Alpha-smooth muscle actin (ACTA2) is required for metastatic potential of human lung adenocarcinoma

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Abstract

Purpose: Metastatic relapse of primary lung cancer leads to therapeutic resistance and unfavorable clinical prognosis; therefore, identification of key molecules associated with metastatic conversion has significant clinical implications. We previously identified a link between early brain metastasis of lung adenocarcinoma (ADC) and amplification of the alpha-smooth muscle actin (ACTA2) gene. The aim of present study was to investigate the prognostic and functional significance of ACTA2 expression in cancer cells for the metastatic potential of lung ADCs. Experimental Design: ACTA2 expression was analyzed in tumor cells from 263 patients with primary lung ADCs by immunohistochemistry, and was correlated with clinicopathological parameters. The expression of ACTA2 in human lung ADC cells was modulated with shRNAs and siRNAs specifically targeting ACTA2. Results: The patients with lung ADCs with high ACTA2 expression in tumor cells showed significantly enhanced distant metastasis and unfavorable prognosis. ACTA2 downregulation remarkably impaired in vitro migration, invasion, clonogenicity, and transendothelial penetration of lung ADC cells without affecting proliferation. Consistent with the in vitro results, depletion of ACTA2 in human lung ADC PC14PE6 cells significantly reduced their metastatic potential without altering their tumorigenic potential. Expression of c-MET and FAK in lung ADC cells was also reduced by ACTA2-targeting siRNAs and shRNAs, and was accompanied by a loss of mesenchymal characteristics. Conclusions: These findings indicate that ACTA2 regulates c-MET and FAK expression in lung ADC cells, which positively and selectively influence metastatic potential. Therefore, ACTA2 could be a promising prognostic biomarker and/or therapeutic target for metastatic lung ADC.
Translational Relevance

The short latency from the initial diagnosis of lung adenocarcinoma (ADC), the most common subtype of non-small cell lung cancer, to metastatic relapse leads to a high mortality rate. This short latency implies that the cancer cells in a primary lung ADCs have already acquired a number of multi-organ metastatic competencies. In this study, using 263 pathologic samples, we determined that ACTA2 expression in lung ADC cells at the primary site is significantly associated with enhanced distant metastasis. This association was translationally interpreted and validated. ACTA2 regulates the expression of the epithelial-mesenchymal transition-associated signaling molecules, c-MET and FAK, and consequently, the in vitro and in vivo metastatic potential of lung ADC cells. These results have high translational and clinical impact because ACTA2 could be utilized for the development of predictive diagnostics and/or anti-metastatic agents for advanced lung ADC.
Introduction

Lung cancer is the most common cancer worldwide with the highest mortality rate (1). The identification of therapeutic targets in non-small cell lung cancer (NSCLC), such as epithelial growth factor receptor (EGFR)-activating mutations, has enabled the development of effective targeted therapies for advanced NSCLC (2). However, the 5-year survival rate for NSCLC remains 15% across all stages of the disease. These unfavorable clinical outcomes originate from its high invasive and metastatic potential (3, 4). In particular, lung adenocarcinoma (ADC) is known to establish distant macrometastases in various organs, within months of diagnosis (5). Current targeted therapeutics have limited efficacy for the treatment of distant metastases in lung ADC.

The short latency of metastatic relapse in lung ADC implies that the cancer cells in the primary tumor have already acquired numerous multi-organ metastatic competencies, including cell motility, invasiveness, resistance to hypoxia, enhanced angiogenesis, survival after detachment, and evasion of immune surveillance (6, 7). Therefore, it might be possible to identify predictive biomarkers of metastasis and novel therapeutic targets in primary ADCs with high metastatic potential. These novel therapeutic targets could then be used to overcome the resistance of NSCLC to currently available targeted treatments.

In our previous study, we compared the gene amplifications and deletions in lung ADCs with synchronous brain metastasis to those in lung ADCs with metachronous brain metastasis to elucidate the genomic alterations associated with early distant metastasis in lung ADC (8). Among several genomic alterations, amplification of alpha-smooth muscle actin ($\alpha$-SMA; hereafter ACTA2) was significantly associated with synchronous brain metastasis. ACTA2 is known to contribute to cell-generated mechanical tension and maintenance of cell shape and movement. Since cell motility is critically dependent on the
actin cytoskeleton, the dynamics of cytoskeletal structures affected by ACTA2 could be essential to invasion and metastasis in lung ADC (9, 10).

