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Growth inhibition of *KRAS*- and *EGFR*-mutant lung adenocarcinoma by cosuppression of STAT3 and the SRC/ARHGAP35 axis

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Abstract. The need for effective treatment of *KRAS*-mutant lung cancer is an emerging issue. Rho GTPase-activating protein 35 (ARHGAP35) is reported to be a possible molecular target for lung adenocarcinoma. We investigated the effect of long-term ARHGAP35 suppression on the proliferation, migration and molecular dynamics of lung adenocarcinomas harboring *KRAS* and *EGFR* gene mutations. Lung adenocarcinoma cell lines A549 (*KRAS*-mutant) and PC9 and H1975 (*EGFR*-mutants) were used, and *ARHGAP35* knockdown was carried out using puromycin. Cell viability, migration and molecular dynamics were assayed 1 month after introducing small hairpin RNA. The compensatory upregulated mechanism was screened by western blotting and confirmed by a specific inhibitor. Finally, we tested the effects of cosuppression of the SRC/ARHGAP35 axis and the identified pathway *in vitro*. ARHGAP35 suppression was attenuated by long-term knockdown of the target genes. Compensatory mechanisms by SRC and STAT3 caused attenuation in A549 cells. After long-term *ARHGAP35* knockdown, both A549 and PC9 cells were more sensitive to treatment with a STAT3 inhibitor. The suppressive effect of *ARHGAP35* knockdown on migration was sustained, but only modest, in all cell lines. Synergistic and strong growth inhibition was observed with concomitant use of an SRC inhibitor and a STAT3 inhibitor in A549 cells. STAT3 activation compensated for *ARHGAP35* knockdown in lung adenocarcinoma with the *KRAS* mutation. Moreover, cosuppression of the STAT3 pathway and SRC/ARHGAP35 axis may be an effective strategy for treating lung adenocarcinoma, especially in the presence of a *KRAS* mutation.

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Key words: lung cancer, signaling pathway, synthetic lethality, ARHGAP35, STAT3, SRC

Introduction

Lung cancer is the leading cause of mortality worldwide, being responsible for 1.59 million patient deaths every year. Although early diagnosis increases the potential for curative surgical resection, more than half of cases present with advanced disease and require treatment with chemotherapy and radiotherapy (1). A recent breakthrough, the discovery of driver mutations and the development of corresponding tyrosine kinase inhibitors (TKIs), has shown significant clinical benefits to date (2,3). However, targeting *KRAS*, the second major driver, is an emerging problem as its activation is different from the usual kinase-based signaling. In addition, overcoming an acquired resistance, including a mutation at exon 20 in *ERBB2*, (4,5) and activation of the HGF and MET signaling pathway, (6,7) has yet been a problem to resolve although the specific inhibitor targeting T790M in the *EGFR* gene (8,9) has been developed.

The concept of synthetic lethality leading to cell death in the presence of a combination of mutations in multiple genes was first advanced in 1945 (10). This was later highlighted in 2005 by reports indicating that breast cancer with the breast cancer susceptibility gene mutation (BRCA1, DNA repair associated) was very sensitive to inhibitors of poly(ADP-ribose) polymerase (PARP) (11,12). Thus, it seemed that simultaneous suppression of two major DNA repair genes could disrupt DNA replication in cancer cells. Since this discovery, other therapeutic approaches based on synthetic lethality have been sought in various malignancies known to have specific gene alterations (13). Notably, the *KRAS* mutation has been a major target for synthetic lethality, and the cosuppression of *MEK* with RAF1 or BCL-XL was identified as being effective at inducing synthetic lethality in cancer cells with this mutation (14,15). To establish effective clinical treatments for lung cancer with *KRAS* mutations, it is important to identify additional combinations that cause synthetic lethality.

Rho GTPase-activating protein 35 (ARHGAP35) is a RhoGAP protein (16,17) reported to be the principal substrate of SRC and controller of RhoA activity (through the binding of RhoA) (18,19) and also known with altered names such as p190RhoGAP, GRF-1 and GRLF1. To date, however, the role of this molecule in cancer has been uncertain, with both tumor

suppressor effects and oncogenic effects reported (20,21). In proteome studies, the phosphorylation status of tyrosine Y1105 in ARHGAP35 has consistently been reported to change, being dramatically suppressed by EGFR-TKI treatment for lung adenocarcinoma in the presence of the EGFR mutation (22,23). Therefore, we aimed to focus on ARHGAP35 as a possible key molecule in the proliferation and metastasis of lung adenocarcinoma. To this end, our group previously showed that ARHGAP35 messenger RNA (mRNA) was overexpressed in lung cancer cell lines compared to normal cells, and that positive protein expression was widely observed in lung cancer cells in surgically resected specimens immunohistochemically (24). It was also observed that *ARHGAP35* knockdown significantly suppressed the viability, migration and invasion of lung adenocarcinoma cells, including KRAS mutants (24).

