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Mechano-signal transduction in mesenchymal stem cells induces prosaposin secretion to drive the proliferation of breast cancer cells

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Abstract

In response to chemical stimuli from cancer cells, mesenchymal stem cells (MSC) can differentiate into cancer-associated fibroblasts (CAF) and promote tumor progression. How mechanical stimuli such as stiffness of the extracellular matrix (ECM) contribute to MSC phenotype in cancer remains poorly understood. Here we show that ECM stiffness leads to mechano-signal transduction in MSC which promotes mammary tumor growth in part through secretion of the signaling protein prosaposin. On a stiff matrix, MSC cultured with conditioned media from mammary cancer cells expressed increased levels of α-smooth muscle actin, a marker of CAF, compared to MSC cultured on a soft matrix. By contrast, MSC cultured on a stiff matrix secreted prosaposin which promoted proliferation and survival of mammary carcinoma cells but inhibited metastasis. Our findings suggest that in addition to chemical stimuli, increased stiffness of the ECM in the tumor microenvironment induces differentiation of MSC to CAF, triggering enhanced proliferation and survival of mammary cancer cells.

Keywords

Mesenchymal stem cells; breast cancer; cancer associated fibroblasts; prosaposin; mechanotransduction

Introduction

Tumor tissue contains multiple components including cancer cells, stromal cells, immune cells, and extracellular matrices(1), which can interact with each other via chemical and mechanical stimuli and create complex biochemical and biophysical properties of tumor microenvironment. For example, chemical cues such as cytokines and growth factors secreted from stromal and immune cells stimulate cancer cells to promote or suppress tumor

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progression(1). Mechanical stimuli are also critical for cancer progression, as cancer cells response to mechanical cues, such as tissue stiffness, and as a result, acquire malignant phenotypes(2,3). Many previous studies reported that interaction between cancer cells and chemical or mechanical cues play a role in cancer progression or suppression, however, interaction between non-cancerous cells and these cues in the tumor microenvironment has not been clearly defined.

In the tumor microenvironment, carcinoma associated fibroblasts (CAFs) are thought to contribute to cancer progression via chemical stimuli and modification of the extracellular environment(4,5). The origin of CAFs remains controversial, and may include resident fibroblasts, adipocytes, and infiltrating mesenchymal stem cells (MSCs). MSCs are multipotent cells present in many types of tissues(6). In cancer, it is thought that MSCs are recruited to the tumor microenvironment and show both pro-tumor and anti-tumor phenotypes(7,8). Recently it is reported that, in breast cancer, MSCs differentiate to CAFs and promote tumor progression in response to chemical stimuli, such as soluble factors secreted from cancer cells(9–11). However, the contribution of mechanical stimuli including stiffness of ECMs, to differentiation of MSCs in the tumor microenvironment has not been shown clearly.

Prosaposin (PSAP, Psap) is the precursor protein for saposin A–D, which are lysosomal activator proteins that facilitate the hydrolysis of sphingolipids(12). In addition to the function in lysosome, PSAP is also secreted as full-length prosaposin that regulates neurotrophic action in the nerve system(12). Recently, PSAP was reported to promote ERK (Mapk1) activation and breast cancer growth(13) and saposin C, active domain of PSAP, enhances survival via Akt activation in prostate cancer cells(14). In contrast, PSAP inhibits metastasis via stimulating the expression of thorombospondin-1 in fibroblasts present in tumor(15). However, the source of PSAP and its role in tumor microenvironment are not yet fully understood.

In this study, we investigated whether MSCs respond to ECM stiffness and regulate growth or progression of mammary carcinoma. We revealed that stiff ECM induces differentiation of MSCs to CAFs via mechano-signal transduction, including regulation of YAP (Yap1) and myosin light chain (MLC, Myl9) activation. While there has been documentation that MSCs respond to matrix stiffness to control different normal cell fates (e.g. osteogenic, neurogenic, and myogenic)(16), this is the first study to show that a stiff matrix regulates the pro-carcinoma phenotype of mesenchymal stem cells to promote cancer growth. We also identified that PSAP is secreted by MSCs in a stiff tumor microenvironment and promotes proliferation and survival in mammary cancer cells via Akt activation. Conditioned media of mammary cancer cells induced growth and morphological changes in MSCs, suggesting that bi-directional communication between mammary cancer cells and MSCs in stiff tumor microenvironment is critical for mammary cancer progression.

Materials and Methods

Cell culture

Mesenchymal stem cells (MSCs) were established from a BALBc mouse (BALB/cByJ, female, 7 weeks old, from Jackson laboratory) and a B6 mouse (C57BL/6, female, 7 weeks old, Jackson laboratory). As reported previously(17), we isolated femurs from mice and flushed the bone marrow from both ends with 1 mL of MSC-media (Dulbecco's modified Eagle medium (DMEM, Gibco, 11885092) supplemented with 20% fetal bovine serum (FBS, GEMINI bio-products, 100–106) and 1% antibiotics and antimycotics (Sigma-Aldrich, A5955)). Then, the bone marrow suspension was filtered with a 70 mm pore cell strainer and seeded onto a 75 mL cell culture flask or a 6 cm cell culture plastic dish with MSC-media. After 24 hours, cells were washed with phosphate buffered saline (PBS) and cultured with MSC-media. Media was changed every 3 days until the cells reached 70% confluence. At this point, we treated the cells with 0.25% trypsin-EDTA for 2 min to collect and propagate the floating cells in a new dish with MSC-media as MSCs. The differentiation potential to adipocytes and osteocytes was confirmed by differentiation assay. For this assay, we cultured the cells with adipogenic media (cyagen Biosciences, GUXMX-90031) or osteogenic media (cyagen Biosciences, GUXMX-90021) and stained with Oil Red O or Alizarin Red S to confirm adipocytes and osteocytes, respectively. 4T1 cells (ATCC, 2012) were cultured with RPMI 1640 media (Gibco, 11875119) supplemented with 10% FBS and 1% antibiotics and antimycotics. E0771 cells (kind gift from the Dr. Friedl lab, 2016, University of Wisconsin-Madison) were cultured with RPMI 1640 media supplemented with 10% FBS, 10 mM HEPES, and 1% antibiotics and antimycotics. The cells cultured for less than 30 passages were used. 4T1 cells were authenticated and tested for mycoplasma contamination. The other cell lines were not authenticated and tested for mycoplasma contamination. 1, 2.5, and 4 mg/mL Collagen gels were prepared as previously reported(18) and used for experiments shown in a schematic chart of Supplementary Fig. S1. Briefly, cells were cultured within collagen gels at appropriate cell and collagen densities. After 24 hours the gels were washed with PBS three times, then released for floating conditions into 4T1-conditioned media or control media with 1% FBS and 1% antibiotics and antimycotics for up to 6 days. Every third day half of the media was exchanged for fresh conditioned media. After indicated days in culture, the gels were processed for the appropriate end point. Polyacrylamide gels for cell culture were prepared as previously reported(19,20) and used for western blot. For capturing phase-contrast images and fluorescence images, we used a phase-contrast microscope (Nikon-TMS, Nikon) and an epi-fluorescence microscope (TE300, Nikon) with ×10, ×20, or ×40 objectives.

Conditioned media

For the preparation of 4T1- or E0771- conditioned media, we prepared sub-confluent cells on a 10 cm plastic culture dish. The cells were washed with PBS three times and cultured with DMEM (non-serum, 1% antibiotics and antimycotics) for 24 hours. The media was collected and centrifuged (1,300 rpm, 3 min). Supernatant was collected and filtered with 0.22 μm pore filter. For the preparation of MSC-conditioned media, we seeded 7×10^4 MSCs onto 1 mL collagen gels as described above and in Supplementary Fig. S1. After 6 days, the cells were washed with PBS three times and cultured for an additional 24 hours with DMEM

(non-serum, 1% antibiotics and antimycotics). Then, the media was collected and centrifuged (1,300 rpm, 3 min). The supernatant was filtered through a 0.22 μm pore filter. The concentration of conditioned media is adjusted to MSC cell number quantified in collagen gels by fluorescence staining of nuclei or calcein AM (3.4 μM) treatment. The conditioned media were stored at −20 °C or −80°C.

