Fibronectin Stimulates Non–Small Cell Lung Carcinoma Cell Growth through Activation of Akt/Mammalian Target of Rapamycin/S6 Kinase and Inactivation of LKB1/AMPK-Activated Protein Kinase Signal Pathways

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Abstract

The Akt/mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (p70S6K) pathway is considered a central regulator of protein synthesis and of cell proliferation, differentiation, and survival. However, the role of the Akt/mTOR/p70S6K pathway in lung carcinoma remains unknown. We previously showed that fibronectin, a matrix glycoprotein highly expressed in tobacco-related lung disease, stimulates non–small cell lung carcinoma (NSCLC) cell growth and survival. Herein, we explore the role of the Akt/mTOR/p70S6K pathway in fibronectin-induced NSCLC cell growth. We found that fibronectin stimulated the phosphorylation of Akt, an upstream inducer of mTOR, and induced the phosphorylation of p70S6K1 and eukaryotic initiation factor 4E–binding protein 1 (4E-BP1), two downstream targets of mTOR in NSCLC cells (H1792 and H1838), whereas it inhibited the phosphatase and tensin homologue deleted on chromosome 10, a tumor suppressor protein that antagonizes the phosphatidylinositol 3-kinase/Akt signal. In addition, treatment with fibronectin inhibited the mRNA and protein expression of LKB1 as well as the phosphorylation of AMP-activated protein kinase (AMPKα), both known to down-regulate mTOR. Rapamycin, an inhibitor of mTOR, blocked the fibronectin-induced phosphorylation of p70S6K and 4E-BP1. Akt small interfering RNA (siRNA) and an antibody against the fibronectin-binding integrin α5β1 also blocked the p70S6K phosphorylation in response to fibronectin. In contrast, an inhibitor of extracellular signal–regulated kinase 1/2 (PD98095) had no effect on fibronectin-induced phosphorylation of p70S6K. Moreover, the combination of rapamycin and siRNA for Akt blocked fibronectin-induced cell proliferation. Taken together, these observations suggest that fibronectin–induced stimulation of NSCLC cell proliferation requires activation of the Akt/mTOR/p70S6K pathway and is associated with inactivation of LKB1/AMPK signaling. (Cancer Res 2006; 66(1): 315-23)

Introduction

Tumor growth and invasion are not only the result of malignant transformation but also depend on environmental influences from their surrounding stroma, local growth factors, and systemic hormones. In particular, the composition of the extracellular matrix is believed to affect malignant behavior in vivo. Fibronectin, a matrix glycoprotein expressed in several carcinoma cell types, has been implicated in carcinoma development (1–5). In lung carcinoma, fibronectin expression is increased, especially in non–small cell lung carcinoma (NSCLC; refs. 4, 5). Interestingly, the adhesion of lung carcinoma cells to fibronectin enhances tumorigenicity and confers resistance to apoptosis induced by standard chemotherapeutic agents (6). We previously showed that fibronectin stimulates NSCLC cell growth and reduced apoptosis through activation of the integrin α5β1, induction of cyclooxygenase-2 (COX-2), and inhibition of cyclin-dependent kinase inhibitor p21Waf1/Cip1 gene expression (7). These effects were associated with activation of several kinase signaling pathways, including extracellular signal–regulated kinase 1/2 (ERK1/2) and Rho kinase (7, 8). Importantly, these effects are not shared by other matrix components, such as type 1 collagen. More recently, fibronectin was shown to stimulate the phosphatidylinositol 3-kinase (PI3K), which is capable of controlling the expression of cyclin D and related genes involved in cell cycle control (9, 10). The above information suggests that fibronectin promotes lung tumor growth/survival and resistance to therapy and could represent a novel target for the development of new anticancer drugs. This prompted our interest in elucidating the mechanisms by which fibronectin stimulates human lung carcinoma cell growth and led us to investigate the role of the Akt/mammalian target of rapamycin (mTOR)/p70S6K signaling pathway in this process.

