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Metabolic remodeling of the tumor microenvironment

Migration stimulating factor (MSF) reprograms myofibroblasts toward lactate production, fueling anabolic tumor growth

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Keywords: migration stimulating factor (MSF), myofibroblast, metabolic coupling, tumor stroma, cancer-associated fibroblasts, aerobic glycolysis, TGF- β signaling

Migration stimulating factor (MSF) is a genetically truncated N-terminal isoform of fibronectin that is highly expressed during mammalian development in fetal fibroblasts, and during tumor formation in human cancer-associated myofibroblasts. However, its potential functional role in regulating tumor metabolism remains unexplored. Here, we generated an immortalized fibroblast cell line that recombinantly overexpresses MSF and studied their properties relative to vector-alone control fibroblasts. Our results indicate that overexpression of MSF is sufficient to confer myofibroblastic differentiation, likely via increased TGF- β signaling. In addition, MSF activates the inflammation-associated transcription factor NF κ B, resulting in the onset of autophagy/mitophagy, thereby driving glycolytic metabolism (L-lactate production) in the tumor microenvironment. Consistent with the idea that glycolytic fibroblasts fuel tumor growth (via L-lactate, a high-energy mitochondrial fuel), MSF fibroblasts significantly increased tumor growth, by up to 4-fold. Mechanistic dissection of the MSF signaling pathway indicated that Cdc42 lies downstream of MSF and fibroblast activation. In accordance with this notion, Cdc42 overexpression in immortalized fibroblasts was sufficient to drive myofibroblast differentiation, to provoke a shift towards glycolytic metabolism and to promote tumor growth by up to 2-fold. In conclusion, the MSF/Cdc42/NF κ B signaling cascade may be a critical druggable target in preventing "Warburg-like" cancer metabolism in tumor-associated fibroblasts. Thus, MSF functions in the metabolic remodeling of the tumor microenvironment by metabolically reprogramming cancer-associated fibroblasts toward glycolytic metabolism.

Introduction

An important relationship exists between tumor cells and their local extracellular microenvironment.¹⁻⁴ Indeed, tumor-associated stromal cells critically influence cancer progression and metastasis.¹⁻⁴ Thus, tumor progression is the product of interactions between cancer cells and adjacent stromal cells, such as immune cells, endothelia and fibroblasts, although the exact mechanism(s) still remain poorly understood.⁵⁻⁷

More specifically, stromal myo fibroblasts are now considered active metabolic drivers of tumor growth.⁸ In our recent studies, we proposed that stromal fibroblasts fuel epithelial tumor cells via a unilateral transfer of energy-rich nutrients from the tumor

stroma to cancer cells.⁹ In accordance with this assertion, the recycled nutrients produced by stromal fibroblasts, via autophagy/mitophagy, provide a steady-stream of energy-rich metabolites to cancer cells, inducing mitochondrial biogenesis.¹⁰⁻¹⁵

Normal stromal fibroblasts are converted into carcinoma-associated fibroblasts (CAFs) by complex interactions with adjacent cancer cells.^{5,16-19} These CAFs show a fetal-like phenotype, characterized by the expression of molecules typically expressed during embryonic development. In addition, CAFs develop a myofibroblast phenotype, with the expression of smooth muscle cell markers and the local production of transforming growth factor β (TGF- β), which can actively spread the CAF phenotype.²⁰⁻²⁶

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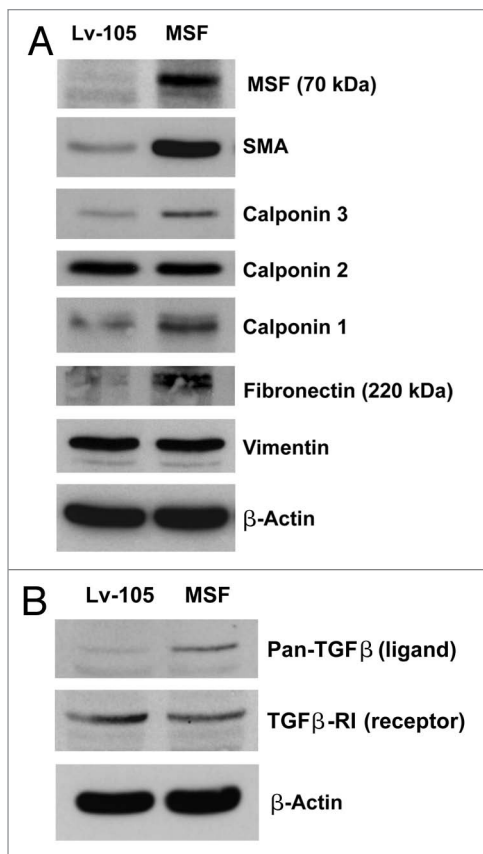


Figure 1. MSF overexpression confers the cancer-associated fibroblast phenotype, characterized by the expression of myo fibroblast markers and activated TGF- β signaling. (A) To assess the functional role of MSF stromal expression in tumor growth, we stably overexpressed the MSF protein in immortalized human fibroblast cell line (hTERT-BJ1 cells). Successful overexpression of the MSF protein was verified by immunoblot analysis. Empty vector control (Lv-105) or MSF fibroblasts were also subjected to immunoblot analysis with different myofibroblast marker proteins. Equal protein loading was assessed by immunoblotting with β -actin. Note that MSF overexpression strongly induces the expression of myofibroblast markers such as α -SMA, calponin 1 and 3 and fibronectin, while no increases in vimentin protein expression were observed in the fibroblasts. (B) MSF overexpression induces the activation of TGF- β signaling. Note that immunoblot analysis of control and MSF fibroblasts reveals that TGF- β ligand protein expression is significantly increased in MSF fibroblasts. Conversely, MSF fibroblasts are characterized by a reduction in the protein expression of TGF β -RI (type I receptor), as compared with control fibroblasts.

Fetal-like fibroblasts and myo fibroblasts are also both viewed as “activated fibroblasts,” due to their increased expression of both ECM components and inflammatory cytokines.²⁷⁻³⁴ Fetal-like fibroblasts also secrete a soluble, genetically truncated form of fibronectin, termed migration stimulating factor (MSF).²⁷⁻³⁴ Interestingly, MSF is highly expressed in both fetal epithelial and stromal cells and in cancer patients, but its expression is somehow suppressed in normal adults.²⁷⁻³⁴

Detailed molecular characterization of MSF indicates that it is a 70-kDa protein that is essentially identical to the N-terminal domain of full-length fibronectin, with the addition of an MSF-specific 10 amino-acid C-terminal sequence.^{35,36} MSF changes

the behavior of many target cell populations (fibroblasts, vascular and epithelial cells) by stimulating migration/invasion, matrix remodelling and neo-angiogenesis.³⁷⁻⁴⁶

Here, we generated a new hTERT-immortalized fibroblast cell line overexpressing MSF in order to clarify the functional role of MSF in driving the cancer-associated fibroblast phenotype. Now, we demonstrate that MSF-expressing fibroblasts create an autophagic/catabolic tumor stroma, which then provides high-energy nutrients to epithelial cancer cells via a paracrine mechanism.

Results

To directly assess the role of MSF in tumor growth, we stably overexpressed MSF in an immortalized human fibroblast cell line (hTERT-BJ1 cells) (Fig. 1A). Empty vector (Lv-105) control fibroblasts were produced in parallel. Figure 1A shows that transduction with MSF lentiviral particles successfully increased the stable expression of the MSF protein.

