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The mechanism of NGEF function

조선대학교 대학원

생물신소재학과

김 민 지

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NGEF의 작용 기작

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이 논문을 이학석사 학위신청 논문으로 제출함.

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The mechanism of NGEF function

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ABSTRACT

Ras oncogenes are thought to play a role at multiple stages of tumorigenesis. Althogh the oncogenic effect of Ras are well known, downstream target molecules of oncogenic Ras, Which is involved in tumor progression, are not fully elucidated. In this report, we found that the levels of neuronal guanine nucleotide exchange factor (NGEF) mRNA and protein are significantly increased in oncogenic H-RasV12-transformed NIH3T3 cells. The levels of NGEF mRNA and protein were decreased in H-RasV12-transformed cells transient transfected with a dominant negative form H-RasN17, and treatment of ERK and PI3K inhibitors led to significant suppression of oncogenic H-Ras-induced NGEF expression. In addition, the expression of NGEF were capable of activating the small GTPase (Rho, Rac1, Cdc42), and transfection of NGEF siRNA into H-RasV12-transformed cells resulted in a decrease in the activity of small GTPase (Rho, Rac1, Cdc42). To investigate the biological function of oncogenic H-Ras-induced NGEF expression, we examined whether NGEF is involved in oncogenic H-Ras-mediated increase in cancer progression. We found that the abilities of cellular proliferation, colony formation in soft agar and aggregation of NGEF expressing cells were significantly increased as compared with those of empty vector transfected cells. The abilities of cellular proliferation, colony formation and aggregation of H-RasV12-transformed NIH3T3 cells were significantly suppressed by transfection of NGEF siRNA. We further demonstrated that the transfection of NGEF siRNA into H-RasV12-transformed NIH3T3 cells led to suppression of *in vitro* cellualr migration, invasion and angiogenesis. In addition, NGEF

siRNA transfected H-RasV12-transformed cells exhibited significant reduction of animal tumor growth, wherase control siRNA transfectants did not. These results suggest that NGEF is a novel downstream target protein of oncogenic H-Ras, and oncogenic H-Ras-induce NGEF expression may be important role for oncogenic H-Ras-mediated tumor progression.

I. INTRODUCTION

RAS PROTEINS FUNCTION AS MEMBRANE-ASSOCIATED GTPASE SWITCHES

The three human *ras* genes encode four highly homologous 188-189 amino acid (21kDa) proteins: H-Ras, N-Ras, K-Ras4A and K-Ras4B (due to alternative exonutilization) proteins (Barbacid, 1987). Mutated *ras* genes are associated with 30% of all human cancers, with highest frequencies associated with pancreatic, lung, and colon carcinomas. These mutated *ras* genes encode structurally mutated proteins, most commonly with single amino acid substitutions at residues 12, 13, or 61. H-Ras, N-Ras and K-Ras have identical sequences covering the effector, exchange factor and guanine-nucleotide-binding domains. The only region of the Ras isoforms that exhibits significant sequence divergence is the final 24 residues of the protein, the hypervariable region (HVR), which exhibits approximately 10-15% conservation compared with >90% identity over the N-terminal 165 residues (Figure.1).

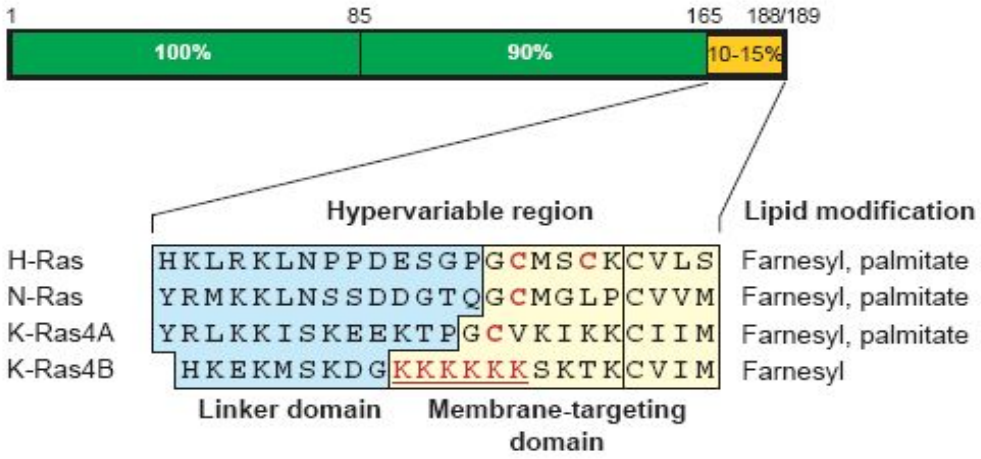

Fig.1. Ras hypervariable region

1. Ras functions as a GTP/GDP-regulated molecular switch

Ras proteins are GTPases that act as molecular switches, transmitting signals from activated receptors to downstream effectors to mediate cell proliferation, survival and differentiation (Figure.2). Ras proteins cycle between a GTP-bound (active) and GDPbound (inactive) state (Bourne et al., 1990). In resting cells, approximately 5% of Ras proteins are GTP-bound. Upon activation by extracellular stimuli, there is a rapid and transient increase (up to 70%) in Ras-GTP levels. Ras proteins have the intrinsic ability to undergo GDP/GTP cycling. GTPase activity hydrolyzes bound GTP in order to limit proliferative signaling, and nucleotide exchange activity releases GDP to allow GTP binding and activation. However, these intrinsic activities are too low for rapid GDP/GTP cycling therefore two distinct classes of regulatory proteins accelerate Ras protein cycling (Bourne et al., 1990). First, intrinsic GDP/GTP exchange is enhanced by guanine exchange factors (GEFs) (Figure.2). Ras GEFs include Sos, RasGRF, and RasGRP. Second, intrinsic GTPase activity is stimulated by GTPase activating proteins (GAPs). These include p120 RasGAP and neurofibromin, the gene product of the NF1 tumor suppressor protein. Mutant Ras proteins are insensitive to GAP-induced GTP hydrolysis, rendering Ras constitutively GTP-bound and active in the absence of extracellular signals (Figure.2).

2. Association with the plasma membrane is critical for Ras function

In addition to GDP/GTP-binding, a second key requirement for Ras function is its association

with the inner face of the plasma membrane (Cox and Der, 1997). Ras proteins are synthesized initially as cytosolic, inactive proteins. They then undergo a rapid series of posttranslational modifications that facilitate their association with the inner face of the plasma membrane. These modifications are signaled by a carboxyl terminal CAAX tetrapeptide motif found on all Ras proteins, where C = cysteine, A = aliphatic amino acid and X = serine or methionine. First, farnesyltransferase (FTase) catalyzes the addition of a C15 farnesyl isoprenoid to the cysteine residue of the CAAX motif. Second, proteolysis of the AAX residues is mediated by endoprotease activity. Finally, carboxymethylation of the now terminal farnesylated cysteine occurs. H-Ras, N-Ras and K-Ras4A are modified further by carboxyl terminal palmitylation at a cysteine residue(s) positioned upstream of the CAAX motif, whereas the second localization signal for K-Ras4B is provided by a lysine-rich polybasic sequence. The CAAX-mediated modifications, together with these second signals, are necessary and sufficient for plasma membrane localization and Ras function. The critical requirement for Ras association with the plasma membrane has prompted considerable effort to identify pharmacologic approaches to block the CAAX-mediated modifications to then block Ras function (Oliff, 1999; Cox, 2001). Of these efforts, the development of FTase inhibitors (FTIs) has been the most intensively evaluated and developed. Currently, several FTIs are under evaluation in phase I/II clinical trials. However, a surprising outcome in these efforts has been that, while FTIs have shown impressive anti-tumor activity in preclinical studies, FTIs are believed to inhibit tumor growth by blocking the function of a farnesylated protein(s) either in addition to, or instead of, Ras.

Therefore, inhibitors of Ras signaling have been considered as another approach to block Ras function, making a clear delineation of the critical signaling events involved in Ras-mediated oncogenes imperative for the success of these efforts.

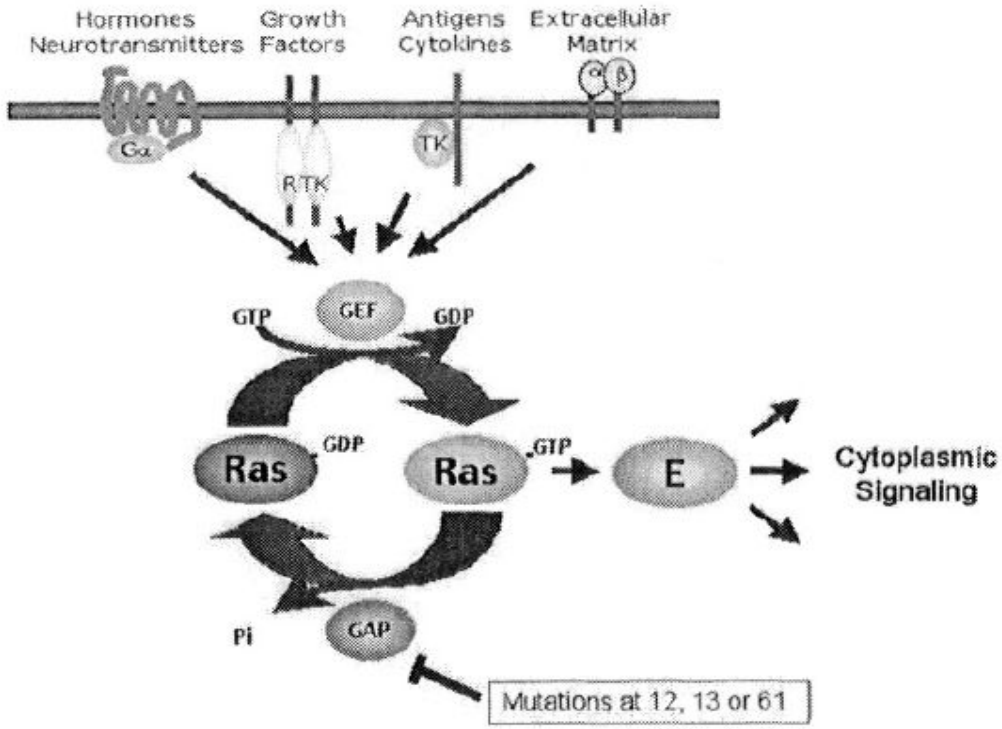

Fig.2. The Ras pathway in cancer

3 Multiple Ras proteins

The different Ras isoforms share significant sequence identity (85%) and biochemical function (common regulators and effectors), and mutated forms of each show comparable transforming activities. This and other evidence initially led to the belief that Ras proteins were functionally identical. However, there are a limited number of observations that suggest some

functional differences. For example, mutations in K-*ras* and N-*ras* occur more frequently than H-*ras* in human tumors (Bos, 1989; Clark and Der, 1993). Recently, evidence has arisen that there is differential intracellular trafficking of Ras proteins as well as isoform-specific differences in their association with specific regions of the plasma membrane (Reuther and Der, 2000; Wolfman, 2001). Also, gene knockout studies in mouse models revealed that K-*ras* is necessary for development, whereas H-r*as* and N-*ras* are not (Bar-Sagi, 2001). Finally, whereas H-Ras activity is sensitive to inhibition by FTIs, K-Ras and N-Ras functions are not (Oliff, 1999; Cox, 2001). While these various observations support functional distinctions, clear and significant functional differences important for the mechanism of Ras-mediated oncogenesis remain to be identified (Figure.3).

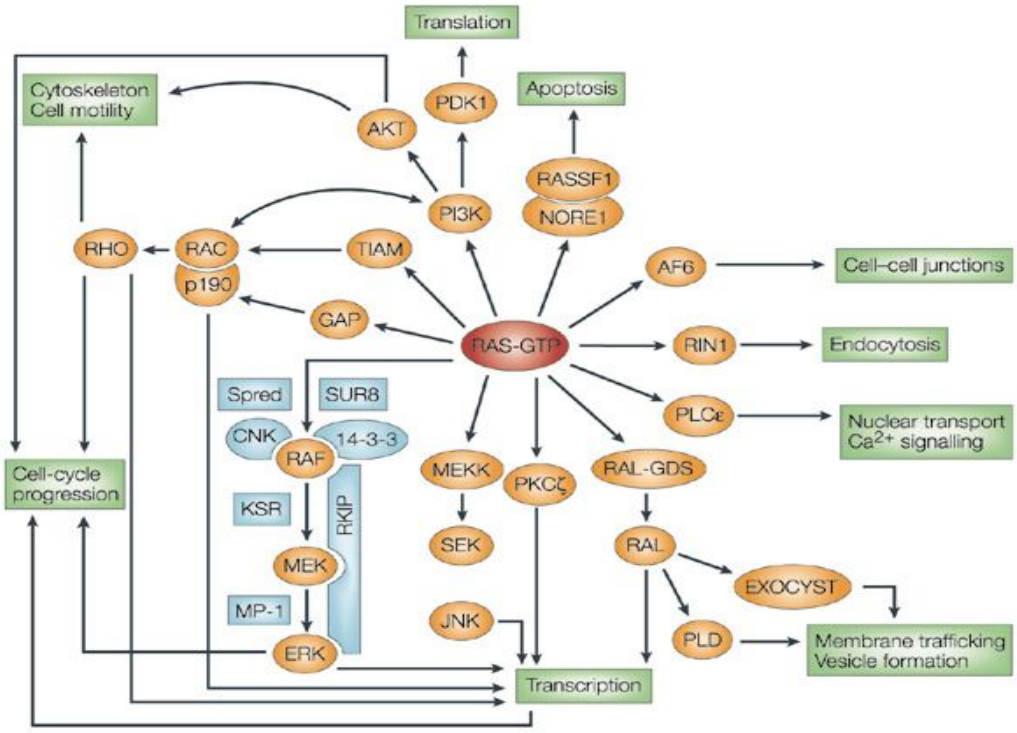

Fig.3. Multiple Ras effectors

At the cellular surface, many different receptors are expressed that allow cellular response to extracellular signals provided by the environment. After ligand binding, receptor activation leads to a large variety of biochemical events in which small guanosine triphosphate hydrolases (GTPases; eg, Ras) are crucial. Ras proteins are prototypical G-proteins that have been shown to play a key role in signal transduction, proliferation, and malignant transformation. G-proteins are a superfamily of regulatory GTP hydrolases that cycle between 2 conformations induced by the binding of either guanosine diphosphate (GDP) or GTP (Sprang, 1997; Bos,

1998; Rabollo and Martinez, 1999) (Figure.4). The Ras-like small GTPases are a superfamily of proteins that include Ras, Rap1, Rap2, R-Ras, TC21, Ral, Rheb, and M-Ras. The *RAS* gene family consists of 3 functional genes, H-*RAS,* N-*RAS*, and K-*RAS*. The *RAS* genes encode 21-kd proteins, which are associated with the inner leaflet of the plasma membrane (H-Ras, N-Ras, and the alternatively spliced K-RasA and K-RasB). Whereas H-Ras, N-Ras, and K-RasB are ubiquitously expressed, K-RasA is induced during differentiation of pluripotent embryonal stem cells in vitro (Pells, Divjak, Romanowski et al., 1997).

