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The study of oncogenic Ras-induced cancer development

조선대학교 대학원

생물신소재학과

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암유전자인 **Ras**의 암유발기작규명

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지도교수 유 호 진

이 논문을 이학박사학위신청 논문으로 제출함.

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조선대학교 대학원
생물신소재학과
김 나 희

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2011년 6월

조선대학교 대학원

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CAAX	C, cysteine; A, aliphatic amino acid; X, any amino acid
CAK	CDK activating kinase
CDK	cyclin-dependent kinases
CKIs	CDK inhibitors
DEGs	Differentially Expressed Genes
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EGF	epidermal growth factor
FITC	fluorescein isothiocynate -
FPP	farnesyldiphosphate
FTase	farnesyltransferase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAPs	GTPase activating proteins
GEF	guanine exchange factor
GGPP	geranylgeranyldiphosphate
GGTase I	geranylgeranyltransferase type I
GGTase II	geranylgeranyltransferase type II
GTPases	guanosine triphosphate hydrolases
HIF-1	hypoxia-induced factor-1
IL-1	interleukin-1
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MTT	(3-94,5-dimethylthiazole-2-yl)-2,5-diphrnyl tetrazolium

NLEF	Neuronal nucleotide exchange factor
Pak1	p21-activated protein kinase 1
PBD	p21-binding domain
PBS	phosphate-buffered saline
PI	phosphotidylinositols
PIP2	phosphatidylinositol 4,5-phosphate
PIP3	phosphatidylinositol 3,4,5-phosphate
PI3K	phosphatidylinositol 3-kinases
Rb	retinoblastoma
RBD	Ras-binding domain
RCE1	Ras converting enzyme 1
RDA	representational difference analysis
SAPK/JNK	stress-activated protein kinase/c-Jun Nterminal kinase
SSH	subtractive suppression hybridization
TGF-α	transforming growth factor-α
TIMP-2	tissue inhibitor of matrix metalloproteinase-2
VEGF	vascular endothelial growth factor

<국문초록>

암유전자인 Ras의 암유발기작규명

김 나 희
지도교수 : 유 호 진
조선대학교 대학원 생물신소재학과

Ras oncogenes는 human cancers의 많은 부부에서 돌연변이 되어 있고, 다른 cancer에서는 다양한 메커니즘을 통해 Ras pathway는 활성화 되어 있다. Ras가 유도하는 종양의 진행 메커니즘은 충분히 밝혀지지 않았다. 이번 연구에서 NLEF level은 human fibroblast GM00637 cells에서 oncogenic H-Ras발현에 의해 상당히 증가되었다. NLEF protein level는 Ras-dominant active cancer cells인 A549, H1299, H460, Sw480, DLD1, Capan-1, and Panc-1 cells에서 감소되어 있고 H-RasN17는 이러한 cell들의 NLEF발현은 억제시킨다. 게다가 ERK and PI3K 억제제의 처리는 Ras-dominant active cancer cells에서 NLEF발현은 상당히 억제시켰다. 이러한 결과는 NLEF는 oncogenic H-Ras의 새로운 downstream target molecule이고 H-Ras-mediated 에 의해 증가되는 ERK and PI3K 활성는 NLEF발현이 필요하였다. Oncogenic-H-Ras-induced NLEF 발현의 생물학적 기능을 연구하기 위해서 우리는 oncogenic Ras-mediated 종양발생의 증가에 NLEF가 관련되어 있는지를 조사하였다. NLEF의 이소성 발현이 GM00637 cells 에서 ERK1/2 and Akt의

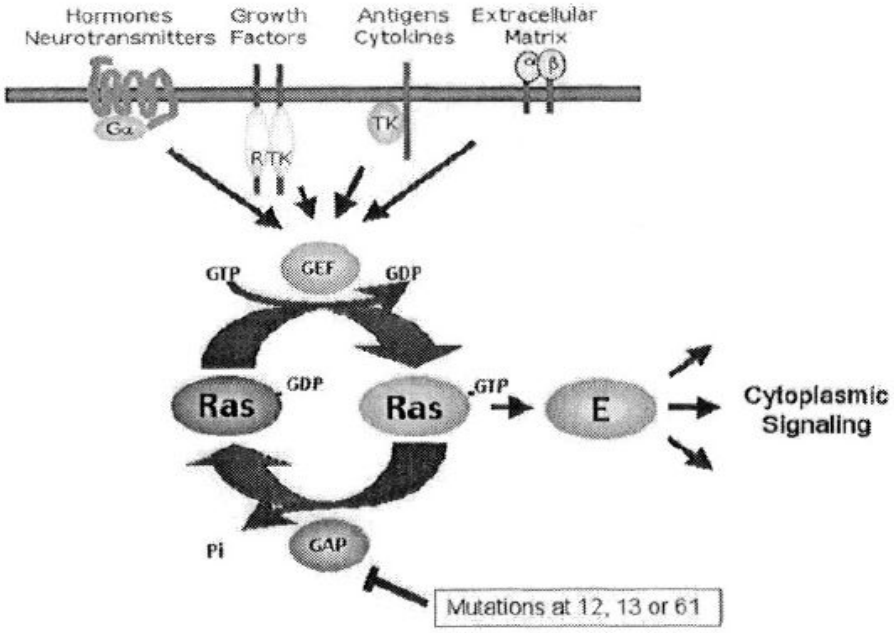
serum-stimulated phosphorylation이 증가 시킴을 찾아냈다. Human colon cancer cells인 HCT116, DLD1, and SW480 cells에서 NLEF shRNA 처리는 몇 개의 cell cycle progression proteins인 p-histone-H3, p-Rb, and p-cdc2의 phosphorylation의 억제의 원인이 되었다. 나는 NLEF 발현이 부드러운 배지에서 집단형성을 증가 시킴을 이끌었다. 더욱이, NLEF shRNA를 형질도입 시킨 A569, H460, H1299, HCT116, and DLD1 cells은 anchorage-independent 성장이 상당히 감소함을 보여주었다. 본 연구에서는 huamn cancer cells의 세포 증식 능력은 NLEF shRNA를 형질 도입 시킴으로서 현저히 억제됨을 보여주고 있다. 이러한 결과들로 NLEF의 upregulation은 Ras에 의해 유도된 매개종양변환발생의 중요한 역할을 수행할 것으로 생각한다.

I. Introduction

Ras proteins are GTPases that act as molecular switches, transmitting signals from activated receptors to downstream effectors to mediate cell proliferation, survival and differentiation. 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 nucleotide exchange factors(GEFs). 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. 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.

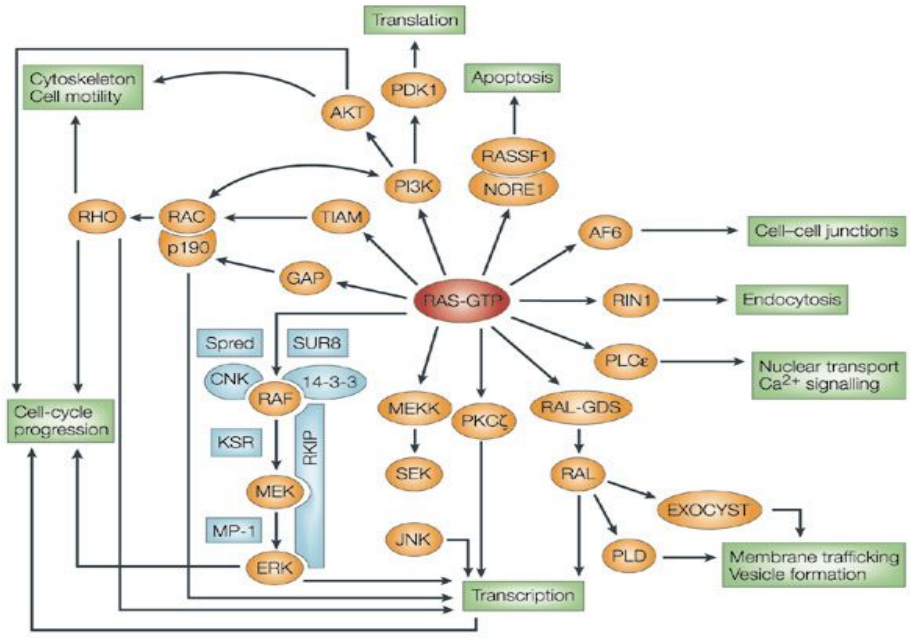
The critical requirement for Ras association with the plasma membrane has prompted considerable effort to identify pharmacologic approaches to block the CAAX-mediated modifications to then block Ras function (Oliff, 1999; Cox, 2001). Of these efforts, the development of FTase inhibitors (FTIs) has been 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.



The Ras pathway in cancer

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

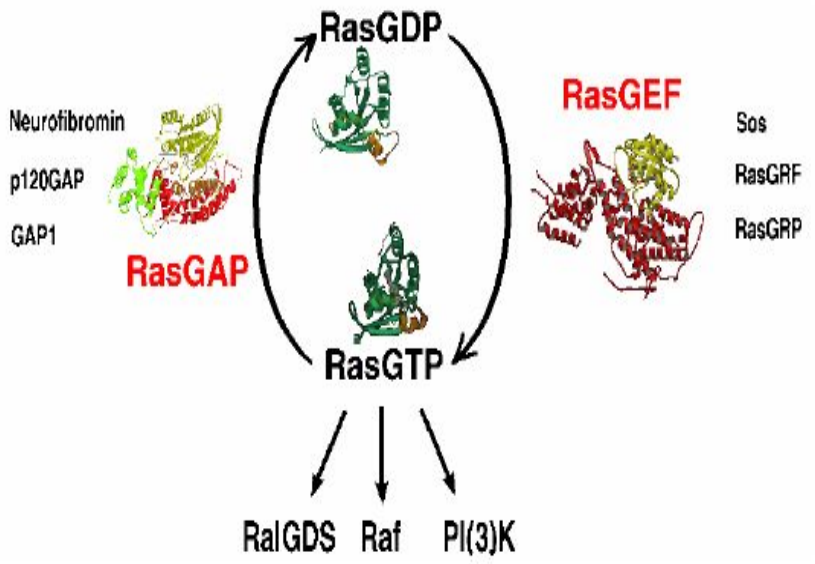
(Oliff, 1999; Cox, 2001). While these various observations support functional distinctions, clear and significant functional differences important for the mechanism of Ras-mediated oncogenesis remain to be identified.



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). 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).



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.

Ras serves as a point of convergence of signaling initiated by diverse extracellular stimuli. This includes stimuli that recognize receptor tyrosine kinases, cytokine receptors, G protein-coupled receptors and integrins. Once activated Ras interacts with and regulates a complex spectrum of functionally distinct effectors to stimulate a multitude of signaling cascades that regulate cytoplasmic (e.g., actin organization) and nuclear (e.g., gene expression, cell cycle progression) processes important for many normal cellular processes. Normal and oncogenic Ras mediate their biological functions by binding to downstream effectors (Shields et al., 2000). All effectors bind to a core effector loop of Ras proteins (residues 32-40), with additional involvement of residues that change in conformation during GDP/GTP cycling; the switch I (residues 30-38) and switch II (residues 59-76) domains (Marshall, 1996; Campbell et al., 1998). The GTPbound form displays a significantly greater affinity for effectors. In recent years, the number of Ras effectors and the complexity of downstream pathways that they regulate have grown considerably. We will focus on the contribution of three key Ras effectors to Ras-mediated signaling and transformation.

