Published in final edited form as:
J Dermatol Sci. 2011 December ; 64(3): . doi:10.1016/j.jdermsci.2011.08.005.

Caveolin-1 is a negative regulator of MMP-1 gene expression in human dermal fibroblasts via inhibition of Erk1/2/Ets1 signaling pathway

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Abstract

Background—Caveolar raft domains, also termed caveolae, are flask shaped invaginations that require the expression of the structural protein caveolin-1 (cav-1). Matrix metalloproteinase 1 (MMP-1) is a collagenase capable of degrading insoluble triple helical collagens. Deregulation of MMP-1 contributes to various pathological processes, including tissue fibrosis and impaired wound healing.

Objective—In this study we investigated the role of cav-1 in MMP-1 gene regulation in human dermal fibroblasts.

Methods—Fibroblasts were isolated from healthy subjects. Western blot was used to analyze protein levels and quantitative real time RT-PCR was used to measure mRNA expression. Cells were transiently transfected with siRNA oligos against acid sphingomyelinase (ASMase) and cav-1, or transduced with adenoviruses overexpressing ASMase and cav-1. The specific pharmacological inhibitors UO126 and SP600125 were used to block Erk1/2 and JNK activity.

Results—This study shows that siRNA-mediated depletion of ASMase or cav-1, results in upregulation of MMP-1 gene expression. Similarly, MMP-1 expression was decreased after overexpresssion of cav-1 via an adenoviral vector. Depletion of cav-1 had no effect on JNK phosphorylation, while it resulted in an increase in Erk1/2 and Ets1 phosphorylation levels. Furthermore, in cav-1 depleted cells treated with the Erk inhibitor UO126, there was no increase in the levels of phospho-Erk1/2, phospho-Ets1, and MMP-1, suggesting that cav-1 mediated effects on MMP-1 and phospho-Ets1 are Erk1/2 dependent.

Conclusions—In conclusion, this study has revealed an important role for cav-1 as a negative regulator of MMP-1 gene expression via inhibition of Erk1/2/Ets1 signaling. Cav-1 could potentially be a therapeutic target in diseases with deregulated extracellular matrix (ECM) turnover.

Keywords

Caveolin-1; MMP-1; Extracellular matrix

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Conflict of interest
The authors have no conflict of interest to declare.

1. Introduction

Non-caveolar and caveolar lipid raft domains are low-density detergent resistant domains enriched in cholesterol and sphingolipids, present in the membrane of most cells, which play important roles in signal transduction and endocyotsis. Caveolar raft domains, also termed caveolae, are flask shaped invaginations that require the expression of the structural protein caveolin. Unlike caveolae, lipid rafts (non-caveolar lipid rafts domains) are flat domains that lack caveolin [1]. Among the three caveolin isoforms identified to date (cav-1, 2, and 3), cav-1 is the most extensively studied and key to the formation of caveolae. Thus, cav-1 knockout mice show a complete loss of caveolae invaginations [2], while cav-1 expression in cells lacking caveolae leads to de novo formation of caveolae [3].

Acid sphingomyelinase (ASMase) is a sphingolipid enzyme with important roles in lipid metabolism and cellular stress response. Studies have shown that during signaling, lipid raft sphingomyelin is converted to ceramide via the action of acid sphingomyelinase, thus allowing signaling molecules to cluster into signaling platforms. Ceramide has been recognized as a key signaling molecule, and published reports suggest that caveolar signaling may also be a ceramide-regulated event [4–6]. This suggests that depletion of either cholesterol or ceramide can result in aberrant signaling in the lipid rafts and caveolae, while cav-1 depletion only affects the later.

The matrix metalloproteinases (MMPs) are enzymes pivotal to the turnover of the extracellular matrix, playing a major role in the physiological processes that are involved in development and morphogenesis. Aberrant expression or activity of these enzymes have also been implicated in disease states such as abnormal wound healing, joint destruction, tumor metastasis and fibrosis [7]. According to their preferred substrate in the extracellular matrix (ECM), MMPs are classified as collagenases, gelatinases and stromelysins. MMP-1 is the main collagenase secreted by fibroblasts, capable of degrading native fibrillar collagen types I, II, III, and IV. Depending on cell type and conditions, various signaling pathways have been shown to induce MMP-1 gene expression, including JNK, p38 MAPK, Erk1/2, Ets1, and Fli1 [7–9].

Due to the crucial roles of cav-1 and MMPs in regulating important processes involved in fibrosis and tumor progression and metastasis, a growing number of studies have investigated a possible relationship between these molecules. Published data suggests that cav-1 can be an important regulator of the expression and activity of MMPs. Thus, in melanoma cells cav-1 upregulated MMP-2 and 9 [10], while treatment of scleroderma monocytes with cav-1 scaffolding domain peptide inhibited MMP-9 release from these cells [11]. Published studies have shown that MMP-2 and MMP-14 are localized in caveolae and that their activity is regulated by interaction with cav-1 [12,13].