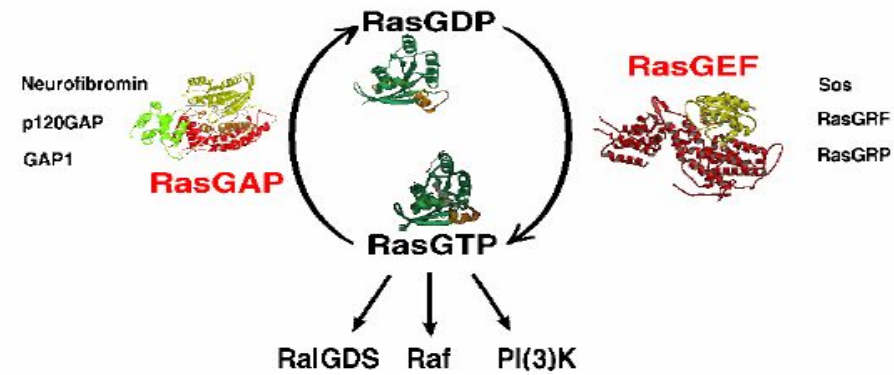

Fig.4. The switch function of Ras

Regulatory proteins that control the GTP/GDP cycling rate of Ras include GTPase activating proteins (GAPs), which accelerate the rate of GTP hydrolysis to GDP, and guanine nucleotide exchange factors (GEFs; eg, SOS and CDC25), which induce the state, Ras couples the signals of activated growth factor receptors to downstream mitogenic effectors. By definition, proteins that interact with the active GTP-bound form of Ras (and thus become GTP-dependently activated) to transmit signals are called Ras effectors (Wittinghofer, 1998; Van

Aelst, 1994; Marshall, 1996; Katz and McCormick, 1997). Mechanisms by which GTP-Ras influences the activity of its effectors include direct activation (eg, B-Raf, PI-3 kinase) recruitment to the plasma membrane (eg, c-Raf-1) and association with substrates (eg, Ral-GDS). Other candidates for Ras effectors include protein kinases, lipid kinases, and GEFs.

Although the oncogenic effect of Ras are well known, downstream target molecules of oncogenic Ras, which is involved in tumor progression, are not fully eluciated. In this study, we sought to determine which genes are regulated by oncogenic H-Ras, particularly those that might be involved in oncogenic Ras-mediated cancer progression, using Gene Fishing RT-PCR analysis. This study reports an oncogenic H-Ras target genes, neuronal guanine nucleotide exchange factor (NGEF), which were identified through this screening, contributes to the oncogenic H-Ras-mediated increase in tumor cell proliferation, migration, angiogenesis and invasion. This study is the first to indicate that NGEF is a downstream target molecule of oncogenic Ras and is regulated for oncogenic Ras-induced tumorigenesis.

II. MATERIALS AND METHODS

1. Reagents and Antibodies

The following pharmacological inhibitor at indicated working concentrations were employed in our studies; 20μM MEK1/2 inhibitor U0126 (Calbiochem); 50μM MEK1/2 inhibitor PD98059 (Calbiochem); 20μM JNK inhibitor SP600125 (Calbiochem); 20μM p38 MAPK inhibitor SB203580 (Calbiochem); 30μM PI3K inhibitor LY294002 (Calbiochem): All inhibitors were dissolved as concentrated stock solutions in DMSO and diluted at the time of treatment with medium. Control cells were treated with medium containing an equal concentration of DMSO. Anti-H-Ras (F235), anti-MMP-2(2C1), anti-MMP-9(C-20), anti-VEGF(C-1), anti-α−tubulin antibody was purchased from Santa cruz. Anti-phospho (Thr202/Tyr204)-p44/42, anti-p44/42, anti-phospho (Thr180/Tyr182)-p38, anti-p38, anti-phospho (Thr183/Tyr185)-SAPK/JNK, anti-SAPK/JNK, anti-phospho (Ser473)-Akt, anti-Akt antibody was purchased from Cell Signaling Technology. Anti-Ras, anti-Rho, anti-Cdc42, anti-Rac1 antibody was purchased from PIERCE.

2. Cell culture

The mouse embryo fibroblast NIH3T3 cells were purchased from American Type Culture Collection (ATCC number CRL-1658) and grown in DMEM medium supplemented with 10% fetal bovine serum, 100 units penicillin/mL, and 100 μg streptomycin/mL (Invitrogen, Carlsbad,

CA). The cells were maintained in 5 % CO_2 -95% air at 37 ^{o}C in a humidified incubator.

3. Plasmid constructs and Transfection

Mouse NGEF cDNA were amplified by RT-PCR using the mNgef oligo primer (sense 5'-ATG GAG ACC AAA AAC TCT GAA GAC-3' and antisense 5'-TTG CCG ATT CCG GCT GCC C-3') from mouse fibroblast NIH3T3 cells. After confirming the sequence, the mNGEF cDNA was cloned into a pcDNA3.1/V5-His-Topo mammalian expression vector, which was driven by the CMV promoter (Invitrogen). After confirming the sequence, the mNGEF cDNA was cloned into a pcDNA3.1/V5-His-Topo mammalian expression vector, which was driven by the CMV promoter (Invitrogen). The mNgef construct was transfected into cells using the Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. After transfection, cells were incubated with complete medium containing 400ug/ml G418 for 5 weeks. The cell clones resistant to G418 were isolated and analyzed

4. Small interfering RNA (siRNA)-based experiments

Sequence information regarding the mNgef mRNA was extracted from the NCBI Entrez nucleotide data base. Three target sites within the mNgef gene were chosen from the mouse NGEF mRNA sequences (Gene Bank^{TM} accession no.BC039279). Following selection, each target site was searched with NCBI BLAST to confirm specificity only to the mNGEF. The sequences of the 21-nucleotide sense and antisense RNA are follows: mNGEF siRNA 5'-

GUUUGUAUCCUUCACAUCUUU-3' (sense) and 5'-AGAUGUGAAGGAUACAAACUU-3' (antisense) for the mNGEF gene (nt 2064-2084); LacZ siRNA, 5'-CGUACG-CGGAAUACUUCGAUU-3' (sense), 5'-AAUCGAAGUAUUCCGCGUACGUU-3' (antisense) for the LacZ gene. These siRNAs were prepared using a transcription-based method with a Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. LacZ siRNA was used as the negative control. Cells were transiently transfected with siRNA duplexes using Oligofectamine (Invitrogen). The siRNA expression vector (pSilencer hygro) for mNGEF and a control vector were employed. The construction of siRNA-expression plasmid was base on the pSilencer hygro vector (Ambion, Texas, USA). The vector included a human U6 promoter, a hygromycin resistance gene. We purchased synthetic oligo-nucleotides (Xenotech, Korea). After anneling, DNA fragments were ligated into the pSilencer hygro. Cells were transfected with the siRNA vector by using Lipofectamine (Invitrogen). After transfection with the hygromycin-resistance vector, resistant colonies were grown in the presence of Hygromycin (200ug/ml) (Invitrogen).

5. Semiquantative reverse transcriptase–polymerase chain reaction

RNA extraction was conducted using the RNA-STAT-60 according to the manufacturer's instructions (TEL-TEST, Inc., Friendswood, TX). Briefly after homogenizing cells in the RNA-STAT-60, the homogenate was mixed with chloroform (5:1,v/v) shaken vigorously for 15 s , and then centrifuged at 13,000 rpm for 15min at 4°C. The RNA present in the upper colorless

aqueous phase was precipitated by adding isopropylalcohol, which was washed twice with 70% ethanol and then air-dried for 10 min. The RNA was then resuspended in DEPC, 10-μl RNA aliquots were prepared and stored at -70°C until needed. 3μg of the total RNA was reverse-transcribed using an M-MLV cDNA synthesis system (Invitrogen), and the reverse-transcribed DNA was subjected to PCR. The profile of replication cycles was denaturation at 94 °C for 30 seconds, annealed at 60 °C for 30 seconds, and polymerized at 72 °C for 1 min. In each reaction, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. The primers used for PCR are as follows mNGEF forward, 5'-ATT GAA ACA CGG AGG CAG CAG G-3'; mNGEF reverse, 5'-TCT AGC TCC AGC AAA AAC CGC TC -3' designed to amplify a 357-bp region; mRAS forward, 5'-GGA AGC AGG TGG TCA TTG AT-3'; mRAS reverse, 5'-TCA GGA CAC ACA CTT GC-3' designed to amplify a 447-bp region; GAPDH forward, 5'-CCA TGG AGA AGG CTG GGG-3'; and GAPDH forward, 5'-TGA CCA CAG TCC ATG CCA TC-3'; and GAPDH reverse 5'-TTA CTC CTT GGA GGC CAT GT-3' designed to amplify a 492-bp region (total number of cycles: 28). The PCR products were resolved on 1 % agarose gels, stained with ethidium bromide, and then photographed.

6. Western blotting

The cell were washed with phosphate-buffered saline (PBS) and lysed on ice for 10 minutes in the M-PER mammalian protein Extraction Reagent (PIERCE) added protease Inhibitor

Cocktail tablet (Roch). After incubation, extracts were vortexed for 5min and centrifuged at 13,000rpm for 15min. The supernatant was diluted with 5X SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 20ug of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (Amersham Biosciences, Piscataway, NJ). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20(TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes were rinsed with TBS-T and then incubated with appropriate primary antibodies in TBS-T at 4℃ overnight. All antibodies used in this study are mhNGEF polyclonal antibody (pAb) and H-Ras monoclonal antibody (santa cruz) and α-tubulin monoclonal antibody (Santa Cruz) and phospho(Thr202/Tyr204)-p44/42 polyclonal antibody, phosphor(Thr180/Tyr182)-p38 polyclonal antibody, phospho (Thr 183/Tyr185)-SAPK/JNK polyclonal antibody, phospho(Ser473)-Akt polyclonal antibody (Cell Signaling Technology) and Rho monoclonal antiby, Cdc42 monoclonal antibody, Rac1 monoclonal antibody (PIERCE) and MMP-2(2C1) monoclonal antibody, MMP-9(C-20) polyclonal antibody, VEGF(C-1) monoclonal antibody (Santa Cruz) ; We followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:4,000) in a blocking buffer for 2 hours at room temperature, and washed again. The blotted proteins were developed using an enhanced

chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea)

7. DEG (Differentially Expressed Gene) experiments

DEG stands for Differentially Expressed Gene and refers to all the genes that are expressed differentially in mRNA level of two or more samples. RT is conducted using oligo dT-ACP to synthesize first-strand cDNAs from samples, wherein the 3'-end core portion of the oligo dT-ACP comprises a hybridizing sequence complementary to a poly A region of mRNA transcripts. 1st stage PCR for only one cycle is conducted using an arbitrary ACP to synthesize second-strand cDNAs under conditions that the 3'-end core portion of the oligo-dT ACP is prevented from annealing to the first-strand cDNAs and only the 3'-end core portion (10-mer) of the arbitrary ACP comprising a hybridizing sequence sufficiently complementary to a region of the first-strand cDNAs is involved in annealing to the first-strand cDNAs. 2nd stage PCR follows under high stringency conditions to amplify only the arbitrary-primed second cDNA strands generated from Step 2. Both the oligo-dT and arbitrary ACP set are involved in annealing only to the sites or complementary sites of 3'- and 5'-ends of the second cDNA strands, which results in the amplification of only real PCR products with NO false products.

8. Small GTPase activation assay

The activation of Ras, Rac1, and Rho was assayed using the EZ-Detect^{TM} Ras, Rac1, Cdc42 or Rho activation kits according to manufacturer's instruction (PIERCE). NIH3T3 cells were

plated in 100-mm plates at a density of 2×10^6 cells/plate. The next day cells were serum-starved by incubation in DMEM supplemented with 0.2% FBS for an additional 24h. Cells were stimulated with 10% FBS for the times indicated. After treatment cells were chilled on ice, washed once with ice-cold TBS and lysed in the ice-cold EZDetect™ lysis/binding/washing buffer containing a protease inhibitor cocktail. Cell lysates were clarified by centrifugation at 13,000g at 4℃ for 15 min and quantified using micro BCA protein assay with BSA as the standard. Equal amounts of lysates (1 mg) were incubated with GST-Raf1-RBD, GTS-PAK1-PBD, or GST-Rhotekin-RBD and one SwellGel™ Immobilized Glutathione disc in a spin cup with a collection tube at 4℃ for 1h. The resin was washed three times with lysis/binding/washing buffer. Bound proteins were eluted by incubation in 50μl 2X SDS sample buffer at 100°C for 5 min. Half (25μl) of the sample volume was analyzed by Western blot using the antibody against Ras, Rac1, Cdc42 or Rho provided in the kit.

9. Co - Immunoprecipitation

Cells were washed with PBS and then lysed in lysis buffer (50mM Tris, PH 7.4~7.5, 150mM NaCl, 1% Nonidet P-40, and protease inhibitor EDTA free). The resulting cell lysates were spun at 15,000g for 10min at 4℃ to pellet the unbroken cells. Protein aliquots of 2mg cell lysates were incubated overnight at RT with the desired antibodies (2ug, H-Ras, RhoA, Cdc42 or Rac1). And captured with 30ul of protein-G-agarose (Santa cruz) for 1hr at RT. The beads were collected by centrifugation, washed three times with lysis buffer, and resuspended in

sample buffer. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti- H-Ras, RhoA, Cdc42, Rac1 or NGEF antibodies.

1*0. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay*

Cell viability was determined by MTT [3-94,5-dimethylthiazole-2-yl)-2,5-diphrnyl tetrazolium bromide] assay. After treatment, 10 ㎕ of MTT (1mg/ml) in PBS was incubated with cells in a 96-well plate for 4 h at 37℃. Subsequently, the medium containing MTT was removed, and 100 ㎕ of DMSO (dimethyl sulfoxide) added. Cells were incubated for a further 10 min at 37°C with gentle shaking. The absorbance was read on an ELISA plate reader using a 540nm filter.

11. Serum stimulation and BrdU labeling

A cell proliferation enzyme-linked immunosorbent assay, BrdU (chemiluminescence) kit (Roche Diagnostics) was used to measure the incorporation of BrdU during DNA synthesis according to the manufacturer's protocol. Briefly, cells were seeded overnight in black 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson) at a density of 10,000 cells per well in 100 μL medium containing 10% BCS (4–6 wells per treatment point). Cells were treated with the desired concentrations of tempo or vehicle control (0.1% ethanol) for 24 or

48hours in medium containing 5% BCS. BrdU (10 μM) was added to the culture medium 2 hours before the termination of tempo treatment. BrdU-labeled adherent cells were fixed and DNA was denatured in FixDenat (Roche Diagnostics) for 30minutes at room temperature. Cells were incubated with Peroxidase Conjugated anti-BrdU antibody (anti-BrdU-POD) for 90 minutes at room temperature and washed 3 times with PBS. The immune complex was detected by the luminol substrate reaction, followed by measurement of chemiluminescence using a luminometer

12. Cellular aggregation

Aggregation assays were performed as described previously. For slow aggregation, single-cell suspensions were seeded on top of a semi-solid agar medium with or without NGEF. After 48h, aggregate formation was evaluated subjectively under an inverted phase-contrast microscope at a magnification of ×40. For the fast aggregation assay, single-cell suspensions were prepared with an E-cadherin-saving procedure. Particle diameters were measured in a Coulter LS200 counter and plotted as a percentage of the volume distribution.

13. Soft agar colony formation analysis

Cell transformation was evaluated with a soft-agar assay. Stable transfectants or control cells were plated 2×10^4 in duplicates in 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% bottom low-melt agarose (Bacto agar; Difco, Detroit, MI, USA)

supplemented with DMEM 10% CS. Medium was replaced every 2 to 3 days, and the cells were left for 14 days after reaching confluence. Macroscopically visible foci were then counted and photographed.

