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The study of oncogenic Ras-induced tumor development

조선대학교 대학원

생물신소재학과

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Ras 에 의한 암 발생 연구

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지도교수 유 호 진

이 논문을 이학석사학위신청 논문으로 제출함.

2008 년 8 월

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김나희의 석사학위 논문을 인준함

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조선대학교 대학원

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Ras에 의한 암 발생 연구

김 나 희

(지도교수 : 유 호 진)

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Clast1/LR8 의 조절억제는 Ras 매개 종양의 변환과 종양생성에 관련되며 Ras 단백질은 암 생성을 증진시키는 다양한 downstream 신호 전달체계를 통해 세포를 변화시킨다. 그러나, downstream targets 을 조절하는 정확한 세포기전은 완전히 알려지지 않았다. 이에 본 연구에서는 mouse fibroblast NIH3T3 cell 에서 Ras 가 Clast1/LR8 transcript 생성을 억제함을 알아보고자 하였다. Clast1/LR8 transcript 는 Ras 활성이 높은 H460, A549, H1299 cell 에서 확인되지 않았다. 상대적으로 Ras 활성이 낮은 DMS53 cell 에서는 높은 발현 수준을 보였다. 이는 Clast1/LR8 mRNA 수준과 Ras활성 사이의 inverse correlation 를 보여주고 있다. 또한, 우리는 NIH3T3 cell 에서 MEK1

억제제인 PD98059의 첨가 후 H-Ras 매개 Clast1/LR8 transcript 의 억제가 역전되는 것으로 확인하였다. 또한, histone deactylase inhibitor인 trichostatin A 와 DNA methyltrasnferase 억제제인 5'-Azacytine 사용 후 Ras에 의한 Clast1/LR8 transcript 의 억제가 회복됨을 확인하였다. Clast1/LR8 의 이소 발현은 H-RasV12-transformed NIH3T3 cell 에서 EGF 에 의해 유도된 Akt의 phosphorylation 을 억제시켰다. Clast1/LR8 의 과 발현이 H-RasV12-transformed NIH3T3 cell 와 사람의 폐암세포인 H-RasV12-transformed NIH3T3 cell 의 anchorage-independent 성장과 잠재적인 전이 능력을 억제시킴을 확인하였다. 본 연구에서는 Clast1/LR8 가 Ras 에 의해 유도된 세포성장과 종양형성을 억제함을 보여주고 있다. 따라서 이러한 결과를 바탕으로 Clast1/LR8 의 조절 억제가 Ras 매개 종양변환발생의 중요한 기작으로 추측할 수 있었다.

ABSTRACT

Oncogenic Ras-mediated down-regulation of Clast1/LR8 is involved in Ras-mediated neoplastic transformation and tumorigenesis Oncogenic Ras proteins transform cells via multiple downstream signaling pathways that promote the genesis of human cancers. However, the exact cellular mechanisms that regulate the downstream targets are not fully understood. Here we show that oncogenic Ras decreases Clast1/LR8 transcript production in mouse fibroblast NIH3T3 and human fibroblast WI38 cells. Clast1/LR8 transcript was undetectable in H460, A549, and H1299 cells exhibiting high Ras activity, but was relatively abundant in DMS53 cells containing low Ras activity, indicating that there is an inverse correlation between Clast1/LR8 mRNA levels and Ras activity. Furthermore, we demonstrate that the addition of MEK1 inhibitor PD98059 reversed oncogenic H-Ras-mediated suppression of Clast1/LR8 transcript in NIH3T3 cells. Additionally, a histone deactylase inhibitor trichostatin A and DNA methyltrasnferase inhibitor 5'-Azacytine potently restored the inhibition of Clast1/LR8 transcript by oncogenic Ras. The ectopic expression of Clast1/LR8 inhibited the epidermal growth factor (EGF)-stimulated Akt phosphorylation in H-RasV12-transformed NIH3T3 cells. We further

demonstrated that overexpression of Clast1/LR8 significantly inhibited anchorage-independent growth and the invasive potential of H-RasV12-transformed NIH3T3 cells and human lung cancer H460 and A549 cells. Finally, our results show that Clast1/LR8 inhibits Ras-induced cell proliferation and tumor formation by oncogenic H-RasV12-transformed NIH3T3 cells in vivo. Thus, the down-regulation of Clast1/LR8 appears to be an important mechanism by which oncogenic Ras-mediated neoplastic transformation occurs.

I. INTRODUCTION

The *ras* protooncogenes encode several 21-kDa GTP-binding proteins that act as pivotal mediators of cell signaling pathways (1, 2). These proteins help transfer extracellular signaling information from the cell membrane through the cytosol and finally into the nucleus. In normal cells, Ras proteins, including H-, K-, and N-Ras, are transiently activated in response to extracellular signals and help control cell proliferation, differentiation and survival in all multicellular organisms (3-6). Consequently, proper regulation of Ras signaling is critically important for normal development. In tumor cells, the oncogenic activation of *ras* is a consequence of point mutations that either impair the GTPase activity or enhance the GTP binding affinity, resulting in a highly active proliferative signal (7). Indeed, constitutively active Ras has been implicated in many aspects of malignant phenotypes, including proliferation, neoplastic transformation, invasion, and metastasis (8-10). Oncogenic Ras proteins are commonly detected in human cancers, including ~ 90% of pancreatic cancers, ~ 70% of malignant neoplasias and ~ 30% of all human cancers, suggesting that ras plays a key role in the development of cancer (9, 11, 12).

Oncogenic Ras proteins transform cells via multiple downstream signaling cascades, including the Raf/MEK/ERK and phosphoinositide 3-kinase (PI3K)/Akt kinase

pathways. The Raf/MEK/ERK pathway is one of the best-characterized kinase cascades that leads to the phosphorylation and activation of pro-proliferative transcription factors, such as Elk-1, Ets-2, and c-Myc (5, 13-15). The inhibition of Raf/MEK/ERK pathway suppresses Ras-mediated transformation, indicating that this pathway plays a major role in this transformation process. PI3K/Akt kinase-regulated pathway is another important Ras effector signaling pathway, activation of which generally involves the PI3K-dependent phosphorylation of the inositol lipids to activate downstream target kinases, such as Akt. Activated Akt phosphorylates a wide range of downstream substrates, many of which are involved in the regulation of cell survival, cell cycle progression, and cell metabolism (16-19). Similar to the Raf/MEK/ERK pathway, inhibition of the PI3K/Akt pathway reduces Ras-induced transformation (20), suggesting that PI3K/Akt pathway activation is also required for transformation.

Signaling events downstream of Ras are complex, nonlinear, and dynamic (21, 22). Genome-wide surveys have found numerous putative Ras target genes mediating Ras transformation (4, 6, 23-26). Despite the identification of these diverse Ras targets, it is still difficult to precisely determine which downstream signaling components represent the best therapeutic targets for blocking Ras-mediated neoplastic transformation/tumorigenesis. Thus, the identification of Ras downstream targets in

cancer cells is of considerable significance.

