LC3-mediated fibronectin mRNA translation induces fibrosarcoma growth by increasing connective tissue growth factor

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Summary
Previously, we related fibronectin (Fn1) mRNA translation to an interaction between an AU-rich element in the Fn1 3′ UTR and light chain 3 (LC3) of microtubule-associated proteins 1A and 1B. Since human fibrosarcoma (HT1080) cells produce little fibronectin and LC3, we used these cells to investigate how LC3-mediated Fn1 mRNA translation might alter tumor growth. Transfection of HT1080 cells with LC3 enhanced fibronectin mRNA translation. Using polysome analysis and RNA-binding assays, we show that elevated levels of translation depend on an interaction between a triple arginine motif in LC3 and the AU-rich element in Fn1 mRNA. Wild-type but not mutant LC3 accelerated HT1080 cell growth in culture and when implanted in SCID mice. Comparison of WT LC3 with vector-transfected HT1080 cells revealed increased fibronectin-dependent proliferation, adhesion and invasion. Microarray analysis of genes differentially expressed in WT and vector-transfected control cells indicated enhanced expression of connective tissue growth factor (CTGF). Using siRNA, we show that enhanced expression of CTGF is fibronectin dependent and that LC3-mediated adhesion, invasion and proliferation are CTGF dependent. Expression profiling of soft tissue tumors revealed increased expression of both LC3 and CTGF in some locally invasive tumor types.

Key words: mRNA translation, Microtubule-associated protein, Tumor invasiveness, LC3, Fibronectin, Connective tissue growth factor

Introduction
Fibronectin is a homodimeric secreted glycoprotein present in the plasma or associated with the extracellular matrix. It influences cell adhesion, migration, proliferation, differentiation, and cytoskeletal organization (Hynes and Lander, 1992). An increase in fibronectin synthesis is associated with migration of vascular smooth muscle cells contributing to intimal cushion formation in the developing ductus arteriosus (Mason et al., 1999b) and to the formation of the neointima in the post-cardiac transplant coronary arteriopathy (Claussell et al., 1993; Salomon et al., 1991) and in other occlusive vascular diseases (Jones et al., 1997). Studies investigating the mechanism regulating enhanced production of fibronectin in ductus arteriosus smooth muscle cells led to the discovery that light chain 3 (LC3, also known as MLP3B) of microtubule-associated proteins 1A and 1B binds to the AU-rich element (ARE) in the 3′ untranslated region (UTR) of the Fn1 mRNA (Zhou et al., 1997), and increases the efficiency of translation by ribosomal recruitment (Zhou and Rabinovitch, 1998). Transfection of smooth muscle cells with a construct containing the chloramphenicol acetyl transferase (CAT) coding region and the wild-type fibronectin 3′ UTR results in increased CAT activity when compared with a construct in which the ARE is mutated at the ARE (Zhou et al., 1997). LC3 co-distributes with heavy polysomes containing Fn1 mRNA, and when microtubules are disrupted, the association of both LC3 and Fn1 mRNA with polysomes is lost and Fn1 translation is suppressed (Zhou and Rabinovitch, 1998).

AREs are common regulatory sequences within the 3′UTR of mRNAs encoding inflammatory mediators, cytokines and oncogenes (Caput et al., 1986). Although their documented role appears to be related to mRNA destabilization (Shaw and Kamen, 1986; Treisman, 1985) or translational repression (Kruys et al., 1989; Kruys et al., 1987), AREs also enhance mRNA translation. For example, enhanced translation of tumor necrosis factor α (TNFα; gene symbol: Tnf) mRNA is associated with the interaction of a 55 kDa protein with an ARE (Lewis et al., 1998). Although a number of amino acid motifs in various RNA-binding proteins are known to interact with mRNA regulatory sequences, none have been specifically identified in association with the ARE. The arginine-rich motif (ARM) is a common RNA-binding domain present in RNA-binding proteins such as Rev and Tat (Iwai et al., 1992). It is the location of the ARM in the complex secondary structure of the binding protein, rather than the amino acid sequence per se, that appears be the major determinant of its interaction with RNA (Dayton et al., 1989; Malim et al., 1989). We have observed, using northwestern immunoblotting, that the ARM in LC3 is necessary for its binding to the ARE in the 3′UTR of Fn1 mRNA (our unpublished data).

To further elucidate the importance of LC3 in mediating cell function related to mRNA translation, we screened cell lines for
low levels of expression of LC3 and found that the HT1080 fibrosarcoma cell line also produces little fibronectin (Dean et al., 1988). In this study, we assess the functional significance of increasing LC3 in HT1080 cells on Fn1 mRNA translation and on growth of the tumor cells in culture and in SCID mice. We show that adhesion, invasion and proliferation are enhanced by LC3 in a fibronectin-dependent manner, and that these properties are modulated by fibronectin-mediated production of connective tissue growth factor (CTGF). Gene expression profiling of 239 soft tissue tumors showed coordinated expression of both LC3 and CTGF in some tumor types with locally invasive properties.

Results

Enhanced fibronectin protein in HT1080 stable transfectants expressing LC3

Previous studies showed that HT1080 cells synthesize low levels of fibronectin and do not exhibit cell-surface fibronectin matrix deposits, a feature associated with the tumorigenicity of the cells (Dean et al., 1988). To establish that LC3-ARE binding can mediate enhanced Fn1 mRNA translation in these cells, we constructed vectors encoding the wild-type LC3 (LC3-WT), and a mutant form of LC3, in which the triple arginine motif of the ARM was replaced with glutamine, thus abolishing the positive charge of the ARM (LC3-R/Q). These constructs, as well as a vector-only construct, were stably transfected into HT1080 cells, and LC3 protein (Fig. 1A), fibronectin protein (Fig. 1B), fibronectin biosynthesis (Fig. 1C) and steady-state Fn1 mRNA levels (Fig. 1D) were compared. LC3-WT and LC3-R/Q transfected cells expressed abundant LC3 protein in the form of a doublet compared with vector-transfected cells where the protein was barely detectable (Fig. 1A). The R/Q mutant LC3 protein doublet, when compared with the WT, was comprised of slightly downward shifted upper band that was increased in intensity and a lower band of reduced intensity. There was little LC3 present in cells transfected only with the vector.

