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# The study for the oncogenic effect of NGEF

조선대학교 대학원

생물신소재학과

김 홍 범

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NGEF의 발암효과에 관한 연구

2008 년 2 월 25 일

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# The study for the oncogenic effect of NGEF

지도교수 유 호 진

이 논문을 이학박사학위신청 논문으로 제출함.

2007 년 10 월 일

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# 김 홍 범의 박사학위 논문을 인준함

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| | |
|---|---|
| CAAX | C, cysteine; A, aliphatic amino acid; X, any amino acid |
| CAK | CDK activating kinase |
| CDK | cyclin-dependentkinases |
| 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 |
| NGEF | Neuronal guanine 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-$\alpha$ |

| | |
|---|---|
| TIMP-2 | tissue inhibitor of matrix metalloproteinase-2 |
| VEGF | vascular endothelial growth factor |

# The study for the oncogenic effect of NGEF

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## ABSTRACT

The activated *Ras* oncogene can transform various mammalian cells and has been implicated in development of a high population of malignant human tumors. The mechanism by which Ras induces tumor progression is, however, 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, angiogenesis and metastasis, wherase control siRNA transfectants did not. Moreover, silencing of NGEF in H-RasV12-transformed cells led to increase of animal survival rate. 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

## 1. 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).

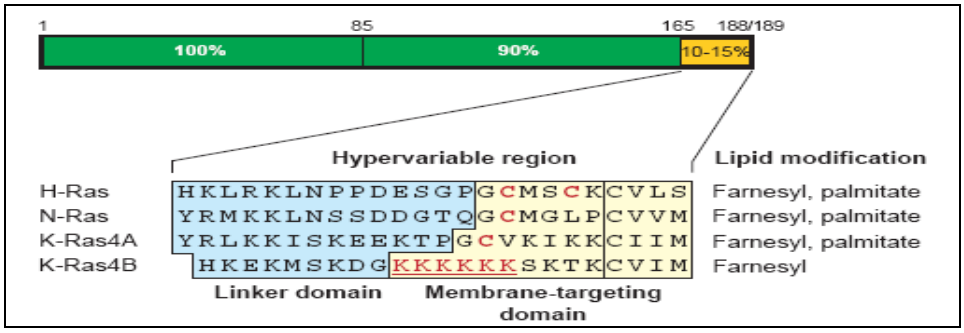


***Fig.1. Ras hypervariable region***

### *1.1 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).

### *1.2 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.

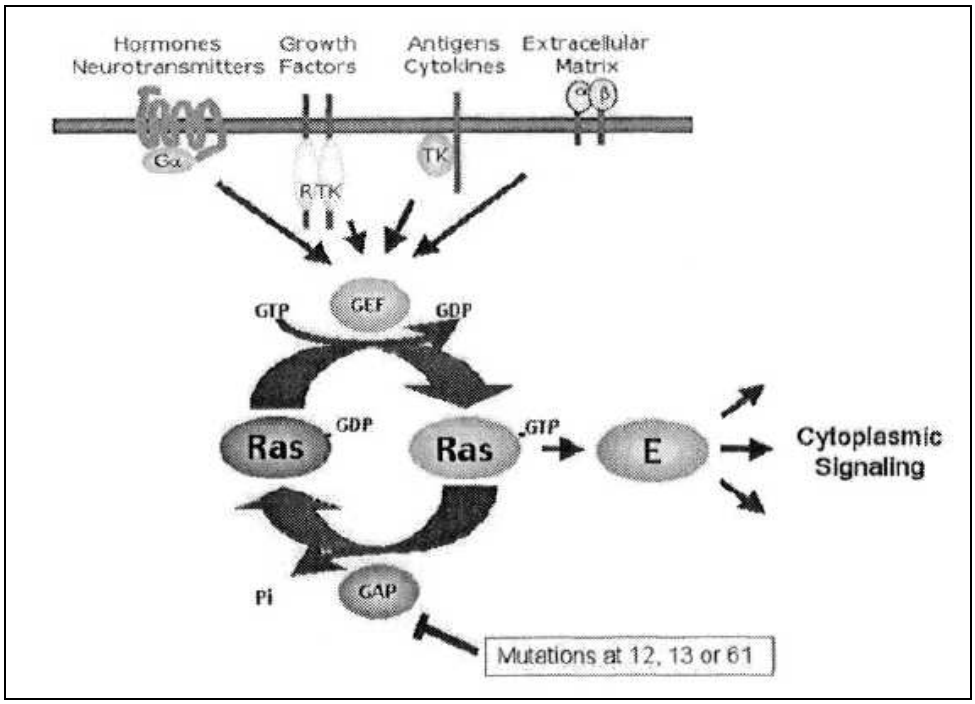


***Fig.2. The Ras pathway in cancer***

### *1.3 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).

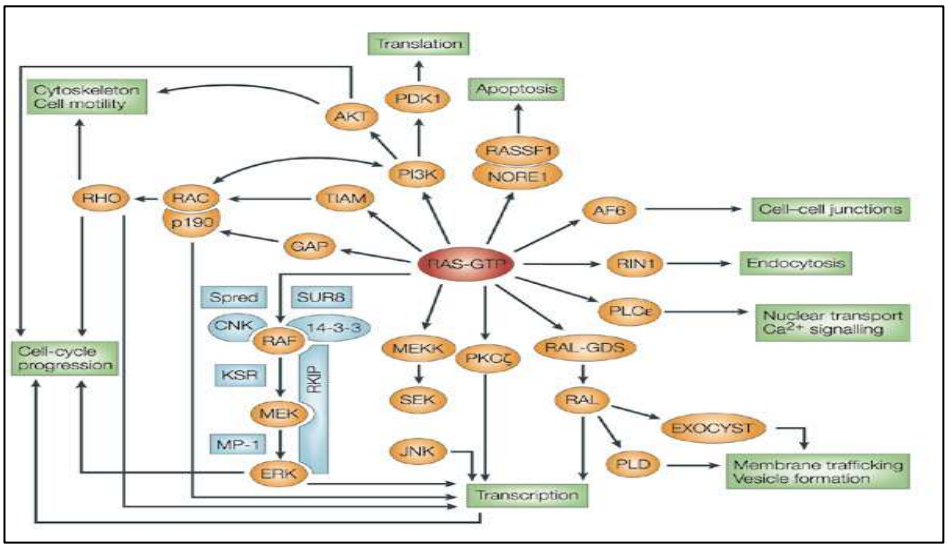


***Fig.3. Multiple Ras effectors***

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).

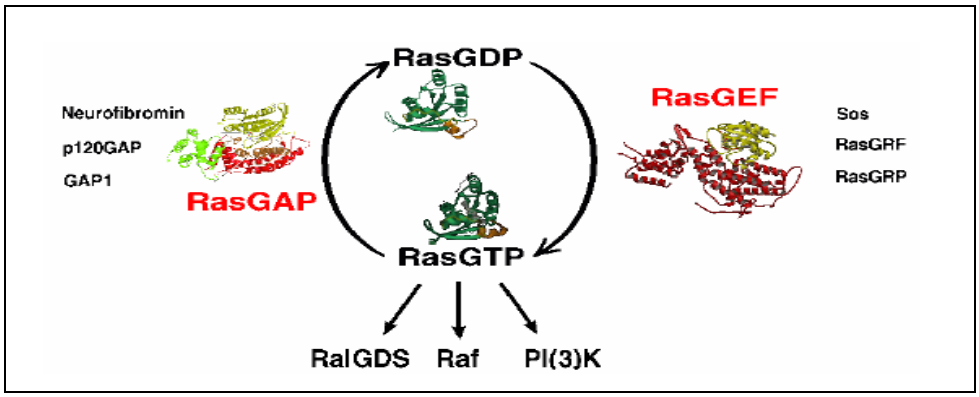


***Fig.4. The switch function of Ras***

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.

### *1.4 Posttranslational modification of Ras*

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) (Figure.5).

Prenylation of proteins by intermediates of the isoprenoid biosynthetic pathway represents a newly discovered form of posttranslational modification and is catalyzed by 3 different enzymes: protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase I), and geranylgeranyltransferase type II (GGTase II) (Glomset and Famsworth, 1994; Zhang and Casey, 1996; Gelb, 1997; Mumby, 1997; Casey and Seabra, 1996). Prenylated proteins share characteristic carboxy-terminal consensus sequences and can be separated into the proteins with a CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid) motif and proteins containing a CC or CXC sequence (Reiss et al, 1990; Raiss and Stadley, 1991; Yokoyama et al, 1991; Moores et al, 1991). FTase I transfers a farnesyl group from farnesyldiphosphate (FPP), and GGTase I transfers a geranylgeranyl group from geranylgeranyldiphosphate (GGPP) to the cysteine residue of the CAAX motif (Trueblood and Ohya, 1993). GGTase II transfers the geranylgeranyl groups from GGPPs to both cysteine residues of CC or CXC motifs.

Farnesylation is the first step in the posttranslational modification of Ras. This modification occurs by covalent attachment of a 15-carbon farnesyl moiety in a thioether linkage to the carboxyterminal cysteine of proteins that contain the CAAX

motif. The reaction is catalyzed by FTase, a heterodimer consisting of a 48-kd and a 45-kd subunit (aF/GGI and bF). Binding sites for the substrates, FPP and the CAAX motif, are located on the aF- and bFsubunits (Pellicena and Scholten, 1996; Trueblood and Boyartchuk, 1997; Park and Boduluri, 1997). Substrates for FTase include all known Ras proteins, nuclear lamins A and B, the g-subunit of the retinal trimeric G-protein transducin, rhodopsin kinase, and a peroxisomal protein termed PxF.

Farnesylation of Ras proteins is followed by endoproteolytic removal of the 3 carboxy-terminal amino acids (AAX) by a cellular thiol-dependent zinc metallopeptidase (Akopyan and Couedel, 1994). This endoproteolytic activity (RACE, or Ras and a-factor converting enzyme) is a composite of 2 different CAAX proteases: a zinc-dependent activity encoded by AFC1 and the type IIb signal peptidase-like RCE1 (Ras converting enzyme 1) (Boyartchck and Ashby, 1997). The final step in the carboxy-terminal modification of proteins with a CAAX motif (eg, Ras) is the methylation of the carboxyl group of the prenylated cysteine residue by an as yet uncharacterized methyltransferase.

Some Ras proteins (H-Ras, N-Ras, Ras2) are further lapidated by palmitoylation at 1 or 2 cysteines near the farnesylated carboxy-terminus (Hancock and Megee, 1989; Milligan and Palmitoylation, 1995; Ross, 1995; Dudler and Gelb, 1996). Like farnesylation, H-Ras palmitoylation plays an important role for signaling functions in vivo (Dudler and Gelb, 1996). A microinjection experiment in Xenopus oocytes

revealed that palmitoylation of H-Ras dramatically enhances its affinity for membranes as well as its ability to activate mitogen-activated protein kinase (MAPK) and initiate meiotic maturation. Both a Ras-specific protein (palmitoyltransferase) and a palmitoyl-protein (thioesterase) have been characterized (Liu and Dudler, 1996; Camp and Verkruyse, 1994). In contrast to farnesylation and proteolysis, palmitoylation and methylation of Ras are thought to be reversible and may have a regulatory role.

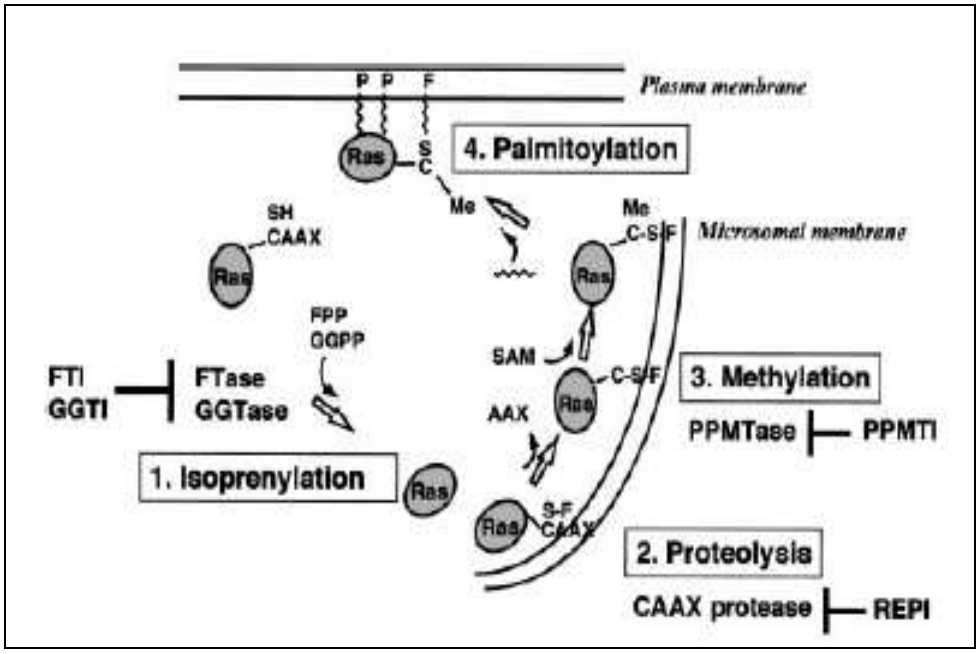


***Fig.5. Overview of the posttranslational modifications of Ras proteins in cells***

## 2. RAS FUNCTIONS AS A SIGNALING NODE

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.

