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Role of ATF5 in the invasive potential of diverse human cancer cell lines

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Abstract

Activating transcription factor 5 (ATF5) is a member of the ATF/cAMP response element-binding protein family. Our research group recently revealed that ATF5 expression increases the invasiveness of human lung carcinoma cells. However, the effects of ATF5 on the invasive potential of other cancer cells lines remain unclear. Therefore, in this study, we investigated the role of ATF5 in the invasive activity of diverse human cancer cell lines. Invasiveness was assessed using Matrigel invasion assays. ATF5 knockdown resulted in decreased invasiveness in seven of eight cancer cell lines tested. These results suggest that ATF5 promotes invasiveness in several cancer cell lines. Furthermore, the roles of ATF5 in the invasiveness were evaluated in three-dimensional (3D) culture conditions. In 3D collagen gel, HT-1080 and MDA-MB-231 cells exhibited high invasiveness, with spindle morphology and high invasion speed. In both cell lines, knockdown of ATF5 resulted in rounded morphology and decreased invasion speed. Next, we showed that ATF5 induced integrin-α2 and integrin-β1 expression and that the depletion of integrin-α2 or integrin-β1 resulted in round morphology and decreased invasion speed. Our results suggest that ATF5 promotes invasion by inducing the expression of integrin-α2 and integrin-β1 in several

human cancer cell lines.

Keywords

Activating transcription factor 5

Integrin-α2

Integrin-β1

Invasion

Spindle morphology

Highlights

ATF5 enhances invasiveness of various cancer cell lines.

ATF5 promotes integrin-α2 and integrin-β1 expression.

Function of integrin-α2β1 contributes to invasiveness.

ATF5 expression correlates to invasiveness in breast cancer cells.

ATF5 regulates cell morphology in a 3D collagen gel via integrin-α2β1 activity.

1. Introduction

Tumor metastasis is the most common cause of death among cancer patients. Metastasis is a crucial process in cancer progression. The first step in metastasis is

cancer cell invasion into adjacent normal tissues, which is caused by neoplastic cells obtaining motility and degrading the extracellular matrix. The next step is penetration into the blood or lymphatic circulation, which is followed by exit from the circulation, settling in distant organs (extravasation), and proliferation in these distant organs [1]. Inhibiting the first step of metastasis is crucial for cancer therapy.

Activating transcription factor 5 (ATF5) is a member of the ATF/cAMP response element-binding (CREB) protein family of basic leucine zipper proteins [2]. ATF5, which is also called ATFx, is expressed in many types of adult tissues, with particularly high expression in the liver. ATF5 is widely detected upon immunohistochemical staining of carcinoma tissue microarrays, and nuclear localization of ATF5 is higher in neoplastic tissues than in non-neoplastic tissues [3]. Recent studies have shown that ATF5 plays an important role in promoting cell survival in a variety of cell types, including breast cancer, glioblastoma, cervical cancer, lymphocytes, and cardiomyocytes [3,4,5,6], while several other studies reported that ATF5 is not required for cell survival in lung cancer, neuro progenitors, adrenal glands, embryonic kidney cells, astrocytes, oligodendrocyte precursors, mouse embryonic stem cells, or non-neoplastic breast cells [3,7,8,9,10,11]. Although the role of ATF5 as a survival-related protein has been extensively reported, few studies have been performed

to reveal the relationship between ATF5 expression and cancer cell invasiveness. Our group previously reported that ATF5 increases invasiveness of A549 lung cancer cells [11]. However, the effects of ATF5 on invasiveness have not been studied in other cancer cell lines. Therefore, the purpose of this study was to examine whether ATF5 generally plays a crucial in the invasive activity of a variety of cancer cell lines.

2. Material and methods

Cell culture

A549 human lung adenocarcinoma cell line, MDA-MB-231 human breast adenocarcinoma cell line, H1299 human non-small cell lung carcinoma cell line, HT-29 human colorectal adenocarcinoma cell line, MCF7 human breast adenocarcinoma cell line, and T84 human colorectal adenocarcinoma cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa human cervical adenocarcinoma cell line, HT-1080 human fibrosarcoma cell line, MIAPaCa-2 human pancreatic carcinoma cell line, and MKN1 human gastric adenocarcinoma cell line were purchased from Riken Cell Bank (Ibaraki, Japan). HSC4 human oral squamous cell carcinoma cell line was purchased from JCRB Cell Bank (Osaka, Japan). A549, H1299, HeLa, HT-1080, HT-29, MCF7, MDA-MB-231, MIAPaCa-2, and T84 cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich Co. LLC, St Louis,

MO, USA) supplemented with 10% fetal bovine serum (Biowest SAS, Nuaillé, France) and 1% antibiotic/antimycotic solution (Sigma-Aldrich Co. LLC). HSC4 and MKN1 were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Biowest SAS) and 1% antibiotic/antimycotic solution (Sigma-Aldrich Co. LLC). The cells were cultured in a humidified incubator at 37°C with 5% CO_2. We used type-I collagen (1.6 mg/mL, Cell matrix I-P, Nitta Gelatin, Osaka, Japan) for collagen gel overlay culture conditions as previously described [12].