Here, we attempt to identify genes that are differentially expressed in control NIH3T3 cells and oncogenic H-Ras expressing NIH3T3 cells, using annealing control primers (ACP)-based differential display reverse transcription-polymerase chain reaction (RT-PCR) (27, 28). We report the isolation of a gene that was down-regulated in H-RasV12 expressing NIH3T3 cells, but not in control NIH3T3 cells. The sequence of this gene was identical to mouse *Clast1/LR8* (Genbank Accession number: AB031386). The isolation and identification of *Clast1/LR8* through differential display RT-PCR suggests that *Clast1/LR8* may be involved in tumorigenesis by oncogenic Ras. Our results showed that Clast1/LR8 expression suppressed EGF-stimulated Akt signaling pathway in oncogenic Ras overexpressing cells. In addition, Clast1/LR8 prevented H-RasV12-induced cell transformation, invasion, and anchorage-independent cell growth as well as tumor growth in nude mice. These results indicate that Clast1/LR8 may play a relevant role as a suppressor of oncogenic Ras-dependent tumorigenesis.

II. MATERIALS AND METHODS

Cell culture

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% FBS. A549 line was maintained in Ham′s F12 medium (Gibco) supplemented with 10% FCS. The H1299 and H460 lines were maintained in RPMI 1640 (Gibco) supplemented with 10% FCS and 5% glutamine. All cells were maintained in cell-specific media at 37 °C in a humidified atmosphere of 5% CO_2. All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Preparation of constructs and clones

The constructs of the dominant-positive H-RasV12 are described elsewhere (29). The mouse and human *Clast1/LR8* cDNAs were amplified by reverse transcription-PCR (RT-PCR) using the following primers: mouse Clast1-sense, 5′-ATGGTCCAGAGCACAGTGAC-3′; mouse Clast1-antisense, 5′-TCACAGGATAGCAGGGATCT-3′; human Clast1-sense, 5′-ATGACGCAAAACACGGTGAT-3′; human Clast1-antisense, 5′-

TCACAGGACAATGGCAGTGG -3′. After confirming the DNA sequence by a Genomelab GeXP system (Beckman Coulter, Inc., Fullerton, CA, USA), the mouse and human *Clast/LR8* cDNAs were cloned into a pcDNA3.1-hygro mammalian expression vector driven by the CMV promoter (Invitrogen, Carlsbad, CA, USA). To create stable Clast/LR8-expressing cells, H-RasV12 NIH3T3, H460 and A549 cells transfected with the Clast1/LR8 expression vector using the Lipofectamine plus method (Promega Corp., Madison, WI, USA) according to the manufacturer′s instructions. After transfection, the cells were incubated with complete medium containing 300 μg/ml hygromycin for 4 weeks. The cell clones resistant to hygromycin were isolated and analyzed.

ACP-based differential display RT-PCR

The procedure used was as previously described with minor modifications (27, 28). First-strand cDNA synthesis was performed for 1.5 h at 42 °C in a final reaction volume of 20 μl containing 3 μg of purified total RNA, 4 μl of reaction buffer, 5 μl of dNTPs (each 2.5 mM), 2 μl of 10 μM cDNA synthesis primer dT-ACP1, 0.5 μl of RNAse inhibitor (40 U/μl; Promega), and 1 μl of Superscript II Reverse Transcriptase (200 U/μl; Invitrogen). The samples were then diluted by the addition of 100 μl of ultra-purified water. PCR amplification was subsequently conducted using a

GeneFishing™ DEG 101 Kit (Seegene, Seoul, Korea) in a 50-μl reaction volume, consisting of 10x PCR buffer without $MgCl_2$, 25 mM $MgCl_2$, 5 μM arbitrary ACPs, 10 μM dT-ACP2, 2 mM dNTPs, 2.5 U of Taq DNA Polymerase (Promega), and 1 μl of the first-strand cDNA. Each kit contains 20 different arbitrary annealing control primers. The program for amplification was one cycle at 94 °C for 5 min, 50 °C for 3 min, and 72 °C for 1 min, followed by 40 cycles at 94 °C for 40 s, 65 °C for 40 s, 72 °C for 40 s, and 72 °C for 5 min. The products were separated on 2% agarose gels and photographed using Polaroid film under ultraviolet light after ethidium bromide staining. Differentially expressed bands were extracted and cloned into a pGEM-T easy cloning vector (Promega). To confirm the identities of the insert DNAs, isolated plasmids were sequenced using a Genomelab GeXP system (Beckman Coulter). The complete sequences were analyzed for similarities using BLASTX (National Center for Biotechnology Information [NCBI]).

RT-PCR analysis

The inhibition of DNA methylation and histone deacetylation was achieved by culturing the cells in 10 μM 5-aza-2′-deoxycytidine (AZC; Sigma, St. Louis, MO, USA) for 2~4 days and 30-100 nM trichostatin A (TSA; Sigma) for 24 h, respectively. Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Hilden,

Germany) according to the manufacturer′s protocol. The following primers were used: mouse Clast1-sense, 5′-TCTCTAGGGGTGACCCAGATATT-3′; mouse Clast1-antisense, 5′-AGGATACAGACCACTGTGAGCAT-3′; human Clast1-sense, 5′-GTTCTTGGAGTGTGTCTCAGCTT-3′; human Clast1-antisense, 5′-GACACAATGACCTTCAAGACACA-3′; mouse GAPDH-sense, 5′-TAAAGGGCATCCTGGGCTACACT-3′; mouse GADPH-antisense, 5′-TTACTCCTTGGAGGCCATGTAGG-3′; human GAPDH-sense, 5′-GGTGAAGGTCGGTGTGAACGGATTT-3′; human GADPH-antisense, 5′-AATGCCAAAGTTGTCATGGATGACC-3′. The RT-PCR assays were carried out in duplicate and confirmed in two independent experiments.

Western blot analysis

Whole-cell lysates were prepared in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % NP-40, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail (Roche Diagnostics Corporation,Indianapolis, IN, USA)]. Protein concentrations from each sample were determined using the Bradford protein assay. Equal amounts of protein were loaded onto a 10% SDS-polyacrylamide gel, separated by electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Following

transfer, the membrane was blocked with 5% skim milk for 1 h and then incubated for 16 h with an anti-phospho-AKT, anti-AKT, anti-phospho-ERK1/2 and anti-ERK1/2 antibodies (Cell Signaling Technology, Beverly, MA). The membranes were then washed, incubated with the appropriate secondary antibodies (1:4,000) in a blocking buffer for 2 hours, and washed again. The blotted proteins were detected using an enhanced chemiluminescence detection system (iNtRON Biotech, Seoul, Korea).

Anchorage-independent growth assay

Cells (2×10^4) were mixed with cell-specific medium in 0.3% agar, plated on top of a 0.5% agar base, and covered with 10% FBS medium. Cultures were maintained at 37 °C in an incubator for 20 days. The medium was changed every 3 days, and the number of colonies in five random fields was counted.