This study aims to investigate the specific contribution of ASMase and cav-1 to MMP-1 gene expression in human dermal fibroblasts. To answer this question, we selectively manipulated levels of cav-1 and ASMase by siRNA or by overexpressing cav-1 and ASMase using an adenoviral vector. Our results demonstrate that cav-1 and ASMase are negative regulators of MMP-1 transcription. Additionally we also show that cav-1 inhibits MMP-1 expression via inhibition of Erk1/2 and Ets1 signaling pathways and that JNK activity is not required for the cav-1-mediated MMP-1 gene regulation.

2. Materials and methods

2.1. Reagents

The following antibodies were used: anti-phospho-Erk1/2 (T202/Y204), anti-Erk1/2, anti-phospho-c-jun (S63), anti-c-jun, anti-phospho-JNK (T183/Y185) (Cell Signaling, Beverly, MA, USA), monoclonal β actin (Sigma Aldrich, St Louis, MO, USA), monoclonal anti-MMP-1 (Millipore, Billerica, MA, USA), anti-cav-1 (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-phospho-Ets1 (T38) (Invitrogen, Carlsbad, CA, USA). Dulbecco's modified Eagle medium (DMEM) and 100× antibiotic-antimycotic solution (penicillin streptomycin and amphotericin B) were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from HyClone (Logan, UT, USA). The Erk1/2 inhibitor UO126 was purchased from Cell Signaling and the ASMase inhibitor Imipramine was obtained from Sigma. The JNK inhibitor SP600125 was purchased from Enzo Life Sciences. Enhanced chemiluminescence reagent and bovine serum albumin (BSA) protein assay reagent were obtained from Pierce (Rockford, IL, USA). TriReagent was purchased from the Molecular Research Center (Cincinnati, OH, USA). Primers were purchased from Operon (Huntsville, AL, USA).

2.2. Cell culture

Human dermal fibroblast cultures were established from biopsy specimens obtained from the dorsal forearms of healthy donors, upon informed consent and in compliance with the Institutional Review Board. Dermal fibroblasts were cultured from the biopsy specimens as described previously [14]. Normal and SSc skin fibroblasts were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution.

2.3. Adenovirus transfection

The cav-1Ad human adenovirus overexpressing cav-1 and utilizing a CMV promoter, and the LucAd (luciferase) control vector were purchased from Vector Biolabs (Philadelphia, PA, USA). An adenoviral vector expressing ASMase or GFP was generated using the method described by He et al. [15]. cDNA encoding ASMase (a gift from Dr. Y. Hannun) was cloned in the shuttle vector pAdTRACK-CMV, which contains a GFP expression cassette driven by a separate CMV promoter, and was used to generate recombinant adenoviruses. A control adenovirus expressing GFP alone was generated via the same method for use as a control vector. Dermal fibroblasts were grown to 80–90% confluence, changed to serum free media, and treated with adenovirus for 48 h before mRNA was collected.

2.4. RNA interference

SMARTpool siRNA against cav-1 and ASMase were purchased from Dharmacon RNA Technologies (Lafayette, CO, USA). Negative-control siRNA and Hiperfect siRNA transfection reagent were purchased from Qiagen (Germantown, MD, USA). Dermal fibroblasts were grown to 70–80% confluence and transiently transfected using 50 nM of gene-specific siRNA, or scrambled nonsilencing siRNA. Transfection was performed in serum containing media according the manufacturer's protocol, 48 h later cultures were changed to serum free DMEM containing 0.1% BSA, and left for 24 h. A second transfection was performed in the same manner, and cell lysates were collected 72 h later. For inhibition of Erk1/2 or JNK, pharmacologic inhibitors were applied in the serum free media 1 h prior to any additional treatment.

2.5. Western blot analysis

Cells were washed with PBS on ice and collected. Cell pellets were suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM glycerophosphate with freshly added phosphatase inhibitors (5 mM sodium fluoride and 1 mM Na_3VO_4) and a protease inhibitor mixture (Sigma Aldrich). Protein concentration was quantified using the BCA Protein Assay kit (Pierce). Equal amounts of total protein for each sample were separated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 2% milk in TBST for 1 h and incubated with primary antibodies overnight at 4 °C in 2% milk. After three TBST washes, membranes were probed with HRP-conjugated secondary antibody against the appropriate species for 1 h at room temperature. Protein was visualized using ECL reagent (Amersham Biosciences, Piscataway, NJ).