Materials

Antibodies against ATF5 (ab184923, Abcam Plc, Cambridge, UK), CD29 (integrin-β1, BD Biosciences, San Jose, CA, USA), CD49b (integrin-α2, BD Biosciences), α-tubulin (T6199, Sigma-Aldrich Co. LLC), horseradish peroxidase (HRP) anti-mouse IgG (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and HRP anti-rabbit IgG (Cell Signaling Technology, Inc., Danvers, MA, USA) were used for western blotting.

MFP 488-phalloidin (MoBiTec GmbH, Göettingen, Germany) or Alexa Fluor-488 Phalloidin (Thermo Fisher Scientific, Inc.) was used to stain F-actin, and SYTOX Green

(Thermo Fisher Scientific, Inc.) was used for nuclear staining.

Western blotting

Cells were fixed in cold 10% trichloroacetic acid (Sigma-Aldrich Co. LLC) for 5 min on ice. Cells were washed 3 times with cold phosphate buffered saline (PBS) for 3 min on ice, then lysed in sodium dodecyl sulfate (SDS) buffer (0.125 M Tris-HCl, 0.2 M dithiothreitol, 4% SDS, 20% glycerol, and 0.01% bromophenol blue, pH 6.8). The cell lysates were treated with ultrasonic fragmentation and heated at 95°C for 5 min. The cell lysates were separated on 8, 10, 12, or 14% SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride membranes. The membranes were then incubated with the appropriate primary antibody (integrin-β1: 1:500, integrin-α2: 1:500, ATF5: 1:10,000, and α-tubulin: 1:20,000) in Tris buffered saline with Tween 20 (TBS-T, 10 mM Tris-HCl containing 150 mM NaCl and 0.05% Tween 20, pH 7.5) at 4°C overnight. Membranes were then washed 3 times with TBS-T and incubated with the appropriate secondary antibody in TBS-T (HRP anti-rabbit IgG: 1:5,000 for ATF5, HRP anti-mouse IgG: 1:10,000 for integrin-β1 and integrin-α2, and α-tubulin) for 1 h at room temperature. Protein signals were detected with Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA).

Small interfering RNA (siRNA)

Cells were transfected with the appropriate siRNA or random RNA with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Inc.). The following target sequences were used: ATF5: 5′-GTCCAAATCATGAAATGTTTG-3′ (sense sequence), integrin-α2: 5′-CCAAAGAAGAAATGATTGTAG-3′ (sense sequence), and integrin-β1: 5′-TTCAACTGTGATAGATCCAAT-3′ (sense sequence).

Inhibitor Treatment

Cells were cultured in collagen gel overlay conditions. Treatment with BHA2.1 (EMD Millipore) was performed at 2000 ng/mL for HT-1080 cells or 800 ng/mL for MDA-MB-231 cells, or dimethyl sulfoxide (DMSO) as a control (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added just before time-lapse imaging. After one day, the cells were fixed for roundness assays.

Roundness assays

To analyze cell roundness, we observed F-actin staining images of the cells by the following procedures: cells cultured in collagen gel overlay conditions were fixed with

4% paraformaldehyde for 10 min at room temperature and then washed 3 times with PBS. The cells were permeabilized with 0.5% Triton X-100 for 10 min at room temperature and then were washed 3 times with PBS. Next, the cells were incubated with MFP 488-Phalloidin (1:400) or Alexa Fluor-488 Phalloidin (1:400) at 4°C overnight. Images were captured with a Nikon C1 confocal imaging system (Nikon Instech Co., Ltd, Tokyo, Japan). Roundness was calculated by the equation $4\pi S$ (surface area)/L^2 (perimeter length).

Matrigel invasion assays

For invasion assays, 50,000 cells were seeded on the top of an 8.0 μm pore insert (24 well insert, Corning Inc., Corning, NY, USA) with a 1:50 diluted Matrigel (BD Biosciences)-coated membrane. In the upper chamber, the appropriate media without FBS was added, and media with 10% FBS was added to the lower well, and the cells were incubated for 24 h. Then, cells on the upper surface of the membrane (i.e., non-invasive cells) were removed with a cotton swab. The cells on the lower surface of the membrane were stained with SYTOX Green (nuclear staining dye) and counted.