Fibroblasts overexpressing MSF develop a cancer-associated fibroblast phenotype, characterized by the expression of myo-fibroblast marker proteins and activated TGF- β signaling. Cancer-associated fibroblasts exhibit a myo fibroblastic phenotype, characterized by the synthesis of intracellular smooth muscle markers, in particular α -smooth muscle actin (α -SMA). To evaluate if MSF expression promotes myo fibroblastic differentiation, MSF-expressing fibroblasts were subjected to immunoblot analysis, using a panel of myo fibroblastic markers (Fig. 1A). The results show that MSF is indeed sufficient to induce the increased protein expression of SMA, Calponin (particularly, isoforms 1 and 3) and Fibronectin (full-length).

Several lines of evidence indicate that activated fibroblasts increase their expression and secretion of TGF- β , thereby promoting tumor growth. Thus, we next tested if MSF overexpression upregulates the expression of TGF- β . Consistent with this hypothesis, Figure 1B shows that MSF-overexpressing fibroblasts are characterized by an increase in TGF- β expression and a downregulation of its receptor, TGF β -RI, both indicative of activated TGF- β signaling.

Fibroblasts overexpressing MSF migrate to a significantly greater extent than do control cells, and they also function as chemo-attractants, stimulating cancer cell migration. MSF is a potent motogenic factor, which is able to stimulate the migration of fibroblasts, epithelial as well as endothelial cells.^{35,46} Here, we demonstrate that MSF overexpression stimulates the migration of fibroblasts, validating the motogenic activity of the MSF protein (Fig. 2A). MSF could also influence the migration of cancer cells, by acting on these cells as a chemo-attractant. In support of this notion, Figure 2B shows that cancer cells, in the presence of MSF-overexpressing fibroblasts, migrate to a greater extent (1.4-fold) than do cancer cells in presence of normal control fibroblasts.

This migratory activity could be induced by the increased expression and/or activation of proteins that play a fundamental role in cytoskeletal organization. Small GTPases, such as Rac1 and Cdc42, play a central role in regulating cell movement and

migration by interacting with other proteins that more directly confer cytoskeletal rearrangements. As predicted, **Figure 2C** shows that MSF overexpression upregulates the expression levels of both these small-GTPases, which are associated with remodeling the actin cytoskeleton.

Fibroblasts overexpressing MSF activate NFκB, exhibit the induction of autophagy and cell cycle arrest. Small GTPases are strong activators of the transcription factor NFκB,^{47,48} so we next validated that MSF is able to induce not only the upregulation of Cdc42 and Rac1, but also the activation of NFκB. As shown in **Figure 3A**, MSF overexpression resulted in increased levels of p-NFκB, suggesting that MSF could influence the stromal fibroblasts through the activation of a number of different signaling pathways, including the NFκB signaling pathway.

NFκB plays a pivotal role as a signal integrator, which controls the autophagic process. For this purpose, we evaluated if the activation of NFκB in stromal MSF fibroblasts is sufficient to promote the autophagic process. Therefore, fibroblasts overexpressing MSF were analyzed by immunoblot analysis, using a panel of autophagy markers. **Figure 3B** shows that MSF increases the expression of several classical autophagy markers, such as Beclin1, BNIP3 and LC3-I. These results suggest that MSF augments or activates the autophagic process in stromal fibroblasts, probably via increased activation of the NFκB pathway. This pro-autophagic phenotype is associated with cell cycle arrest, as evidenced by the upregulation of CDK inhibitors, such as p21(CIP1/WAF1), p19(ARF) and p16(INK4A) (**Fig. 3C**).

Under hypoxic conditions, MSF fibroblasts generate elevated levels of L-lactate and show decreased mitochondrial activity, consistent with a shift toward glycolytic metabolism. We have previously shown that stromal fibroblasts promote and fuel tumor growth via activation of an autophagic program in the tumor stroma.^{8,9,12-15} Autophagy leads to the generation of recycled catabolic nutrients that can be used to power the anabolic growth of cancer cells. Because L-lactate is a critical fuel that provides continued energetic support for cancer cells, we next examined if MSF fibroblasts are able to induce L-lactate accumulation. As shown in **Figure 4A**, fibroblasts overexpressing MSF display increased L-lactate production (~2-fold; $p = 0.004$; 1.5-fold $p = 0.03$, values expressed as nmoles/μg or pmoles/cells, respectively). However, the ability of MSF fibroblasts to secrete L-lactate was observed only under hypoxic conditions.

That L-lactate accumulation is indicative of a shift toward predominantly glycolytic metabolism. This observation was validated by assessing the status of mitochondrial activity in MSF fibroblasts. **Figure 4B** shows decreased mitochondrial activity, as predicted, as visualized using MitoTracker staining. Note that MSF induces a dramatic reduction in MitoTracker staining, indicative of a loss of healthy functional mitochondria, both under normoxic, as well as hypoxic conditions.

As shown in **Figure 4C**, MSF overexpression leads to Akt activation, which likely protects these cells against apoptosis. MSF fibroblasts were subjected to immunoblot analysis, using phospho-specific antibodies directed against different protein components of the Akt pathway.

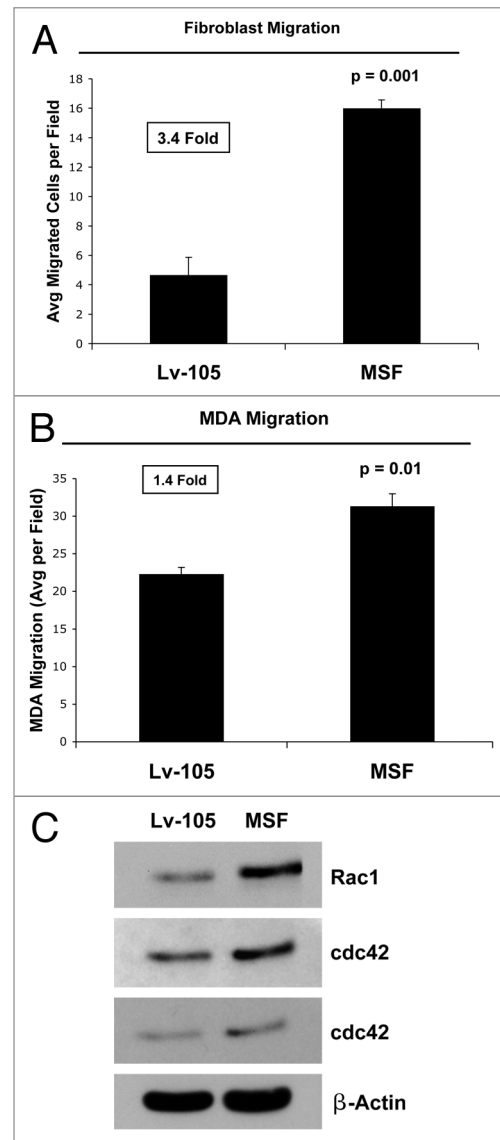


Figure 2. MSF stimulates the migration of fibroblasts and acts as chemo-attractant, stimulating cancer cell migration. (A) The migration of fibroblasts across an 8 μm pore uncoated membrane was assessed after a 6-h period, using a modified “Boyden Chamber” assay, employing Transwell cell culture inserts. Interestingly, the motility of fibroblasts overexpressing MSF was increased by ~3.4-fold, as compared with fibroblasts transfected with the empty-vector (Lv-105); $p = 0.001$ relative to control migration (Student’s t-test). (B) MSF overexpression stimulates cancer cell migration. To assess whether MSF fibroblasts can function as chemo-attractants, MDA-MB-231 cells were placed in the upper chambers and fibroblasts [empty vector (Lv-105) fibroblasts or MSF fibroblasts] were seeded into the lower chambers. Note that MSF fibroblasts promote cancer cell migration by ~1.4-fold. $p = 0.01$, control vs. MSF fibroblasts (Student’s t-test). (C) MSF is sufficient to increase the expression of the two small GTPases, namely Rac1 and Cdc42. Cells [empty vector (Lv-105) fibroblasts or MSF fibroblasts] were subjected to immunoblot analysis. The loading control shown for (C) is the same as in **Figure 1B**, as the same nitrocellulose membrane(s) were stripped and re-probed.

