Eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1) function is suppressed by Src and PP2A on extracellular matrix

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Running title: Integrin-collagen interaction suppresses 4EBP-1 function

Human lung fibroblasts utilize integrins to attach and proliferate on type I collagen. β1 integrin is the major integrin subunit for this attachment. Integrins coordinate cellular responses to cell-cell and cell-extracellular matrix interactions that regulate a variety of biological processes. Although β1 integrin-mediated signaling pathways in lung fibroblasts have been studied, a detailed molecular mechanism regulating translational control of gene expression by 4EBP-1 is not understood. 4EBP-1 inhibits cap-dependent translation by binding to the eIF4E translation initiation factor. We found that when lung fibroblasts attach to collagen via β1 integrin, high Src activity suppresses 4EBP-1 expression via PP2A and the decrease of 4EBP-1 is due to protein degradation. The inhibition of Src activity dramatically increases PP2A and 4EBP-1 expression. Furthermore ectopic expression of PP2A, or PP2A silencing using PP2A siRNA confirmed that 4EBP-1 is regulated by PP2A. In addition, we found that 4EBP-1 inhibition by fibroblast attachment to collagen increases cap-dependent translation. Our study showed that when lung fibroblasts are attached to collagen matrix, β1-integrin/Src/PP2A-mediated 4EBP-1 regulatory pathway is activated. We suggest that β1-integrin-mediated signaling pathway may be a crucial event in regulating fibroblast translational control machinery on collagen matrix.

Introduction
Integrins coordinate cellular responses to cell-cell and cell-extracellular matrix interactions that regulate a variety of biological processes including proliferation and viability (1-4). The α and β chains cooperate in signaling such that ligand binding specificity resides predominantly within the α chain and intracellular signaling molecules largely associate with the intracellular domain of the β chain (5). Among integrins, β1 integrin is the major integrin subunit that mediates fibroblast attachment to type I collagen (6). Seminal studies showed that once fibroblasts attach to type I collagen, several crucial signaling pathways are activated, conferring fibroblast fates such as proliferation, migration and apoptosis. Among them, eIF4E-binding protein-1 (4EBP-1) is a PI3K/Akt downstream target protein and negatively regulates the translational function of eIF4E, the mRNA cap-binding protein, and prevents binding of eIF4E to eIF4G, a large scaffolding protein (7,8). Studies showed that non phosphorylated 4EBP-1 binds tightly to eIF4E, thereby inhibiting a key step in translation initiation (9-12).

When fibroblasts attach to type I collagen, PI3K/Akt activity increases via reduced PTEN function (13,14). High Akt activity allows cells to progress G0 to G1 by inhibiting expression of the cell cycle inhibitor protein p27, promoting fibroblast proliferation (14,15). Furthermore, serine/threonine phosphatase PP2A activity is also suppressed by fibroblast attachment to type I collagen (14). One of the upstream proteins that is known to regulate PP2A function is Src kinase (16-18). Src phosphorylates tyrosine 307 residue of PP2A and inactivates its activity (19,20). These studies suggested that β1 integrin-collagen interaction may inhibit PP2A by high Src activity. Furthermore a prior study suggested that 4EBP-1 can be a potential substrate of PP2A (21) and the inhibition of 4EBP-1 increases cell proliferation (22,23). Thus these findings provide a possibility that type I collagen-integrin interaction inhibits 4EBP-1 via a Src/PP2A-dependent pathway. Therefore we hypothesized that
when fibroblasts attach to collagen, 4EBP-1 function is inhibited through the suppression of PP2A as a result of high Src activity, thereby increasing the activity of cap-dependent translational control machinery.

To examine our hypothesis, we measured 4EBP-1 expression in response to fibroblast attachment to collagen as a function of time. Fibroblast interaction with type I collagen via a β1 integrin promotes a decrease in 4EBP-1 protein expression. The decrease of 4EBP-1 is due to protein degradation. We also found that β1 integrin is a crucial integrin subunit that is responsible for the suppression of PP2A expression. Furthermore, PP2A function is inhibited by high Src kinase activity when fibroblasts attached to collagen, which subsequently inhibits 4EBP-1 expression. In contrast, ectopic expression of PP2A enhanced 4EBP-1 level in response to fibroblast attachment to collagen and the inhibition of Src kinase increased 4EBP-1 via high PP2A activity. Our study suggests that in response to type I collagen matrix, β1 integrin modulates translational control machinery through a Src/PP2A/4EBP-1-dependent mechanism.

Materials and Methods

Cell culture and type I collagen matrices. For this study, the normal human lung fibroblast line HLF-210 was used (purchased from ATCC). HLF-210 fibroblasts were cultured in high glucose DMEM containing 10% fetal calf serum (FCS). The fibroblasts were used between passages 5-8 for all experiments. Type I collagen matrix was purchased from Advanced Biomatrix, CA and prepared by coating petri dishes with 100 µg/ml of type I collagen solution. The dishes were washed twice with DMEM prior to plating the cells. Fibroblasts were cultured on the surface of the collagen coated dishes as previously described (24).

Antibodies and Chemicals. Anti-4EBP-1, Thr 35/46 4EBP-1, Thr 70 4EBP-1, 4EBP-2, Src, non-phosphorylated-Src-tyr 527 and phosphorylated-Src-tyr-416 antibodies were obtained from Cell Signaling Technologies, MA. eIF4E antibody was purchased from BD Transduction Laboratories. Rabbit anti-eIF4G antibody was a gift from Nahum Sonenberg. PP2A antibody was obtained from Millipore, MA. GAPDH and actin antibodies were purchased from Santa Cruz Biotechnologies, CA. β1 integrin activating monoclonal antibody TS2/16 was produced from hybridoma culture (ATCC, HB-243). β1 integrin blocking antibody P5D2 was also produced from hybridoma culture. α1 to α5 and αv, β3 integrin blocking antibodies were purchased from Chemicon, MA. Src inhibitor 1, PP2A inhibitor (okadaic acid), lactacystin and proteasome inhibitor were purchased from Calbiochem, CA.