Time-lapse imaging

The cells were cultured in collagen gel overlay conditions. We performed time-lapse imaging for cell tracking and evaluated invasion speed using previously reported methods [13].

Statistical analysis

Data are shown as mean ± SEM. Statistical analyses were performed with Welch's t-tests. P-values of < 0.05 were considered statistically significant.

3. Results

3.1. ATF5 is required for the invasive activity of cancer cell lines derived from various tissues

First, to select highly invasive cancer cell lines suitable for this study, we performed Matrigel invasion assays for 11 cancer cell lines (Fig. 1A). We observed that human HT-1080 fibrosarcoma, A549 lung, MDA-MB-231 breast, H1299 lung, HSC4 oral squamous, HeLa cervix, MKN1 gastric, and MIAPaCa-2 cancer cells were categorized as highly invasive cancer cells, whereas human T84 colorectal, HT-29 colorectal, and MCF7 breast cancer cells showed low levels of invasiveness (Fig. 1A). In order to investigate whether invasiveness correlated with the level of ATF5 expression, we

compared the ATF5 expression in the highly invasive breast cancer line MDA-MB-231 with that in the less invasive breast cancer line MCF7. ATF5 expression was greater in MDA-MB-231 cells than in MCF7 cells (Fig. 1B). This result indicates a positive correlation between ATF5 expression and invasiveness of breast cancer cells. To further examine the role of ATF5 in cancer cell invasion, we tested whether downregulation of ATF5 decreased the invasiveness of various cancer cell lines. To accomplish this, we used 8 highly invasive cancer cells: HT-1080, A549, MDA-MB-231, H1299, HSC4, HeLa, MKN1, and MIAPaCa-2 cells. We transfected these cells with short interfering RNA (siRNA) targeting ATF5 mRNA and performed Matrigel invasion assays to compare the invasiveness of the ATF5 knockdown (siATF5) cells and the negative control (siCtrl) cells. The siATF5 cells of HT-1080, A549, MDA-MB-231, H1299, HeLa, MKN1, and MIAPaCa-2 cell lines exhibited lower invasiveness than the corresponding siCtrl cells, whereas depletion of ATF5 had no effect on the invasiveness of HSC4 cells (Fig. 1C).

These findings suggest that the levels of ATF5 expression correlate with breast cancer cell invasiveness, and that ATF5 enhances the invasiveness of various cancer cell lines.

3.2. ATF5 enhances invasiveness via induction of spindle morphology in a 3D collagen

gel

To further investigate the effect of ATF5 on invasiveness, we performed 3D collagen gel invasion assays. We used HT-1080 and MDA-MB-231 cells because these cells exhibited high levels of invasiveness among the cell lines tested (Fig. 1A). These cells exhibited spindle morphology when cultured in collagen gel overlay conditions, indicating an invasive phenotype. To estimate invasiveness in 3D culture conditions, we evaluated spindle cell ratio and invasion speed in collagen gel overlay conditions. We transfected cells with ATF5-specific or NC siRNA, and then compared the invasiveness of the siATF5 cells with siCtrl cells in collagen gel overlay conditions. Fig. 2A shows phase contrast images for siCtrl and siATF5 cells at 0, 2, 4, and 6 h. First, we analyzed the effect of ATF5 knockdown on cell trajectory in collagen gel overlay conditions. siCtrl cells exhibited a wide range of movement, whereas ATF5 knockdown strikingly suppressed the invasion range in both cell lines tested (Fig. 2B). Since we observed that siATF5 cells exhibited round morphology in both cell lines, we further explored ATF5-mediated changes in cell morphology. To examine alterations in cell morphology, we calculated cell roundness. ATF5 depletion increased cell roundness, that is, the ratio of spindle cells was diminished (Fig. 2C). In addition, we analyzed cancer cell invasion speed in a 3D collagen matrix. Downregulation of ATF5 significantly decreased

invasion speed in both cell lines tested (Fig. 2D).

These findings suggest that ATF5 enhances cancer cell invasion in a 3D collagen matrix via inducing spindle morphology in HT-1080 and MDA-MB-231 cells.