Western immunoblots showed a marked increase in steady-state levels of fibronectin protein in the WT-LC3 when compared with the R/Q mutant and with the vector transfected cells (P<0.01 for both comparisons) (Fig. 1B). Similar results were obtained upon assessment of newly synthesized fibronectin secreted into the cultured medium and measured following [35S]methionine metabolic labeling. As shown in Fig. 1C, fibronectin synthesis in LC3-WT was considerably elevated when compared with vector (P<0.005) or LC3-R/Q transfected clones (P<0.05), although there was also increased production of fibronectin in LC3-R/Q transfected cells when compared with those transfected with vector alone (P<0.01). Quantitative RT-PCR (Fig. 1D) revealed no significant differences in steady-state Fn1 mRNA levels in the different transfected cells, supporting the idea that LC3 mediates enhanced Fn1 mRNA translation.

Polysome analysis of Fn1 mRNA in stably transfected HT1080 cells

We investigated the relative distribution of Fn1 mRNA on polysomes using sucrose gradient analysis, to establish that the increase in fibronectin synthesis in LC3-WT transfected HT1080 cells is related to mRNA translation, and requires both the ARM of LC3 and the ARE of Fn1 mRNA. The location of the monosomes and/or preinitiation complexes and of the polysomes was determined by ethidium bromide staining (data not shown). The polysome RNA profile at 260 nm absorbance is illustrated in Fig. 2A (top). Similar profiles were observed in LC3-WT and LC3-R/Q mutant transfected cells. RNA was extracted from each fraction and analyzed by quantitative RT-PCR.

In cells transfected with LC3-WT, a major portion of human FN1 mRNA is associated with heavy polysomes and is thus actively translated (specifically fractions 8, 9 and 10), whereas in cells transfected with LC3-R/Q, more FN1 mRNA is associated with the lighter fractions (Fig. 2B). As a negative control, we performed

Fig. 1. Stable transfection of HT1080 cells with LC3 induces fibronectin synthesis. (A) Western immunoblot of LC3 protein expression (top) and densitometric analyses (bottom) in HT1080 fibrosarcoma cells stably transfected with wild-type LC3 (LC3-WT), R/Q mutant LC3 (LC3-R/Q) and vector alone. A doublet appears at 16 kDa in LC3-WT but a more intense slightly downward shifted upper band in LC3-R/Q transfected cells. Upper bands were measured. (B) Western immunoblot of fibronectin protein relative to β-actin (top) with densitometric analyses below in LC3-WT, LC3-R/Q and vector-transfected cells. (C) Representative autoradiographs of fibronectin (top) following [35S]methionine labeling of cells and gelatin Sepharose purification of fibronectin, followed by radioactivity measurement (bottom) in LC3-WT, LC3-R/Q and vector-transfected cells. (D) Quantitative RT-PCR for Fn1 mRNA levels in LC3-WT, LC3-R/Q and vector-transfected cells. In all panels, bars represent mean ± s.e.m. of n=3 different experiments each carried out in triplicate. *P<0.05; **P<0.01; ***P<0.005.
LC3, fibronectin and fibrosarcoma growth

Polysome analysis on Myc, a transcript with an ARE that is not regulated by LC3. Transfection of LC3-WT compared with the LC3-R/Q mutant did not alter the polysome profile of Myc mRNA (Fig. 2C), confirming the specificity of LC3 for the ARE in fibronectin in enhancing mRNA translation.

We saw an equivalent low level of Fn1 mRNA across the sucrose gradient in cells that were stably transfected with vector only (data not shown). Treatment with EDTA resulted in the disruption of polysomes with little Fn1 mRNA in fractions at the bottom of the sucrose gradient (data not shown). To address whether the ARE of the Fn1 mRNA is important for fibronectin mRNA translation, rat wild-type or ARE-deleted Fn1 mRNA were transiently transfected into HT1080 cells that stably express LC3, and the distribution of rat Fn1 mRNA on polysomes was analyzed. Wild-type rat Fn1 mRNA, but not mutant ARE-deleted rat Fn1 mRNA, was associated with heavy polysomes in HT1080 cells stably expressing LC3 (Fig. 2D).

To further establish the importance of the interaction between the ARM motif in LC3 and the ARE element in Fn1 mRNA, we performed RNA electromobility gel shift assays. Cytoplasmic extracts from LC3-WT, LC3-R/Q and vector-transfected HT1080 cells were incubated with radiolabeled wild-type fibronectin ARE (wtARE) RNA oligonucleotides and competition assessed using extracts from LC3-transfected cells and an excess of cold wt ARE or an irrelevant oligonucleotide. Fig. 2E is a representative blot of two different experiments, with a histogram of the intensities of the highlighted bands. The binding complexes formed between cytoplasmic extracts from LC3-WT-transfected cells and radiolabeled wtARE are more abundant than those formed when using cytoplasmic extracts of cells transfected with LC3-R/Q or vector only. The binding (indicated by the arrow in Fig. 2E) is specific, as it was competed completely by excess of unlabeled fibronectin ARE (LC3-WT+cold probe) and much less well by an oligonucleotide without the consensus ARE (LC3-WT+irrelevant). These data are consistent with the idea that optimization of LC3 interaction with Fn1 mRNA requires the ARM motif in LC3 and the ARE element in Fn1 mRNA.