Herein, we report that high ACTA2 expression in tumor cells in primary lung ADC is closely associated with progression of lung ADC, and provide the first evidence of its novel roles in the metastatic potential of lung ADC. To our knowledge, this is the first report suggesting that ACTA2 is a promising prognostic biomarker and/or therapeutic target for advanced lung ADC.
Materials and Methods

Study population

Surgical samples were obtained from 263 patients with lung ADC who underwent surgical resection at the Samsung Medical Center (SMC, Seoul, Korea) between November 1994 and August 2004 (11, 12). Written informed consent for the use of paraffin-embedded tissues, as approved by the Institutional Review Board at SMC, was obtained from each patient before surgery. Postoperative follow-up was scheduled at 1 and 2 months, every 3 months during the first 2 years after surgery, and then every 6 months thereafter, or more frequently if needed. The patients consisted of 140 males (53%) and 123 females (47%), 36~80 years of age. The mean age at surgical resection was 58.9 years. Of the 263 samples, 71, 134, 41, and 7 were stage I, II, III, and IV, respectively.

Immunohistochemistry and immunofluorescence staining

Tissue microarray sections generated from the above-mentioned surgical samples were immunostained with mouse monoclonal antibodies against human α-SMA (N1584; Dako, clone 1A4 or MA5-15806; Thermo Scientific Pierce, clone 4F4). Briefly, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide. The antigen was retrieved by heating sections in 10 mM sodium citrate (pH 6.0) at 95°C for 30 minutes (mins). The primary antibodies, biotinylated secondary antibody (Vector Laboratories), and then avidin-biotin complex (Vector Laboratories) were incubated with the sections at 4°C overnight, for 1 hour (h) at room temperature (RT), and for 1 h at RT, respectively. ACTA2 immunoreactivity was categorized as low (<50% ACTA2-positive tumor cells, ACTA2-Low)
or high grade (>50% ACTA2-positive tumor cells, ACTA2-High) in areas with maximal staining.

To confirm the expression of ACTA2 in lung ADC tumor cells, immunofluorescence was performed with the mouse monoclonal anti-ACTA2 (Dako, 1:250) and rabbit polyclonal anti-thyroid transcription factor 1 (TTF-1) (abcam, 1:200) antibody against antigen-retrieved lung ADC paraffin sections. TTF-1 has been reported to be one of several markers differentiating ADC from squamous cell carcinoma of the lung (13). The antibodies were visualized by Alexa Fluor® 488-labeled goat anti-mouse IgG and Alexa Fluor® 594-labeled goat anti-rabbit IgG antibody (Life Technologies), respectively. Samples were then incubated with DAPI (1:1,000) for 5 mins at RT to reveal cell nuclei.

**Cell culture, transfection, and generation of stable cell lines**

PC14PE6 cells were established from a pleural effusion developed in a nude mouse injected intravenously with parental human lung ADC PC14 cells (Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, TX). The karyotypic analysis of the PC14PE6 cells ruled out contamination with murine cells. PC14PE6 cells were maintained at 37°C and 5% CO₂ in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Gibco). H322, H1299, H460, and A549 human NSCLC cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and all experiments were performed within 6 months of purchase. Authentication of these cell lines was performed by Short Tandem Repeat (STR) profiling to exclude cross-contamination between the cell lines. H322, H1299, H460, and A549 cells were grown in RPMI (Gibco) supplemented with 10% FBS and penicillin/streptomycin (Gibco). For transfection, human NSCLC cells were plated at a density of 5×10⁵ cells/dish and transfected with the indicated
siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

SiRNAs directed against ACTA2, Focal adhesion kinase (FAK), and c-MET were purchased from Santa Cruz Biotechnology. Control siRNA (siCTRL) was synthesized by Genolution.

For stable cell lines, cells were transfected with an ACTA2-specific shRNA (shACTA2, Sigma) or empty pLKO vector (shCTRL) using Lipofectamine 2000, and then selected by culturing in puromycin (5 μg/mL).

**Western blot analysis and quantitative real-time PCR (RT-qPCR)**

For western blot analysis, cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors (Roche). Equal amounts of protein were subjected to SDS-polyacrylamide gels electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking in 5% bovine serum albumin (BSA), membranes were incubated with the indicated primary antibodies overnight, and then with the appropriate secondary antibodies. For RT-qPCR, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and then used to synthesize cDNA with Superscript III Reverse Transcriptase (Invitrogen). Expression of mRNA was quantified by RT-qPCR (ABI Prism 7600) using power SYBR® Green PCR Master Mix (Applied Biosystems). The primers used in this study were summarized in supplementary table S1.