***2.1 Ras utilizes multiple effectors to mediate diverse cytoplasmic signaling cascades***

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 (Figure.6).

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) (Figure.6.).

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 (Figure.6).

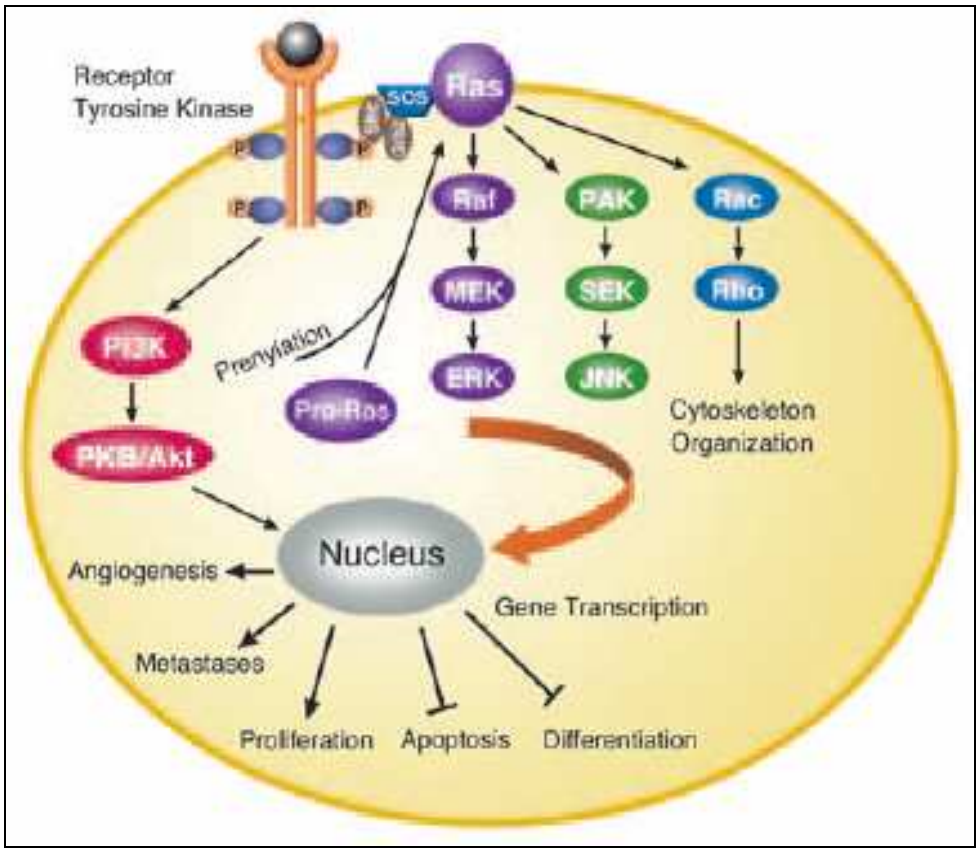


***Fig.6. Ras-mediated signal transduction pathways***

### *2.2 Dissecting Ras signal transduction: tools of the trade*

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 (Figure.7). 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 (Figure.7). 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). The ability of activated PI3K or RalGEF to cooperate with activated Raf and cause synergistic transformation of NIH 3T3 cells has provided evidence for the contribution of each effector to Ras transformation. While activated Raf alone can cause transformation of NIH 3T3 mouse fibroblasts, activated Raf failed to cause transformation of a variety of epithelial cell types, indicating the critical requirement for Raf-independent effectors in transformation of some cell types (Oldham et al., 1996; Gire et al., 1999; Schulze et al., 2001). Constitutively activated substrates of Raf [e.g., MEK(ED)], PI3K (e.g., membrane-targeted Akt; Myr-Akt), and RalGEF (e.g., GTPase-deficient mutants of Ral) have also been used for similar analyses (Figure.7).

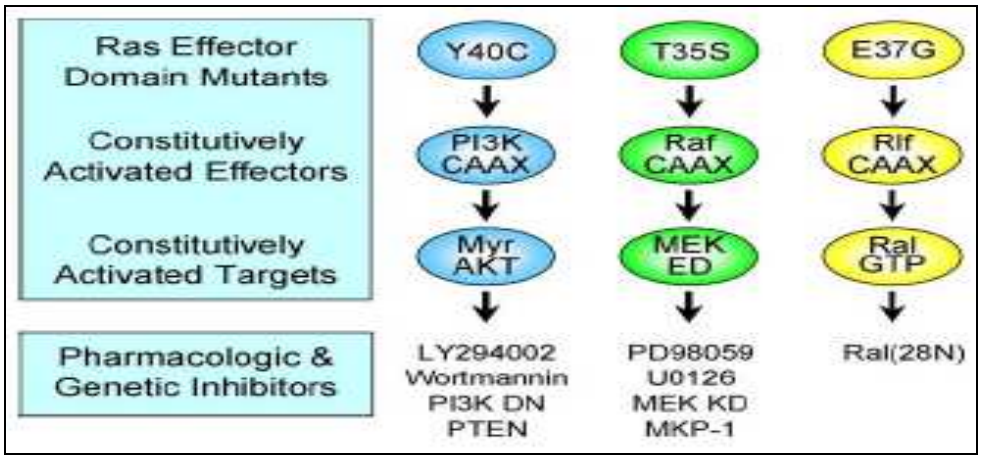


***Fig.7. Effectors of Ras function***

Pharmacologic or genetic inhibitors of specific effector signaling pathways have also been useful reagents for defining the contribution of specific effectors in Ras transformation (Figure.7). 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).

### *2.3 The MAPK signaling cascades*

MAPK pathways are well-conserved major signaling systems involved in the transduction of extracellular signals into cellular responses in a variety of organisms, including mammals (Treisman, 1996; Fanger et al, 1997; Robinson and Cobb, 1997; Garrington and Johnson, 1999; Schaeffer and Weber, 1999; Elion, 1998). The core components of the MAPK signaling cascades are 3 sequential kinases, including MAP kinase (MAPK, or extracellular signalregulated kinase, ERK), MAPK kinase (MAPKK, or MAPK/ERK kinase, MEK), and MAPKK kinase (MAPKKK, or MEK kinase, MEKK) (Figure.8). The MAPKs are activated by dual phosphorylation on

tyrosine and threonine residues by upstream dualspecificity MAPKKs. MAPKKs are also phosphorylated and activated by serine- and threonine-specific MAPKKKs. At least 6 MAPK cascades have been clearly identified in mammalian cells (Treisman, 1996; Fanger et al, 1997; Robinson and Cobb, 1997; Garrington and Johnson, 1999; Schaeffer and Weber, 1999; Elion, 1998). The best characterized MAPK signaling pathways are the Ras-to-MAPK signal transduction pathway (or ERK pathway), which is responsive to signals from receptor tyrosine kinase, hematopoietic growth factor receptors, and some heterotrimeric G-protein–coupled receptors, which promote cell proliferation or differentiation; the stress-activated protein kinase/c-Jun Nterminal kinase (SAPK/JNK) pathway, which is activated in response to stresses such as heat, high osmolarity, UV irradiation, and proinflammatory cytokines such as tumor necrosis factor–a and interleukin-1 (IL-1); and the p38 pathway, which is responsive to osmotic stress, heat shock, lipopolysaccharide, tumor necrosis factor–a, and IL-1 (Figure.8). Scaffolding/adapter proteins such as MP-1, JSAP-1, and JIP-1 route MAPK modules in mammals by binding ERK-1 and MEK-1, JNK-3 and SEK-1 and MEKK-1, or JNK and MKK-7 and MLKs (Schaeffer and Weber, 1999; Elion, 1998).

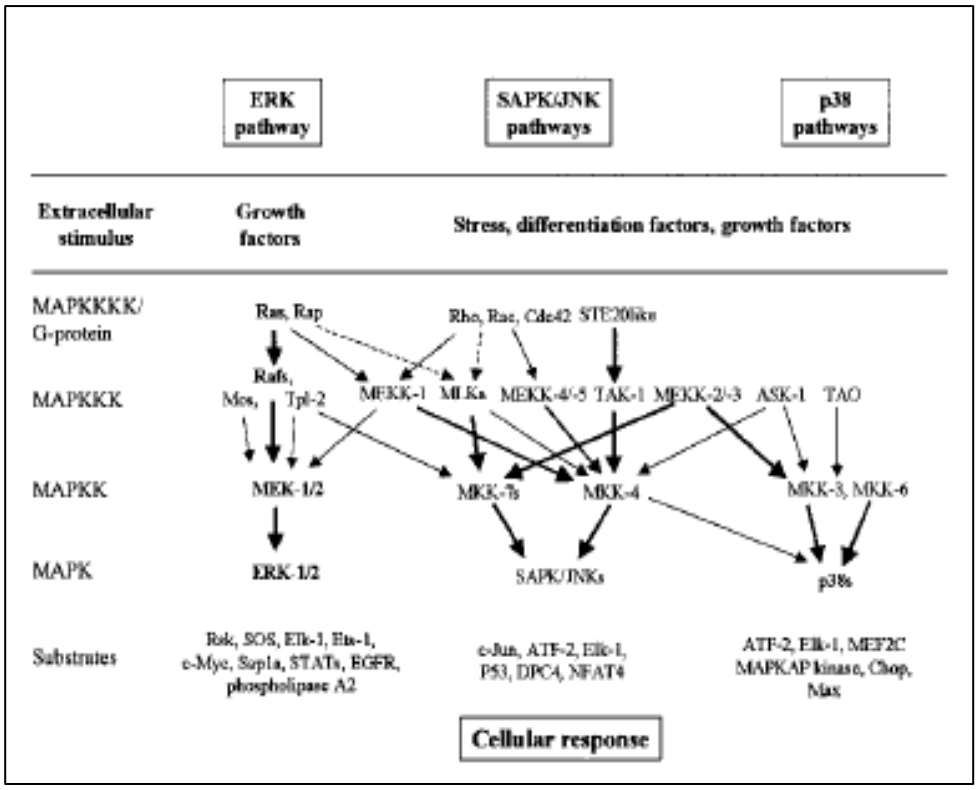


***Fig.8. The best-characterized MAPK modules are the ERK pathway, the SAPK/JNK pathway, and the p38 pathway***

### *2.4 Ras deregulation of gene expression and transformation*

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) (Figure.9). 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.

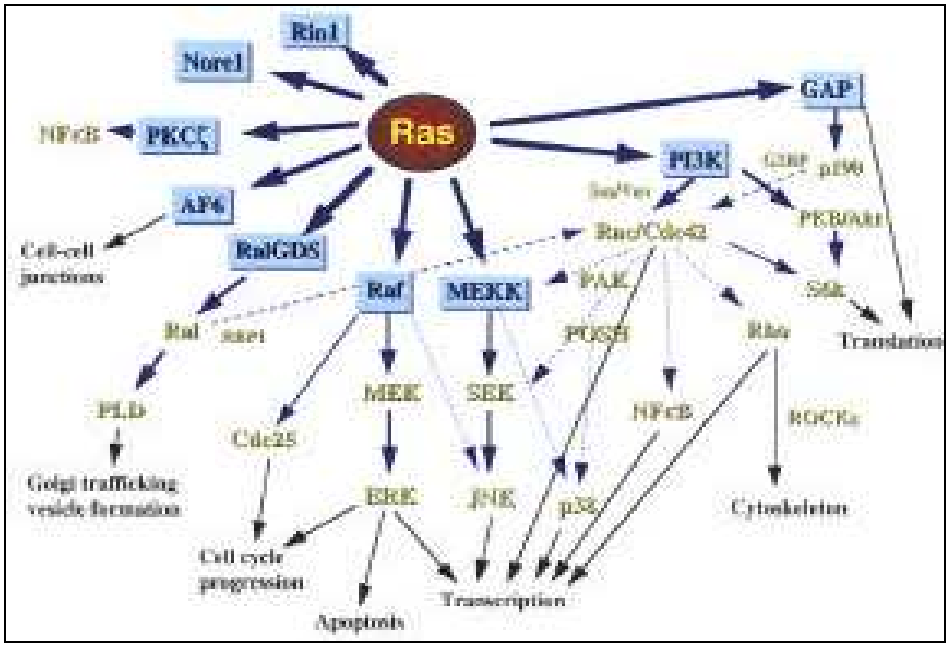


***Fig.9. Ras effectors and downstream pathways***

## 3. RAS DEREGULATION OF CELL PROLIFERATION

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.