Note that MSF induces the activation of Akt-downstream effectors, such as phospho-mTOR and phospho-p70S6 kinase, both involved in protein biosynthesis. Akt normally activates

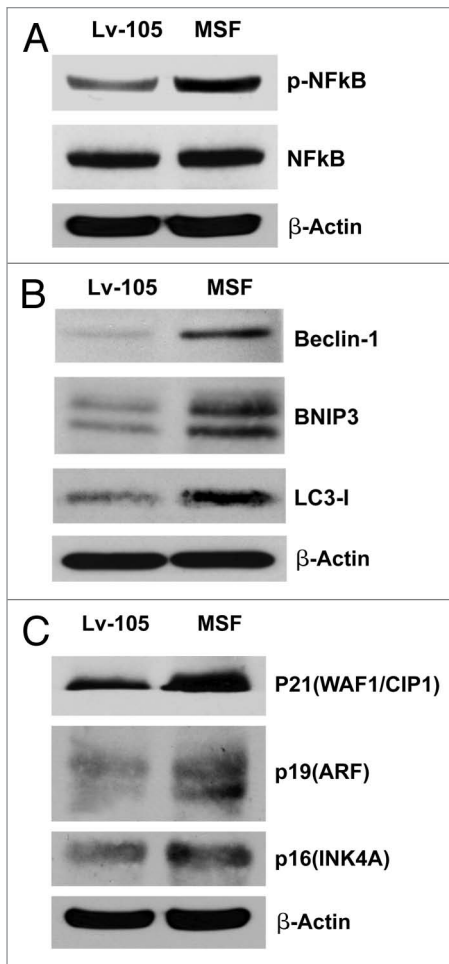


Figure 3. MSF overexpression in fibroblasts promotes the activation of NFκB, increases autophagy, and induces CDK inhibitors. (A) Since the human Rac-1 and Cdc42 proteins efficiently induce the transcriptional activity of nuclear factor kappaB (NF-kappaB), we next evaluated if MSF is able to induce not only the upregulation of the two small GTPases, but also the activation of NFκB. Note that immunoblot analysis of control or MSF fibroblasts revealed that the levels of p-NFκB are significantly increased in MSF fibroblasts, as compared with control fibroblasts. (B) NFκB can induce autophagy, so we verified if activation of NFκB by MSF overexpressed fibroblasts is sufficient to induce autophagy. Cells were lysed and subjected to immunoblot analysis using antibodies directed against a panel of autophagy markers. β-actin was used as equal loading control. Note that MSF increases the expression of several key autophagy markers, namely Beclin-1, BNIP3, and LC3-I. (C) To determine if MSF expression is also associated with senescence, we investigated whether known CDK inhibitors are upregulated. Immunoblot analysis shows that p21 (WAF1/CIP1), p19(ARF) and p16(INK4A) are all upregulated, consistent with cell cycle arrest and/or the onset of senescence.

mTOR, leading to p70S6K activation. Activation of Akt pathway by MSF in stromal fibroblasts may lead to activation of protein synthesis, as a compensatory mechanism to prevent apoptotic cell death in cells undergoing constitutive autophagy/mitophagy.

Fibroblast overexpressing MSF promote tumor growth, without any increases in tumor angiogenesis. Because MSF fibroblasts are able to increase L-lactate production and have a strong autophagic phenotype, we evaluated whether MSF is able

to promote tumor growth. For this purpose, we developed a human tumor xenograft model. MSF-overexpressing fibroblasts were co-injected with MDA-MB-231 breast cancer cells into the flanks of immunodeficient nude mice. **Figure 5A** demonstrates that MSF overexpression in stromal fibroblasts is sufficient to promote tumor growth, as evidenced by significant increases in both tumor weight and volume.

Stromal expression of MSF may contribute to tumor pathogenesis by a number of mechanism(s), including the stimulation of angiogenesis. To address this issue, frozen tissue sections derived from tumor xenografts were subjected to immunostaining with a well-established vascular marker, namely CD31. As shown in **Figure 5B**, MSF overexpression in stromal fibroblasts does not have a significant effect on tumor neo-vascularization, indicating that the tumor-promoting effects of MSF in cancer-associated fibroblasts are independent of tumor angiogenesis.

SMA, Rac1 and Cdc42 overexpression in fibroblasts induces myo fibroblast differentiation. We demonstrated above that MSF fibroblasts show increased expression of SMA and two small GTPase proteins, namely Rac1 and Cdc42. To determine if there is a cause-effect relationship here, we employed a genetic approach by overexpressing SMA, Rac1, and Cdc42 in an immortalized human fibroblast cell line (**Fig. 6AC**) Then, these fibroblast cell lines were subjected to immunoblot analysis, employing a panel of myo fibroblast markers, in order to characterize their phenotype.

Note that Rac1- and Cdc42-overexpressing fibroblasts display the upregulation in SMA protein expression (**Fig. 7A**), and all three overexpressing cell lines show increases in the calponin and vimentin (**Fig. 7B**), consistent with a myo fibroblast phenotype. Similarly, both GTPases, Rac1 and Cdc42, were able to induce the reorganization of the F-actin cytoskeleton, as evidenced by an increase in the density of actin stress fibers, as visualized by Phalloidin staining (**Fig. 7C**); note the bundles of parallel fibers aligned along the cell axis.

Cdc42-overexpression induces NFκB-activation, with increased autophagy and a shift toward glycolytic metabolism. Small GTPases are strong activators of the transcription factor NFκB.^{47,48} Thus, we evaluated the effects of expressing SMA, Rac1 and cdc42 in fibroblasts, on the status of NFκB and p-NFκB. Our results demonstrate that the p-NFκB protein levels are significantly increased only in Cdc42-overexpressing fibroblasts (**Fig. 8A**).

For this and all subsequent experiments, we chose to examine only the fibroblasts overexpressing SMA and Cdc42; SMA was used as a negative control and Cdc42 was used, as it is the GTPase that activates NFκB. To evaluate if this Cdc42-driven NFκB-activation promotes autophagy, fibroblasts overexpressing SMA and Cdc42 were subjected to immunoblot analysis, using a panel of autophagy markers.

Figure 8B demonstrates that Cdc42 overexpression in fibroblasts drives the increased expression of mitophagy (BNIP3) and autophagy markers (Beclin-1, LAMP1 and Cathepsin B).