Establishment of transgenic cells

We constructed pBABE-puro-EGFP-hYAP vector for establishment of EGFP-hYAP-overexpressed MSCs. For the construction, we cloned the EGFP-hYAP sequence from pEGFP-C3-hYAP vector (a gift from Marius Sudol, Addgene plasmid # 17843)(21) by PCR with Pfu ultra polymerase (Agilent Technologies, 600670) and inserted it into multi cloning site of pBABE-puro vector (a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg, Addgene plasmid # 1764)(22) by using sequence- and ligation-independent cloning method(23) with the primers (forward: 5'-tccttctctaggcgccggccggatctataagcagagctggtttagtgaaccgtca-3', reverse: 5'-actgacacacattccacagggtcgacctgagggctctataaccatgtaagaaagc-3'). The construction was confirmed by diagnostic digestion and sequencing. IRES-EGFP-overexpressed MSCs (EGFP-MSCs, control for MSCs with hYAP) and EGFP-hYAP-overexpressed MSCs (YAP-MSCs) were established by retrovirus transfection of pBABE-puro-IRES-EGFP (a gift from L. Miguel Martins, Addgene plasmid # 14430) and pBABE-puro-EGFP-hYAP, respectively. Empty pGIPZ-transgenic MSCs (empty-MSCs), non-silencing RNA-pGIPZ-transgenic MSCs (shNS-MSCs, control for MSCs with shRNA), shYAP-pGIPZ-transgenic MSCs (shYAP-MSCs), and shPSAP1 and 2-pGIPZ-transgenic MSCs (shPSAP1-MSCs, shPSAP2-MSCs) were established by lentivirus transfection of blank pGIPZ vector (Open Biosystems), pGIPZ-non-silencing control vector (Dharmacon RNAi Technologies, RHS4346), pGIPZ-shYAP vector (Dharmacon RNAi Technologies, RMM4532-EG22601), and pGIPZ-shPSAP vector (Dharmacon RNAi Technologies, RMM4532-EG19156), respectively. For viral production, HEK Phoenix-Ampho cells (obtained from National Gene Vector Biorepository) were used. The virus-transfected cells were selected by culturing with MSC-media supplemented with puromycin (30 μg/mL, Sigma-Aldrich, P8833) and established transgenic MSCs. Expression of fluorescence proteins and differentiation potential of transgenic MSCs were confirmed with observation by an epi-fluorescence microscope and differentiation assay, respectively (Supplementary Fig. S2A and B).

Western blot

7×10^4 or 3×10^4 MSCs, 1×10^5 4T1, or 1×10^5 E0771 cells were seeded onto collagen gels as described above and in Supplementary Fig. S1 or a 6 well plates with or without collagen-coating and cultured the cells for 24 hours. For the cells on collagen gels, the gel diameter was measured on day 6 to assess cell contraction, the gel were fixed cells with 10% trichloroacetic acid (TCA) in PBS, and washed the cells with PBS three times. Then, the cells were lysed with 2× RIPA buffer(18) and 5× Laemmli sample buffer (62.5 mM Tris pH = 6.8, 20% glycerol, 2% sodium dodecyl sulfate, 10% β-mercaptoethanol, 0.3125 mg/mL bromophenol blue) and boiled for 5 min. For the cells on a 6 well plates, the cells were washed with PBS twice, cultured with appropriate media for 15 min, 3 hours, 1 day, or 2 days, lysed with 2× RIPA buffer and 5× Laemmli sample buffer, and boiled for 5 min. For

the cells on polyacrylamide gels, we seeded 1×10^4 MSCs onto the gels in a 6 well plates and cultured the cells. After 24 hours, gels were washed with PBS three times and incubated with 4T1-conditioned media containing 1% FBS and 1% antibiotics and antimycotics. Every 3rd day half of the media was changed to fresh conditioned media. After 1 day, cells were lysed with 2× RIPA buffer and 5× Laemmli sample buffer and boiled for 5 min. Conditioned media was directly lysed with 2× RIPA buffer and 5× Laemmli sample buffer and boiled for 5 min. Western blot was performed with Mini Gel Tank (Life Technologies, A25977) and the bands were detected with Luminia Crescendo Western HRP Substrate (Millipore, WBLUR0100) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095). RUBY staining of a gel was performed with SYPRO Ruby protein gel stain (Invitrogen, S12000). Relative expression of proteins was calculated by normalizing the intensity of target proteins with the intensity of β-actin, except conditioned media (normalized with cell number), pAkt-T308 (normalized with total-Akt), and pERK (normalized with total-ERK).

Luciferase assay

Sub-confluent MSCs on a 6 cm plastic dish were transfected with 8×GTIIC-luciferase (reporter vector of YAP, a gift from Stefano Piccolo, Addgene plasmid # 34615)(24) and pRL-TK (control vector, Promega, E2241) with Lipofectamine 3000 (Life Technologies, L3000008). After 1 day, the cells were detached and seeded on a 0.5 mL collagen gel prepared in a 12 well culture plate as previously described. Luciferase activity was determined after 3 days in culture with Dual Glo Luciferase (Promega, E2920). Relative activity of YAP was calculated by normalizing the activity of YAP with the activity of HSV-thymidine kinase promoter.

Mouse studies

Mice were bred and maintained under the approval of the University of Wisconsin Animal Care and Use Committee (protocol #: M01668). The B6 mice heterozygous for the *Col1a1*^{jaetm1} mutation mice (a kind gift from the Jaenisch lab, Massachusetts Institute of Technology, which show increased collagen I deposition(25), were crossed to female wild type (wt) BALBc mice. The resulting mice were either wt/wt (WT) or wt/*Col1a1*^{jaetm1} mutation (COL). Genotyping was performed by polymerase chain reaction in DNA extracted from tail biopsies. 4T1 cells (1×10^5) with or without MSCs (1×10^5) suspension in PBS were orthotopically injected into a fat pad of at least 6-week-old female WT or COL mice. Macroscopic observation and tumor volume measurement with a caliper were performed twice a week after injection. The volume of tumor was determined by the formula: tumor volume (mm^3) = length (mm)×(width (mm))^2/2, length>width. The mice were sacrificed 21 or 28 days after injection and the tumor tissues were dissected and weighed. Lung tissues were also dissected and fixed with 4% formalin. Number of metastasis of 4T1 cells to lung tissues was determined by microscopic observation of metastatic tumors on the lung surface. For staining of nuclei and PSAP, tumors were fixed with 4% formalin, embedded in paraffin, and sectioned. After deparaffinized by heating at 60°C for 30 min and rehydrated, the sections were boiled with citrate buffer (pH = 6.0) for antigen retrieval. Then, the sections were blocked with 1% BSA and 1% DS in PBS for 30 min at room temperature. The sections were incubated with primary antibody in PBS with

1% DS overnight at 4°C, followed by three washes with TBS-T. Next, the cells were incubated with secondary antibody in PBS with 1% DS for 1 hour at room temperature, followed by three washes with TBS-T and two wash with TBS. Then, the sections were counter stained with DAPI (1:10,000) in TBS and washed with TBS and distilled water. The sections were covered with CC/mount media (Sigma-Aldrich, C9368) and cover slip. The images were captured with an epi-fluorescence microscope (TE300, Nikon) with ×20 objective and analyzed with ImageJ software. No randomization was performed. No blinding was performed except metastasis analysis.

Data analysis of patients

Kaplan-Meier curves of relapse free survival in breast cancer patients were analyzed by using the Kaplan-Meier plotter(26) at http://kmplot.com/analysis/index.php?p=service&cancer=breast.

Antibodies, reagents, growth and morphology assays, survival assay, fluorescence staining, proteomics analysis, qPCR, and statistics are described in Supplementary Methods.

Results

MSCs differentiate to CAFs on a stiff matrix with soluble factors from mammary cancer cells

First, we isolated MSCs from BALBc mice and confirmed the cell type by morphology (Supplementary Fig. S3A), and the ability to differentiate into either adipocytes or osteocytes with appropriate stimuli (Supplementary Fig. S3B). These MSCs were next cultured on collagen gels of defined stiffness with cancer cell-conditioned media to examine the effect of stiffness and soluble factors in the tumor microenvironment (experimental design is shown in Supplementary Fig. S1). The conditioned media was derived from 4T1 cells, a well characterized mammary carcinoma cell line established from BALBc mice(27). To test the effect of ECM stiffness we cultured MSCs on floating collagen gels of increasing collagen concentration, as it is well established that gels with a greater content of collagen are stiffer(28). Concentrations were chosen that allowed MSCs to contract the gel, as well as concentrations too stiff for contraction. Thus, a 1 mg/mL collagen gel (approximately 10 kPa) was sufficiently soft for MSCs as the cells contracted the gel, while a 2.5 mg/mL stiff collagen gel (approximately 20 kPa) showed less contraction, in contrast, a 4 mg/mL collagen gel (approximately 40 kPa) was not contracted at all (Supplementary Fig. S3C). Therefore, we used 1, 2.5, 4 mg/mL collagen gels as soft, mid, and stiff collagen gels, respectively. When MSCs were cultured with 4T1-conditioned media, they showed high growth and spread morphology with large cell area on a stiff collagen gel whereas MSCs on a soft collagen gel demonstrated low growth and a spindle morphology with small cell area (Fig. 1A and B; Supplementary Fig. S3D). In addition, MSCs cultured with control media (DMEM) displayed low growth and a round morphology, independent of the stiffness of the collagen gels (Fig. 1A and B; Supplementary Fig. S3D). In control medium, there was a decrease in viability of MSCs, with approximately 25% cell death compared to only approximately 5% for MSCs cultured with 4T1-conditioned media (Fig. 1C). These results

suggest that MSCs survive with soluble factors from mammary cancer cells and display enhanced growth and spreading morphology with stiff ECMs.