**Detection of metastatic activity in vitro**

**Migration and invasion assay**
Migratory activity was measured by the wound closure assay. Briefly, a confluent cell surface was scratched with a pipette tip and migration distance was measured by wound closure. For the invasion assay, Matrigel coated transwells (BD) were utilized. Equal numbers of cells in serum free media were added to the upper chamber. The addition of complete media containing 10% FBS to the lower compartment stimulated the cells to invade. After 24 h incubation, cells were fixed with 100% methanol and stained with H&E. The number of invading cells on the lower surface of the membrane was counted.

Transendothelial migration assay

Human umbilical vein endothelial cells (HUVECs) were seeded (at 1×10^5 cells/well) in fibronectin-coated 24-well transwell inserts with a pore size of 8 μm (Corning Costar Corp.), and grown to confluence. Calcein AM-labeled cancer cells (1×10^5 cells) were suspended in serum free medium and added to the endothelial monolayer. After 24 h incubation, cells remaining in the upper chamber were completely removed and the tumor cells that migrated through the endothelial monolayer to the lower face of the filter were fixed with 4% paraformaldehyde (PFA). The transmigrated tumor cells were counted in 10 random fields at 100× magnification.

Focus forming assay

Cells were seeded (at 300 cells/well) in 6-well plates and maintained in complete medium for 3 weeks. The number of viable colonies per well was counted after staining with 0.2% crystal violet.
**In vivo tumorigenesis and metastasis assay**

All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources published by the National Institutes of Health and according to the Animal Experiment Guidelines of Samsung Biomedical Research Institute.

**Tumorigenicity assay**

The effect of ACTA2 silencing on tumorigenic potential of lung ADC cells was analyzed in two different *in vivo* models. For subcutaneous model, 1x10⁶ cancer cells in 100 μL Hank’s balanced salt solution (HBSS) were subcutaneously injected into the posterior flank of 6~8 week-old female BALB/c nude mice ( Orient, 7 mice per group). The volume of subcutaneous tumor (0.5 × length × width², mm³) was measured on 14, 17, 21, 24, and 28 days after the implantation. At 28 days post-implantation subcutaneous tumors were excised, photographed, and weighted. For orthotopic model, female 6~8 week-old female BALB/c nude mice were anesthetized in the right lateral decubitus position. A 3 mm incision was made at the lateral dorsal axillary line, 1.5 cm above the lower rib line, just below the inferior border of the scapula. Cancer cells (1x10⁵) in 40 μL HBSS were injected into the left lung with a 1 mL insulin syringe and a 30-gauge needle, and then the incision was repaired. The body weights of the animals were measured every day, and an autopsy was performed immediately after a 25% weight loss. Lungs were fixed in formalin, embedded in paraffin, and then stained with H&E.

**Metastasis assay**
For experimental metastasis \textit{in vivo} model, \(2 \times 10^5\) cancer cells in 100 \(\mu\)L of HBSS were directly injected into the left ventricle of the heart of 6~8 week-old female BALB/c nude mice with a 1mL insulin syringe. Four weeks later, the animals were sacrificed. Brains, lungs, lower leg bones, adrenal glands, and abnormal organs were harvested, fixed in formalin, and embedded in paraffin. Paraffin-embedded tissues were stained with H&E.

\textbf{Statistical analysis}

Overall survival and metastasis-free survival were calculated by the Kaplan-Meier method and compared by the log-rank test. Group comparisons were analyzed with Student's two-tailed t-test. Tumor metastasis rates were compared by Fisher's exact test, two-tailed. Differences with \(P\) values less than 0.05 were considered statistically significant.
Results