14. In vitro Migration assay using transwell

In vitro migration experiments were performed using transwell (Costar), which consist of a 24-well companion plate with cell culture inserts containing 8 mm pore size filters. Briefly, transfected NIH3T3 cells (5×10^4) with serum-free medium were added to each insert (upper chamber), and the chemoattractant (10% FCS) was placed in each well of a 24-well companion plate (lower chamber). After 24h incubation at 37°C in a 5% CO2 incubator, the upper surface of the filter was wiped with a cotton-tipped applicator to remove nonmigratory cells. Cells that had migrated through the filter pores and attached on the under surface of the filter were fixed and stained. The membranes were mounted on glass slides, and the cells from 10 random microscopic fields were counted. All experiments were run in triplicate.

15. Wound healing migration assay

Wound healing migration assay was performed as previously described. Briefly, cells were pretreated with mitomycinC (25 mg/ml) for 30 min before the injury line was made. The injury line was made on the cells plated in culture dishes at 90% confluence. After they were rinsed with phosphate-buffered saline, cells were allowed to migrate in complete media, and

photographs were taken (×40) at the indicated time points

16. In vitro Invasion assay

In vitro invasion assay was performed using BD BioCoat Invasion Assay System (BD Biosciences) according to the protocol of the manufacturer. Briefly, transfected NIH3T3 cells (5×10^4) with serum-free medium were seeded into the upper chamber of the system. The lower compartment was filled with serum-free media containing 5% bovine serum albumin. Cells were placed in the upper part of the Transwell plate, incubated for 24h, fixed with methanol, and stained with hematoxylin for 10min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×40. Ten random fields were counted for each filter, and each sample was assayed in triplicate. Invasiveness was calculated as the percentage of cells that had successfully invaded through the matrix-coated membrane to the lower wells relative to the total number of cells seeded into the upper wells. The invasion assays were done in triplicate and repeated once.

17. In vitro Angiogenesis assay

Angiogenesis assay on Matrigel was performed as previously described. HUVECs cultured for 24 hours in EBM-2 with 0.5% FCS were then plated at 3.5×10^5cells/well in 24-well plates precoated with 250 ml of Matrigel (BD Bioscience) in conditioned medium was collected fresh

and cleared of cellular debris by using low-speed centrifugation (1500 *g*, 4 °C for 10 minutes). After 24 hours of incubation in a 5% CO2-humidified atmosphere at 37°C, the cell threedimensional organization was examined under an inverted photomicroscope. Each treatment was performed in triplicate wells.

18. Statistical Analysis

All experiments were repeated at least three times to ensure reproducibility. Data in all experiments are represented as mean ± S.E. Statistical comparisons were carried out using two-tailed Student's *t* test. *p* values <0.05 were considered to be statistically significant.

III. RESULTS

1. Identification of differential gene expression in NIH3T3 cells and oncogenic H-RasV12-transformed NIH3T3 cells

To apply the ACP primer system to differential display, first-strand cDNAs are synthesized by reverse transcription using oligo (dT) 15 ACP as a primer. This method requires only a single cDNA synthesis for each different RNA sample, in contrast to the multiple cDNA reactions required for differential display methods. Using first-strand cDNAs as templates, second-strand cDNAs are synthesized during one cycle of first-stage PCR using an arbitrary ACP primer and an initial annealing temperature (50°C –53°C). Second-strand cDNAs are then amplified during second-stage PCR at a second annealing temperature (65°C), which are high-stringency conditions, using the sequences at the 3′ and 5′ ends of the second-strand cDNAs as the templates for the amplification priming sequences. During the second-stage PCR, the 3′-end core region sequences alone of the oligo (dT) 15 ACP or the arbitrary ACP primer cannot anneal to the cDNA templates in such high-stringency conditions, another selective hybridization feature of Gene Fishing technology. To identify the genes specifically or predominantly expressed at two type cells, we compared the mRNA expression profiles of NIH3T3 cell and NIH3T3+H-RasV12 cell. Accordingly, mRNA sequences from both types of embryos were extracted and subjected to ACP-based RT-PCR, using a combination of 60 arbitrary primers and two anchored oligo (dT) primers of ACP-based Gene Fishing PCR kit

(See-Gene, Seoul, Korea). Gene Ontology annotations, BLASTN, and BLASTX searches of all 14 sequences against the Gene Bank database revealed that all of these DEGs have been well characterized in other species. We examined the expression patterns of selected genes in NIH3T3 cell and NIH3T3+H-RasV12 cells. Sequence-specific primers were designed to amplify products with lengths ranging from to 438~881bp. Using RT-PCR, we confirmed that NGEF were highly expressed in the NIH3T3+H-RasV12 cell (Figure.5).

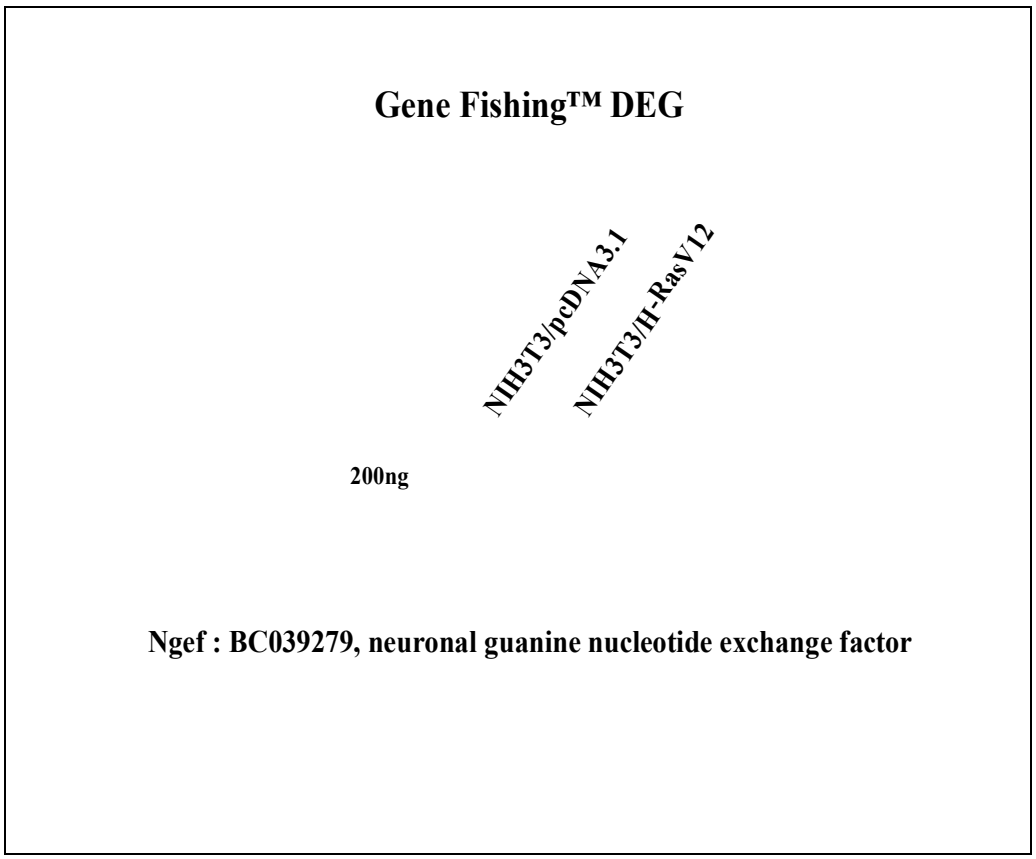

Fig.5. Result of GeneFishing PCR for the identification of differentially expressed genes (DEGs)

Expression patterns of DEGs (A22, A24 primer) assessed by RT-PCR were compared with NIH3T3 and NIH3T3/H-RasV12 cells. Amplified DNA products were separated on a 1.2% standard agarose gel, and stained with ethidiumbromide.

2. Oncogenic H-Ras induces expression of the NGEF

To confirm the Gene Fishing RT-PCR result, semiquantitative RT-PCR analysis of the control vector and H-RasV12 transfected NIH3T3 cells were performed. Semiquantitative RT-PCR analysis using the NGEF specific primers showed that the expression level of the NGEF genes was increased dramatically by transfecting them with H-RasV12 expressing vector, but not with control empty vector (Figure.6A and C). In order to determine if this increase in the NGEF mRNA levels correspond to an increase in the NGEF protein level, western blot was carried out using an antibody against the NGEF. SDS-polyacrlyamide-gel electrophoresis (SDS-PAGE) was used to separate the whole-cell extracts of the protein from the H-RasV12-transformed cells, as well as separate the protein from the empty vector-transfected cells. Western blot analysis with the NGEF antibody showed that the NGEF protein levels were higher in the H-RasV12-transfected cells than the empty vector-transfected cells (Figure.6B and D). Thus, oncogenic H-Ras expression induces NGEF expression in murine fibroblast cells.

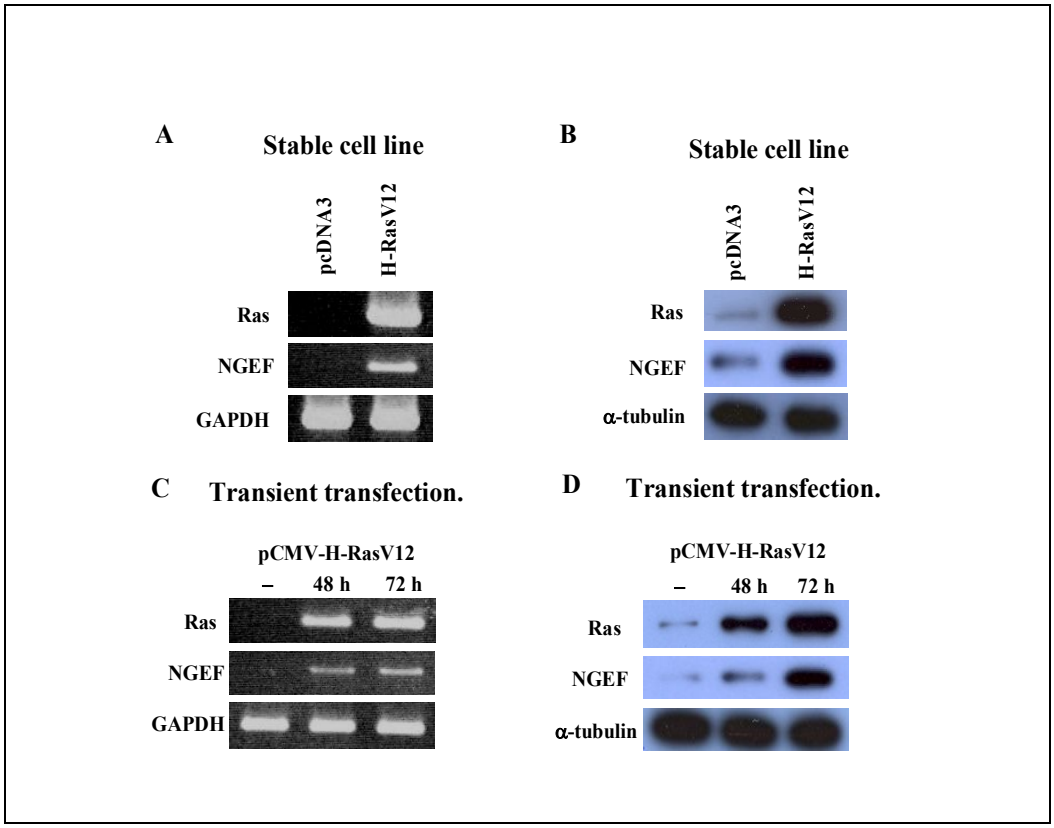

Fig.6. Oncogenic H-Ras induces expression of the NGEF

Expression levels of NGEF analysis in control empty vector- and H-RasV12 expressing vector- transfected NIH3T3 cells. (A) The total RNA was extracted from NIH3T3 cells stably expressing H-RasV12 or pcDNA3 alone and subjected to RT-PCR analysis using Ras, NGEF, GAPDH specific primers to the indicated genes. GAPDH was used as the loading control. (B) Protein extracts prepared from NIH3T3 cells stably expressing H-RasV12 or pcDNA3 vector alone and the Ras, NGEF, and α-tubulin were analyzed by western blotting. (C) RNA was isolated from NIH3T3 cells transiently transfecting H-RasV12 expressing vector or pcDNA3 alone and subjected to RT-PCR analysis using Ras, NGEF, GAPDH specific primers to the indicated genes. GAPDH was used as the loading control. (D) Total cell extracts from stable cells were used to detect Ras, NGEF, α-tubulin protein by western blotting analysis. α-tubulin was used as the loading control.

3. Dominant negative H-RasN17 supprssed H-RasV12-induced NGEF expression

To confirm whether oncogenic H-Ras is indeed involved in the NGEF expression in NIH3T3 cells, dominant negative H-RasN17 was transiently transfected into H-RasV12-expressing NIH3T3 cells. The H-RasN17 mutant, which has a 100-fold higher affinity for GDP than for GTP and can inhibit Ras effects in a dominant negative manner. Expression of the H-RasN17 mutant was verified by western blot analysis of protein lysates prepared from transfected cells. The protein expressed by H-RasN17 is the same size as endogenous p21 Ras, but it is expressed at significantly higher levels. Expression of the Dominant-negative Ras mutant in H-RasV12 infected cell was confirmed by the high level of Ras protein expression detected in cells transfected with H-RasN17 in comparison to that in cells transfected with the vector alone (Figure.7A and 7B). We found that transiently introducing a dominant negative form of H-RasN17 into oncogenic H-RasV12 transformed cell resulted in the downregulation of NGEF expression. We also confirmed the ability of H-RasN17 construct to inhibit a well-characterized Ras signaling pathway (Figure.7 C).

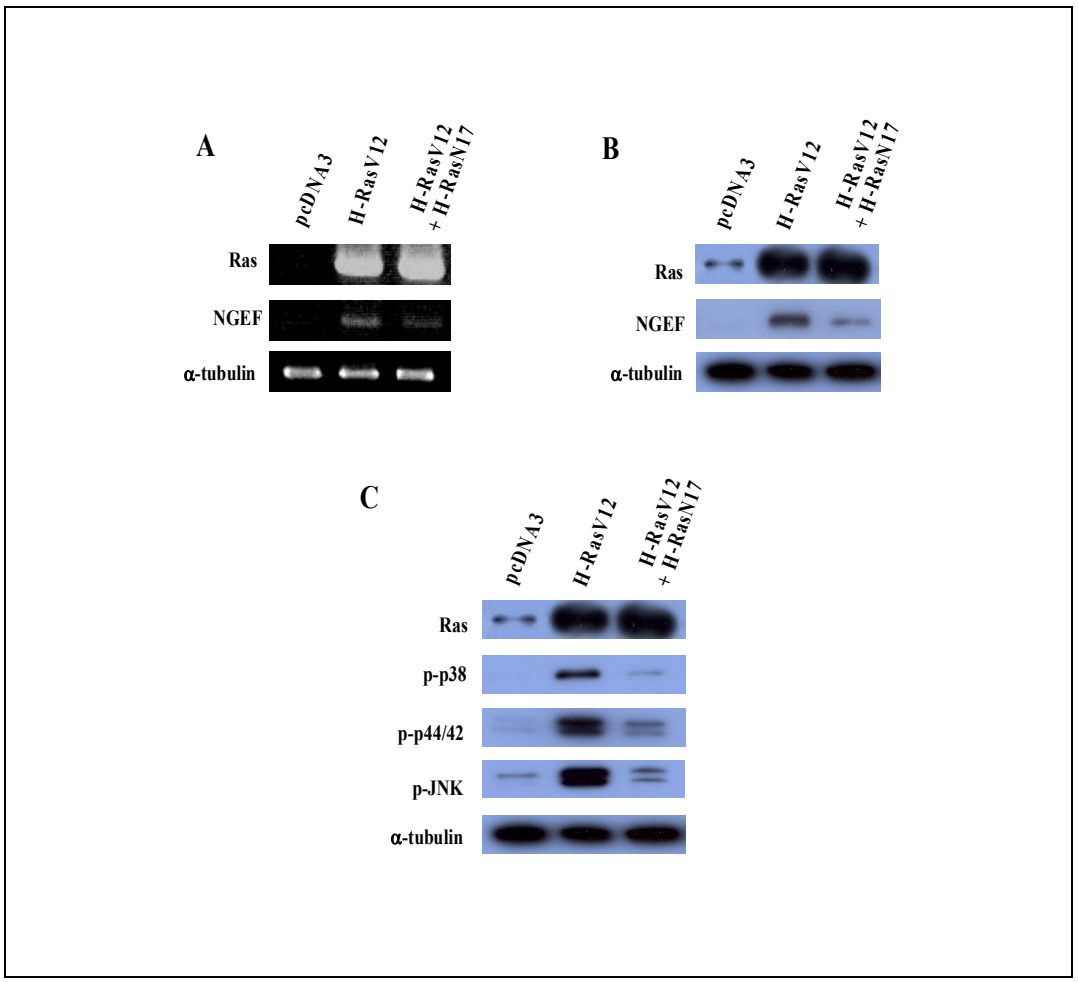

Fig.7. Dominant negative H-RasN17 transfection led to suppression of H-RasV12-mediated increased NGEF expression

Expression of NGEF in oncogenic H-RasV12 transformed cells transiently transfecting H-RasN17. (A) RT-PCR analyses for Ras, NGEF and GAPDH. GAPDH was used as the loading control. (B) Western blot for Ras, NGEF and GAPDH. α-tubulin was used as the loading control. (C) The dominant-negative RasN17 interferred endogenous Ras major signaling. Western blots ananlysis phospho-p38, phospho-p44/42 and phospho-SAPK/JNK. α-tubulin was used as the loading control.