2.6. Real-time PCR

Total RNA was isolated from dermal fibroblasts using TriReagent (Molecular Research Center) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed in a 20-μl reaction using random primers and Transcriptor First Strand synthesis kit (Roche Applied Sciences Indianapolis, IN). cDNA was diluted 10-fold and Quantitative (q)PCR was carried out using IQ SYBR Green mix (Bio-Rad, Hercules, CA) on an iCycler PCR machine (Bio-Rad) using 1 μl of cDNA in triplicate, with β actin as the internal control. The primers used are as follows. β2-Microglobulin forward (GCC GTG TGA ACC ATG TGA CTT T), β2-microglobulin reverse (CCA AAT GCG GCA TCT TCA AA); MMP-1 forward (TCT GGG GTG TGG TGT CTA), MMP-1 reverse (GCC TCC CAT CAT TCT CAG GTT); caveolin-1 forward (ACA GCC CAG GGA AAC CTC), caveolin-1 reverse (GAT GGG AAC GGT GTA GAG ATG); MMP-14 forward (TAC TTC CCA GGC CCC AAC), MMP-14 reverse (GCC ACC AGG AAG ATG TCA TT).

3. Results

3.1. Depletion of ASMase up-regulates MMP-1 gene expression

To investigate the role of ASMase in MMP-1 gene regulation in dermal fibroblasts, we used a specific pharmacological ASMase inhibitor (Imipramine) and siRNA against ASMase. Upon treatment of cells with Imipramine (30 μM) increased MMP-1 protein levels were observed by western blot analysis (Fig. 1a). For the siRNA experiments, we first confirmed successful blockade of ASMase at the mRNA level (Fig. 1b). Normal fibroblasts treated with ASMsi showed a significant increase in MMP-1 protein expression (Fig. 1c). Analysis of MMP-1 mRNA expression showed similar increases after ASMase suppression (Fig. 1d). To further confirm the role of ASMase in MMP-1 regulation in dermal fibroblasts, we overexpressed ASMase by transducing cells with adenovirus expressing full length ASMase (AdASM) or a control adenovirus (AdG0) Western blot analysis of the extracts showed a significant decrease in MMP-1 expression after ASMase overexpression (Fig. 1e).

These results are consistent with a study by Kim and co-workers, in which cholesterol depletion in human dermal fibroblasts was found to enhance MMP-1 expression, suggesting a role for lipid rafts/caveolae as negative regulators of MMP-1 gene expression. Taken together, these data suggest that depletion of ASMase has effects similar to cholesterol depletion in the regulation of MMP-1 gene expression.

3.2. Cav-1 is a negative regulator of MMP-1 gene expression

Caveolae are distinguished from lipid rafts by the presence of the coating protein cav-1, which is considered a marker for caveolae. It is widely accepted that downregulation of cav-1 results in a reduction in the number of caveolae at the plasma membrane. Using cav-1

specific siRNA we investigated whether deficiency in cav-1 protein levels affects the expression of MMP-1. Specific cav-1-siRNA treatment was able to consistently suppress the protein levels of cav-1 by over 90%, as quantified by densitometry analysis (Fig. 2a and b). Analysis of extracts from control (scrambled) and cav-1 siRNA treated human dermal fibroblasts revealed a significant increase in the levels of MMP-1 protein upon cav-1 downregulation (up to 4-fold, Fig. 2a and b). Similar results were obtained in the conditioned media after cav-1 blockade (data not shown). To determine whether changes in MMP-1 protein might be due to transcriptional regulation, we evaluated the MMP-1 mRNA levels after cav-1 silencing, using quantitative real-time RT-PCR. As presented in Fig. 2c, in cells treated with cav-1 siRNA, the mRNA levels of MMP-1 were significantly increased, to an extent similar to the results obtained for the protein levels. These data are consistent with an inhibitory role for cav-1 in the regulation of MMP-1 gene expression. Based on this evidence, we decided to perform further experiments to evaluate the effects of cav-1 upregulation on MMP-1. For this approach we transduced cells with incremental doses of either an adenovirus overexpressing full-length cav-1 protein (cav-1Ad) or a control adenovirus (LucAd). The control adenovirus did not affect cav-1 levels, and a dose-dependent increase in the mRNA levels of cav-1 was obtained after cav-1Ad treatment, as analyzed using real time RT-PCR (Fig. 3a, left panel). For subsequent experiments, cells were treated with the optimal dose of adenovirus, leading to increases in cav-1 protein levels within the physiological range (2–4-fold, Fig. 3a, right panel). Real time RT-PCR analysis of cells treated with cav-1Ad revealed a significant decrease in MMP-1 mRNA levels compared to control cells (Fig. 3b). Collectively, these results show that cav-1 is a negative regulator of MMP-1 gene expression in human dermal fibroblasts in vitro.

