akt and HIF-1α 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-HIF-1α from BD Biosciences; anti-Akt, anti-phospho-Akt (S473), anti-phospho-EGFR (Y1068), anti-phospho-GSK-3α/β (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;
vascular endothelial growth factor (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. cDNA constructs expressing HIF-1α-ΔODD, Myr-Akt, and pBI-GL-V6L (VEGF hypoxia response element) were kindly provided by Dr. Zhen Fan (UT MD Anderson Cancer Center).

**Immunohistochemical (IHC) and immunocytochemical staining**

Both of them were carried out as described in our previous work (35). In 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 a priori 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 mM BS3 in PBS for 30 minutes at RT with gentle shaking. The reaction was quenched at RT with 90 mM glycine, 9 mM 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, Mannheim, Germany). EGFR forward primer: 5'-GCGTCTCTTGGCAATGT-3, reverse primer: 5'-CTTGGCTCACCCTCCAGAAG-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 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
PDAC patients, 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 1). For example, patients with
stromal-only lumican staining were less likely to have lymph node metastasis within the surgical
specimen compared to all other expression subgroups (p=0.006) (Supplementary Table 1).
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, 1C, 1D). This observation persisted after
performing a univariate Cox proportional hazards model of risk factors (Supplementary Table 2),
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 3).

**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). Additionally, 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 Fig. 2D (left), we also detected the protein expressions of EGFR, Akt, and HIF-1α, 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 HIF-1α expression. Fig. D (right) showed that the secreted lumican from pancreatic stromal cell line HPSCT is much more than that from the four
primary cell lines. These findings suggest that lumican and EGFR, Akt, and HIF-1α 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 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, MDA-PATC53 (shown in Fig. 2D, left) showed better response to lumican treatment in cell MTT assay (Fig. 3A, upper). This suggested EGFR may be involved in lumican-induced cell inhibition. MDA-PATC53 and MDA-PATC50 cells exposed to extracellular lumican exhibited marked downregulation 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, lower). 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. Fig. 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 at 30 and 120 minutes (Fig. 4A, upper). Since 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 abolishes Akt activity (Fig. 4A, lower). We next investigated whether lumican reduces Akt activity via EGFR. As demonstrated in previous studies (39), the proteosome 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 HIF-1α activity through inhibition of the Akt pathway**

HIF-1α 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 HIF-1α and VEGF (Fig. 4C, left) and secretion of VEGF in the conditioned medium (Fig. 4C, right), the protein product of a major HIF-1α target gene. To determine whether inhibition of Akt mediates the observed downregulation of HIF-1α, the effect of lumican on HIF-1α 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 HIF-1α and resistance to the decrease in HIF-1α and VEGF levels previously observed after exposure to lumican (Fig. 4D, upper). Levels of secreted VEGF (Fig. 4D, lower-left) and HIF-1α activity (Fig. 4D, lower-right) were similarly affected, indicating a critical role of Akt inhibition in the observed lumican-mediated decrease in HIF-1α. Additionally, we also tested the effect of lumican on downstream survival markers of Akt/ HIF-1α, 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 HIF-1α 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 HIF-1α downregulation**
HIF-1α 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 HIF-1α inhibition, we measured glucose consumption, lactate production, and intracellular ATP level after lumican exposure in MDA-PATC50 and MDA-PATC53 cells that did or did not overexpress a HIF-1α mutant (HIF-1α/ΔODD). This mutant retains the majority of the transcriptional activity of full-length HIF-1α 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 HIF-1α/ΔODD increased glucose consumption and lactate production in MDA-PATC53 cells, which is consistent with established knowledge that HIF-1α 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-HIF-1α/ΔODD cells treated with lumican was only modestly lower (P>0.05) when compared to untreated cells (Fig. 5D).

To determine whether these effects on HIF-1α levels could mediate the antitumor activity of lumican, we examined whether lumican exposure induces apoptotic cell death and whether experimental elevation of the HIF-1α level would mitigate the pro-apoptotic effects of lumican. Using three complementary methods to detect apoptotic death, we found that lumican induced marked apoptosis in MDA-PATC50 and MDA-PATC-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 HIF-1α/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 HIF-1α.

**DISCUSSION**

The results of our study highlight two complementary findings: 1) a positive association between stromal lumican in primary PDAC tumors and prolonged survival after tumor resection; and 2) 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 HIF-1α. Reduced HIF-1α 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. Immunohistochemical 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 three-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) (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 1). 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 while MDA-PATC53 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/ HIF-1α 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. Since HIF-1α/VEGF plays a pivotal role in progression of pancreatic cancer, in further studies we need 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.

REFERENCES


**Figure Legends**
Figure 1. Characteristic IHC staining pattern of lumican in 123 resected human pancreatic ductal adenocarcinomas. 