3.3. ITGA2 and ITGB1 expression is controlled by ATF5 to promote cell invasion in a 3D collagen matrix

Integrins are a major family of cell surface-adhesion receptors that interact with the extracellular matrix to translate environmental cues into cellular responses. Previous studies have suggested that integrins are required for cell invasion and migration [14]. In addition, we previously reported that ATF5 enhances integrin-β1 (ITGB1) expression, and ITGB1 downregulation in A549 cells results in rounded morphology [11]. Integrin-α2 (ITGA2) can form a heterodimer with ITGB1, which is a known collagen receptor [15]. Furthermore, we reported that ITGA2 and ITGB1 regulate lung cancer cell invasion in a 3D collagen matrix [12]. Therefore, we determined whether ATF5 upregulated invasiveness via ITGB1 expression in HT-1080 and MDA-MB-231 cells. First, we transfected cells with ATF5-specific or NC siRNA and compared ITGB1 expression between siATF5 cells and siCtrl cells in collagen gel overlay conditions. ATF5 depletion decreased ITGA2 and ITGB1 expression in both HT-1080 and

MDA-MB-231 cells. Next, we investigated whether ITGA2 or ITGB1 regulates cancer cell morphology and invasive activity in a 3D collagen gel. Fig. 3B shows phase contrast images for each sample at 0, 2, 4, and 6 h. These results indicate that downregulation of ITGA2 or ITGB1 slowed 3D cancer cell invasion and decreased the spindle cell ratio in both cell lines. Furthermore, to determine whether these results depend on an ITGA2B heterodimer, we used the ITGA2B1-specific blocking antibody, BHA2.1. Treatment with BHA2.1 immediately induced rounded morphology in a 3D collagen gel (Supplemental Fig. 1A and 1B). Moreover, BHA2.1 treatment significantly decreased invasive activity of both cell lines (Supplemental Fig. 1C). This result shows that ITGA2B1 function is required for maintaining cancer cell spindle morphology and invasion in a 3D collagen matrix.

From these results, we found that the inhibition of integrins and the knockdown of ATF5 showed similar results in terms of invasion speed and cell morphology. These findings suggest that ATF5 promotes spindle morphology and cancer cell invasion via expression of ITGA2 and ITGB1.

4. Discussion

In this study, we showed that ATF5 increases invasiveness in various cancer cell lines in Matrigel invasion assays, and ATF5 increases the spindle morphology ratio and

invasiveness via ITGA2 and ITGB1 expression in a 3D collagen matrix (Fig. 4). Furthermore, we demonstrated that ATF5 expression correlated with invasiveness in breast cancer cell lines MDA-MB-231 and MCF7. To generalize these results, future studies should be conducted to confirm the relationship between ATF5 expression and invasiveness in other cancer cell types. Previous studies suggested that ATF5 mRNA expression is observed in many different mouse [16] and human [17] tissues. We also showed that ATF5 increased the invasive activity of various cancer cell lines such as fibrosarcoma, lung, breast, cervical, gastric, and pancreatic cells. These results suggest that ATF5 is required for invasiveness in various cancer tissues, and ATF5 could be new biomarker for cancer progression. In addition, these findings suggest that ATF5 is a potential therapeutic target for treating cancer invasion.

We showed that ATF5 knockdown in HT-1080 and MDA-MB-231 cells induced round morphology in a collagen gel. We previously reported that ATF5 overexpression in A549 cells resulted in spindle morphology [11]. These findings suggested that ATF5 can induce an amoeboid to mesenchymal transition. However, the molecular mechanism by which ATF5 enhances spindle morphology remains unclear. In general, spindle morphology requires activity of the small GTPase Rac [18], whereas rounded morphology is induced through Rho and Rho-associated protein kinase (ROCK)

pathway-dependent myosin regulatory light chain (MRLC) phosphorylation, resulting in actomyosin contractility [19]. Our group has suggested that ATF5 suppresses MRLC phosphorylation in 3D culture. These studies suggest that ATF5 may activate Rac and inhibit Rho-ROCK pathways in many cancer cell lines.

In this study, we identified downstream effectors of ATF5 that regulate cancer cell invasion, but it remains unclear how ATF5 expression itself is controlled. An earlier study suggested that fibroblast growth factor receptor or epidermal growth factor receptor (EGFR) can enhance RAS-mitogen-activated protein kinase or phosphoinositide-3-kinase signaling pathways, resulting in activation of CREB protein 3-like 2 that upregulates ATF5 expression in malignant glioblastoma [4]. We previously reported that EGFR increases the expression of ITGA2 and ITGB1 in colon cancer cells [20]. Therefore, there is possibility that EGFR signaling enhances ITGA2 and ITGB1 expression via ATF5 activation in our experimental system. This remains to be determined in future studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at_____

Transparency document

Transparency document related to this article can be found online at_____

References

[1] I.J. Fidler, Timeline - The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited, Nature Reviews Cancer 3 (2003) 453-458.

[2] T. Hai, M.G. Hartman, The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis, Gene 273 (2001) 1-11.

[3] S.E. Monaco, J.M. Angelastro, M. Szabolcs, L.A. Greene, The transcription factor ATF5 is widely expressed in carcinomas, and interference with its function selectively kills neoplastic, but not nontransformed, breast cell lines, International Journal of Cancer 120 (2007) 1883-1890.