In the present study, we investigated the effect of long-term ARHGAP35 suppression in lung cancer cells. Our aim was to identify a compensatory pathway that could be both a potential mechanism of acquired resistance for SRC/ARHGAP35 inhibition and a candidate for synthetic lethality when used in combination with inhibition of the SRC/ARHGAP35 axis.

Materials and methods

Cell lines and culture. A549 and H1975 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VI, USA). PC9 was obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). All cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37˚C. These cells are histologically adenocarcinomas, and A549 has the wild-type (WT) *EGFR* and *KRAS* mutations (codon 12), PC-9 has the *EGFR* mutation (exon 19 del), and H1975 has the *EGFR* mutation (L858R and T790M). A549 and H1975 cells have been reported to be EGFR-TKI resistant, whereas PC9 is EGFR-TKI sensitive. To test whether there was concomitant inhibition of STAT3 and SRC, cells were treated with combinations of different concentrations of a STAT3 inhibitor (S3I-201; Santa Cruz Biotechnology, Dallas, TX, USA) and SRC inhibitor (SKI-1; Abcam, Cambridge, UK). Drug concentrations of S3I-201 were adjusted to 0, 100, 200 and 400 μM and those for SKI-1 were adjusted to 0, 2.5, 5 and 10 μM.

RNA interference (RNAi). Lentiviral transfection with small hairpin RNA (shRNA) was conducted using MISSION® shRNA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were transfected with shRNA directed to ARHGAP35 or negative control shRNA using hexadimethrine bromide. Transfected cells were cultured with media containing puromycin and selected clones were maintained with puromycin over 1 month.

Western blot analysis. Cell lines were washed with ice-cold PBS and lysed in ice-cold lysis buffer (pH 8.0 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM $Na_4P_2O_7H_2O$, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol and 1% Triton X-100) which was added Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and PhosSTOP phosphatase Inhibitor Cocktail (Roche). The cell lysates were centrifuged at 15,000 x g for 20 min at 4˚C to collect the supernatant. We calculated protein concentrations by Bradford method using Bio-Rad Protein assay (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Protein samples (10 μg) were separated by SDS-PAGE Mini-PROTEAN TGX Gel (GE Healthcare Life Sciences) and transferred onto a polyvinylidene difluoride membrane (PVDF; Hybond-P®; GE Healthcare Life Sciences). The PVDF membranes with proteins were blocked with 5% non-fat dry milk (NFDM) for 1 h at room temperature. The primary antibodies dissolved in 5% bovine serum albumin (BSA) were used to detect the target protein blots at 4˚C overnight for incubation. Protein were incubated on the membranes with primary antibodies dissolved in 5% BSA (or 5% NFDM) at 4˚C overnight and secondary antibodies labeled with horseradish peroxidase dissolved in blocking buffer for 1 h at room temperature. The proteins were visualized on ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences), using the enhanced chemiluminescence western blot detection system. The primary antibodies used in the present study included: ARHGAP35 (1:1,000; cat. no. 2860), SRC (1:1,000; cat. no. 2109), AKT (1:1,000; cat. no. 4691), STAT3 (1:2,000; cat. no. 4904), MEK (1:1,000; cat. no. 9126), PRKCD (1:1,000, 5% NFDM; cat. no. 9616), PRKCZ (1:1,000; cat. no. 9368), p-SRC (1:1,000; cat. no. 2101), p-AKT (1:2,000; cat. no. 4060), p-STAT3 (1:2,000; cat. no. 9145), p-MEK (1:1,000; cat. no. 9121) and β-actin (1:1,000; cat. no. 4967) all from Cell Signaling Technology (Boston, MA, USA).

Quantitative assay for Rho kinase activity. Measurement of Rho kinase (ROCK) activity was performed using a ROCK Activity Immunoblot kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

RT-qPCR. Total RNA isolation from cell lines and complementary DNA synthesis was performed using TaqMan® Gene Expression Cells-to-Ct™ Kits (Thermo Fisher Scientific, Inc.). All mRNA was measured by qRT-PCR using an ABI PRISM 7000 Sequence Detection System (Thermo Fisher Scientific) with primers (Thermo Fisher Scientific; ARHGAP35: Hs00534180_m1, SRC: Hs01082246_m1, STAT3: Hs00374280_m1, 18S rRNA: Hs99999901_s1). SRC, STAT3 and 18S ribosomal RNA primer (Thermo Fisher Scientific). PCR reaction conditions were performed as follows: 95˚C for 10 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

Cell viability assay. Measurement of cell viability was performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were seeded onto a 96-well plate at a concentration of 3,000 cells/well and were incubated at 37˚C. At 72 h, the optical density was measured at 490 nm using a microtiter plate reader, and the rate of cell survival was expressed as the absorbance. To assess the effect of an inhibitor, cell viability was evaluated 48 h after treatment.