### *3.1 Ras regulation of cyclin D1 and cell cycle progression*

A number of studies determined that normal Ras is required for mitogen-induced cell cycle progression, while oncogenic Ras promotes growth factor-independent entry into the cell cycle (Marshall, 1999; Pruitt and Der, 2001). Similarly, the mitogenstimulated regulation of positive (e.g., cyclin D1) and negative (e.g., $p21^{CIP1}$, $p27^{KIP1}$) regulatory components of the cell cycle machinery is well understood (Sherr and Roberts, 1999). Of these, the role of Ras regulation of cyclin D1 expression and function has been the best characterized.

Growth factor stimulation promotes entry into the cell cycle from $G_0$ to $G_1$ and facilitates the $G_1$/S transition partly through D-type cyclin upregulation (Sherr, 1996). Cyclin D1 binds cyclin-dependent kinases (CDKs), enhancing their ability to phosphorylate the Rb tumor suppressor protein that functions as a negative regulator of cell cycle progession. Phosphorylation inactivates Rb, which permits E2F-dependent gene expression necessary for cell proliferation.

Ras mediates upregulation of cyclin D1 by transcriptional activation in a wide variety of cell types (Arber et al., 1996; Filmus et al., 1994; Liu et al., 1995). Transient activation of Ras in rodent fibroblasts and epithelial cells is accompanied by upregulation of cyclin D1 transcription and protein expression (Filmus et al., 1994; Shao et al., 2000; Winston et al., 1996). Serum-stimulated upregulation of cyclin D1 expression is Ras-dependent, and constitutive expression of cyclin D1 overcomes the requirement for Ras activation in NIH 3T3 cell proliferation (Aktas et al., 1997).

Finally, Ras transformation of a variety of cell types is associated with sustained upregulation of cyclin D1 protein (Arber et al., 1996; Liu et al., 1995; Shao et al., 2000; Takuwa and Takuwa, 2001; Pruitt et al., 2000).

Oncogenic Ras upregulates cyclinD1 by Raf-dependent and Raf-independent signaling. Although Raf/ERK activation is sufficient to stimulate cyclin D1 gene expression in rodent fibroblasts (Liu et al., 1995; Lavoie et al., 1996; Kerkhoff and Rapp, 1997; Greulich and Erikson, 1998; Cheng et al., 1998; Ladha et al., 1998) additional Ras-mediated pathways may be necessary for cyclin D1 regulation in other cell types (Pruitt et al., 2000; Lavoie et al., 1996). For example, PI3K activation may promote cell cycle entry via post-transcriptional as well as transcriptional regulation of cyclin D1 (Gille and Downward, 1999). Ral GEF-mediated activation of Ral may stimulate the cyclin D1 promoter through activation of NF-$_{K}$B (Henry et al., 2000). These and other findings suggest that several Ras effector pathways may contribute to distinct aspects of Ras deregulation of the cell cycle in a cell-type specific manner.

***3.2 Ras regulation of TGF-α and autocrine growth***

In addition to circumventing growth arrest machinery, Ras-transformed cells become independent of growth factors in order to ensure proliferation. One such mechanism may be oncogenic Ras-induced upregulation of transforming growth factor- *α* (TGF-α*)* in a variety of cell types (Oldham et al., 1996; Marshall et al., 1985; Ciardiello et al., 1988; Godwin and Lieberman, 1990; Glick et al., 1991; Filmus et al., 1993). TGF-α is

a member of the epidermal growth factor (EGF) family of mitogens that activate the EGF receptor (EGFR) to promote cell proliferation (Normanno et al., 2001). TGF-α-mediated autocrine signaling has been shown to be at least partially responsible for Ras transformation (Filmus et al., 1993; Ciardiello et al., 1990; Gangarosa et al., 1997). Activation of the Raf-MEK-ERK pathway is sufficient for upregulation of TGF-α gene expression in some, but not other, cell types (Oldham et al., 1996; Schulze et al., 2001). Although these findings implicate multiple Ras-mediated pathways in the stimulation of the TGF-α autocrine loop, the mechanism of TGF-α gene upregulation and contribution of TGF-α stimulation of EGFR to malignant transformation remain to be determined.

## 4. RAS TARGETS INVOLVED IN TUMOR CELL ANGIOGENESIS, INVASION AND METASTASIS

In addition to deregulating cell growth and proliferation, oncogenic Ras causes changes in genes that promote malignant transformation. In this section, we highlight several gene targets of Ras whose protein products may contribute to tumor cell angiogenesis (vascular endothelial growth factor; VEGF), invasion and metastasis (matrix metalloproteases; MMPs).

### *4.1 Ras, VEGF and tumor cell angiogenesis*

Oncogenic Ras has been observed to be a potent stimulator of vascular endothelial

growth factor (VEGF) gene expression (Rak et al., 1995a; Konishi et al., 2000; White et al., 1997). VEGF is one of a number of soluble factors that are mitogens specific for vascular endothelial cells, mediating both normal and pathological angiogenesis. Angiogenesis is required for the growth of microscopic solid tumors beyond 1-2 mm in diameter, providing adequate oxygen and nutrient supplies as well as access to distant sites of metastasis. Tumor cells under hypoxic conditions either commandeer existing vasculature or stimulate endothelial cells to undergo angiogenesis.

The effectors that mediate oncogenic Ras stimulation of VEGF gene expression exhibit significant cell-type differences. For example, the Raf/ERK pathway is sufficient to promote VEGF upregulation in rodent fibroblasts (Grugel et al., 1995; Milanini et al., 1998). Phosphorylation of hypoxia-induced factor-1 (HIF-1) by ERKs may represent one level of integration between Ras-mediated and hypoxiainduced VEGF gene expression. In contrast, in epithelial or other cell types, PI3K is also necessary for Ras-mediated VEGF expression, suggesting that Ras regulation of VEGF may involve several Ras effectors and show cell-type specific differences (Mazure et al., 1997; Rak et al., 2000b).

While upregulation of VEGF may be important for angiogenesis, Ras must regulate the expression of other factors as well to promote tumor angiogenesis. For example, one study found that oncogenic Ras was required for upregulated expression and secretion of VEGF in human colorectal carcinoma cell lines (Okada et al., 1998).

Suppression of VEGF expression impaired the tumorigenic growth of these cells, showing the importance of this factor in Ras-induced tumor angiogenesis. However, forced overexpression of VEGF in the absence of mutated Ras was not sufficient to fully restore tumorigenic growth. Similarly, evaluation using a mouse melanoma model showed the importance of continued expression of oncogenic Ras in tumor maintenance (Chin et al., 1999). Expression of Ras was associated with increased tumor vascularization and upregulated expression of VEGF. Loss of Ras expression resulted in apoptosis of endothelial cells lining the tumor vasculature and subsequent tumor cell apoptosis and regression. However, forced VEGF overexpression alone was not sufficient to overcome the need for Ras activity, suggesting that other angiogenic factors in addition to VEGF are regulated by Ras activation. For example, Ras has been shown to downregulate angiogenesis inhibitors such as thrombospondin-1 and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), adding further complexity to the molecular mechanism for Ras-mediated angiogenesis (Zuber et al., 2000; Laderoute et al., 2000; Tokunaga et al., 2000), Further studies are needed to determine which Rasmediated pathways are important for VEGF expression in various tumors and to establish the contribution of Ras upregulation of VEGF as well as other factors to angiogenesis.

***4.2 Ras and tumor cell invasion/metastasis***

Oncogenic Ras can also promote tumor metastasis of a variety of cell types

(Thorgeirsson et al., 1985; Vousden et al., 1986; Collard et al., 1987; Treiger and Isaacs, 1988). Metastasis accounts for approximately 90% of cancer mortalities but is the least understood step in the multi-step model of cancer (Woodhouse et al., 1997; Fidler, 1999). The processes that render a benign cancer cell locally invasive as well as metastatic are complex and not yet completely defined. Invading cells must overcome barriers such as basement membranes and interstitial stroma through precisely regulated on-off cycling of adhesion to surrounding matrix and degradation of matrix by proteases.

The contribution of different effector signaling pathways to Ras-induced metastasis has been evaluated. For example, one study utilized Ras effector domain mutants and determined that activation of Raf, but not PI3K or RalGEF, was sufficient for Ras-mediated induction of metastasis of NIH 3T3 mouse fibroblasts (Webb et al., 1998). In contrast, a similar study also used effector domain mutants but found instead a critical role for the RalGEF pathway in promoting metastatic growth in nude mice (Ward et al., 2001). In NIH 3T3 fibroblasts, as well as mouse and human mammary epithelial cells, RalGEF activation promoted aggressive, infiltrating metastases whereas Raf-induced metastases have non-infiltrating borders. While ERK or PI3K activation alone was not sufficient to promote metastasis, it was found that ERK activity was required and cooperated with RalGDS for metastasis. In contrast to these studies, for the *ras* mutation positive HT1080 human fibrosarcoma cell line, PI3K/Akt pathway activation

was implicated in mediating increased cell motility and invasion (Kim et al., 2001). Based on these studies, Ras proteins appear to promote invasion through the cooperation or selective activation of several key pathways. However, whether the pathways mediating invasion are cell-type specific or tumor-type specific is still unclear. Furthermore, the mechanisms by which Ras effector activation induces the invasive phenotype and the contribution of gene deregulation to the development of this phenotype remains largely to be determined.

Oncogenic Ras may promote tumor cell invasion and metastasis by causing deregulation of gene expression (Chambers and Tuck, 1993). This includes increased expression or activity of degradative enzymes such as matrix metalloproteinases (MMPs) and cysteine proteinases (cathepsins) as well as decreased expression or activity of their inhibitors (e.g., TIMPs). Of these, MMPs have been relatively well studied as targets for Ras-mediated gene upregulation of invasion-promoting proteins.

MMPs are zinc-dependent endopeptidases that degrade the extracellular matrix (ECM) as well as cleave cell surface molecules to mediate tumor progression, invasion, and angiogenesis. The MMP superfamily is divided into collagenases, stromelysins, gelatinases, transmembrane MMPs, and other MMPs (Coussens and Werb, 1996; Shapiro, 1998; Westermarck and Kahari, 1999; Matrisian, 1999). Most MMPs are secreted as latent precursors that are activated by an initial cleavage of an amino terminal propeptide followed by autocatalytic amino terminal cleavage resulting in full

exposure of the catalytic site and protease activity. Four members of specific MMP inhibitors known as tissue inhibitors of MMPs (TIMPs) bind the MMP catalytic domains to inhibit protease activity.

The evidence linking MMP upregulation with invasion and metastasis in a large variety of cancers of different tissue origins is quite extensive. Furthermore, mouse models deficient in specific MMPs exhibit decreased tumor growth, angiogenesis and invasion in response to various carcinogens and tumor promoting protein expression (Shapiro, 1998; McCawley and Matrisian, 2001). Despite the strong correlation between MMP overexpression and tumor invasion, few mechanistic studies are available that demonstrate the direct role of MMPs in oncogene-stimulated invasion. Furthermore, though most MMPs are induced at the transcriptional level by growth factors, hormones, cell contact to ECM, and oncogenes activation, recent studies have focused on transcription factors and not the cytoplasmic signaling pathways that mediate MMP promoter regulation. This section will focus on the transcriptional upregulation of MMP-2, -3, -7, -9 and –10 by activated Ras and its key effectors.

The best evidence for linking Ras to upregulation of MMPs involves MMP-9/type IV collagenase/gelatinase B (Yanagihara et al., 1995; Ballin et al., 1988; Himelstein et al., 1997; Giambernardi et al., 1998; Bernhard et al., 1990; Baruch et al., 2001; Yang et al., 2001; Gum et al., 1997). Ras-mediated upregulation of MMP-9 enzymatic activity is due primarily to upregulation in gene expression. The MMP-9 promoter contains a

variety of Ras-responsive promoter elements, including Ets, AP-1 and binding sites (Himelstein et al., 1997; Gum et al., 1996). Although clear cell-type differences in regulation are seen, an important contribution of the Raf/MEK/ERK effector pathway to Ras-mediated MMP-9 upregulation has been determined, but Raf-independent effector function (e.g., PI3K) is also involved (Gum et al., 1997; Gum et al., 1996; Arbiser et al., 1997). Evidence for a functional role for MMP-9 is provided by the observation that forced upregulation of MMP-9 promoted metastasis, whereas suppression of MMP-9 expression in Ras-transformed rodent fibroblasts caused a loss of metastatic growth but not tumorigencity (Bernhard et al., 1994; Hua and Muschel, 1996).