3.3. Cav-1 is a suppressor of Erk1/2 and Ets1 signaling

It has been shown that Erk1/2 is a major regulator of MMP-1 gene transcription, and Erk1/2 activation was linked to MMP-1 gene regulation in cholesterol-depleted dermal fibroblasts [16]. To address the mechanism of MMP-1 regulation by cav-1 we evaluated the effects of cav-1 inhibition on Erk1/2 activation status. A prominent upregulation in the levels of phosphorylated Erk1/2 was noted in cells depleted of endogenous cav-1 protein via siRNA treatment. Conversely, Erk1/2 signaling was attenuated in response to overexpression of cav-1 (Fig. 4). This downregulation was consistent with previously published reports on the inhibitory effect of caveolin-1 and caveolae on Erk1/2 signaling in other cell types [17,18]. Ets1 is a transcription factor of the ETS family that is activated by Erk1/2 via phosphorylation on threonine 38 [19]. It has been reported that activation of MMP-1 expression by the Erk pathway in dermal fibroblasts involves activation of Ets1 [14,20,21]. To further investigate the mechanism of cav-1 mediated regulation of MMP-1 gene expression we evaluated the effects of cav-1 depletion on the levels of phosphorylated Ets1. As shown in Fig. 4b, cav-1 inhibition resulted in a potent upregulation of phospho-Ets1. Furthermore, in cells with low cav-1 levels, upregulation of MMP-1 protein correlated with concomitant increases in the levels of phosphorylated Erk1/2 and Ets1, suggesting a connection between these events (Fig. 4b). To our knowledge, this is the first report that indicates the importance of cav-1 in the regulation of Ets1 phosphorylation.

3.4. Down-regulation of cav-1 does not alter the levels of P-JNK

Previous reports have revealed that cav-1 can bind to and negatively regulate JNK phosphorylation in various cell types [11,22–25], and JNK activation has been linked to MMP-1 gene regulation at the basal levels and after treatment with cholesterol depleting agents [8,16]. We next investigated the effects of cav-1 downregulation on the levels of phosphorylated JNK. Experiments were performed as previously described using siRNA to deplete endogenous cav-1, and then cell lysates were analyzed by western blot. As a positive control for JNK phosphorylation fibroblasts were pretreated with the JNK inhibitor

SP600125 for an hour, followed by incubation in hyperosmolar media containing 0.5 M NaCl, for an additional hour. SP600125 completely prevented the hyperosmolarity-activated phosphorylation of JNK (Fig. 5). However, cav-1 downregulation had no effect on JNK activation, suggesting that, contrary to previous reports in other cell types, cav-1 is not modulating phosphorylation of JNK in dermal fibroblasts.

3.5. Cav-1 controls MMP-1 gene expression via an Erk1/2/Ets1 dependent pathway

To investigate whether up-regulation of MMP-1 in cav-1 depleted cells is mediated via activation of Erk1/2/Ets1, we pretreated cells with UO126, a specific inhibitor of MEK (MAPK/Erk kinase) and examined the effects of siRNA mediated cav-1 downregulation on MMP-1 protein levels. Fig. 6 shows that UO126 prevented Erk1/2 activation and significantly inhibited the phosphorylation of Ets1 in response to cav-1 depletion. Additionally, treatment of cells with the UO126 inhibitor completely abolished the up-regulation of MMP-1 following cav-1 depletion, suggesting that Erk1/2 and Ets1 are the downstream mediators of these effects.

4. Discussion

In the present study we investigated the role of cav-1 in the regulation of MMP-1 gene expression in human dermal fibroblasts. Using siRNA-mediated depletion of endogenous cav-1 as well as overexpression of cav-1 via an adenoviral vector, we demonstrated that cav-1 downregulates MMP-1 in these cells via an Erk1/2/Ets1 dependent mechanism.