Also, we evaluated if Cdc42-overexpressing fibroblasts are able to induce L-lactate accumulation and a shift toward glycolytic metabolism. **Figure 8C** demonstrates that Cdc42

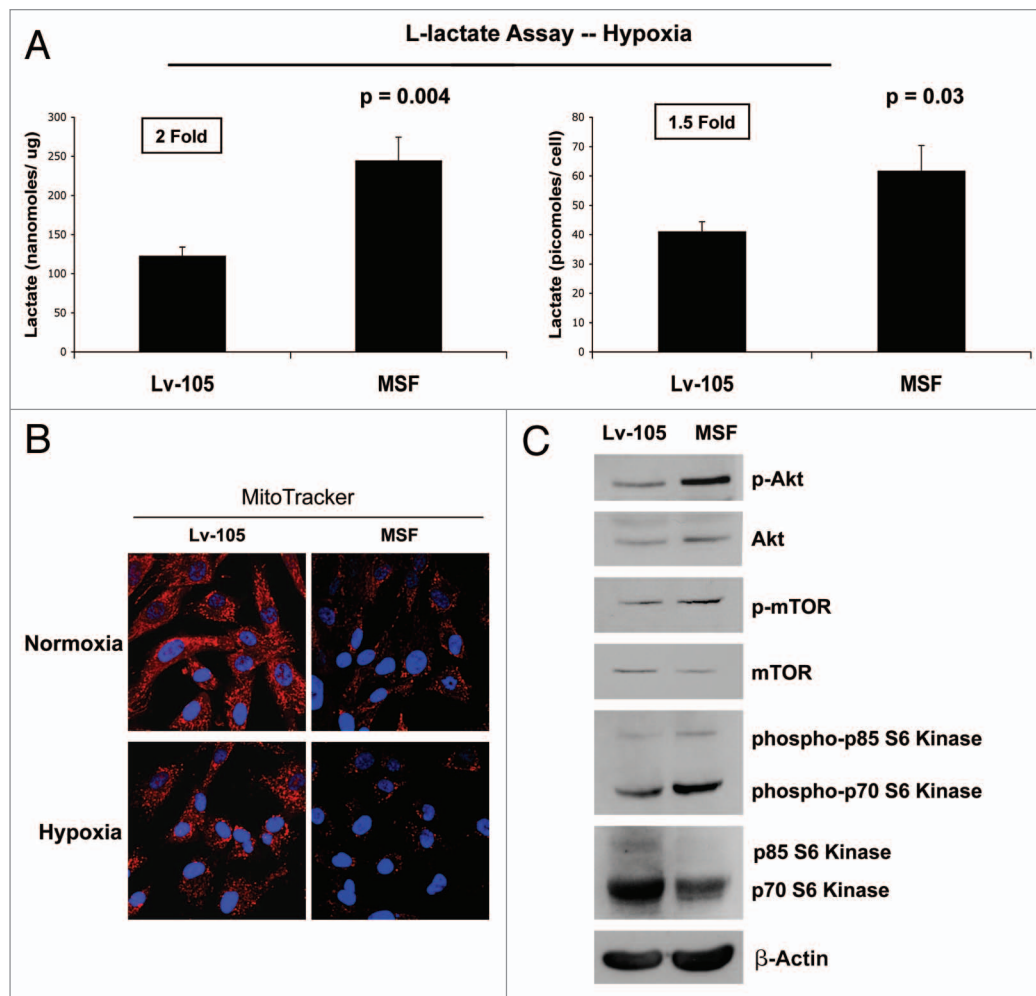


Figure 4. MSF overexpression in fibroblasts induces a shift toward glycolytic metabolism. (A) Under hypoxic conditions, MSF fibroblasts secrete elevated levels of L-lactate, consistent with increased aerobic glycolysis. As shown in the figure, MSF fibroblasts secrete increased levels of L-lactate (~2-fold, $p = 0.004$; normalized for protein content; ~1.5-fold, $p = 0.03$; normalized for cell number), relative to control fibroblasts processed in parallel. This suggests that MSF-overexpressing fibroblasts could provide essential energetic support for adjacent tumor epithelial cells, via the secretion of L-lactate, which is a critical mitochondrial fuel for the tumor growth. (B) MSF overexpression induces a decrease in mitochondrial activity, as visualized using MitoTracker staining. Note that MSF decreases mitochondrial activity, both under normoxic condition (upper panels) and under hypoxic condition (lower panels). MitoTracker (red); nuclei/DAPI (blue). Original magnification, 60 \times . (C) MSF activates the Akt pathway. Control or MSF fibroblasts were subjected to immunoblot analysis with antibodies to phospho-Akt, phospho-mTOR and phospho-p70 S6 kinase. The phospho-Akt, phospho-mTOR and phospho-p70 S6 kinase immunoblots were then reprobbed with Akt, mTOR and p70 S6 kinase antibodies, respectively. Note that MSF overexpression is associated with Akt pathway activation. This reaction is probably an anti-apoptotic response, to protect the fibroblasts from autophagy-induced cell death.

expression is sufficient to induce an ~80% increase in L-lactate production, under hypoxic condition and after treatment with Metformin, a specific inhibitor of mitochondrial complex I. This shift toward glycolytic metabolism was further validated by MitoTracker staining, showing that Cdc42 expression strongly decreases mitochondrial activity under hypoxic conditions (Fig. 8D).

Stromal expression of Cdc42 promotes increased tumor growth in vivo. To evaluate if Cdc42 expression in stromal cells is able to promote tumor growth in vivo, we used a human tumor xenograft model. Control, SMA or Cdc42 fibroblasts were co-injected with MDA-MB-231 breast cancer cells in the flanks of immunodeficient nude mice. Figure 9A shows that overexpression

of Cdc42 in stromal fibroblasts consistently promotes tumor growth, over a 25-d time course. Figure 9B shows that, at 4 weeks post-injection, Cdc42 fibroblasts increased tumor volume by ~1.75-fold, as compared with vector-alone control fibroblasts cells, directly demonstrating that stromal Cdc42 is able to support tumor growth in vivo.

Finally, to determine the role of neo-vascularization in Cdc42-mediated tumor growth, we quantified neo-vascularization via immunostaining with CD31 (Fig. 9C). However, a 25% increase of tumor angiogenesis in Cdc42 tumors is not sufficient to account for a near 2-fold increase in tumor growth. Instead, metabolic reprogramming of the tumor microenvironment toward L-lactate production is a more likely mechanism (Fig. 10).