Cell migration assay. Cell migration was evaluated by scratch assay, as described in a previous report (25). Cells were seeded onto a 24-well plate at a concentration of 150,000 cells/well and were incubated at 37˚C. At 24 h, the cell monolayer

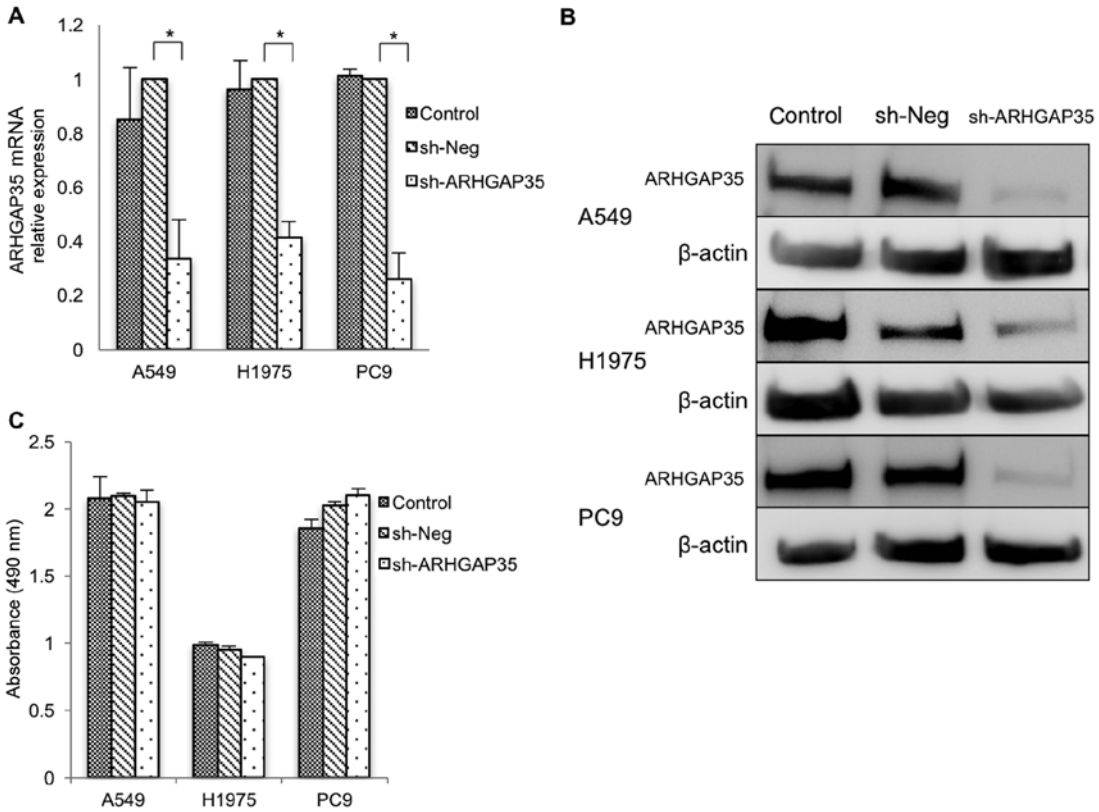

Figure 1. (A) ARHGAP35 mRNA and (B) protein in cells with and without ARHGAP35 gene knockdown and (C) cell viability with long-term ARHGAP35 gene knockdown. We evaluated ARHGAP35 mRNA and protein levels in cell lines with introduced shRNA and selected by quantitative real-time PCR and western blot analysis. ARHGAP35 mRNA was decreased and protein levels were suppressed in sh-ARHGAP35 compared with all sh-Neg cell lines (*P<0.02). We examined cell viability in cells with long-term *ARHGAP35* knockdown by MTS assays. Cell proliferative ability of cells with long-term *ARHGAP35* knockdown was at the same level as without knockdown in all of the cell lines. Control, cells with no transfection; sh-Neg, cells transfected with negative control shRNA; sh-ARHGAP35; cells transfected with ARHGAP35 shRNA.

was scratched in a straight line to create a scratch with Cell Scratcher (AGC, Tokyo, Japan). Twenty-four and 48 h later, we measured the width of the scratch using an optical microscope without stain, and calculated the rate of cell migration.