In normal cells, cav-1 transits from the Golgi apparatus, where it exists as monomers, to the plasma membrane where it oligomerizes and associates with lipid raft domains, thus forming caveolae. Addition of cholesterol accelerates, while sphingolipid depletion inhibits transport of cav-1 from the Golgi apparatus to the membrane. Furthermore, cholesterol extraction via MβCD has been shown to disrupt caveolae [1,26–28]. In our study, inhibition of caveolae, either by ceramide disruption, or by depletion of the protein component cav-1, resulted in upregulation of MMP-1 gene expression, thus suggesting an inhibitory role for caveolae in this process. Our results are consistent with previous studies in human dermal fibroblasts showing an inverse correlation between cholesterol depletion and MMP-1 gene expression [16]. However, caveolae and lipid rafts represent two different cholesterol-rich membrane fractions that are both disrupted upon cholesterol extraction. Cholesterol and ceramide depletion do not clearly distinguish between the effects of lipid rafts versus caveolae on the regulation of MMP-1 gene expression. By directly manipulating cav-1 levels within the cells, we clearly demonstrated herein that caveolae contribute to the regulation of MMP-1 protein in human dermal fibroblasts. Since depletion of ASMase did not alter the mRNA levels of cav-1 (data not shown), we concluded that ceramide disruption and cav-1 downregulation are both separate events that control MMP-1 gene expression by interfering with the organization and proper function of caveolae.

Published studies show that members of the MAPK family are important regulators of MMP-1 gene expression, via a proximal AP1 response element in the MMP-1 promoter. Various signaling molecules can localize to caveolae and interact with cav-1, and these interactions may fine-tune the signaling cascades. In human lung fibroblasts [22], human renal tubular cells [25] and mouse macrophages [23], cav-1 had a negative effect on JNK phosphorylation. It has been demonstrated that cav-1 negatively regulates Erk1/2 activation in a variety of cells. Consistent with these results, we have observed an inverse correlation between the levels of cav-1 and the activation status of Erk1/2. However, in our study there was no difference in JNK activation after depleting cav-1 levels in human dermal fibroblasts. This suggests that cav-1 effects on JNK activity are cell type dependent. In our system, cav-1 mediated regulation of MMP-1 levels was dependent on Erk1/2 activation and

independent of JNK. Since cholesterol depletion resulted in activation of JNK [16], our results imply the possibility that cholesterol extraction in human dermal fibroblasts could modulate JNK activity via a non-caveolar, lipid raft-dependent mechanism.

We have previously demonstrated that in human dermal fibroblasts Erk1/2 controls MMP-1 gene expression via phosphorylation and activation of Ets1 [14,20]. In here we show that cav-1 is a negative regulator of Ets1 phosphorylation and that activation of the Erk1/2/Ets1 signaling pathway in response to cav-1 depletion is responsible for the effects on MMP-1. To the best of our knowledge this is the first report to associate cav-1 with Ets1 activation. In our study, the presence of Erk inhibitor, UO126, completely abrogated the phosphorylation of both Erk1/2 and Ets1 and the up-regulation of MMP-1 in response to cav-1 depletion. This suggests that Ets1 activation downstream of cav-1 is Erk1/2 dependent.

MMP-1 is one of the most abundant enzymes in the MMP family and one of only four MMPs capable of degrading insoluble triple helical collagens (types I, II and III). Tight regulation of MMP-1 secretion and activity is important for tissue development and homeostasis, and deregulation of MMP-1 contributes to various pathological processes, including tissue fibrosis and impaired wound healing, cancer cell invasion and metastasis [7].

Cav-1 is involved in the pathogenesis of tissue fibrosis and its expression is altered in various fibrotic diseases. Low levels of cav-1 were found in lungs from idiopathic pulmonary fibrosis [24] and SSc patients [29], while higher levels of cav-1 were demonstrated in the cirrhotic liver [30]. The antifibrotic effects of cav-1 are mediated via inhibition of the canonical TGFβ/Smad2/3 signaling [31]. Interestingly, a recent report showed that cav-1 could also have profibrotic functions by enhancing signaling through the PI3K/Akt/mTOR pathway in dermal fibroblasts [32]. Adding more complexity to the role of cav-1 in ECM deposition and remodeling, our data shows that cav-1 is a negative regulator of the matrix-degrading enzyme MMP-1, thus an indirect promoter of collagen deposition. Although deletion of cav-1 in mice leads to lung [33] and skin fibrosis [29], mice do not harbor a homologue of human MMP-1, making them imperfect models for an accurate assessment of the role of cav-1 in human fibrosis. Further studies are required to elucidate the exact role of cav-1 in the pathogenesis of fibrotic diseases.

A growing body of evidence suggests that cav-1 plays important roles in oncogenic transformation, cancer progression and metastasis. In a recent study, stromal fibroblasts isolated from breast cancer patients had decreased cav-1 expression. Furthermore there was an inverse correlation between cav-1 levels and early tumor recurrence, metastasis and decreased survival in these patients [34]. Changes in the tumor microenvironment play a critical role in human breast cancer onset and progression. MMP-1 derived from cancer-associated fibroblasts cleaves PAR1 on breast cancer cells, thus promoting migration and invasion of cancer cells [35]. Our data suggests that cav-1 mediated downregulation of MMP-1 could contribute to the tumor suppressive effects of cav-1. Thus, a decrease in cav-1 would stimulate MMP-1 production, leading to increased ECM degradation, accelerated tumor growth and metastasis.