To identify the critical factor of 4T1-conditioned media for growth and morphological change in MSCs, we performed western blotting of the media. We found that 4T1-conditioned media contains transforming growth factor β (TGFβ. Supplementary Fig. S3E), which contributes to tumor progression in multiple aspects(1). Indeed, stimulation of TGFβ promoted growth and cell spreading in MSCs cultured on a collagen gel, similar to 4T1-conditioned media (Supplementary Fig. S3F and G). These results indicate that TGFβ is one of the critical factors secreted from mammary cancer cells, which stimulates MSCs in tumor microenvironment.

To determine whether ECM stiffness regulates differentiation of MSCs to CAFs we assessed expression of α-smooth muscle actin (αSMA, Acta2), a major marker of CAFs(4,5). We used β-actin as a loading control for western blotting because this protein reflected total protein amount in MSCs cultured on soft or stiff collagen gels, compared with Histone-H3 (Supplementary Fig. S3H). αSMA was highly expressed in MSCs cultured with 4T1-conditioned media on stiff collagen gels as compared to soft collagen gels. (Fig. 1D). This result is similar to a previous study, which suggests that TGF-β1 and a stiff matrix can induce MSCs to differentiate into myofibroblasts, which are also defined by αSMA expression(29).

To control for ligand density, we cultured MSCs on a floating 1 mg/mL collagen gel (soft collagen gel) and an attached 1 mg/mL collagen gel, which MSCs experience as stiff since the cells cannot contract an attached gel. On an attached 1 mg/mL collagen gel, MSCs showed greater cell growth, spreading morphology, and higher αSMA expression when cultured with 4T1-conditioned media (Fig. 2A–C), similar to the 4 mg/mL floating gel. In addition, αSMA and vimentin (Vim), a marker of fibroblasts(30), were localized to fibrous structures in spreading MSCs on a stiff collagen gel, whereas these markers were not localized fibrously in spindle cells cultured on a soft collagen gel (Fig. 2D). In contrast, expression of vimentin and fibroblast specific protein 1 (S100a4), also a marker of CAFs(30), were not significantly different between MSCs on soft and stiff gels (Fig. 1D and 2C; Supplementary Fig. S3I). We also cultured MSCs on collagen-coated polyacrylamide gels of different stiffness. On stiffer polyacrylamide gels, MSCs cultured with 4T1-conditioned media showed the spread morphology and higher expression of αSMA consistent with a CAF phenotype, but did not express this phenotype on the softer polyacrylamide gels (Fig. 2E and F). There was a trend toward a decrease in vimentin levels on stiff gels but the change did not achieve significance (Fig. 2F). Taken together, these data suggest that MSCs cultured with conditioned media from mammary carcinoma cells show the spreading morphology and high αSMA expression that are typical phenotypes of CAFs(5,31).

YAP and MLC are critical for differentiation of MSCs to CAFs

In order to better understand the underlying mechanism, we analyzed the role of the transcription factor, YAP in the differentiation of MSCs cultured with 4T1-conditioned media on matrices of varying stiffness. YAP is known to contribute to CAF functions and

mechano-signal transduction of MSCs(24,32,33). On stiff collagen matrices, YAP was highly expressed in MSCs cultured with 4T1-conditioned media (Fig. 3A–C). Since activated YAP functions as a transcription factor in nucleus(34), we investigated localization and transcriptional activity of YAP in MSCs. With 4T1-conditioned media, MSCs on stiff matrices showed increased nuclear-localization and high transcriptional activity of YAP compared with cells on a soft matrix (Fig. 3D and E; Supplementary Fig. S4A).The activity of YAP in YAP-overexpressing MSCs was higher than control MSCs (Supplementary Fig. S4B), showing the range of YAP activity measured by luciferase assay in this study.

MLC contributes to and is a marker of mechano-signal transduction in several cell types(32,35,36). Elevated di-phosphorylated MLC (2P-MLC) and mono-phosphorylated MLC (1P-MLC), both active forms of MLC, were detected in MSCs on stiff vs soft matrices when they were cultured with 4T1-conditioned media (Fig. 3F and G). To block MLC phosphorylation, we treated MSCs with the Rho kinase-inhibitor, H1152(37) (Fig. 4A). H1152 treatment inhibited the spreading morphology (Supplementary Fig. S5A), localization of YAP in nuclei (Fig. 4B), and expression of αSMA (Fig. 4A) in MSCs cultured on stiff matrices with 4T1-conditioned media. Importantly, YAP-overexpression increased αSMA expression (Fig. 4C) whereas YAP-knockdown by shRNA or siRNA decreased αSMA and 2P-MLC expression in MSCs (Fig. 4D; Supplementary Fig. S5B) linking YAP to down-stream signaling through 2P-MLC. In our experiments, 2P-MLC in MSCs was not increased by YAP overexpression. One possibility for this result is due to the culturing condition, the YAP-overexpressing MSCs are cultured on a plastic dish and in this situation phosphorylation of MLC may be saturated in MSCs due to a stiff matrix. On the other hand, YAP-overexpression and YAP-knockdown did not change morphology of MSCs, suggesting that differentiation of MSCs to CAFs by YAP is independent of cell spreading (Supplementary Fig. S5C).

We also investigated the expression and activity of molecules related to YAP regulation. Large tumor suppressor kinase 1 (LATS1, Lats1), a regulator of YAP degradation, was not activated in MSCs cultured on either stiff or soft matrices as determined by western blot of phosphorylation of LATS1 at S909 and T1079 (Supplementary Fig. S5D). The total LATS1 was highly expressed in MSCs on stiff matrices, however, the difference was not significant. Transcriptional coactivator with PDZ-binding motif (TAZ, Wwtr1) a homolog of YAP, showed no significant difference in expression in MSCs cultured on stiff or soft matrices (Supplementary Fig. S5E). YAP knockdown did not change TAZ expression, whereas H1152 treatment reduced TAZ expression in MSCs (Supplementary Fig. S5F and G) indicating MLC is a critical regulator of both YAP and TAZ. These results suggest that in the presence of mammary carcinoma cell conditioned media, YAP and MLC are activated in MSCs by a stiff matrix, which induces differentiation to CAFs independent of LATS1 and TAZ.

MSCs activated by a stiff microenvironment induce growth of mammary cancer cells

Since MSCs showed CAF phenotypes on stiff matrices, we next investigated whether MSCs on stiff matrices feedback to induce malignant phenotypes in mammary carcinoma cells. After inducing the CAF phenotype in MSCs, we washed the cells and cultured them with

control media (DMEM). After 1 day, we collected and filtered the media from MSC culture, and cultured mammary carcinoma cells with this media (experimental design is shown in Supplementary Fig. S1). 4T1 numbers increases in response to conditioned media from MSCs cultured on stiff matrices compared to conditioned media from MSCs on a soft matrix (Fig. 5A). This suggests that MSCs on a stiff matrix promote the growth of mammary carcinoma cells. To further investigate of the roles in MSCs as related to tumor growth in soft and stiff tissues, we performed *in vivo* experiments. We crossed BALBc mice with the B6 *Col1a1*^{jaetm1} model, in which a mutation in the collagenase cleavage site of *Col1a1* leads to increased collagen I deposition and tissue stiffening(25,28,38). 7 days after the injection of 4T1 cells into the mammary fat pad with or without MSCs into wt/wt (WT) mice, there were no significant differences in the tumor volume between the two conditions (Fig. 5B). On the other hand, in wt/*Col1a1*^{jaetm1} (COL) mice, the tumor volume of 4T1 cells with MSCs was significantly larger than that of 4T1 cells only (Fig. 5B). However, in both WT and COL mice, the difference in tumor volume and weight between 4T1 cells only and 4T1 cells + MSCs was not significant 28 days after injection (Supplementary Fig. S6A and B). These results suggest that MSCs within stiff tissue significantly support tumor growth in the early stage of mammary cancer.