[4] Z. Sheng, L. Li, L.H.J. Zhu, T.W. Smith, A. Demers, A.H. Ross, R.P. Moser, M.R. Green, A genome-wide RNA interference screen reveals an essential CREB3L2-ATF5-MCL1 survival pathway in malignant glioma with therapeutic implications, Nature Medicine 16 (2010) 671-U673.

[5] S.P. Persengiev, L.R. Devireddy, M.R. Green, Inhibition of apoptosis by ATFx: a novel role for a member of the ATF/CREB family of mammalian bZIP transcription factors, Genes & Development 16 (2002) 1806-1814.

[6] H. Wang, G. Lin, Z. Zhang, ATF5 promotes cell survival through transcriptional activation of Hsp27 in H9c2 cells, Cell Biology International 31 (2007) 1309-1315.

[7] J.M. Angelastro, T.N. Ignatova, V.G. Kukekov, D.A. Steindler, G.B. Stengren, C. Mendelsohn, L.A. Greene, Regulated expression of ATF5 is required for the progression of neural progenitor cells to neurons, Journal of Neuroscience 23 (2003) 4590-4600.

[8] J.M. Angelastro, J.L. Mason, T.N. Ignatova, V.G. Kukekov, G.B. Stengren, J.E. Goldman, L.A. Greene, Downregulation of activating transcription factor 5 is required for differentiation of neural progenitor cells into astrocytes, Journal of Neuroscience 25 (2005) 3889-3899.

[9] J.M. Angelastro, P.D. Canoll, J. Kuo, M. Weicker, A. Costa, J.N. Bruce, L.A. Greene, Selective destruction of glioblastoma cells by interference with the activity or expression of ATF5, Oncogene 25 (2006) 907-916.

[10] J.L. Mason, J.M. Angelastro, T.N. Ignatova, V.G. Kukekov, G. Lin, L.A. Greene, J.E. Goldman, ATF5 regulates the proliferation and differentiation of oligodendrocytes, Molecular and Cellular Neuroscience 29 (2005) 372-380.

[11] S. Ishihara, M. Yasuda, A. Ishizu, M. Ishikawa, H. Shirato, H. Haga, Activating transcription factor 5 enhances radioresistance and malignancy in cancer cells, Oncotarget 6 (2015) 4602-4614.

[12] X. Li, S. Ishihara, M. Yasuda, T. Nishioka, T. Mizutani, M. Ishikawa, K. Kawabata, H. Shirato, H. Haga, Lung Cancer Cells That Survive Ionizing Radiation Show Increased Integrin alpha 2 beta 1-and EGFR-Dependent Invasiveness, Plos One 8 (2013) 12.

[13] Y. Iguchi, S. Ishihara, Y. Uchida, K. Tajima, T. Mizutani, K. Kawabata, H. Haga, Filamin B Enhances the Invasiveness of Cancer Cells into 3D Collagen Matrices, Cell Structure and Function 40 (2015) 61-67.

[14] J.D. Hood, D.A. Cheresh, Role of integrins in cell invasion and migration, Nature Reviews Cancer 2 (2002) 91-+.

[15] J.D. Humphries, A. Byron, M.J. Humphries, Integrin ligands at a glance, Journal of Cell Science 119 (2006) 3901-3903.

[16] M.B. Hansen, C. Mitchelmore, K.M. Kjaerulff, T.E. Rasmussen, K.M. Pedersen, N.A. Jensen, Mouse Atf5: Molecular cloning of two novel mRNAs, genomic organization, and odorant sensory neuron localization, Genomics 80 (2002) 344-350.

[17] M. Pascual, M.J. Gomez-Lechon, J.V. Castell, R. Jover, ATF5 is a highly abundant liver-enriched transcription factor that cooperates with constitutive androstane receptor in the transactivation of CYP2B6: Implications in hepatic stress responses, Drug Metabolism and Disposition 36 (2008) 1063-1072.

[18] D. Yamazaki, S. Kurisu, T. Takenawa, Involvement of Rac and Rho signaling in cancer cell motility in 3D substrates, Oncogene 28 (2009) 1570-1583.

[19] E. Sahai, C.J. Marshall, Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis, Nature Cell Biology 5 (2003) 711-719.

[20] A. Nukuda, C. Sasaki, S. Ishihara, T. Mizutani, K. Nakamura, T. Ayabe, K. Kawabata, H. Haga, Stiff substrates increase YAP-signaling-mediated matrix metalloproteinase-7 expression, Oncogenesis 4 (2015) 11.