LC3 regulates cell growth

Increased production of fibronectin in tumor cells has been previously reported to revert the transformed phenotype by enhancing adhesion of cells to the substrate, changing their morphology from rounded to spread, and decreasing their growth rate (Dean et al., 1988). We therefore assessed whether the increased Fn1 mRNA translation in LC3-transfected HT1080 cells would alter the growth rate of the cells. As shown in Fig. 3A, as early as 3 days after plating, and at 7 days, LC3-WT-transfected cells, despite
increased production of fibronectin, exhibited significantly faster growth compared with both vector and LC3-R/Q transfectants ($P<0.05$ for each comparison at 3 days, and $P<0.001$ at 7 days). The difference in growth between vector-transfected and LC3-R/Q-transfected cells was not significant on day 7 (Fig. 3A). Similar results were observed using multiple clones (data not shown). To determine whether LC3-WT-transfected HT1080 cells could also promote tumor growth in an intact animal, SCID mice were injected subcutaneously with LC3-WT, LC3-R/Q or vector-only stably transfected HT1080 cells. Tumors were monitored in the mice as described in the Materials and Methods. The LC3-WT-transfected HT1080 tumors reached a significantly larger volume when assessed at 14, 18 and 21 days after injection when compared with both vector transfected and LC3-R/Q mutant cells ($P<0.005$ for each comparison) (Fig. 3B).

To determine whether the increase in growth in LC3-WT-transfected HT1080 cells was dependent on the increase in fibronectin, RNAi was used to repress $Fn1$ mRNA in both LC3-WT and vector-transfected cells. Efficient knockdown of fibronectin protein (Fig. 4A, top panel) was observed at 72 hours, and in $Fn1$ mRNA (Fig. 4A, bottom panel) at 48 hours after transfection of fibronectin siRNA. We then showed, using the MTT assay, that the increase in cell proliferation in the LC3-WT compared with vector-transfected cells observed with control siRNA ($P<0.05$) was lost following transfection with fibronectin siRNA (Fig. 4B). In vector-transfected cells in which fibronectin was reduced by siRNA, cell proliferation was also reduced ($P<0.01$) (Fig. 4B).
We observed that LC3-WT-transfected cells treated with control siRNA were more adhesive on plastic compared with vector-transfected cells when cultured in medium containing bovine serum (Fig. 4C,D) and also without serum (data not shown). This feature was also related to production of fibronectin, because cell adhesion was reduced by fibronectin siRNA in LC3-WT cells ($P<0.01$) to levels observed in the control vector-transfected cells. Treating vector-transfected cells with fibronectin siRNA further reduced their adhesion when compared with cells treated with control siRNA ($P<0.01$). Moreover, culturing vector-transfected cells on fibronectin-coated dishes enhanced their adhesion to the level observed with LC3-WT-transfected cells (Fig. 4D), and this was not significantly changed with fibronectin siRNA. LC3 WT cells cultured on fibronectin showed a further increase in adhesion compared with their culture on plastic, which was also not significantly reduced by fibronectin siRNA. These experiments confirm the ability of exogenous fibronectin to rescue the loss of adhesion attributed to reduced endogenous production of fibronectin by siRNA.

To assess the contribution of fibronectin to invasion, we carried out Matrigel invasion assays on LC3-WT and vector-transfected HT1080 cells. Tumor cell invasion was increased in LC3-WT-transfected cells treated with control siRNA compared with vector-transfected cells ($P<0.05$); however, following treatment with fibronectin siRNA, levels were reduced to those in vector-transfected cells ($P<0.05$). Invasion was further reduced in vector-transfected cells using fibronectin siRNA compared with control siRNA ($P<0.05$) (Fig. 4E). The presence of some invasion

| Function       | Gene          | Abbreviation | Fold change |
|----------------|---------------|--------------|-------------|
| Adhesion       | Cadherin 11, type 2 | CDH11 | 2           |
|                | Collagen, type VI, alpha 3 | COL6A3 | 2           |
|                | Collagen, type XII, alpha 1 | COL12A1 | 2.1         |
| Connective tissue growth factor | CTGF | 2.3         |
| Desmocollin 1  | DSC1          | 1.8         |
|                | Inhibin, beta A | INHBA       | 2.6         |
| Differentiation| Thrombospondin 1 | THBS1       | 2.6         |
| Proliferation  | Thrombospondin 1 | THBS1       | 2.6         |
| Desmocollin 1  | DSC1          | 1.8         |

*Genes differentially expressed in LC3-WT stably transfected HT1080 cells versus vector-transfected cells identified using SAM (significant analysis of microarray) with false discovery rate <5. Differentially expressed genes were confirmed by qPCR using TaqMan probes. The probe IDs are described in the Materials and Methods.
despite very low levels of fibronectin induced by siRNA in LC3-transfected HT1080 cells, suggests that additional LC3-dependent features might contribute to invasion in these cells.

Gene-expression profile of HT1080 cells stably transfected with LC3
To identify the specific genes regulated by LC3 in HT1080 cells, we performed analyses using the significance analysis of microarrays (SAM). Nine upregulated and eight downregulated genes were confirmed by qRT-PCR. Confirmed upregulated genes are listed in Table 1. Of these, there was a significant increase in expression of six genes associated with adhesion in the LC3-WT-transfected HT1080 cells, one of which, CTGF, has been consistently implicated in tumor growth and invasion (Aikawa et al., 2006; Planque and Perbal, 2003).

We therefore first confirmed an increase in expression of CTGF in LC3-WT versus vector-transfected cells by both western immunoblot (Fig. 5A) and qRT-PCR (Fig. 5B). To relate the increase in CTGF to fibronectin, we showed that loss of fibronectin in LC3-WT-transfected cells induced by fibronectin siRNA resulted in a 72% reduction in CTGF protein, which was similar to levels in vector-transfected cells. In vector-transfected cells, fibronectin siRNA further reduced CTGF protein by 70% (P<0.001) (Fig. 5B).