Statistical analysis. All experiments were performed in triplicate and analyzed using JMP Pro 11 (SAS Institute, Inc., Cary, NC, USA). Unpaired Student's t-tests were used for comparisons between two groups. P<0.05 were considered to indicate a statistically significant result. The Chou-Talalay method was used to evaluate synergistic effect of drug combination as described in a previous report (26). Combination index (CI) was calculated based on the effect ratio of cell viability under various drug concentrations using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). CI <0.9, 0.9-1.1 and >1.1 were regarded as synergism, additive effect and antagonism, respectively (27).

Results

Suppressive effects of cell viability are attenuated by long-term ARHGAP35 knockdown in lung adenocarcinoma cell lines. We established lung adenocarcinoma cell clones by continuous exposure to puromycin for a month after introducing shRNA against ARHGAP35 into cells. qRT-PCR (Fig. 1A) and western blot (Fig. 1B) analyses showed that ARHGAP35 was significantly suppressed in these cells. In the MTS assay, cell viability with long-term *ARHGAP35* knockdown was comparable to the cell viability without knockdown in all cell lines (Fig. 1C). This suggested that the suppressive effects of cell viability were attenuated by long-term knockdown.

Molecular dynamics in EGFR signaling pathway changes after long-term ARHGAP35 knockdown. We also evaluated the molecular dynamics of the RAS/RAF1/MAPK, STAT, PI3K, MET and ERBB2 pathways (Figs. 2 and 3). Western blots showed increases in SRC and STAT3 total protein, and increased levels of phosphorylated STAT3 in A549 cells after long-term *ARHGAP35* knockdown (Fig. 2). PRKCZ, MET and ERBB2 levels were also increased with *ARHGAP35* knockdown in the A549 cells (Fig. 2 and data not shown). By contrast, PRKCD, PRKCZ, MET and ERBB2 levels were decreased after long-term *ARHGAP35* knockdown in the H1975 cells (Fig. 2 and data not shown). SRC, MEK, STAT3, AKT, PRKCD, PRKCZ and MET levels were decreased with long-term *ARHGAP35* knockdown in the PC9 cells (Fig. 2 and data not shown). qRT-PCR showed increased SRC mRNA in A549 cells and decreased SRC (Fig. 3A) and STAT3 (Fig. 3B) mRNA in the H1975 cells. ROCK activity, which is associated with cell migration, was within normal limits in all cell lines with *ARHGAP35* knockdown (Fig. 4).