Members of the mTOR subfamily belong to the PI3K-related kinase family and are inhibited by rapamycin, a feature that has facilitated efforts to study their functions in eukaryotic cells (11, 12). The mTOR serine/threonine kinases play an important role in the translational control of gene expression during cell growth and the overall rate of protein synthesis in response to nutrients and other environmental factors (12, 13). mTOR is also a key intermediary in multiple mitogenic signaling pathways and plays a central role in modulating cellular proliferation and angiogenesis in normal tissues and neoplastic processes (13, 14). The primary pathway by which growth factors and cytokines activate mTOR and its downstream targets seems to be the PI3K/Akt pathway. Accordingly, targeting the Akt/PI3K pathway with mTOR antagonists may increase the therapeutic efficacy of cancer therapy (14).

The signals that up-regulate mTOR activity are frequently activated in human cancers. In mammals, the two best-characterized targets of mTOR are the ribosomal S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF4E)–binding protein 1 (4E-BP1). mTOR activity leads to S6K1/2 phosphorylation and activation and to 4E-BP1 phosphorylation and release from the cap-dependent translation initiation factor

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eIF4E. These two events, likely combined with other mTOR targets, lead to an increase in ribosomal biogenesis and the selective translation of specific mRNA populations (11–15). This ability to increase the protein synthesis capacity of the cell is responsible, at least in part, for the ability of the mTOR proteins to drive cell growth and proliferation (15) and their implication in lung cancer progression (16).

Herein, we report that fibronectin stimulates the phosphorylation of Akt and inhibits LKB1 and AMP-activated protein kinase (AMPK) in NSCLC, thereby promoting activation of mTOR and its downstream targets, such as p70S6K. This result is also evidenced in vitro using a lung fibroblast (glioblastoma cell line). In controls, the primary antibodies were omitted or replaced with a control (Amersham, Arlington Heights, IL) for 1 minute, and exposed to X-ray film. horseradish peroxidase (1:2,000, Cell Signaling). The blots were washed, and the 5% (Temecula, CA). The LightCycler/FastStart DNA Master SYBR Green I kit in the Cepheid Smart-Cycler real-time PCR cycler (Sunnyvale, CA; ref. 7). Experiments were done in triplicate for each data point.

**Treatment with Akt small interfering RNA.** The Akt small interfering RNA (siRNA) duplex target sequences were synthesized by Sigma-Genosys based on published data (18). The Akt target sequence was 5′-CAGCAUGAGGUCUCCAGGCT-3′. Nonspecific control siRNA was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For the transfection procedure, cells were grown to 50% confluence, and Akt or control siRNAs were transfected using the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, oligofectamine reagent was incubated with serum-free medium for 10 minutes. Subsequently, a mixture of siRNA was added. After incubation for 15 minutes at room temperature, the mixture was diluted with medium and added to each well. The final concentration of siRNA in each well was 100 nmol/L. After culturing for 30 hours, cells were washed, resuspended in new culture media in the presence or absence of fibronectin, and/or incubated with 1 μCi/mL [methyl-3H]thymidine (Amersham; specific activity, 250 Ci/nmol) for an additional 24 hours for Western blot or [methyl-3H]thymidine incorporation assays.

**Molecular Biochemicals (Indianapolis, IN). All reverse transcription-PCR (RT-PCR) kit components were obtained from Perkin-Elmer Co. (Foster City, CA). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