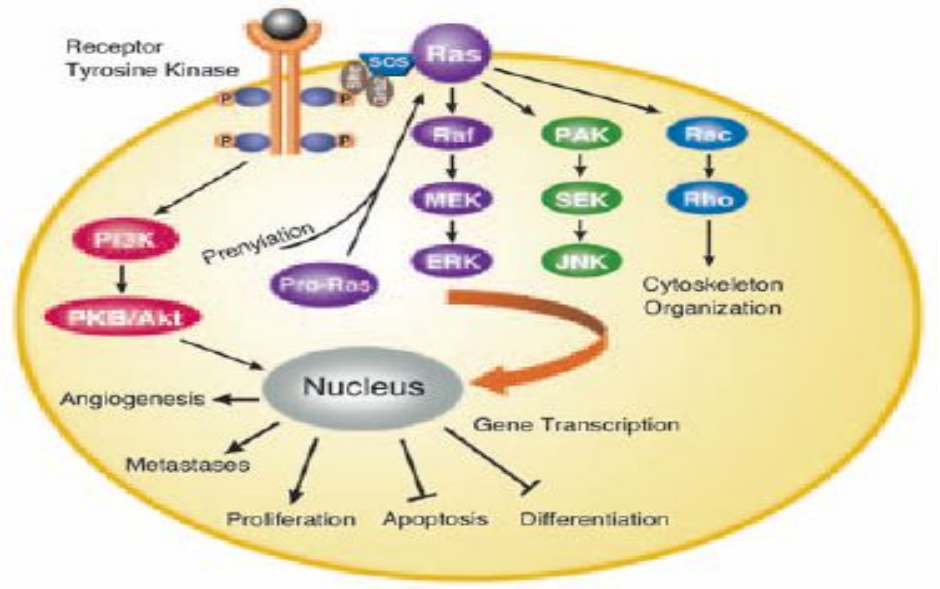
The first Ras-induced signal transduction cascade to be identified was the Raf>MEK>ERK protein kinase cascade (Marshall, 1996; Campbell et al., 1998) Activated Ras binds to and promotes the activation of Raf serine/threonine kineses (c-Raf-1, A-Raf and B-Raf). Ras causes activation of Raf, in part, by promoting a translocation of Raf to the plasma membrane, where additional binding and phosphorylation events are necessary for complete Raf activation (Morrison and Cutler, Jr., 1997). Once activated, Raf phosphorylates and activates the MEK1/2 dual specificity kinases that in turn phosphorylate and activate ERK1/2 mitogen activated protein kinases (MAPKs). Activated ERKs translocate to the nucleus and phosphorylate various transcription factors that include the Ets family member Elk-1.

The second best characterized effectors of Ras are phosphatidylinositol 3-kinases (PI3Ks), lipid kinases consisting of a p85 regulatory and a p110 catalytic subunit (Rodriguez, Viciana et al., 1994; Rodriguez-Viciana et al., 1997). PI3K phosphorylates integral membrane phosphotidylinositols (PI) at the 3' position (e.g., phosphatidylinositol 4,5-phosphate; PIP2) to generate various short-lived second messenger products (e.g., phosphatidylinositol 3,4,5-phosphate; PIP3) (Vanhaesebroeck et al., 1997). Membrane-associated PIP3 in turn can regulate the activity of a diverse array of signaling molecules that include the Akt serine/threonine kinase. Akt activation results in complex signaling cascades that lead to the phosphorylation of diverse substrates such as caspases, transcription factors (ATX), and proapoptotic proteins (BAD) that regulate cell survival (Chan et al., 1999). PI3K also mediates antiapoptotic signaling, as well as actin organization, by activating the Rac small GTPase (Bar-Sagi and Hall, 2000). The importance of PI3K in Ras transformation is best characterized in NIH 3T3 mouse fibroblasts. However, PI3K is

not required for Ras transformation of other cells, reflecting cell-type differences in Ras effector utilization in transformation (McFall et al., 2001).

The third best understood Ras effectors are Ral GEFs (RalGDS, Rgl, Rlf/Rgl2, etc.) that function as activators of the Ras-related RalA and RalB small GTPases (Feig et al., 1996). RalGEF activation by Ras leads to a GTPase cascade in which activated, GTP-bound Ral binds RalBP1, a putative Rho family GAP. Activated Ral also mediates phosphorylation of the fork head transcription factor AFX, which may provide a link between Ras and the cell cycle (Medema et al., 2000). Whether the effects of RalGEF activation are mediated solely by Ral activation or whether RalGEF has other functions is not clear. RalGEF binding to Ras has been shown to stimulate transcription of transcription factors, proteases and cell cycle components (Reuther and Der, 2000).

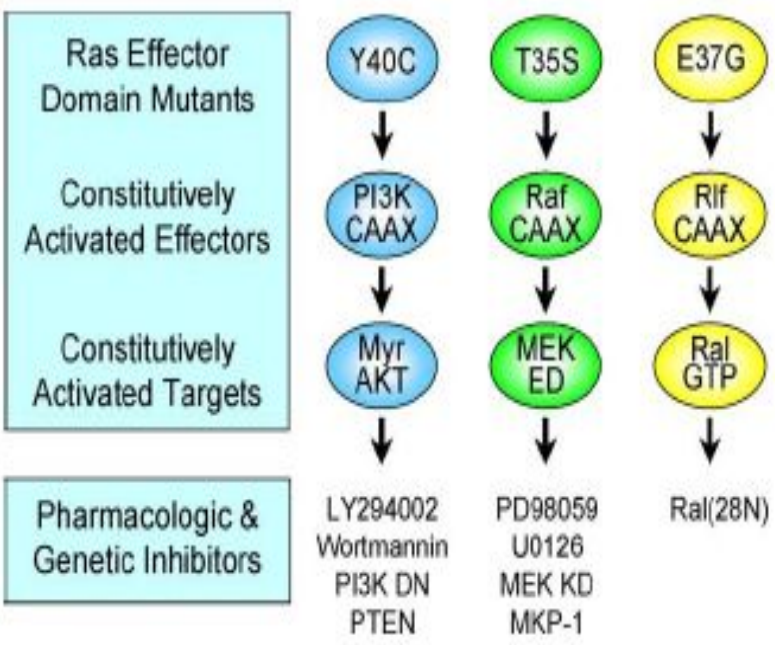
Ras proteins bind a large number of other effectors including AF-6, PLCε, PKCζ, Nore1, and RASSF1 (Cullen, 2001; Feig and Buchsbaum, 2002). The roles of these effectors in Ras function are only now being studied. Each different effector pathway contributes distinct aspects of Ras-mediated tumor progression and metastasis. Dissecting these pathways and determining the level of crosstalk has become staggeringly complex but may ultimately increase our understanding of the role of Ras in carcinogenesis and invasion. We will focus on an overview of the contribution of the three main effectors Raf, PI3K and RalGEF to Ras deregulation of proliferation, apoptosis, angiogenesis and invasion/metastasis through gene deregulation .



Ras-mediated signal transduction pathways

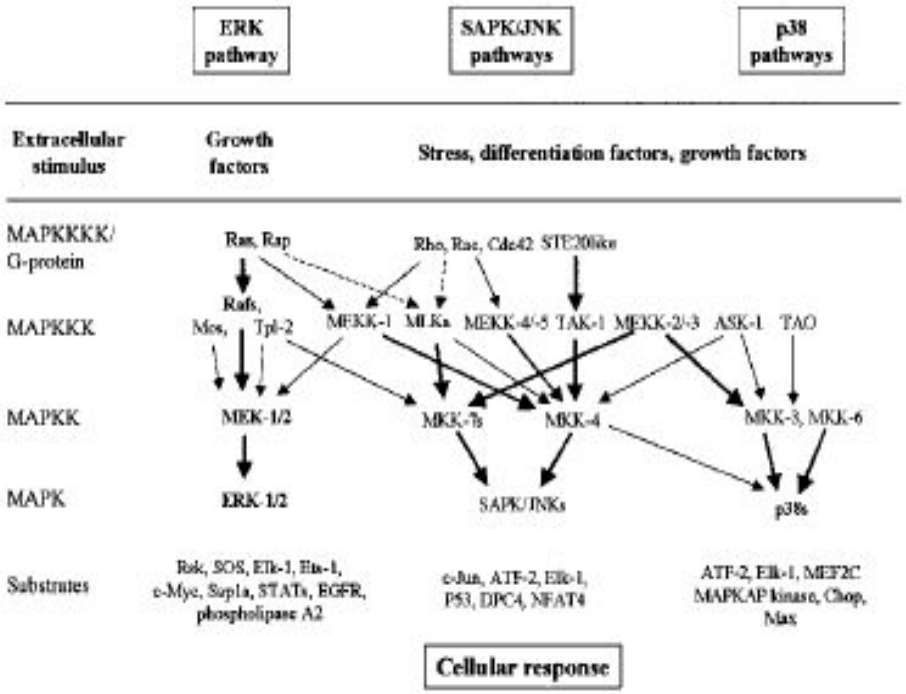
In light of the interaction of Ras with multiple effectors, one important issue has been to determine the contribution of each effector in mediating the diverse actions of oncogenic Ras. The ability of activated Raf or MEK alone to cause transformation of NIH 3T3 mouse fibroblasts initially suggested that the Raf>MEK>ERK cascade alone was sufficient for Ras transformation (Marshall, 1996; Campbell et al., 1998). However, it is now clear that Ras causes transformation by utilization of Raf-dependent as well as Raf-independent effector signaling. Another facet that has emerged from these studies is that there can be striking cell-type differences in the contribution of specific effectors to Ras transformation. One important experimental approach that demonstrated the involvement of Raf independent effectors in Ras transformation was the identification of effector domain mutants of Ras that showed impaired interaction with a subset of effectors (Rodriguez-Viciana et al., 1997; White et al., 1995; Joneson et al., 1996; Khosravi- Far et al., 1996). These mutants have single mutations at

residues E35, E37, and Y40. The E35S mutant retains the ability to bind to and activate Raf but is impaired in binding to RalGEF and PI3K. The E37G mutant also lost the ability to activate Raf and PI3K, but retained the ability to activate RalGEF, whereas the Y40C mutant retained the ability to activate PI3K but not Raf or RalGEF. The E37G and Y40C mutants showed impaired ability to bind to and activate Raf, yet they retained the ability to cause tumorigenic transformation of NIH 3T3 cells (Khosravi-Far et al., 1996; Webb et al., 1998). Hence, the transforming activity of 37G or 40C has been attributed to their ability to activate RalGEF or PI3K, respectively. These mutants have been very useful reagents to assess the role of Raf, RalGEF, and PI3K in Ras function. Constitutively activated effectors have also been useful reagents for assessing the role of each effector in Ras function. Since Ras promotes effector activation, in part, by promoting their membrane association, the addition of the carboxyl terminal plasma membrane-targeting sequence of Ras onto effectors has been a useful approach to generate constitutively-activated variants of Raf-1, the p110 catalytic subunit of PI3K, and various RalGEFs (Rodriguez-Viciana et al., 1997; Leevers et al., 1994; Stokoe et al., 1994; Wolthuis et al., 1997).



Effectors of Ras function

Pharmacologic or genetic inhibitors of specific effector signaling pathways have also been useful reagents for defining the contribution of specific effectors in Ras transformation. For example, LY294002 is a specific inhibitor of PI3K, whereas PD98059 and U0126 are specific inhibitors of MEK activation of ERK (Davies et al., 2000). LY294002, but not PD98059, treatment reversed the ability of oncogenic Ras to inhibit suspension-induced apoptosis, or anoikis, in MDCK canine kidney epithelial cells (Khwaja et al., 1997). This demonstrated the critical role of PI3K but not Raf in mediating this important facet of anchorage-independent growth. Finally, kinase-dead mutants of Raf-1, MEK, ERK, Akt, and dominant negative Ral have been useful genetic inhibitors of specific effector signaling pathways (Rodriguez-Viciana et al., 1997; Brtva et al., 1995; Cowley et al., 1994; Khosravi-Far et al., 1995).