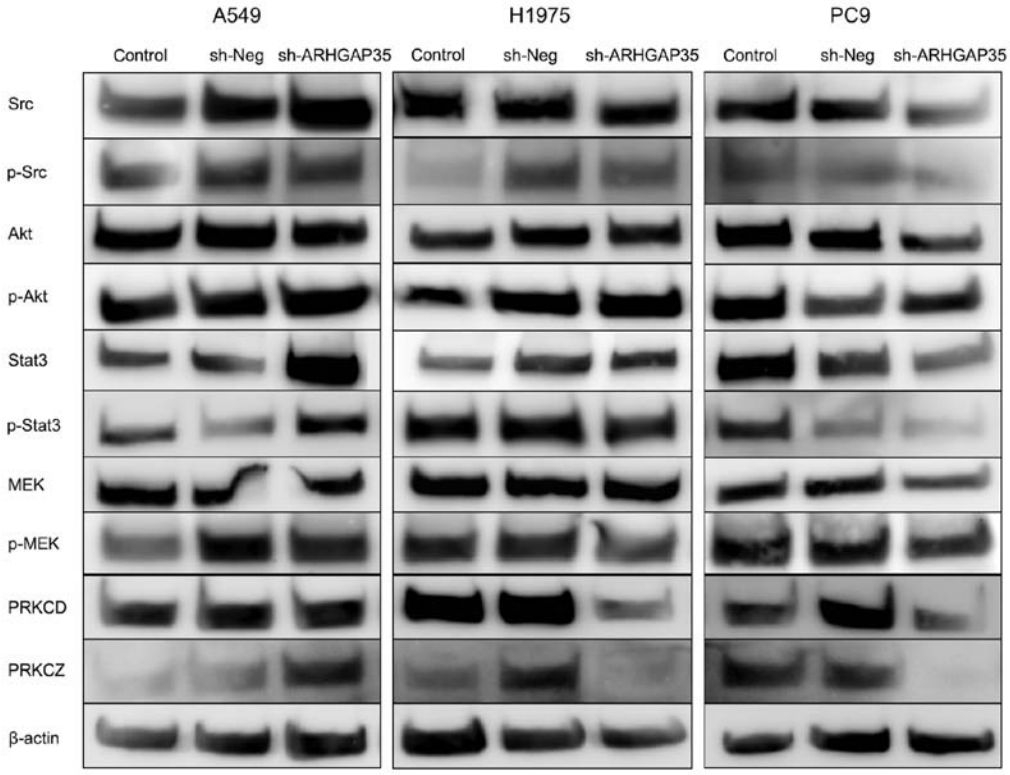

Figure 2. Dynamics of proteins in signaling pathways associated with ARHGAP35. We evaluated the protein dynamics of signaling pathways associated with ARHGAP35 by western blot analysis when *ARHGAP35* gene knockdown was continued in the long term. Increases in SRC and STAT3, and consequently phosphorylated STAT3, are shown following long-term *ARHGAP35* knockdown in A549 cell line. PRKCZ, MET (data not shown) and ERBB2 (data not shown) were also increased in A549 cells with *ARHGAP35* knockdown. PRKCA (data not shown), PRKCZ, MET (data not shown) and ERBB2 (data not shown) were decreased in H1975 cells after long-term *ARHGAP35* knockdown. SRC, MEK, STAT3, AKT, PRKCD, PRKCZ and MET (data not shown) were decreased in PC9 cells after long-term *ARHGAP35* knockdown.

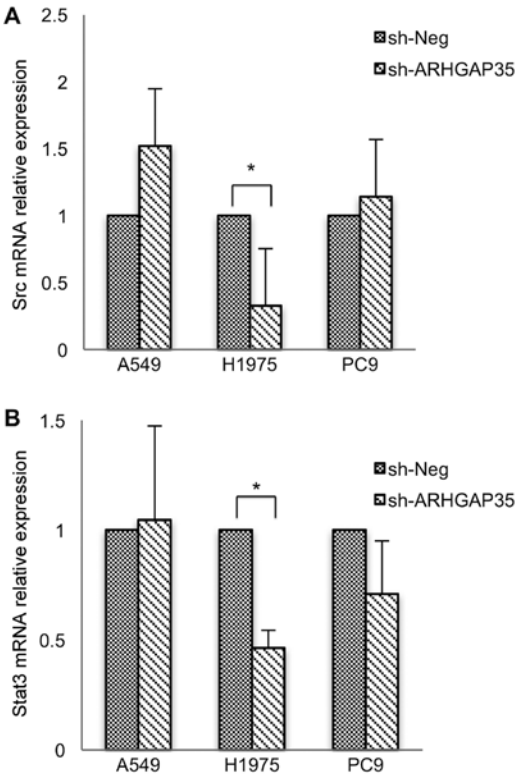

Figure 3. Dynamics of mRNA of the signaling pathways associated with ARHGAP35. We measured the mRNA dynamics of the signaling pathways associated with ARHGAP35 by quantitative real-time PCR after stable knockdown treatment. SRC mRNA was increased in the A549 cells (A), and SRC and STAT3 mRNA were significantly (*P<0.001) decreased in the H1975 cells (B).

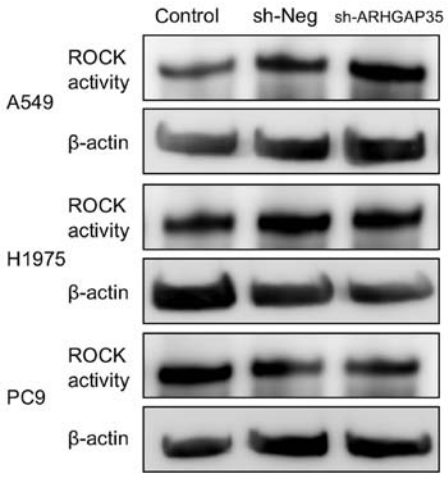

Figure 4. Rho kinase (ROCK) activities of cells after long-term ARHGAP35 knockdown. We evaluated the ROCK activities of cells with long-term *ARHGAP35* knockdown by ROCK Activity Immunoblot kit. ROCK activity was within normal limits in all cell lines with *ARHGAP35* knockdown.

STAT3 inhibitor is more effective after long-term ARHGAP35 knockdown in A549 and PC9 cells. Using MTS assay, we measured cell viability after long-term *ARHGAP35* knockdown in cells treated with a STAT3 inhibitor (Fig. 5). A549 and PC9 cells were more sensitive to the STAT3 inhibitor after long-term *ARHGAP35* knockdown than when there was no knockdown, but the opposite was noted in the H1975 cells.