**Western blot analysis.** The procedure was done as previously described (17). Protein concentrations were determined by the Bio-Rad (Richmond, VA) protein assay. Equal amounts of protein from whole-cell lysates were solubilized in 2× SDS/sample buffer and separated on SDS/10% polyacrylamide gels. Blots were incubated with rabbit antibodies raised against mouse LKB1, AMPKα, phosphorylated AMPKα (p-AMPKα, Thr172), p70S6K, p70S6K (Thr42/ Ser40), Akt, Akt (Ser473), and phosphatase and tensin homologue deleted on chromosome 10 (PTEN), 4E-BP1, and p-4E-BP1/Thr37/46 were purchased from Cell Signaling (Beverly, MA). Fibronectin derived from human fibroblasts was purchased from Sigma-Aldrich (St. Louis, MO). The mouse monoclonal anti-human α5(3) integrin (J558) and anti-human α3(1) integrin (AB1944) antibodies were purchased from Chemicon International, Inc. (Temecula, CA). The LightCycler/FastStart DNA Master SYBR Green 1 kit and the 5′ DNA Terminus Labeling System were purchased from Roche Molecular Biochemicals (Indianapolis, IN). All reverse transcription-PCR (RT-PCR) kit components were obtained from Perkin-Elmer Co. (Foster City, CA). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

**Results**

**Fibronectin stimulates Akt protein phosphorylation.** The PI3K/Akt signaling pathway is involved in the control of cell cycle progression and cell proliferation. A link between fibronectin and activation of PI3K has been reported in other studies (10, 19), but its role in NSCLC is unknown. Here, we report that fibronectin increased the phosphorylation of Akt, a downstream target of PI3K, in a time-dependent and dose-dependent manner starting at 30 minutes and reaching maximal stimulation at 1 hour at 20 μg/mL concentration (Fig. 1A and B). Total Akt protein was not affected by fibronectin. Fibronectin also decreased the protein levels of the tumor suppressor gene PTEN in a dose-dependent and time-dependent manner with optimal reduction at 8 hours with 20 μg/mL concentration (Fig. 1C and D). Inhibition of PTEN mRNA accumulation is also noted in the presence of fibronectin (data not shown). Similar results were also found in another NSCLC cell line, H1838 (data not shown).

**Fibronectin inhibits LKB1 and AMPK expression.** The tumor suppressor LKB1 is an activating upstream kinase for AMPK (20). Here, we show that fibronectin inhibits LKB1 mRNA and protein expression in a time-dependent manner as determined by RT-PCR and Western blot analysis, respectively (Fig. 2A and C). The inhibition of LKB1 mRNA by fibronectin was confirmed by real-time RT-PCR analysis (Fig. 2B). Fibronectin also inhibited the
phosphorylation of the AMPKα protein in a dose-dependent and time-dependent manner, whereas it had no effect on the total content of AMPKα protein as determined by Western blot analysis (Fig. 3A and B). We also examined whether inhibition of either PI3K/Akt or mitogen-activated protein/ERK kinase-1 (MEK-1)/ERK signaling would block the effect of fibronectin on AMPK protein expression. As shown in Fig. 3C, the effect of fibronectin on reduction of AMPK protein expression was unaffected by inhibitors of PI3K (wortmannin) or MEK-1/ERK (PD98095), suggesting that PI3K and ERK signaling pathways are not involved in this process. Similar effects were observed in H1838 cells (data not shown).

**Fibronectin stimulates phosphorylation of p70S6K and 4E-BP1 protein levels in a dose-dependent and time-dependent manner: role of α5β1.** mTOR activation stimulates the phosphorylation of downstream kinases, such as p70S6K and 4E-BP1. Therefore, we examined the effects of fibronectin on p70S6K and 4E-BP1 expression. As shown in Fig. 4A and B, H1792 cells exposed to the indicated concentrations of fibronectin showed increased phosphorylation of p70S6K protein. The stimulatory effect of fibronectin was dose and time dependent with optimal increases noted when 20 μg/mL of fibronectin were used for 24 hours; no effect was detected in the abundance of p70S6K total protein. Fibronectin also increased the phosphorylation 4E-BP1 (Thr37/46), another downstream target of mTOR, in a time-dependent and dose-dependent manner with maximal induction at 24 hours, whereas it had little effect on total 4E-BP1 protein (Fig. 4C and D). Similar results were obtained in an additional NSCLC cell line, H1838 (data not shown).