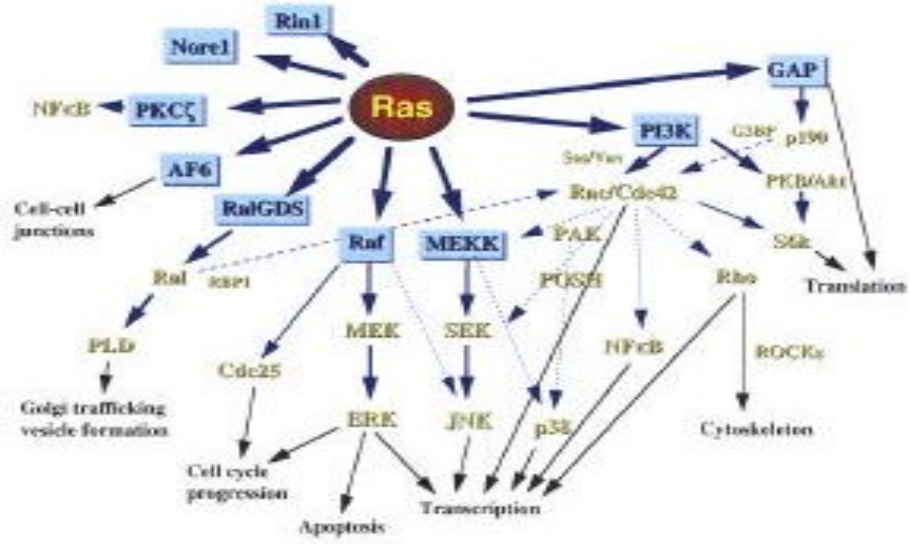
The best-characterized MAPK modules are the ERK pathway, the SAPK/JNK pathway, and the p38 pathway

As indicated above, signaling initiated by the three main Ras effectors results in the stimulation of a variety of transcription factors (Campbell et al., 1998). Therefore, it is not surprising that Ras transformation has been shown to be dependent on the function of many of these transcription factors. For example, depletion of c-*myc* with specific antisense sequences (Sklar et al., 1991) or expression of dominant negative mutants of Ets (Wasylyk et al., 1998; Langer et al., 1992; Wasylyk et al., 1994), c-Fos (Wick et al., 1992) or c-Jun (Granger-Schnarr et al., 1992) have been shown to block Ras-mediated transformation of NIH 3T3 fibroblasts. Similarly, c-*jun* null mouse embryo fibroblasts were found to be insensitive to Ras-mediated transformation (Johnson et al., 1996). An

essential requirement for c-*fos* in Rasmediated skin tumor formation was shown in c-*fos* knockout mice carrying an H-*ras* transgene (Saez et al., 1995). Finally, inhibition of NF-$_K$B blocked Ras-mediated transformation and resulted in apoptosis of rodent fibroblast cell lines (Finco et al., 1997; Mayo et al., 1997). Taken together, these observations demonstrate the essential role of gene expression changes in Ras-mediated oncogenesis.

At least two broad approaches have been utilized to define the gene targets involved in Ras transformation. First, several techniques to study genome-wide changes in gene expression have been applied to study the transcriptional changes associated with Ras- or Raf -mediated expression or transformation. These techniques include differential display (Liang et al., 1994; McCarthy et al., 1995; Zhang et al., 1998), subtractive suppression hybridization (SSH) (Baba et al., 2000; Zuber et al., 2000), representational difference analysis (RDA) (Shields et al., 2001b; Shields et al., 2001a), and microchip array analyses (Schulze et al., 2001; Habets et al., 2001). These approaches reveal the complexity of gene expression changes associated with Ras transformation. For example, SSH was also employed by Schafer and colleagues to identify genes whose expression was upregulated or downregulated in H-Ras-transformed 208F rat fibroblasts (Zuber et al., 2000). They identified transcriptional stimulation or repression of 244 known genes, 104 ESTs, and 45 novel sequences. Overall, it was estimated that 3 to 8% of all expressed genes were altered in Ras-transformed cells. Interestingly, only a fraction of these gene expression changes were reversed by inhibition of MEK, indicating that Raf>MEK>ERK independent pathways contribute significantly to gene deregulation. This possibility is also supported by RDA analyses that identified gene expression changes caused by activated Ras but not Raf (Shields et al., 2001b; Shields et al., 2001a).

A second approach for defining gene targets of Ras has involved an evaluation of whether the expressions of specific genes whose products may contribute to transformation are altered by oncogenic Ras. Included among these are genes encoding proteins that regulate cell proliferation and cell cycle progression, tumor cell invasion and metastasis, and angiogenesis. In the sections below, we summarize some of the findings that have come from these studies. We have not provided a complete summary of this topic. Instead, we have chosen to highlight specific examples of gene targets that may promote oncogenic Ras deregulation of cell proliferation and induction of tumor cell invasion, metastasis, and angiogenesis. These examples also further highlight the role of Raf-independent effectors in Ras oncogenesis as well as cell-type differences in Ras signaling.



Ras effectors and downstream pathways

The significant role of aberrant Ras activation in increased cancer cell growth and proliferation has been well-established. In examining the contribution of Ras to stimulus-independent growth and the inhibition of growth arrest pathways, two themes emerge: deregulation of the cell cycle and induction of growth factor autocrine loops. The first allows Ras-transformed cells to overcome growth arrest imposed by cell cycle checkpoints; the second renders cells self-sufficient by providing a constant stimulus to proliferate. Deregulation of key components of both vital cell regulatory mechanisms can be achieved, in part, by Ras-mediated changes in gene expression.

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. This study reports an oncogenic H-Ras target genes, neuronal nucleotide exchange factor (NLEF), which were identified through this screening, contributes to the oncogenic H-Ras-mediated increase in tumor cell proliferation aggregation. This study is the first to indicate that NLEF is a downstream target molecule of oncogenic Ras and is regulated for oncogenic Ras-induced tumorigenesis.

II. Materials and Methods

(A) Cell culture

The human cancer cells were purchased from American Type Culture Collection (ATCC number 30-2001) and grown in RPMI1640 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 °C in a humidified incubator.

(B) 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. Monoclonal NLEF antibody was purchased from ATGen. Anti-H-Ras (F235), phosphor-Histone H3(Ser10), phosphor-Rb(Ser795), Cyclin B1, CDK6(DCS83), phosphor-cdc2(Tyr15), p21 Waf1/Cip(12D1), Myt1, anti-α-tubulin, 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, anti-p15 INK4B antibody was purchased from Cell Signaling Technology. Anti-Ras was purchased from PIERCE.

(C) Plasmid constructs and Transfection

The human GM00637 fibroblasts cell ransfected with the pCMV-AC-human NLEF (Origene), pCMV-RasV12(Clontech) expression vector using the Lipofectamine method according to the manufacturer′s instructions. Two target sequences for shRNA TRCN47655 5'CCGGCCAAGA ACCTCAAGGAATGTTTCTCGAGAAACATTCCTTGAGGTTCTGGTTTTTG and TRCN47657 5'-CCGGAGACAAGTACCAGGTATTCTCGAGAATACCTGGTAGTCTCCT TTTTTG(SIGMA) was transfected into A549, H460, H1299, HCT116, DLD1cells. The hNLEF 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.

(D) 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 mhNLEF 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 CDK4 monoclonal antibody, CDK6 monoclonal antibody, CyclinD3 monoclonal antibody, Rb2 monoclonal antibody, p15 INK4B polyclonal antibody, p27 Kip1 polyclonal antibody, phospho(Ser807/811)-Rb polyclonal antibody (Cell Signaling Technology) 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)

(E) Small GTPase activation assay

The activation of Ras was assayed using the EZ-Detect^{TM} Ras activation kits according to manufacturer's instruction (PIERCE). Human cancer 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 RPMI 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 provided in the kit.

(F)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.

(G) 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.

(H) 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 RPMI 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.

(I) 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

(A) Oncogenic H-RasV12 induces expression of the NLEF

In order to determine expression levels of NLEF after H-RasV12-transfection, western blot was carried out using an antibody against the NLEF. 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 the protein from the empty vector-transfected cells. Western blot analysis with the NLEF antibody showed that the NLEF protein levels were higher in the H-RasV12-transfected cells than the empty vector-transfected cells (Figure.1). Thus, oncogenic H-RasV12 expression induces NLEF expression in GM00637 cells.

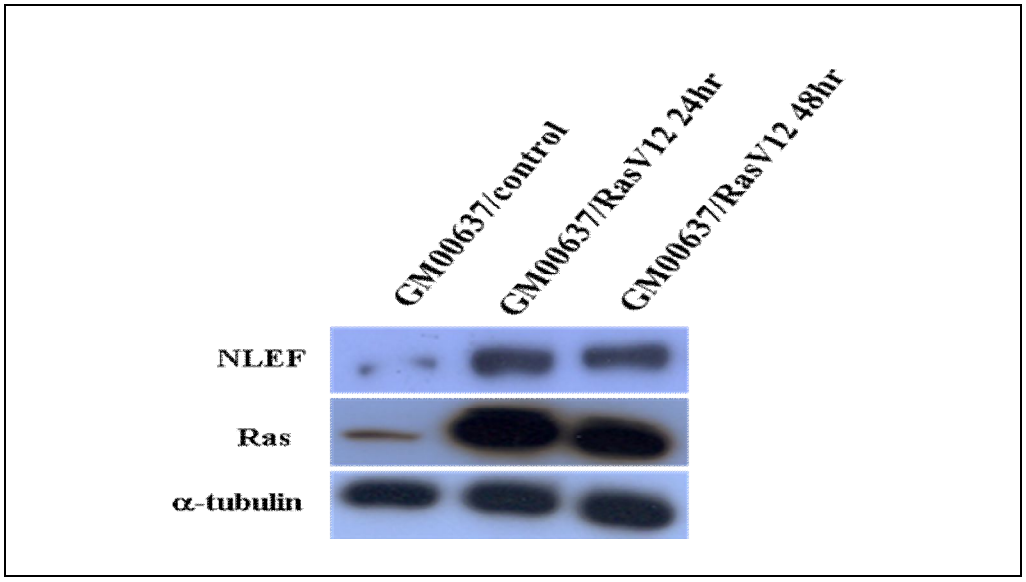


Fig.1. Oncogenic H-RasV12 induces expression of the NLEF
Expression levels of NLEF in control empty vector- and H-RasV12 expressing vector-transfected GM00637 cells. Total cell extracts were used to detect Ras and NLEF by western blotting analysis. α-tubulin was used as the loading control.

(B) Effect of NLEF on the aggregation in GM00637 and oncogenic H-RasV12-transforming GM00637 cells

To investigate whether NLEF 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 showed that wild-type GM00637 cell did not have any morphological change (Figure.2A). By contrast, expression of NLEF in GM00637 cells resulted in the aggregation of these cells under subconfluent conditions (Figure.2B). In addition, GM00637/H-RasV12 cells have an increased tendency to aggregate, and NLEF transfection led to significant increase of cellular aggregation of these cells.

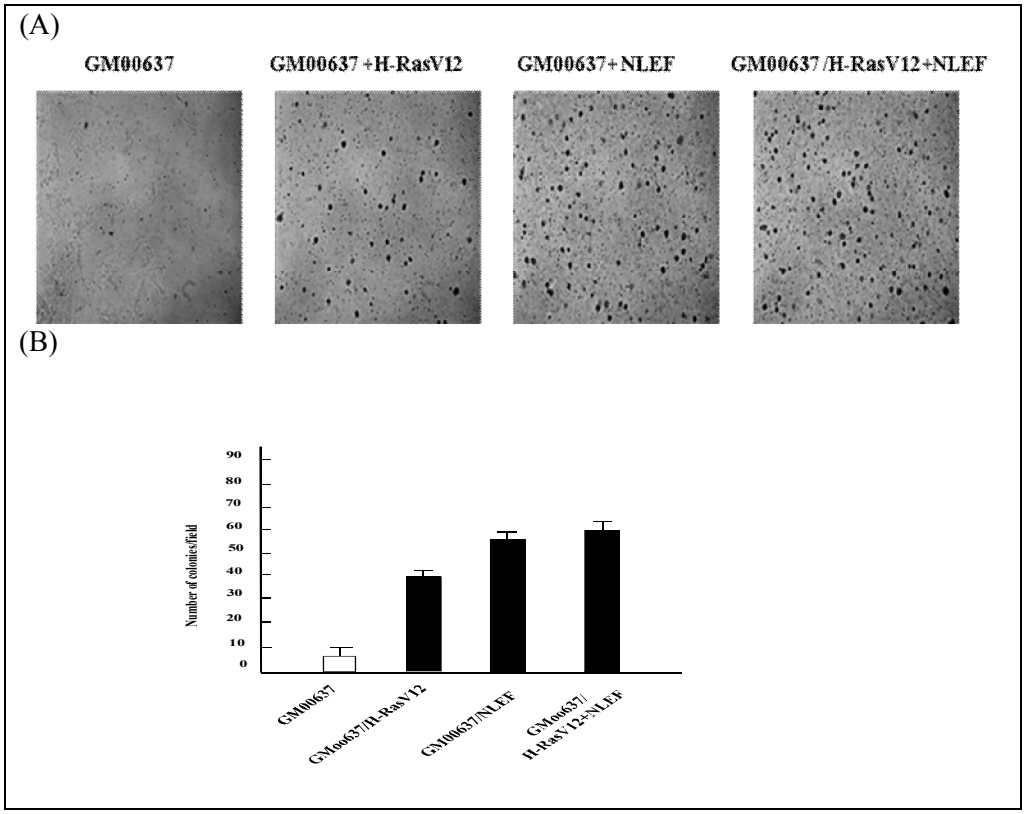


Fig.2. *In vitro* Aggregation assay

Single-cell suspensions of GM00637, GM00637/H-RasV12, GM00637/NLEF, GM00637/H-RasV12+NLEF 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 and recorded as digital images using Adobe Photoshop. Mean values±s.e.m are shown of three independent experiments.