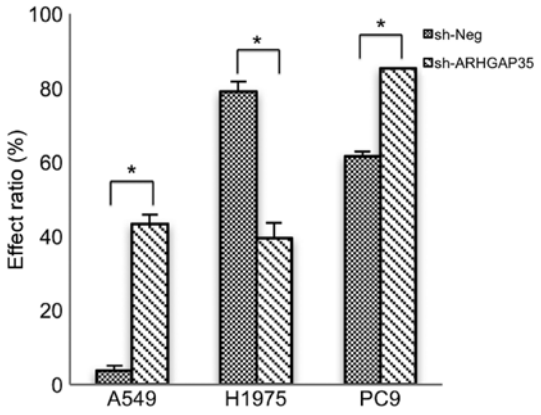

Figure 5. The effects of a STAT3 inhibitor on cell viability after long-term ARHGAP35 knockdown. We examined viability in cells with long-term *ARHGAP35* knockdown by MTS assay, using 200 μM of a STAT3 inhibitor. A549 and PC9 cells with long-term *ARHGAP35* knockdown were more sensitive to STAT3 inhibitors than were cells without knockdown; however, the opposite result was noted in the H1975 cells. The effect ratio was calculated as the absorbance of the control groups and STAT3-inhibitor groups: Effect ratio (%)=(1-A^S/A^C) x100, where A^C is the absorbance of the control groups and A^S is the absorbance of the STAT3-inhibitor groups (*P<0.001).

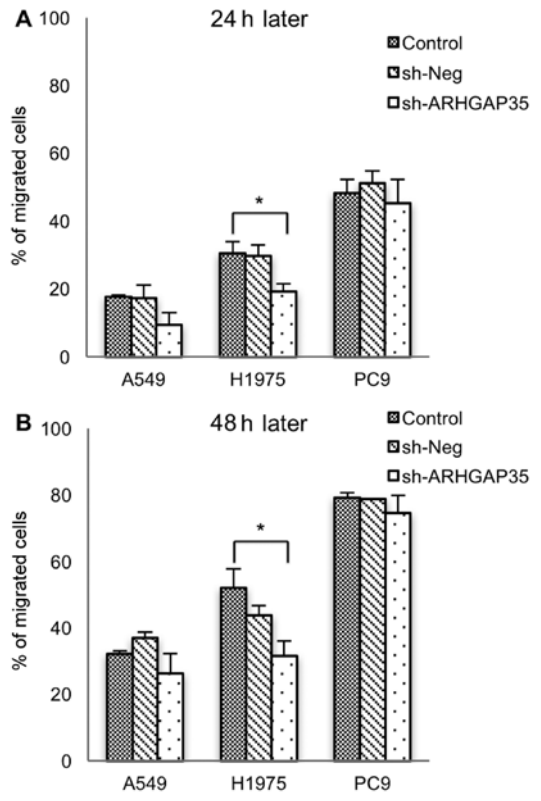

Figure 6. The migratory ability of cells after long-term ARHGAP35 knockdown. We measured cell migration at 24 (A) and 48 h (B) following scratch assay. The cell migratory ability was reduced in the cell lines with *ARHGAP35* knockdown than in those without, for all cell lines. % of migrated cells was calculated as the wound widths at each assessment point: % of migrated cells=(1-W^t/W^0) x100, where W^0 is the wound width at the initial time and W^t is the wound with at a subsequent time (t) (*P<0.05).

Suppressive effect of ARHGAP35 knockdown on migration is maintained for a long time. Using scratch assays, we measured cell migration after long-term *ARHGAP35* knockdown in the cell lines (Fig. 6A and B). This demonstrated that cell migratory ability was reduced in cancers with *ARHGAP35* knockdown when compared with cancers without knockdown. This was the case for all cell lines, and suggested that the suppressive effect of *ARHGAP35* knockdown on migration was maintained for a long time.

SRC inhibitor and STAT3 inhibitor synergistically suppress cell growth in lung adenocarcinoma cell lines. Finally, we measured cell viability under combination treatment with an SRC inhibitor and a STAT3 inhibitor in each cell line (Fig. 7). CIs of SRC inhibitor and STAT3 inhibitor were 0.61, 0.08 and 0.17 for A549, H1975 and PC9 cell lines, respectively; suggesting that the two inhibitors synergistically suppressed cell growth.

Discussion

In our previous study, we showed that *ARHGAP35* knockdown by means of chemically modulated small interfering RNA suppressed the proliferation of lung adenocarcinoma cell lines with *EGFR* or *KRAS* mutations (24). In the present study, we obtained clones in which *ARHGAP35* was stably knocked down, and we hypothesized that attenuated viability would be seen as a compensatory mechanism for survival in those cells.

RAS activates the RAF1/MAPK pathway and EGFR activates several downstream pathways, including the RAS/RAF1/MAPK, PI3K/AKT and STAT pathways, and these play important roles when regulating proliferation, invasion and migration (28). Among these pathways, ARHGAP35 has been reported to be inactivated by the RAS/RAF1/MAPK pathway and to regulate RhoA (24,29,30). Importantly, ARHGAP35 is also activated by SRC, which is a potential signal mediator of the EGFR pathway (19). We assumed that the RAS/RAF1/MAPK and SRC pathways were compensatory mechanisms. In addition, based on acquired resistance for EGFR-TKIs, we decided to screen MET, ERBB2, PKC, AKT and STAT3 (31-33).