In previous work, we showed that fibronectin-induced NSCLC cell proliferation was mediated via the integrin α5β1 (7). Therefore, we tested the effects of blocking α5β1 in our system. We found that antibodies against the integrin α5β1 blocked fibronectin-induced p70S6K phosphorylation (Fig. 4E). In contrast, neither control IgG (data not shown) nor antibodies against integrin α2β1 (a collagen-binding integrin) mimicked this effect (Fig. 4E). In separate experiments, we found that a specific inhibitor of ERK1/2...
(PD98095) did not affect fibronectin-induced p70S6K phosphorylation (Fig. 4F). These results suggest that the effect of fibronectin on p70S6K protein activity is mediated, at least in part, through PI3K/Akt activation but not through ERK1/2 signaling. Similar effects were observed in H1838 cells (data not shown).

Akt siRNA and rapamycin inhibit fibronectin-induced phosphorylation of p70S6K and 4E-BP1 and lung carcinoma cell proliferation. We next tested whether knockdown of Akt expression could prevent the stimulatory effect of fibronectin on p70S6K phosphorylation. To this end, we depleted expression in cells using the small RNA interference method. As shown in Fig. 5A, Akt siRNA greatly diminished endogenous Akt protein production; no changes were noted in cells transfected with control siRNA. The Akt siRNA also blocked the stimulatory effects of fibronectin on p70S6K phosphorylation; the Akt siRNA alone or a control siRNA had no effect (Fig. 5D).

Rapamycin, an inhibitor of mTOR, has been shown to efficiently inhibit the de novo phosphorylation of p70S6K induced by different stimuli and the phosphorylation of previously activated p70S6K (21). Therefore, we next tested whether rapamycin affected fibronectin-induced phosphorylation of p70S6K. As shown in Fig. 5C, rapamycin (10 nmol/L) not only inhibited the phosphorylation of previously activated p70S6K but also blocked the phosphorylation of p70S6K induced by fibronectin. Fibronectin-induced phosphorylation of 4E-BP1 was also abrogated in the presence of rapamycin, whereas the latter alone had no effect on 4E-BP1 expression (Fig. 5D).

Next, we investigated whether Akt played a role in lung carcinoma cell proliferation induced by fibronectin. H1792 cells were transfected with Akt siRNA duplexes. Afterwards, the cells were exposed to fibronectin for an additional 24 hours followed by evaluation of cell proliferation. As shown in Fig. 5E, Akt siRNA significantly reduced fibronectin-induced cell proliferation; the control siRNA had no effect. We next examined if blocking mTOR affects the stimulatory effect of fibronectin on NSCLC cell growth. The mTOR inhibitor, rapamycin, inhibited, at least in part, fibronectin-induced NSCLC cell growth; rapamycin alone showed a small inhibitory effect on cell growth when used for 48 hours. A higher dose of rapamycin had no further effect (data not shown). Furthermore, rapamycin enhanced the inhibitory effect of Akt siRNA on cell proliferation (Fig. 5E). Similar effects were observed in H1838 cells (data not shown).