Adenovirus constructs, siRNA and quantitative RT-PCR. The PP2A adenovirus expressing HA-tagged PP2A catalytic subunit was kindly provided by Dr. Alexander Verin (University of Chicago). The cells were infected with adenoviral vectors at a multiplicity of infection (MOI) of 20. Adenovirus expressing HA tagged Src or wild type or transactivation domain deleted FoxO3a was purchased from Vector BioLabs, PA. For 4EBP-1 expression, pShuttle-CMV-4EBP-1-construct and empty vector were kindly provided from Dr. Karen Smith at the University of Minnesota. For quantitative PCR (qPCR) assay, human lung cells were serum starved for 2 days followed by plating on a collagen coated plate for the indicated time as described. Cells were then collected and total RNA was isolated using TRIzol. cDNA was prepared using random hexamer primers with purified total RNA (1 µg) from each sample, and qPCR was carried out with 4EBP-1 sense primer, 5'-AAATTCCTGATGGAGTGTCG-3’; 4EBP-1 antisense primer, 5'-CATCTCAAACGATGACTTCTT-3’ and GAPDH sense primer, 5’-TGC ACC ACC AAC TGC TTA GC-3’; GAPDH antisense primer, 5’-GTC ATC GAC TGT GGT CAT GAG-3’. Quantitative PCR was carried out using the Roche Light-Cycler with SYBR Green (Roche). An annealing temperature for 4EBP-1 and GAPDH was 55°C and 58°C, respectively. Relative mRNA levels were normalized with GAPDH. For siRNA transfection experiments, human lung fibroblasts were transfected with 100 nM of pre-made human PP2A siRNA, Src siRNA, β1 integrin siRNA, α10 and α11 integrin subunit siRNA or a control non-silencing siRNA obtained from Santa Cruz Biotechnology, Inc. using X-tremeGENE siRNA transfection reagent (Roche, Germany) according to manufacturer’s protocol.

PP2A, Src activity and cap-binding assay. For PP2A activity assay, fibroblasts treated with various doses of a Src inhibitor were lysed in lysis buffer
containing 2 mM imidazole (pH 7.0), 2 mM EDTA, 2 mM EGTA, 2% NP-40 and 1X protease inhibitor cocktail (Calbiochem, La Jolla, CA). Total protein levels were measured from the resulting lysates. PP2A catalytic subunit protein was then immunoprecipitated from lysates containing equal amounts of protein by incubation with 2 µg of anti-PP2A antibody overnight at 4°C. The immunoprecipitates were washed twice with TBS buffer followed by two additional washes with phosphatase reaction buffer (50 mM Tris pH 7.4; 10 mM NaCl; 1 mM EDTA; 1 mM DTT). PP2A activity was assayed using a Malachite green phosphatase kit (Echelon) according to the manufacturer’s instructions. Src activity based on tyrosine phosphorylated 416 in fibroblasts on collagen was performed using STAR Src activity assay kit according to manufacture’s protocol (Millipore, MA). Briefly, fibroblasts attached on collagen were collected as a function of time followed by lysis using cold 1X RIPA buffer containing protease inhibitors. 100 µl of standard or samples were then added to 96 well plates coated with a Src capture antibody and incubated for 2 h at room temperature. 100 µl of the detection antibody was added to each well and further incubated for 1 h. After washing, 1:100 diluted anti-rabbit IgG HRP conjugate was added to each well and further incubated for 45 min. After TMB solution was added to each well and brief incubation for 15 min, the reaction was stopped and read at 450 nm using a light microscopy further demonstrated that cell attachment progressively increased on collagen coated plate whereas cells were not able to efficiently attach to non coated plate (Fig 1B). These data show that fibroblast attachment to type I collagen decreases 4EBP-1 expression as a function of time. We next examined whether cell attachment to extracellular matrices such as fibronectin or laminin also inhibits 4EBP-1 expression levels. Like type I collagen, 4EBP -1 levels decreased rapidly when fibroblasts attach to fibronectin coated plates (Fig 1C, upper left and right). In contrast, 4EBP -1 levels moderately decreased on laminin coated plates (lower). These data suggest that the degree of 4EBP-1 protein decrease was dependent upon ECM.

Fibroblasts utilized integrins to attach type I collagen (2). Since α2β1 integrin is a major receptor for type I collagen, we next examined the role of α2β1 integrin on collagen matrix. Serum starved lung fibroblasts were pre-incubated with α2 and/or β1 integrin blocking antibody and allowed to attach to collagen. 4EBP-1 expression level was higher when fibroblasts were pre-incubated with a β1 integrin blocking antibody (Fig 2A upper lane 3 and lower). 4EBP-1 level marginally increased in the presence of α2 integrin blocking antibody when compare to an isotype control antibody (lane 2). The combination of α2 and β1 integrin blocking antibodies had a synergistic effect in preventing the decrease in 4EBP-1 protein expression (lane 4). In contrast, 4EBP-1 expression levels were low in fibroblasts pre-incubated with isotype control antibody (lane 5). These data demonstrated that when fibroblasts are attached to type I collagen, β1 integrin is mainly responsible for the suppression of 4EBP-1 expression.