Upregulation of the related MMP-2 (gelatinase A), often together with MMP-9, has also been observed in a variety of cell types transformed by oncogenic Ras (Yanagihara et al., 1995; Baruch et al., 2001; Arbiser et al., 1997; Meade-Tollin et al., 1998; Charvat et al., 1999). Little is known regarding the effector signaling involved in MMP-2 upregulation, and the MMP-2 promoter lacks the Rasresponsive elements seen in the MMP-9 promoter (Westermarck and Kahari, 1999). Evidence for the importance of MMP-2 upregulation in Ras oncogenesis is suggested by the observation that for H-Ras-transformed MCF-10A human mammary epithelial cells, antisense inhibition of MMP-2 gene expression decreased Ras-mediated *in vitro* invasion (Moon et al., 2000). Interestingly, N-Ras transformation of MCF-10A cells

preferentially upregulated MMP-9 rather than MMP-2 and did not promote invasion, indicating cell-type differences in MMP-9 involvement in invasion.

MMP-3 (stromelysin-1), a member of the stromelysin subfamily of MMPs, has also been shown to be regulated by Ras in rodent fibroblast cells (Engel et al., 1992; LoSardo et al., 1995). Analyses of differentially-expressed genes identified MMP-3, as well as the related MMP-10 (stromelysin-2), gene as a MEKdependent upregulated gene in Ras-transformed 208F rat fibroblasts (208F) (Zuber et al., 2000) or as Raf-induced genes in Rat-1 rat fibroblasts (Heinrich et al., 2000). These studies suggest that in fibroblasts, Raf/MEK/ERK pathway activation may be sufficient for MMP-3 and MMP-10 upregulation. Similar to promoter studies performed on the human MMP-9 promoter, MMP-3 promoter analysis revealed Raf/ERK-dependent MMP-9 activation via Ets binding sites and Raf-independent activation via AP-1 binding sites (Kirstein et al., 1996; Jayaraman et al., 1999). The role of activated Ras and its effectors in upregulation of MMP-3 in epithelial cells remains to be clarified.

Perhaps the lease well-studied MMP discussed in this section is MMP-7 (matrilysin). In pancreatic carcinoma cells, MMP-7 transcriptional upregulation is associated with aberrant K-Ras activation (Ohnami et al., 1999; Fukushima et al., 2001). For example, antisense downregulation of K-*ras* expression in a pancreatic cancer cell line was associated with a downregulation of MMP-7 transcript levels. Similarly, K-Ras activation in colon carcinoma cells upregulated MMP-7 transcript in an AP-1

dependent manner (Yamamoto et al., 1995). Although these preliminary studies link Ras activation to MMP-7 upregulation, what effectors may mediate? Ras upregulation of MMP-7 has not been identified, nor has the role of MMP-7 in Rasmediated invasion been determined.

Although many studies illustrate Ras-induced MMP transcriptional upregulation as well as the correlation between Ras-mediated invasion and MMP upregulation, substantial evidence demonstrating MMP upregulation as a mechanism for Ras-mediated invasion is not available. Another complex question that remains unanswered is the vital role that Ras-induced TIMP downregulation may play in the regulation of MMPs by activated Ras. And finally, the interplay between epithelial cells expressing invasion-promoting oncoproteins and their surrounding stroma has only recently come under close scrutiny. Although this review focused on MMP upregulation in tumor cells, recent studies suggest that tumor cells may secrete factors that enhance MMP expression in neighboring stromal tissue. These secreted proteases may then localize to the tumor cell surface or surrounding extracellular environment to promote tumor cell invasion.

## 5. RHO GTPASES IN TUMORIGENESIS

Rho GTPases mediate many aspects of cell biology including cell size, proliferation, apoptosis/survival, morphology, polarity, adhesion, and membrane trafficking (Van,

1997; Aznar et al, 2001; Bar-Sagi et al, 2000; Ridley, 2000; Schmit et al, 2002; Settleman, 2001; Settlemen, 2000). They do so as signaling switches that regulate lipid signaling, microtubules and actin cytoskeleton, epithelial cell-junctions, cell cycle and apoptosis regulatory proteins, and transcription factors. As well, in the past 5 years an increasing amount of evidence indicates that several members of the Rho family are important players in tumor biology and several other human pathologies (Boettner et al, 2002)..

One of the major differences between Ras and Rho GTPases with respect to human cancers is the lack of dominant point mutations that result in constitutive binding to GTP and a quantitative overall increase in downstream signaling (Nakamoto et al, 2001; Suwa et al, 1998). However, overexpression of wild type Rho GTPases does indeed result in an increase in the turn over of GTP-loading, with the same final outcome, a net increase in the subsequent downstream signaling. It is interesting to note that overexpression of either the GTPase itself or some upstream or downstream element of Rho signaling has been detected in many human tumors suggesting that they might be key elements in the process of tumorigenesis (Benitah et al, 2003).

A role of Rho GTPases in tumorigenesis was first shown with murine fibroblasts that overexpressed dominant positive mutant RhoA, Rac1 and Cdc42 (Ballestero et al, 2000; Perona et al, 1993). Besides developing tumors when inoculated subcutaneously, these Rho-transformants develop distant lung metastasis in experimental metastasis

experiments (Van et al, 1995; Michiels et al, 1995; Del peso et al, 1997). In addition, Rho GTPases mediate essential aspects of tumorigenesis induced by many oncogenic signals. This is the case for Ras, since anchorage independent growth, tumor growth and invasion of oncogenic Ras-transformed cells depend on proper signaling from Rho GTPases (Pruitt et al, 2001; McCormick, 1998; Khosravi-Far et al, 1998; Qiu et al, 1995). In addition, Rho signaling is necessary for the oncogenic phenotype elicited by other pathways including receptor tyrosine kinases such as EGFR, IGFR, MET, or RETand G-protein coupled receptors (Boerner et al, 2000; Kim et al, 1998; Sachdev et al, 2001; Barone et al, 2001; Royal et al, 2000; Kamei et al, 1999; Whitehead et al, 2001).

In the past years much effort has been put in elucidating the mechanisms that underlie Rhoinducedeffects. Many proteins that bind the GTPloaded form of Rho GTPases have been cloned and classified according to their structure and function. There are two main groups of effectors to Rho GTPases: kinases (serine/threonine kinases, tyrosine kinases, and lipid kinases), and non-kinases. The role in cytoskeleton organization with respect to epithelialintegrity and motility, and their role in transformation,has provided enough information to develop drugs S. Aznar et al. / Cancer Letters 182 206 (2004) 181–191 with putative antineoplastic activity targeted to inhibit their functions. In the following sections a general view of the potential of some of these effectors and the GTPases themselves as targets for the development of

new drugs with antineoplastic properties will be presented.

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, anti-CDK6, anti-CyclinD3, anti-Rb2, anti-p15 INK4B, anti-p27 Kip1, anti-phospho (Ser807/811)-Rb 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™ 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 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)

### *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™ 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 $2\times10^6$ 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. 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.

### *10. 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

### *11. 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.

### *12. Soft agar colony formation analysis*

Cell transformation was evaluated with a soft-agar assay. Stable transfectants or control cells were plated $2\times10^4$ 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.

### *13. 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 ($5\times10^4$) 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.

### *14. 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

### *15. 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 ($5\times10^4$) 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.

### *16. 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 $3.5\times10^5$cells/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.

### *17. Tumorigenesis in nude mice*

Five-week-old female athimic nu/nu mice were purchased from ORIENT (CHARLES RIVER TECHNOLOGY, KOREA). Mice were housed in germ-free conditions of controlled temperature (23℃±1℃), humidity (55±10%) and lighting (0700 - 1900 h), received adlibitum sterilized food pellets and sterilized water

containing Josamicine (65 μg/ml) and Gentamicine Sulfate (270μ g/ml) during both acclimation period (1 week) and experiments. All the procedures were performed in the animal facilities according to the ethical guidelines for the conduct of animal research. For tumorigenicity assay mice were randomly assigned to single treatments (four animals /group) and challenged subcutaneously in the left inguinal region with 0.2 ml of and $2.5\times10^5$ cells in phosphate buffered saline. Cages were coded and the size of tumors was evaluated for 36 days by biweekly measurement of the average of two perpendicular diameters of the neoplastic masses with linear caliber. At the end of this time period the mice without tumors were classified as survivors. Latency and survival times were respectively the time period in days to reach a neoplastic mass diameter >3 mm and >10 mm, and the growth was considered the time between the latency and the survival. Tumorigenicity was reported as the per cent of mice with tumors after 6 weeks. For histology, tumor samples were fixed in formalin and stained with ematoxylin-eosin by standard techniques. All the tumors showed comparable histological characteristics and were classified as sarcomas.

### *18. Immunofluorescent and immunohistochemical staining*

Tumors were excised from mice and fixed in 10% neutral buffered formalin for 24 hours. Frozen tissues were cut into 10μm sections in a microtome at 20℃. Frozen tissue sections on glass slides were fixed in acetone at 20℃ for 20 min and rinsed in

phosphate buffered saline (PBS). A rat antimouse CD31 monoclonal antibody (BD PharMingen, San Diego, CA), diluted 1:100 (v/v) in PBS, was applied to the slides which were incubated at room temperature for 2 h. The slides were rinsed three times in PBS and then incubated with a fluorescein isothiocynate (FITC)-conjugated polyclonal goat anti-rat IgG antibody (Pharmingen), in 1:50 (v/v) dilution in PBS, for 1 h at room temperature in a dark room. The slides wererinsed in PBS and mounted under a cover slip with a mounting medium gel mount (Biomeda, Foster, California). The slides were kept at 4 ℃ in the dark until scanning with laser microscopy.

### *19. Laser scanning microscopy and image analysis*

The FITC-labeled slides were scanned with a Zeiss model LSM510 computer-assisted CLSM equipped with an argon laser (Zeiss, Germany). Images were acquired and analyzed using the LSM510 software 3D (Zeiss, Germany). The channel settings of pinhole, detector gain, amplification offset and gain, and % laser transmission were adjusted to provide an optimal balance of fluorescent intensity of the targeted microvessels and background. The same settings were then used for scanning all the slides prepared on the same day. Tissue sections, in series with those examined previously with light microscopy, were scanned at 400× magnification with an excitation wavelength of 568 nm. With the z-stack function, a composite image was created as previously described from nine serial 6μm fluorescent images acquired

vertically for each microscopic field identical to that examined with light microscopy. Background fluorescence was subtracted from the image. The total area of fluorescence in mm2 in a microscopic field, detected above the background, was integrated automatically by the computer. Thus, the fluorescent area was a numerical representation of the total area of microvessels labeled by the CD31 antibody

### *20. Animal micrometastasis assay*

A total of $1\times10^6$ NIH3T3 transfectant cells were injected into the tail vein of 5-week old female. Five-week-old female athimic nu/nu mice were purchased from ORIENT (CHARLES RIVER TECHNOLOGY, KOREA). Four mice were injected with each cell type. After 42 days, mice were sacriWced, dissected, and analyzed by gross examination. The lungs were excised and placed in saline solution. Metastatic foci were counted at the lung surface under a dissecting microscope.

### *21. Animal survival assay*

Survival rate of nude mice bearing intraabdominally implanted NIH3T3 transfectant cells. A suspension of 0.2 ml and $2\times10^6$ cells in phosphate buffered saline wasinjected into the abdomen of nude mice. The condition of these mice was checked once daily. Survival time was compared between 4 groups of nude.

### *22. 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.10).

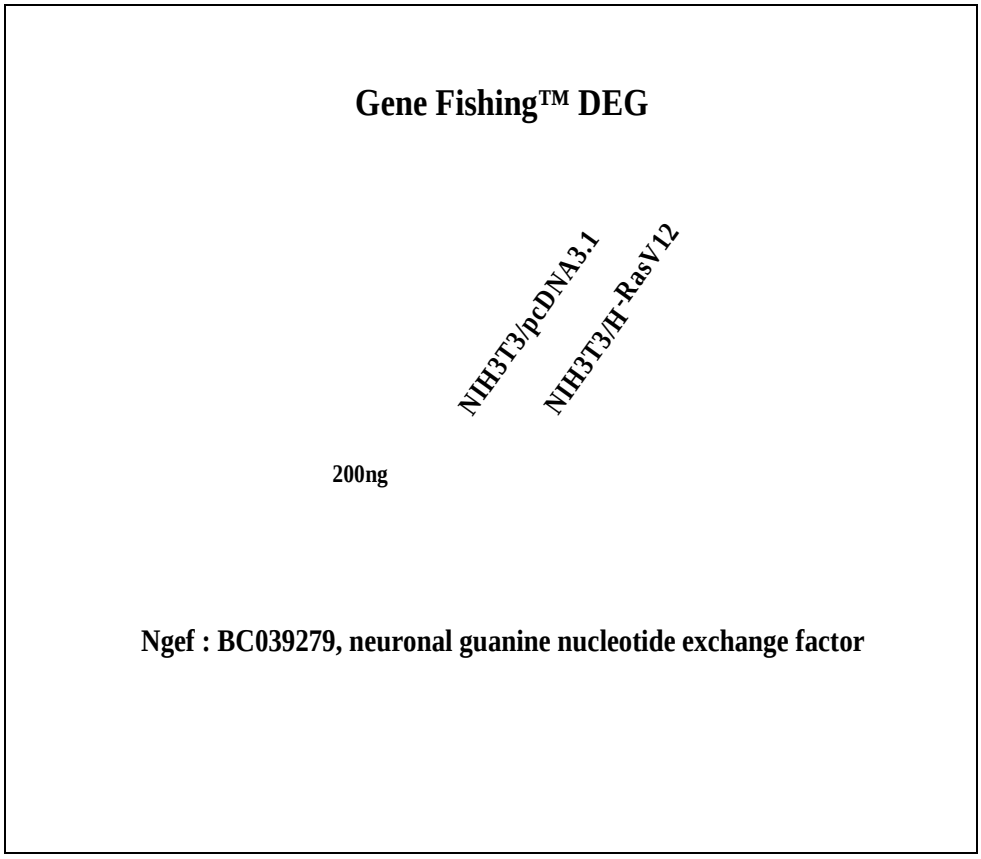


**Fig.10. Result of GeneFishing PCR for the identification of differentially expressed genes (DEGs)**

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.11A 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.11B and D). Thus, oncogenic H-Ras expression induces NGEF expression in murine fibroblast cells.