Discussion

Interactions between tumor cells and the microenvironment are increasingly recognized to influence cancer progression. The extracellular matrix in tumor tissue is thought to be of critical importance in controlling the migratory behavior of tumor cells as well as the delivery of therapeutic agents into the tumor (22). Fibronectin is a matrix glycoprotein that plays an important role in cellular attachment and cell spreading, and its expression is increased in carcinomas, especially in NSCLC (4, 5). Studies from this group have shown that fibronectin stimulates human lung carcinoma cell growth through up-regulation of both COX-2 expression and prostaglandin E2 production (7). This is consistent with other reports showing the positive correlation between expression of fibronectin and aggressive lung carcinoma behavior (7–11). However, other than signals related to COX-2 activation, the intracellular pathways mediating the effect of fibronectin have not been entirely elucidated. In this study, we show that fibronectin stimulates the phosphorylation of Akt, an upstream positive modulator of mTOR, and down-regulates the expression of LKB1 and AMPK, upstream down-regulators of mTOR. These events induce the activation of mTOR as highlighted by the phosphorylation of a downstream target of mTOR, p70S6K, and result in increased NSCLC proliferation. The demonstration that fibronectin stimulates Akt phosphorylation supports a role for fibronectin in activating the PI3K signaling pathway. Activation of PI3K seems to play a key role in...
both malignant transformation and resistance to chemotherapy by perpetuating processes that positively influence cell survival, growth, migration, and angiogenesis. Aberrations of PI3K/Akt, which increase signaling through mTOR, are found in several types of malignancies, most notably prostate and breast cancers (12). Moreover, gene amplifications of these kinase signals have been identified in cervical, ovarian, gastric, pancreatic, head and neck, and NSCLCs (12, 23). Of note, it seems that the primary pathway by which most growth factors and cytokines activate mTOR and its downstream targets is the PI3K/Akt pathway. Consistent with this statement, PI3K inhibitors, wortmannin or LY294002, have been shown to inhibit mTOR in several cell systems (24, 25). For example, wortmannin reduced the epidermal growth factor (EGF)–stimulated phosphorylation of both Akt and p70S6K, whereas it had no effect on EGF-induced ERK phosphorylation in human trophoblast cells (26). LY294002 was shown to inhibit mTOR and block the human inducible nitric oxide synthase gene transcription in lung epithelial adenocarcinoma (A549) cells (25). As we found in NSCLCs (this report), fibronectin, through its integrin α5β1, has been shown to activate the PI3K/Akt pathway in several other cell systems (27, 28). Recently, others have shown that wortmannin and LY294002 blocked fibronectin-induced activation of mTOR/S6K signaling in human arterial smooth muscle cells (29). We also found that fibronectin reduced the protein levels of PTEN. PTEN suppression tumor cell growth by antagonizing protein tyrosine kinases, such as PI3K/Akt, and regulates tumor cell invasion and metastasis through interactions at focal adhesions (30). Loss of PTEN function results in Akt phosphorylation with subsequent stimulation of cell proliferation (30). PTEN heterozygous mice spontaneously develop neoplasia, associated with loss of the normal PTEN allele and increased activation of Akt/mTOR/P70S6K signaling (31). Therefore, up-regulation of mTOR can be related to loss of the tumor suppressor gene PTEN and activation of Akt, and both seem to be affected by fibronectin.

To test the role of Akt in fibronectin-induced NSCLC proliferation, we blocked Akt expression using Akt siRNA. The targeting of Akt by Akt siRNA inhibited basal NSCLC cell growth and significantly blocked fibronectin-induced cell proliferation suggesting a role for Akt activation in this process. This is particularly interesting in view of related work showing that knocking out Akt using specific siRNAs increases the sensitivity of cancer cells towards cisplatin and enhances programmed cell death (27).