Results

4EBP-1 expression decreases on type I collagen via integrin. We have previously shown that when fibroblasts attach to collagen, PI3K/Akt activity increases while PP2A expression and activity was suppressed, promoting fibroblast proliferation (13). Since 4EBP-1 is an inhibitor of eIF4E and a potential downstream target of PP2A, we hypothesized that integrin-collagen interaction suppressed 4EBP-1, thereby increasing cap-dependent protein translation. To test this hypothesis, serum starved lung fibroblasts were first attached to type I collagen coated plates and 4EBP-1 expression levels were measured as a function of time. 4EBP-1 expression progressively decreased on collagen (Fig 1A, left and right) while 4EBP-1 protein levels remained relatively unaltered on non coated plate. The attachment to type I collagen measured by a light microscopy further demonstrated that cell attachment progressively increased on collagen coated plate whereas cells were not able to efficiently attach to non coated plate (Fig 1B). These data show that fibroblast attachment to type I collagen decreases 4EBP-1 expression as a function of time. We next examined whether cell attachment to extracellular matrices such as fibronectin or laminin also inhibits 4EBP-1 expression levels. Like type I collagen, 4EBP-1 levels decreased rapidly when fibroblasts attach to fibronectin coated plates (Fig 1C, upper left and right). In contrast, 4EBP-1 levels moderately decreased on laminin coated plates (lower). These data suggest that the degree of 4EBP-1 protein decrease was dependent upon ECM.

Fibroblasts utilized integrins to attach type I collagen (2). Since α2β1 integrin is a major receptor for type I collagen, we next examined the role of α2β1 integrin on collagen matrix. Serum starved lung fibroblasts were pre-incubated with α2 and/or β1 integrin blocking antibody and allowed to attach to collagen. 4EBP-1 expression level was higher when fibroblasts were pre-incubated with a β1 integrin blocking antibody (Fig 2A upper lane 3 and lower). 4EBP-1 level marginally increased in the presence of α2 integrin blocking antibody when compare to an isotype control antibody (lane 2). The combination of α2 and β1 integrin blocking antibodies had a synergistic effect in preventing the decrease in 4EBP-1 protein expression (lane 4). In contrast, 4EBP-1 expression levels were low in fibroblasts pre-incubated with isotype control antibody (lane 5). These data demonstrated that when fibroblasts are attached to type I collagen, β1 integrin is mainly responsible for the suppression of 4EBP-1 expression.

A prior study showed that 4EBP-1 is phosphorylated by up-stream kinases which
facilitates the dissociation from eIF4E or subsequent phosphorylation of 4EBP-1 (26-29). To test whether fibroblast attachment to collagen increases phosphorylation of 4EBP-1 via α2 or β1 integrin subunit, we measured the phosphorylated 4EBP-1 levels in the presence or absence of α2, β1 or both integrin blocking antibodies on collagen. Unlike 4EBP-1 expression on collagen matrix, thr 37/46 phosphorylation level was relatively low and did not significantly change in the presence of α2 or β1 integrin blocking antibody (Fig 2B, upper and lower). Thr 70 phosphorylation was also barely detected in the presence or absence of integrin blocking antibody. Furthermore we found that 4EBP-2 level was slightly elevated when cells were treated with α2 and β1 integrin blocking antibodies together. These data suggested that unlike 4EBP-1, the phosphorylation of 4EBP-1 or 4EBP-2 expression may not be significantly altered in response to fibroblast attachment to collagen. Collectively, our data suggest that when lung fibroblasts attach to collagen, 4EBP-1 is predominantly regulated by a β1 integrin.

**PP2A decreases when fibroblasts are attached to collagen via β1 integrin.** Prior studies suggested that PP2A has been implicated with the regulation of 4EBP-1 (30,31). Therefore, in order to test integrin function on PP2A expression, we first measured PP2A catalytic expression on collagen coated plates as a function of time. PP2A progressively decreased when fibroblasts attached to collagen coated plates while PP2A level remained relatively unaltered on a non coated plate (Fig 3A, upper and lower). We next examined whether PP2A decrease is also due to cellular attachment to collagen via various α or β integrin subunits. To test this, serum starved lung fibroblasts were attached type I collagen in the presence or absence of α or β integrin subunit specific blocking antibodies and PP2A expression was measured by Western analysis. Like 4EBP-1 expression level, PP2A protein expression was preserved when fibroblasts were attached to collagen in the presence of β1 integrin blocking antibody (Fig 3B, upper lane 7 and lower). In contrast, when fibroblasts attached to collagen in the presence of α1, α2, α5 integrin blocking antibodies, PP2A proteins were moderately increased (Fig 3B, lower). We further tested other collagen binding α integrin subunits such as α10 and α11 on PP2A protein regulation. When α10 protein was silenced, PP2A protein was highly elevated (Supplemental data Fig A and Fig B upper). In contrast, the inhibition of α11 subunit protein had very minimum effect on PP2A expression. These findings strongly suggest that although β1 integrin-collagen interaction plays a crucial role in the suppression of PP2A expression, PP2A regulatory signaling pathway is also dependent upon a variety of α subunits such as α1, α2, α5 and α10, and the variable combinations of α and β1 integrin subunits differently modulate PP2A protein expression.

