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 and amplification of the α-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 adenocarcinomas.

**Experimental Design:** ACTA2 expression was analyzed in tumor cells from 263 patients with primary lung adenocarcinomas by immunohistochemistry, and was correlated with clinicopathologic parameters. The expression of ACTA2 in human lung adenocarcinoma cells was modulated with short hairpin RNAs (shRNA) and siRNAs specifically targeting ACTA2.

**Results:** The patients with lung adenocarcinomas 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 adenocarcinoma cells without affecting proliferation. Consistent with the *in vitro* results, depletion of ACTA2 in human lung adenocarcinoma PC14PE6 cells significantly reduced their metastatic potential without altering their tumorigenic potential. Expression of c-MET and FAK in lung adenocarcinoma 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 adenocarcinoma cells, which positively and selectively influence metastatic potential. Therefore, ACTA2 could be a promising prognostic biomarker and/or therapeutic target for metastatic lung adenocarcinoma. *Clin Cancer Res; 19*(21); 5879–89. ©2013 AACR.

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 EGFR 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 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 adenocarcinoma.

The short latency of metastatic relapse in lung adenocarcinoma 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).
Translational Relevance
The short latency from the initial diagnosis of lung adenocarcinoma, 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 adenocarcinomas have already acquired a number of multi-organ metastatic competencies. In this study, using 263 pathologic samples, we determined that ACTA2 expression in lung adenocarcinoma 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 adenocarcinoma cells. These results have high translational and clinical impact because ACTA2 could be used for the development of predictive diagnostics and/or antimetastatic agents for advanced lung adenocarcinoma.

Therefore, it might be possible to identify predictive biomarkers of metastasis and novel therapeutic targets in primary adenocarcinomas 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 adenocarcinomas with synchronous brain metastasis to those in lung adenocarcinomas with metachronous brain metastasis to elucidate the genomic alterations associated with early distant metastasis in lung adenocarcinoma (8). Among several genomic alterations, amplification of α-smooth muscle actin (α-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. As 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 adenocarcinoma (9, 10).

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

Materials and Methods

Study population
Surgical samples were obtained from 263 patients with lung adenocarcinoma 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 mmol/L sodium citrate (pH 6.0) at 95°C for 30 minutes. 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 at room temperature, and for 1 hour at room temperature, 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 adenocarcinoma tumor cells, immunofluorescence was conducted 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 adenocarcinoma paraffin sections. TTF-1 has been reported to be one of several markers differentiating adenocarcinoma 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 minutes at room temperature 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 adenocarcinoma 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 Dulbecco’s modified Eagle medium (HyClone) supplemented with 10% FBS and 1% antibiotic–antimycotic solution (GIBCO). H322, H1299, H460, and A549 human NSCLC cell lines were purchased from American Type Culture Collection and all experiments were conducted within 6 months of purchase. Authentication...
of these cell lines was conducted 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 × 10^5 cells per 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

For Western blot analysis, cells were lysed with radio-immunoprecipitation buffer containing protease inhibitors and phosphatase inhibitors (Roche). Equal amounts of protein were subjected to SDS-PAGE and transferred to polyvinylidene difluoride 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 quantitative real-time PCR (qRT-PCR), 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 qRT-PCR (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 used. Equal numbers of cells in serum-free media were added to the top chamber. The addition of complete media containing 10% FBS to the bottom compartment stimulated the cells to invade. After 24-hour incubation, cells were fixed with 100% methanol and stained with hematoxylin and eosin (H&E). The number of invading cells on the bottom surface of the membrane was counted.

Transendothelial migration assay. Human umbilical vein endothelial cells (HUVEC) 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-hour incubation, cells remaining in the top chamber were completely removed and the tumor cells that migrated through the endothelial monolayer to the bottom 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 NIH and according to the Animal Experiment Guidelines of Samsung Biomedical Research Institute.

Tumorigenicity assay. The effect of ACTA2 silencing on tumorigenic potential of lung adenocarcinoma cells was analyzed in two different in vivo models. For subcutaneous model, 1 × 10^6 cancer cells in 100 μL Hank balanced salt solution (HBSS) were subcutaneously injected into the posterior flank of 6 to 8-week-old female BALB/c nude mice (Orient, 7 mice/group). The volume of subcutaneous tumor (0.5 × length × width^2, mm^3) was measured on 14, 17, 21, 24, and 28 days after the implantation. At 28 days postimplantation, subcutaneous tumors were excised, photographed, and weighted. For orthotopic model, female 6 to 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 (1 × 10^6) 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 conducted 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 in vivo model, 2 × 10^5 cancer cells in 100 μL of HBSS were directly injected into the left ventricle of the heart of 6 to 8-week-old female BALB/c nude mice with a 1 mL 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.

Statistical analysis

Overall survival and metastasis-free survival were calculated by the Kaplan–Meier method and compared with the log-rank test. Group comparisons were analyzed with Student two-tailed t test. Tumor metastasis rates were compared by Fisher exact test, two-tailed. Differences with P values less than 0.05 were considered statistically significant.

Results

Clinical implications of ACTA2 in lung adenocarcinoma

To confirm the role of ACTA2 in the metastasis of lung adenocarcinoma, we assayed ACTA2 expression in 263 lung adenocarcinomas 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 adenocarcinoma cells was confirmed by colocalization of ACTA2 with a lung adenocarcinoma-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 TFF-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 adenocarcinomas. 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 with 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 adenocarcinoma cells are significantly associated with enhanced metastatic potential and worse clinical prognosis of lung adenocarcinoma.