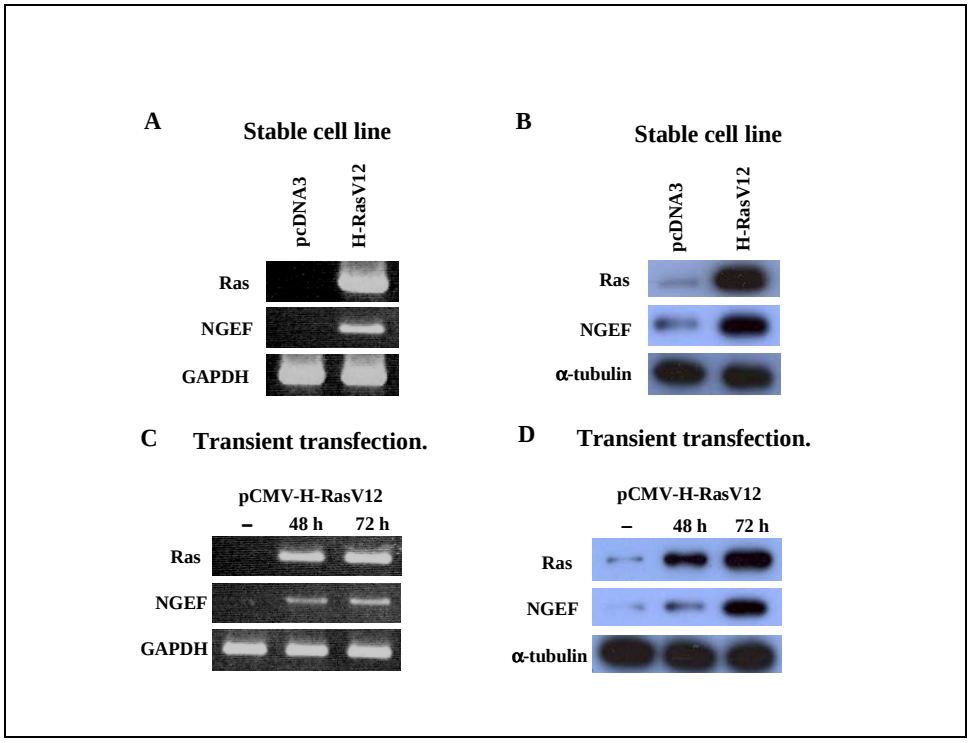


**Fig.11. Oncogenic H-Ras induces expression of the NGEF**

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.12A and 12B). 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.12 C).

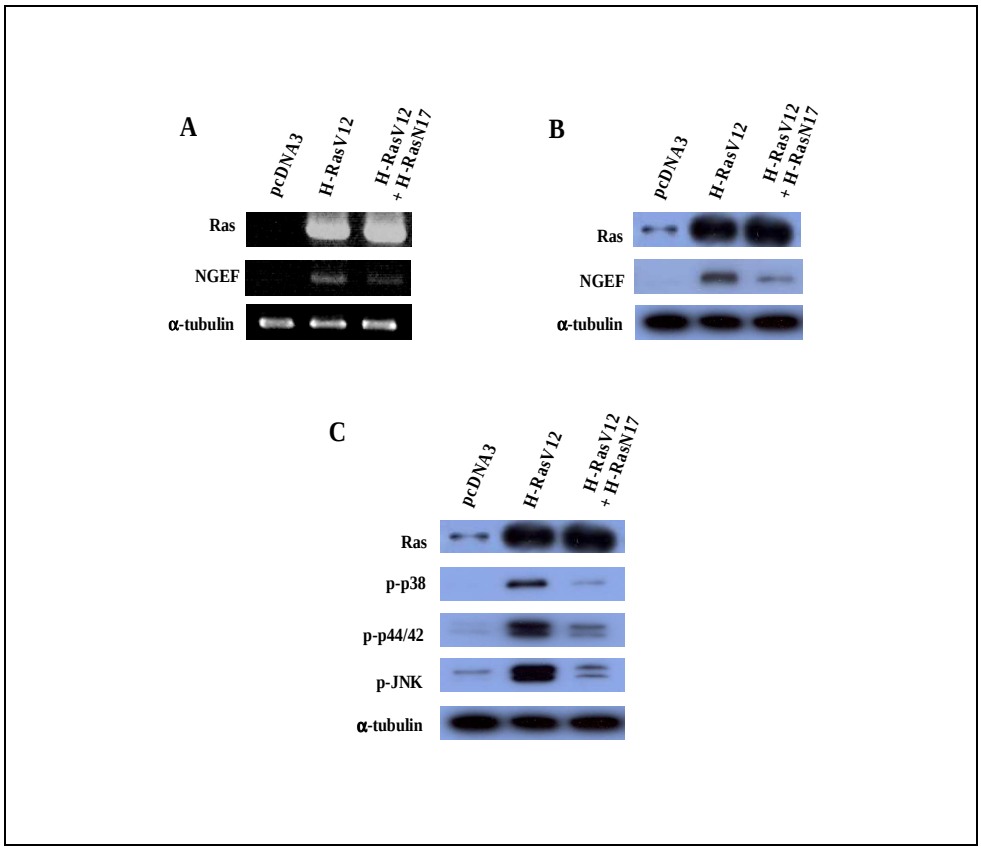


**Fig.12. Dominant negative H-RasN17 transfection led to suppression of H-RasV12-mediated increased NGEF expression**

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.13A). 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.13B). 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.

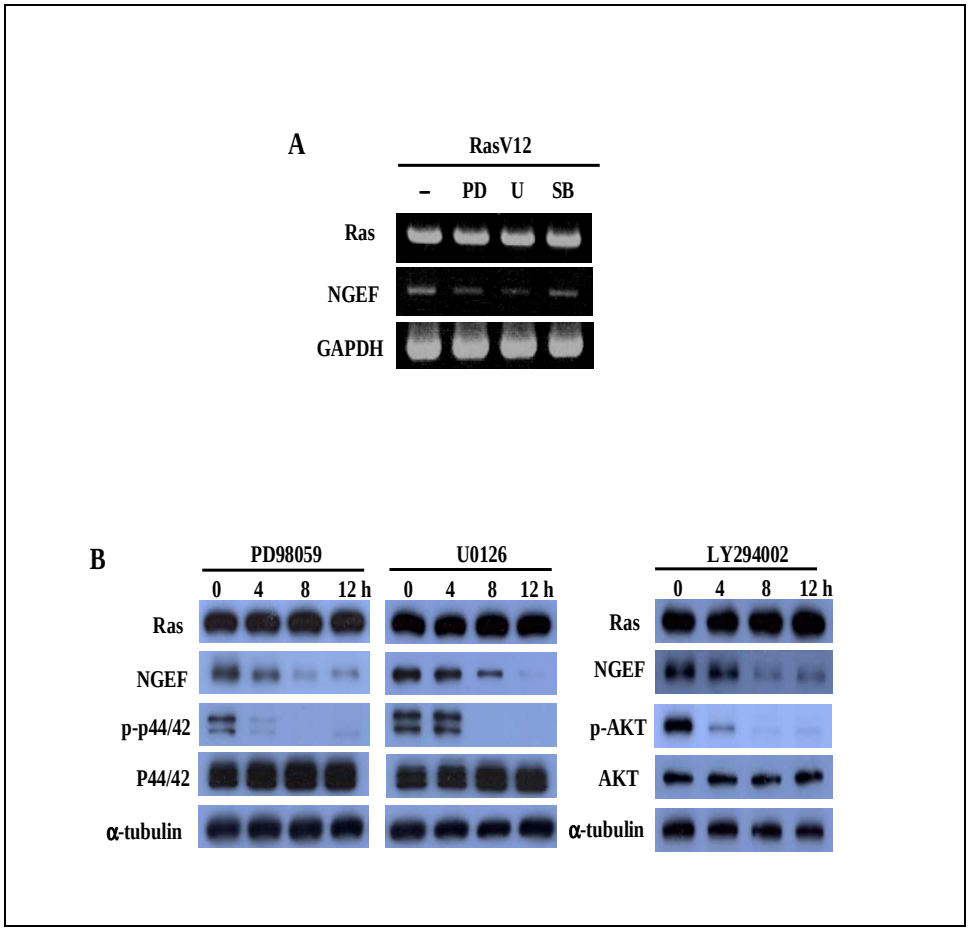


**Fig.13. Effect of Ras signal inhibitor on NGEF expression in oncogenic H-Ras transformed cells**

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.14). These data suggest that expression of NGEF were capable of activating Rho, Rac1 and Cdc42, but not Raf activity.

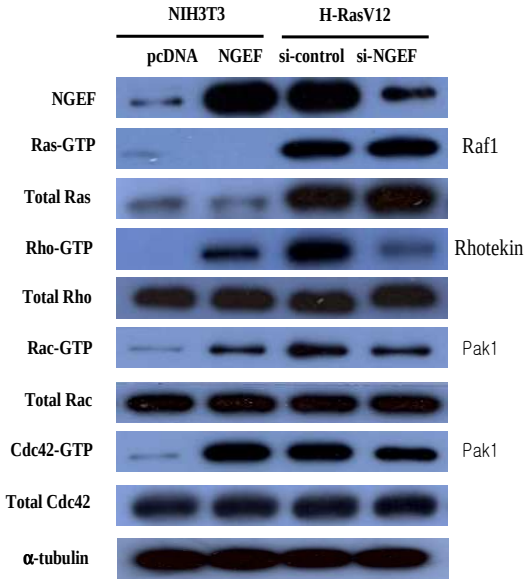


**Fig.14. Effect of NGEF on the Raf, Rho, Rac1 and Cdc42 activity**

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. EGF-stimulated induction of NGEF is dependent upon ERK, PI3K

In the activated GTP-bound form, Ras activate several downstream targets, including ERK and PI3K. EGF-induced activation of ERK is dependent on Raf and EGF-induced activation of Jun kinase is dependent on another Ras effector, phosphatidylinositol-3-kinase. Therefore, we examined the effect of NGEF expression on ERK and PI3K activation in response to EGF stimulation in NIH3T3 or NIH3T3/H-Rasv12 cells. The results indicate that both of ERK and PI3K signaling pathway required for the upregulation of NGEF protein in response to EGF (Figure.15).

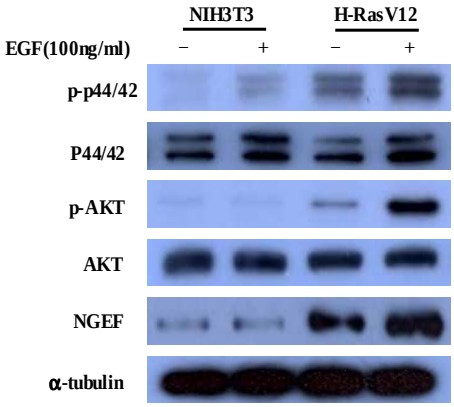


**Fig.15. NGEF expression was induced by EGF-stimulated MEK-ERK and PI3K-AKT activation**

Control and H-RasV12-transforming NIH3T3 cells were serum-starved for 12h and then stimulated with 100ng/ml EGF for 30 min. Cell lysates were directly immunoblotted with anti-phopho-p44/42, anti-p44/42, anti-phospho-Akt and anti-Akt and anti-NGEF antibody. α-tubulin was used as the loading control.

## 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.16A) and MTT assay (Figure16B). These findings strongly indicate that NGEF plays an essential role in mediating the proliferative activity of NIH3T3 cell or oncogenic H-RasV12 NIH3T3 cell.