### High Src activity suppresses PP2A function.

Previous studies showed that Src kinase suppresses PP2A, and the expression of non phosphorylated Src tyr-527 and/or phosphorylation of tyr 416 in the activation loop of the kinase domain increases its activity (32,33). Therefore we hypothesized that high Src activity in fibroblasts on collagen inhibits PP2A via β1 integrin. To test this, we first measured non-phosphorylated Src tyr 527 expression in response to fibroblast attachment to collagen as a function of time. Non phosphorylated Src level progressively increased on collagen (Fig 4A, upper and middle), showing that Src activity increases when fibroblasts attach to collagen. To verify Src activity by the phosphorylation of tyr 416 on collagen, we utilized a Src activity assay kit. Interestingly, tyr 416 phosphorylation levels were high at 15 min time point and gradually decreases at later time points (Fig 4A, lower). These data suggest that when lung fibroblasts attach to type I collagen, Src becomes active at early time point by predominately phosphorylating tyr 416 residue whereas Src activity by dephosphorylating Src tyr 527 increases at later time points. Collectively these results show that Src activity increases when fibroblasts attach to collagen by utilizing different tyr phosphorylation residues of Src protein. We next examined whether Src functions to regulate PP2A expression on collagen by using a Src inhibitor. PP2A expression remained relatively unaltered when cells pre-incubated with the Src inhibitor were attached to collagen (Fig 4B). Furthermore, PP2A expression progressively increased in a dose-dependent fashion (Fig 4C, upper and lower). These data suggest that Src inhibits PP2A expression on collagen. To confirm these findings, we performed a PP2A activity assay in the presence of various doses of Src inhibitor. PP2A activity also progressively
increased when Src inhibitor concentration was increased on collagen (Fig 4D). These data demonstrated that Src has a function in inhibiting PP2A expression and activity on collagen.

**Src suppresses PP2A and 4EBP-1 expression.** Our results suggest the possibility that high Src activity inhibits 4EBP-1 via PP2A when fibroblasts attach to type I collagen. To explore this, we first examined whether PP2A can regulate 4EBP-1 expression. We ectopically expressed PP2A catalytic subunit and measured whether PP2A protein induction can increase 4EBP-1 expression on collagen. 4EBP-1 expression was high when PP2A was over-expressed (Fig 5A). This finding showed that when PP2A protein is reconstituted in cells cultured on collagen, 4EBP-1 expression remains high. Our data showed that when fibroblasts were serum starved, PP2A and 4EBP-1 expression levels were high. To further elucidate the role of PP2A in regulating 4EBP-1, we next inhibit PP2A under serum starved condition and examined 4EBP-1 level. 4EBP-1 expression was low when PP2A was silenced by PP2A siRNA (Fig 5B, left panel). Likewise, we found that 4EBP-1 expression was also reduced when PP2A was silenced in the presence of serum (Fig 5B, right). These data demonstrated that PP2A regulates 4EBP-1 expression. To elucidate whether Src regulates 4EBP-1 via PP2A, we next examined 4EBP-1 expression in the presence of various doses of Src inhibitor on collagen matrix. 4EBP-1 expression progressively increased as Src inhibitor concentration increased (Fig 5C, left and right). To confirm this finding, we utilized adenovirus expressing Src protein and measured PP2A and 4EBP-1. When Src was over-expressed, PP2A protein level was low (Fig 5D). Likewise, 4EBP-1 expression level was also suppressed. We further examined PP2A and 4EBP-1 levels on collagen coated plates using Src siRNA. Unlike PP2A and 4EBP-1 expression levels on collagen coated plates, PP2A expression increased when Src protein was silenced (Fig 5E). 4EBP-1 protein level was also high in the presence of Src siRNA. Collectively our results showed that when fibroblasts attach to collagen, high Src activity suppresses PP2A, and this low PP2A activity can be responsible for 4EBP-1 inhibition.

**β1 integrin regulates 4EBP-1 through Src/PP2A-dependent pathway.** Our data suggest that β1 integrin inhibits 4EBP-1 via PP2A. In order to confirm this, we first examined the levels of 4EBP-1 using PP2A siRNA in the presence or absence of β1 integrin blocking antibody on collagen. When control siRNA transfected cells were attached to collagen in the presence of β1 integrin blocking antibody, 4EBP-1 level was high (Fig 6A, lane 2 and right panel). However when cells transfected with PP2A siRNA were attached on collagen, 4EBP-1 protein level remained low in the presence of β1 integrin blocking antibody (lane 3). Similarly we found that 4EBP-1 level was low when PP2A protein was silenced by PP2A siRNA in the presence of isotype control antibody (lanes 4, 5, right panel). We further examined this finding using β1 integrin blocking antibody and β1 integrin siRNA. Cells pre-incubated with β1 integrin blocking antibody were cultured on a collagen-coated plate and 4EBP-1 level was examined. 4EBP-1 level was high when β1 integrin blocking antibody was used (Fig 6B, upper). To confirm this, β1 integrin was silenced by β1 integrin siRNA and 4EBP-1 expression was measured on collagen coated plates. Like the case of β1 integrin blocking antibody, 4EBP-1 level was also low when β1 integrin siRNA was used (Fig 6B, lower, 4EBP-1 level in the presence of α10 and α11 siRNA is also shown in the supplemental data). Taken together, these data demonstrated that β1 integrin inhibits 4EBP-1.