Figure legends

Fig. 1. ATF5 promotes invasive activity of various cancer cell types (A) Several cancer cell lines were subjected to Matrigel invasion assays. (B) Representative western blots (upper) and quantification (lower) of ATF5 levels in MDA-MB-231 and MCF7 cells. (C) Eight cancer cell lines were subjected to Matrigel invasion assays after transfection with siCtrl or siATF5. The bars represent mean ± SEM. $n = 3$ independent experiments. *$P < 0.05$, **$P < 0.01$, unpaired *t*-test.

Fig. 2. ATF5 increases cancer cell invasiveness by regulating cell morphology in a 3D collagen gel (A) Time-lapse phase-contrast images of HT-1080 and MDA-MB-231 cells in a collagen gel after transfection with siCtrl or siATF5. Scale bars = 100 μm. (B) The cell invasion trajectories of three representative HT-1080 and MDA-MB-231 cells in a collagen gel after transfection with siCtrl or siATF5 for 24 h. (C) Quantification of cell roundness from Fig. 2(A). (D) Quantification of invasion speed from Fig. 2(A). The bars represent mean ± SEM. $n = 3$ independent experiments. $^{**}P < 0.01$, unpaired *t*-test.

Fig. 3. ATF5 enhances ITGA2 and ITGB1 expression to increase invasiveness in HT-1080 and MDA-MB-231 cells (A) Representative western blots (upper) and quantification (lower) of ITGA2 and ITGB1 levels after transfection with siCtrl or siATF5. (B) Time-lapse phase-contrast images of HT-1080 and MDA-MB-231 cells in a collagen gel after transfection with siCtrl, siITGA2, or siITGB1. Scale bars = 100 μm. (C) Quantification of cell roundness from Fig. 3(B). (D) Quantification of invasion speed from Fig. 3(B). The bars represent mean ± SEM. *n* = at least 3 independent experiments. **P* < 0.05, ***P* < 0.01, unpaired *t*-test.

Fig. 4. ATF5 contributes to upregulation of invasiveness via ITGA2 and ITGB1 expression in a 3D collagen matrix Schematic of our findings that ATF5 is required for the invasive activity of various cancer cell lines and that ATF5 promotes spindle morphology and invasiveness via ITGA2 and ITGB1 expression in a 3D collagen gel.

Supplementary Fig. 1. ITGA2B1 heterodimer function contributes to invasiveness in HT-1080 and MDA-MB-231 cells (A) Time-lapse phase-contrast images of HT-1080 cells and MDA-MB-231 cells in a collagen gel after treatment with or without BHA2.1. Scale bars = 100 μm. (B) Quantification of cell roundness from Supplementary Fig. 1(A). (C) Quantification of invasion speed from Supplementary Fig. 1(A). The bars represent mean ± SEM. $n = 3$ independent experiments. $^{**}P < 0.01$, unpaired *t*-test.