We also showed, by immunohistochemistry, that there was an increase in both fibronectin and CTGF in LC3-WT compared with vector-transfected tumors, which was associated with a greater number of blood vessels as shown by PECAM immunostaining (P<0.05) (Fig. 5C).

Fibronectin-mediated proliferation, adhesion and invasion of HT1080 cells is CTGF dependent
To determine whether fibronectin mediates the increase in proliferation, adhesion and invasion of LC3-WT through heightened production of CTGF, these features were assessed in cells treated with CTGF siRNA. We reduced levels of CTGF protein and mRNA more than 80% by siRNA, as assessed by western immunoblot (Fig. 6A, top panel) and by quantitative RT-PCR (Fig. 6A, bottom panel) in both LC3-WT- and vector-transfected cells. Adhesion, invasion and proliferation of HT1080 cells transfected with LC3-WT were reduced by CTGF siRNA to values below those observed in vector-transfected cells treated with control siRNA either in the presence of serum (Fig. 6B,C,D) or in its absence (not shown). HT1080 cells transfected with vector and LC3-WT showed reduced adhesion following treatment with CTGF siRNA in the presence or absence of a fibronectin coating on the plates (P<0.05 for both) (Fig. 6B).

Following treatment with control siRNA, vector-transfected cells shown increased adhesion on fibronectin-coated plates, similar to the level observed in LC3-WT-transfected cells. The decreased adhesion of cells transfected with vector or LC3-WT that was evident following treatment with CTGF siRNA, was not significantly rescued by plating the cells on fibronectin. This confirms that CTGF acts downstream of fibronectin.

Gene expression profile of LC3 and CTGF in soft tissue tumors
As the HT1080 cell line was derived from a fibrosarcoma, we looked at a number of human soft tissue tumors (benign mesenchymal tumors and sarcomas) for expression of LC3 and CTGF. Data from previous expression-profiling studies on soft tissue tumors (West and van de Rijn, 2006) indicated increased expression of both LC3 and CTGF in some tumor types (e.g. desmoid-type fibromatosis and epithelial hemangiopericytoma) but not in other soft tissue tumors such as myxoid liposarcoma (Fig. 7). TNFα and TGFβ stimulate production of LC3 (O’Blennes et al., 2001) and fibronectin (Dean et al., 1988), respectively. There was, however, no convincing evidence that mRNA expression of LC3 and CTGF corresponded with the presence of TGFβ or TNFα.

Fig. 6. CTGF is necessary for enhanced adhesion, invasion and proliferation of HT1080 cells stably transfected with LC3-WT. (A) Western blot of CTGF protein (top) and qRT-PCR of Ctgf mRNA (bottom) in vector- and LC3-WT-transfected HT1080 cells transiently transfected with control siRNA or CTGF. Both CTGF protein and mRNA were efficiently knocked down following fibronectin siRNA transfection for 72 hours, in both vector- and LC3-WT-transfected HT1080 cells. (B-D) Adhesion (B), invasion (C) and proliferation (D) of HT1080 cells stably transfected with LC3-WT (as judged by MTT assay) compared in transfected cells transiently cotransfected with control siRNA or CTGF siRNA. Cell adhesion was assessed for cells cultured with or without fibronectin coating of the dishes (B). Bars denote mean ± s.e.m. of three experiments; *P<0.05; ** P<0.01; ***P<0.005.
Fig. 7. LC3 and CTGF expression in soft tissue tumors. cDNA gene-profiling analysis of MAP1LC3B (LC3), CTGF, TGFβ and TNFα expression in soft tissue tumors. Truncated heat map and expression values are shown for selected tumors. Mean centered expression is shown for representative gene spots. Red represents high expression; black represents median expression; green represents low expression; and gray represents no data. Fold change values from significant analysis of microarray (SAM) relative to controls for corresponding samples are shown below. DTF, desmoid-type fibromatosis; EHE, epithelioid hemangioendothelioma; AS, angiosarcoma; MLPS, myxoid liposarcoma; SFT, solitary fibrous tumor; SS, synovial sarcoma.

Discussion

Here, we show that LC3, as a consequence of enhancing Fn1 mRNA translation, increases CTGF levels, which produces fibrosarcoma cells that are faster growing, more adhesive and more invasive, both in culture and in a living animal. Data from previous expression profiling of 239 soft tissue tumors showed that coordinated expression of both LC3 and CTGF was present in some invasive tumor types.

In our previous studies, we produced a variety of peptides from a recombinant LC3-GST fusion protein and determined that a 27 bp region of LC3 appears to be required for binding to the ARE of Fn1 mRNA (our unpublished data). An RNA-binding motif containing three consecutive arginine residues is present in this region, and this influences mRNA translation (Lewis et al., 1998) as well as mRNA stability (Shaw and Kamen, 1986; Treisman, 1985). However, these arginine motifs (ARMs) were previously shown to have RNA-binding properties only in proteins from bacteriophages and viruses (Weiss and Narayana, 1998). As a control for our experiments, we constructed an LC3 transcript in which the ARM was mutated with an arginine to glutamine (LC3-R/Q) substitution that neutralized the positive charge, which greatly reduced ARE-binding activity. The mutant LC3 protein was stable and formed a doublet at 16 kDa, but it appeared somewhat different to the WT protein in that the upper band was more intense than the lower band and it was also shifted slightly downward. Our previous studies related the lower band of the doublet to the phosphorylated form of LC3 that is associated with the pelleted membranes of the cell and with the polyribosomes necessary for mRNA translation (O’Blenes et al., 2001). The lower band is also associated with a longer sequence (Kabeya et al., 2000). The inability of LC3-R/Q to increase fibronectin synthesis and thus maintain steady-state levels of fibronectin protein similar to those observed in LC3-WT-transfected HT1080 cells, is consistent with a relative impairment in translation of Fn1 mRNA. This is supported by the polysome analysis, which shows that there is an increase in Fn1 mRNA transcripts in the heavy polysomes in LC3-WT-transfected cells compared with LC3-R/Q mutant cells. Transfection of LC3-WT cells with the rat WT fibronectin construct, but not with an ARE-deleted mutant construct, also showed increased distribution of the rat WT Fn1 mRNA in the heavy polysomes. This confirms previous studies by our group in vascular smooth muscle cells, which showed that the ARE is crucial to the function of LC3 in increasing Fn1 mRNA translation (Zhou et al., 1997). However, it is also clear from these and previous studies that the presence of an ARE alone does not confer increased translation in the presence of LC3. For example, the Myc transcript, has an ARE known to be an mRNA stability element, but LC3 does not regulate translation of Myc mRNA.