In vitro invasion assays

An *in vitro* invasion assay was performed using 24-well trans-well units with polycarbonate filters (pore size, 8 μm; Costar, Corning Incorporated, Corning, NY, USA) coated on the upper side with Matrigel (Becton Dickinson Labware, Bedford, MA). Cells (5×10^4) were placed in the upper part of the transwell unit and were allowed to be invasive for 48 h. The lower part of the transwell unit was filled with

10% FBS medium. After incubation, the non-invaded cells on the upper surface of the membrane were removed from the chamber, and the invaded cells on the lower surface of the membrane were stained with Diff-Quick (Kokusai Shiyaku, Kobe, Japan). Five randomly selected microscopic fields (200×) per membrane were then counted to determine the number of invaded cells.

Cell proliferation analysis

To analyze growth curves, cells (2×10^4) were plated on day 0 in 10% FBS medium. On day 2, the cells were washed twice in phosphate-buffered saline (PBS) and then incubated in serum-free medium for 13 h to synchronize the cells. Subsequently, the growth medium was re-integrated with 10% FBS, and the cells were grown for 3 additional days. Cell growth was quantified using a hematocytometer at the indicated time points. BrdU incorporation assays were performed using an assay kit (Roche Diagnostics Corporation) according to the manufacturer′s protocol. The percentage of cells incorporating BrdU after 18 h was determined. Data are presented as mean ± standard deviation (SD) for a minimum of three experiments.

GST-RBD-Raf Pull-Down

The RAS binding domain (RBD) was coupled to GST, and a pull-down experiment

was performed using an EZ-Detect Ras Activation Kit (Pierce, Rockford, IL, UAS) according to the manufacturer′s protocol. Briefly, cell lysates were prepared by lysing cells with commercial buffer and incubating with pre-coupled GST-RBD for 30 min at 4 °C. Beads were then collected by centrifugation, washed three times with lysis buffer, and re-suspended in SDS loading buffer. Samples were then analyzed by Western blot analysis using an anti-pan-RAS antibody.

In vivo tumor formation assays

Six-week-old nude female mice, purchased from Orient (Charles River Korea, Seoul, Korea), were used for the *in vivo* tumor formation assays. Tumor cells (1×10^6) were re-suspended in 150 μl of sterile PBS and injected subcutaneously into the flank of the nude mouse. Tumor size was measured every two to five days using calipers. The length (l) and width (w) of the developing tumor was converted to volume using the equation $(w^2 \times l)/2$. Mice were sacrificed when tumors reached a maximum diameter of 2 cm.

Data Analysis

The data represents the mean ± SD. Statistical comparisons were carried out using an unpaired *t* test. *p* values < 0.01 were considered significant.

III. RESULTS

Oncogenic H-Ras-mediated down-regulation of Clast1/LR8.

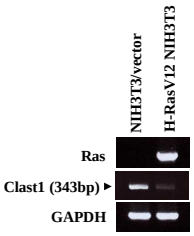
In an attempt to discover novel Ras downstream effectors responsible for Ras-mediated cellular transformation, we conducted ACP-based RT-PCR analysis and the expression profiles of mouse fibroblast NIH3T3 cells stably transfected with H-RasV12 (H-RasV12 NIH3T3) or empty pcDNA3 vector (NIH3T3/vector) were compared. We identified a partial cDNA that was significantly down-regulated in H-RasV12-expressing cells, but not in empty vector-transfected cells (Fig. 1*A*). The 343-bp amplicon displayed complete homology to *Clast1/LR8* (Fig. 1*B*). The *Clast1/LR8* gene is ubiquitously expressed in various organs of adult mice and its expression is induced in splenic B cells activated with CD40 ligand (30). However, its physiological function is largely unknown. To confirm our ACP-based RT-PCR results, semiquantitative RT-PCR analyses of the H-RasV12 and empty vector-transfected NIH3T3 cells were performed. Semiquantitative RT-PCR analysis using the *Clast1/LR8* primers showed that the expression of the *Clast1/LR8* genes decreased dramatically after transfection with H-RasV12 expressing vector, compared with the parental NIH3T3 cells and vector control (Fig. 2A). We also investigated the effect of

oncogenic Ras on human *Clast1/LR8* transcription in human lung fibroblast WI38 cells, and found that oncogenic H-RasV12 down-regulated *Clast1/LR8* mRNA expression in WI38 cells (Fig. 2B).

Because *Clast1/LR8* mRNA was originally detected in human lung fibroblasts (31) and active mutations in K-Ras have been frequently noted in lung cancer tissues (32, 33), we next investigated *Clast1/LR8* expression in human lung cancer cell lines including DMS53, H460 A549, and H1299. The H460 and A549 cells harbor a constitutively activate K-RAS gene (mutations in codon 61 and codon 12, respectively) and H1299 cells contain the oncogenic N-RAS mutation (mutations in codon 61). We initially confirmed and compared RAS activity among lung cancer cell lines using pull-down experiments with GST-RAF-RBD. Consistent with previous reports, we also found that the above lung cancer cell lines contained the constitutively active form of RAS, with the exception of DMS53 cells (Fig. 2C, upper panel). We next examined the level of Clast1/LR8 mRNA expressed in these cells lines. As shown in Fig. 2C, lower panel, *Clast1/LR8* expression was undetectable in H460, A549, and H1299 cells with higher levels of Ras activity, but was detectable in DMS53 cells with low Ras activity, indicating that there is an inverse correlation between *Clast1/LR8* mRNA levels and K-RAS activation.

Figure 1

A



cDNA synthesis primer: dT-ACP1: 5'-CTGTGAATGCTGCGACTACGATXXXXX(T)18-3'
Forward primer: arbitrary ACP34: 5'-GTCTACCAGGCATTCGCTTCATXXXXXGCATCCAGCA-3'
Reverse primer: dT-ACP2: 5'-CTGTGAATGCTGCGACTACGATXXXXX(T)15-3'

B

Mus musculus mRNA for Clast1 mRNA (AB031386 (937-1211)

GTCTACCAGGCATTCGCTTCATXXXXXGCATCCAGCACCTGCGTCCCCGGCCAAA
GAGAAGATCCCTGCTATCCTGTGATCCGCCCACACCCACGTGTCCCTGTCCATAGT
AATCCAGGGAGGACGCGACTGTTCCCAGGGCCTCTTCTGGCCCCGCAGCACACAC
CTACTTCTGATGTAGCCACTATTCAGCCCTCTTTCTCTCCCTCTCTCCCCACTGTCC
TCACGCCAATGTCCCTTCTTCCACGAACAGATATTATGTCATTTAATCCAATGCCG
ATAAAGGAGAAACAACTTGAAAAAAAAAAAAAAAAAXXXXXATCGTAGTCGCA
GCATTCACAG

Figure 1. Clast1/LR8 expression is down-regulated by oncogenic Ras activation

A. Differential display of H-RasV12-transfected and empty vector pcDNA3-transfected NIH3T3 cells. NIH3T3 cells were stably transfected with control vector (NIH3T3/vector) or H-RasV12-expressing vector (H-RasV12 NIH3T3), and mRNA extracted from these cells was used for ACP-based differential display RT-PCR. mRNA extracted from the H-RasV12-transfected and empty vector-transfected cells was used for the synthesis of first-strand cDNA with the primer dT-ACP1. Second-strand cDNA was then amplified by PCR using the arbitrary ACP34 primer (forward primer) and dT-ACP2 (reverse primer) to produce a 343-bp product. Arrows indicate differences in mRNA expression between the H-RasV12-transfected and empty vector-transfected cells. The lower panel

shows the sequences of the dT-ACP1 (cDNA synthesis primer), arbitrary ACP34 (forward primer) and dT-ACP2 (reverse primer). *B.* This cDNA band was cloned; the sequence between 937 and 1211 (red bold) was completely homologous to mouse Clast1/LR8 (Genbank Accession number, AB031386).