We also examined the effects of fibronectin on the tumor suppressor gene LKB1, a serine/threonine kinase that is inactivated by mutation in the autosomal-dominant Peutz-Jeghers syndrome (20) as well as in some sporadic lung adenocarcinomas (24). As a major upstream kinase of AMPK, LKB1 directly phosphorylates Thr172 in the activation loop of AMPK, a modification that is absolutely required for AMPK catalytic activity (20). Activation of AMPK inhibits mTOR signaling and is associated with inhibition of cancer cell growth (22, 32). In cell culture–based systems, LKB1 modulation of mTOR is mediated through phosphorylation of...
Figure 4. Fibronectin stimulates phosphorylation of p70S6K and 4E-BP1 protein levels, and blocking α5β1 binding but not ERK1/2 signals prevents fibronectin-induced p70S6K and 4E-BP1 phosphorylation. A, fibronectin stimulation of p70S6K phosphorylation is dose dependent. Cellular protein was isolated from H1792 cells that were cultured with increasing concentrations of fibronectin (0–50 μg/mL) coated in the culture plates for 24 hours followed by Western blot analysis with antibodies against p-p70S6K and total p70S6K protein. B, fibronectin stimulation of p70S6K phosphorylation is time dependent. Cellular protein was isolated from H1792 cells that were cultured with fibronectin (20 μg/mL) coated in the culture plates for increasing periods of time (0–48 hours) followed by Western blot analysis with antibodies against p-p70S6K and total p70S6K protein. C, fibronectin stimulation of 4E-BP1 phosphorylation is dose dependent. Cellular protein was isolated from H1792 cells that were cultured with increasing concentrations of fibronectin (0–50 μg/mL) coated in the culture plates. Afterwards, Western blot analysis was done using antibodies against p-4E-BP1 and total 4E-BP1 protein. D, fibronectin stimulation of 4E-BP1 phosphorylation is time dependent. Cellular protein was isolated from H1792 cells that were cultured with fibronectin (20 μg/mL) coated in the culture plates for up to 48 hours followed by Western blot analysis for p-4E-BP1 and total 4E-BP1 protein. E, effect of anti-integrin antibodies on fibronectin-induced p-p70S6K. Cellular protein was isolated from H1792 cells cultured for up to 24 hours in the presence or absence of anti-α5β1 antibody (JBS5) or anti-α2 antibody (AB1944; 25 μg/mL each) before exposing the cells to fibronectin-coated plates for an additional 24 hours and then subjected to Western blot analysis for p70S6K or p-p70S6K. Note that fibronectin induced p-p70S6K, and this effect was inhibited with the anti-α5 antibody but not by the anti-α2 antibody. F, effects of MEK-1/ERK pathway inhibitor. Cellular protein was isolated from H1792 cells cultured for up to 2 hours in the presence or absence of PD98059 (25 μmol/L) before exposing the cells to fibronectin-coated culture plates for an additional 24 hours and then subjected to Western blot analysis for p70S6K and p-p70S6K. Note that PD98059 did not inhibit the stimulatory effect of fibronectin. Right, summary of results with more than three experiments. Actin served as internal control for normalization purposes. *, P < 0.05, significant difference from control. **, significance of combination treatment compared with fibronectin alone. Con, untreated control cells.
AMPK in response to energy stress. AMPK has been shown to diminish protein synthesis by activating Tsc2, leading to inhibition of mTOR and subsequent inhibition of p70S6K protein activity (20, 33). We found that fibronectin inhibited LKB1 protein expression as well as the phosphorylation of AMPK\(\alpha\) in NSCLC cells. Because this observation was associated with activation of p70S6K phosphorylation, we postulate that fibronectin stimulates mTOR through activation of PI3K and Akt phosphorylation, while concomitantly inhibiting LKB1/AMPK signals. In another work, we explored the connection between the mTOR downstream signal p70S6K and AMPK. We found that AICAR, a specific AMPK activator (34), abrogated not only the effect of fibronectin on reduction of AMPK but also the fibronectin-induced phosphorylation of p70S6K, suggesting a cross-talk between the AMPK and p70S6K in mediating the effect of fibronectin in stimulation of cell growth.\(^4\) In contrast with other studies, which showed cross-talk among Akt, MEK-1/ERK, and AMPK (32, 35, 36), we found that the

\[ p70S6K \text{ and AMPK} \]

\(^4\) S.W. Han et al., in preparation.
effect of fibronectin on reduction of AMPK protein expression was unaffected by inhibitors of PI3K (wortmannin) or MEK-1/ERK (PD98095), suggesting that PI3K and ERK signaling pathways are not involved in this process.