(C) Effect of NLEF on protein levels related to signal pathway of Ras

To check which pathway contributes to Ras-mediated NLEF protein expression, GM00637, GM00637/H-Ras, GM00637/NLEF, GM00637/H-RasV12+NLEF cells were anaysed using western blot. P-p44/42 and p-p38 levels were induced after NLEF overexpression (Figure.3). According to these date, we concluded that Ras-mediated NLEF expression was regulated through MEK and MAPK signal pathway.

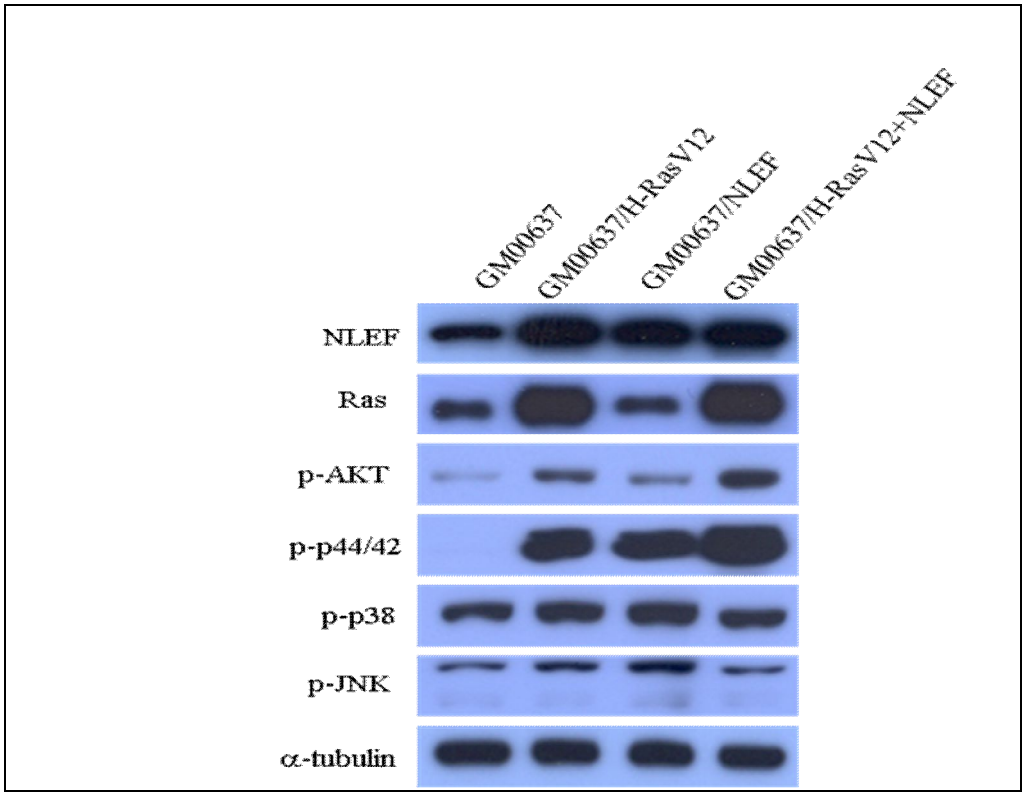


Fig.3. Effect of NLEF on Ras-signal pathway proteins.
Expression levels of proteins related to signal pathway of Ras. GM00637, GM00637/H-RasV12, GM00637/NLEF, GM00637/H-RasV12+NLEF were harvested and the NLEF expression was analyzed by Western blotting. α-tubulin was used as the loading controls of western blotting, respectively.

(D) Effect of NLEF on the cell cycle regulator proteins

A number of experimental approaches have established the importance and requirement for endogenous Ras for cell cycle progression and the ability of oncogenic Ras to promote growth factor-independent cell cycle entry. To assess if NLEF has any effects on the cell cycle, we investigated whether shNLEF could affect the cell cycle checkpoint related genes. We found that shNLEF expression led to decrease of phosphor-Histone H3(Ser10), phosphor-Rb(Ser795), Myt1,

expression in HCT116, DLD-1 and SW480 cells, phosphor-cdc2(Tyr15) expression in SW480 cell (Figure.4). Thus, NLEF may regulate cell cycle progression through expression of several cell cycle progression proteins, and they suggest that NLEF is an important role for cell cycle regulation.

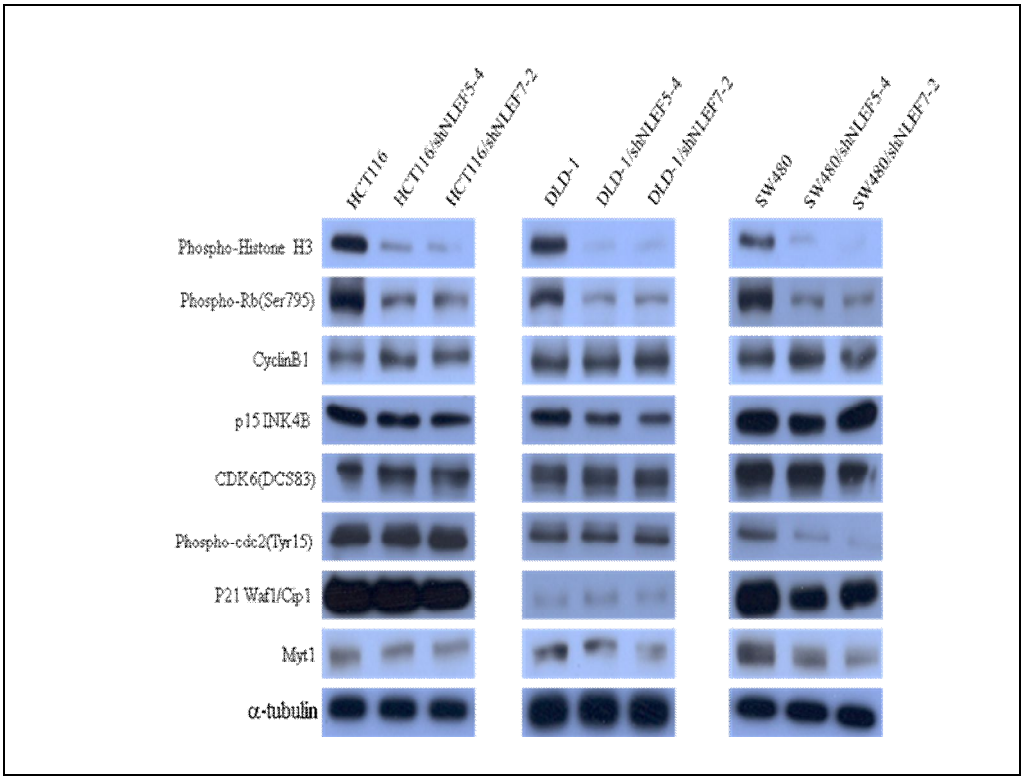
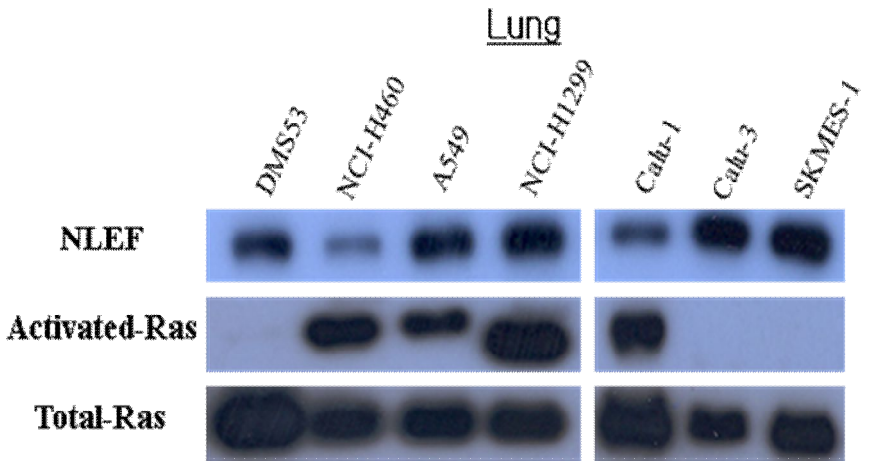


Fig.4. Screening of cell cycle regulator proteins
Cell cycle regulator proteins were analyzed by western blot using the indicated antibodies in indicated cells. The phosphor-Histone H3(Ser10), phosphor-Rb(Ser795), Cyclin B1, p15 INK4B, CDK6(DCS83), phospho-cdc2(Tyr15), p21 Waf1/Cip(12D1), Myt1, and anti-NLEF antibodies were used. α-tubulin was used as the loading control.

(E) Screening of Ras and NLEF expression

To check the expression levels of Ras and NLEF in various cells, western blot analysis was used. The interaction between NLEF and activated Ras was determined. Lung cancer cells, A549, H460 and H1299 showed increased level of NLEF and activated Ras (Figure.5A). Colon cancer cells, HCT116, SW480, and DLD1 cells also showed increased level of NLEF and Ras (Figure.5B). In addition Capan-1, MIA-Capa2 and PANC-1 (Pancreas cancer cells) and HT-1080 showed elevated expression of NLEF and activated Ras (Figure.5C). We selected some cells for futher study according to correlation of NLEF and Ras expression.