We next examined whether the β1 integrin suppresses PP2A and 4EBP-1 expression via Src. Lung fibroblasts were ligated with a β1 integrin activating antibody TS216 in the presence of various concentrations of Src inhibitor and PP2A expression was measured. PP2A level was decreased when cells were ligated with only TS216 (Fig 6C, lane 2, upper and lower). However PP2A protein levels progressively increased when fibroblasts attached to collagen in the presence of increasing doses of Src inhibitor (lanes 3&4). We next measured 4EBP-1 level when cells were ligated with TS216 in the presence of Src inhibitor. 4EBP-1 expression decreased when cells were treated with DMSO control (Fig 6D, upper, lane 4 and lower). However, 4EBP-1 level remained high when cells were pre-treated with Src inhibitor (lane 2). Collectively, our results demonstrated that cell attachment to collagen via β1 integrin suppresses PP2A function via high Src activity, which results in the inhibition of 4EBP-1 protein expression.
Our results showed that when fibroblasts attach to type I collagen, 4EBP-1 expression decreased as a function of time. We next sought to examine the underlying mechanism of the decreases in 4EBP-1 protein expression on collagen coated plates. We first examined whether low 4EBP-1 mRNA is responsible for 4EBP-1 protein levels on collagen. Unlike 4EBP-1 protein level on collagen, quantitative PCR analysis demonstrated that 4EBP-1 mRNA levels did not decrease as a function of time (Fig 6E). These data showed that when fibroblasts attach to collagen, low 4EBP-1 protein expression is not due to transcriptional suppression. A prior study showed that 4EBP-1 is degraded by the proteasome (38). Therefore we next examined whether 4EBP-1 becomes degraded in response to fibroblast attachment to collagen via proteasome. 4EBP-1 levels were not altered when cells were pre-incubated with proteasome inhibitor (Fig 6F, upper lane 3 and lower). 4EBP-1 expression was also moderately high in the presence of lactacystin. Furthermore, 4EBP-1 levels progressively increased when a various doses of proteasome inhibitor was used (Fig 6G, upper and lower). Collectively, our data showed that when fibroblasts attach to collagen, 4EBP-1 protein becomes degraded.

Collagen-β1 integrin interaction increases cap-dependent translation via low 4EBP-1 function on collagen. Our results showed that when fibroblasts attach to collagen, 4EBP-1 expression is suppressed. In order to examine whether β1 integrin-collagen interaction increases eIF4G activity via low 4EBP-1 function thereby promoting cap-dependent translation, we performed cap-binding assay to elucidate the functional role of 4EBP-1 in the presence or absence of α2 or β1 integrin blocking antibody on collagen matrix. eIF4E protein levels were unaltered in the presence of both α2 and β1 integrin blocking antibodies when cells were placed on collagen (Fig 7A, upper panel). In contrast, when cells were treated with β1 integrin blocking antibody, 4EBP-1 level was high and 4EBP-1 expression was substantially higher when cells were pre-incubated with both α2 and β1 integrin blocking antibodies (upper panel lane 4). Similar to 4EBP-1 expression levels on collagen in the presence of integrin blocking antibodies, cap-binding assay demonstrated that 4EBP-1 activity was high when cells were pre-incubated with β1 or α2 and β1 integrin blocking antibodies together (Fig 7A, lower panel, lanes 3&4, respectively). The ratio of eIF4G/4EBP-1 protein levels demonstrated that when β1 integrin function was inhibited, 4EBP-1 activity was high and eIF4G activity was suppressed (Fig 7B). Furthermore, the combination of α2 and β1 integrin blocking antibodies synergistically suppressed eIF4G function by high 4EBP-1 expression. In contrast, when cells were attached to collagen in the presence of isotype control antibody, eIF4G activity was high due to low 4EBP-1 activity. Taken together, these data demonstrated that when fibroblasts attach to collagen, eIF4G activity increases as a result of low 4EBP-1 activity, thereby promoting cap-dependent translational machinery.

Src, PP2A and 4EBP-1 regulate fibroblast proliferation. We have previously shown that fibroblast proliferation increases on type I collagen (14,24). In this study, we further elucidate that when fibroblasts attach to collagen, high Src activity suppresses PP2A function, thereby inhibiting 4EBP-1. Since the suppression of 4EBP-1 promotes cell proliferation (22,23), we next examined whether the inhibition of 4EBP-1 as a result of high Src and low PP2A activities regulates fibroblast proliferation. To examine this, PP2A protein was silenced in fibroblasts and cell proliferation was measured using MTS assay. Fibroblast proliferation was increased 40% in the presence of PP2A siRNA (Fig 8A). In contrast, when PP2A was over-expressed, fibroblast proliferation was suppressed (Fig 8B). Likewise, when cells were infected with adenovirus expressing Src protein approximately 20% of fibroblast proliferation increased (Fig 8C, right). However, the proliferation was low when Src protein was silenced using siRNA (Fig 8C, left). Furthermore, like the case of PP2A protein, when wild type 4EBP-1 was over-expressed, fibroblast proliferation was suppressed (Fig 8D). Taken together, these data demonstrated that when fibroblasts attach to collagen, Src, PP2A and 4EBP-1 functions are important to regulate fibroblast proliferation.

Discussion
Cell attachment to extracellular matrix is a crucial event in matrix biology. Under normal physiological conditions, when β1 integrin interacts with type I collagen, it activates Akt and inhibits PP2A, thereby determining fibroblast’s fate. Our study showed that these events suppress eIF4E inhibitor protein, 4EBP-1, initiating cap-dependent translation by increasing
eIF4G activity. We showed that when fibroblasts interact with type I collagen via β1 integrin, activated Src suppresses PP2A, thereby inhibiting 4EBP-1 expression. 4EBP-1 is an important regulator for protein translation. 4EBP-1 is an inhibitor of eIF4E and the precise control of 4EBP-1 and eIF4E function is required for cells to regulate protein synthesis. Ribosome recruitment to mRNA is mediated by the eIF4 group of initiation factors. eIF4E recognizes the cap structure of mRNAs, initiating the translation process. 4EBP-1 can bind to eIF4E and prevents its association with eIF4G and incorporation into the eIF4F complex, which leads to inhibition of cap-dependent translation. Our prior study showed that when fibroblasts attach to type I collagen, high PI3K/Akt and low PP2A synergistically promotes fibroblast proliferation (13,24). When Akt is activated, eIF4E function is up-regulated, thereby promoting protein synthesis. 4EBP-1 takes part in as an antagonist in this event and a negative regulator of cell growth (34). 4EBP-1 is also known to be a potential PP2A down-stream protein. These findings also indicate that collagen integrin interaction may suppress 4EBP-1 function via low PP2A activity. Therefore we hypothesized that β1 integrin plays a key role in suppressing 4EBP-1 via PP2A, thereby increasing cap-dependent translation. To test our hypothesis, we first measured 4EBP-1 expression levels in lung fibroblasts on type I collagen. When cells were attached to type I collagen, 4EBP-1 level progressively decreased. In contrast, 4EBP-1 levels were high when PP2A was over-expressed. β1 integrin activating and blocking antibodies demonstrated that PP2A and 4EBP-1 function is dependent upon β1 integrin. These results further support our previous findings that β1 integrin plays an important role in conferring cell fate.