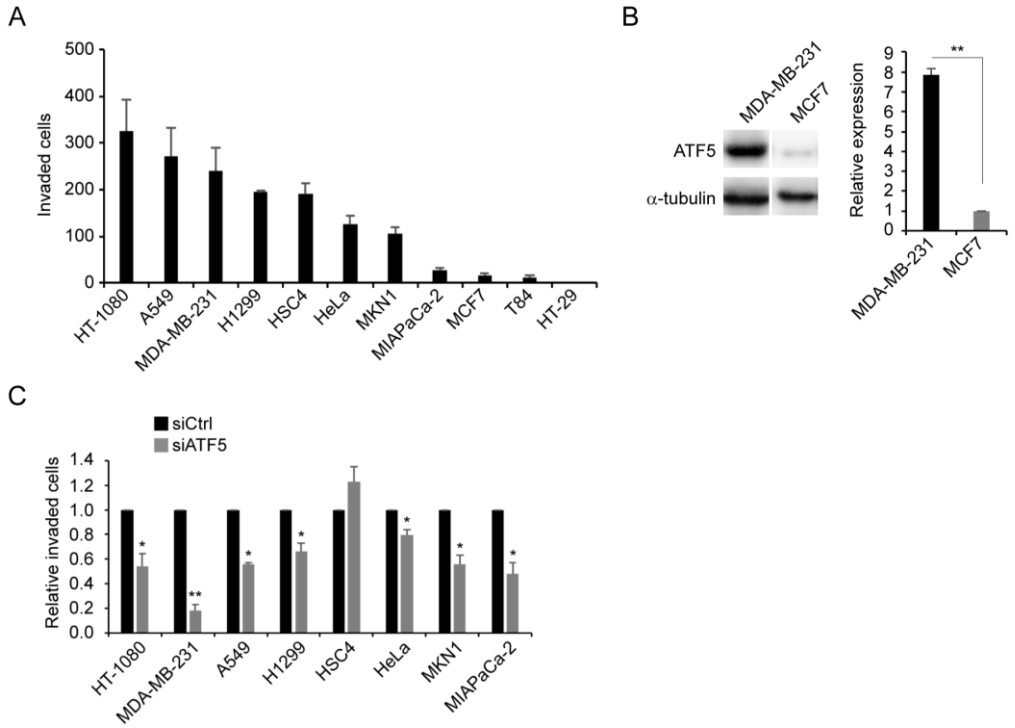
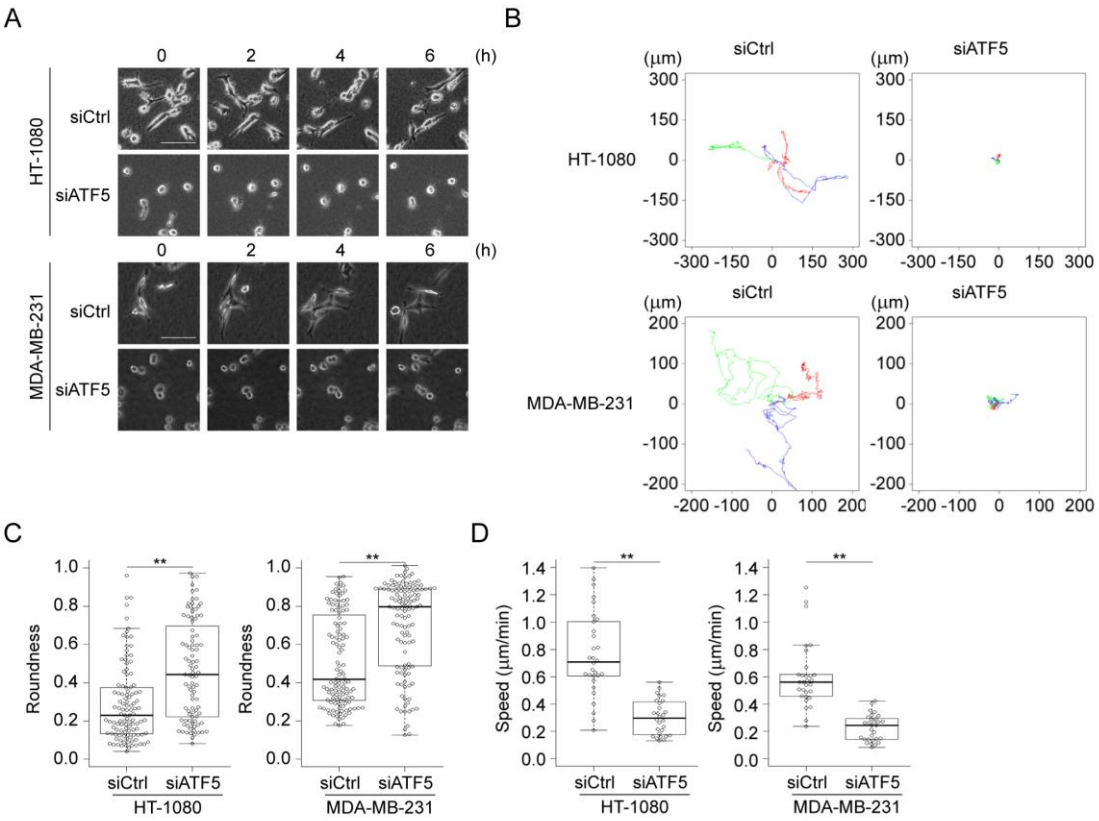


Figure 1. A. Nukuda, *et al*.