4. Effect of Ras signal pathways inhibition on NGEF expression in oncogenic H-RasV12-transformed NIH3T3 cells

The Raf/MEK/ERK and the PI3K/AKT pathway is a major signal transduction pathway activated by Ras. To determine which path way contributes to Ras-mediated NGEF mRNA expression, H-RasV12-expressing NIH3T3 cells were pretreated with the p44/42 MEK inhibitors PD98059 and U0126, or the p38 MAPK inhibitor SB203580. Pretreatment of H-RasV12-expressing NIH3T3 cells with MEK inhibitor PD98059 and U0126 decreased NGEF mRNA expression, whereas p38 MAPK inhibitor SB203580 did not exert any effect on NGEF mRNA expression (Figure.8A). To determine the role of the PI3K pathway in the suppression of NGEF protein expression, LY 294002 was used to block the activation of the PI3K pathway in H-RasV12-expressing NIH3T3 cells. Treatment with PI3K inhibitor LY294002 or MEK inhibitor PD98059, U0126 decreased the amount of NGEF protein in H-RasV12-eapressing NIH3T3 cells (Figure.8B). These results indicate that the elevated expression of NGEF in oncogenic H-RasV12-transformed cells is due to the increased PI3Knase or MEK activities in these cells.

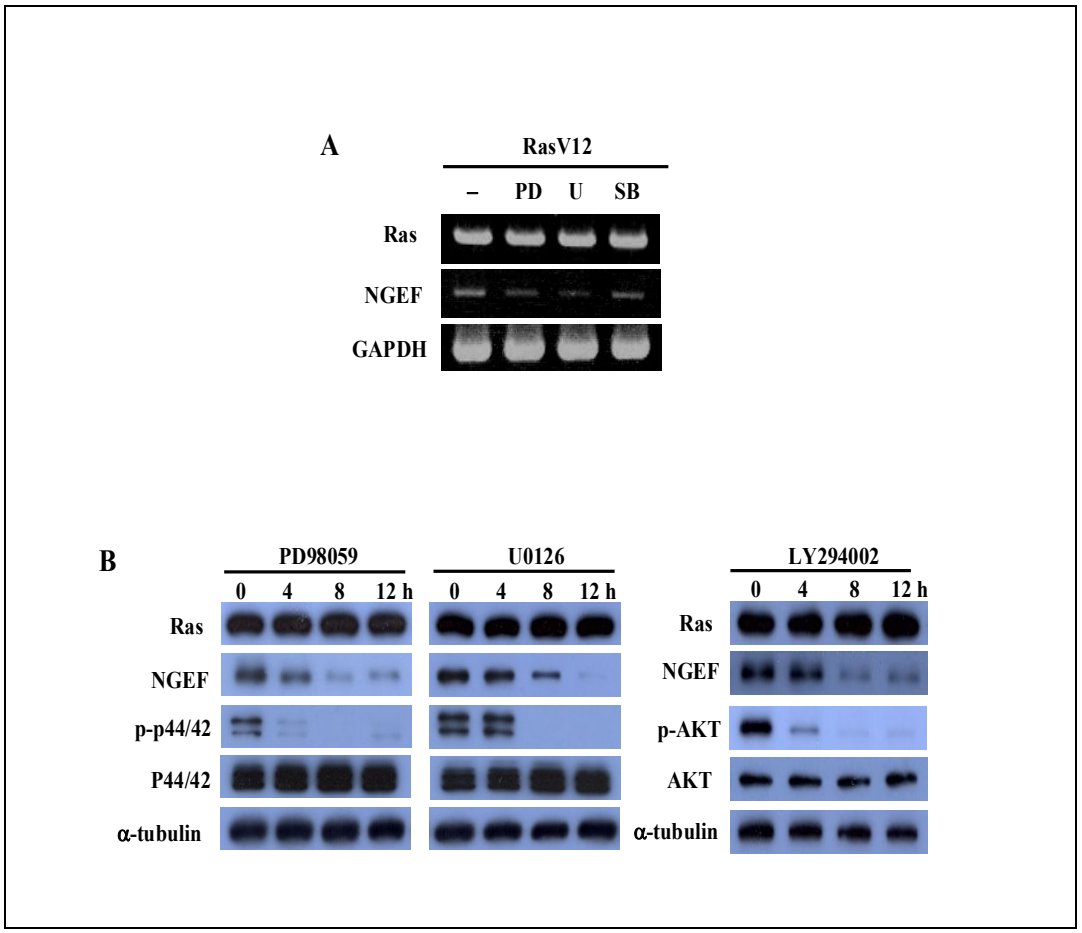

Fig.8. Effect of Ras signal inhibitor on NGEF expression in oncogenic H-Ras transformed cells

Serum-starved NIH3T3/H-RasV12 cells were pretreated MEK inhibitor (50μM PD98059 or 20μM U0126) or PI3K inhibitor (30 μM LY294002) or MAPK inhibitor (20μM SB203580). Cells were harvested and the NGEF expression was analyzed by RT-PCR (A) and Western blotting (B) GAPDH and α-tubulin were used as the loading controls of RT-PCR and western blotting, respectively.

5. Effect of NGEF on the Raf, Rho, Rac1 and Cdc42 activation

Small GTP-binding proteins (or GTPases) serve as molecular switches in signaling transduction pathways. Active Ras binds specifically to the Ras-binding domain (RBD) of Raf1, leading its activation. Therefore, the RBD of Raf1 can be used as a probe to specifically isolate the active form of Ras. Small GTP-binding proteins (or GTPases) serve as molecular switches in signaling transduction pathways. Rho (24 kDa), a small GTPase, regulates stress fiber formation, focal adhesion and cell migration. Upon binding to GTP, Rho interacts with downstream effectors such as Rhotekin. Therefore, the Rhotekin RBD can be used as a probe to specifically isolate active or GTP-Rho. GTPase Rac1 plays an important role in the organization of actin filament networks and in membrane ruffling. Active Rac1 and Cdc42 binds specifically to the p21-binding domain (PBD) of p21-activated protein kinase 1 (Pak1), leading its activation. GTPase Cdc42 (22 kDa) regulates the organization of the actin cytoskeleton and gene transcription. Activation of Cdc42 promotes actin polymerization to form filopodia or microspikes and is associated with integrin complexes. Here we investigate if overexpression of NGEF protein coordinately regulates the induction of constitutively active Ras and Rho GTPases. Control NIH3T3 and H-RasV12-expressing NIH3T3 cells were transiently transfected with control pcDNA3 vector or NGEF expressing vector, respectively; H-RasV12 transfected cells were transiently transfected with control siRNA or NGEF siRNA. To investigate the effect of NGEF on the activation of small GTPase protein, cells were serum starved for 24h and then treated with serum, and incubated additional 3h. Expression of NGEF

coordinately stimulated the expression of active Rho and active Rac1 and active Cdc42. Moreover, NGEF siRNA transfected H-RasV12 transformed cell exhibited suppression of Rho, Rac1 and Cdc42. However, expression of NGEF did not affect Raf activity (Figure.9). These data suggest that expression of NGEF were capable of activating Rho, Rac1 and Cdc42, but not Raf activity.

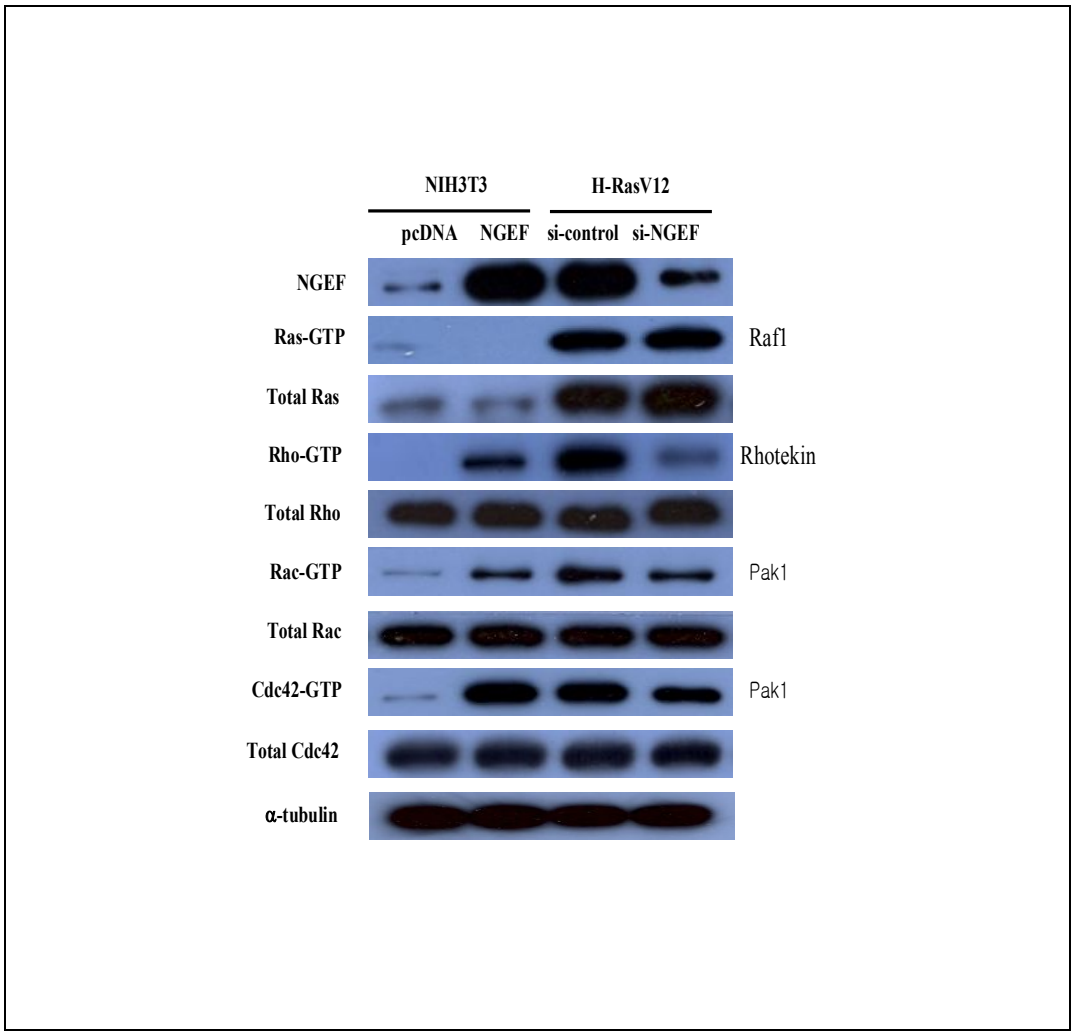

Fig.9. Effect of NGEF on the Raf, Rho, Rac1 and Cdc42 activity

NIH3T3 and H-RasV12-transforming NIH3T3 cells were transfected either with plasmids containing pcDNA3.1/V5-His-Topo, pcDNA3.1/NGEF, control siRNA pCMV/H-RasV12 or pCMV/H-RasV12 NGEF siRNA. The cell lysates (500ug) were then incubated with GST-Raf-PBD, GST-Rhotekin, GST-Pak1 and SwellGel Immobilized Glutathione Disc. Half of the volumes of the eluted samples (25ul) and 20ug of cell lysate were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and probe with anti-Ras, anti-Rho, anti-Rac1 and anti-Cdc42 Antibody. α-tubulin was used as the loading control.

6. Interaction of NGEF with small GTP-binding proteins

The GTPases serve as a molecular switch by converting from an inactive GDP-bound state to an active GTP-bound state and, once activated, they can interact with their specific effectors. GTPases, including RhoA, Cdc42, Rac1 are also important modulators that control the activity of NGEF. We previously found that NGEF was overexpressed by oncogenic H-Ras V12-transformed NIH3T3 cells. In the present study, we examined the role of RhoA family GTPases on NGEF activation in oncogenic H-Ras V12-transformed NIH3T3 cells, so we investigated whether NGEF interacted with GTPase (H-Ras, RhoA, Cdc42, Rac1) co- immunoprecipitation experiments were performed. NIH3T3 and H-Ras V 12 –transformed NIH3T3 cells lysates were immunoprecipitated with either anti H-Ras, RhoA, Cdc42 or Rac1 antibodies, respectively, and the immunoprecipitates were probed with either anti H-Ras, RhoA, Cdc42 or Rac1 antibody by Western blot analysis. As shown in Fig.10 H-Ras and RhoA could see the band, which come from the binding of NGEF in H-Ras V 12 -transformed NIH3T3 cell, but not NIH3T3 cell. However, Cdc42 and Rac1 couldn't see the binding band. (data not shown). We also found that IgG sample completely didn't have any binding.These results suggesting that NGEF is closely interacted H-Ras and RhoA , but not Cdc42 and Rac1. Further study, we will do two experiments confirmed that the interacting of NGEF to H-Ras or RhoA. First, we will investigate NGEF binding expression level with anti H-Ras or RhoA in H-Ras V 12 -transformed NIH3T3 cells and H-Ras V 12 -transformed NIH3T3 cells + NGEF si RNA by using co- immunoprecipitation experiments.

Second, we will investigate NGEF binding expression level with H-Ras V 12 -transformed NIH3T3 cells and H-Ras V 12 -transformed NIH3T3 + Dominant negative H-Ras or H-Ras V 12 -transformed NIH3T3 cells and H-Ras V 12 -transformed NIH3T3 + Dominant negative RhoA.