A, immunohistochemical staining of lumican in the cytoplasm of cancer cells and in adjacent stromal tissues. Original magnification: 200X. B, Kaplan Meier curves for overall survival for stromal-only lumican expression versus all other subgroups. C, recurrence-free survival for stromal-only lumican expression versus all other subgroups. D, liver-specific recurrence-free survival for stromal-only lumican expression versus all other subgroups.

Figure 2. Lumican, EGFR, Akt, and HIF-1α are expressed in direct xenograft tumors and related primary cells

A, flowchart depicts pancreatic tumor implantation, expansion, and primary cell isolation. B, immunohistochemical images of lumican in direct xenograft tumors. C, Western blot analysis of lumican, EGFR, Akt, and HIF-1α 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).

Figure 3. Lumican increases dimerization, internalization, and degradation of EGFR

A, Upper: indicated cells were treated with different dose of lumican for 4 days, cell viability was detected using MTT assay. Lower: MDA-PATC53 and MDA-PATC50 cells were treated with 2μg/mL lumican for the indicated intervals. Cell lysates were subjected to Western blotting with indicated antibodies. Quantification of EGFR protein level was normalized to β-actin. B, immunofluorescent staining of MDA-PATC53 cells with anti-EGFR antibody. Arrows show EGFR protein expression. C, MDA-PATC53 cells were treated with lumican (2μg/mL) or EGF
(10nM) and then cultured with the cross-linker BS3 (3mM) for 30 minutes at room temperature. Cell lysates were subjected to Western blotting with EGFR and β-actin antibodies. EGFR dimers and monomers were quantified using Scion image analysis software. **D**, relative EGFR mRNA levels were tested using real time PCR in treated cells.

**Figure 4.** Lumican downregulates HIF-1α expression and activity through EGFR/Akt signal pathway

**A**, Upper: MDA-PATC53 and MDA-PATC50 cells were treated with lumican (2μg/mL) for the indicated intervals. Cell lysates were subjected to Western blotting with the indicated antibodies. Quantification of Akt phosphorylation was normalized to total Akt. Lower: treated MDA-PATC53 cells were subjected to in vitro Akt kinase assay. Quantification of GSK phosphorylation was normalized to total GSK. **B**, MDA-PATC53 cells were treated with or without lumican (2μg/mL) or MG132 (1μM) for 24 hours. Cell lysates were subjected to Western blotting with the indicated antibodies. Quantification of Akt phosphorylation was normalized to total Akt. **C**, Left: indicated cells were treated with lumican (2μg/mL) for 24 hours. Cell lysates were subjected to Western blotting with the indicated antibodies. Right: the level of VEGF in the conditioned medium was measured by a VEGF ELISA kit. The results shown are the average from three independent experiments. The values are expressed as mean ± SD. **D**, Upper: MDA-PATC53 cells were transiently transfected with Myr-Akt cDNA for 24 hours and treated with lumican (2μg/mL) for another 24 hours. Cell lysates were subjected to Western blotting. Quantification of HIF-1α protein level was normalized to β-actin. Lower left: the VEGF level was detected in the conditioned medium. Lower right: MDA-PATC53 cells were co-transfected with Myr-Akt and
HRE-luciferase reporter (pBI-GL-V6L) for 24 hours and treated with lumican (2µg/mL) for another 24 hours. HIF-1 transcriptional activity was detected by a luciferase assay.

**Figure 5.** Lumican inhibits glucose consumption, lactate production, and intracellular ATP levels in a HIF-1α inhibition-dependent manner.

A, MDA-PATC50 cells were treated with lumican (2µg/mL) for the indicated times in low glucose (1g/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-HIF-1α/ΔODD pool cells were treated with lumican (2µg/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.

**Figure 6.** Lumican-induced cell apoptosis can be overcome by overexpression of HIF-1α/ΔODD

A, Left: MDA-PATC50 and MDA-PATC53 cells were treated with lumican (2µg/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 cell death detection ELISA kit (upper) and Western blotting with the indicated antibodies (lower). Right: Western blotting analysis of PARP and cleaved PARP. B, MDA-PATC53 cells were transfected with HIF-1α/ΔODD for 24 hours and treated with lumican (2µg/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/HIF-1α signaling pathway.
Figure 4

(A) Bar graph showing the percentage change in Akt-p levels in MDA-PATC53 and MDA-PATC50 cells after treatment with 10, 30, and 120 minutes of Lumican. The Akt(S473)-p levels are also shown.

(B) Comparison of Akt-p levels in Lumican and EGFR treated cells with and without MG132 treatment.

(C) Western blot analysis of VEGF, C-IAP1, XIAP, Survivin, Bcl-2, and Bcl-XL expression levels in Lumican treated MDA-PATC53 and MDA-PATC50 cells.

(D) Graph showing the percentage change in HIF-1α expression level with Myr-Akt treatment compared to vector control.
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