Published data shows association between cav-1 and different members of the MMP family. Cav-1 and caveolae have been demonstrated to affect both the activity and expression levels of various MMPs. Relevant to our findings, the function of CD147 (an MMP-1-inducing surface molecule) was negatively regulated by cav-1 in HT1080 cells, and coculture of fibroblasts with mutant HT1080 cells – characterized by decreased cav-1 association with CD147 – resulted in enhanced MMP-1 production by fibroblasts [36]. Recent data suggests

that MMP-14, a membrane type metalloproteinase with important roles in ECM degradation and tumor invasion, associates with cav-1 in caveolae [37]. This association had inhibitory effects on MMP-14-induced cell migration, but a role for cav-1 in modulating MMP-14 expression has not been described. Interestingly, similar to the negative effects of cav-1 on MMP-1 expression, inhibition of cav-1 resulted in a significant (up to 2-folds) induction in the mRNA levels of MMP-14 (data not shown). While further studies are required for a definitive understanding of the molecular basis for this effect, it is important to note that cav-1 may modulate not only the function but also the expression of MMP-14.

In conclusion, this study demonstrates that in human dermal fibroblasts cav-1 is a negative regulator of MMP-1 gene expression via inhibition of Erk1/2/Ets1 signaling. Thus, cav-1 could potentially serve as target for therapeutic intervention in various diseases characterized by deregulated ECM turnover.

Acknowledgments

Funding

Scleroderma Foundation grant (AB) and NIAMS AR42334 (MT).

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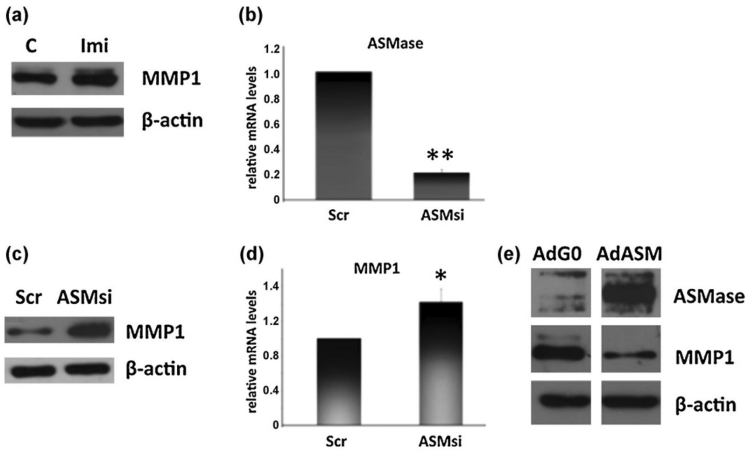

Fig. 1.

Inhibition of ceramide synthesis via depletion of ASMase up-regulates MMP-1 gene expression. (a) Dermal fibroblasts were treated with 30 μM Imipramine for 24 h and protein levels of MMP-1 were measured by western blot; (b) Dermal fibroblasts treated with 20 nM ASMase siRNA for 48 h were analyzed for ASMase expression using real time PCR; (c) protein and (d) mRNA expression of MMP-1 were measured following ASMase siRNA treatment; (e) Dermal fibroblasts transduced with ASMase overexpression adenovirus were analyzed for ASMase and MMP-1 expression using western blot analysis (*$P < 0.05$).

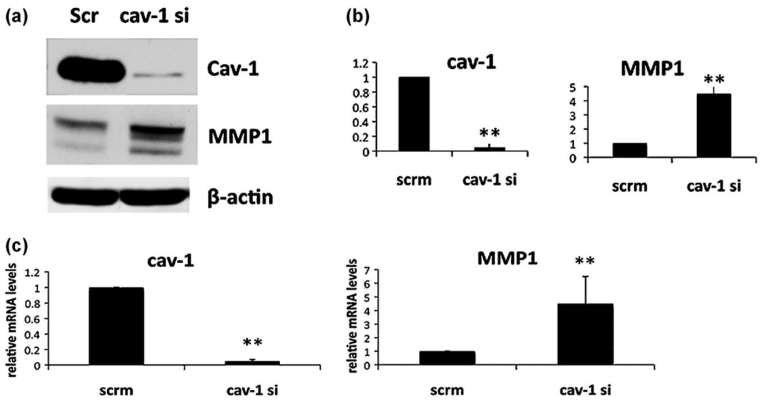

Fig. 2.