LC3 has functions in addition to translation of Fn1 mRNA, and these include possible roles in microtubule assembly (Hammarback et al., 1991), in mRNA transport (Seidenbecher et al., 2004), and in autophagy (Kabeya et al., 2000; Kabeya et al., 2004). In the future, these functions could be assessed in the HT1080 cells transfected with LC3. Interestingly, loss of function of LC3 in a knockout mouse reported by our group, has no autophagy phenotype (Cann et al., 2008).

The LC3-transfected HT1080 cells appeared to be more proliferative, both in culture and when implanted into SCID mice. Studies in cultured cells were subsequently carried out to determine whether we could account, at least in part, for this phenotype by an LC3-mediated increase in fibronectin synthesis alone. Previous studies have attributed a fibronectin-mediated increase in the adhesive properties of tumor cells as being responsible for their reduced proliferation (Dean et al., 1988). More recent studies have shown that the interaction of fibronectin with α5β1 integrin (Aguirre-Ghiso et al., 2003), as well as α3β1 integrin, increases tumor cell growth, as well as invasion, by activating MMP9 and Rac1 (Wei et al., 2007). Hence, the context is clearly important, and suggests that in response to LC3, coordinated regulation of genes at the transcriptional and post-transcriptional level might be necessary to produce the fibronectin-dependent proliferative and invasive responses. In keeping with this, our studies show that LC3, via fibronectin, also promotes cell adhesion, a property previously shown to be necessary for the migration of fibrosarcoma cells (Zaman et al., 2006) and vascular cells (Boudreau et al., 1991) in 3D matrices.

To address whether the fibronectin-cell interaction might be inducing other genes that are required to enhance HT1080 cell adhesion, invasion and proliferation, we carried out microarray analysis to compare HT1080 cells transfected with LC3-WT and vector. Although a number of transcripts were upregulated, one – CTGF – stood out as being crucial to the mechanism associated with our findings, and its upregulation was confirmed by qRT-PCR to be dependent on both LC3 and fibronectin. Although it is possible that LC3 increases Ctgf mRNA by increasing mRNA stability, the dependence of enhanced CTGF expression on fibronectin, suggests an indirect effect of LC3. Overexpression of CTGF has been shown
in a number of cancers (Croci et al., 2004; Koliopoulos et al., 2002; Kubo et al., 1998; Moritani et al., 2003; Pan et al., 2002; Shakunaga et al., 2000; Vorwerk et al., 2002; Wenger et al., 1999; Xie et al., 2001; Xie et al., 2004; Zeng et al., 2004), but its direct role in tumor suppression or progression has not been characterized.

CTGF is a cysteine-rich secreted protein, belonging to a group of immediate-early genes induced by growth factors, such as TGFβ, or certain oncogenes. CTGF promotes proliferation and migration of vascular endothelial cells (Takigawa et al., 2003) and stimulates human mesangial cell adhesion to fibronectin (Weston et al., 2003). CTGF stimulates mesenchymal cells, including fibroblasts, chondrocytes and osteoblasts, to proliferate and to produce connective tissue components, such as collagen type 1 and fibronectin, while also remodeling the extracellular matrix (Blom et al., 2001; Frazier et al., 1996) and promoting granulation tissue formation (Chen et al., 2001). Thus, heightened expression of CTGF might contribute to the angiogenic response that supports HT1080 cell growth in addition to the proliferative and invasive response of the cells per se.

It is interesting that whereas CTGF was shown to induce fibronectin (Blom et al., 2001; Frazier et al., 1996), in our study, the expression of CTGF is dependent on fibronectin. This might imply a positive-feedback mechanism that promotes the features of tumorigenesis by amplifying the interaction of both fibronectin and CTGF with integrin receptors, including αβ3, αIIbβ3 and α3β1 integrins (Chen et al., 2001; Gao and Brigstock, 2006; Jedadayammata et al., 1999; Lym et al., 2002). Future studies could address whether loss of CTGF also reduces fibronectin. In addition, it would be of interest to investigate whether LC3-mediated enhanced synthesis of fibronectin can, by inducing integrin-linked kinase (ILK), mediate transcriptional activity of factors upstream of the CTGF promoter, such as AP1 (Troussard et al., 2000; Troussard et al., 1999). ILK activation downstream of the fibronectin-integrin interaction can induce an invasive phenotype via AP1-dependent upregulation of matrix metalloproteinase MMP9 (Troussard et al., 2000). It is intriguing, however, that fibronectin-induced production of CTGF was sufficient to explain the proliferative and invasive phenotype of the cultured HT1080 cells. Our subsequent studies show coexpression of mRNA encoding LC3 and CTGF in at least some soft tissue tumor types, such as desmoid type fibromatosis but not others. Further prospective phenotyping studies and confirmation of the microarray data by qRT-PCR are necessary to indicate whether these factors could serve as important new biomarkers. Our previous studies have shown that TNFα upregulates fibronectin in coronary artery smooth muscle cells via LC3 (O’Blenes et al., 2001) and TNFα is known to upregulate CTGF (Cooker et al., 2007), so it would be interesting to know whether TNFα could mediate an increase in CTGF and fibronectin via LC3 in HT1080 cells. TGFβ also stimulates production of fibronectin (Dean et al., 1988). Although there is no obvious coordinated expression of these cytokines with CTGF and LC3, it would be interesting to establish whether the subset of tumors with an increase in LC3 and CTGF have a cytokine signature.