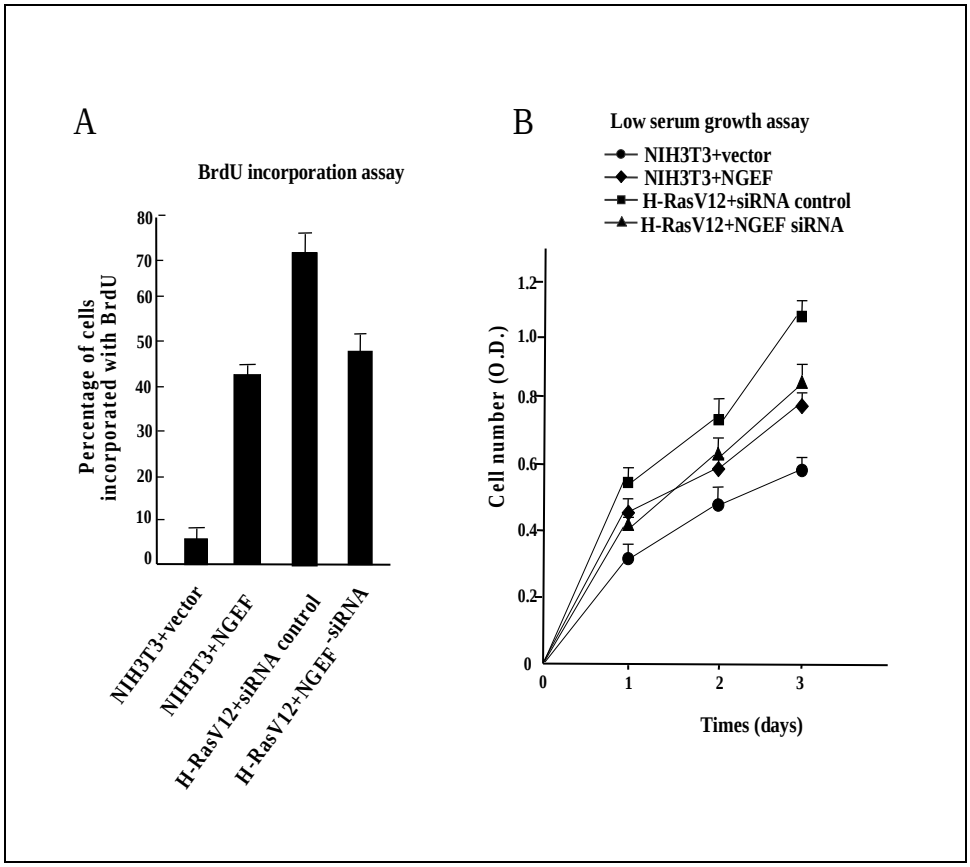


**Fig.16. *In vitro* Proliferation assay**

(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 $5\times10^5$ 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 cell cycle regulator proteins

Mitogenic stimuli promote the entry of quiescent cells into the first gap phase (G1) and initiation of DNA synthesis (S phase) of the cell cycle. Exit from or entry into the G0 quiescent state is controlled by positive and negative regulatory proteins. G1 cyclin-dependent kinases (CDKs) serve as positive regulators. D-type cyclins(D1, D2, D3) complex with CDK4 and CDK6 to stimulate their kinase activities, which in turn cause the phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein. By binding to E2F, Rb recruits histone deacetylases to the promoters of E2F-responsive genes and represses their transcription. Cyclin D1, in part, regulates the kinase activities of both CDK4 and CDK6. These complexes are formed in the cytoplasm and are transported into the nucleus and undergo stimulatory modifications including phosphorylation by CDK activating kinase (CAK) to yield active holoenzymes. Further into G1, cyclinE complexes with CDK2 and causes additional phosphorylation and inactivation of Rb. With sufficient phosphorylation of Rb, E2F is released and transactivates genes required for S phase entry, including cyclins E and A. CDK inhibitors (CKIs) serve as negative regulators of the Rb pathway. CKIs are classified into two distinct families on the basis of their structural and functional characteristics. The members of the INK4 family of CKIs ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$) contain multiple ankyrin repeats and act as negative regulators of CDK4/6 by binding to the catalytic subunit and preventing formation of

the active cyclin-CDK complex. The Cip/Kip family of CKIs ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) is more broadly acting and regulates both CDK4/6 and CDK2 activity. Each member of the family contains a characteristic motif within the amino-terminal region that enables them to bind to both cyclin and CDK subunits. The stoichiometry between CDKs and CKIs is important and determines the activity of Rb and the proliferative state of cells. 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 NGEF has any effects on the cell cycle, we investigated whether NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12, NIH3T3/H-RasV12 siNGEF could affect the cell cycle checkpoint related gene. We found that NGEF expression led to increase in CDK4, cyclinD3, p27 and p-Rb expression in NIH3T3. In addition, transfection of NGEF siRNA into oncogenic H-Ras transfected cells resulted in the suppression of these cell cycle regulator proteins (Figure.17). Thus, NGEF may regulate cell cycle progression through expression of several cell cycle progression proteins, and they suggest that NGEF is an important role for cell cycle regulation in oncogenic H-Ras-transformed cells.

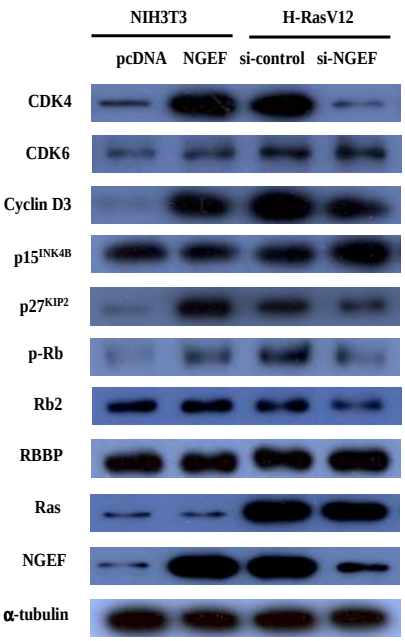


**Fig.17. Screening of cell cycle regulator gene**

The cycling cells were analyzed by immunoblot using the indicated antibodies. The anti-CDK4, anti-CDK-6, anti-CyclinD3, anti-$p15^{Ink4b}$, anti-$p27^{Kip1}$, anti-phospho Rb, anti-Rb2, anti-RBBP, anti-Ras and anti-NGEF antibody were used. α-tubulin was used as the loading control

## 9. 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.18).

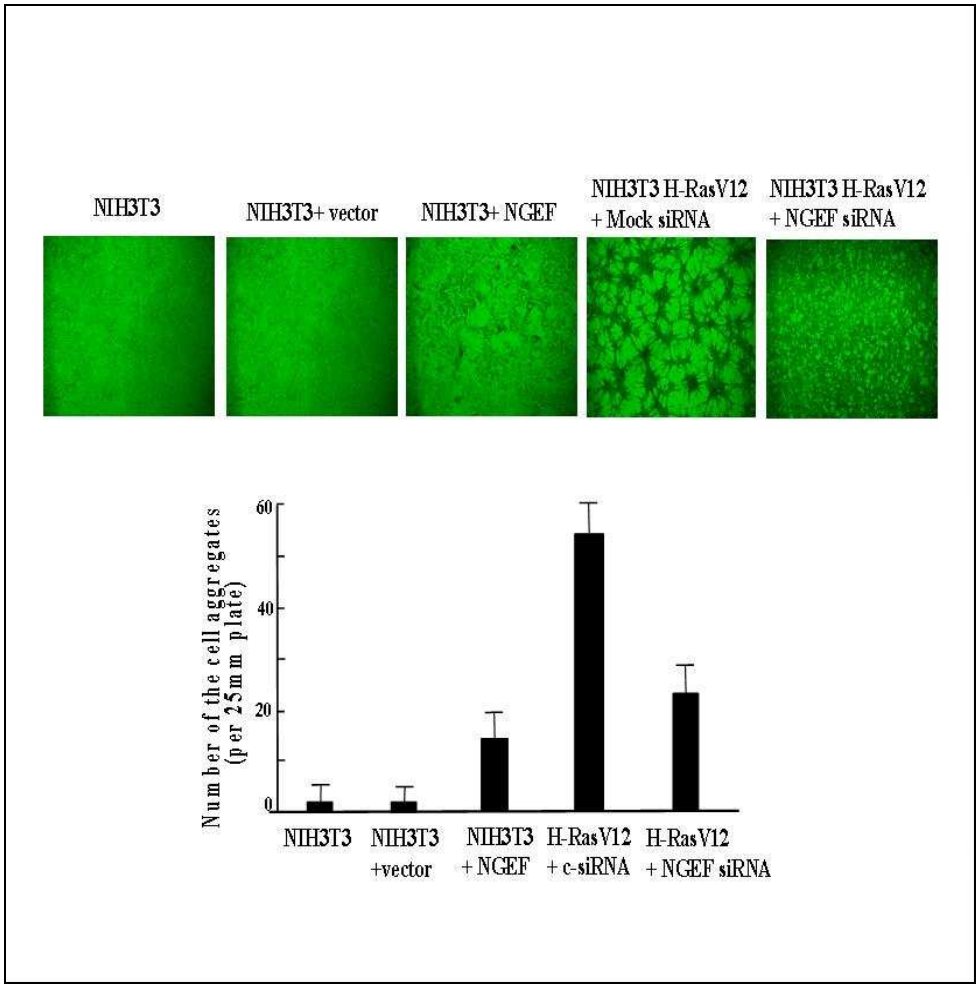


**Fig.18. *In vitro* Aggregation assay**

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.

## 10. 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.10, expression of NGEF caused a 40% induction in transforming activity compared with the activity caused by transfection of the empty vector (Figure.19). 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.19, 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.

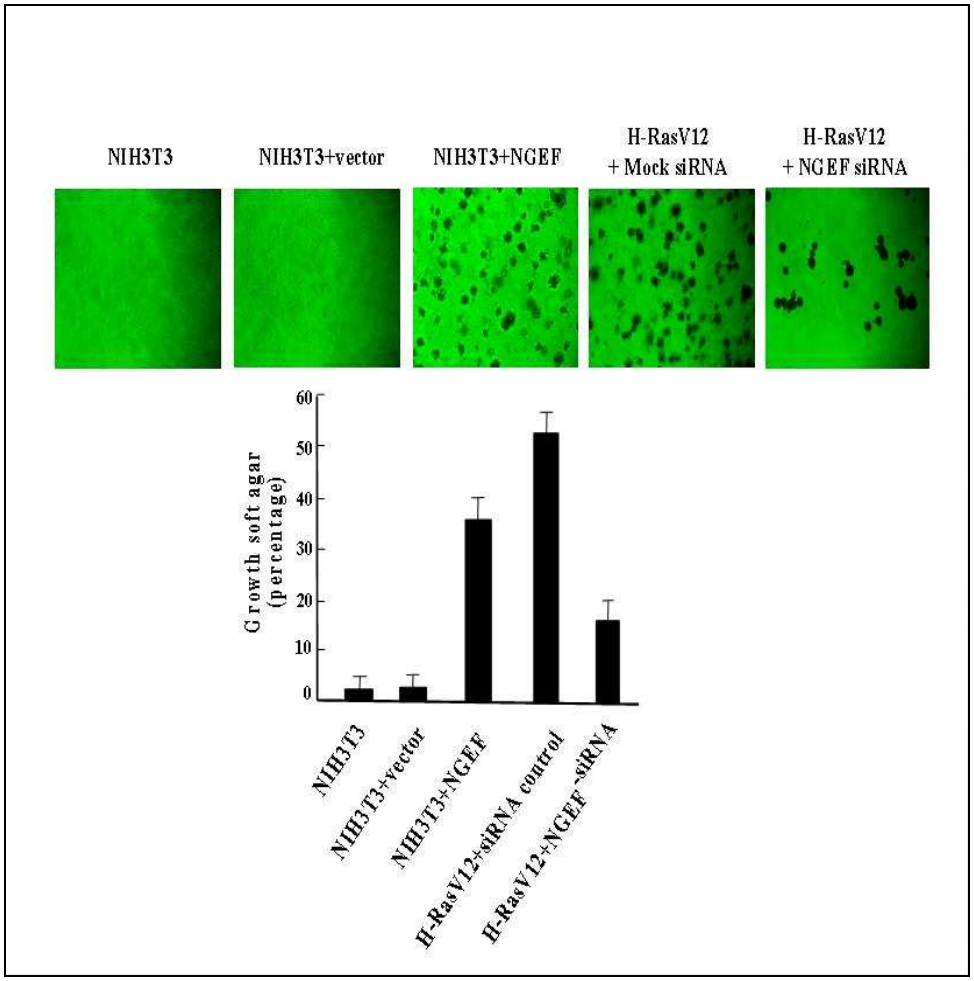


**Fig.19. *In vitro* Colony formation assay**

NIH3T3, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA or NIH3T3/H-RasV12 NGEF siRNA cells were plated $2\times10^4$ 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.

## 11. 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.20, 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.20). We also examined the effect of NGEF over-expression on cell migration by in vitro wound healing assay. As shown in Figure.21, 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.21).