(A)



(B)

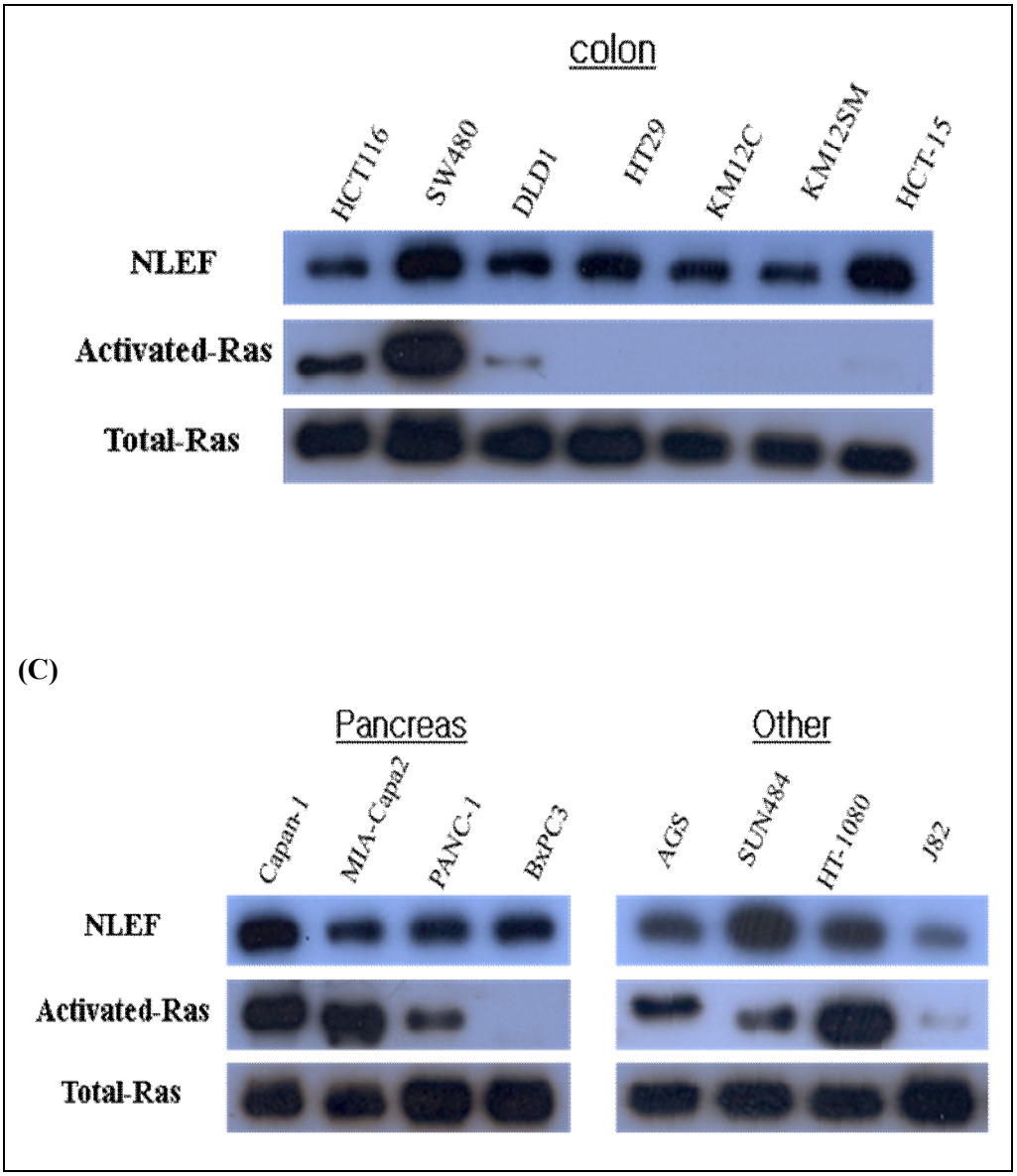
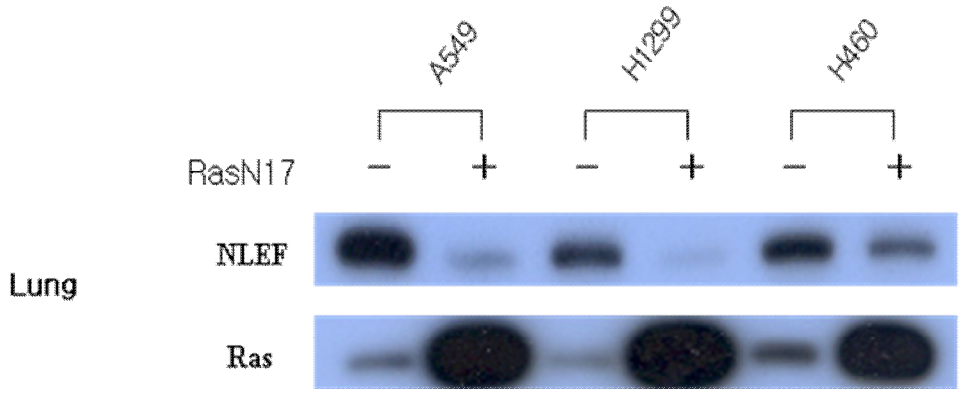


Fig.5. Screening of Ras and NLEF expression in human cancer cells
NLEF and Ras expression levels were detected in various cells. The eluted samples (25ul) and 20ug of cell lysate were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and detected with NLEF and Total-Ras antiboies.

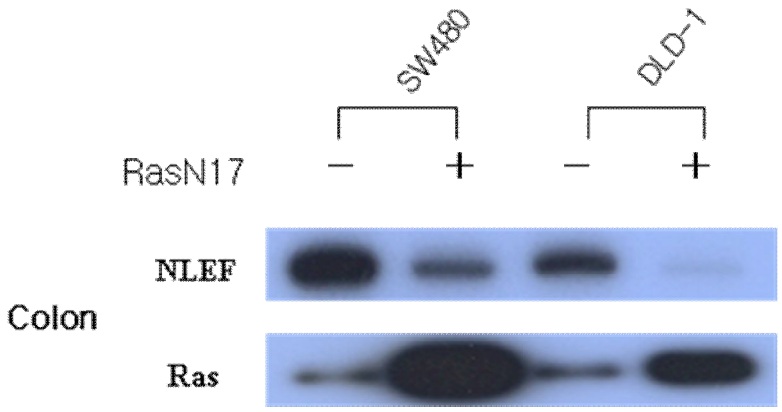
(F) Dominant negative H-RasN17 supprssed Ras-induced NLEF expression in Human cancer cells

To confirm whether oncogenic H-Ras is indeed involved in the NLEF expression in human cancer cells, dominant negative H-RasN17 was transiently transfected into Lung cancer cells (A), Colon cancer cells (B), and Pancreas cancer cells (C). 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 Ras, but it is expressed significantly higher levels. Expression of the Dominant-negative Ras mutant 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. We found that transiently introducing a dominant negative form of H-RasN17 resulted in the downregulation of NLEF expression in human Lung cancer cells (A), Colon cancer cells (B) and Pancreas cancer cells (C).

(A)


A549
H1299
H460
RasN17
– + – + – +
NLEF
Lung
Ras

(B)


SW480
DLD-1
RasN17
– + – +
NLEF
Colon
Ras

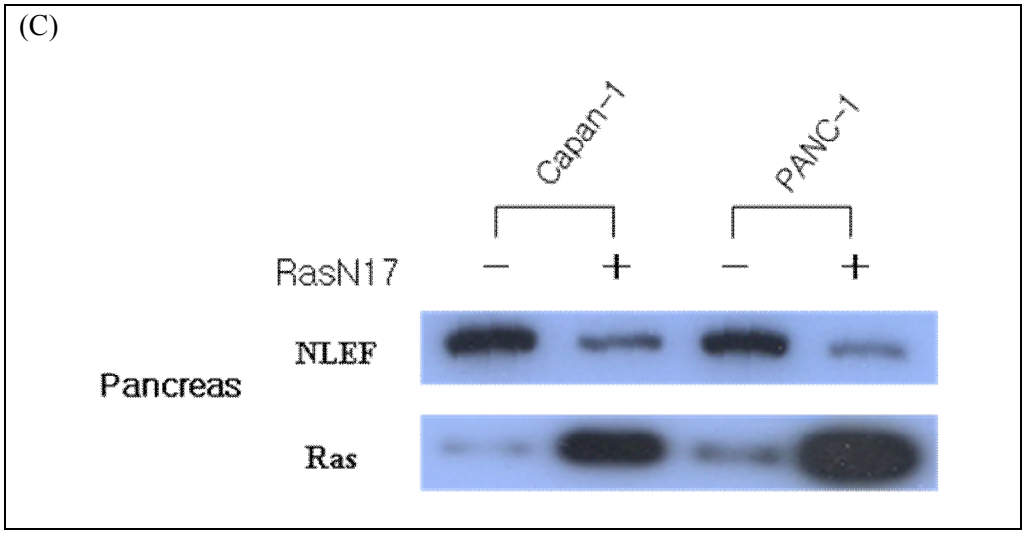


Fig.6. Dominant negative H-RasN17 transfection led to suppression of NLEF expression in human cancer cells

Western blot ananlysis for NLEF and Ras in human cnacer cells after transient transfection of H-RasN17. NLEF and Ras expression levels were detected human cnacer cells. The eluted samples (25ul) and 20ug of cell lysate were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and detected with NLEF and Ras antiboies.

(G) Effect of Ras signal pathways inhibition on NLEF expression in human cancer cells

The Raf/MEK/ERK and the PI3K/AKT pathway is a major signal transduction pathway activated by Ras. To determine which pathway contributes to Ras-mediated NLEF expression, human cancer cells were pretreated with the p44/42 MEK inhibitors PD98059+U0126, PI3K inhibitor LY294002 or the p38 MAPK inhibitor SB203580 or JNK inhibitors SP600125. Human cancer cells, treated with MEK inhibitor PD98059+U0126, and PI3K inhibitor LY294002 showed decreased NLEF protein expression, whereas p38 MAPK inhibitor SB203580, JNK inhibitors SP600125 did not

exert any effect on NLEF expression (Figure.7). These results indicate that the decreased expression of NLEF in human cancer cells is due to MEK or PI3Knase activities in these cells.

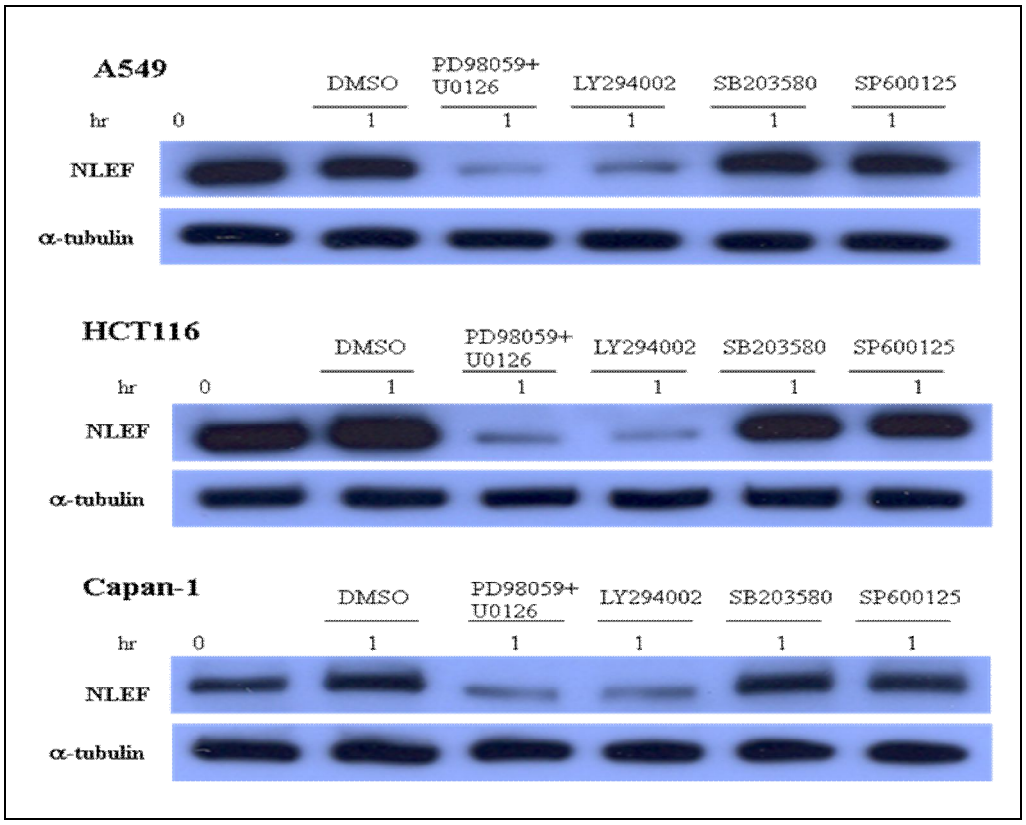


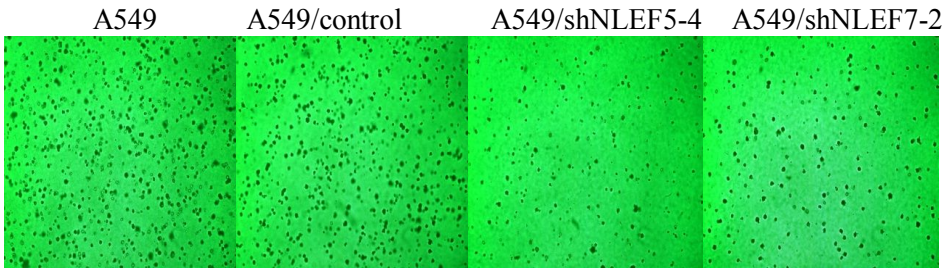
Fig.7. Effect of Ras signal inhibitor on NLEF expression in human cancer cells