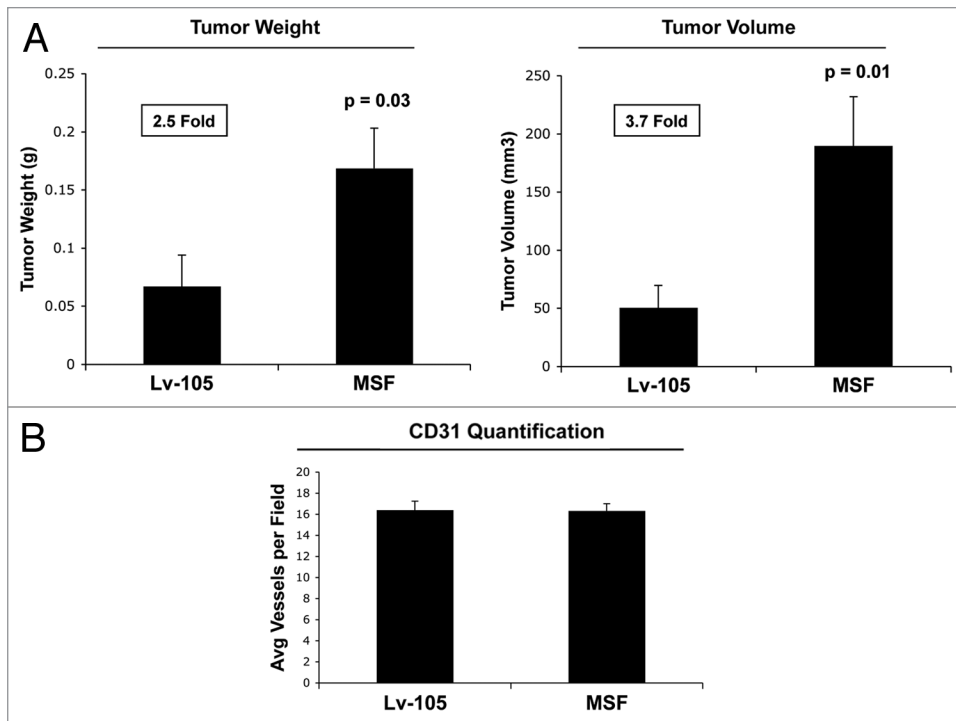


Figure 5. MSF overexpression in fibroblasts dramatically promotes breast cancer tumor growth, without affecting tumor angiogenesis. (A) Control or MSF fibroblasts were co-injected with MDA-MB-231 breast cancer cells into the flanks of nude mice. After 4 weeks post-injection, the resulting tumors were harvested. Interestingly, relative to control fibroblasts (Lv-105), MSF fibroblasts increased tumor weight by ~2.5-fold ($p = 0.03$) and tumor volume by ~4-fold ($p = 0.01$). $n = 10$ tumors per experimental group. (B) MSF overexpressing fibroblasts do not increase tumor angiogenesis. Frozen tumor sections were immunostained with anti-CD31 antibodies to quantify vessel density. As shown in the figure, note that the tumor promoting effect of MSF fibroblasts are independent of angiogenesis, as no significant increases in vessel density were observed.

Discussion

The role of the host stromal microenvironment in promoting tumor initiation and progression is now well-established.¹⁻⁴ However, the exact molecular mechanism(s) of how cancer-associated fibroblasts promote tumor growth remain unknown. Here, we highlight that MSF (migration-stimulating factor) functions to metabolically reprogram stromal fibroblasts toward glycolytic metabolism, resulting in the generation of a catabolic tumor microenvironment that actively “fuels” anabolic tumor growth.

More specifically, MSF-overexpressing fibroblasts were used to mimic the “activated microenvironment” that is now widely known to support tumor growth. We demonstrated that MSF fibroblasts show many characteristics of differentiated myo fibroblasts, including the expression of smooth muscle-specific proteins.

Transforming growth factor- β (TGF- β) is a potent inducer of myo fibroblast differentiation that has been implicated in conferring the tumor-associated fibroblast phenotype.^{3,18,19,49-52} Here, we have demonstrated that MSF overexpression in stromal fibroblasts leads to the increased production of TGF- β and is associated with a reduction in the expression of its receptor, TGF- β -RI.

The expression of TGF- β , specifically TGF- β 1, is upregulated in most tumors and seems to play a key role in cancer progression.^{3,18,19,49-52}

Increased TGF- β expression in fibroblasts benefits cancer progression, likely via paracrine effects on tumor cells.^{18,19,49-52} In particular, the release of TGF- β in the vicinity of cancer cells may result in a more hospitable microenvironment, facilitating tumor growth. Several authors have shown that TGF- β overexpression leads to an increased metabolic rate, due to enhanced glycolysis.^{53,54} MSF may induce glycolysis in stromal fibroblasts via increased endogenous production of TGF- β . The observed increase in glycolytic metabolism may be due to the autophagic destruction of mitochondria in MSF-overexpressing fibroblasts. This assertion is consistent with our previous observations that autophagy in cancer-associated fibroblasts is able to generate a catabolic tumor stroma that drives the anabolic growth of cancer cells.^{8,9} Regardless of the exact mechanism activating glycolysis, MSF is able to produce a catabolic, energy-rich microenvironment that favors tumor growth.

Small GTPases, such as Rac1 and Cdc42, are known to play a critical role in cell migration and invasion.^{55,56} However, their potential roles in myo fibroblast differentiation, autophagy and cellular metabolism are underappreciated. In the current study, we demonstrated that MSF-overexpressing fibroblasts have increased expression both Rac1 and Cdc42. To determine whether increased Rac1 and/or Cdc42 expression influences the activation of tumor microenvironment, we generated Rac1- and Cdc42-overexpressing fibroblasts.

Our results demonstrate that both Rac1 and Cdc42 fibroblasts undergo myo fibroblast differentiation, with characteristic re-organization of the actin cytoskeleton. However, only Cdc42 fibroblasts show activation of NF κ B, with the onset of autophagy and a shift toward predominantly glycolytic metabolism in the tumor stroma resulting in the promotion of tumor growth. Therefore, overexpression and/or activation of Cdc42 is a likely mechanism by which MSF induces NF κ B-activation, leading to increased autophagy and glycolysis due to reduced mitochondrial function. As such, glycolytic/catabolic MSF fibroblasts create a favorable metabolic microenvironment to support tumor growth.

In conclusion, our results highlight the critical functional role of MSF as a driver of cancer progression. This is consistent with

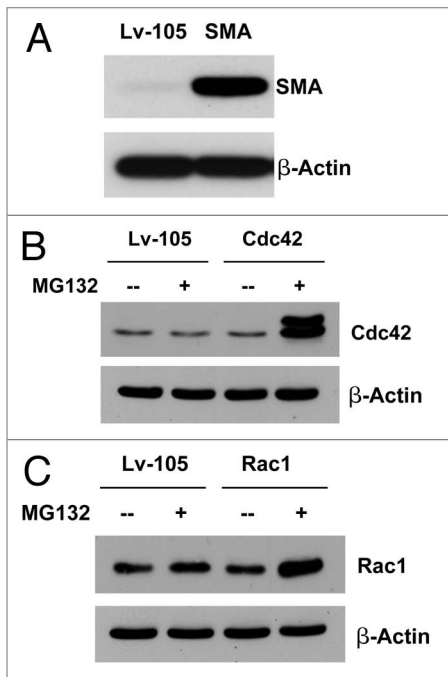


Figure 6. Recombinant expression of SMA, Rac1, and Cdc42 in immortalized fibroblasts. To assess the role of SMA, Rac1, and Cdc42 in the tumor microenvironment, we stably overexpressed SMA, Rac1, and Cdc42 in the immortalized human fibroblast cell line (hTERT-BJ1 cells). Successful protein overexpression of SMA, Rac1, and Cdc42 was validated by immunoblot analysis (A–C). In order to better visualize Cdc42 and Rac1 overexpression, we treated fibroblasts with a protease inhibitor (MG132; 10 μ M for 16 h).

its ability to stimulate the migration/invasion in both stromal and tumor cells and with its effects on the metabolic remodeling of the tumor microenvironment.