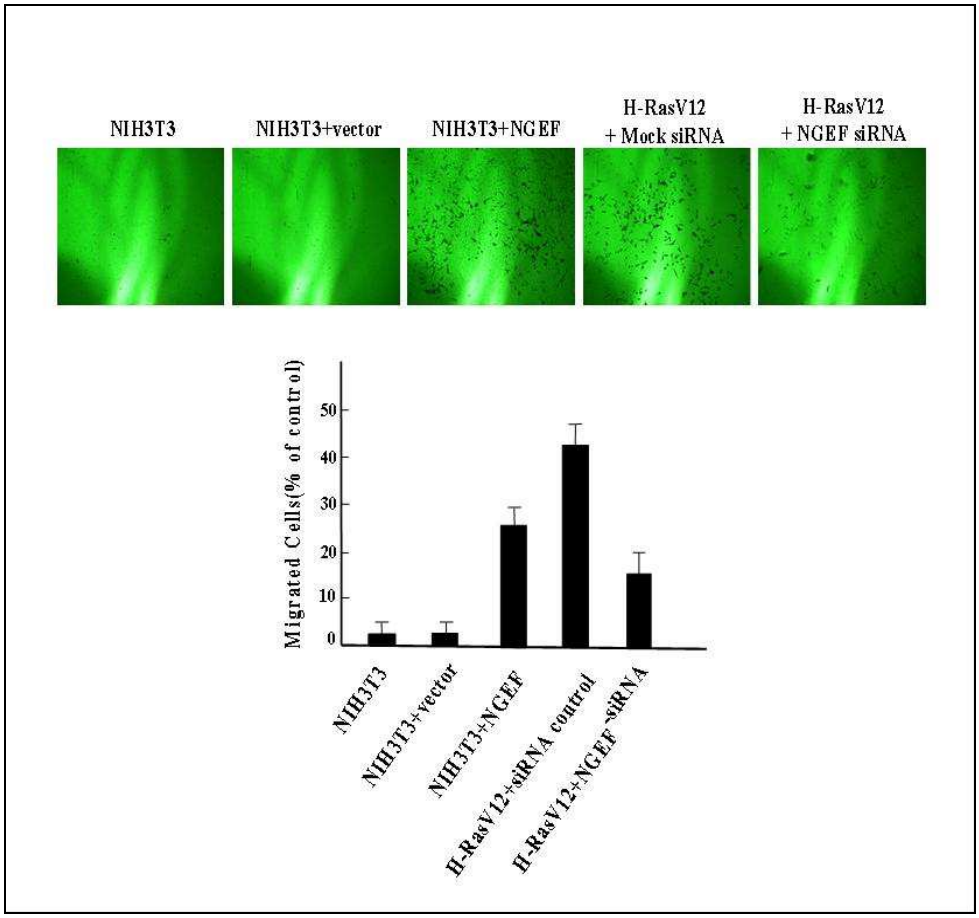


**Fig.20. *In vitro* Migration assay using transwell**

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 ($5\times10^4$) 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.

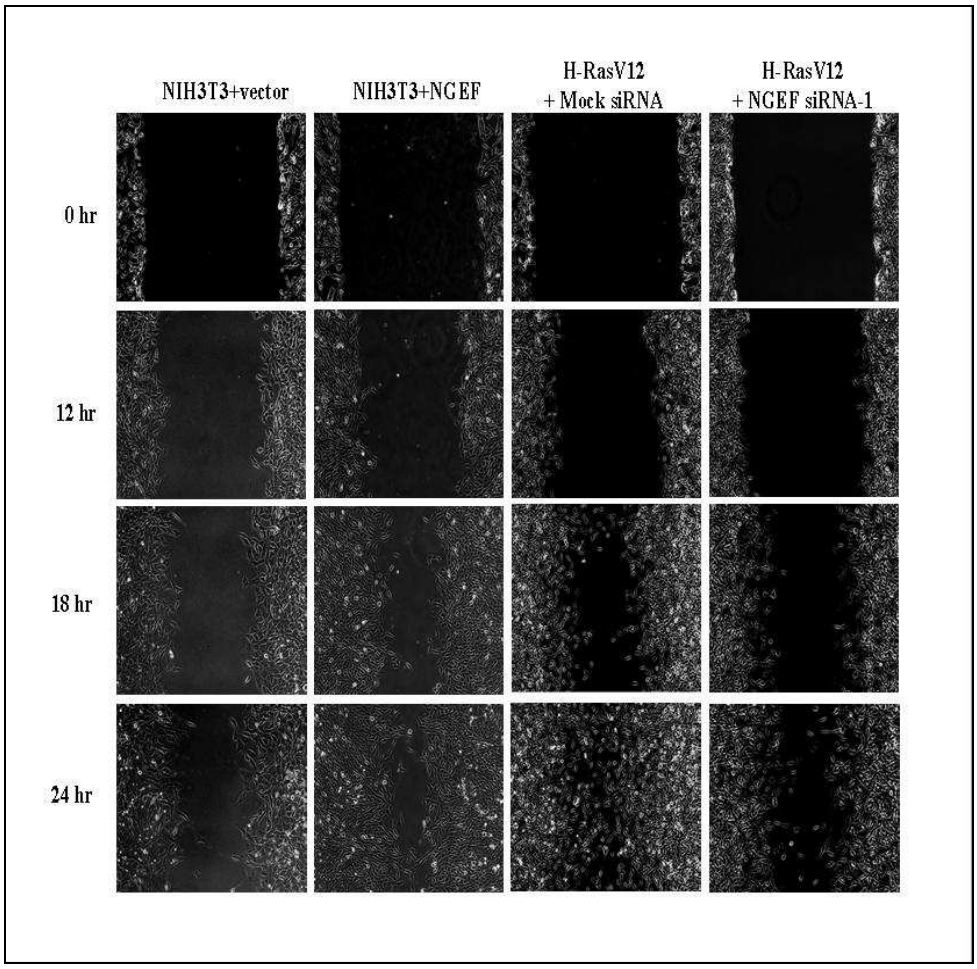


**Fig.21. *In vitro* Wound healing migration assay**

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.

## 12. 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. $5 \times 10^4$ 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.22, 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.22). 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.22, 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.22). 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.

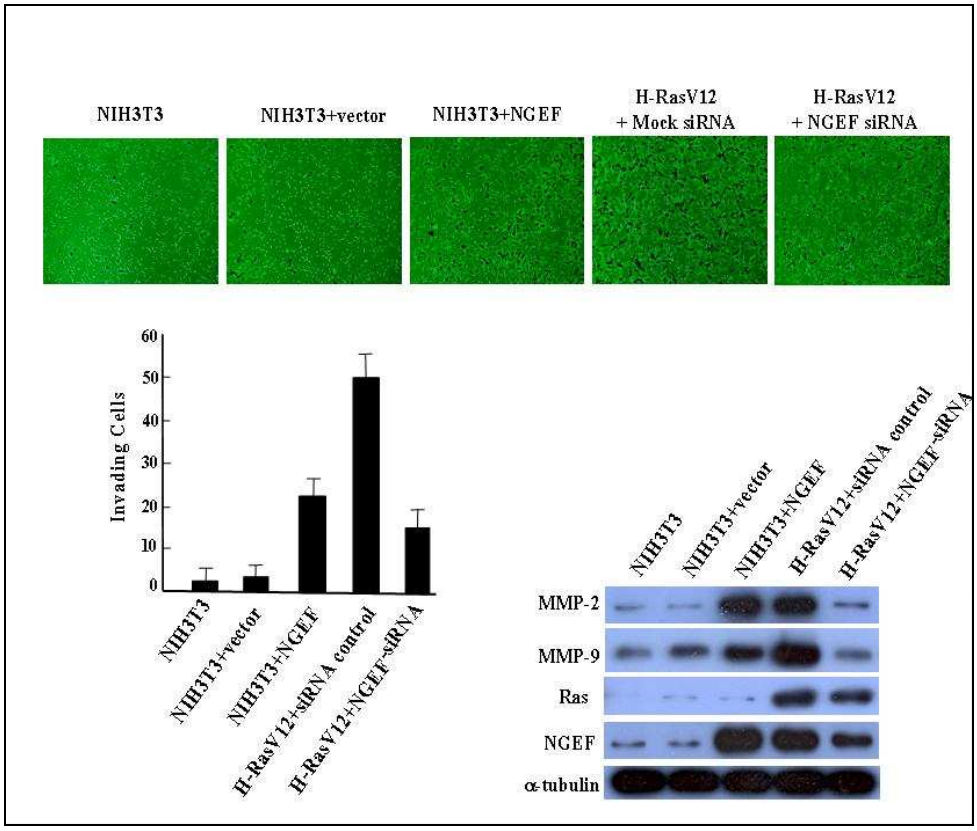


**Fig.22. *In vitro* Invasion assay using matrigel chamber**

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.

## 13. 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.23, 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.23).

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.

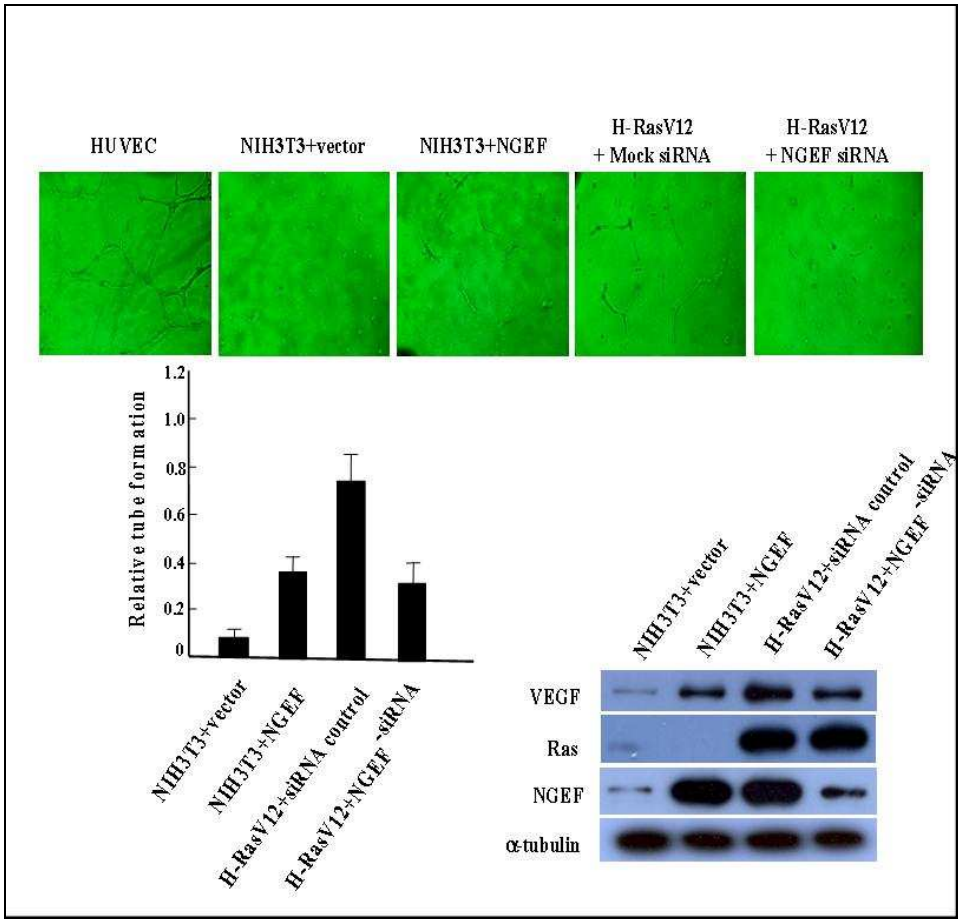


**Fig.23. *In vitro* Angiogenesis assay**

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.

## 14. 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 $2.5\times10^5$ in PBS.

Animals were monitored for subcutaneous tumor formation and the growth rate of the developing tumors was established. As illustrated in Figure.24, 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.24). Animal injected with the expressed NGEF at the tumor site were initiated eight days after cell inoculation. (Figure.25). Moreover, NGEF were stably knocked down by siRNA in the highly tumorigenic H-RasV12-transformed NIH3T3 cells reduced the tumor formation (Figure.24). 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.25). 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.