MSCs with stiff tissues secrete PSAP

The result of Fig. 5A suggests that soluble factors from MSCs on stiff matrices enhance proliferation and/or survival of mammary carcinoma cells. Therefore, to identify the soluble factor, we performed proteomics analysis of conditioned media from MSCs cultured on matrices with different stiffness. We found that the concentration of soluble factor prosaposin (PSAP) was elevated in the conditioned media of MSCs on stiff matrices, but not on soft matrices (Fig. 5C). This result was also confirmed by western blot (Fig. 5D). Interestingly, the levels of PSAP protein decreased in total cell lysates of MSCs on stiff matrices (Fig. 5D), suggesting the effect mainly enhances secretion of PSAP. The effect is complex as mRNA of PSAP in MSCs on a stiff matrix was elevated compared with cells on a soft matrix (Fig. 5E). In contrast, expression of MMP13 (Mmp13) and MMP3 (Mmp3), which were also identified from proteomics analysis (Fig. 5C), were down-regulated at the mRNA level by a stiff matrix (Fig. 5E), suggesting mRNA expression and subsequent secretion of MMPs are regulated by different mechanisms in MSCs. Furthermore, in wt/*Col1a1*^{jaetm1} (COL) mice, mixed 4T1-MSC tumors showed greater PSAP accumulation than 4T1 tumors without MSCs added (Supplementary Fig. S7A and B). This enhancement was not observed in WT mice. These results suggest two possibilities, PSAP secreted from MSCs is taken up by 4T1 cancer cells because MSCs on stiff matrices promoted secretion of PSAP to extracellular space whereas they reduced the expression of PSAP in cytoplasm (Fig. 5D) or co-culture of 4T1-MSC in a stiff tumor microenvironment induce the expression of PSAP in 4T1 cancer cells. It is worth noting that PSAP up-take via endocytosis in certain types of cells, including nerve cells and fibroblasts, was previously reported(12).

To determine if PSAP is regulated by YAP, we probed the conditioned media of cells over-expressing YAP, and found that PSAP was higher than in control MSCs (Fig. 5F). Again, no significant difference was detected in the total cell lysates from YAP-expressing cells (Fig. 5F). PSAP expression in YAP-overexpressing and control MSCs was not significantly

different at the mRNA level (Fig. 5G), suggesting that PSAP secretion by MSCs is not regulated by YAP at the transcriptional level.

PSAP promotes proliferation and survival of mammary cancer cells

Next, we investigated the role of PSAP for proliferation and survival in mammary carcinoma cells. When treated with PSAP, numbers of 4T1 cells increased compared with the control (no PSAP) condition (Fig. 6A). In addition, conditioned media of YAP-overexpressed MSCs, which secrete more PSAP than control MSCs, increased growth of 4T1 cells (Fig. 6B). To further understand the mechanism of action for PSAP, we analyzed phosphorylation of ERK and Akt in 4T1 cells, because PSAP induces phosphorylation of ERK and growth of breast cancer cells(13) and saposin C, including the functional domain of PSAP, promotes survival of prostate cancer cells via Akt phosphorylation(14). PSAP treatment induced phosphorylation of Akt at T308 (Fig. 6C) but not of Akt at S473 (Fig. 6D) or ERK (Supplementary Fig. S8A). These results are consistent with a previous study, which showed that Akt at T308 is highly phosphorylated in high-grade breast cancer whereas Akt at S473 is not(39). Therefore, we inhibited phosphorylation of Akt at T308 and investigated its role in the proliferation and survival of 4T1 cells. We successfully inhibited Akt phosphorylation at T308 in 4T1 cells by treatment of LY294002 (Supplementary Fig. S8B), which inhibits PI3K and prevents Akt phosphorylation(40), whereas Akt phosphorylation at T308 was not decreased by treatment of LY294002 in non-treated 4T1 cells (Supplementary Fig. S8C). PSAP treatment elevated cell proliferation as measured by an increase in the ratio of phosphorylated histone-H3 at S10 (p-histone-H3, Fig. 6E and F). The increase in proliferation was blocked by LY294002 treatment in 4T1 cells. (Fig. 6E and F), and suggests that PSAP induces proliferation of 4T1 cells dependent on Akt phosphorylation at T308. In addition, PSAP treatment for 1 day increased the number of surviving cells in serum-free media. This effect occurred without changing the total amount of DNA in 4T1 cells (Fig. 6G; Supplementary Fig. S8D), implying that DNA was not degraded 1 day after removal of serum, even though the cells did not survive. Similarly, LY294002 treatment reduced the survival of cells, also without altering the total amount of DNA in PSAP-treated 4T1 cells (Fig. 6G; Supplementary Fig. S8D), suggesting that PSAP-induced cell survival in 4T1 cells is also dependent on pT308 Akt. We also quantified the expression of cleaved caspase 3 (Casp3), a major inducer of apoptosis, in 4T1 cells treated with PSAP and LY294002. There were no remarkable differences in expression of cleaved caspase 3 between control and PSAP-treated 4T1 cells (Supplementary Fig. S8E), suggesting that PSAP induces survival of 4T1 cells independent of caspase 3. Collectively these results suggest that PSAP secreted from MSCs in stiff tumor microenvironments promotes proliferation and survival of mammary carcinoma cells via phosphorylation of Akt at T308.

PSAP correlates with poor prognosis in grade 1 breast cancer patients

Next, we injected 4T1 cells with either PSAP knock-down or control MSCs (Supplementary Fig. S9A) into COL mice to examine the contribution of MSC derived PSAP to tumor growth in stiff tissue. Tumor volume of 4T1 cells with PSAP-knock downed MSCs was significantly smaller than control tumors 7 days after injection (Fig. 6H) whereas the volume was not significantly different 21 days after injection, compared with 7 days after injection

(Supplementary Fig. S9B and C), suggesting that PSAP secretion by MSCs in stiff tumor microenvironment is critical for tumor growth in early stage mammary cancer.

We also investigated metastasis to lungs in WT and COL mice, the latter of which develop stiff tissues. Although the differences were not significant, there was a trend toward a reduced number of lung metastasis from 4T1 + MSC tumors in COL mice compared to WT mice (Supplementary Fig. S9D). Interestingly, in COL mice with PSAP knockdown MSCs and 4T1 cells in the primary tumors the number of lung metastasis significantly increased (Fig. 6I). Previous studies showed that PSAP induces mammary cancer growth(13) and inhibits metastasis(15). Our results are consistent with these reports. We propose that MSCs within stiff tissues secrete PSAP, and as a result, promote tumor growth in early stages, whereas in later stages, our data suggest that MSCs might prevent metastasis of mammary cancer.

We also analyzed whether PSAP expression correlated with poor prognosis in breast cancer patients. In grade 1 breast cancer, PSAP is significantly correlated with poor prognosis (Fig. 6J). PSAP expression trends towards predicting poor outcome for grade 2 patients as well, but was not significant (Supplementary Fig. S9E). On the other hand, PSAP expression correlated with improved prognosis for grade 3 patients (Supplementary Fig. S9E), suggesting PSAP regulates tumor progression in a manner that differs with less differentiated breast cancer grade. These results are consistent with our observation that MSCs increase growth of early mammary carcinoma in mice, but decrease metastasis at a later time point (Fig. 5B; Supplementary Fig. S6A and B, S9A).

MSCs from B6 mice respond to ECM stiffness and promote growth of mammary cancer cells

To demonstrate that the effects we observed were not specific to the BALBc-derived cells, we established MSCs from the B6 mouse. These MSCs showed spreading morphology on a plastic dish and multi-potent differentiation to both adipocytes and osteocytes (Supplementary Fig. S10A and B). When the MSCs were cultured with conditioned media from E0771 cells, a mammary carcinoma cell line derived from B6 mice, the cells recapitulated the results found with 4T1 conditioned medium in that they demonstrated a spread morphology on a stiff matrix but were spindle-shaped on a soft matrix (Supplementary Fig. S10C). MSCs on a stiff matrix demonstrated an elevated expression of αSMA and 2P-MLC, secretion of PSAP, and activity of YAP compared to those on a soft matrix cultured with E0771-conditioned media (Supplementary Fig. S10D–F). In addition, E0771 cells cultured with conditioned media from the MSCs on a stiff matrix displayed greater cell growth than on a soft matrix (Supplementary Fig. S10G). These results show that, in a manner similar to cells derived from BALBc mice, MSCs from B6 mice respond to matrix stiffness and promote growth of mammary carcinoma cells derived from a B6 mouse.

Discussion

In this study, we found that soluble factors from mammary cancer cells are critical for MSCs survival (Fig. 7). We revealed that MSCs on stiff ECMs showed CAF phenotypes dependent on up-regulation of YAP and MLC. MSCs on stiff ECMs also secreted PSAP and promoted

mammary cancer proliferation and survival via Akt activation. Furthermore, MSCs increased tumor growth in mice with stiff tissues, but not in wild-type mice. These results indicate that MSCs respond to the stiffness of surrounding tissues, resulting in mammary cancer growth. Previous studies have focused on the interaction between cancer cells and tissue stiffness(2,3). However, so far, the three-way interaction between stromal cells, tumor cells, and tissue stiffness in cancer has been poorly understood. This is the first report to show MSCs clearly respond to tissue stiffness and contribute to cancer growth.