Figure 2

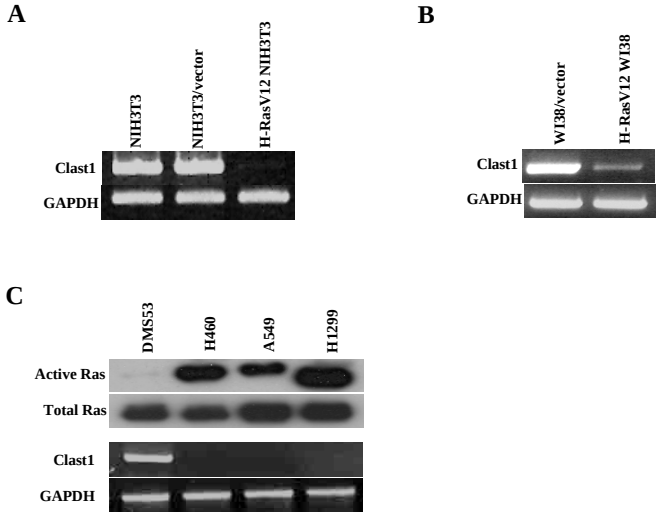


Figure 2. Clast1/LR8 expression is down-regulated by oncogenic Ras activation

A. Total RNA extracts were prepared from parent NIH3T3, NIH3T3/vector, and H-RasV12 NIH3T3 cells, and subjected to semiquantitative RT-PCR using mouse Clast1/LR8-specific primers. B. WI38 was transiently transfected with pBabe-puro-H-RasV12-expressing vector or control vector pBabe-puro. Total RNA was isolated and human Clast1/LR8 mRNA

expression was then examined by RT-PCR using human Clast1/LR8-specific primers. C. Clast1/LR8 mRNA was significantly decreased in Ras activated human pulmonary cancer cells. Upper panel, DMS53, H460, A549, and H1299 cells were lysed and subjected to quantitation of active Ras-GTP by pull-doan assays followed by immunoblotting as described in Material and Methods. Samples of lysates were also subjected to the determination of total levels of Ras by immunoblotting. Lower panel, total RNA was isolated from DMS53, H460, A549, and H1299 cells, and subjected to RT-PCR analysis using human Clast1/LR8-specific primers. GAPDH was used as a loading control.

Oncogenic H-Ras suppresses Clast1/LR8 expression through MEK/ERK pathway

We next clarified the signaling pathway by which oncogenic Ras suppressed clast1/LR8 expression. Because MAPKs and PI3K/Akt are two major downstream signaling pathway for oncogenic Ras, we tested whether theses kinases might be involved in the down-regulation of Clast1/LR8 by oncogenic Ras. H-RasV12 NIH3T3 cells were treated with pharmaceutical inhibitors of specific signaling pathway and approximately 24 h later cells were harvested and measured the level of Clast1/LR8 mRNA. Treatment of H-RasV12 NIH3T3 cells with MEK/ERK inhibitor, PD98059, led to restore the level of Clast1/LR8 mRNA expression suppressed by oncogenic Ras (Fig. 3). However, p38 and JNK pathway inhibitors, SB203580 and SP600125 could not reverse the oncogenic Ras-related suppression of Clast1/LR8 expression. In

addition, treatment the same cells with the PI3K inhibitor, LY294002, had no effect on the suppression of the Clast1/LR8 expression by oncogenic Ras. These results indicate that downregulation of Clast1/LR8 by oncogenic Ras is mainly through activation of MEK/ERK and not PI3K, p38 or JNK signaling pathway.

Figure 3

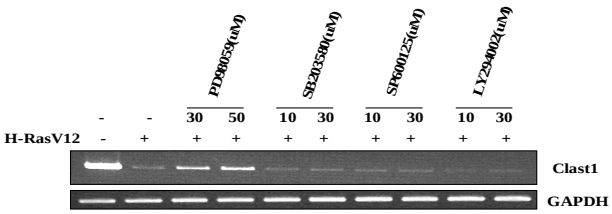


Figure 3. The EMK/ERK signaling pathway participates in the suppression of Clast1/LR8 by oncogenic H-Ras.

H-RasV12 NIH3T3 cells were treated with vehicle (0.02% dimehtyl sulfoxide (DMSO)) or different kinase inhibitors, PD98059 (30 and 50 μM) for MEK11, SB203580 (10 and 30 μM) for p38, SP600125 (10 and 30 μM) for JNK, LY294002 (10 and 30 μM) for PI3K, for 24 h. Clast1/LR8 expression was then examined via RT-PCR. GAPDH was used as a loading control.

DNA methylation and histone deacetylation contribute to the oncogenic Ras-mediated down-regulation of Clast1/LR8 expression.

We next investigated whether activation of histone deacetylase (HDAC) and hypermethylation are involved in the down-regulation of Clast1/LR8 by H-RasV12. NIH3T3/vector and H-RasV12 NIH3T3 cells were treated with or without AZC, an inhibitor of DNA methylation and the histone deacetylase inhibitor, TSA. Clast1/LR8 mRNA levels were then determined by RT-PCR analysis. As shown in Figure 4A, AZC and TSA effectively reversed the H-RasV12-induced down-regulation of Clast1 mRNA in NIH3T3 cells. We next determined whether the above regulatory mechanism in mouse fibroblast NIH3T3 cells occurred in human lung cancer cells harboring the constitutively active K-RAS mutations. We chose three human lung cancer cell lines, H460, H1299, and A549, to address this issue. We found that Clast1/LR8 mRNA expression increased in all three human cancer cells following treatment with AZC and TSA (Fig. 4B). These results suggest that hypermethylation and histone deacetylation may be involved in the oncogenic Ras-mediated down-regulation of Clast1/LR8 transcription in mouse fibroblast and human lung cancer cells.

Figure 4

A

B

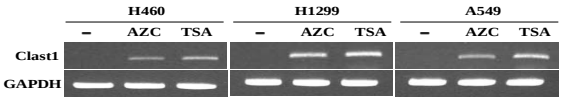


Figure 4. Effect of DNA methylation and histone deacetylase inhibitors on oncogenic Ras-mediated down-regulation of Clast1/LR8.