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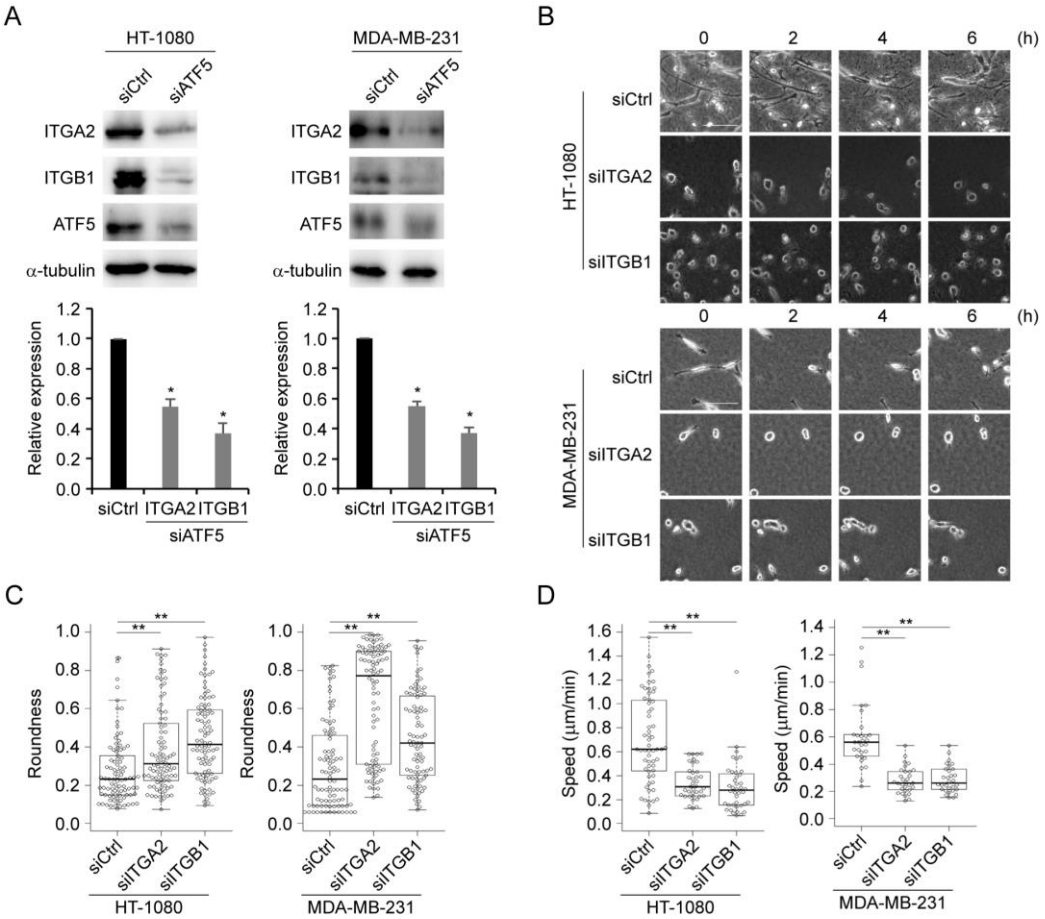

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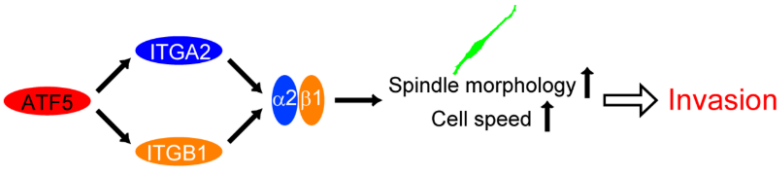

Figure 4. A. Nukuda, *et al*.

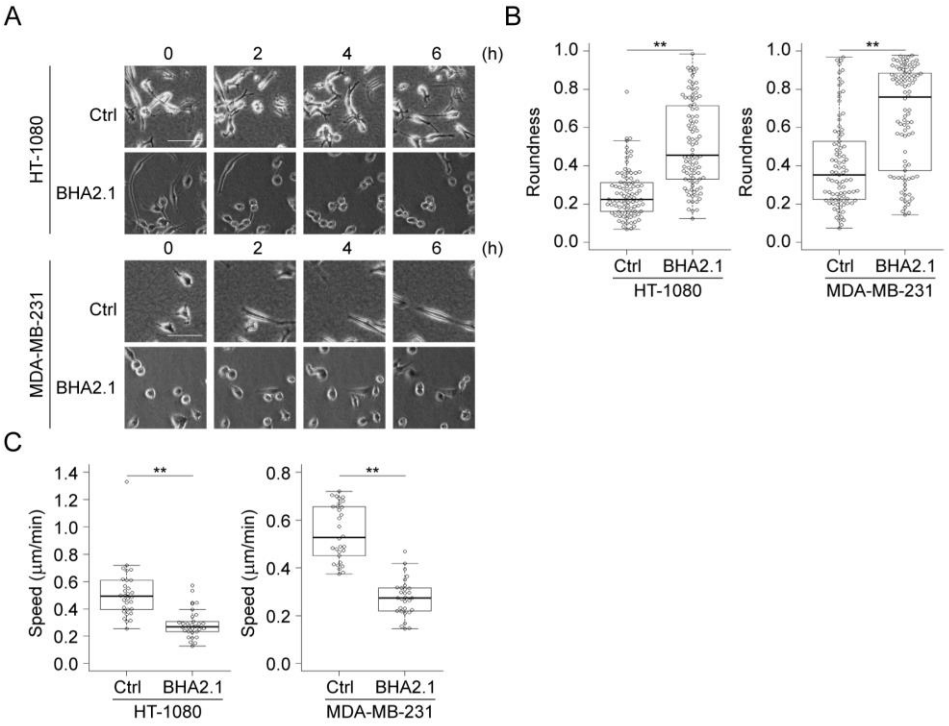

Figure S1. A. Nukuda, *et al*.