Materials and Methods

Materials. Reagents were purchased as follows: the specific and cell-permeable proteasome inhibitor (MG132) was from Calbiochem (used at a final concentration of 10 μ M for 16h); Metformin (1.1-dimethylbiguanide hydrochloride) was from Sigma (D150959); Alexa Fluor 633 Phalloidin (A222284) was from Invitrogen. Antibodies to the following target proteins were also used: Fibronectin N-terminal (Chemicon International, MAB1936; to recognize MSF); Fibronectin (Abcam, ab23750); Vimentin (BD PharMingen, 550513); Calponin 1/2/3 (Santa Cruz Biotech, sc-28545); Smooth Muscle Actin (Dako, M0851); Beclin (Novus Biologicals, NBP1–00085); BNIP-3 (Abcam, ab10433); LC3 (Abcam, ab48394); β -actin (Sigma-Aldrich, A5441); TGF- β (Cell Signaling, 3711); TGF- β -RI (Santa Cruz Biotech, sc-398); phospho-Akt (Cell Signaling, 9271); Akt (Cell Signaling, 2967); phospho-mTOR (Cell Signaling, 2971); mTOR (Cell Signaling, 2972); phospho-p70 S6 kinase (Cell Signaling, 9234); p70 S6 kinase (Cell Signaling, 9202); CD31 (BD Biosciences, 550274); Rac1 (Santa Cruz, sc-95); Cdc42 (Santa Cruz, sc-8401); p-NF κ B (Cell Signaling, 3037); NF κ B (Cell Signaling, 3034); p14ARF (Santa Cruz, sc-53639); p16

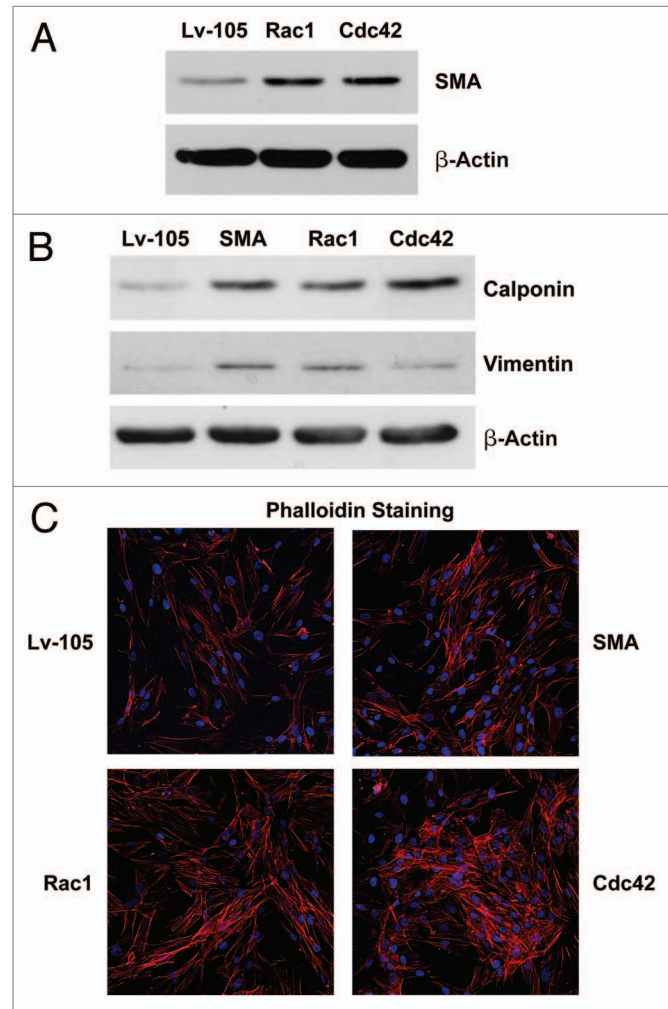


Figure 7. Overexpression of SMA, Rac1, and Cdc42 confers the myo fibroblast phenotype. (A) Rac1- and Cdc42- overexpressing fibroblasts display the upregulation of SMA protein expression. (B) Rac1 and Cdc42 induce the expression of other myofibroblast markers, namely calponin and vimentin. (C) Rac1 and Cdc42 regulate the organization of the actin cytoskeleton, inducing a reorganization of F-actin, as suggested by the increase of actin stress fibers. Equal cell numbers were plated on glass cover sides, and after 24 h, the cells were fixed and actin filaments and nuclei were stained with Phalloidin (red) and DAPI (blue), respectively. Note that SMA-, Rac1- and Cdc42- overexpressing fibroblasts display an increased number of stress fibers, as compared with vector-alone control cells.

(Santa Cruz, sc-759); p21 (Santa Cruz, sc-6246); LAMP1 (Santa Cruz, sc-17768); cathepsin B (Santa Cruz, sc-13985).

Cell culture and stable transfection. Human immortalized fibroblasts (hTERT-BJ1) were used to generate the cell lines overexpressing migration-stimulatory factor (MSF), SMA, Rac1 and Cdc42. Lentiviral plasmids [EX-NEG-Lv105 (empty vector), Ex- Z4998-Lv105 (human MSF), Ex-D0101-Lv105 (human SMA), Ex-A0247-Lv105 (human Rac1), Ex-X0047-Lv105 (human cdc42) (obtained from GeneCopoeia, Inc.)] were used to transfect GeneCopoeia 293Ta lentiviral packaging cells using Lenti-PacTM HIV Expression Packaging Kit following the manufacturer's instructions. After 48 h, lentivirus containing culture