A. NIH3T3/vector and H-RasV12 NIH3T3 cells were treated with or without 10 μM 5-aza-2′-deoxycytidine (AZC) for 48 h or 30 nM trichostatin A (TSA) for 24 h, and Clast1/LR8 expression was examined via RT-PCR. *B*. The human lung cancer cell lines, H460, A549, and H1299 were treated with or without 10 μM AZC for 4 days, or 100 nM TSA for 24 h. Clast1/LR8 expression was then examined via RT-PCR using human Clast1/LR8-specific primers. GAPDH was used as a loading control.

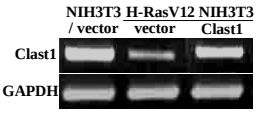
Clast1 prevents EGF-stimulated Akt phosphorylation in H-RasV12-transformed NIH3T3 cells.

To determine the effects of the ectopic expression of Clast1 on these two major signal

transduction pathways, including Raf/MEK/ERK and the PI3K/Akt signaling, we analyzed the effects of Clast1 on EGF-stimulated ERK1/2 and PI3K activation in H-RasV12 NIH3T3 cells stably transfected with control vector or Clast1-over-expressing vector (Fig. 5*A*). Cells were serum-starved for 13 h and then stimulated with EGF (50 ng/ml) for 2 h. H-RasV12 NIH3T3 cells exhibited increased levels of phospho-ERK1/2 and phospho-Akt under EGF stimulation (Fig. 5B). However, Clast1 expression led to significant suppression of EGF-induced phospho-Akt in H-RasV12 NIH3T3 cells compared to vector-transfected cells. In contrast with our results regarding the Akt pathway, Clast1 did not prevent EGF-induced ERK1/2 phosphorylation.

Figure 5

A



B

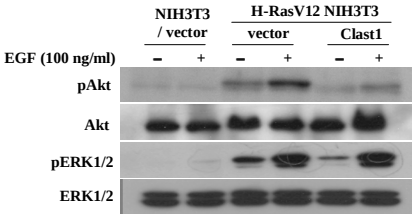


Figure 5. Effect of Clast1/LR8 on the Ras signaling pathway in NIH3T3 cells.

A. Clast1/LR8 expression was analyzed by RT-PCR in H-RasV12 NIH3T3 cells stably transfected with control empty vector and Clast1/LR8 expressing vector. *B*. NIH3T3/vector (NIH3T3), vector-transfected H-RasV12 NIH3T3, and Clast1/LR8 expressing H-RasV12 NIH23T3 cells were were plated on day 0 in DMEM containing 10% FBS, washed twice in PBS on day 2, and then incubated in serum-free medium for 13 h. Cells were then stimulated with (+) or without (–) EGF (50 ng/ml) for 2 h, and whole-cell lysates were separated by SDS-PAGE. Western blot analyses for phosphorylated ERK1/2 and Akt were performed, in addition to blot analyses to determine total ERK1/2 and Akt protein expression. Data are

representative of three independent experiments.

Clast1/LR8 inhibits oncogenic Ras-mediated transformation.

Oncogenic Ras-mediated down-regulation of Clast1/LR8 may be an important event required for oncogenic Ras-induced cellular transformation. To gain further insight into this issue, we co-expressed Clast1/LR8 with oncogenic H-Ras to elucidate whether exogenous Clast1/LR8 would prevent oncogenic-induced cellular transformation. We reasoned that if decreased levels of Clast1/LR8 are a prerequisite for oncogenic Ras to successfully transform cells, then exogenously expressed Clast1/LR8 should alter oncogenic Ras-induced cellular transformation. To determine whether Clast1/LR8 participates in Ras-mediated anchorage-independent growth of NIH3T3 cells in soft agar, we used parent NIH3T3 cells, vector-transfected H-RasV12 NIH3T3 cells, and Clast1/LR8-transfected H-RasV12 NIH3T3 cells. Indeed, colony formation in Clast1/LR8 expressing H-RasV12 NIH3T3 cells was significantly inhibited compared to vector-transfected H-RasV12 NIH3T3 cells (Fig. 6A).

To determine whether Clast1/LR8 also suppresses anchorage-independent tumor growth in Ras-activated human cancer cells, we used K-Ras-activated H460 and A549 cells stably transfected with the Clast1/LR8 expression vector or empty control vector. Stable cell clones overexpressing Clast1/LR8 or a control vector were isolated

following selection with hygromycin, pooled, and used for subsequent gene expression and functional studies. We confirmed that the level of Clast1/LR8 transcripts increased markedly in Clast1/LR8 transfected cells, compared to parent and control vector-transfected cells. Additionally, the over-expression of Clast1/LR8 significantly inhibited anchorage-independent growth in both H460 and A549 cells (Fig. 6B and C). The size and density of anchorage-independent colonies of stable transfectants in both lung cancer cell lines were much lower than in control cells. Taken together, these results suggest that Clast1/LR8 suppresses the oncogenic potential of H-RasV12 NIH3T3 cells and lung cancer cells H460 and A549 cells, as reflected by a much lower anchorage-independent growth rate.