Materials and Methods

Materials

DMEM, aminoglycoside G418 and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). All radiolabeled materials were obtained from Amersham (Arlington Heights, IL) and the HT1080 human fibrosarcoma cell line and MTT Cell Proliferation kit were kindly supplied by James Hammarback, Department of Neuropathology and Anatomy, Wake Forest (Bowman Gray) School of Medicine, Winston-Salem, NC, and was generated and characterized as previously described (Mann and Hammarback, 1994).

The polyclonal antibodies to LC3 were either supplied by James Hammarback or made within the same N-terminal sequence by AnaSpec (San Jose, CA). Peroxidase-conjugated donkey anti-rabbit IgG secondary antibody was from Amersham. Mouse monoclonal anti-fibronectin IgG was from Neomarker (Fremont, CA), rabbit polyclonal antibody to CTGF was from Abcam (Cambridge, MA), fluorescein-conjugated goat-anti-mouse IgG secondary antibody and the enhanced chemiluminescence (ECL) western immunoblotting reagents were from Amersham.

αβ3, αIIbβ3 and α3β1 integrins were either wild-type or containing either the wild-type or the respective allele, respectively, of the predicted mutation, and the mutations were verified by DNA sequencing. To construct a second LC3-R3-R mutation, the mutagenesis was carried out using a kit from Stratagene (La Jolla, CA). Reagents for transfection were Lipofectin 2000 (Invitrogen), SuperFect (Qiagen, Valencia, CA) or FuGENE (Roche Diagnostics, Pleasanton, CA). Anti-fade reagent was purchased from Molecular Probes (Eugene, OR). Gelatin 4B-Sepharose was from Pharmacia Biotech (Piscataway, NJ), Bi-Tris gel and Novex tricine gel was from Invitrogen. Nucleosilicel membranes and BCA protein assay kits were from Bio-Rad Laboratories (Hercules, CA). Qiagen RNA extracting kit and SuperFect Reagent were from Qiagen. NucTrap Probe Purification Column was from Stratagene. All other chemicals unless otherwise specified, were purchased from Sigma.

Plasmid constructs and site-directed mutagenesis

To transfet the HT1080 cells, plasmid pCR3-LC3 was kindly provided by James Hammarback. The mutant pCR3-LC3 vectors containing the full-length LC3 sequence were generated by PCR. The primers for LC3-R/Q are: 5'-ATTCAGAGAACCCTGTTTATGTTGTTG-3' and 5'-GATCCTTCTGTTTATGTTGTTG-3'. Restriction enzyme sites were introduced by a 5' primer, 5'-GAGGCTCGAGATCCACACATGTCGTTGTTG-3' and a 3' primer, 5'-GTCACCAAGCCGCGAGTCATTCTCCGGG-3' flanking the insertion site. The 963 bp BamHI-XbaI fragments containing the mutated sites were then used to replace the corresponding fragment within wild-type pCR3-LC3. All constructs were confirmed by restriction enzyme mapping, and the mutations were verified by DNA sequencing. To construct a second LC3-R3-R mutant stable fraction in HT1080 cells, the primer sequences used were: 5'-GAGGAACTCATCAGCAGAATACATGCAACGCTATTGACTCAGTACAAACCAGG-3', and its complementary sequence 5'-GCTTGTTAGTGGGCATTCGAGTGGAGG-3', and the non-ARE irrelevant consensus sequence (AGAGCGGGAGGGAGU).

Construction of rat fibronectin expression vectors

Rat fibronectin cDNA and its 3' UTR were cloned into a pDEST26 expression vector using the Gateway cloning system (Invitrogen). This was done by first joining the fibronectin coding region and 3' UTR using a pENTr vector. Briefly, a full-length fibronectin cDNA of rat fibronectin was PCR amplified from a retroviral rat fibronectin expression plasmid (a gift from Richard O. Hynes, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA), using 5' primers containing a terminal CACC site for directional cloning (5'-CACCAGGCTCGAGGCGGCGAGC-3') and a 3' primer containing an XbaI site (5'-CTAGCTACGAGGCGTAGCTGAGAAGACG-3'). The fragment was then cloned into pENTr vector to form pENTr/fN. A 3' UTR PCR fragment amplified from rat EST clone #ERN1017 (Open Biosystem, Huntsville, AL) using a forward primer containing an XbaI site (5'-CTACCAGGAGGCGTAGCTGAGAAGACG-3') and a reverse primer 5'-GATAAAGAGGCTCGAGGCGGCGAGC-3'. The fragment was then cloned into pENTr vector to form pENTr/fN. A 3' UTR PCR fragment amplified from rat EST clone #ERN1017 (Open Biosystem, Huntsville, AL) using a forward primer containing an XbaI site (5'-CTACCAGGAGGCGTAGCTGAGAAGACG-3') and a reverse primer 5'-GATAAAGAGGCTCGAGGCGGCGAGC-3'. The fragment was then cloned into pENTr vector to form pENTr/fN. A 3' UTR PCR fragment amplified from rat EST clone #ERN1017 (Open Biosystem, Huntsville, AL) using a forward primer containing an XbaI site (5'-CTACCAGGAGGCGTAGCTGAGAAGACG-3') and a reverse primer 5'-GATAAAGAGGCTCGAGGCGGCGAGC-3'.