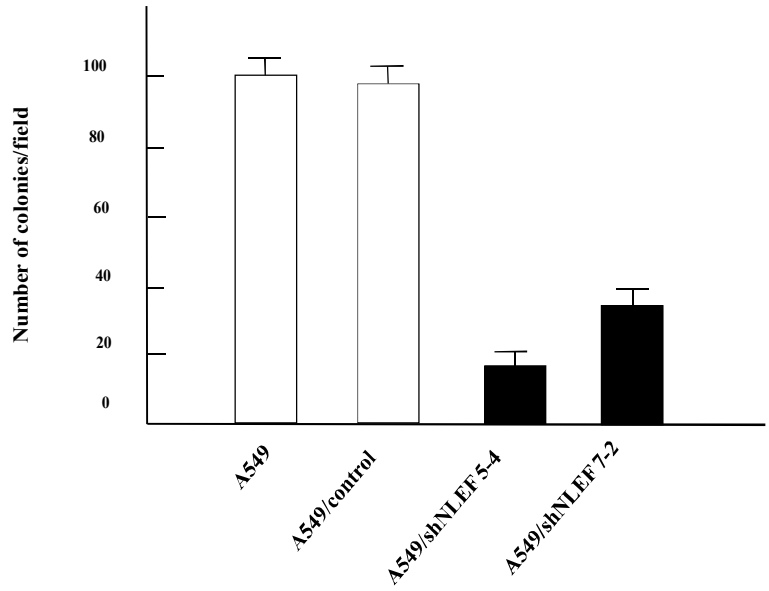
Human cancer cells were pretreated MEK inhibitor (50μM PD98059+20μM U0126), PI3K inhibitor (30 μM LY294002), MAPK inhibitor (20μM SB203580) or JNK inhibitor (20μM SP600125). After 1hour treatment, cells were harvested and the NLEF expression was analyzed using western blotting. The α-tubulin was used as the loading controls of western blotting.

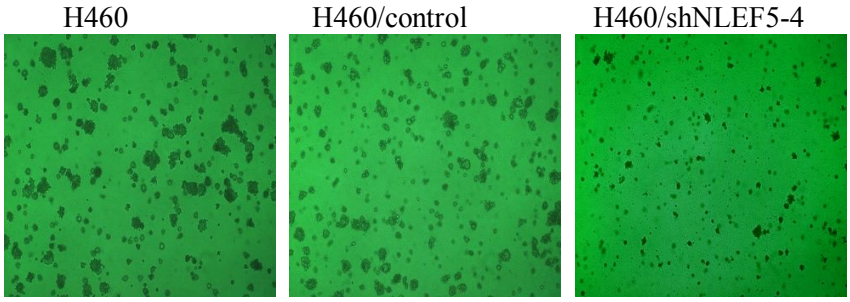
(H)Effect of NLEF on the Colony formation in shNLEF transfected cancer cells

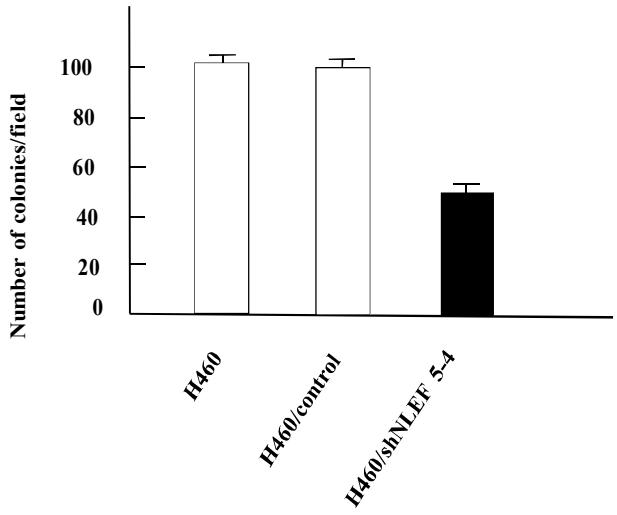
Colony formation assay is a classifical *in vitro* experiment to determine the ability of an oncogene to induce the growth of colonies in soft agar. We observed, that the expression of shNLEF in human lung cancer cells, and colon cancer cells inhibited the growth of colonies in soft agar. These data suggest that elevated expression of NLEF induces transformation and that up-regulation of NLEF is a necessary prerequisite for colony transformation. In these experiment, we transfected an expression vector encoding shNLEF into human lung cancer cells and colon cancer cells (Figure.8). As shown in expression of shNLEF caused a reduction in transforming activity. Expression of NLEF reducted the soft agar growth of the shNLEF-transformed human lung cancer cells and colon cancer cells. These results suggest that NLEF suppresses colony formation in the human lung cancer cells and colon cancer cells, as reflecting a much lower anchorage-independent growth rate.

(A) Lung


A549
A549/control
A549/shNLEF5-4
A549/shNLEF7-2


Number of colonies/field
100
80
60
40
20
0
A549
A549/control
A549/shNLEF5-4
A549/shNLEF7-2


H460
H460/control
H460/shNLEF5-4


Number of colonies/field
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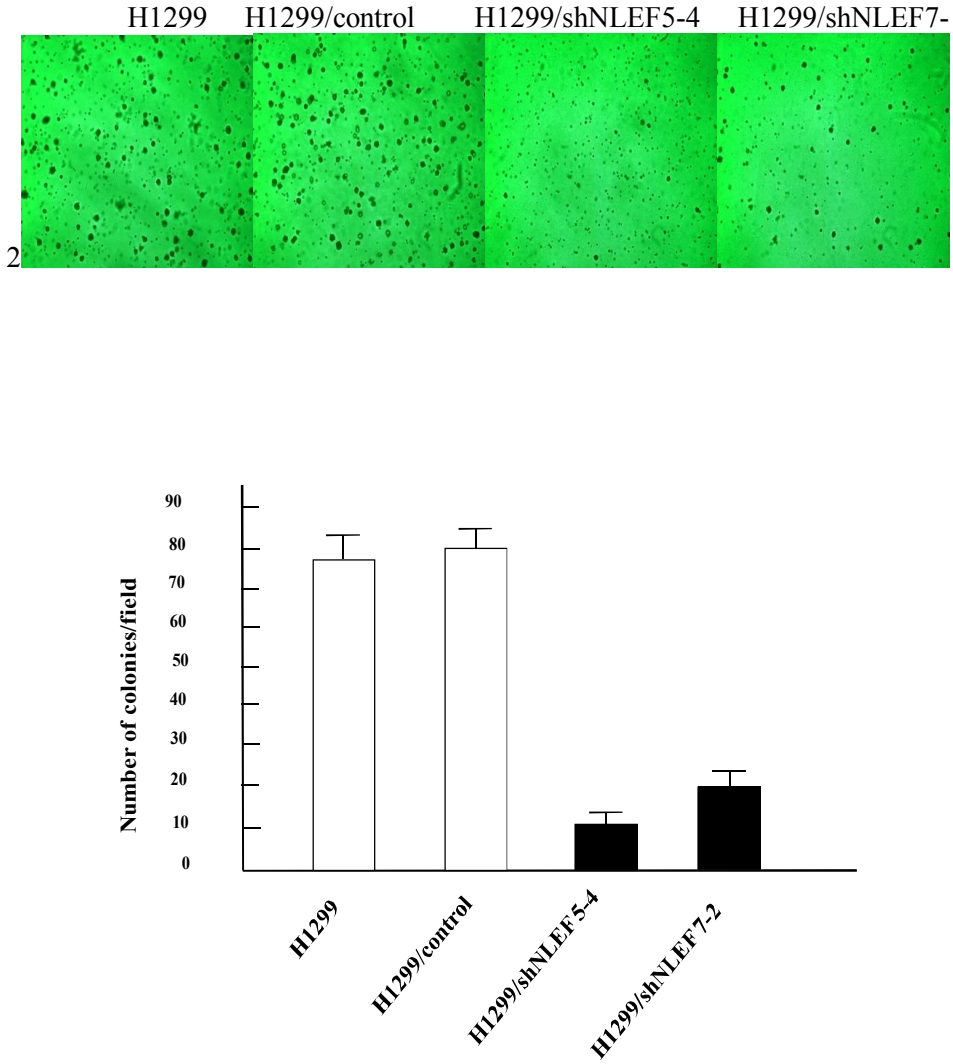
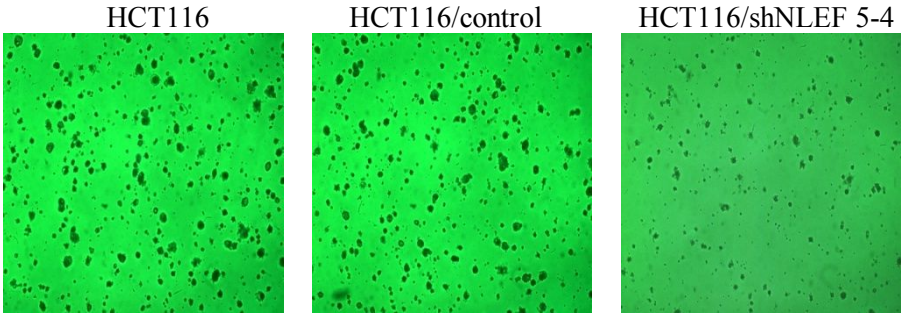
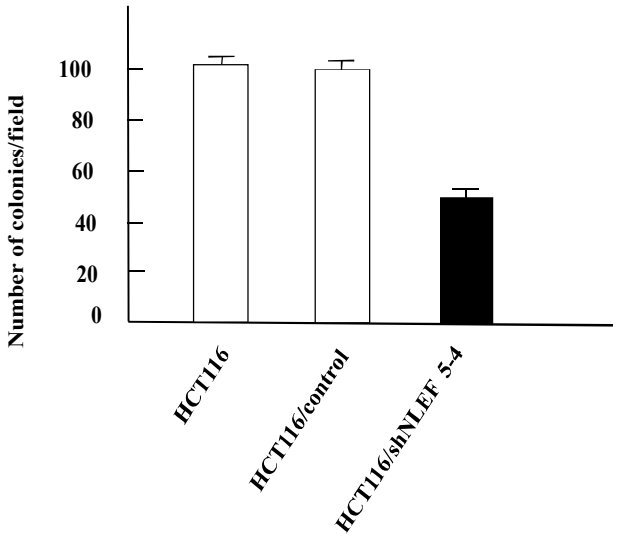
80
60
40
20
0
H460
H460/control
H460/shNLEF 5-4


H1299
H1299/control
H1299/shNLEF5-4
H1299/shNLEF7-
2
Number of colonies/field
90
80
70
60
50
40
30
20
10
0
H1299
H1299/control
H1299/shNLEF5-4
H1299/shNLEF7-2

(B) Colon


HCT116
HCT116/control
HCT116/shNLEF 5-4


Number of colonies/field
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80
60
40
20
0
HCT116
HCT116/control
HCT116/shNLEF 5-4