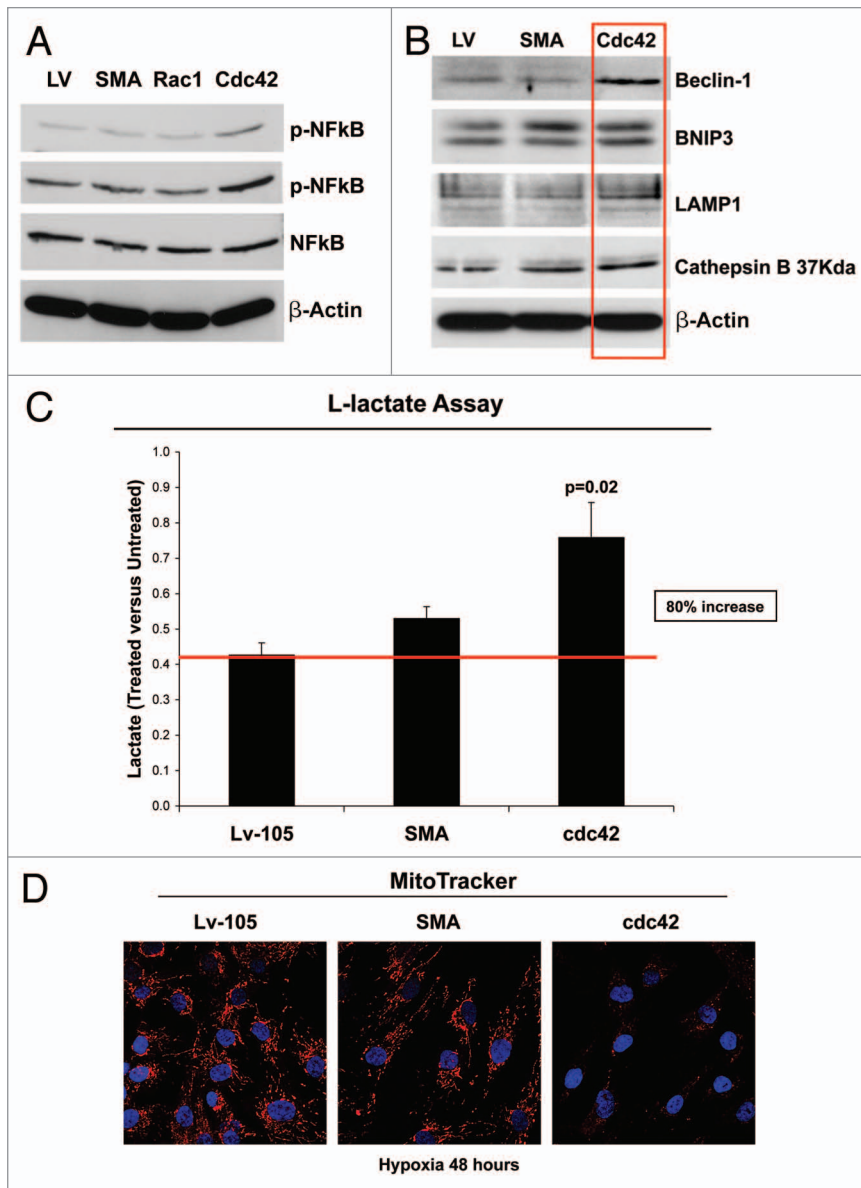


Figure 8. Cdc42-overexpression promotes the activation of NFκB, induces increased autophagy and glycolytic metabolism. (A) GTPases are strong activators of the transcription factor NFκB. As shown in figure, note that immunoblot analysis of control or SMA-, Rac1- and cdc42-overexpressing fibroblasts reveals that p-NFκB protein levels are significantly increased, exclusively in Cdc42 fibroblasts, as compared with control fibroblasts. (B) To validate that Cdc42 induces an autophagic program, cells were subjected to immunoblot analysis using several autophagy markers. β-actin was used as an equal loading control. Note that Cdc42 increases the expression of the autophagy markers, such as Beclin-1, BNIP3, LAMP-1 and Cathepsin B (37kDa). (C) Cdc42-overexpressing fibroblasts display a predominantly glycolytic metabolism, as demonstrated by increased L-Lactate production, under hypoxic conditions. Cells cultured for 48 h under hypoxic conditions (0.5% O₂) were treated with or without metformin (1 mM), and the results are expressed as ratio between treated vs. untreated cells. (D) The shift toward a predominantly glycolytic metabolism is also demonstrated by decreased mitochondrial activity, as visualized using MitoTracker. Note that Cdc42 significantly decreases mitochondrial activity, as compared with vector alone control or SMA overexpressing fibroblasts. MitoTracker (red); nuclei/DAPI (blue). Original magnification, 60x.

medium was added to human fibroblasts in the presence of 5 μg/ml Polybrene. Infected fibroblasts were selected with puromycin (1.5 μg/ml).

All cell lines used in the following experiments were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum in a 37°C humidified atmosphere (5% CO₂) unless otherwise noted.

Immunoblot analysis. For immunoblotting, cultured cells were harvested in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 60 mM n-octyl-glucoside) or RIPA lysis buffer containing protease inhibitors (Roche, 11836153001) and phosphatase inhibitors (Thermoscientific, 78420). The pooled cells were rotated for 40 min at 4°C, centrifuged at 10,000 × g for 15 min at 4°C, and the protein concentration of the supernatant was determined using the BCA reagent (Pierce). Protein samples (30–50 μg total proteins per lane) were then subjected to 12% or 15% SDS-PAGE, and the proteins were then electrophoretically transferred to a nitrocellulose membrane. After blocking for 1 h at room temperature with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) supplemented with 5% nonfat dry milk and 1% BSA, membranes were incubated for 1 h at room temperature with primary antibodies and then for 1 h at RT with specific (HRP)-conjugate secondary antibodies [anti-mouse, 1:6,000 dilution (Pierce) or anti-rabbit, 1:5,000 dilution (BD Pharmingen)]. HRP activity was visualized by enhanced chemiluminescent substrate (Thermo Scientific) followed by exposure of the membrane to X-ray film.

Migration assay. The effects of MSF overexpression on fibroblast migration and the effects of MSF fibroblasts on the migratory potential of MDA-MB-231 cells were measured in vitro using a modified Boyden chamber assay. Briefly, fibroblasts [EV (empty vector) or MSF overexpressing] in 0.5 ml of serum-free Dulbecco's modified Eagle's medium were added to the wells of 8 μm pore uncoated membrane of modified Boyden chambers. The lower chambers contained 10% fetal bovine serum in Dulbecco's modified Eagle's medium to serve as a chemo-attractant. Cells were incubated at 37°C and allowed to migrate throughout the course of 6 h. To assess the effect of fibroblast overexpressing MSF protein on the migratory capacity of MDA-MB-231 cells, fibroblasts were seeded in the lower chambers in DMEM supplemented with 10% NuSerum and used as chemo-attractant. MDA-MB-231 cells were added to the wells of 8 μm pore uncoated membrane of modified Boyden chambers and allowed

to migrate throughout the course of 4 h at 37°C. In both cases, cells were removed from the upper surface of the membrane by scrubbing with cotton swabs. Chambers were stained in 0.5% crystal violet diluted in 100% methanol for 30 min, rinsed in water and examined under a bright-field microscope. Values for migration were obtained by counting five fields per membrane (X20 objective) and represent the average of three independent experiments.

L-lactate assay. Cells (empty vector or MSF-, SMA-, Cdc42-overexpressing fibroblasts) were seeded in quadruplicate (100,000 cells per well) in 12-well plates in 1 ml of complete media. After 18 h, the media was changed to DMEM containing 2% FBS and incubated under hypoxic conditions (0.5% O₂). SMA and cdc42 overexpressing fibroblasts were also with or without metformin (1 mM). After 48 h, the media of each well was collected and the concentration of L-lactate was measured using the EnzyChrom™ L-Lactate Assay Kit (ECLC-100, BioAssay Systems, Inc.). After removing the media, cells were trypsinized, spun down and resuspended in 1 mL of media for quantification. Cells were counted in 4–6 fields, using a 40× objective lens and a hemocytometer. Cells were then lysed, and the protein concentration was determined using the BCA reagent (Pierce). The amount of L-lactate in the media was normalized to total cell number or to total cell protein content.

Mitochondrial staining. To evaluate mitochondrial activity, cells were stained with MitoTracker Orange (CMTMRos; M7510, Invitrogen, Inc.). Lyophilized MitoTracker was dissolved in DMSO to generate a 1 mM stock solution that was then diluted into serum-free DMEM at a final concentration of 25 nM. Briefly, control or MSF-, SMA-, Cdc42-overexpressing fibroblasts (90,000 cells per well) were cultured for 48 h in normoxia or under hypoxic conditions. Then, they were incubated with pre-warmed MitoTracker staining solution for 12 min at 37°C in the dark. Cells were then washed in PBS Ca²⁺/Mg²⁺, three times and fixed with 2% PFA 30 min at RT. Cells were washed again with PBS Ca²⁺/Mg²⁺, incubated with the nuclear stain DAPI and mounted.

Murine xenograft studies. All animals were housed and maintained in a barrier facility at the Kimmel Cancer Center at Thomas Jefferson University under National Institutes of Health (NIH) guidelines. Mice were kept on a 12-h light/dark cycle with ad libitum access to food and water. Animal protocols used for this study were pre-approved by the Institutional Animal Care and Use Committee (IACUC). Briefly, MDA-MB-231-GFP human breast cancer cells (1 × 10⁶ cells) were co-injected with control (empty vector) or MSF-, SMA-, Cdc42-overexpressing fibroblasts (3 × 10⁵ cells) in 100 μl of sterile PBS into the flanks of athymic NCr nude mice (NCRNU; Taconic Farms; 6–8 weeks of age). Mice were then sacrificed at 4 weeks post-injection; tumors were dissected to determine their weight and size using calipers. Tumor volume was calculated using the formula (X²Y)/2, where X and Y are the short and long dimensions, respectively, of the tumor. After the dissection, tumors were fixed with 10% formalin or flash-frozen in liquid nitrogen-cooled isopentane.