Cell culture and transfection

The HT1080 human fibrosarcoma cell line were grown as previously described (Dean et al., 1988) and plated at a density of 10^6 cells per 100 mm dish 24 hours before transfection. Wild-type and mutant LC3 constructs were transfected into HT1080 cells using the following protocols: 10 µg of empty vector (pCR3), wild-type pCR3-LC3 (LC3-WT) plasmid or mutant pCR3-LC3-R68-70Q (LC3-R/Q) plasmid, were transfected into each dish for 3 hours using SuperFect or FuGENE transfection reagent, according to the manufacturer’s instructions.
instructions. The cells were then fed with fresh complete medium containing 200 μg/ml amphotericin B and 4H18. The medium was changed every 2 days with gradual increasing concentrations of 4H18 up to 800 μg/ml. Eight clones transfected with empty vector and 24 clones each transfected with LC3-WT and LC3-R-Q, were selected on the basis of resistance to 4H18 (800 μg/ml) by trypsinization and screened for LC3 expression using western immunoblot analysis. Five clones each were verified to express LC3-WT and LC3-R-Q. These clones, together with three vector-transfected clones were expanded individually and passaged at least three times before use. To determine the role of the ARE in the fibronectin 3'UTR, transient transfections were carried out using the wild-type and mutant rat fibronectin constructs described above. In these experiments, HT1080 cells were grown to 80% confluence in six-well plates and transfected with 4 μg plasmid DNA using Lipofectin 2000 for 48 hours, following the manufacturer’s instructions.

Western immunoblot analysis

LC3-WT- and LC3-R/Q-transfected HT1080 cells were harvested at semi-confluence and cell fractionation and western immunoblots were performed as previously described (Mason et al., 1999a). Protein extracts (20 μg) in Laemmli sample buffer (5% β-mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) were separated on a 4-12% Bis-Tris gel (Novex) and transferred to a nylon membrane (Invitrogen). Membranes were probed for 1.5 hours at room temperature with polyclonal antibodies to LC3 (1:3000) or fibronectin (1:1000) or CTGF (1:1000), β-actin (1:5000) as control, and incubated with goat anti-rabbit HPR (1:5000) for LC3 and β-actin, or anti-mouse HPR (1:5000) for fibronectin and CTGF, using the ECL detection system.

Fibronectin biosynthesis

HT1080 cells individually expanded from vector-transfected, LC3-WT or mutant LC3-R/Q-transfected clones were plated on six-well dishes at a density of 5×10⁴ cells/well. After 24 hours, cells were labeled with [35S]methionine (10 to 18S RNA with similar results. An equal amount of mouse RNA (100 ng) was run on a 4% denaturing agarose gel to check loading and equal loading was confirmed by staining. For quantification of PCR, the LC3-WT and mutant LC3-R/Q, using the NE-PER kit (Pierce Biotechnology, Rockford, IL) and following the manufacturer’s instructions. A total of 60 μg of cytoplasmic extracts were incubated for 30 minutes in a binding reaction containing [γ-32P]-labeled oligonucleotides with the fibronectin ARE sequence (5'-CCUUGUAUUUUAAUCAUU) 100 mM KCl, 20 mM HEPES, 0.5 μM EDTA, 0.5 mM DTT, 20% glycerol, 500 mg salmon sperm and 0.01 U of Poly (dI-dC). Signal specificity was ensured by competition reactions using a 50-fold excess (1.25 pm) of nonradioabeled (cold) fibronectin ARE oligonucleotide, compared with an irrelevant radiolabeled oligonucleotide containing no ARE (5'-TACCGGGAGCGAGGAGG). Samples were run on a 6% polyacrylamide gel in 1X TBE buffer at 150 V for 2 hours. The gel was then dried, and exposed to autoradiographic film overnight at –80°C. The volume of bands was quantified using densitometry.

Cell-proliferation assays

HT1080 cells stably expressing empty vector, LC3-WT or LC3-R/Q were plated on six-well dishes at a density of 1×10⁵ cells/well. Cells were trypsinized every 24 hours for a 3-day course and every 72 hours for a 1-week course. Cell number was determined using a Coulter Flow Cytometer with light scatter (Coulter Cooperation, Miami, FL). The results were confirmed by manually counting the cells using a hemocytometer. The mean cell number from three separate wells was calculated. Each experiment was repeated at least three times. In experiments using siRNA, we assessed cell proliferation by the rate of DNA synthesis using the MTT Cell Proliferation Assay kit (ATCC) in accordance with the manufacturer’s instructions. Briefly, 48 hours following siRNA transfection, 1×10⁵ cells were placed into 96-well plates and incubated at 37°C for 24 hours in 100 μl of serum-free medium. Cell proliferation was assessed by manual counting of the cells using a hemocytometer. The mean cell number from three separate wells was calculated. Each experiment was repeated at least three times.

Fibronectin biosynthesis

HT1080 cells individually expanded from vector-transfected, LC3-WT or mutant LC3-R/Q-transfected clones were plated on six-well dishes at a density of 5×10⁴ cells/well. After 24 hours, cells were labeled with [35S]methionine (10 μCi/ml) for 5 hours, and fibronectin was purified by gelatin Sepharose affinity and analyzed as previously described (Mason et al., 1999b).

RNA isolation

Total RNA was isolated from HT1080 cells using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions, and further purified using Qiagen RNA Clean up kit. RNA quality was assessed by the integrity of RNA bands following gel electrophoresis, and quantified by UV spectrophotometry.

Polysome analysis

Polysome mRNA was prepared as previously described (Johannes et al., 1999). Briefly, cells were incubated with 0.1 mg/ml cycloheximide for 3 minutes at 37°C before being harvested. Cells were washed with PBS, and lysed directly on the plate in high-salt lysis buffer. The supernatants were loaded onto 10-50% sucrose gradients and being harvested. Cells were washed with PBS, and lysed directly on the plate in high-salt lysis buffer. The supernatants were loaded onto 10-50% sucrose gradients and separated on a 4-12% Bis-Tris gel (Novex) and transferred to a nylon membrane (Invitrogen). Membranes were probed for 1.5 hours at room temperature with polyclonal antibodies to LC3 (1:3000) or fibronectin (1:1000) or CTGF (1:1000), β-actin (1:5000) as control, and incubated with goat anti-rabbit HPR (1:5000) for LC3 and β-actin, or anti-mouse HPR (1:5000) for fibronectin and CTGF, using the ECL detection system.