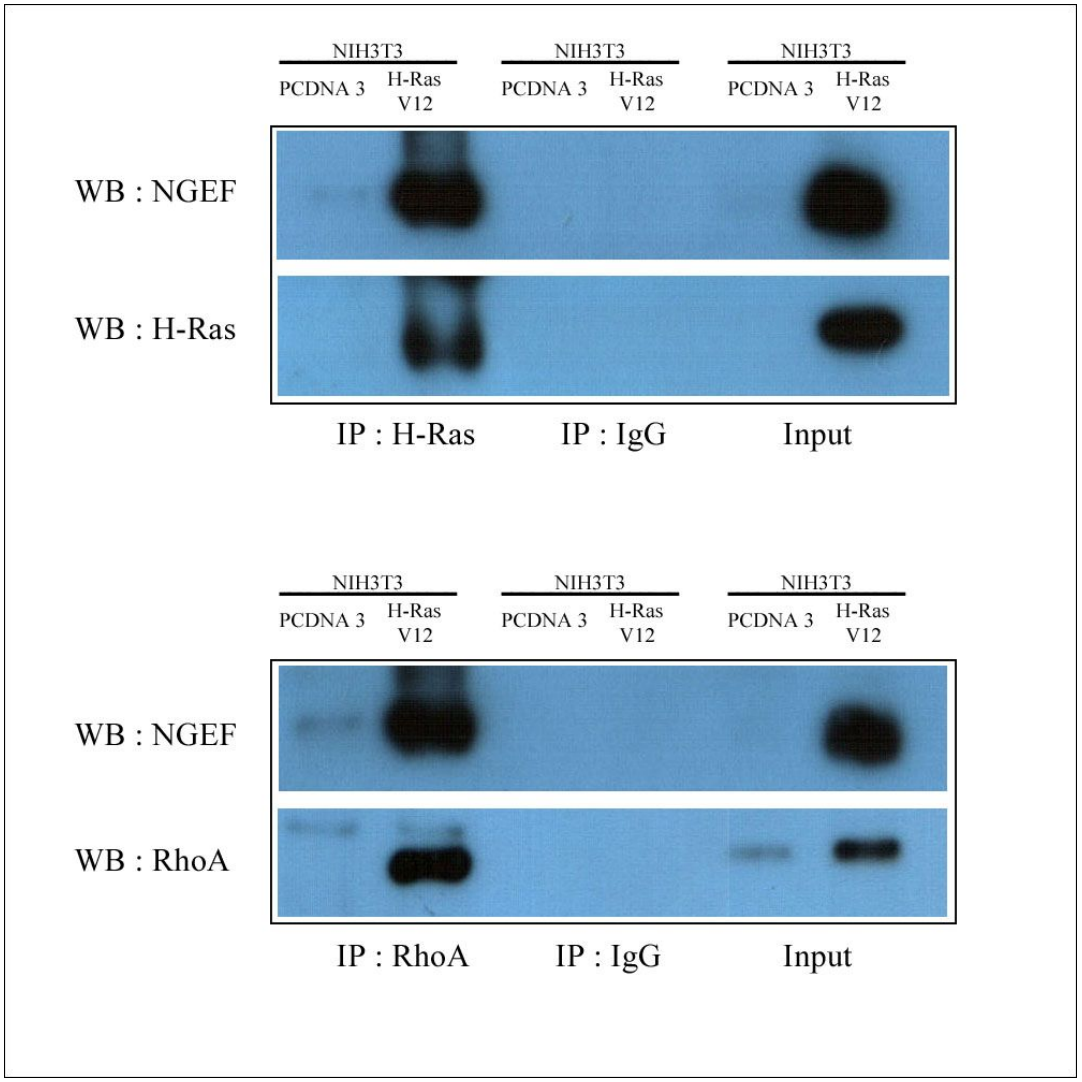

Fig.10. Interaction of NGEF with small GTP-binding proteins

NIH3T3 and H-Ras V12-transforming NIH3T3 cells aliquots of 2mg cell lysates were incubated overnight at RT with the desired antibodies (2ug, H-Ras, RhoA, Cdc42 or Rac1). And captured with 30ul of protein-G-agarose (Santa cruz) for 1hr at RT. The beads were collected by centrifugation, washed three times with lysis buffer, and resuspended in sample buffer. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti- H-Ras, RhoA, Cdc42, Rac1 or NGEF antibodies.

7. NGEF is reguired for oncogenic H-Ras-induced cellular proliferation

To investigate whether NGEF affects cellualr proliferation in NIH3T3 cells, we used control and NGEF expressing NIH3T3 cells. The proliferation rate of NGEF expressing NIH3T3 was significantly induced as compared with that of control cells. To determine whether NGEF affects proliferation rate of oncogenic H-Ras-expressing cells, we inhibited NGEF expression in oncogenic H-RasV12 cells using siRNA and monitored the effect of such treatments. Importantly, the proliferation rate of oncogenic H-RasV12 cells treated with NGEF specific siRNA was significantly reduced as compared to those treated with the control siRNA, determined by BrdU incorporation (Figure.11A) and MTT assay (Figure11B). These findings strongly indicate that NGEF plays an essential role in mediating the proliferative activity of NIH3T3 cell or oncogenic H-RasV12 NIH3T3 cell.

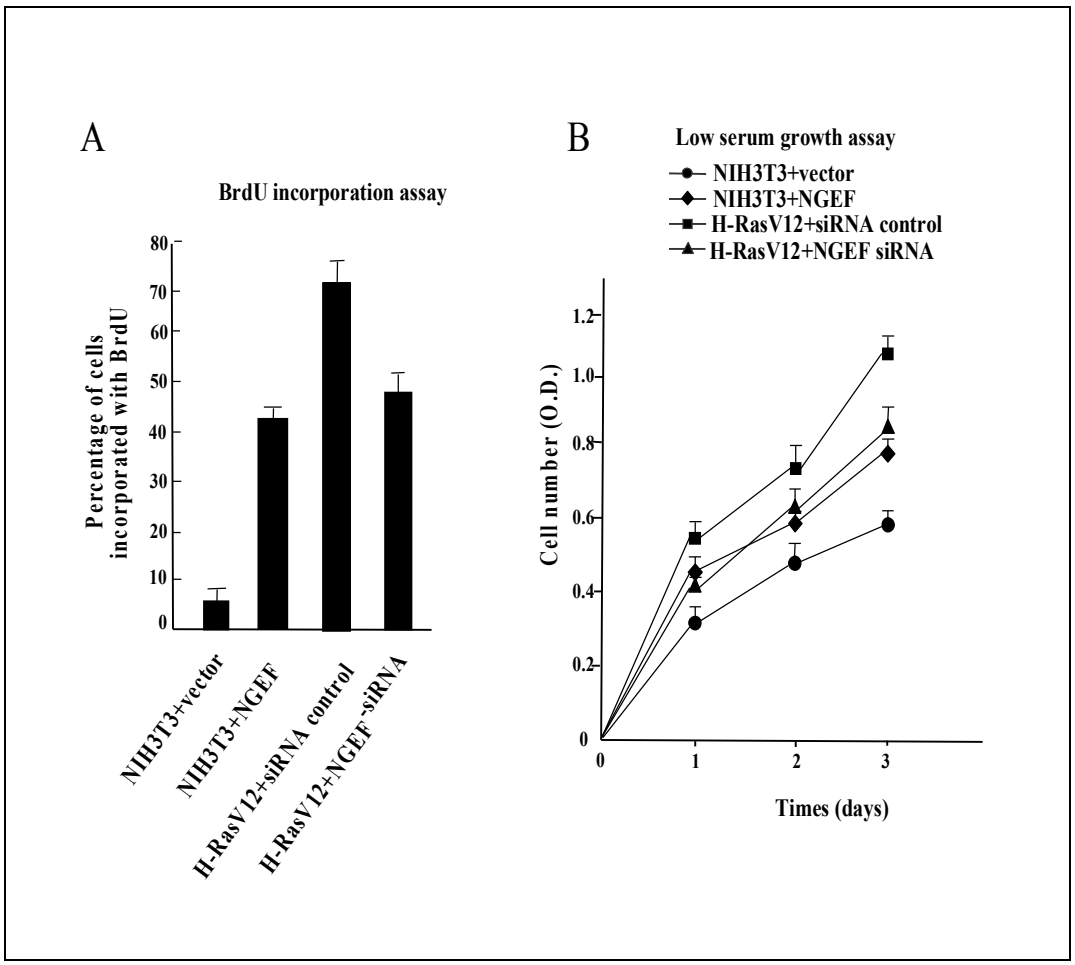

Fig.11. *In vitro* Proliferation assay

(A) Vector and NGEF expressing NIH3T3 cells and control siRNA- or NGEF siRNA-transfected H-RasV12-transforming NIH3T3 cells were grown for 8 h at 37 ℃ and then labeled by adding 10 uM bromodeoxyuridine (BrdU) to each well flat bottoms at a density of 10,000 cells per well. (B) Cells were seeded in 0.25% FBS at day 0 at a density 5×10^5 cells/well and counted for up to 3 days. The number of cells were recored every 24 h. Each value is the mean ± s.d.from three separate experiments.

8. Effect of NGEF on the Aggregation in NIH3T3 and oncogenic H-RasV12-transforming NIH3T3 cells

To investigate whether NGEF is contributed to the oncogenic Ras-induced cellular aggregation, single-cell suspensions were seeded on top of a semi-solid agar medium. After 48hr, aggregate formation was evaluated subjectively under an inverted phase-contrast microscope at a magnification of ×40. The result NGEF did not appear to have any morphological effect on wild-type NIH3T3 cell. By contrast, enforced expression of NGEF in NIH3T3 cells resulted in the aggregation of these cells under subconfluent conditions. In addition, NIH3T3/H-RasV12 cells have an increased tendency to aggregate, and NGEF siRNA transfection led to significant suppression of cellular aggregation of these cells (Figure.12).

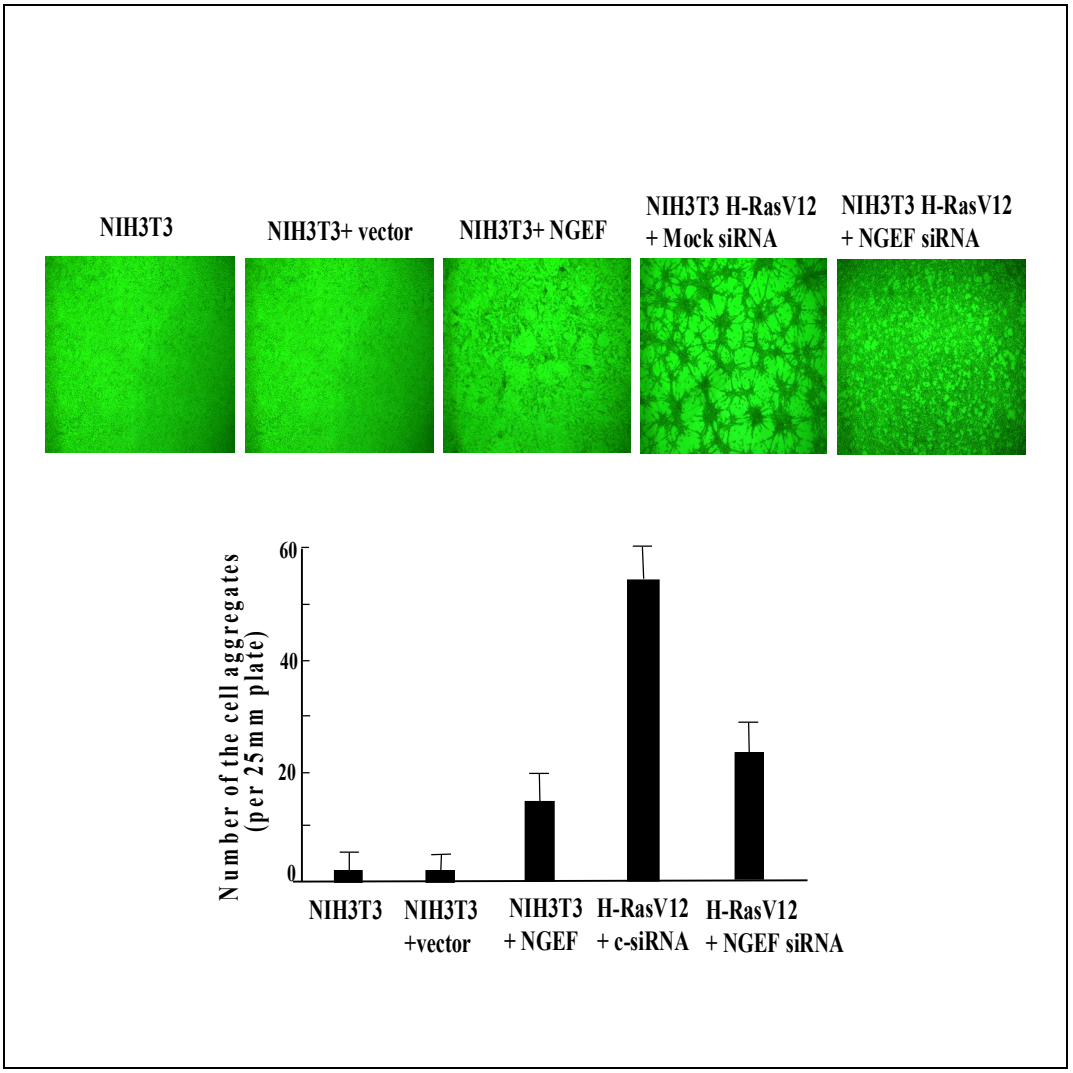

Fig.12. *In vitro* Aggregation assay

Single-cell suspensions were seeded on top of a semi-solid agar medium with NIH3T3/pcDNA3.1, NIH3T3/NGEF or NIH3T3/H-RasV12, NIH3T3/H-RasV12 siNGEF. After 48hr, aggregate formation was evaluated subjectively under an inverted phase-contrast microscope at a magnification of ×40 and recorded as digital images using Adobe Photoshop. Mean values±s.e.m are shown of three independent experiments.

9. Effect of NGEF on the Colony formation in NIH3T3 and oncogenic H-RasV12-transforming NIH3T3 cells

The ability of an oncogene to induce the growth of colonies in soft agar is a classifical *in vitro* experiment to determine its transforming potential. We observed, as expected, that the expression of NGEF in NIH3T3 cells induced the growth of colonies in soft agar. Also, as expected, that the inhibited of NGEF in NIH3T3/H-RasV12 cells reduced the growth of colonies in soft agar. First, we examined the ability of NGEF to induced normal cell forcus forming (density dependence) activity. In these experiment, we transfected NIH3T3 cells with an expression vector encoding activated NGEF. As shown in Figure.13, expression of NGEF caused a 40% induction in transforming activity compared with the activity caused by transfection of the empty vector (Figure.13). These data suggest that elevated expression of NGEF induces transformation and that up-regulation of NGEF is a necessary prerequisite for colony transformation. To further evaluate how NGEF antagonizes transformation, we generated stable cell lines that ectopically express NGEF to determine whether expression of NGEF could induce colony transformation. Second, we examined the ability of NGEF to inhibited Ras-tansformed cell focus forming activity. In these experiment, we transfected NIH3T3/H-RasV12 cells with an expression vector encoding siRNA NGEF. As shown in Figure.13, inhibited expression of NGEF caused a 30% reduction in transforming activity compared with the activity caused by oncogenic H-Rasv12 transfected cells to grow in semisolid agar by 55%. In addition, inhibited expression of NGEF reduced not only the number

of colonies, but also the size of the colonies. Thus, because forced inhibited expression of NGEF reduction the soft agar growth of the Ras-transformed cells, we conclude that the increased expression of NGEF observed in Ras-transformed represents an important mechanism by which Ras mediates transformation.