Figure 6

A

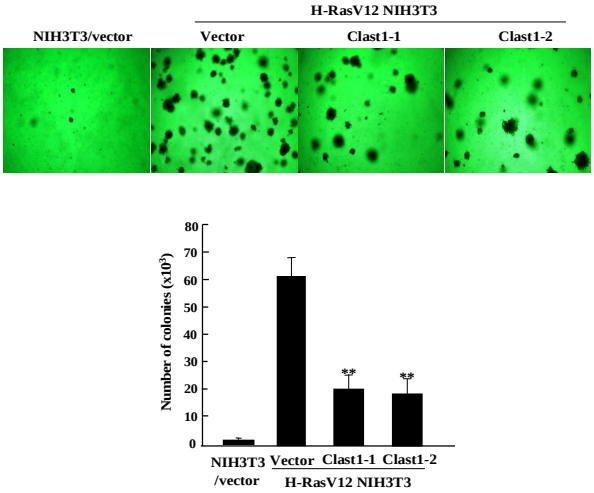


Figure 6 B

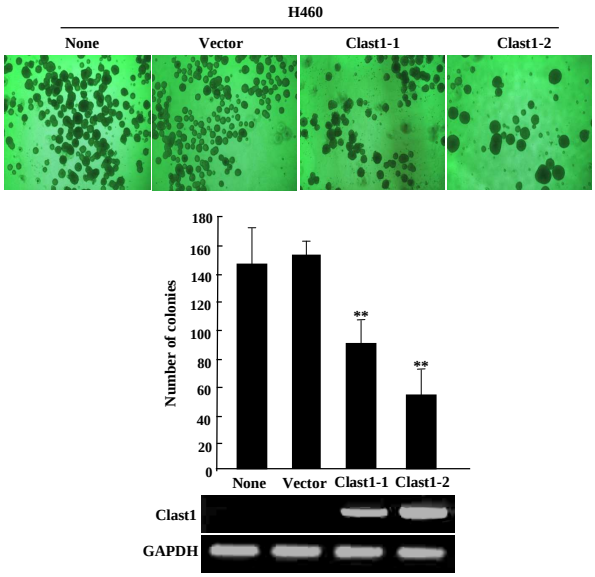


Figure 6 C

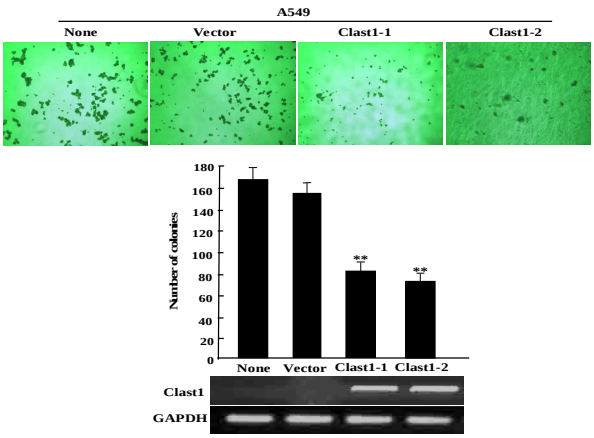


Figure 6. Expression of exogenous Clast1/LR8 inhibits oncogenic Ras-mediated cellular transformation.

A. Clast1/LR8 expression inhibited H-RasV12-induced anchorage-independent tumor growth. Cells (2×10^4) were plated in 0.3% agar containing 10% FBS on 0.5 % base agar. Photographs of representative experiments were taken after 20 days of growth. Each value is the mean ± s.d. from three separate experiments. **, $P < 0.01$. (B, C) Clast1/LR8 expression inhibited anchorage-independent tumor growth in H460 and A549 cells. H460 (B) and A549 (C) cells were stably transfected with control vector or Clast1/LR8-expressing vector. A soft agar assay was performed as shown in (A). Photographs of representative experiments were taken after 20 days of growth. Each value is the mean ± s.d. from three separate experiments. **, $P < 0.01$. RT-PCR data showed that Clast1/LR8 expression increased significantly in Clast1/LR8-transfected H460 and A549 cells.

Clast1/LR8 overexpression interferes with cell invasion.

To investigate potential inhibitory effects of Clast1/LR8 on oncogenic Ras-induced NIH3T3 cell invasion, we performed an *in vitro* invasion assay using 24-well trans-well units with polycarbonate filters coated on the upper side with Matrigel. Invasive cells found on the lower surface of the inserts were quantified after 48 h. Our results showed that Clast1/LR8 expressing H-RasV12 NIH3T3 cells were much less invasive than control vector-transfected H-RasV12 NIH3T3 cells (Fig. 7*A*).

We next examined whether Clast1/LR8 decreases invasiveness in lung cancer H460

and H549 cells. As shown in Figure 7, the over-expression of LR8 significantly inhibited the invasive potential of both H460 and A549 cells when compared to control cells (Fig. 7*B* and *C*). The number of cells that crossed the Matrigel barrier to the lower surface of the insert was approximately 2.4- and 1.9-fold lower in Clast1/LR8 stable transfectants of H460 and A549 cells, respectively, compared to control vector-transfected cells.

Figure 7

A

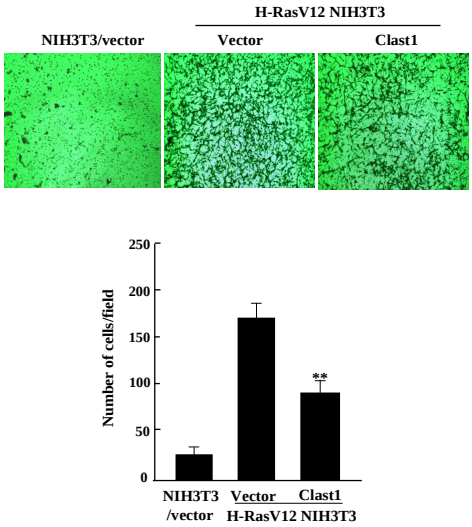


Figure 7

B

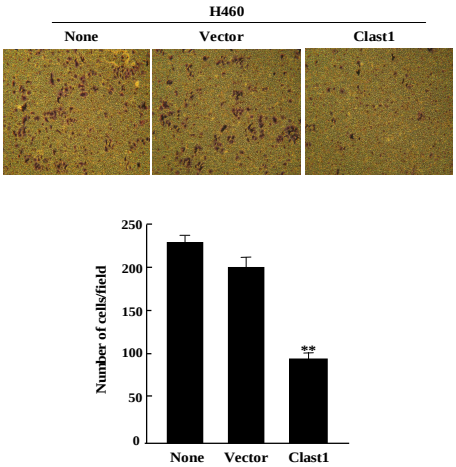


Figure 7

C

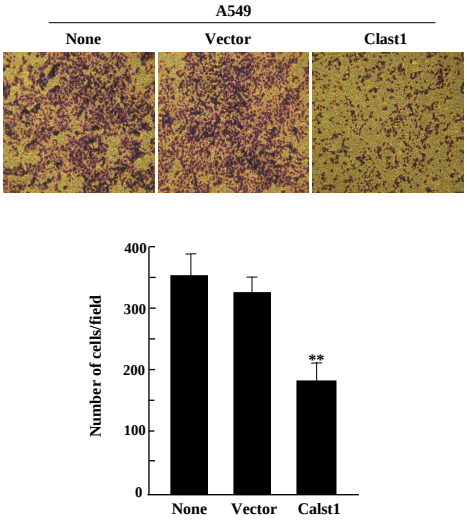


Figure 7. Clast1 over-expression suppress H-RasV12-induced cell invasion.

An *in vitro* invasion assay was performed using 24-well trans-well units with polycarbonate filters coated on the upper side with Matrigel. 5×10^4 NIH3T3 (*A*), H460 (*B*), and A549 (*C*) transfectants were placed in the upper part of the transwell unit and incubated for 48 h. The figures are representative for cell invasion in each group. Cells that penetrated to the bottom surface of the membrane were fixed, stained, and counted under a microscope. The histograms show the average number of invading cells. Each value is the mean ± s.d. from three separate experiments. **, $P < 0.01$.

Clast1/LR8 suppresses cell proliferation and in vivo tumorigenesis by the H-Ras oncogenes.

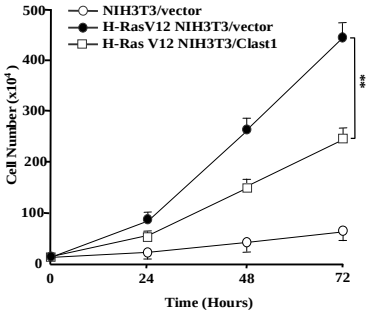
To investigate whether the down-regulation of Clast1/LR8 contributes to the oncogenic Ras-induced cell proliferation in NIH3T3 cells, we compared the proliferation rates of control NIH3T3 cells, clast1-expressing H-RasV12 NIH3T3 cells, and vector-transfected H-RasV12 NIH3T3 cells under normal culture conditions. Equal numbers of cells were seeded and grown for up to 3 days. Under these conditions, Clast1/LR8 expression led to the suppression of H-RasV12 NIH3T3 cell proliferation (Fig. 8*A*). Moreover, BrdU incorporation assays revealed that Clast1/LR8 expression prevented DNA synthesis in H-RasV12 NIH3T3 cells (Fig. 8*B*), indicating

that Clast1/LR8 inhibits Ras-induced cell proliferation.