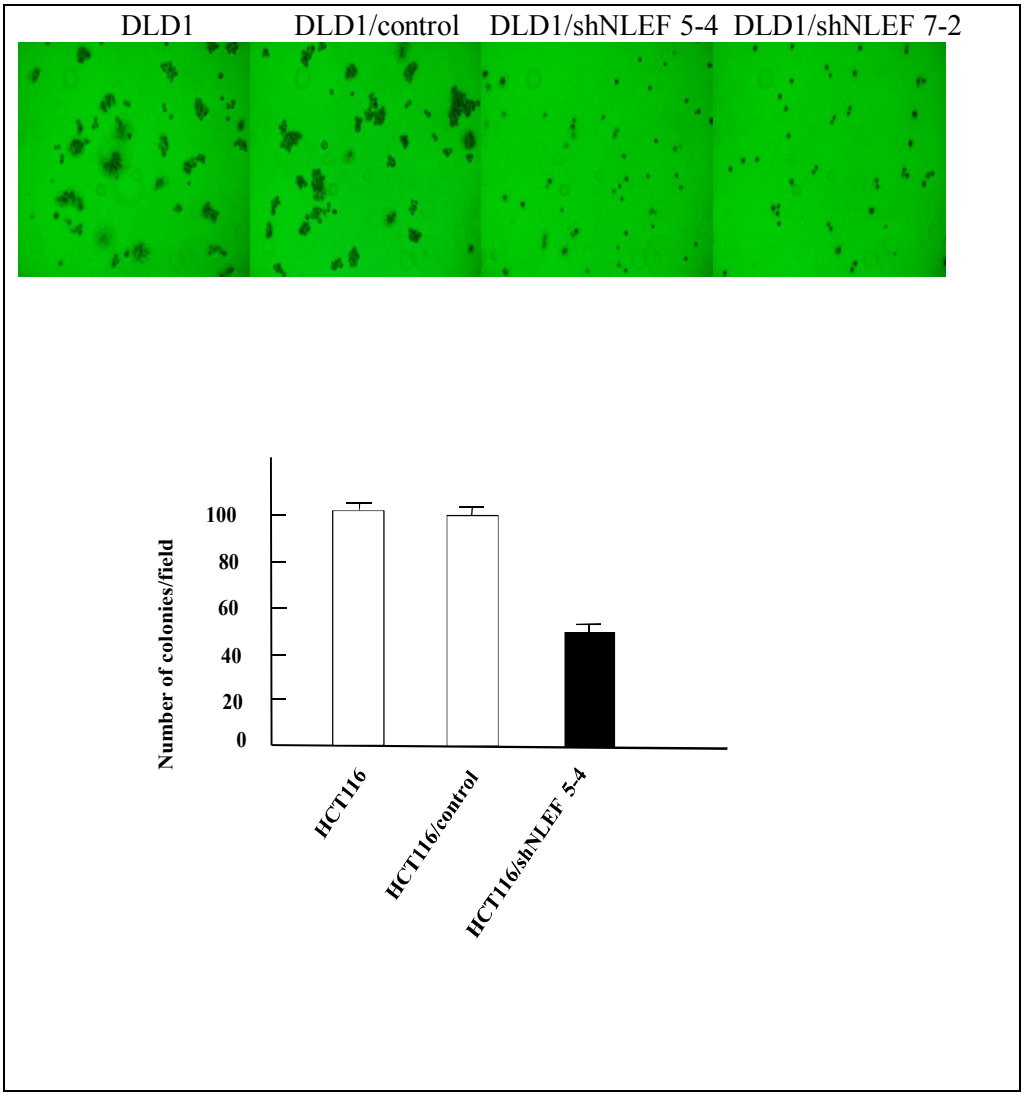


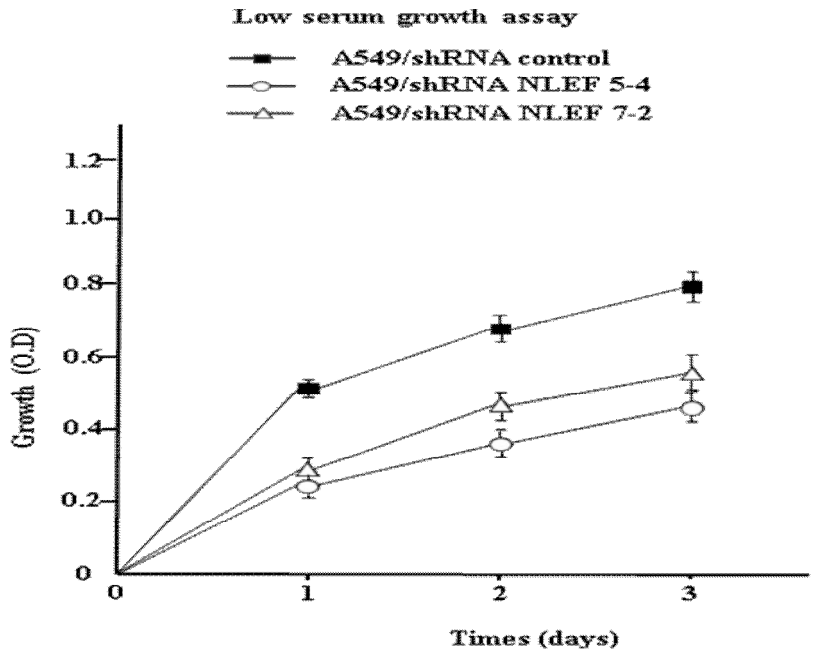
Fig.8. *In vitro* Colony formation assay

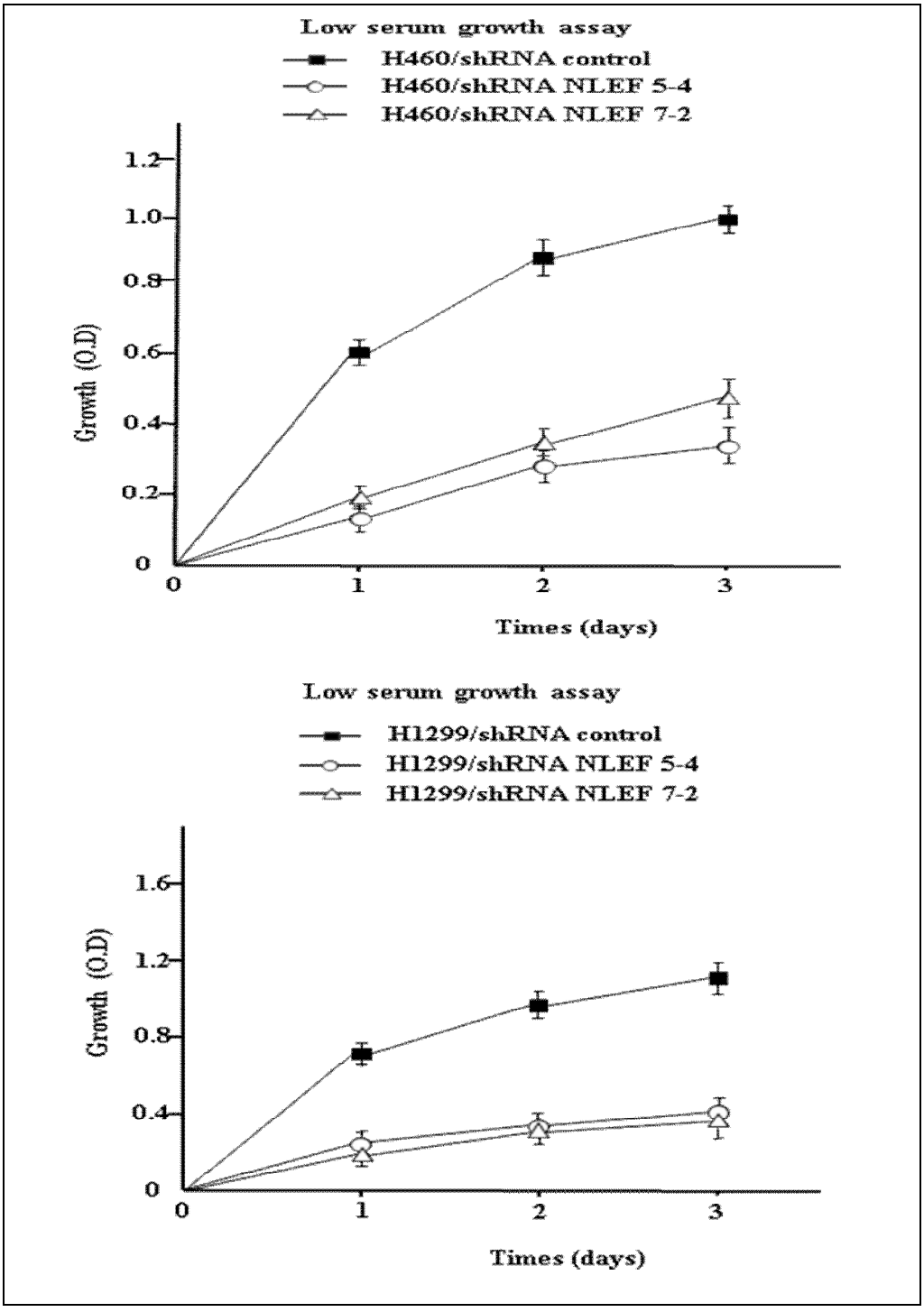
After shNLEF-transfection Human lung cancer and colon cancer cells, were plated 2×10^4 in duplicates in 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% bottom low-melt agarose. After 2 weeks of incubation, colonies of >1 mm in size were counted. Colony formation was observed 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.

(I) NLEF is reguired for human cancer cells proliferation

To determine whether NLEF affects proliferation rate of human lung cancer cells and colon cancer cells, we inhibited NLEF expression using shRNA and monitored the effect of such treatments. Importantly, the proliferation rate of human lung cancer cells and colon cancer cells treated with NLEF specific shRNA was significantly reduced as compared to those treated with the control shRNA. These findings strongly indicate that NLEF plays an essential role in mediating the proliferative activity of human lung cancer cells and colon cancer cells.

(A) Lung




Low serum growth assay
H460/shRNA control
H460/shRNA NLEF 5-4
H460/shRNA NLEF 7-2
Growth (O.D)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
Times (days)
Low serum growth assay
H1299/shRNA control
H1299/shRNA NLEF 5-4
H1299/shRNA NLEF 7-2
Growth (O.D)
1.6
1.2
0.8
0.4
0
0
1
2
3
Times (days)