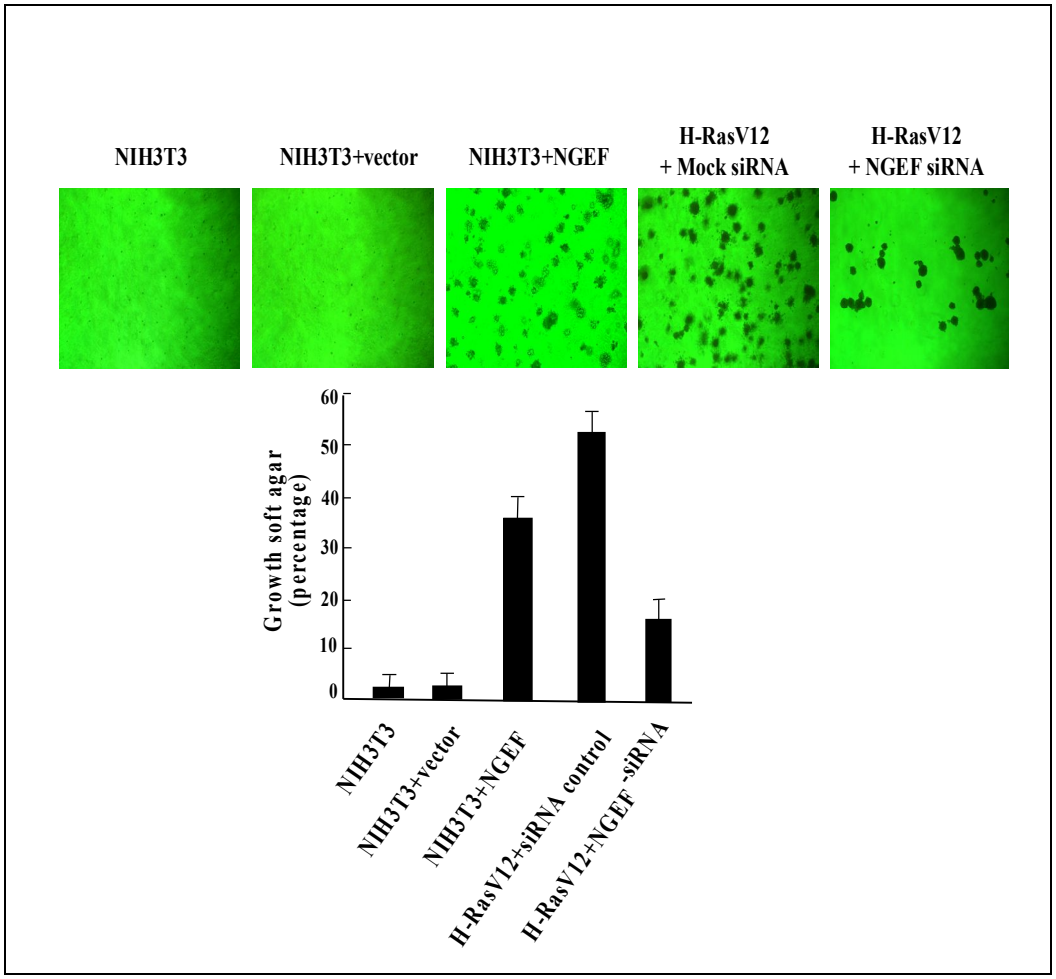

Fig.13. ***In vitro*** **Colony formation assay**

NIH3T3, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA or NIH3T3/H-RasV12 NGEF siRNA cells were plated 2×10^4 in duplicates in 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% bottom low-melt agarose. After 2 weeks of incubation, colonies of >1 mm in size were counted. Cellular migration was observed with light microscope(×40). Data represent the mean ± standard deviation of triplicate samples derived from a typical experiment and similar were performed at least three times.

10. Effect of NGEF on the Migration in NIH3T3 and oncogenic H-RasV12-transforming NIH3T3 cells

Cell migration is an essential process in physiological conditions such as wound healing, tissue regeneration, and in pathological conditions such as tumor invasion. The role of the Ras oncogene in the pathophysiology of cell transformation and the pathogenesis of various cancers is well established. Ras oncoproteins are thought to contribute to the proliferative, invasive, metastatic properties of transformed cells. For example, an overexpression of H-Ras oncoprotein has been reported to induce metastatic potentiality in H-Ras transfected murine NIH3T3 cells. To examine the functional roles of NGEF in NIH3T3 cell migration, we performed both modified Boyden chamber assays and wound healing assays using NGEF stable cell lines (NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12, NIH3T3/H-RasV12 siNGEF). In order to test whether the expression of NGEF cell migration, we performed modified Boyden chamber migration aasay. As shown in Figure.14, expression of NGEF in NIH3T3 cell significantly enhanced the migration activity, whereas did not effect the migration of parental NIH3T3/pcDNA3.1 cells. Furthermore, inhibited expression of NGEF caused reduction in migration activity compared with the migration activity caused by oncogenic H-Rasv12-transformed cells (Figure.14). We also examined the effect of NGEF over-expression on cell migration by in vitro wound healing assay. As shown in Figure.15, expression of NGEF in NIH3T3 cell significantly enhanced wound healing activity, whereas delayed wound healing activity of parental NIH3T3/pcDNA3.1 cells. Furthermore, inhibited

expression of NGEF caused a delayed in wound healing activity compared with wound healing activity caused by oncogenic H-Rasv12-transformed cells (Figure.15).

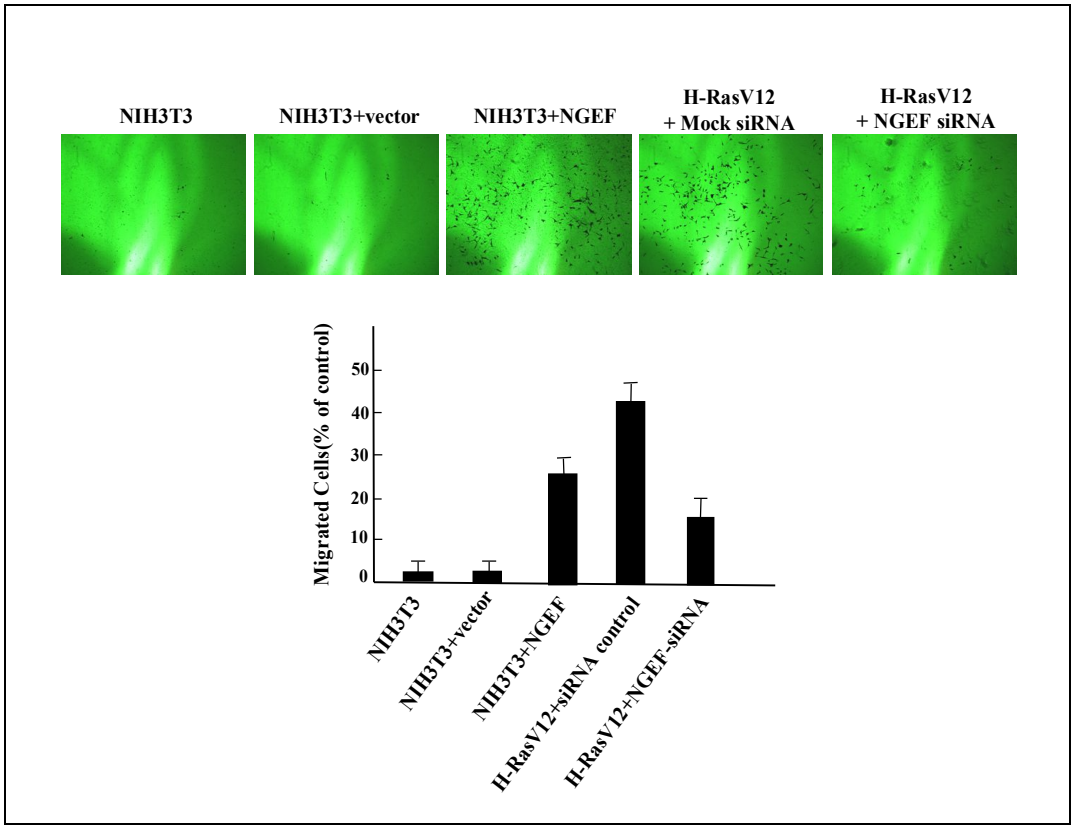

Fig.14. *In vitro* Migration assay using transwell

Cell migration abilities of NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were analysed using uncoated transwell cell culture inserts (5×10^4) with 8 μm pores. After inhibition of cell proliferation by treatment with 10μg/ml mitomycin-C or solvent were added to lower compartment. After 24 hours of incubation of cells, which had migrated through the pores, was estimated by counting 5 independent visual fields. Cellular migration was observed with light microscope(×40). Three independent assays were performed in triplicate. Mean values ±s.d. are shown.

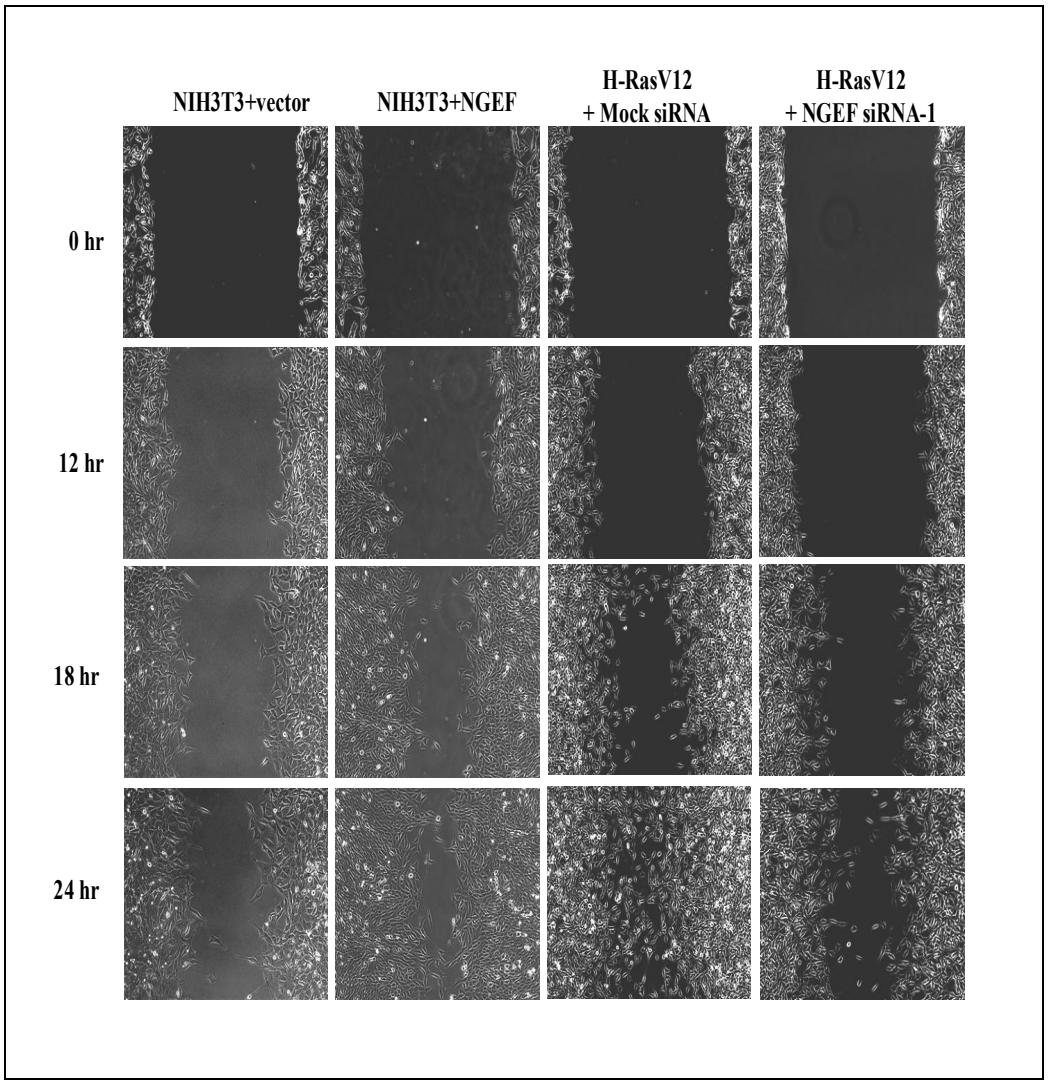

Fig.15. *In vitro* Wound healing migration assay

Cell Migratory abilities of the NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were determined by wound healing assay. Width of injury line from three independent experiments was measured and plotted. After inhibition of cell proliferation by treatment with 10 μg/ml mitomycin-C or solvent were added to lower compartment. Cellular migration was observed with light microscope (×40) at indicated time points. The results presented were means of triplicates.

11. Effect of NGEF on the Invasion in NIH3T3 and oncogenic H-RasV12-transforming NIH3T3 cells

Invasion through the extracellular matrix (ECM) is an important step in tumor metastasis. Cancer cells initiate invasion by adhering to and spreading along the blood vessel wall. Proteolytic enzymes, such as MMP collagenases, dissolve tiny holes in the sheath-like covering (basement membrane) surrounding the blood vessels to allow cancer cells to invade. To evaluate the invasive phenotype of expression of NGEF in NIH3T3 cells and inhibited expression of NGEF in oncogenic H-RasV12-transformed NIH3T3 cells, we performed an in vitro invasion assay. Matrigel invasion chamber are hydrated for at least 2hr in the culture incubator with 500ul serum free DMEM in the bottom of the well and 500μl in the top of the chamber. After hydration of Matrigel, the DMEM in the bottom of the well is replaced with DMEM containing 10% FBS. 5×10^4 cells are plates in 500μl DMEM supplemented with 10% FBS in the top of the chamber. The invasion assay is carried out for 24hr in the culture incubator. The cells are fixed by replaced the culture medium in the bottom and top of the chamber with 4% formaidehyde dissolved in PBS. After fixing for 15min at room temperature, the chambers are rinsed in PBS and stained with 0.2% crystal violet for 10 min. After washing the chamber 5 times by dipping the chambers in a large beaker filled with dH_2O the cells at the top of the Matrigel membrane are removed with several cotton-tips. It is safe to assume that all cells are removed when no more blue dye can be removed with the cotton-tip. These cells are counted using an inverted microscope equipped with either a ×40 objective and plotted as the

percentage of invading cells of the total number of plated cells. As shown Figure.16, Transfection with NGEF significantly induced the number of invaded cells (25%) compared with transfection control vector. We also evaluated in the invasive activity of H-RasV12-transfored NIH3T3 cells by *In vitro* invasion assay. Oncogenic H-RasV12-transformed NIH3T3 cells (50%) invasion was significantly reduced by inhibited (15%) expression of NGEF (Figure.16). Invasive phenotype of cancer cells is often associated with increased expression of MMP-2 and MMP-9, which can degrade type IV collagen, the major structural collagen of the basement membrane. Thus, we next examined the involvement of MMP-2, MMP-9 in NGEF-induced and NGEF-reduced invasive phenotypes in NIH3T3 cells. As shown Figure.16, activity of MMP-9 and MMP-2 was increased in NGEF over-expression cells. Moreover, blocking the expression of NGEF markedly decreased the protein levels MMP-2 and MMP-9 in H-R asV12-transformed NIH3T3 cells (Figure.16). These results demonstrated that NGEF is reguired for the NGEF expression could elicit invasion of normal murine fibroblast NIH3T3 cells ans is an essential role for oncogenic H-Ras-induced increase invasion ability.