Previous studies showed that MSCs respond to chemical stimuli, such as soluble factors secreted from cancer cells and promote tumor progression(9–11). Another study found that matrix stiffness regulates differentiation of MSCs to multiple lineage in non-cancerous condition(16). However, to date there has not been a study investigating the effect and mechanisms of matrix stiffness on MSCs in cancer. To our knowledge, this is the first study to show that MSCs contribute to cancer progression by responding to ECM stiffness, by performing *in vitro* and *in vivo* experiments. Furthermore, we identified PSAP, secreted from MSCs in stiff tumor microenvironment, as a key factor for mammary cancer growth.

Here we showed that PSAP secreted from MSCs promotes proliferation and survival in mammary cancer cells *in vivo*. We also demonstrated that PSAP from MSCs increases tumor size 7 days after injection but inhibit metastasis *in vivo*. These results suggest that PSAP has different functions in tumor progression. Previous studies revealed that PSAP modulates immune response (41) and saposin C reduces angiogenesis (42). Thus, PSAP promotes proliferation and survival of mammary tumor cells by directly stimulating the cells, whereas, prevents metastasis potentially via regulating tumor microenvironment, such as to stimulate the immune response and to suppress angiogenesis.

MSCs have been reported to show both pro-tumorigenic and anti-tumorigenic potentials(7,8). This discrepancy may be caused by a difference in the origin of MSCs, the types of cancer cells, or by differences in the interaction of cancer cells, MSCs, immune cells, and the other stroma cells in cancer. Our results suggest that tissue stiffness and tumor stages are critical factors in the contribution of MSCs to cancer progression. In WT mice, MSCs did not contribute to tumor growth. In COL mice, which have increased collagen deposition and tissue stiffness, MSCs promoted early stage tumor growth. Thus, inhibition of MSCs may be a therapeutic target during early stages of breast cancer. Although the results were not significant, MSCs reduced metastasis in COL mice. This implies that in late stages, MSCs inhibit tumor progression by reducing metastasis in stiff tissues. MSCs and PSAP may be good therapeutic targets for breast cancer, however, this line of treatment should be carefully considered for each patient based on breast tissue density, cancer stage, and grade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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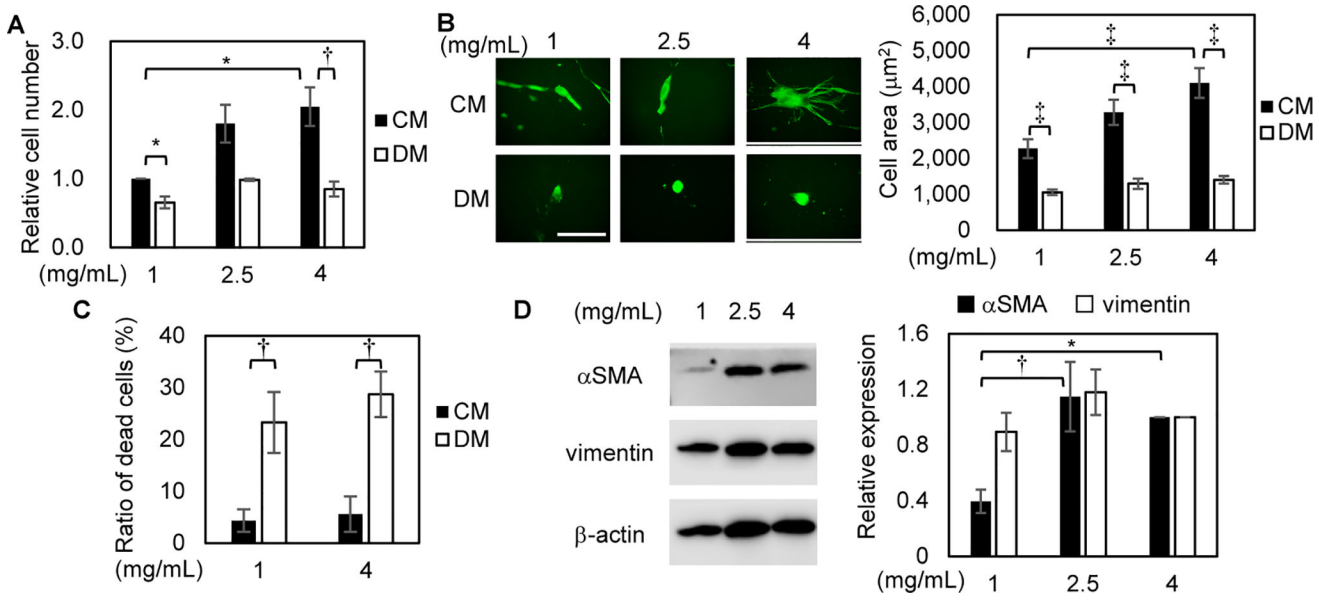

Figure 1. Collagen density is critical for differentiation of MSCs to CAFs

A, Growth assay of MSCs cultured with 4T1-conditioned media (CM) or control DMEM (DM) on floating collagen gels of 1, 2.5, or 4 mg/mL collagen. n = 3 (1, 4 mg/mL), 2 (2.5 mg/mL) experiments. B, Morphology assay of MSCs. Green: F-actin. Cells were quantitated for overall area, n = 30 cells in 3 (1, 4 mg/mL), 2 (2.5 mg/mL) experiments. C, Viability assay of MSCs using calcein AM. n = 3 experiments. D, Western blot of αSMA, vimentin, and β-actin in MSCs. n = 4 (1, 4 mg/mL) or n = 3 (2.5 mg/mL) experiments. Statistical significance was determined as Supplementary Materials and Methods. Mean±S.E. are shown. Bar = 100 μm. MSCs were established from BALBc mice.