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Quantitative RT-PCR

For polysome RNA, quantitative RT-PCR was performed using FAM-labeled TaqMan probes for either human or rat fibronectin following verification that the probes could distinguish the different transcripts. We also assessed Myc, a control transcript with an ARE. Equal volumes (11 µl) of RNA containing samples from each fraction were used for reverse transcription (RT) using Superscript III (Invitrogen) in a final reaction volume of 20 µl following the manufacturer’s protocol. RT-PCR reactions were carried out in 384-well plates in a 20 µl volume containing 4 µl DNA, 5 µl water, 1 µl TaqMan probe and 10 µl super master mix buffer (ABI, Foster City, CA). The PCR reactions were run in an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA) under cycle conditions following the manufacturer’s instructions. The relative amount of mRNA expression was calculated using a comparative Ct method. Values were assessed un-normalized or normalized to 18S RNA with similar results. An equal amount of mouse RNA (100 ng) was added to each sample to monitor the efficiency.

To quantify selected transcripts from HT1080 stable cells, 2 μg total RNA was reverse transcribed through the RT reaction. Quantitative PCR probes were used for qRT-PCR were: FN1 (Hs00451506tm1), CTGF (Hs0071014_1m1), THBS1 (Hs00170236_m1), CDH11 (Hs00156438_m1), COL6A3 (Hs00189128_m1), COL12A1 (Hs00189184_m1), DSC1 (Hs00245189), INHBA (Hs00710013_m1), EGFR (Hs00193306_m1) and TRIB1 (Hs00179769_m1).

RNA-protein binding assay

Cytoplasmic extracts were obtained from fibroblasts cells transfected with vector, LC3-WT and mutant LC3-R/Q, using the NE-PER kit (Pierce Biotechnology, Rockford, IL) and following the manufacturer’s instructions. A total of 60 μg of

Invasion assays

HT1080 cells grown to 80-90% confluence and 5×10⁵ cells/ml were resuspended in culture medium containing 0.1% BSA. Cell invasion was evaluated using growth factor reduced (GFR) Matrigel Invasion Chambers (Becton Dickinson) with an 8 μm pore size PET membrane. The membrane has a thin layer of GFR Matrigel matrix that serves as a reconstituted basement membrane in vitro. We added 0.75 ml of 5% FBS to each well as a chemoattractant, and 0.5 ml of the cell suspension (2.5×10⁵ cells) were added to each well. The Matrigel Invasion Chambers were then incubated for 24 hours in a humidified 5% CO₂ tissue culture incubator at 37°C. Noninvasive cells were removed from the upper surface of the membrane with a cotton swab before staining. Invasive cells on the underside of the membrane were stained with DIFF QUICK Staining kit (MEB, San Marcos, CA). Cells in five random fields in each well were counted, and the mean value was calculated. Values from three separate wells were calculated for each experimental condition.

RNAi transfection

RNA interference (RNAi) was induced by transient transfection using 100 nM short-interfering RNA (siRNA) oligonucleotides complexed with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Stealth selected RNAi were purchased from Invitrogen (catalog numbers: FN1, HS103782; CTGF, HS102444).


Microarray analyses
Gene-expression profiling was performed essentially as reported previously (Perou et al., 2000), and detailed protocols for array fabrication and hybridization are available online (http://brownlab.stanford.edu/protocols/). Briefly, Cy5-labeled cDNA was prepared using total RNA from HT1080 cells or from frozen sarcoma tumor specimens and Cy3-labeled cDNA was prepared using equal amount of total RNA common reference total RNA (Strategene). For each experimental sample, Cy5- and Cy3-labeled samples were hybridized to a cDNA microarray containing 42,000 human cDNAs, representing 28,000 different genes or ESTs (http://www.microarray.org/sgf/). Microarrays were imaged using an Axon GenePix 4000 scanner (Axon Instruments). Fluorescence ratios for array elements were extracted using GenePix software. Control and empty spots on the arrays were not included for the analyses, as well as those spots flagged as bad spots because of technical errors. Differentially expressed genes were identified using the significance analysis of microarrays (SAM) method (Tusher et al., 2001).

Immunofluorescence
Frozen tissues were sectioned and fixed with ice cold methanol for 20 minutes at 4°C before incubation with monomolecular mouse anti-fibronectin (1:500) (NeoMarker) and rabbit anti-CTGF (1:500) (Abcam) antibodies at 4°C overnight. Immunofluorescence was detected using goat anti-rabbit Alexa Fluor 488 nm and goat anti-mouse Alexa Fluor 594 nm secondary antibodies (Molecular Probes, Eugene, OR) and slides were mounted using SlowFade anti-fade with DAPI (Molecular Probes). Images were captured using a Leica DMR2A microscope.

Immunohistochemistry
To detect tumor blood vessels, the frozen sections were stained with rat anti-CD 31 (PECAM) antibody. Slides were fixed in cold acetone, air-dried and then incubated in 1% H2O2 for 10 minutes. Then PECAM-TDR antibody was added (1:100) for 1 hour followed by incubations with goat anti-rat-biotinylated (1:100) and Streptavidin-HRP (1:500) antibodies for 30 minutes each. The mean density of PECAM staining per surface area was measured using ImageJ software, in five random areas per slide and three slides per tumor, and mean PECAM density per tumor surface area was calculated.

Statistical analyses
For comparisons between groups, data were subjected to ANOVA followed by Bonferroni’s test of multiple comparisons to determine which groups were different. P < 0.05 was judged to represents a statistically significant difference.

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