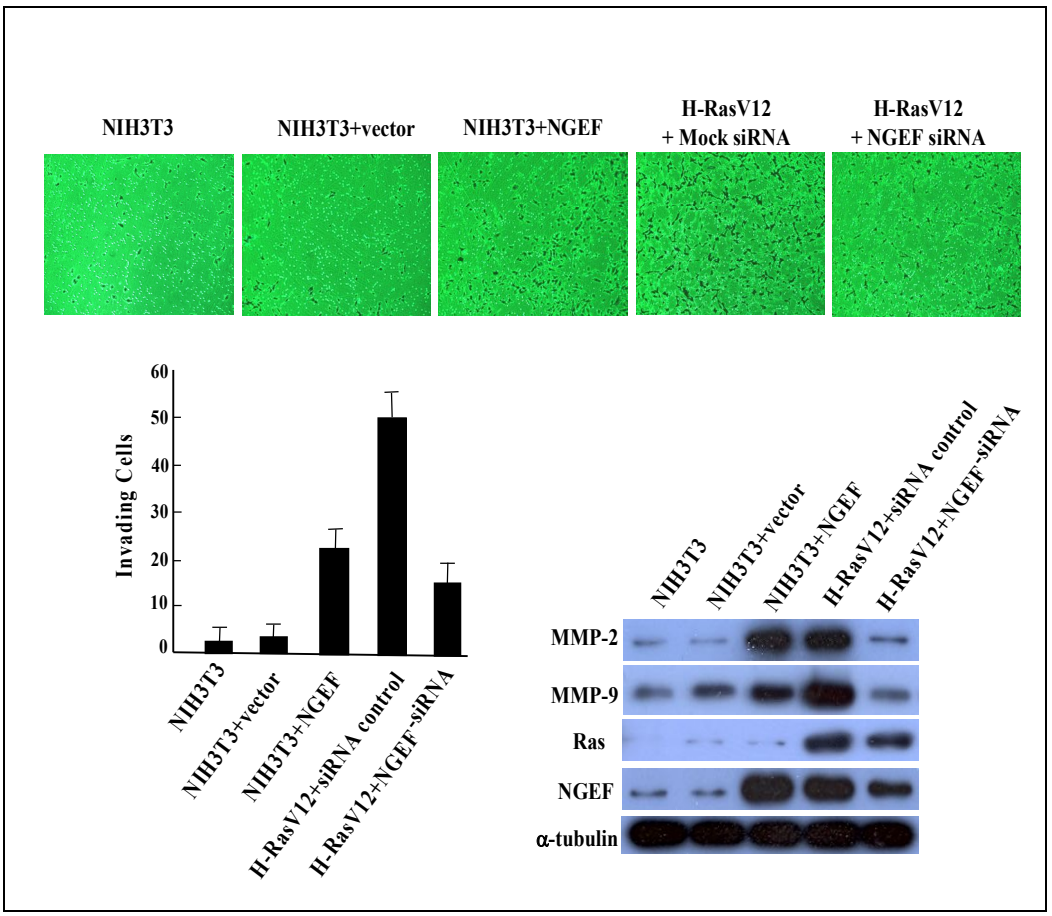

Fig.16. *In vitro* Invasion assay using matrigel chamber

Cell Invasive abilities of the NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were determined by In vitro Invasion assay using matrigel chamber. The filters were fixed by immersion in 4 % formaldehyde and then stained with 0.2 % crystal violet. The upper surface of the membrane was cleaned with a cotton swab to remove all noninvasive cells. The stained, number of invaded cells per field was counted in 13 fields under light microscope (×40). Three independent assays were performed in triplicate. Total Cell lysates for the indicated cell lines were resolved by SDS-PAGE and western blot analysis using anti-MMP-2, anti-MMP-9, anti-CyclinD3, anti-Ras and anti-NGEF antibody. α-tubulin was used as the loading control.

12. Effect of NGEF on the Angiogenesis in NIH3T3 and oncogenic H-RasV12-transforming NIH3T3 cells

Angiogenesis is the process of generating new capillary blood vessels. It is a fundamental component of a number of normal (reproduction and wound healing) and pathological processes (diabetic retinopathy, rheumatoid arthritis, tumor growth and metastasis). To evaluate the angiogenesis phenotype of expression of NGEF in NIH3T3 cells and inhibited expression of NGEF in oncogenic H-RasV12-transformed NIH3T3 cells, we performed an in vitro angiogenesis assay. To determine whether the increased levels of VEGF protein induced by NGEF gene correspond to increases in functional VEGF protein, we collected conditioned medium from NGEF transfected cell, as well as from control nontransfected cell. We used an endothelial tube forming assay to assess VEGF activity: HUVEC cells were embedded in three-dimentional collagen (Type1) mesh and the extent of tube-forming network was evaluated after application of various conditioned medium. These data demonstrate that activated NGEF-expressing cells secrete high levels of functional VEGF. To determine the effect of NGEF on the oncogenic H-Ras-induced VEGF expression, protein from transformed and control NIH3T3 cells was examined for VEGF expression. As shown Figure.17, a marked induction in VEGF protein expression was observed in NIH3T3 cells transfected with H-RasV12 oncogene. Moreover, only low levels of VEGF protein levels was observed in oncogenic H-Ras-transformed cells with inhibited expression of NGEF (Figure.17). Our results suggest that NGEF is able to stimulate angiogenesis, and is contributed to oncogenic H-Ras-induced *in vitro*

angiogenesis. We demonstrate here the ability of NGEF to elicit angiogenesis in vitro shown by the endothelial tube forming assay.

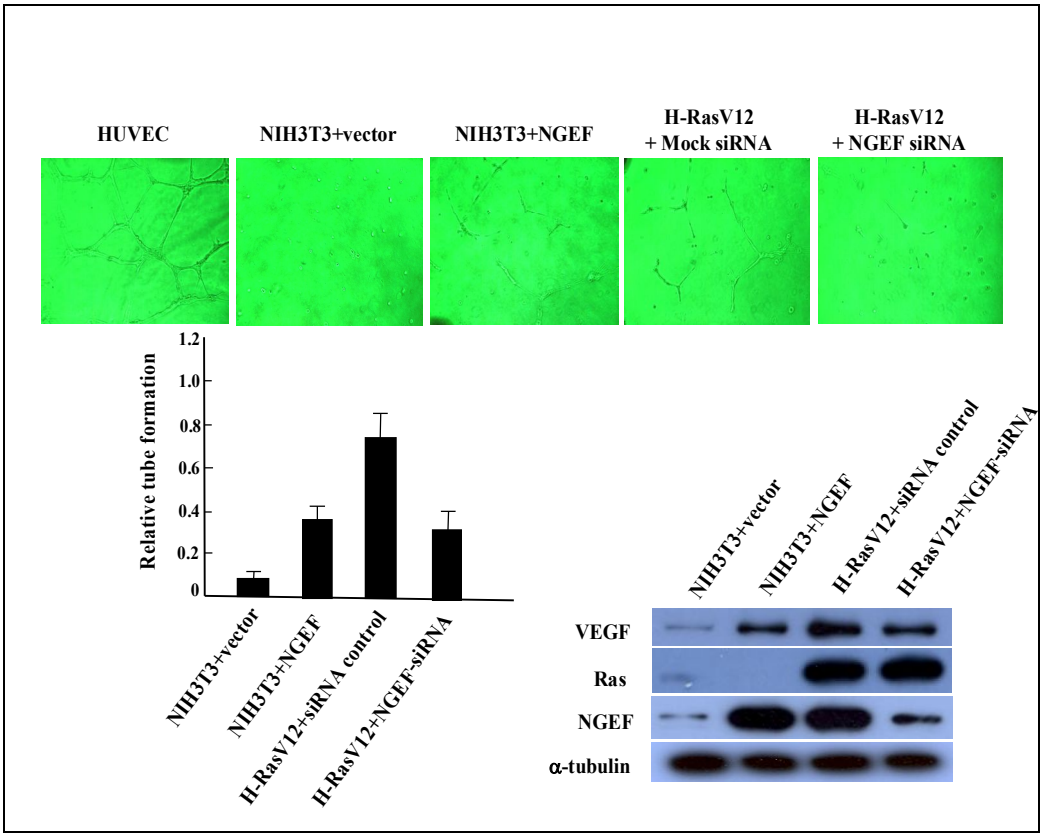

Fig.17. *In vitro* Angiogenesis assay

Cell angiogenesis abilities of NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were determined by In vitro angiogenesis assay. Conditional media was collected after 48hr and HUVEC cells grown in conditioned media were stained with H&E after 72hr and then examined under a confocal scanning laser microscope. Three independent assays were performed in triplicate. Total Cell lysates for the indicated cell lines were resolved by SDS-PAGE and western blot analysis using anti-VEGF, anti-CyclinD3, anti-Ras and anti-NGEF antibody. α-tubulin was used as the loading control.

13. Effect of NGEF on the Animal tumorigenesis

We next sought to determine whether the induction and secretion of NGEF in response to RasV12 signaling contributes to Ras-mediated tumorigenesis. To investigate this, we conducted xenograft studies using immunocompromised nude mice. Animals were divided into four experimental groups. The first cohort was injected subcutaneously with NIH3T3/pcDNA3.1 and NIH3T3/NGEF cells. The second cohort was injected subcutaneously with NIH3T3/H-RasV12 and NIH3T3/H-RasV12 siNGEF cells. These groups cells injected 2.5×10^5 in PBS.

Animals were monitored for subcutaneous tumor formation and the growth rate of the developing tumors was established. As illustrated in Figure.18, NIH3T3/NGEF expression significantly enhanced tumor growth in experimental animals. In contrast, we could not see any tumor growth, when control vector transfected NIH3T3 cells were injected (Figure.18). Animal injected with the expressed NGEF at the tumor site were initiated eight days after cell inoculation. (Figure.19). Moreover, NGEF were stably knocked down by siRNA in the highly tumorigenic H-RasV12-transformed NIH3T3 cells reduced the tumor formation (Figure.18). In addition, inhibited expression of NGEF in oncogenic H-RasV12-transformed NIH3T3 cells demonstrated an approximate 60% reduction in tumor growth rate at the time of sacrifice when compared with oncogenic H-RasV12-transformed NIH3T3 cells (Figure.19). This analysis revealed a significant enhancement of tumorigenesis in NGEF expressing injected with compared to the stunted tumor formation found in NIH3T3 control vector. Injection of H-RasV12-expressing tumors with stably knocked down by siRNA NGEF drastically reduced the

increase in tumor formation induced by H-RasV12. We conclude that NGEF function is specifically required for Ras-induced or normal cell tumor formatiom.

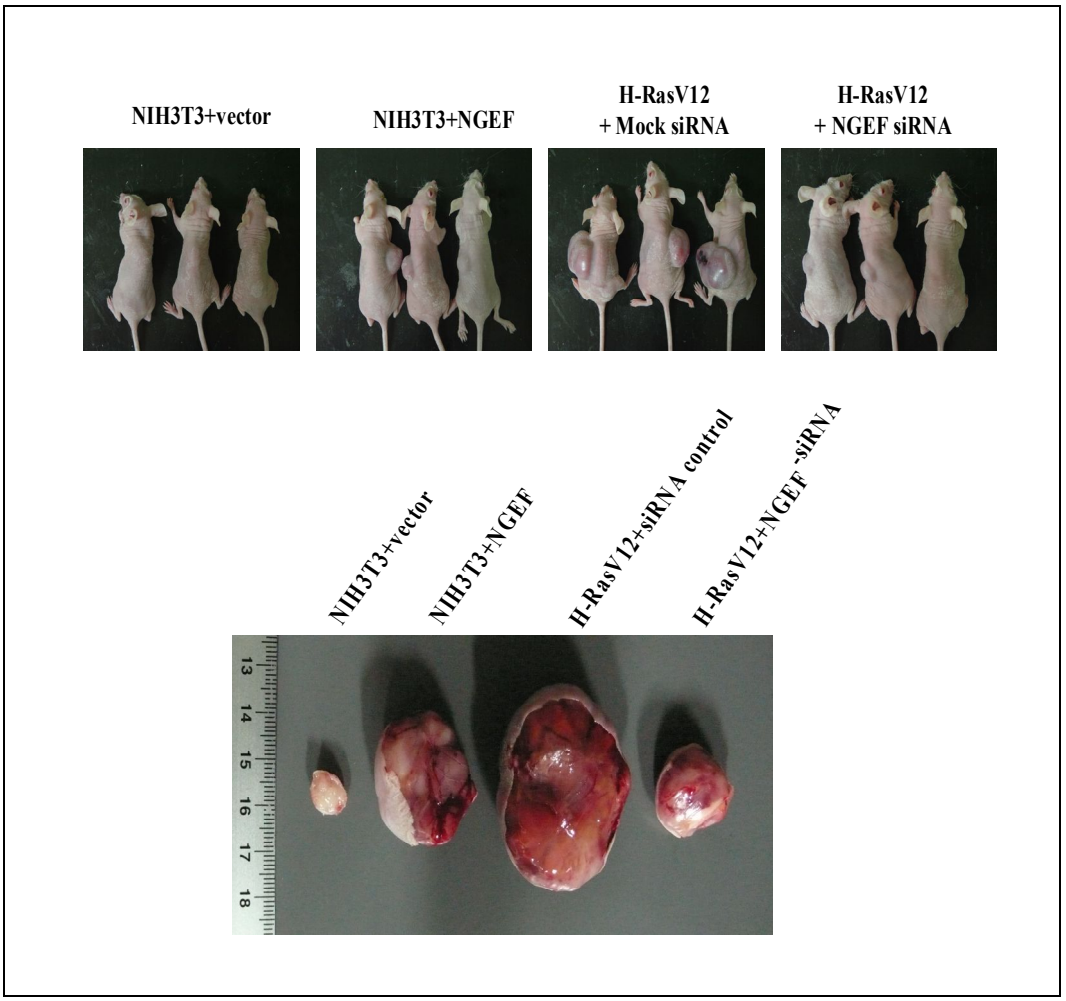

Fig.18. Animal tumor formation assay

NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were subcutaneously injected into immunocompromised nude mice. Tumors were excised from animals at day 40 after cell inoculation when the largest tumor had reached a diameter of 6 cm. Representative examples for each experimental group are shown.

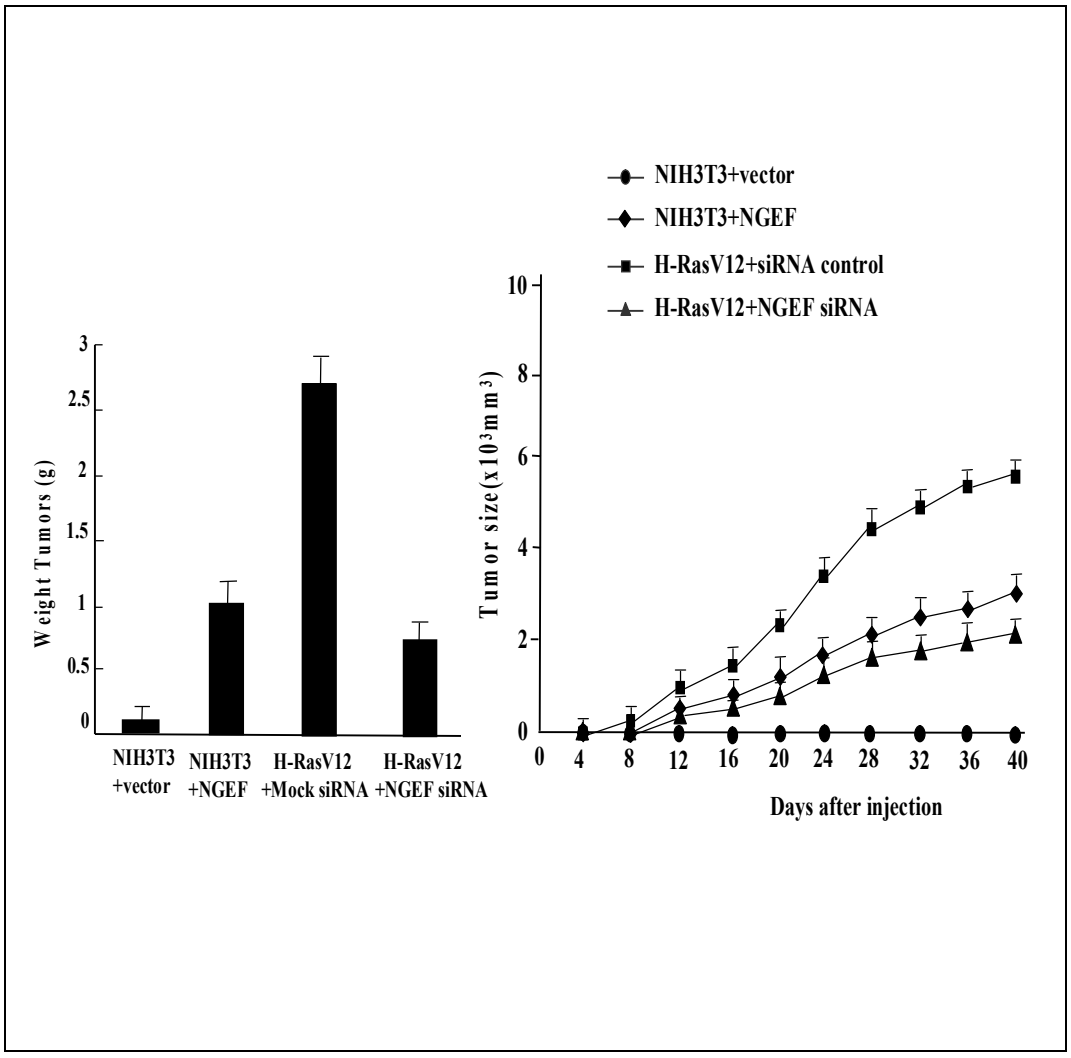

Fig.19. Animal tumor growth assay

NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were subcutaneously injected into immunocompromised nude mice. NIH3T3/pcDNA3.1(●), NIH3T3/NGEF(◆), NIH3T3/H-RasV12(■) and NIH3T3/H-RasV12 siNGEF (▲) injected into a flank of immunocompromised nude mice. The graphs indicate the mean tumor rates ± SD of three animals per experimental condition.