Studies have shown that the down-regulation of 4EBP-1 activity increases cell proliferation by activating eIF4E. In particular, the role of 4EBP-1 has been studied in cancer models and 4EBP-1 protein levels are frequently altered in a variety of tumors (35-37). This observation suggested us that fibroblast attachment to collagen matrix may alter 4EBP-1 function, thereby promoting cap-dependent translation. Since Src activity increases when fibroblasts attach to collagen, and PP2A and 4EBP-1 expression are inhibited via the β1 integrin, we further hypothesized that high Src activity inhibit 4EBP-1 function via PP2A when cells attach to collagen via β1 integrin. Our data showed that when fibroblasts attach to collagen via β1 integrin, 4EBP-1 function is inhibited by a Src/PP2A-dependent pathway which increases cap-dependent translation and cell proliferation (Fig 9). Cap-binding assay further demonstrated that low 4EBP-1 activity increases eIF4G activity when fibroblasts are interacted with collagen matrix and this event is a β1 integrin-dependent. Although PP2A function has been implicated with the regulation of cell growth by modulating 4EBP-1, to our knowledge, this is the first report that 4EBP-1 function is inhibited via β1 integrin on type I collagen. Our data support the notion that when fibroblasts are cultured on collagen, the precise orchestration of a series of kinase(s) and phosphatase(s) facilitates cap-dependent translational machinery via inhibiting 4EBP-1. In summary, we demonstrated that β1 integrin-collagen interaction is an important event in promoting cap-dependent translation by utilizing Src, PP2A and 4EBP-1. Based on our study, future study of β1 integrin function can reveal the significant molecular mechanisms of how cells respond to extracellular matrix and how the alteration of this integrin function can contribute to certain types of diseases.
References

Figure legends

Figure 1. 4EBP-1 protein expression decreases on extracellular matrix. A) Left, serum starved human lung fibroblasts were attached to collagen coated plates (100 μg/ml) as a function of time. 4EBP-1 protein levels were then measured. GAPDH was used as a loading control. P: non coated plastic plate as a negative control. Right, 4EBP-1/GAPDH expression ratio as a function of time on collagen. Assay was repeat at least three times. B) Shown are the phase-contrast microscopic cell morphologies as a function of time after plating lung fibroblasts on type I collagen coated plates. C) Left, human lung fibroblasts were attached to either fibronectin (100 μg/ml) or laminin coated plates (100 μg/ml) as a function of time and Western analysis was carried out to measure 4EBP-1 protein levels. Right, 4EBP-1 expression (4EBP-1/GAPDH expression) was measured. Assay was repeated 3 times.

Figure 2. β1 integrin collagen interaction suppresses 4EBP-1 expression. A) Upper panel, lung fibroblasts were serum starved followed by pre-incubation with α2 or β1 integrin or both blocking antibodies (1 μg/ml, respectively) for 45 min. Cells were then attached to type I collagen for 30 min and 4EBP-1 levels were measured. Actin was used as a loading control. α2 : α2 integrin blocking antibody. β1 : β1 integrin blocking antibody. α2+β1 : α2 and β1 blocking antibodies. IgG : isotype control antibody (1 μg/ml). Lower panel, 4EBP-1/GAPDH expression was measured. Assay was repeated 3 times. B) Western analysis was carried out to measure phospho-4EBP-1 (thr 37/46), phospho-4EBP-1 (thr 70) and 4EBP-2 expression levels on type I collagen in the presence of α2 or β1 integrin or both blocking antibodies (1 μg/ml, respectively). GAPDH was used as a loading control. Assay was repeated 3 times.

Figure 3. β1 integrin regulates PP2A expression on collagen. A) Upper, serum starved human lung fibroblasts were attached to collagen coated plates as a function of time and PP2A expression was measured. GAPDH was used as a loading control. P: non-coated plastic plate. Lower, PP2A expression (PP2A/GAPDH levels) was measured on collagen as a function of time. B) Upper, serum starved human lung fibroblasts were pre-incubated with α1 to α5 and αv integrin blocking antibodies or β1 and β3 integrin blocking antibodies (1 μg/ml, respectively) for 45 min followed by the attachment to type I collagen for 30 min. PP2A levels were then measured. GAPDH was used as a loading control. IgG : isotype control antibody. Lower, PP2A/GAPDH expression was measured in the presence of α or β integrin blocking antibodies on collagen as described above. Assay was repeated 3 times. *p=0.03 versus IgG isotype control.