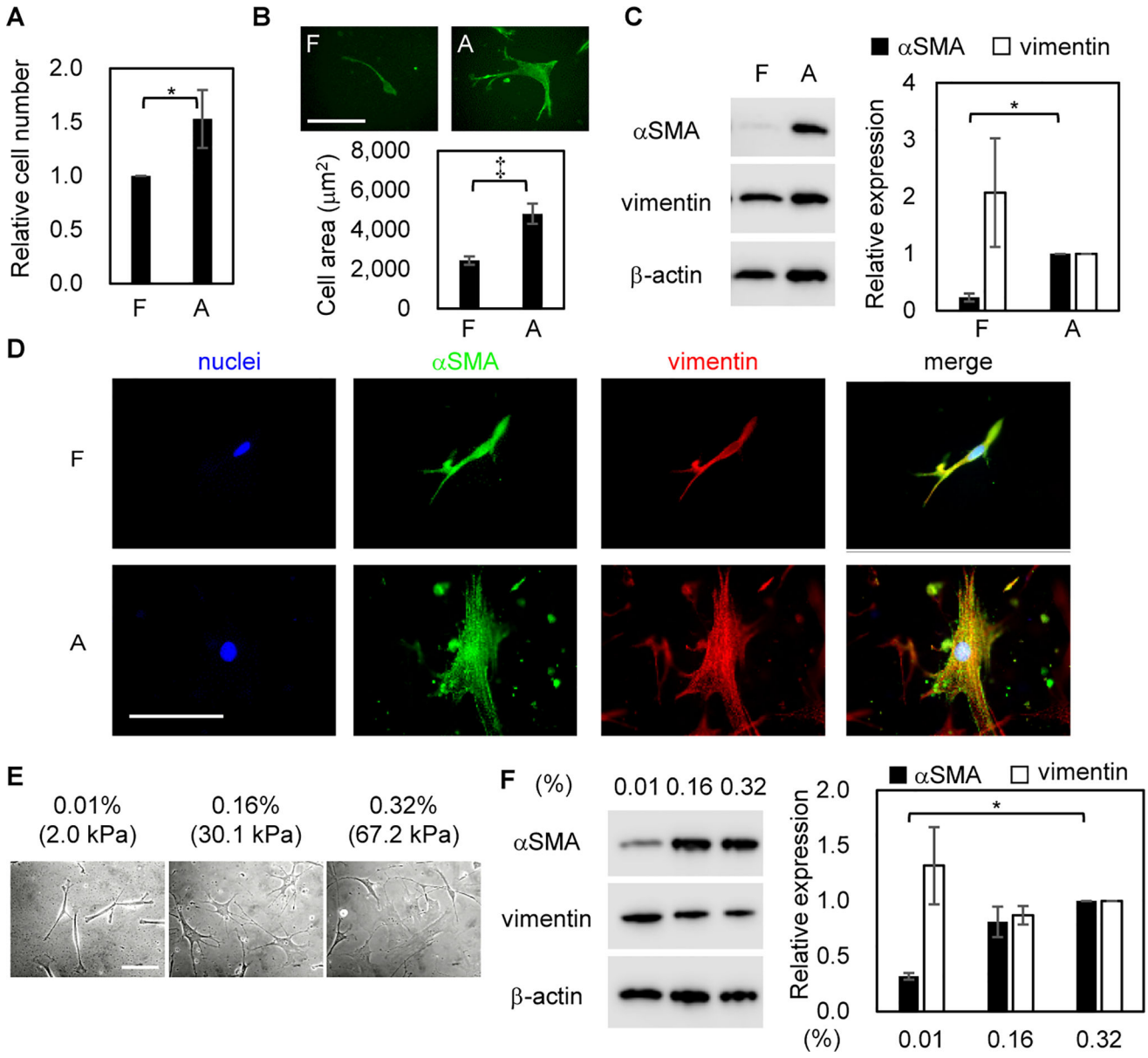

Figure 2. Matrix stiffness regulates differentiation of MSCs to CAFs

A, Growth assay of MSCs cultured with 4T1-conditioned media (CM) on floating or attached collagen gels of 1 mg/mL collagen. n = 3 experiments. B, Morphology assay of MSCs. Green: F-actin. n = 30 cells in 3 experiments. C, Western blot of αSMA, vimentin, and β-actin in MSCs. n = 3 experiments. D, Fluorescence images of nuclei, αSMA, and vimentin in MSCs. E, Phase contrast images of MSCs cultured with CM on polyacrylamide gels of 0.01, 0.16, 0.32% BIS concentration and the corresponding stiffness of the gels. F, Western blot of αSMA, vimentin, and β-actin in MSCs. n = 3 experiments. Statistical significance was determined as Supplementary Materials and Methods. Mean±S.E. are shown. Bar = 100 μm. MSCs were established from BALBc mice. F: floating collagen gels, A: attached collagen gels.

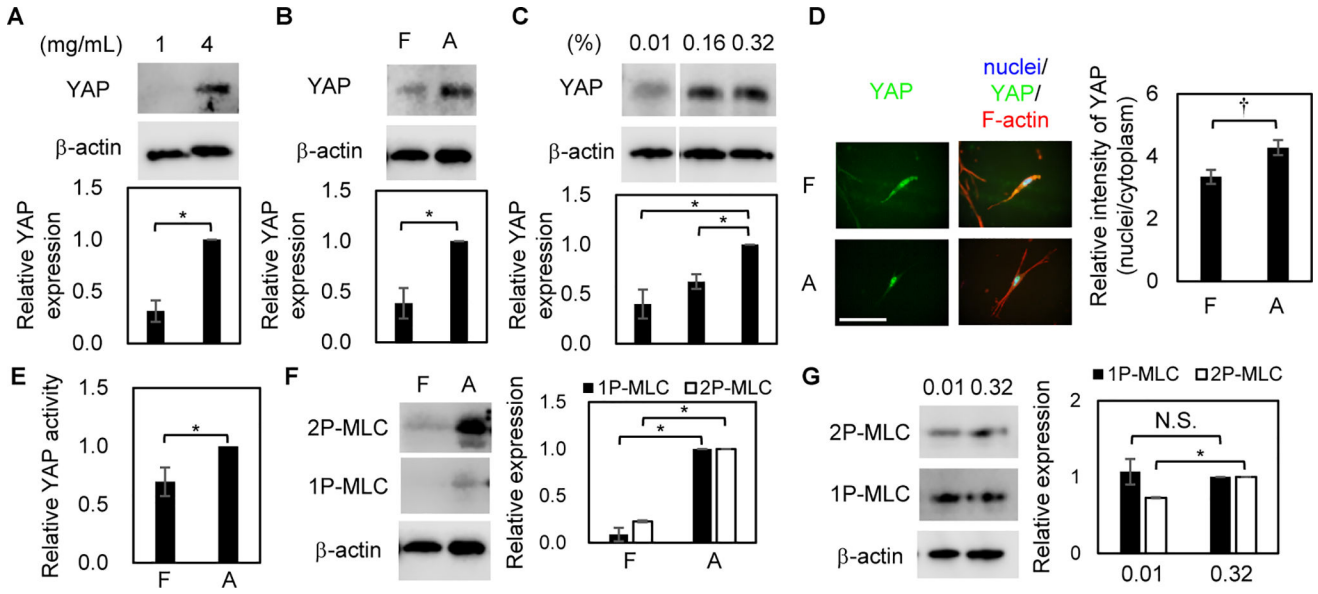

Figure 3. YAP and MLC are regulated by matrix stiffness in MSCs

A, Western blot of YAP and β-actin in MSCs cultured with 4T1-conditioned media (CM) on floating collagen gels of 1 or 4 mg/mL collagen. n = 3 experiments. B, Western blot of YAP and β-actin in MSCs cultured with CM on floating (F) or attached (A) collagen gels of 1 mg/mL collagen. n = 4 experiments. C, Western blot of YAP and β-actin in MSCs cultured with CM on polyacrylamide gels of 0.01, 0.16, 0.32% BIS concentration. n = 3 experiments. D, Fluorescence images of nuclei, YAP, and F-actin in MSCs. Relative intensity of YAP in nuclei was quantified in n = 30 cells in 3 experiments. E, Luciferase assay of a YAP reporter construct in MSCs. n = 3 experiments. F, Western blot of 2P-MLC, 1P-MLC, and β-actin in MSCs. n = 3 experiments. G, Western blot of 2P-MLC, 1P-MLC, and β-actin in MSCs cultured with CM on polyacrylamide gels of 0.01 and 0.32% BIS concentration. n = 3 experiments. Statistical significance was determined as Supplementary Materials and Methods. N.S.: no significance with 95% confidence interval. Mean±S.E. are shown. Bar = 100 μm. MSCs were established from BALBc mice.

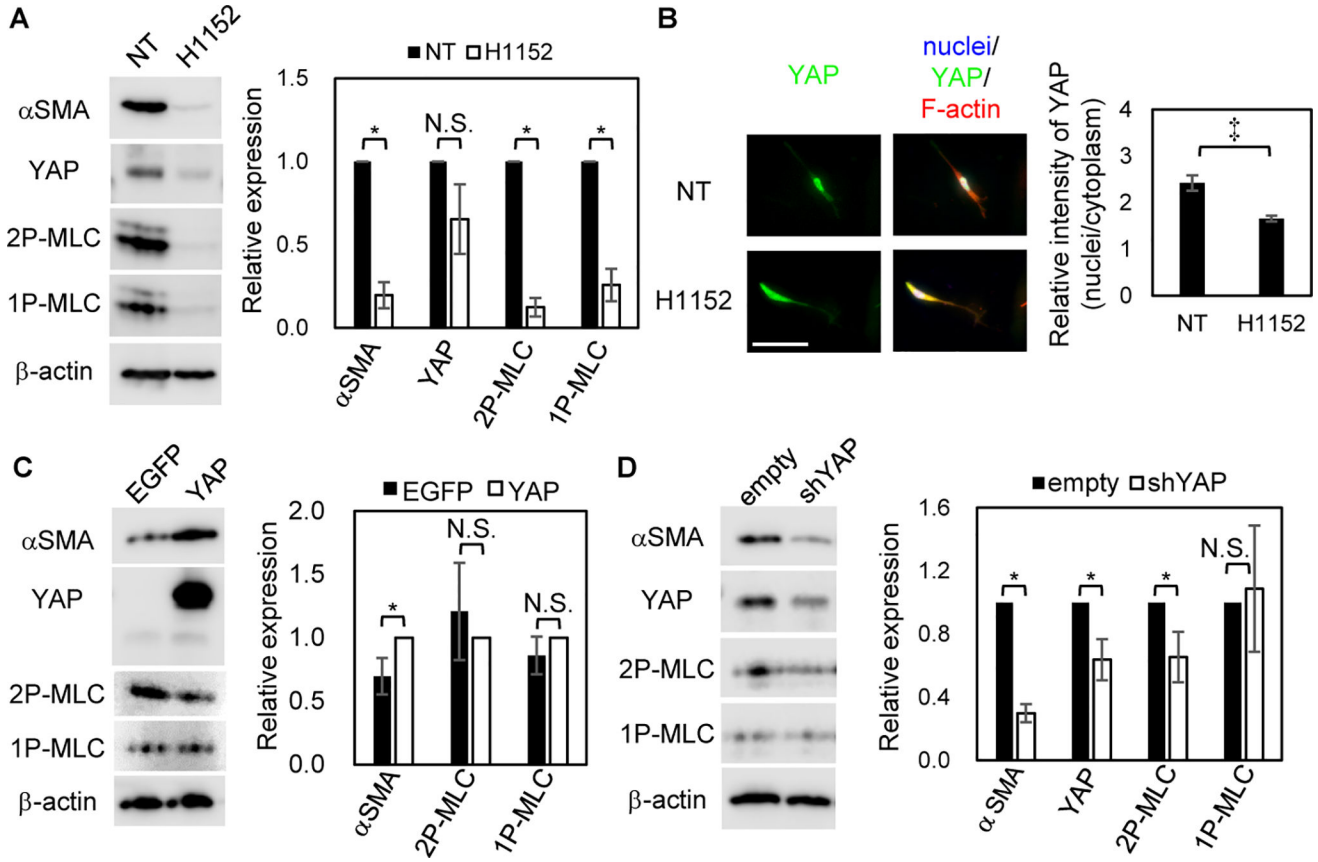

Figure 4. Mechano-signal transduction is critical for differentiation of MSCs into CAFs in response to cancer cell conditioned medium