Clinical implications of ACTA2 in lung ADC

To confirm the role of ACTA2 in the metastasis of lung ADC, we assayed ACTA2 expression in 263 lung ADCs by immunohistochemistry (Fig. 1A). When more than 50% of tumor cells expressed ACTA2, the patients were considered as ACTA2-High (n=14). Specific detection of ACTA2 protein was verified by same staining patterns of two different antibodies against human ACTA2 (Supplementary Fig. S1). Moreover, ACTA2 protein in lung ADC cells was confirmed by co-localization of ACTA2 with a lung ADC specific marker, TTF-1 (Fig. 1B). The stromal ACTA2 expression in immunohistochemistry (Fig. 1A) and the presence of ACTA2-positive, TTF-1-negative cells in immunofluorescence staining (Fig. 1B) suggested concomitant ACTA2 expression in tumor stromal cells (14). ACTA2 staining was also observed in close proximity to the nucleus and/or nuclear domain in TTF-1 positive cancer cells in agreement with previous report (15), although its functional roles need to be further elucidated. Kaplan–Meier survival analysis was conducted to determine the prognostic significance of ACTA2 expression in patients with lung ADCs. ACTA2-High patients had significantly worse overall survival [OS; median (95% CI): 28.0 (6.0-50.0) months, \( P=0.011 \); Fig. 1C] and metastasis-free survival [MFS; 16.0 (7.6-24.4) months, \( P=0.022 \), Fig. 1D], compared to those of ACTA2-Low patients [n=249; OS=71.0 (55.8-86.2) months; MFS=53.0 (37.4-68.6) months]. These results indicate that not only ACTA2 gene amplification (8), but also ACTA2 overexpression in lung ADC cells are significantly associated with enhanced metastatic potential and worse clinical prognosis of lung ADC.

In vitro effects of shRNA-mediated downregulation of ACTA2
We generated 7 PC14PE6 lung ADC cell subclones that stably express 6 different ACTA2 shRNAs or a control vector (pLKO) to assess the effect of ACTA2 silencing on the metastatic potential of lung ADC cells. Downregulation of ACTA2 mRNA and protein was confirmed by RT-qPCR (Fig. 2A and Supplementary Fig. S2) and western blot analysis (Fig. 2B), respectively. When ACTA2 expression was downregulated by shRNA, the migration (Fig. 2C and Supplementary Fig. S2) and invasion (Fig. 2D and Supplementary Fig. S2) of PC14PE6 cells were significantly inhibited. Since PC14PE6 cells transfected with shACTA2#6 showed the lowest expression of ACTA2 mRNA and strong suppression of migration and invasion, PC14PE6/shACTA2#6 cells (hereafter, PC14PE6/shACTA2) were used for all subsequent experiments. The inhibitory effects of ACTA2 silencing on the in vitro migration potential of lung ADC cells were confirmed using various lung ADC cell lines and a specific siRNA targeting ACTA2 (Supplementary Fig. S3).

ACTA2 downregulation also significantly decreased transendothelial migration (TEM, Fig. 3A), and clonogenicity (Fig. 3B) of PC14PE6 cells, properties that are required for distant metastasis of lung cancer. In contrast, proliferation (Supplementary Fig. S4A), anoikis-resistance (Supplementary Fig. S4B), and adhesion to fibronectin [an extracellular matrix (ECM) component] (Supplementary Fig. S4C) of PC14PE6 cells were not affected by the shRNA-mediated ACTA2 downregulation. Therefore, the inhibitory effects of ACTA2 silencing on the migration and invasion of PC14PE6 cells did not result from decreased proliferation and/or cellular growth.

Specific implications of ACTA2 in the metastasis of lung ADC in vivo

Metastasis is a sequential, multi-step process that includes invasion, intravasation, survival in circulation, extravasation, and colonization. In vitro downregulation of ACTA2
significantly affected those processes, which indicated that ACTA2 could be required for distant metastasis of lung ADC. To investigate whether ACTA2 has the effect on primary lung tumorigenesis in vivo, $1 \times 10^6$ control (PC14PE6/shCTRL) or ACTA2 knockdown (PC14PE6/shACTA2) cells were implanted into the subcutaneous tissue (PC14PE6/shCTRL, n=7; PC14PE6/shACTA2, n=7) or left lung (PC14PE6/shCTRL, n=7; PC14PE6/shACTA2, n=6) of nude mice. In the subcutaneous model, both tumor growth rate ($P=0.43$) and final tumor weight ($P=0.49$) were not significantly affected by ACTA2 downregulation (Fig. 4A). ACTA2 downregulation also made no effects on the tumor formation in the lung cancer orthotopic model, since the survival of mice with intralung injection of PC14PE6/shACTA2 cells (median survival length=32 days) was comparable to that of the control group (median survival length=25 days) (Fig. 4B, $P=0.318$). These results are consistent with that of the in vitro cell proliferation assay, which firmly validates no significant role of ACTA2 in lung tumorigenesis.