We next examined whether Clast1/LR8 participates in oncogenic Ras-mediated tumorigenesis *in vivo*. First, we asked whether Clast1/LR8 expression inhibits H-Ras-mediated tumor formation in NIH3T3 cells implanted into in nude mice. Parent NIH3T3 cells and H-RasV12 NIH3T3 cells expressing either pcDNA3 or Clast1/LR8 were subcutaneously implanted into nude mice and subsequent tumor formation was monitored over 20 days to determine the efficacy of Clast1/LR8 molecules in blocking Ras-mediated murine fibroblast tumorigenesis *in vivo*. Our results showed that vector-transfected H-RasV12 NIH3T3 cells gave rise to significantly larger tumors, whereas Clast1-overexpressing H-rasV12 NIH3T3 cells exhibited markedly diminished tumor formation (Fig. 8*C* and *D*). These results clearly show that Clast1/LR8 expression drastically reduces tumor formation by oncogenic H-RasV12-transformed NIH3T3 cells *in vivo*.

Figure 8

A



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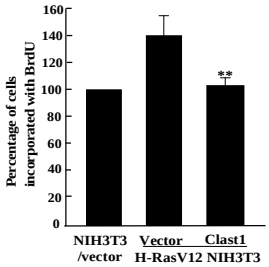
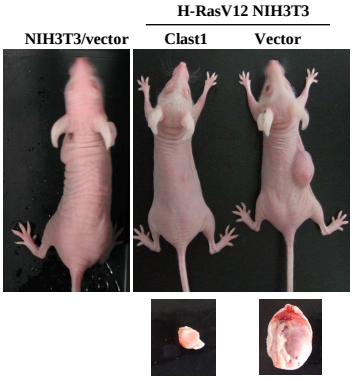


Figure 8

C



D

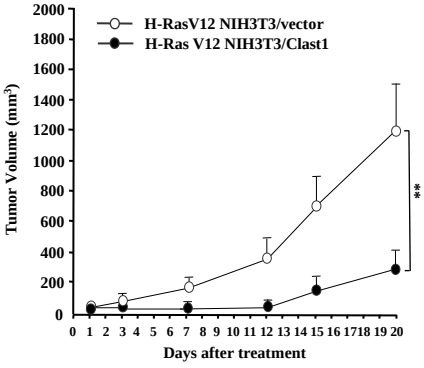


Figure 8. Ectopic expression of Clast1 inhibits H-RasV12-induced cell proliferation and tumor formation in nude mice.

A. NIH3T3/vector, vector-transfected H-RasV12 NIH3T3, and Clast1/LR8 expressing H-RasV12 NIH3T3 cells were plated in DMEM containing 10% FBS, washed twice in PBS on day 2, and then incubated in serum-free medium for 13 h to synchronize cells. Subsequently, the growth medium was re-integrated with 10% FBS, and the cells were grown for an additional 3 days. Data are presented as mean ± SD of three experiments. . **, $P < 0.01$. *B*. BrdU incorporation assays were performed using the representative Clast1-over-expressing clones indicated in *A*. The percentage of cells incorporating BrdU after 18 h was shown. Data are presented as the mean ± SD three experiments. . **, $P < 0.01$. *C*. One million Clast1/LR8 expressing H-RasV12 NIH3T3 cells were injected subcutaneously into the right flank of 5-week-old nude mice and tumor volume was assessed every 2 to 5 days for 20 days. One million NIH3T3/vector and control vector-transfected H-RasV12 NIH3T3 cells were also injected as controls. The upper panels show representative mice from each injection group and the low panels show the corresponding tumors that developed at the injected sites. The expression of Clast1/LR8 dramatically suppressed tumor growth from H-RasV12 NIH3T3 cells in nude mice when compared to H-RasV12 NIH3T3 cells transfected with the control vector alone. *D*. The graph displays tumor growth rates for H-RasV12 NIH3T3 transfected with empty vector and H-Ras V12 NIH3T3 cell expressing Clast1/LR8 in nude mice. Data are presented as the mean ± SD three experiments. . **, $P < 0.01$.

IV.DICUSSION

In this study, we identified Clast1/LR8 as one of several genes down-regulated in oncogenic H-Ras expressing NIH3T3 cells using differential display RT-PCR analysis. Clast1/LR8 was originally cloned in two human lung fibroblast subpopulations using mRNA differential display analysis (31). Mouse Clast1/LR8 shares 62% similarity with human LR8 and contains four predicted transmembrane domains. Clast1 mRNA is ubiquitously expressed in various organs, particularly in the lung, liver, kidney, and colon of adult mice (30). Clast1 mRNA was induced by the CD40 ligand in B lymphocytes. Although Clast1-knockout mice did not exert any functional B cell abnormality, they did exhibit severe ataxia, indicating that Clast1/LR8 may be involved in the development of cerebellar granule cells. However, its physiological function is largely unknown.

The expression of Clast1/LR8 in NIH3T3 cells was dramatically reduced in the presence of H-RasV12. H-RasV12 expression also markedly suppressed Clast1/LR8 expression in normal human fibroblast WI38 cells. Mutations in K-Ras have been

frequently noted in lung cancer tissues and cell lines (32, 33) and the over-expression of K-Ras oncogene in mice causes lung tumor development (34). Thus, we investigated the correlation between Clast1/LR8 expression and Ras activation in human lung cancer cells, including H460, A549, DMS53, and H1299 cell lines. H460 and A549 cells contain an activated K-Ras gene mutation, and H1299 cells contain an activated N-Ras gene mutation. Thus, these three human cancer cell lines exhibited high Ras activity. In contrast, DMS53 cells have a wild-type Ras gene, resulting in low Ras activity. The Clast1/LR8 transcript was undetectable in H460, A549 and H1299 cells bearing high Ras activity, but was relatively abundant in DMS53 cells containing low Ras activity, suggesting that the constitutive activation of Ras is involved in the suppression of Clast1/LR8 expression in human lung cancer cells.

We have investigated the role of Ras signal machinery in Clast1/LR8 suppression and observed that activation of the MEK/ERK signaling pathway suppressed the Clast1/LR8 transcription in oncogenic H-Ras expressing NIH3T3 cells. Using the MEK-specific inhibitor PD98059 revealed that MEK/ERK signal was participated in the down-regulation of Clast1/LR8 by oncogenic Ras. These results suggest that the constitutive activation of Ras might be involved in the suppression of Clast1/LR8 expression through MEK/ERK signaling pathway.