A, Western blot of αSMA, YAP, 2P-MLC, 1P-MLC, and β-actin in non-treated (NT) or 10 μM H1152-treated MSCs cultured with 4T1-conditioned media (CM) on attached collagen gels of 1 mg/mL collagen. n = 3 experiments. B, Fluorescence images of nuclei, YAP, and F-actin in NT or 10 μM H1152-treated MSCs. Relative intensity of YAP in nuclei was quantified from n = 24 cells in 3 experiments. C, Western blot of αSMA, YAP, 2P-MLC, 1P-MLC, and β-actin in EGFP- or YAP-MSCs cultured on a collagen-coated plastic dish for 1 day. n = 3 experiments. D, Western blot of αSMA, YAP, 2P-MLC, 1P-MLC, and β-actin in empty- or shYAP-MSCs cultured with CM on attached collagen gels of 1 mg/mL collagen. n = 3 experiments. Statistical significance was determined as Supplementary Materials and Methods. N.S.: no significance with 95% confidence interval. Mean±S.E. are shown. Bar = 100 μm. MSCs were established from BALBc mice.

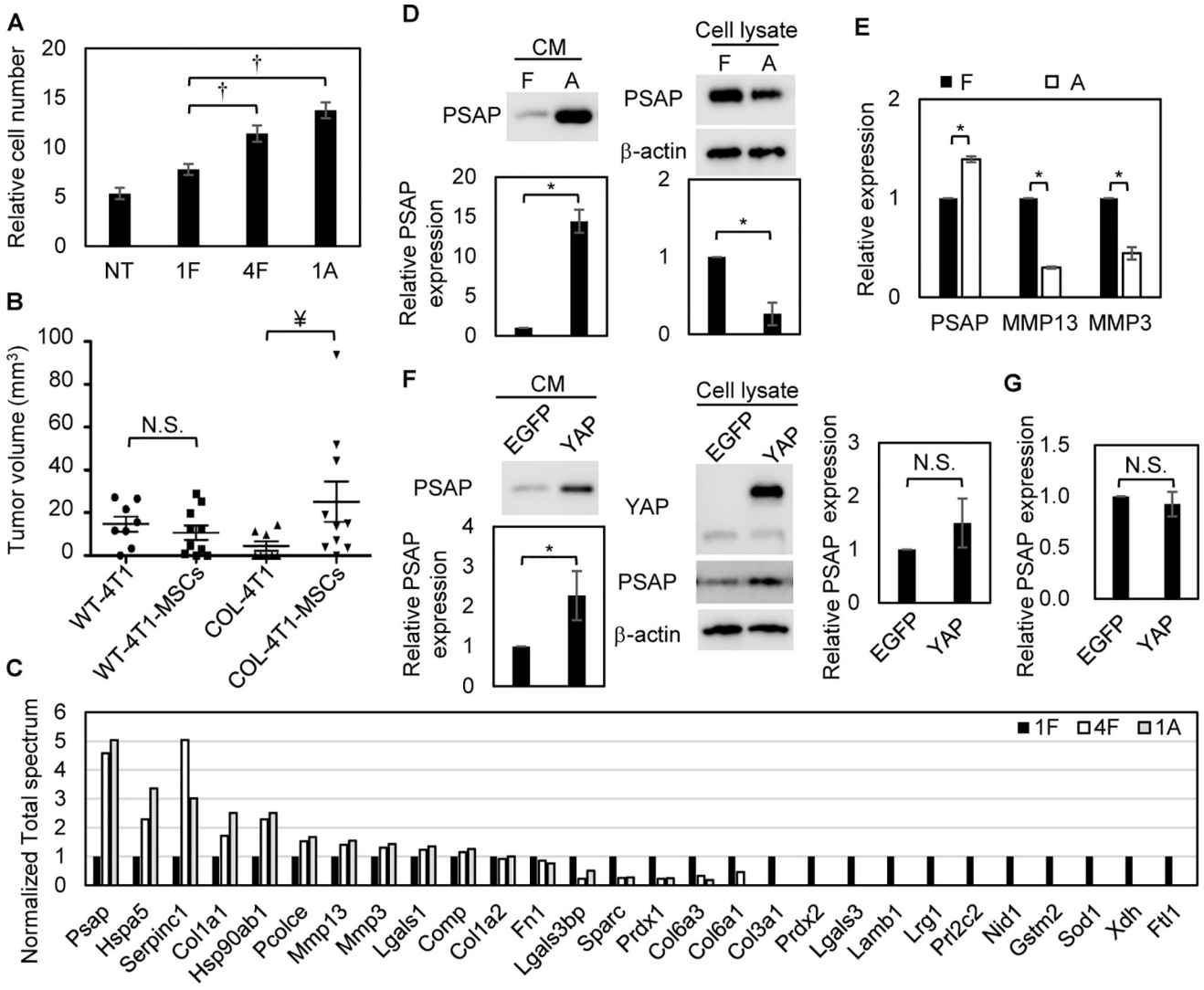

Figure 5. MSCs on stiff matrices promote growth of mammary cancer cells via PSAP secretion

A, Growth assay of 4T1 cells cultured with control DMEM (NT), conditioned media of MSCs cultured with 4T1-conditioned media on floating collagen gels of 1 mg/mL collagen (1F), on floating collagen gels of 4 mg/mL collagen (4F), or on attached collagen gels of 1 mg/mL collagen (1A), n = 9 samples in 3 experiments. B, Volume of tumors 7 days after injection. WT or COL mice were injected with 4T1 cells only (4T1) or 4T1 cells with empty-MSCs (4T1-MSCs). n = 8 tumors in 4 mice (4T1), 10 in 5 mice (4T1-MSCs). C, Proteomics analysis of conditioned media from MSCs. D, Western blot of PSAP and β-actin in conditioned media (CM) or cell lysate of MSCs cultured with 4T1-conditioned media on floating (F) or attached (A) collagen gels of 1 mg/mL collagen. CM: n = 3 technical replicates. Cell lysate: n = 3 experiments. E, qPCR of PSAP, MMP13, and MMP3 in MSCs. n = 3 experiments. F, Western blot of YAP, PSAP, and β-actin in CM or cell lysate of EGFP- or YAP-MSCs cultured with 4T1-conditioned media on floating collagen gels of 1 mg/mL collagen. CM: n = 6 samples in 3 experiments. Cell lysate: n = 6 samples in 2 experiments. G, qPCR of PSAP in control EGFP or YAP-MSCs on a collagen-coated plastic dish. n = 4 experiments. Statistical significance was determined as Supplementary Materials and

Methods. N.S.: no significance (P>0.05) with Wilcoxon rank sum test (B). or no significance with 95% confidence interval (D, F, and G). Mean±S.E. are shown. MSCs were established from BALBc mice.