To further examine the in vivo biological role of ACTA2 in the metastatic potential of lung ADC cells, we injected $1 \times 10^6$ PC14PE6/shCTRL or PC14PE6/shACTA2 cells into the left ventricle [systemic metastasis animal model; PC14PE6/shCTRL (n=9), PC14PE6/shACTA2 (n=7)] of nude mice. In contrast to the tumorigenicity assays, significantly less metastatic potential was observed in mice that received an intracardiac injection of PC14PE6/shACTA2 cells (Fig. 4C). These mice had a sole metastatic tumor in the adrenal gland (14% incidence, 1/7 mice), whereas mice that received an intracardiac injection of PC14PE6/shCTRL cells had a 78% incidence (7/9 mice) of metastatic tumors, and the metastatic sites varied [lung, adrenal gland, bone, brain, and eyeball] (Fig. 4C). Each metastatic tumor was confirmed pathologically (Fig. 4D). These in vivo assays indicated that ACTA2 promotes lung ADC metastasis without affecting primary tumor formation potential, which would be mediated by increased migration, invasion, and clonogenicity of lung ADC cells.
Effects of ACTA2 on FAK and c-MET expression

From the above results, we determined that ACTA2 is a critical factor for the acquisition of metastatic potential in lung ADC cells. To address the mechanism whereby ACTA2 regulates metastatic potential, we analyzed the expression of EMT-associated proteins, such as FAK and c-MET, since EMT is a critical process for acquisition of metastatic potential. PC14PE6 cells were transfected with an ACTA2-specific siRNA. Forty eight hours after the transfection, mRNA and protein of both FAK and c-MET were significantly decreased (Fig. 5A). ACTA2 silencing also significantly increased E-cadherin expression and decreased vimentin expression (Fig. 5B). These results indicated that the expression of FAK and c-MET, which are major signaling molecules for metastasis, is suppressed by ACTA2 downregulation, and that ACTA2 expression is required for maintenance of the mesenchymal characteristics of PC14PE6 cells. To ensure that ACTA2 is associated with FAK and c-MET expression, we checked the downregulation effects again with several shRNAs specifically targeting ACTA2. As shown in Supplemental Fig. S5, shRNA-elicited knockdown of ACTA2 also reduced the expression of FAK and c-MET.

To determine if FAK and c-MET are required for ACTA2 silencing-mediated suppression of migration and invasion, PC14PE6 cells were transfected with FAK and c-MET-specific siRNAs (Supplementary Fig. S5). Similarly to ACTA2 silencing, silencing of either FAK and c-MET inhibited migration (Fig. 5C) and invasion (Fig. 5D) of PC14PE6 cells. In accordance with data obtained from PC14PE6, ACTA2 silencing also inhibited migratory and invasive activity of lung ADC H322 cells through downregulation of FAK and c-MET (Supplemental Fig. S6 and S7). These results suggest that decreased expression of FAK and c-MET is involved in ACTA2 downregulation-mediated suppression of metastatic potential.
Discussion

Distant metastasis decreases the survival of patients with NSCLC. While targeted molecular therapies have greatly improved the management of primary NSCLC, these therapies are ineffective for the treatment of distant metastases. Because systemic metastasis is induced by additional metastasis-specific genetic alterations, identifying key molecules that contribute to lung cancer cell dissemination is essential for the development of new predictive biomarkers and/or anti-metastatic therapeutic strategies. In this study, we demonstrated that ACTA2 plays a critical role in lung ADC metastasis. Clinically, high expression of ACTA2 in lung ADC cells was significantly associated with worse prognostic outcome and early distant metastasis of lung ADC patients. Experimentally, ACTA2 expression was closely correlated with the metastatic potential of human lung ADC cell line, PC14PE6 both in vitro and in vivo. Moreover, our results suggested that the decreased metastatic potential induced by ACTA2 silencing is mediated by downregulation of FAK and c-MET expression.