Figure 4. High Src activity inhibits PP2A function on collagen. A) Upper, serum starved human lung fibroblasts were attached to collagen coated plates as a function of time and non-phosphorylated tyrosine Src 527 (NP-527-Src) and total Src (Src) levels were measured. Middle, NP-527-Src /Src protein levels were measured as a function of time. Assay was repeated at least 3 times. Lower, tyrosine phosphorylated-Src activity on collagen was measured as a function of time using a kit as described in the Materials and Methods. Note that when fibroblasts attach to collagen, Src activity based on tyr-416 phosphorylation increases early time point followed by decrease in later time point while Src activity based on NP-527-Src was low at early time point and progressively increased at later time point. *p=0.002, **p=0.0002, ***p=0.01 versus control. P: non coated plastic plate. B) Lung fibroblasts were pre-incubated with a 100 nM of Src inhibitor (SI) for 45 min followed by the attachment to 100 μg/ml of collagen coated plates for 30 min. PP2A and GAPDH levels were then measured. DMSO: DMSO control. C) Upper, serum starved human lung fibroblasts were pre-incubated with a 10 or 100 nM of Src inhibitor (SI) for 45 min. Cells were then attached to collagen coated plates for 30 min and Western analysis was carried out to measure PP2A expression levels. GAPDH was used as a loading control. S/S : serum starved cells. Lower, PP2A expression was measured in the presence of various doses of Src inhibitor on collagen. Assay were repeated 3 times. *p=0.03 versus DMSO control. D) Lung fibroblasts were pre-incubated with various doses of Src inhibitor (SI, 0.1 to 10 nM) followed by attachment to collagen coated plates for 30 min. Cells were then collected and PP2A activity assay was carried out as described in the Materials and Methods. *p=0.04, **p=0.03 versus DMSO control.
Figure 5. Src suppresses 4EBP-1 expression via PP2A. A) Serum starved lung fibroblasts infected with an adenovirus expressing PP2A or empty vector were attached to collagen coated plates for 30 min and Western analysis was carried out to measure PP2A, 4EBP-1 levels. GAPDH was used as a loading control. Ad : Adenovirus expressing PP2A or GFP protein. B) Left, lung fibroblasts transfected with 100 nM of PP2A (PP2A) or control siRNA (Con) for 24 h. Cells were then serum starved for a day and Western analysis was carried out to measure PP2A and 4EBP-1 levels. SD: serum starved. Right, lung fibroblasts transfected with 100 nM of PP2A or control siRNA were collected in the presence of serum and PP2A and 4EBP-1 levels were then measured. Actin was used as a loading control. Un : Untransfected cells, Con : control siRNA, PP2A : PP2A siRNA. C) Left, serum starved human lung fibroblasts (S/S) were pre-incubated with various doses of Src inhibitor (SI) or DMSO for 45 min. Cells were then attached to collagen for 30 min and PP2A, 4EBP-1 and GAPDH levels were measured. Right, 4EBP-1/GAPDH expression was measured under the same condition. Assay was repeated 3 times. *p=0.03 versus DMSO control. D) Fibroblasts were infected with an adenovirus constructs expressing Src (Src) or empty vector GFP. After 48h post-infection, cells were then collected and Western analysis was performed to measure Src, PP2A, 4EBP-1 and GAPDH levels. Ad : Adenovirus expressing Src or GFP protein. E) Fibroblasts transfected with Src or control siRNA (100 nM) were serum starved for 24 h. Cells were then attached to collagen coated plates for 30 min. Src, PP2A and 4EBP-1 expression were then measured. GAPDH was used as a loading control.

Figure 6. β1 integrin promotes 4EBP-1 degradation through Src/PP2A-dependent pathway. A) Left, fibroblasts transfected with 100 nM of control (C) or PP2A siRNA (PP2A) were attached to collagen coated plates in the presence of 1 µg of β1 integrin blocking antibody (BA) or IgG isotype control antibody (IgG) for 30 min. Cells were then collected and Western analysis was carried out to measure 4EBP-1 and Actin levels. Right, fold changes in 4EBP-1 expression (4EBP-1/Actin expression) in control or PP2A transfected cells were measured by densitometry. B) Upper, serum starved lung fibroblasts were pre-incubated with 1 µg/ml of β1 integrin blocking antibody for 45 min followed by the attachment to collagen for 30 min. 4EBP-1 and GAPDH levels were measured. Lower, β1 integrin siRNA (100 nM) transfected cells were serum starved for 24 h. Cells were then attached to collagen for 30 min. β1 integrin, 4EBP-1 levels were then measured using Western analysis. GAPDH was used as a loading control. β1 : β1 integrin siRNA, Con : control siRNA. C) Upper, serum starved fibroblasts pre-incubated with various doses of Src inhibitor ranging from 0 to 100 nM were ligated with a β1 integrin activating antibody (3 µg/ml) for 30 min followed by the attachment to collagen. PP2A levels were then measured. GAPDH was used as a loading control. TS216 : β1 integrin activating antibody. Lower, PP2A/GAPDH expression under the same condition was measured. Assay was repeated 3 times. *p=0.01 versus IgG isotype control. **p=0.007 versus IgG isotype control. D) Upper, Lung fibroblasts were pre-incubated with Src inhibitor (SI, 100 nM), Okadaic acid (OA, 10 nM) or DMSO for 45 min. Cells were then ligated with β1 integrin activating antibody for 30 min. 4EBP-1 and GAPDH levels were then measured. Lower, 4EBP-1 expression (4EBP-1/GAPDH expression) was measured under the same experimental condition. Note that 4EBP-1 level was high in the presence of Src inhibitor whereas 4EBP-1 level remained low when cells were treated with Okadaic acid due to low PP2A function on collagen. E) The mRNA levels of 4EBP-1 were assessed by quantitative RT-PCR as described in the Materials and Methods. Relative mRNA levels were normalized with GAPDH. F) Upper, serum starved human lung fibroblasts (S/S) were pre-incubated with 5 nM of Lactacyctin (Lac) or 100 µM of proteasome inhibitor (PSI) for 45 min. Cells were then attached to collagen for 30 min and 4EBP-1 levels were then measured. Lower, 4EBP-1/GAPDH expression in the presence of lactacystin or proteasomic inhibitor was measured. Assay was repeated 3 times. G) Upper, human lung fibroblasts pre-incubated with various doses of proteasome inhibitor as described and 4EBP-1 level was measured. Lower, 4EBP-1/GAPDH expression was measured. Assay was repeated 3 times. *p=0.012 versus DMSO control.