Cav-1 down-regulation induces MMP-1 levels in dermal fibroblasts. (a) Human dermal fibroblasts were treated with scrambled siRNA (Scr) or siRNA targeted against cav-1 (cav-1 si) as described, and protein levels of cav-1, MMP-1, and β-actin were determined by western blot. (b) Bar graph representing the densitometric analysis of western blots from three experiments, with results expressed as arbitrary units ± standard deviation. (c) mRNA isolated from normal dermal fibroblasts treated with scrambled or cav-1 siRNA was analyzed by qRT-PCR for expression of cav-1 and MMP-1. β2-microglobulin was used as an internal control (*$P < 0.05$, **$P < 0.01$).

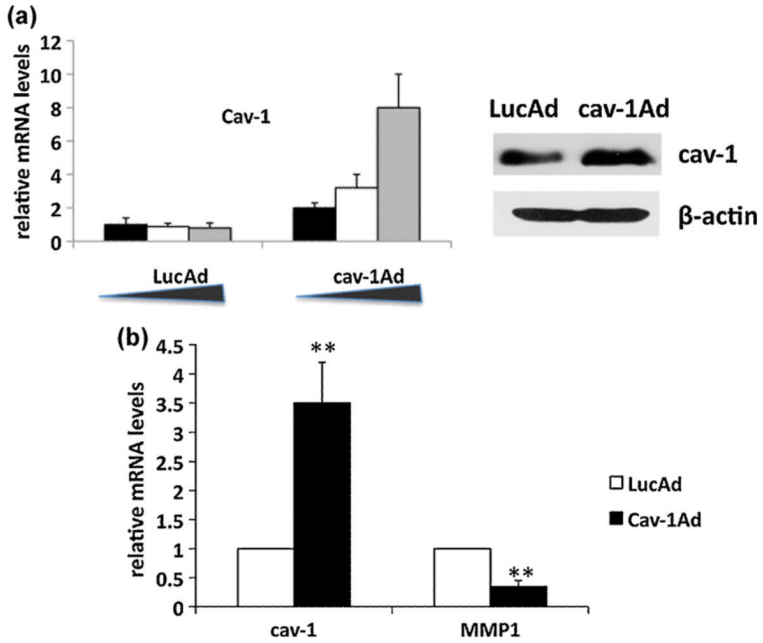

Fig. 3.

Adenovirus-mediated cav-1 overexpression negatively regulates MMP-1 mRNA levels. Human dermal fibroblasts were grown to confluence, serum starved overnight and transduced with either cav-1Ad or control adenovirus (LucAd) for 72 h. (a) A dose response experiment was performed to determine the optimal concentration of virus for a 2–4-fold increase in cav-1 expression. Left bar graphs shows dose dependent up-regulation of cav-1 mRNA levels (as assessed by qRT-PCR). Western blot (right pannel) showing that the selected dose up-regulates protein levels of cav-1 in the desired range. (b) qRT-PCR analysis shows a corresponding decrease in MMP-1 mRNA after cav-1 up-regulation by adenovirus (*$P < 0.05$, **$P < 0.01$).

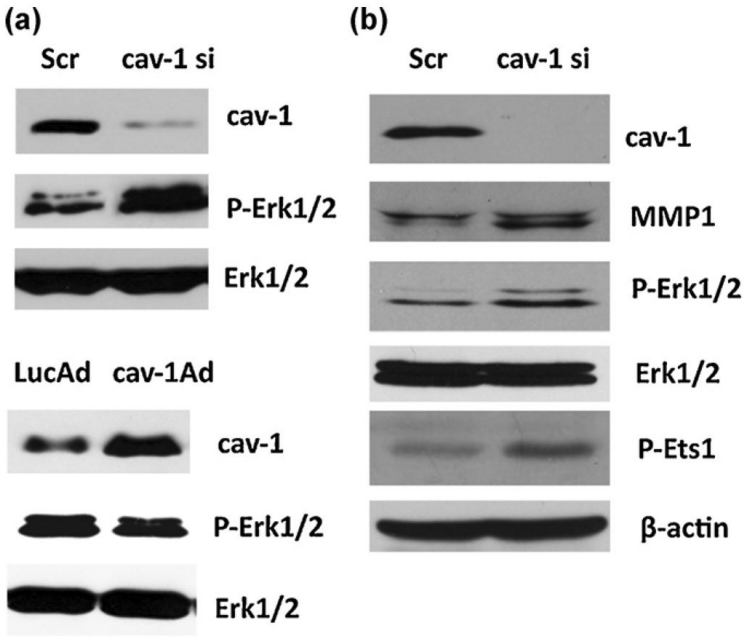

Fig. 4.