Although ACTA2 is generally expressed in the smooth muscle cells and activated cancer-associated fibroblasts (CAFs), tumor cells could also use actin bundles to allow them to break away from a primary tumor and invade the surrounding tissue (9, 10). In many epithelial cancers, transforming growth factor (TGF)-β-elicited EMT induces the expression of ACTA2 (16), increases tumor invasion, and worsens patient survival (17). The connection between EMT and epithelial stemness also suggests that EMT can confer clonogenicity and resistance to apoptosis on primary tumor cells (18). Finally, the presence of ACTA2 in the nuclear proximity of some cells indicated that ACTA2 relate the change of mechanical force to an altered gene expression via nuclear transcription regulators (15, 19). Therefore, ACTA2 expression and/or amplification of the ACTA2 gene in lung ADC cells could be used
to predict clinical aggressiveness of lung ADC, which were demonstrated in this report and our previous study (8), respectively.

However, the possibility that ACTA2 expressing CAFs could synergistically contribute the progression and metastasis of lung ADCs with adjacent ACTA2 positive cancer cells could not be ruled out. In recent studies, lung CAFs has a significant role in the metastatic potential of NSCLC via the direct regulation of gene expression at the invasive front of cancer nests and induction of EMT (20, 21). Although the present study and previous reports highlight the importance of ACTA2 expression of both cancer cells and CAFs in NSCLC progression and metastasis, further studies are needed to elucidate detail mechanisms how ACTA2 contributes to tumor promoting effects in CAFs and lung ADC cells.

Inhibition of ACTA2 expression in lung ADC PC14PE6 and H322 cells downregulated the transcription of c-MET and FAK, which decreased the in vitro migration and invasion of PC14PE6 and H322 cells. Hepatocyte growth factor (HGF)/c-MET signaling activates a number of downstream pathways, including Mitogen-activated protein kinase (MAPK), Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (PKB/Akt), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), in epithelial cancer cells (17), and induces proliferation, motility, cell survival, and EMT. Recent studies demonstrated that HGF is an indicator of poor prognosis in lung ADC (22). FAK is activated by integrins that connect the cytoskeleton to the extracellular matrix and act as nucleation sites for the assembly of cell adhesions (23, 24). In addition, FAK plays a critical role in TGF-β1-induced EMT. Its overexpression was significantly associated with positive lymph node metastasis and worse overall survival of patients with lung ADC (25). Because ACTA2 not only mediates alterations in the mechanical properties of cancer cells, but also regulates the expression of signaling proteins that significantly influence the clinical outcome of lung ADC, this protein could be used as a therapeutic target for lung ADCs.
Maintenance of stemness is critical for lung ADC cells to make distant metastases since establishment of new colonies in unfamiliar microenvironments is the final step of sequential metastatic processes. According to elaborate analysis of genomic features of primary and metastatic tumors, metastasis is a result from clonal selection of heterogeneous cancer cells in the primary tumor (26, 27). Therefore, clonogenicity is one of key phenotypes of stemness, which is required for distant metastasis. Our results indicate that ACTA2 is involved in the clonogenicity of lung ADC PC14PE6 cells. Moreover EMT, a transdifferentiating process, was also affected by ACTA2 expression, in this study. One study postulated that EMT and stem cell properties are combined in invasive cancer cells—which often coexpress EMT and stem cell markers (28). Moreover, EMTs additionally equip more differentiated epithelial cells with stem-cell traits through molecular linking of EMT-inducing transcription factors to self-renewal programs (29, 30). This connection between EMT and epithelial stemness indicates that by imparting mesenchymal traits to carcinoma cells, an EMT can confer motility, invasiveness, are resistance to apoptosis and metastatic dissemination from primary tumors (18). Accordingly, stemness of lung ADC cells might be regulated by ACTA2, which could in turn influence the metastatic potential of lung ADC cells. However, the detailed mechanism of how ACTA2 mediates this regulation remains to be elucidated further.

In this study, we demonstrated for the first time that ACTA2 regulates FAK and c-MET expression in lung ADC cells, which positively influences in vitro and in vivo metastatic potential. Since ACTA2 expression in lung ADC cells is significantly associated with poor clinical outcome and early distant metastasis, ACTA2 could be utilized as a possible therapeutic target and a prognostic biomarker for metastasis.
References


Figure Legends

Figure 1. ACTA2 expression is closely associated with early metastasis and poor prognosis of lung adenocarcinoma (ADC). A, Representative results of immunohistochemical staining against ACTA2 in 263 lung ADCs (top, low-expression, ACTA2-Low; bottom, high-expression, ACTA2-High). B, Colocalization of ACTA2 (green) with lung ADC specific marker, Thyroid transcription factor 1 (TTF-1, red) in lung ADC cells. ACTA2+/TTF-1+ lung ADC cells were indicated by arrows. C and D, Kaplan-Meier analysis of overall survival (OS) and metastasis free survival (MFS) of 263 patients with lung ADC. Immunohistochemical staining of ACTA2 was performed using Tissue Microarray (TMA) sections from 263 patients. Lung ADCs were categorized by the frequency of ACTA2-stained cancer cells (>50% tumor cells = ACTA2-High). P values were determined by the log-rank test.