A genome-wide survey of oncogenic Ras transformation targets suggests that both

gene activation and repression are critical for the transformation by oncogenic Ras (26). Although the molecular mechanism controlling gene silencing in tumor suppressor genes is still unknown, recent studies suggest that DNA methyltransferases (DNMTs) may be involved in this process. For example, DNA methyltransferase (DNMT) was involved in Ras-mediated suppression of tumor suppressor genes, such as *FHIT*, *TLSC1*, and *RASSFIA* (35). Increased DNMT activity was critical for the transformation and gene repression by oncogenic Ras (36). In addition, Ras-induced loss of the proapoptotic response protein PAR-4 was mediated by DNA hypermethylation through Raf-dependent and Raf-independent signaling pathway in epithelial cells (37). More recently, Chang et al. reported oncogenic Ras suppressed the metastatic suppressor RECK through upregulation of DNMT expression (38). In the present study, we found that oncogenic Ras-mediated Clast1/LR8 gene suppression was reversed in NIH3T3 cells after treatment with a DNA methylation inhibitor (AZC). Treatment with AZC also up-regulated Clast1/LR8 mRNA expression in the human lung cancer cell lines, H460, A549, and H1299. Because MEK/ERK is contributed to the down-regulation of Clast1/LR8 by oncogenic Ras, and because the ERK signaling pathway was involved in the activation of DNMT transcription by oncogenic Ras (38), we think that DNA methylation might be contributed to the suppression of Clast1/LR8 expression via activation of ERK signaling cascades in oncogenic Ras expressing cells.

The detailed mechanism by which DNA methylation inhibitor up-regulates Clast1/LR8 in oncogenic Ras activated NIH3T3 and human cancer cells are under investigation in this laboratory.

Ras oncogene also suppressed tumor suppressor gene via histone deacetylation (39). Thus, we investigated whether this potential mechanism reduced Clast1/LR8 expression in oncogenic Ras activating cells. We found that suppression of Clast1/LR8 transcription was restored in oncogenic H-Ras expressing NIH3T3, H460, A549, and H1299 cells, after treatment with a histone deacetylase inhibitor (TSA). These results suggested that histone deacetylation might be also involved in the Clast1/LR8 expression in Ras-activated cancer cells. A recent work indicated that oncogenic Ras might activate ERKs to phosphorylated HDACs *in vitro* and in cells and might increase nuclear accumulation of HDACs (40). Thus, it is possible that oncogenic Ras may activate MEK/ERK to phosphorylate HDACs and induce binding HDACs to transcription factors to suppress Clast1/LR8 expression. Experiments are now undertaking to test this hypothesis.

Cellular transformation is a complex process that involves a series of cellular and molecular changes. Ras proteins exert their tumorigenic effects through the activation of an intricate signaling network consisting of multiple downstream effectors (4, 41, 42). The two major downstream effector pathways believed to play a critical role in

Ras-mediated transformation and tumorigenesis are the Raf/MEK/ERK pathway (13-15) and the PI3K-dependent pathway (16-18, 43, 44). Activation of the Raf/MEK/ERK pathway is sufficient to transform NIH3T3 mouse fibroblasts (45, 46). Ras-transformed NIH3T3 cells induced metastatic growth in mice after being injected into the tail vein of mice, which was mediated by a Raf-dependent mechanism (47). It has been also shown that oncogenic Ras down-regulated RhoB expression through the PI3K/Akt pathway, leading to the RhoB-mediated suppression of Ras/PI3K/Akt-induced transformation, invasion, and metastasis in various cancer cell lines and NIH3T3 fibroblasts (48). The observed inverse correlation between Clast1/LR8 expression and Ras activation in lung cancer cells and the down-regulation of Clast1/LR8 expression induced by H-RasV12 in NIH3T3 cells suggest that the inactivation of Clast1/LR8 expression/function may be involved in oncogenic Ras-induced neoplastic transformation. Therefore, we investigated whether Clast1/LR8 is involved in Ras-mediated cellular transformation. Recent evidence indicates that Ras down-regulates the expression of several molecules, including Par-4, Bak, and GADD153 (49-51). The exogenous expression of the transcription repressor Par-4 or GAD153 has been reported to suppress oncogenic Ras-induced transformation (50, 51). Similarly, the ectopic expression of Bak in Ras-transformed cells inhibited Ras-induced tumorigenicity (49). Here, we report that the down-regulation of Clast1/LR8

appears to be a key event during Ras-induced cellular transformation. The transfection of Clast1/LR8 cDNA resulted in a dramatic inhibition of anchorage-independent growth and cellular invasion in oncogenic H-Ras expressing NIH3T3 cells, and human lung cancer cell lines, H460 and A549. Moreover, the expression of Clast1/LR8 in H-RasV12 NIH3T3 cells significantly suppressed cell proliferation and tumor formation in mice. Thus, Clast1/LR8 is an important molecule whose down-regulation may be a prerequisite for cellular transformation induced by oncogenic Ras. Although exactly how the down-regulation of Clast1/LR8 may facilitate cellular transformation need to be elucidated, we found that the ectopic expression of Clast1/LR8 inhibited EGF-stimulated Akt phosphorylation in H-RasV12-transformed NIH3T3 cells. Because the Ras/PI3K/Akt signaling pathways are responsible for transducing oncogenic Ras-mediated tumorigenic signals (15-19, 43, 44, 48), it is possibility that the down-regulation of Clast1/LR8 expression induced by oncogenic Ras might contribute to the activation of the Ras downstream PI3K/Akt signaling pathway, leading to facilitate cellular transformation and invasion. Given the fact that Clast1/LR8 negatively modulates oncogenic Ras-induced cell growth and invasion, decreases in expression and function are likely to have profound consequences. It is possible that the oncogenic activation of growth stimulatory signals, such as Raf/MEK/ERK and PI3K/Akt, and the suppression of growth inhibitory signals, such as Par-4, Bak,

GADD153 and Clast1/LR8, may occur simultaneously and contribute to oncogenic transformation. In this context, low levels of growth inhibitory molecules would provide crucial information for the initiation and maintenance of oncogenic Ras transformation.

In summary, the data obtained in this study provide information on one of the novel Ras downstream target molecules, Clast1/LR8, which may play an important role in tumor suppression in Ras-activated cancer cells. The involvement of Clast1/LR8 has been demonstrated as a phenotypic suppressor of transformation induced by oncogenic Ras. Thus, Clast1/LR8 may represent another potential target for pharmacological re-expression as a novel mode for cancer treatment, specifically with regards to Ras-activated lung cancer cells.

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Footnotes

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The Abbreviations used are: PI3K, phosphoinositide 3-kinase; EGF, epidermal growth factor; ACP, annealing control primers; AZC, 5-aza-2′-deoxycytidine; TAS, trichostatin A; RBD, RAS binding domain; HDAC, histone deacetylase.

저작물 이용 허락서

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의 여부: 동의 (O) 반대 ( )

2008 년 8 월

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