Western blots showed increased SRC, STAT3, PRKCZ, MET and ERBB2 levels in A549 cells after long-term *ARHGAP35* knockdown. A possible explanation for this is that increased SRC, MET and ERBB2 might have accelerated STAT3 and PRKCZ downstream, ultimately attenuating *ARHGAP35* knockdown. This was supported by the RT-qPCR findings. In contrast to this, the protein and mRNA levels of the molecules associated with the EGFR pathway were decreased in both the H1975 and PC9 cell lines after long-term *ARHGAP35* knockdown. We therefore assume the involvement of another pathway.

Levels of STAT3 and phosphorylated STAT3 increased in the A549 cell line after long-term *ARHGAP35* knockdown. STAT3 is a 92-kDa protein, encoded by *STAT3* on 17q21, and is a member of the STAT family of transcriptional activators (34-36). This protein is associated with cell proliferation, differentiation, invasion and apoptosis in the EGFR signaling pathway (36-38). In lung cancer, STAT3 promotes cell proliferation and invasion, while its inhibition leads to the suppression of tumor proliferation *in vitro* and *in vivo* (39-41).

After long-term *ARHGAP35* knockdown, MTS assay indicated that A549 cells were more sensitive to the STAT3 inhibitor. This was consistent with the hypothesis that increased

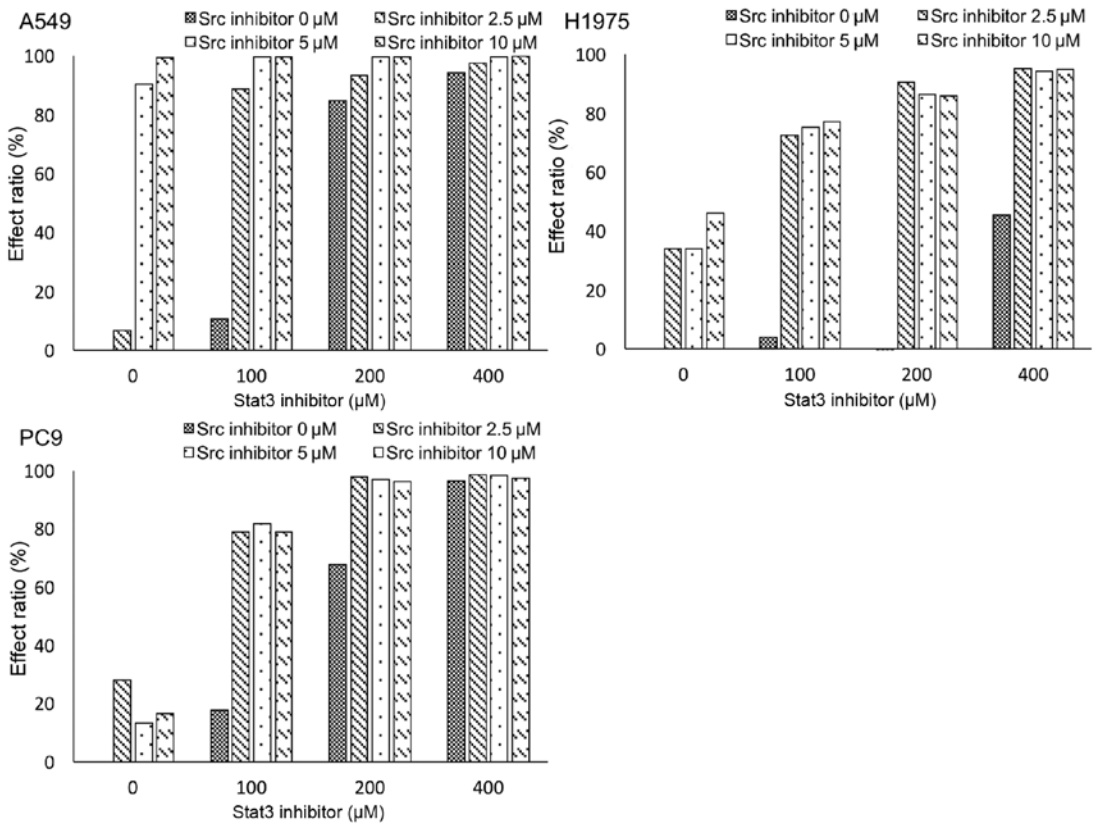

Figure 7. The effects of concomitant SRC and STAT3 inhibitor use in lung adenocarcinoma. We examined viability in lung adenocarcinoma cell lines by MTS assay, using Src and STAT3 inhibitors. A synergistic and strong effect was observed on growth inhibition after concomitant use of an SRC inhibitor and a STAT3 inhibitor for the lung adenocarcinoma cell lines, especially cell line A549. The effect ratio was calculated as the absorbance of the control groups and inhibitor groups: Effect ratio (%)=(A^I/A^C) x100, where A^C is the absorbance of the control groups and A^I is the absorbance of the inhibitor groups.