Quantitation of tumor angiogenesis. Immunohistochemical staining for CD31 was performed on frozen tumor sections using

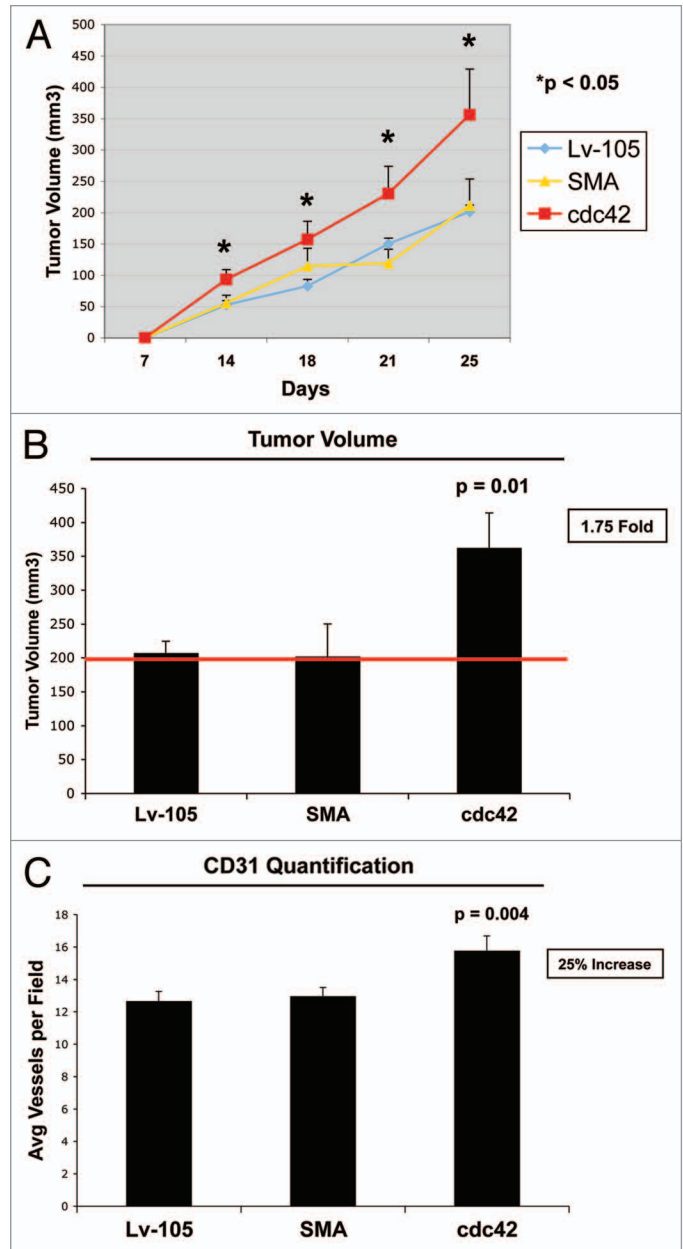


Figure 9. Cdc42 overexpressing fibroblasts promote tumor growth in vivo. We used a xenograft model employing MDA-MB-231 breast cancer cells injected into the flanks of athymic nude mice. MDA-MB-231 breast cancer cells were co-injected with either the empty vector (Lv-105), SMA- or Cdc42- overexpressing fibroblasts. (A) Comparative trend measurements for tumor growth (days 7–25 post-injection). Tumor volumes were measured with callipers about twice a week and mean tumor volume is plotted vs. time, for each experimental group. (B) Tumor growth. Tumor volumes were also measured at 4 weeks post-injection. Note that fibroblasts overexpressing Cdc42 significantly promote tumor growth, resulting in a 1.75-fold increase in tumor volume. p = 0.01; n = 10 tumors per experimental group. (C) Tumor angiogenesis. Tumor frozen sections were cut and immunostained with anti-CD31 antibodies. Then, vascular density (number of vessels per field) was quantified. The observed 25% increase in tumor angiogenesis in Cdc42 tumors is not sufficient to account for a near 2-fold increase in tumor growth. Instead, metabolic reprogramming of the tumor microenvironment, toward L-lactate production, is a more likely mechanism.

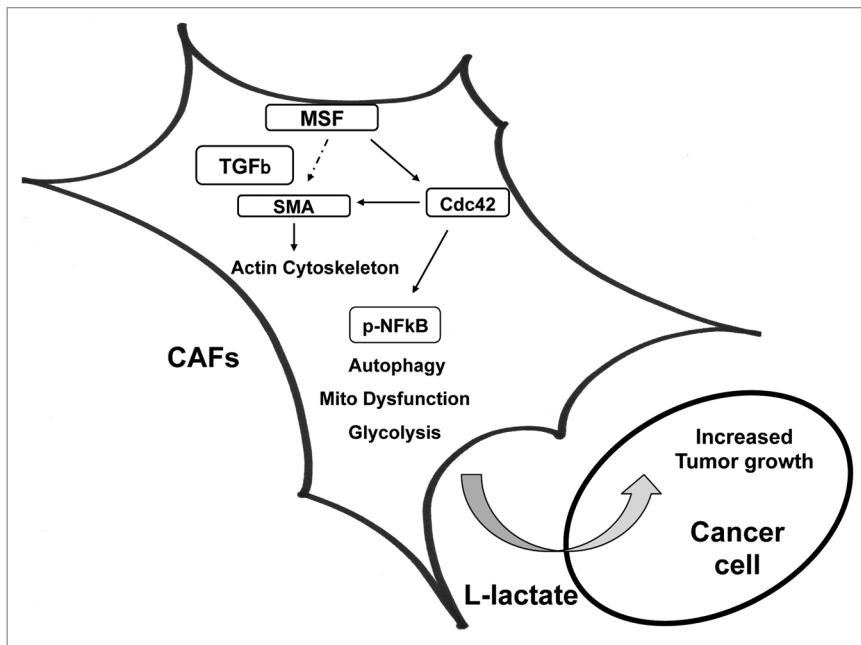


Figure 10. MSF expression in cancer-associated fibroblasts drives tumor growth. MSF leads to the upregulation of SMA and a number of other myo fibroblast marker proteins, conferring a myo fibroblast phenotype. The fibroblast activation by MSF is probably induced by TGF- β signaling and the increased expression of the small GTPase Cdc42, driving the activation of NF κ B that, in turn, induces autophagy, mitophagy and aerobic glycolysis, thereby promoting tumor growth.

a three step biotin-streptavidin-horseradish peroxidase method. Frozen tissue sections (6 μ m) were fixed in 4% paraformaldehyde in PBS for 10 min and washed with PBS. After blocking with 10% rabbit serum, the sections were incubated overnight at 4°C with rat anti-mouse CD31 antibody (BS Biosciences) at a dilution of 1:200, followed by biotinylated rabbit anti-rat IgG (Vector Labs, 1:200) antibody and streptavidin-HRP (Daki, 1:1000). Immunoreactivity was revealed with 3,3'-diaminobenzidine (DAB).

Phalloidin staining. Cell monolayers were stained with Alexa Fluor 633-phalloidin to examine the structure of filamentous F-actin. Washed cells were fixed with paraformaldehyde 2%, washed again with PBS $\text{Ca}^{2+}/\text{Mg}^{2+}$ and permeabilized for 10 min

with TBP buffer (0.1% Triton X-100, 0.2% BSA in PBS $\text{Ca}^{2+}/\text{Mg}^{2+}$). The cells were stained with Phalloidin Staining Solution (diluted 1:40 in PBS 1% BSA) 30 min at RT in dark conditions. Stained F-actin was visualized using a Zeiss LSM510 meta-confocal system. Images were acquired with a 20 \times objective.

Statistical analysis. Statistical significance was examined using the Student's t-test. Values of $p \leq 0.05$ were considered significant. Values were expressed as means \pm SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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