We previously reported that fibronectin induced NSCLC proliferation through effects on its integrin receptor $\alpha_5\beta_1$ (7). This is consistent with data reported here showing that antibodies against $\alpha_5\beta_1$ blocked the stimulatory effect of fibronectin on p70S6K phosphorylation. In another work, we also reported that fibronectin/ $\alpha_5\beta_1$ binding results in ERK activation, and this is the reason we tested the role of ERK in this system (7). However, the MEK-1/ERK inhibitor PD98095 did not block fibronectin-induced p70S6K phosphorylation, suggesting the induction of an ERK-independent pathway. This is consistent with another work showing that the MEK-1/ERK inhibitor U0126 decreased cell migration and ERK phosphorylation but did not influence p70S6K phosphorylation in response to EGF in human trophoblast cells (25).

We assume that fibronectin stimulates mTOR on the observed phosphorylation of the mTOR downstream targets p70S6K and 4E-BP1. p70S6K is a key regulator of cell size and growth that is modulated via both PI3K and mTOR. Overexpression of p70S6K has been found in many tumor cells and thus results in increased tumor size in vivo, whereas its blockade of p70S6K prevents cell proliferation (37–40). Interestingly, we also found increased phosphorylation of 4E-BP1 in cells exposed to fibronectin. elf4F consists of three polypeptides (elf4A, elf4G, and elf4E) and is responsible for recruiting ribosomes to mRNA. elf4E recognizes the mRNA 5'-cap structure (m7GpppN) and plays a pivotal role in control of translation initiation, which is the rate-limiting step in translation. Phosphorylation of 4E-BP1 results in the release of elf4E, and activation of elf4E is considered an essential component of the malignant phenotype in breast carcinoma (41). Furthermore, growth and survival factors positively regulate assembly of the elf4F complex by promoting sequential phosphorylation of the 4E-BPs on six serine/threonine sites through multiple Ras-dependent protein kinase cascades (42).

We then tested the effects of the mTOR inhibitor rapamycin in our system. Rapamycin and its analogues are highly specific inhibitors of mTOR (12). Treatment with rapamycin inhibits EGF-induced phosphorylation and activation of p70S6K (43). Recently, Sullivan et al. found that fibronectin induced rapid activation of mTOR and p70S6K in human arterial smooth muscle cells, and rapamycin inhibited this effect (34). Consistent with this, we found that rapamycin blocked the fibronectin-induced phosphorylation of p70S6K and 4E-BP1, and these effects were associated with inhibition of fibronectin-induced NSCLC cell growth. Blockade of Akt with Akt siRNA enhanced the effect of rapamycin, suggesting that they participate in these pathways. This work and a recent study showing that dominant-negative Akt expression or inhibitors of PI3K/Akt are sufficient to increase the sensitivity of tumor cells to rapamycin show that disruption of the PI3K/Akt signaling pathway could greatly enhance the effectiveness of mTOR inhibitors (44). Therefore, therapeutic targeting of mTOR might be a more efficient strategy against malignancies driven by activation of Akt and/or PTEN mutations (45). Rapamycin and several of its analogues with more favorable pharmacological properties have shown impressive growth inhibitory effects against a broad range of human cancers in both preclinical and early clinical evaluations, including NSCLC (12).

Taken together, these findings show that fibronectin, by binding to its integrin receptor $\alpha_5\beta_1$, stimulates NSCLC proliferation through activation of the Akt/mTOR/p70S6K pathway, while inhibiting the PTEN and LKB1/AMPK signals. These studies raise new questions about the potential role of the lung extracellular matrix in lung cancer. Furthermore, they suggest that targeting these signaling pathways might aid in the development of novel agents with therapeutic potential for the prevention and treatment of human lung carcinoma. As inhibitors of mTOR and Akt are brought forward into the clinic, agents blocking fibronectin-mediated lung cancer signaling may provide intriguing complementary effects.

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