**In vitro effects of shRNA-mediated downregulation of ACTA2**

We generated 7 PC14PE6 lung adenocarcinoma cell subclones that stably express different ACTA2 shRNAs or a control vector (pLKO) to assess the effect of ACTA2 silencing on the metastatic potential of lung adenocarcinoma cells. Downregulation of ACTA2 mRNA and protein was confirmed by qRT-PCR (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. As 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 adenocarcinoma cells were confirmed using various lung adenocarcinoma cell lines and a specific siRNA targetting ACTA2 (Supplementary Fig. S3).

ACTA2 downregulation also significantly decreased trans-endothelial migration (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 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 adenocarcinoma in vivo**

Metastasis is a sequential, multi-step process that includes invasion, intravasation, survival in circulation, extravasation, and colonization. In **in vitro** downregulation of A-CTA2 significantly affected those processes, which indicated that ACTA2 could be required for distant metastasis of lung adenocarcinoma. 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 down-regulation (Fig. 4A). ACTA2 downregulation also made no effects on the tumor formation in the lung cancer orthotopic model, as the survival of mice with intralung injection of PC14PE6/shACTA2 cells (median survival length = 33 days) was comparable with 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** biologic role of ACTA2 in the metastatic potential of lung adenocarcinoma 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 with 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

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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 adenocarcinomas (top, low-expression, ACTA2-Low; bottom, high-expression, ACTA2-High). B, colocalization of ACTA2 (green) with a lung adenocarcinoma-specific marker, Thyroid transcription factor 1 (TTF-1, red) in lung adenocarcinoma cells. ACTA2+/TTF-1+ lung adenocarcinoma 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 adenocarcinoma. Immunohistochemical staining of ACTA2 was conducted using tissue microarray (TMA) sections from 263 patients. Lung adenocarcinomas were categorized by the frequency of ACTA2-stained cancer cells (>50% tumor cells = ACTA2-High). P values were determined by the log-rank test.
adenocarcinoma metastasis without affecting primary tumor formation potential, which would be mediated by increased migration, invasion, and clonogenicity of lung adenocarcinoma 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 adenocarcinoma cells. To address the mechanism whereby ACTA2 regulates metastatic potential, we analyzed the expression of EMT-associated proteins, such as FAK and c-MET, as 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 down-regulation, 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 Supplementary Fig. S5, shRNA-elicited knockdown of ACTA2 also reduced the expression of FAK and c-MET.
To determine whether 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 adenocarcinoma H322 cells through downregulation of FAK and c-MET (Supplementary Figs. S6 and S7). These results suggest that decreased expression of FAK and c-MET is involved in ACTA2 down-regulation-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 antimetastatic therapeutic strategies. In this study, we showed that ACTA2 plays a critical role in lung adenocarcinoma metastasis. Clinically, high expression of ACTA2 in lung adenocarcinoma cells was significantly associated with worse prognostic outcome and early distant metastasis of patients with lung adenocarcinoma. Experimentally, ACTA2 expression was closely correlated with the metastatic potential of human lung adenocarcinoma 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 (CAF), 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, 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 adenocarcinoma cells could be used to predict clinical aggressiveness of lung adenocarcinoma, which were shown 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 adenocarcinomas with adjacent ACTA2-positive cancer cells could not be ruled out. In recent studies, lung CAFs have 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 adenocarcinoma cells.

Inhibition of ACTA2 expression in lung adenocarcinoma 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 NF-κB, in epithelial cancer cells (17), and induces proliferation, motility, cell survival, and EMT. Recent studies demonstrated that HGF is an

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 adenocarcinoma in subcutaneous model. A total of $1 \times 10^6$ PC14PE6 control (PC14PE6/shCTRL) or ACTA2 knockdown (PC14PE6/shACTA2) cells were implanted into the subcutaneous tissue of nude mice ($n=7$). The volume of xenograft tumors and relative tumor weight at 28 days postimplantation are shown. Data $\pm$ SEM. Representative photographs of the excised tumors at 28 days postimplantation. B, the effect of ACTA2 on primary tumor growth was evaluated in an orthotopic lung cancer animal model by an intra-lung injection of $1 \times 10^6$ 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 adenocarcinoma PC14PE6 cells were evaluated by injection of $2 \times 10^5$ 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.
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 adenocarcinoma (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 adenocarcinoma, this protein could be used as a therapeutic target for lung adenocarcinomas.

Maintenance of stemness is critical for lung adenocarcinoma 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

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**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 analysis (left) and qRT-PCR (right), respectively. GAPDH = internal control. B, the protein and mRNA levels of the EMT makers, E-cadherin and vimentin, were determined by Western blot analysis (left) and qRT-PCR (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 SD from 3 separate experiments. * \( P < 0.05 \).
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 adenocarcinoma 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 resistant to apoptosis and metastatic dissemination from primary tumors (18). Accordingly, stemness of lung adenocarcinoma cells might be regulated by ACTA2, which could in turn influence the metastatic potential of lung adenocarcinoma cells. However, the detailed mechanism of how ACTA2 mediates this regulation remains to be elucidated further.

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

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Development of methodology: H.W. Lee, M.-S. Kang
Writing, review, and/or revision of the manuscript: H.W. Lee, J.-I. Lee, H.H. Kim, K.M. Joo

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