(B) Colon

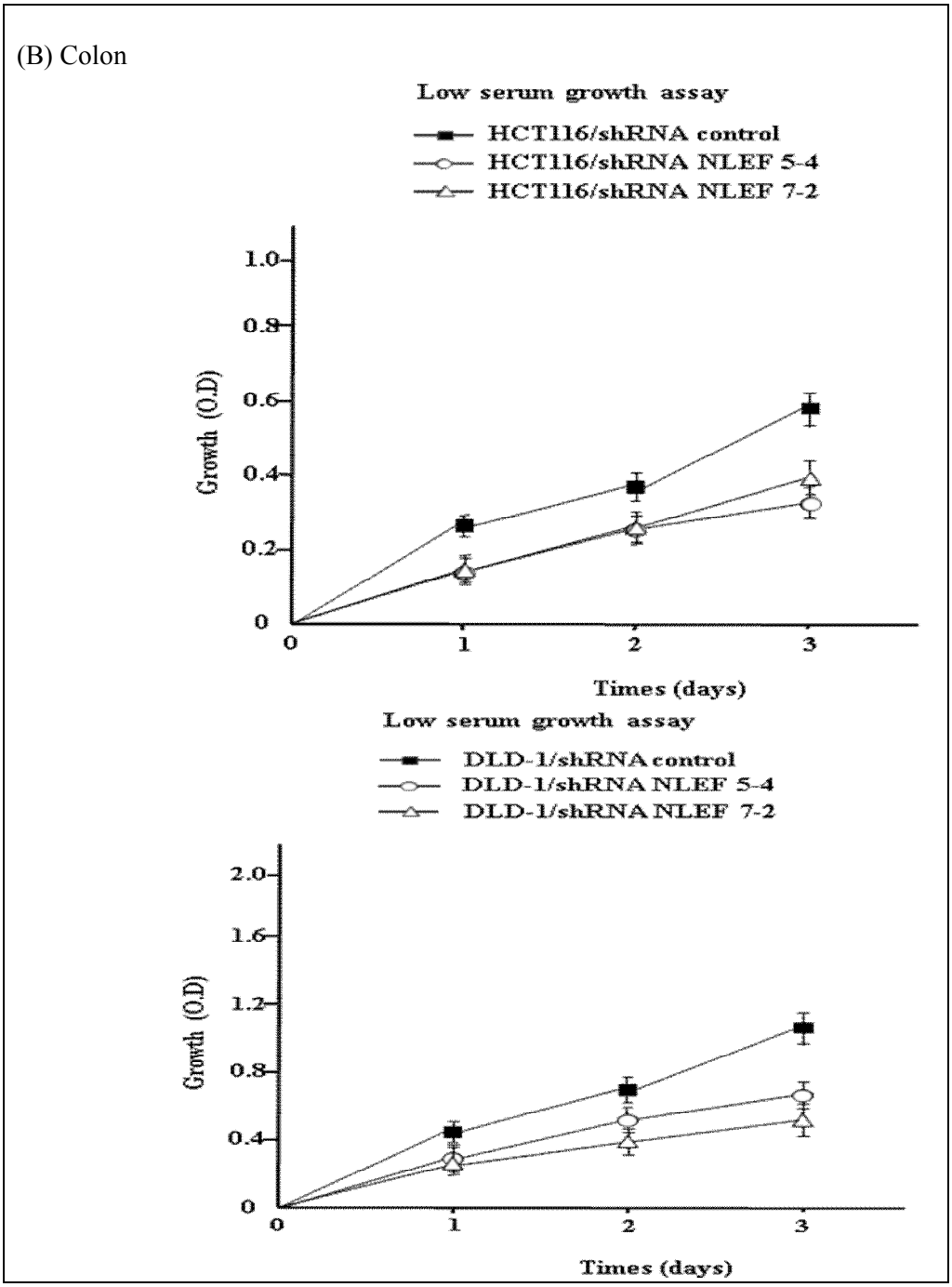

Low serum growth assay
HCT116/shRNA control
HCT116/shRNA NLEF 5-4
HCT116/shRNA NLEF 7-2
Growth (O.D)
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
Times (days)
Low serum growth assay
DLD-1/shRNA control
DLD-1/shRNA NLEF 5-4
DLD-1/shRNA NLEF 7-2
Growth (O.D)
2.0
1.6
1.2
0.8
0.4
0
0
1
2
3
Times (days)

Fig.9. *In vitro* proliferation assay

Proferation assay in NLEF shRNA-transfected human lung cancer cells (A) and colon cancer cells (B). Cells were seeded in 0.25% FBS at day 0 with 5×10^5 cells/well density and counted for up to 3 days. The number of cells was recorded every 24 h. Each value is the mean ± s.d.from three separate experiments.

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. 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. Ras proteins are produced as cytoplasmatic precursor proteins and require several posttranslational modifications to acquire full biologic activity. These modifications include prenylation, proteolysis, carboxymethylation, and palmitoylation (Glomset and Famsworth, 1994; Zhang and Casey, 1996; Gelb, 1997; Mumby, 1997; Casey and Seabra, 1996). 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).

In the present study, we have characterized the functional significance of the upregulation of NLEF (guanine nucleotide exchange factor) by oncogenic H-Ras. We demonstrated that the expression of oncogenic Ras upregulate NLEF expression levels not only in stable H-RasV12 transformed GM00637 cell but also in transiently H-RasV12 transfected GM00637 cell. Importantly, the relative abundance of the NLEF 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 NLEF expression. These findings indicated that NLEF is a direct target of oncogenic H-Ras.

Normal and oncogenic Ras mediate their biological functions by binding to downstream effectors (Shields et al., 2000). All effectors bind to a core effector loop of

Ras proteins (residues 32-40), with additional involvement of residues that change in conformation during GDP/GTP cycling; the switch I (residues 30-38) and switch II (residues 59-76) domains (Marshall, 1996; Campbell et al., 1998). The GTPbound form displays a significantly greater affinity for effectors. In recent years, the number of Ras effectors and the complexity of downstream pathways that they regulate have grown considerably. We will focus on the contribution of three key Ras effectors to Ras-mediated signaling and transformation. The first Ras-induced signal transduction cascade to be identified was the Raf>MEK>ERK protein kinase cascade (Marshall, 1996; Campbell et al., 1998) Activated Ras binds to and promotes the activation of Raf serine/threonine kineses (c-Raf-1, A-Raf and B-Raf). Ras causes activation of Raf, in part, by promoting a translocation of Raf to the plasma membrane, where additional binding and phosphorylation events are necessary for complete Raf activation (Morrison and Cutler, Jr., 1997). Once activated, Raf phosphorylates and activates the MEK1/2 dual specificity kinases that in turn phosphorylate and activate ERK1/2 mitogen activated protein kinases (MAPKs). Activated ERKs translocate to the nucleus and phosphorylate various transcription factors that include the Ets family member Elk-1. The second best characterized effectors of Ras are phosphatidylinositol 3-kinases (PI3Ks), lipid kinases consisting of a p85 regulatory and a p110 catalytic subunit (Rodriguez, Viciana et al., 1994; Rodriguez-Viciana et al., 1997). PI3K phosphorylates integral membrane phosphotidylinositols (PI) at the 3' position (e.g., phosphatidylinositol 4,5-phosphate; PIP2) to generate various short-lived second messenger products (e.g., phosphatidylinositol 3,4,5-phosphate; PIP3) (Vanhaesebroeck et al., 1997). Membrane-associated PIP3 in turn can regulate the activity of a diverse array of signaling molecules that include the Akt serine/threonine kinase. Akt activation results in complex signaling cascades that lead to the

phosphorylation of diverse substrates such as caspases, transcription factors (ATX), and proapoptotic proteins (BAD) that regulate cell survival (Chan et al., 1999). PI3K also mediates antiapoptotic signaling, as well as actin organization, by activating the Rac small GTPase (Bar-Sagi and Hall, 2000). The importance of PI3K in Ras transformation is best characterized in NIH 3T3 mouse fibroblasts. However, PI3K is not required for Ras transformation of other cells, reflecting cell-type differences in Ras effector utilization in transformation (McFall et al., 2001)

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 MEK and PI3Knase activity as measured by the levels of p-ERK and p-AKT, was much higher in H-RasV12-transformed cells than untransformed human cancer cells. Significantly, inhibition of MEK 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 NLEF protein. These findings provide strong evidence that NLEF is regulated by an activated MEK 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, 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 NLEF expression, human cancer cells were pretreated with the p44/42 MEK inhibitors PD98059+U0126, PI3K inhibitor LY294002 or the p38 MAPK inhibitor SB203580 or JNK inhibitors SP600125. Human cancer cells, treated with MEK inhibitor PD98059+U0126, and PI3K inhibitor LY294002 showed decreased NLEF protein expression, whereas p38 MAPK inhibitor

SB203580, JNK inhibitors SP600125 did not exert any effect on NLEF expression. These results indicate that the decreased expression of NLEF in human cancer cells is due to MEK or PI3Knase activities in these cells.

We next investigated whether or not NLEF 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 transfection of NLEF-expressing cells led to increase of the proliferation, colony formation and aggregation, compared with those of the mock- and control empty vector transfected cells. These results strongly suggest that NLEF is involved in oncogenic H-Ras-mediated increase of cellular proliferation, colony formation and aggregation.

Pharmacologic or genetic inhibitors of specific effector signaling pathways have also been useful reagents for defining the contribution of specific effectors in Ras transformation. For example, LY294002 is a specific inhibitor of PI3K, whereas PD98059 and U0126 are specific inhibitors of MEK activation of ERK (Davies et al., 2000). LY294002, but not PD98059, treatment reversed the ability of oncogenic Ras to inhibit suspension-induced apoptosis, or anoikis, in MDCK canine kidney epithelial cells (Khwaja et al., 1997). This demonstrated the critical role of PI3K but not Raf in mediating this important facet of anchorage-independent growth. Finally, kinase-dead mutants of Raf-1, MEK, ERK, Akt, and dominant negative Ral have been useful genetic inhibitors of specific effector signaling pathways (Rodriguez-Viciana et al., 1997; Brtva et al., 1995; Cowley et al., 1994; Khosravi-Far et al., 1995).

In the present study, we have demonstrated that NLEF 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 NLEF protein. Moreover, transfection of

NLEF in human cancer cells resulted in exhibition of tumorogenetic phenotypes. These results suggest that NLEF 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. To determine which path way contributes to Ras-mediated NLEF expression, human cancer cells were pretreated with the p44/42 MEK inhibitors PD98059+U0126, PI3K inhibitor LY294002 or the p38 MAPK inhibitor SB203580 or JNK inhibitors SP600125. Human cancer cells, treated with MEK inhibitor PD98059+U0126, and PI3K inhibitor LY294002 showed decreased NLEF protein expression, whereas p38 MAPK inhibitor SB203580, JNK inhibitors SP600125 did not exert any effect on NLEF expression. These results indicate that the decreased expression of NLEF in human cancer cells is due to MEK or PI3Knase activities in these cells.

The significant role of aberrant Ras activation in increased cancer cell growth and proliferation has been well-established. In examining the contribution of Ras to stimulus-independent growth and the inhibition of growth arrest pathways, two themes emerge: deregulation of the cell cycle and induction of growth factor autocrine loops. The first allows Ras-transformed cells to overcome growth arrest imposed by cell cycle checkpoints; the second renders cells self-sufficient by providing a constant stimulus to proliferate. Deregulation of key components of both vital cell regulatory mechanisms can be achieved, in part, by Ras-mediated changes in gene expression.

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. The proliferation rate of human lung cancer cells, and colon cancer cells

treated with NLEF specific shRNA was significantly reduced as compared to those treated with the control shRNA. These findings strongly indicate that NLEF plays an essential role in mediating the proliferative activity of human lung cancer cells and colon cancer cells. This study reports an oncogenic H-Ras target genes, neuronal nucleotide exchange factor (NLEF), which were identified through this screening, contributes to the oncogenic H-Ras-mediated increase in tumor cell proliferation aggregation. This study is the first to indicate that NLEF is a downstream target molecule of oncogenic Ras and is regulated for oncogenic Ras-induced tumorigenesis.

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ABSTRACT

The study of the oncogenic Ras-induced cancer development

Kim Na Hee
Advisor: Prof. Ho Jin You, Ph.D., M.D.
Department of Bio-Materials
Graduate School of Chosun University

Ras oncogenes are mutated in a large proportion of human cancers, and Ras pathway are activated by a variety of other mechanisms in may other cancers. The mechanisms by which Ras induces tumor progression is , however, not fully elucidated. In this report, I found that the levels of nucleotide exchange factor (NLEF) protein are significantly increased in oncogenic H-Ras expression in human fibroblast GM00637 cells. The levels of NLEF protein was decreased in Ras-dominant active cancer cells, including A549, H1299, H460, Sw480, DLD1, Capan-1, and Panc-1 cells, and dominant negative form H-RasN17 suppressed NLEF expression in these cells. In addition, treatment of ERK and PI3K inhibitors led to significant suppression of NLEF expression in those Ras-dominant active cancer cells. These results suggest that NLEF is a novel downstream target molecule of oncogenic H-Ras, and H-Ras-mediated increase in ERK and PI3K activity is required for NLEF expression.

To investigate the biological function of oncogenic-H-Ras-induced NLEF expression, I examined whether NLEF is contributed to oncogenic Ras-mediated increase in cancer

progression. I found that ectopic expression of NLEF increased serum-stimulated phosphorylation of ERK1/2 and Akt in HGM00637 cells. NLEF shRNA treatment caused suppression of phosphorylation of several cell cycle progression proteins, such as p-histone-H3, p-Rb, and p-cdc2 in human colon cancer cells, HCT116, DLD1, and SW480 cells. I further showed that expression of NLEF led to increase in colony formation in soft agar. Furthermore, NLEF shRNA-transfected A569, H460, H1299, HCT116, and DLD1 cells exhibited significant reduction of anchorage-independent growth rate. Finally, our results showed that the ability of cellular proliferation of huamn cancer ccells was significantly suppressed by transfection of NLEF shRNA. These results suggest that the upregulation of NLEF induced by oncogenic Ras may be an important role for oncogenic Ras-mediated neoplastic transformation.