IV. **DISCUSSION**

Ras oncogenes are mutated in a large proportion of human cancers, and Ras pathways are activated by a variety of other mechanisms in many other cancers (Bos, 1989; Clark and Der, 1995). Rational therapies that target Ras downstream signaling molecules essential for malignant cancer cell behavior, but less critical for normal cell function, would therefore have a potential impact (Downward, 2003). Because tumorigenesis contributes to most cancer deaths, and because therapies that target Ras and its downstream signaling pathways are under active development as anticancer agents, an understanding of the biological role of Ras in the tumor progression is clinically important. The mechanisms whereby Ras oncogenes maintain the transformed characteristics of human cancer cells are poorly understood and may differ from those required for tumor initiation. In the present study, we have characterized the functional significance of the upregulation of NGEF (neuronal guanine nucleotide exchange factor) by oncogenic H-Ras (Fig.5-6). We demonstrated that the expression of oncogenic Ras upregulate NGEF expression levels not only in stable H-RasV12 transformed NIH3T3 cells but also in transiently H-RasV12 transfected NIH3T3 cells. Importantly, the relative abundance of the NGEF mRNA and protein was correlated to that of H-Ras in the transformed cells. We confirmed dominant negative form of H-RasN17 suppressed oncogenic H-RasV12-induced NGEF expression (Fig.7). These findings indicated that NGEF is a direct target of oncogenic H-Ras. Ras activation is accompanied by the stimulation of several downstream cascades,

which result in subsequent transcriptional regulation within the cell (Shields et al, 2000). Consistent with these studies, our study also showed that MAPK and PI3Knase activity as measured by the levels of p-ERK and p-AKT, was much higher in H-RasV12-transformed cells than untransformed NIH3T3 cells. Significantly, inhibition of ERK and PI3K activity in the H-Ras-transformed cells by two MEK inhibitors (PD98059 and U0126), PI3K inhibitor (LY294002), resulted in a considerable reduction in the levels of NGEF mRNA and protein (Fig.7-8). These findings provide strong evidence that NGEF is regulated by an activated MAPK and PI3K pathway elicited by oncogenic H-Ras. This effect is mediated by the coordinated activation of several Ras effector pathways, thus offering multiple potential targets for therapeutic intervention. We found that increasing amount of activated, oncogenic H-RasV12-transformed in NIH3T3 cells led to increased Raf, Rho, Cdc42, Rac1 activity. To investigated whether NGEF is involved in the oncogenic H-Ras-mediated increase of small GTPase activity. NIH3T3 cells were transfected with plasmids containing NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA individually or in combination. Surprisingly, expression of NGEF coordinately stimulated the expression of active Rho, active Rac1, active Cdc42. In addition, NGEF siRNA transfected H-RasV12 transformed cell coordinately decreased the expression of active Rho, active Rac1 and active Cdc42. However, expression of NGEF did not affect the Raf activity (Fig.9). These result suggest that NGEF increase of Rho, Rac1 and Cdc42 activity in NIH3T3 and oncogenic H-Ras-transforming NIH3T3 cells. we previously found that the role of GTPases on NGEF

activation in oncogenic H-Ras V12-transformed NIH3T3 cells. To investigate whether NGEF interacted with GTPase (H-Ras, RhoA, Cdc42, Rac1) co- immunoprecipitation experiments were performed. We found that H-Ras and RhoA could see the band, which come from the binding of NGEF in H-Ras V 12- transformed NIH3T3 cell, but not NIH3T3 cell (Fig10). But not Cdc42 and Rac1 couldn't see the binding band (data not shown). These results suggesting that NGEF is closely interacted H-Ras and RhoA , but not Cdc42 and Rac1. Further study, we will do two experiments confirmed that the interacting of NGEF to H-Ras or RhoA. First, we will investigate NGEF binding expression level with anti H-Ras or RhoA in H-Ras V 12 -transformed NIH3T3 cells and H-Ras V 12 -transformed NIH3T3 cells + NGEF si RNA by using co- immunoprecipitation experiments. Second, we will investigate NGEF binding expression level with H-Ras V 12 -transformed NIH3T3 cells and H-Ras V 12 -transformed NIH3T3 + Dominant negative H-Ras or H-Ras V 12 -transformed NIH3T3 cells and H-Ras V 12 -transformed NIH3T3 + Dominant negative RhoA. We next investigated whether or not NGEF plays an important role for oncogenic H-Ras-induced tumor progression, using cell proliferation, colony formation in soft agar, and cellular aggregation assay. We have shown that the stable transfection of NGEF-expressing cells led to increase of the proliferation, colony formation and aggregation, compared with those of the mock- and control empty vector transfected cells (Fig.11, Fig.12-13). In addition, the transfection of the activated H-Ras-expressing cells with NGEF siRNA causes the cells to reduce the oncogenic H-Ras-mediated increased in the proliferation, colony formation and aggregation, compared with those of the

mock- and control siRNA-transfected cells (Fig.11, Fig12-13). These results strongly suggest that NGEF is involved in oncogenic H-Ras-mediated increase of cellular proliferation, colony formation and aggregation. Elevated levels of the Ras protein are often associated with abnormal cell migration, invasion and angiogenesis in multistage carcinogenesis. We surmise that the tumorigenesis caused by oncogenic H-RasV12 could at least in part be due to NGEF overexpression. Indeed, inhibition of NGEF expression using NGEF-specific siRNA in H-RasV12-transformed cells impeded *in vitro* cell migration, invasion and angiogenesis (Fig.14-17). In addition, ability of *in vitro* cellular migration, invasion and angiogenesis of NIH3T3 cells was significantly induced by NGEF overexpression (Fig.14-17). Moreover, protein expression levels of invasion marker MMP-2 and MMP-9 proteins were increased in NGEF expressing cells as compared with the mock- and control empty vector transfected cells (Fig.16). Whereas, MMP-2 and MMP-9 expression levels were significantly decreased in NGEF-specific siRNA transfected H-RasV12-transformed cells as compared with those of the mock- and control siRNA-transfected cells (Fig.16). Expression levels of angiogenesis marker VEGF were increased in NGEF expressing cells, and transfection of NGEF siRNA in H-RasV12-transformed cells led to significant reduce the VEGF expression (Fig.17). Taking together, these results indicate that increased NGEF expression is contributed to the oncogenic H-Ras-mediated cell tumor progression, such as migration, Invasion and angiogenesis (Fig.14-17). Activation of Ras signaling pathway has been shown to be involved in the induction of NGEF, which may contribute to tumorigenesis. Xenograft studies in immunocompromised

nude mice have been used extensively to analyze cellular and molecular mechanisms of tumorigenesis. We have shown that NGEF, which is the downstream target of oncogenic H-Ras in NIH3T3 cells, has been shown to play an important role of tumor progression. We also demonstrate that the ability of NGEF to elicit *in vivo* tumor progression, such as animal tumor growth. The ability of animal tumor growth of NIH3T3 cells with NGEF overexpressing was increased (Fig.18-19), and H-RasV12-transformed NIH3T3 cells with NGEF siRNA expression exhibited significant suppression of *in vivo* tumor progression. These results suggested that NGEF has an essential role for oncogenic H-Ras-induced in vivo tumorigenesis.

In the present study, we have demonstrated that NGEF is an important downstream target molecule, and is required for the oncogenic H-Ras-mediated *in vivo* tumor progression. The MEK-ERK and PI3K-AKT signaling pathway induced by oncogenic H-Ras is contributed to the expression of NGEF mRNA and protein. Moreover, transfection of NGEF in NIH3T3 cells resulted in exhibition of tumorogenetic phenotypes. These results suggest that NGEf expression induced by oncogenic H-Ras seems to play an important role in tumor progression. Nevertheless, future studies using mouse models that more closely recapitulate *in vivo* progression of spontaneously arising human tumors will be instrumental in strengthening the implications of our observations for the pathogenic mechanism of cancer development.

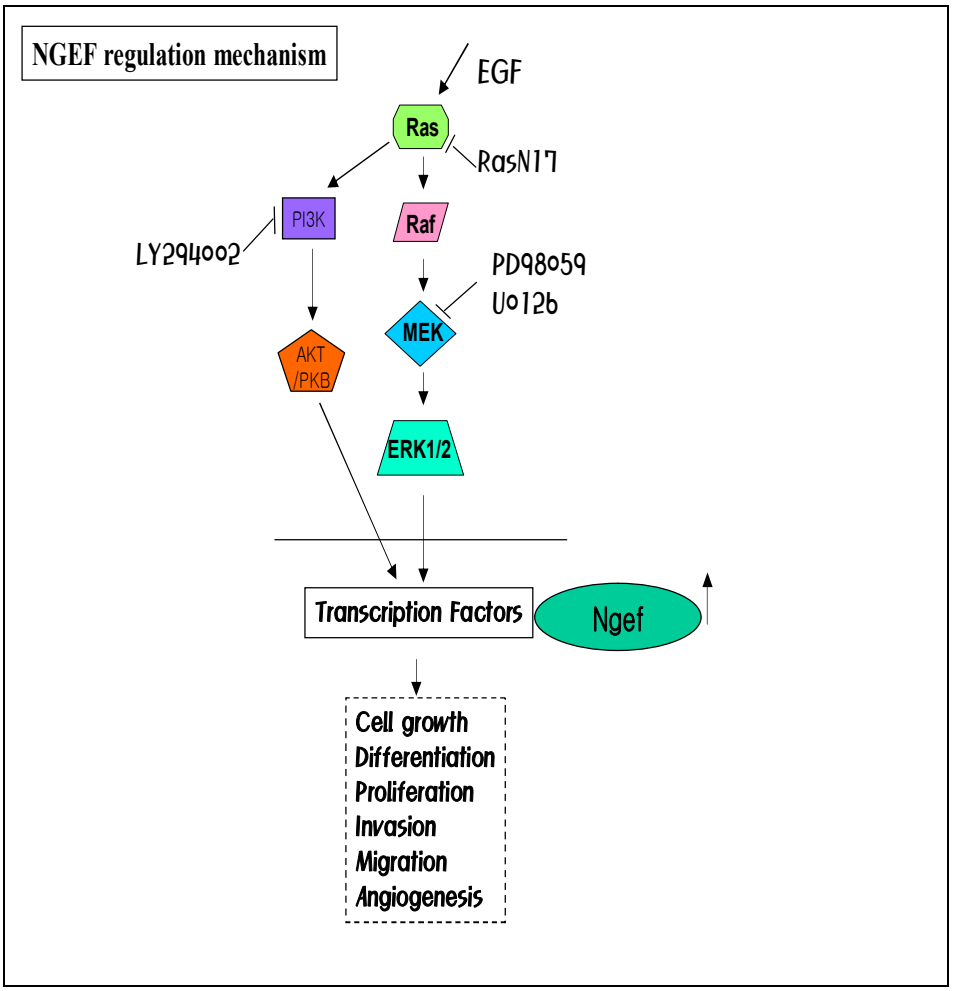

Fig.20. Schematic representation of NGEF-regulation mechanism by induced oncogenic H-RasV12

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<국문초록>

NGEF의 작용 기작

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Ras 종양유전자는 종양발생의 여러 단계에서 역할을 한다고 생각한다. 비록 종양 성장력을 가지고 있는 하류 표적 분자 종양유전자 Ras는 종양력을 가지고 있는 것으로 잘 알려져 있지만 아직 완전히 밝혀지지 않았다. 이번 연구에서는 oncogenic H-RasV12-transformed NIH3T3 세포들에서 NGEF mRNA전사와 단백질의 발현 정도가 현저히 증가되었음을 증명하였다. Oncogenic H-RasV12-transformed NIH3T3 세포들에 월등히 부정적인 형태의 H-RasN17을 일시적으로 형질 도입시키면 NGEF의 mRNA 전사와 단백질의 발현 정도가 감소하였고 ERK와 PI3K 억제제들을 처리함에 Oncogenic H-RasV-induced NGEF의 발현은 현저히 억제되었다. 게다가 NGEF의 과발현은 small GTPases (Rho, Rac1, Cdc42)를 활성화 시킬 수 있고 H-RasV12-transformed 세포들에 NGEF siRNA를 형질 도입 시키면 small GTPases (Rho, Rac1,

Cdc42)의 발현이 감소하였다. Oncogenic H-Ras-induced NGEF 발현의 생물학적 기능을 연구하기 위해서 우리는 oncogenic H-Ras-mediated 종양 발생에서 NGEF가 관련되어 있는지를 조사하였다. NGEF 과 발현 세포들의 세포 증식, 부드러운 배지에서의 집단 형성, 세포 집합의 능력이 빈 운반체를 형질 도입시킨 세포들과 비교할 때 현저히 증가하였다. H-RasV12-transformed NIH3T3 세포의 세포 증식, 부드러운 배지에서 집단 형성, 세포 집합의 능력은 NGEF siRNA를 형질도입 시킴으로서 현저히 억제되었다. 게다가 H-RasV12-transformed NIH3T3 세포들에 NGEF siRNA 형질도입이 시험관내 세포 이동, 침윤, 신생혈관형성 감소를 나타냄을 증명하였다. 또한 H-RasV12-transformed 세포들에 형질도입한 NGEF siRNA는 동물 종양 성장을 현저히 감소하는 것으로 나타났고 반면에 control siRNA 형질도입은 감소하지 않았다. 이러한 결과들로 NGEF는 oncogenic H-Ras의 새로운 하위 표적 단백질이고 oncogenic H-RasV12에 의해 유도된 NGEF의 과 발현은 종양 형성에 있어 중요한 역할을 수행 할 것으로 생각한다.

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 (0) 반대 ( )

2010 2 월 일

저작자: 김 민 지 서명 또는 인)

조선대학교 총장 귀하