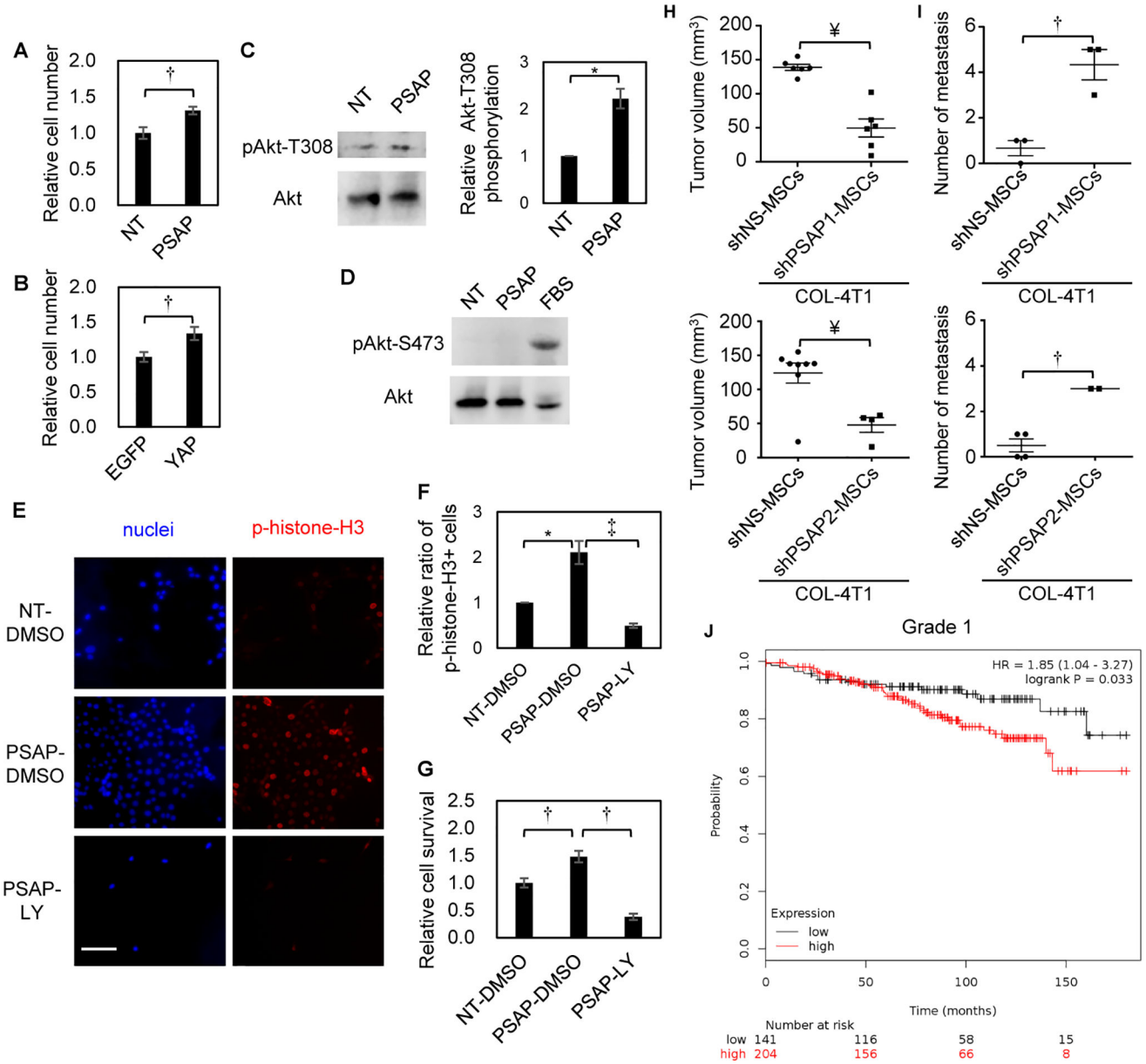

Figure 6. PSAP promotes proliferation and survival of mammary cancer cells

A, Growth assay of 4T1 cells cultured with control DMEM (NT) or DMEM with exogenously added 20 ng/mL PSAP (PSAP) for 4 days. n = 9 samples in 3 experiments. B, Growth assay of 4T1 cells cultured with conditioned media from EGFP- or YAP-MSCs for 4 days. n = 12 samples in 4 experiments. C, Western blot of phosphorylated Akt at T308 (pAkt-T308) and total-Akt (Akt) in 4T1 cells cultured with DMEM (NT) or DMEM with PSAP (PSAP) for 15 min. n = 4 experiments. D, Western blot of phosphorylated Akt at S473 (pAkt-S473) and Akt in 4T1 cells cultured with DMEM (NT), DMEM with PSAP (PSAP), or RPMI with 10% FBS (FBS) for 15 min. E, Fluorescence images of nuclei and phosphorylated histone-H3 at S10 (p-histone-H3) in 4T1 cells cultured with DMEM exogenously added with DMSO (NT-DMSO), DMEM with PSAP+DMSO (PSAP−DMSO), or DMEM with PSAP+LY294002 (PSAP−LY) for 1 day. F, Relative ratio of phosphorylated

histone-H3 at S10 positive (p-histone-H3+) 4T1 cells in e. n = 3 experiments. G, Survival assay of 4T1 cells. n = 9 samples in 3 experiments. H, Volume of tumors 7 days after injection. COL mice were injected with 4T1 cells with shNS-MSCs, shPSAP1-MSCs, or shPSAP2-MSCs. n = 6 tumors in 3 mice (upper panel), n = 8 tumors in 4 mice (lower panel, shNS-MSCs), or n = 4 tumors in 2 mice (lower panel, shPSAP2-MSCs). I, Number of metastatic lung lesions 21 days after injection. n = 3 mice (upper panel), n = 4 mice (lower panel, shNS-MSCs), or n = 2 mice (lower panel, shPSAP2-MSCs). J, Kaplan-Meier curves of relapse free survival in relation to the gene expression of PSAP in grade 1 breast cancer patients. Statistical significance was determined as Supplementary Materials and Methods. Mean±S.E. are shown. MSCs were established from BALBc mice. Bar = 100 μm.

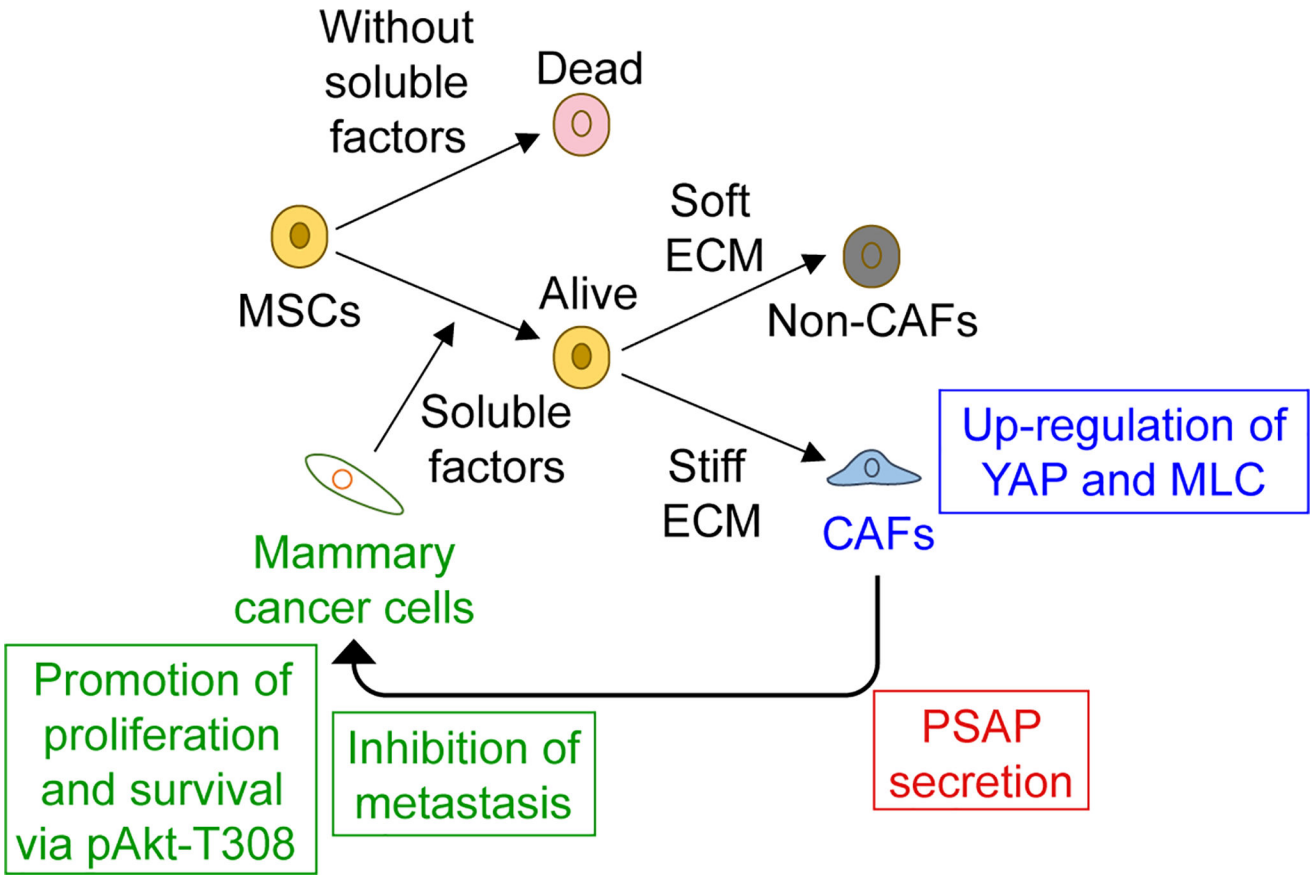

Figure 7.
Bi-directional communication between MSCs and mammary cancer cells in stiff tumor microenvironment.