Cav-1 is a negative regulator of Erk1/2/Ets1 signaling in human dermal fibroblasts. (a) Human dermal fibroblasts were treated with cav-1 siRNA (upper panel) or adenovirus overexpressing cav-1 (cav-1Ad, lower panel), and corresponding controls – scrambled siRNA (Scr) and luciferase adenovirus (LucAd). Protein levels of cav-1, phospho-Erk1/2, and total Erk1/2 were analyzed by western blot. (b) Human dermal fibroblasts were treated with cav-1 siRNA or Scr and the levels of cav-1, MMP-1, P-Erk1/2, total Erk1/2 and P-Ets1 were analyzed by western blot. β-actin was used as a loading control.

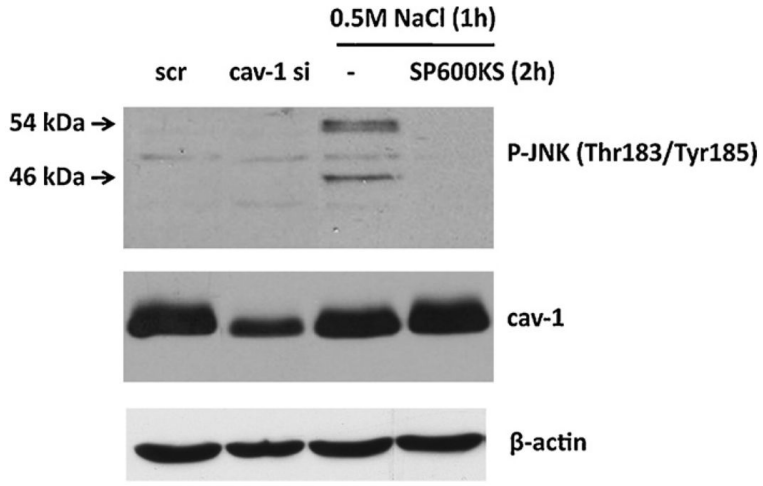

Fig. 5.

Down-regulation of cav-1 does not alter the levels of P-JNK. Human dermal fibroblasts treated with scrambled siRNA or siRNA targeted against cav-1 were analyzed by western blot for phosphorylated JNK and cav-1 protein. 0.5 M NaCl treatment for 1 h was used as a positive control for induction of JNK phosphorylation, and JNK activation was blocked by 1 h pre-treatment with the JNK inhibitor SP600125 (10 μM). Phospho-JNK and cav-1 protein levels were analyzed by western blot and β-actin was used as a loading control.

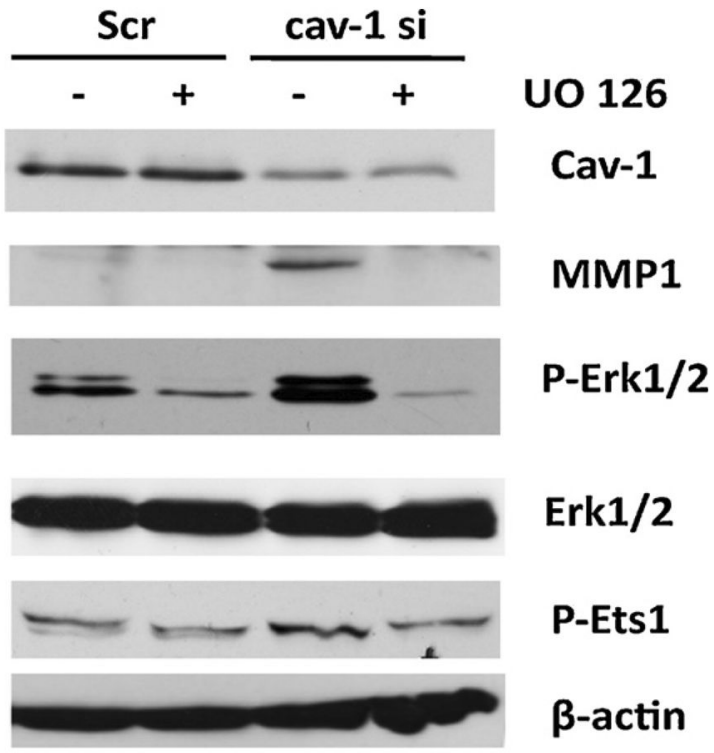

Fig. 6.

Cav-1 controls MMP-1 gene expression via an Erk1/Ets1 dependent pathway. Human dermal fibroblasts were treated with the Erk1 inhibitor, UO126 (10 μM), 1 h before transfection with scrambled siRNA or siRNA targeted against cav-1. Western blot shows that cav-1 siRNA increases MMP-1, phopho-Erk1/2, and phospho-Ets1 protein only in the absence of the Erk1/2 inhibitor. β-actin was used as a loading control.