STAT3 levels in A549 cells after long-term *ARHGAP35* knockdown would be a critical factor for cell survival. Similar but modest results were obtained in the MTS assay for the PC9 cell line, although STAT3 was not increased after long-term *ARHGAP35* knockdown. A possible explanation for the different impact of the inhibitor in these two cell lines may be the different dependency on the STAT3 pathway for proliferation. We speculate that A549 cells depended heavily on the STAT3 pathway as a compensatory mechanism for viability. This presented the possibility of tumor suppression by combining STAT3 inhibition and ARHGAP35 inhibition for lung cancer with the *KRAS* mutation.

In contrast to A549 cells, however, opposite results were obtained in H1975 cells after STAT3 inhibition. Although we cannot explain these phenomena, we speculate that: i) Any specific underlying molecular mechanisms may cause unexpected results after STAT3 inhibition in lung cancer with acquired resistance for the *EGFR* mutation or ii) sh-Neg treatment may become a stress for H1975 since elevated protein levels of protein kinase C delta (PRKCD) and zeta (PRKCZ) in sh-Neg treated H1975 cells were observed (Fig. 2). The former could be important because STAT3 inhibitors have been studied in clinical trials for both hematological and solid malignancies (42,43). For the latter, PKCs have been reported to be upregulated by Toll-like receptors and positively regulate STAT3 in response to stress (44,45). Accordingly, sh-Neg-treated H1975 cells may have relatively strong dependence to STAT3 and respond strongly to STAT3 inhibitor. Our results that the ARHGAP35 protein level was slightly decreased and the STAT3 protein level was increased by sh-Neg treatment in H1975 cells (Figs. 1 and 2) and discrepancy between mRNA expression and protein translation for SRC and STAT3 (Figs. 1 and 3) may be affected by same reason. However, our important finding is that STAT3 inhibitor and SRC inhibitor synergistically suppressed cell growth even in EGFR-mutant cells (Fig. 7).

ARHGAP35 is poor therapeutic target because it lacks a kinase domain. A natural alternative strategy is therefore to target an upstream mediator, such as SRC, that can suppress ARHGAP35 activity. Unfortunately, a phase II clinical trial of a single-use SRC inhibitor for lung cancer has presented only modest clinical benefit (46). Based on the concept of synthetic lethality, we therefore, tested the effects of cosuppression of the SRC/ARHGAP35 axis with both a STAT3 inhibitor and an SRC inhibitor *in vitro*. Concomitant treatment caused a synergistic and strong effect on growth inhibition in the *KRAS*- and *EGFR*-mutant cell lines, especially in the *KRAS*-mutant cell line. Thus, cosuppression of the STAT3 pathway and SRC/ARHGAP35 axis may be an effective strategy for treating *KRAS* and *EGFR* mutant lung adenocarcinoma. A further study is needed to confirm whether this effect can be generalized to other cell lines.

Notably, a synergistic effect was observed for the SRC and STAT3 inhibitors in both *EGFR*-mutant cell lines, despite the fact that the H1975 cell line is usually resistant to STAT3 inhibitors and the PC9 cell line is relatively resistant

to SRC inhibitors. Unfortunately, we cannot illuminate the mechanism underlying this synergistic effect, but based on previous reports of synthetic lethality, it is possible that mitosis regulation or DNA duplication systems could be involved (11,12,14). In a previous research, ARHGAP35 has been shown to regulate mitosis by controlling RhoA (47). In addition, a recent report showed that STAT3 upregulated TPX2, a microtubule-associated protein known to be involved in mitosis, by binding the 5'-flanking sequence of the *TPX2* gene (48). Further study is warranted on this topic as STAT3 and SRC have many potential targets.

In our previous study, short-term *ARHGAP35* knockdown was also shown to suppress migration in lung cancer (24). In the present study, cell migration ability was reduced in all three cell lines after *ARHGAP35* knockdown, suggesting that the suppressive effect on migration may be maintained in the long term. ROCK activity was within normal limits, however, so this conflicting result will need to be resolved in future research.

In conclusion, cosuppression of STAT3 and the SRC/ARHGAP35 axis may be an effective strategy for treating *KRAS*- and *EGFR*-mutant lung adenocarcinoma.

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Availability of data and materials

The analysed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KO and HN performed the experiments. KO wrote the manuscript. AS and CE made substantial contributions to conception, design and intellectual content of the studies. TW, YM and MN made key contributions to the analysis and interpretation of the data. AS and YO reviewed and edited the manuscript. YO also contributed to the planning of the research. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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