Figure 2. Short hairpin (sh) RNA-mediated ACTA2 knockdown inhibited the migratory and invasive activity of PC14PE6 cells. A and B, Knockdown of ACTA2 mRNA and protein was verified by RT-qPCR (A) and western blot (B), respectively. C and D, The effect of ACTA2 silencing on in vitro migration and invasion was assessed by the wound closure assay (C) and Matrigel coated transwell assay (D), respectively. Migration distance was measured 24 hours (h) after wound formation on a confluent cell surface and invaded cells were counted under a microscope in more than 6 fields for each group. Data shown are the mean plus the standard deviation.*, $P<0.05$. 
Figure 3. Effects of shRNA mediated ACTA2 knockdown on transendothelial migration and clonogenicity of PC14PE6 cells. A, For transendothelial migration, cells were added to a endothelial monolayer. The transmigrated cells were counted under a fluorescent microscope in more than 10 fields for each group. B, To assess clonogenicity, cells were seeded in 6-well plates and cultured in complete media for 3 weeks. Colonies were stained with 0.2% crystal violet and counted. Data shown are the mean plus the standard deviation.*, $P<0.05$.

Figure 4. Decreased expression of ACTA2 specifically suppressed systemic metastasis of PC14PE6 cells in vivo. A, Downregulation of ACTA2 had no effect on tumorigenic potential of lung ADC in subcutaneous model. $1\times10^6$ PC14PE6 control (PC14PE6/shCTRL) or ACTA2 knockdown (PC14PE6/shACTA2) cells were implanted into the subcutaneous tissue of nude mice ($n=7$ per group). The volume of xenograft tumors and relative tumor weight at 28 days post-implantation are demonstrated. Data = mean±standard error of means (SEM). Representative photographs of the excised tumors at 28 days post-implantation. B, The effect of ACTA2 on primary tumor growth was evaluated in an orthotopic lung cancer animal model by an intra-lung injection of $1\times10^6$ of PC14PE6 control (PC14PE6/shCTRL) ($n=7$) or ACTA2-knockdown (PC14PE6/shACTA2) cells ($n=6$). C, The in vivo effects of ACTA2 on the metastatic potential of lung ADC PC14PE6 cells were evaluated by injection of $2\times10^5$ PC14PE6/shCTRL ($n=9$) or PC14PE6/shACTA2 cells ($n=7$) into the left ventricle of female BALB/c nude mice. The incidence of systemic metastases to each organ was compared.*, $P<0.05$. D, Representative illustrations of metastatic foci in each organ.
Figure 5. Silencing of ACTA2 suppressed c-MET and FAK expression. A, PC14PE6 cells were transfected with control (siCTRL) or ACTA2 siRNA (siACTA2). At 48 hours after transfection, protein and mRNA levels of ACTA2, c-MET, and FAK were determined by western blot (left) and RT-qPCR (right), respectively. GAPDH=internal control. B, The protein and mRNA levels of the EMT makers, E-cadherin and vimentin, were determined by western blot (left) and RT-qPCR (right), respectively. C and D, PC14PE6 cells were transfected with c-MET or FAK siRNA. At 48 hours after transfection, in vitro migration (C) and invasion (D) were determined by wound closure assay and Matrigel transwell assay, respectively. The data shown are the mean plus standard deviation from 3 separate experiments. *, P<0.05.
Figure 3

A

B

shCTRL | shACTA2

shCTRL | shACTA2

TEM activity

Clonogenicity
Figure 5

A

ACTA2
c-MET
FAK
GAPDH

siCTRL siACTA2 c-MET FAK

B

ACTA2
E-cadherin
Vimentin
GAPDH

siCTRL siACTA2

0h

siRNA CTRL ACTA2 FAK c-MET

24h

siRNA CTRL ACTA2 FAK c-MET

Migration distance

CTRL ACTA2 FAK c-MET

Invasion

CTRL ACTA2 FAK c-MET

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