Figure 7. Collagen-β1 integrin interaction increases eIF4G activity via low 4EBP-1 function on collagen. A) Upper panel, serum starved human lung fibroblasts were pre-incubated with a 1 µg/ml of α2, β1, or α2 and β1 integrin blocking antibodies for 45 min and cells were attached to collagen coated plates for 30 min. eIF4E and 4EBP-1 levels were then measured using Western analysis. GAPDH was used as a loading
control. Lower panel, cap-binding assay was carried out as described in the Materials and Methods. eIF4E, eIF4G and 4EBP-1 levels were then measured. Note that 4EBP-1 activity was high when integrin blocking antibody was used whereas low 4EBP-1 activity was found when isotype control antibody (IgG) was used on collagen. B) eIF4G/4EBP-1 expression ratio was measured using densitometry and plotted. Note that eIF4G/4EBP-1 expression ratio was low when cells were pre-incubated with α2 and β1 integrin blocking antibody together while eIF4G/4EBP-1 expression ratio was high when cells were treated with IgG isotype control antibody on collagen coated plates. *p=0.05 versus IgG control.

Figure 8. Fibroblast proliferation increases when cells attach to collagen via Src, PP2A and 4EBP-1.
A) Fibroblasts transfected with 100 nM of PP2A or control siRNA were grown in 96 well plates for 48 h. Cells were then incubated with MTS reagent for 3 h and absorbance at 490 nm was measured using 96 well reader for fibroblast proliferation assay. *p=0.03 versus control. B) Fibroblasts infected with adenovirus expressing PP2A or empty GFP vector were grown in 96 well plates for 48 h and cell proliferation assay was performed using MTS reagent as described. *p=0.03 versus control. Ad : Adenovirus expressing PP2A or GFP protein. C) Left, Src protein was silenced by using 100 nM of Src siRNA or control siRNA in fibroblasts. Cells were then grown for 48 h and fibroblast proliferation was measured using MTS reagent. siRNA: Src siRNA or control siRNA. Right AD: Src protein was over-expressed in fibroblasts by adenovirus construct and cell proliferation was measured in 96 well plates at 48 h as described. Ad : Adenovirus expressing Src or GFP protein. Note that when Src protein was silenced, cell proliferation was low. In contrast, when Src was over-expressed, fibroblast proliferation increased. *p=0.005 versus control. D) Wild type 4EBP-1 protein or empty vector was over-expressed in fibroblasts as described in the Materials and Methods. Cell proliferation was then measured at 490 nm using 96 well plate reader. *p=0.02 versus control.

Figure 9. Cap-dependent translation increases via β1 integrin/Src/PP2A/4EBP-1-dependent pathway.
When fibroblasts attach to type I collagen via β1 integrin or β1 integrin becomes activated by β1 integrin activating antibody, high Src activity suppresses PP2A function which results in the inhibition of 4EBP-1. The suppression of 4EBP-1 increases cap-dependent translation. The activation of this pathway promotes fibroblast proliferation on collagen.↑Activity increases, ↓Activity decreases.
Fig 1.

A. Collagen

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<tr>
<th>0</th>
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<th>30</th>
<th>60</th>
<th>P (min)</th>
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4EBP-1

GAPDH

B. 15 min 30 min 60 min 120 min Non coated

C. Collagen

<table>
<thead>
<tr>
<th>0</th>
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Fibronectin

4EBP-1

GAPDH

Laminin

4EBP-1

GAPDH

0 15 30 60 (min)

4EBP-1 expression (4EBP-1/GAPDH expression)

Fibronectin

Laminin
Fig 2.

A. 

Collagen

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<th>α2</th>
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<th>α2+β1</th>
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4EBP-1

Actin

![Image]

B. 

Collagen

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p-4EBP-1 (thr 37/46)

p-4EBP-1 (thr 70)

4EBP-2

GAPDH

![Image]
Fig 3.

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B. Collagen

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PP2A expression (PP2A/GAPDH expression)
Fig 4.

A. 

Collagen

0  15  30  60  120 (min)

NP-527-Src

Src

B. 

Collagen

S/S   SI   DMSO

PP2A

GAPDH

C. 

Collagen

S/S   10   100 (nM)   DMSO

PP2A

GAPDH

D. 

NP-527-Src activity

Src expression

PP2A expression (PP2A/GAPDH expression)

Pulldown in PP2A activity

S/S   0.1   1   10   DMSO

*  

**  

***
Fig 5.

A. Collagen

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B. SD

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C. SI

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D. Ad

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E. Collagen

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Fig 6.

A.

B.

C.

D.

E.

F.

G.
Fig 7.

A.

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B.

[Bar graph showing eIF4G/4EBP-1 ratio for different conditions: S/S, α2, β1, α2+β1, IgG.]

* denotes statistical significance.
Fig. 8.

A. siRNA

B. Ad

C. siRNA

D. Ad

% change in MTS assay (absorbance at 490 nm)
Fig 9.

β1-integrin → Src → PP2A → 4EBP-1 → Cap-dependent translation → Cell proliferation
Eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1) function is suppressed by Src and PP2A on extracellular matrix
Richard Seonghun Nho and Mark Peterson

J. Biol. Chem. published online July 22, 2011

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