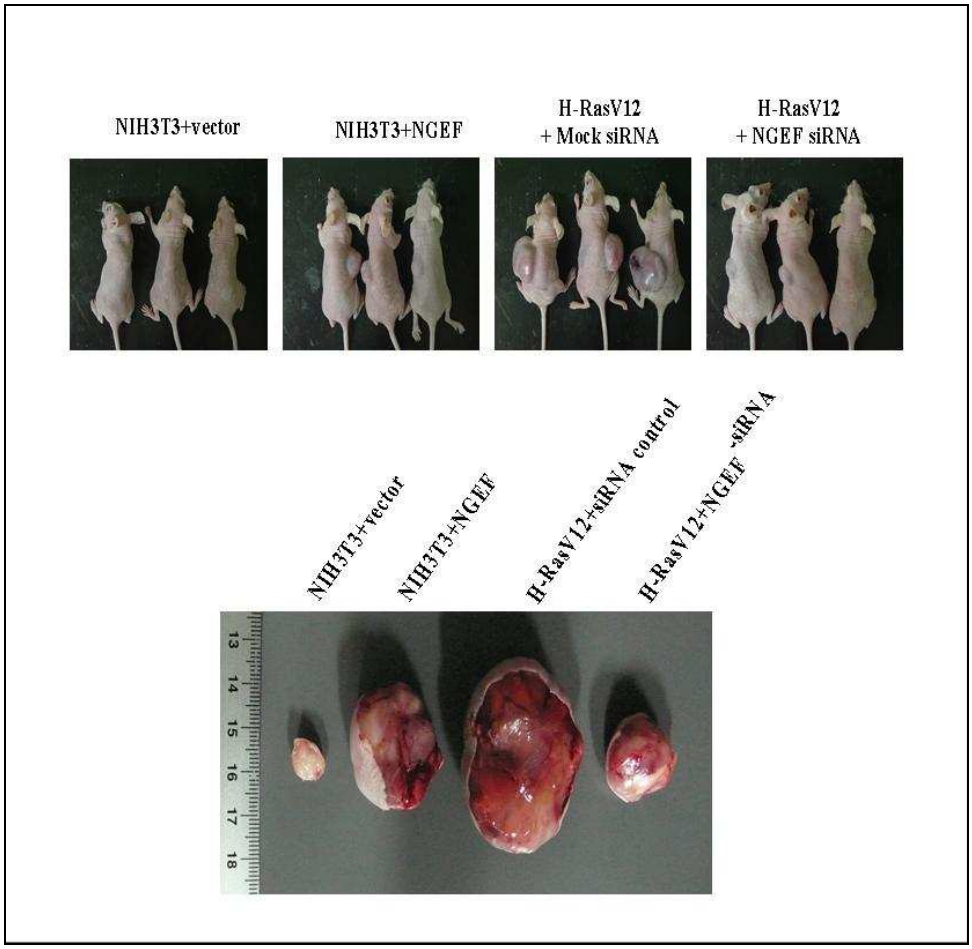


**Fig.24. Animal tumor formation assay**

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.

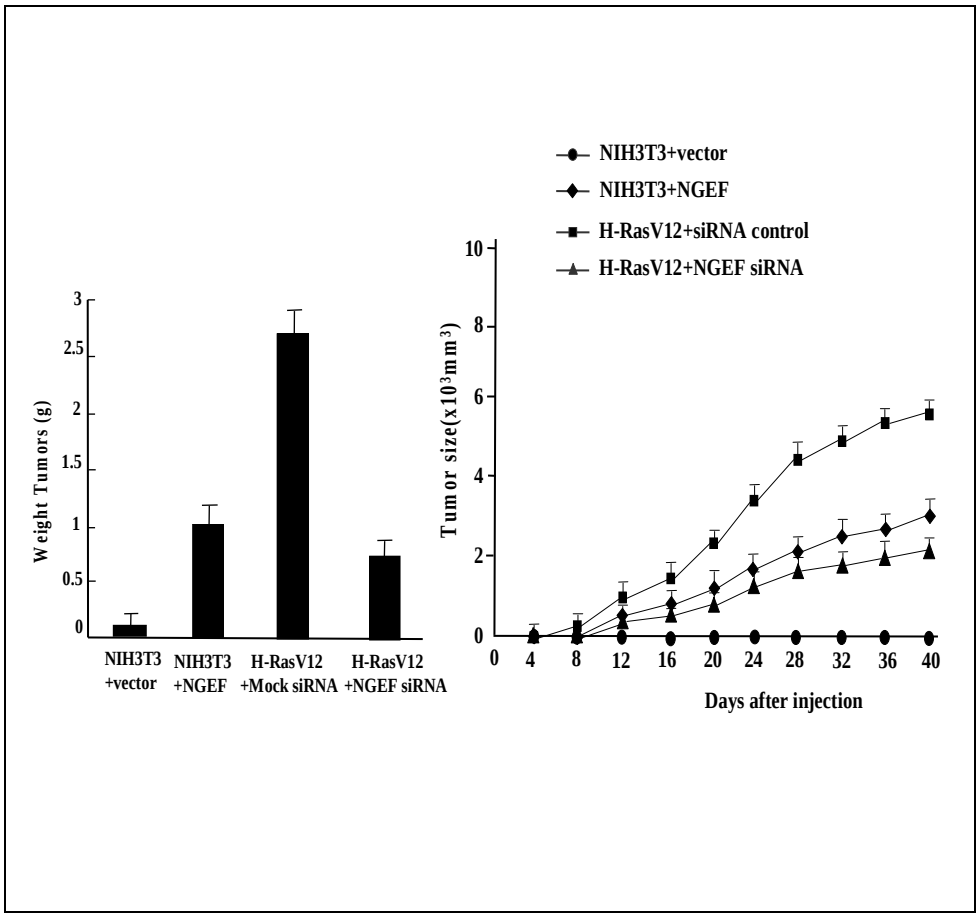


**Fig.25. Animal tumor growth assay**

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.

## 15. Effect of NGEF on the Animal survival

Kaplan-Meier survival curves were generated to compare survival time distributions of animals treated with each specific regimen. Then 0.1 ml of this intraperitoneal mixture was injected into the nude mice. Prolonged mouse survival was tested during the following 2-month period compared to the control group cells injected with NIH3T3/NGEF, NIH3T3/H-RasV12 NGEF siRNA cells pre-incubated only with PBS. Following tumor cell implantation, mice were monitored at least twice every week as described in Materials and Methods for tumor growth up to 2-months when the experiments were terminated. The result of tumor-free survival is shown in Figure.26.There was significant inductive effect of NGEF gene expression such as, compared with control vector animal The median survival time for the H-RasV12-transformed group was approximately 14 days. In contrast, H-RasV12/siNGEF group by i.p. injection significantly prolonged the animal's survival. The median survival time for the H-RasV12/siNGEF animals was 23 days, representing a 40% increase over that of the H-RasV12-transforned group (Figure.26).

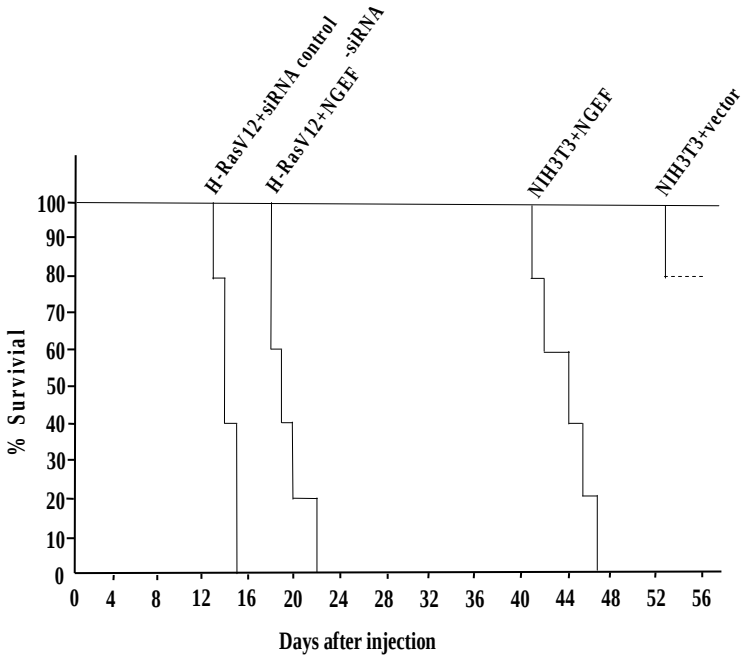


**Fig.26. Animal survival assay**

Animal survival of NIH3T3/vector, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA were determined by Kaplan-Meier analysis. Mice were either injected intraperitoneally with phosphate-buffered saline/each cells. Animals were monitored for 2 month; no deaths occurred after 72 hr. Statistical analysis was performed using log-rank test.

## 16. Effect of NGEF on the Animal angiogenesis

To establish whether impaired tumor growth was associated with a paucity of histologic sections of the tumors were stained using monoclonal CD31 antibodies to visualize endothelial cells and vascular pericytes, respectively. CD31 is expressed in both tumorigenic lymph and angiogenic endothelial cells of both early and mature vasculature. We evaluated the capillary density by immunohistochemistry, since this is a measure of tumorassociated capillary angiogenesis. Immunohistologic staining was carried out with antibody against CD31. To evaluate the level of microvessel density in more detail, the fluorescent images of the anti-CD31-stained tumor sections were digitally recorded and used for computer-assisted image analysis. This analysis indicated that the NGEF had significantly induced tumor angiogenesis, showing induction in vessel ends, vessel nodes, and total vessel length. The same experiment done using NIH3T3/H-RasV12 transformed cells showed that H-RasV12 expression was associated with induced angiogenesis (Figure.27). However, we investigated the levels of tumor induced microvessel density in tumor xenografts; the microvessel density was significantly reduced in xenografts of NIH3T3/H-RasV12 siNGEF cells compared with cells transfected with NIH3T3/H-RasV12 (Figure.27). These data strongly suggest that NGEF is involved in the oncogenic H-Ras-medated increase *in vivo* angiogenesis.

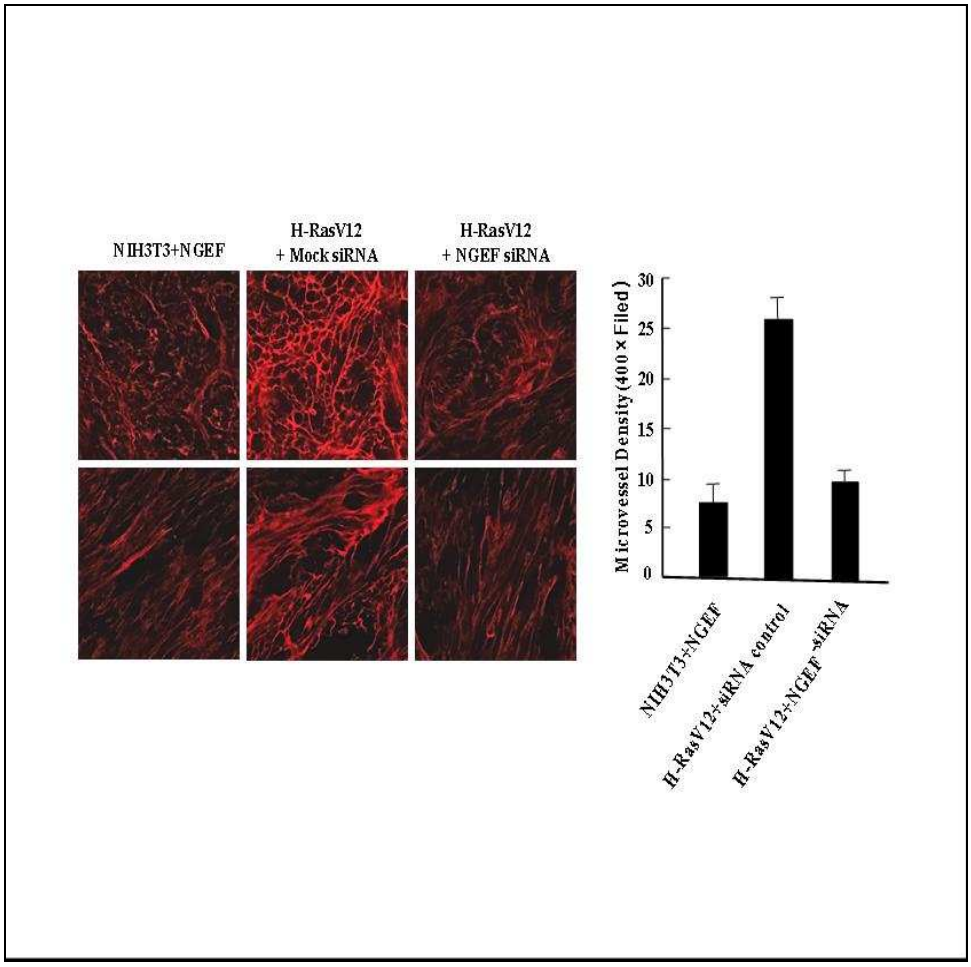


**Fig.27. Animal angiogenesis staining assay**

Immunofluorescence an analysis of tumor sections in NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA injected mice. Fluorescence microscopy of tumor sections stained with an anti-CD31 antibody (*red*) and showing microvascular morphology. Microvessels of mouse skeletal muscle detected by CD31 immunohistochemical staining and light microscopy (magnification×400).

## 17. Effect of NGEF on the Animal metastasis

To determine which steps in the metastatic process are sensitive to ras expression in NIH3T3 cells transformed by activated H-Ras, we injected the NIH3T3/pcDNA3.1, NIH3T3/NGEF, NIH3T3/H-RasV12 control siRNA and NIH3T3/H-RasV12 NGEF siRNA cells in the nude mice intravenously (i.v.) via tail vein and assayed the ability of these cells to establish tumors in the lung. The experimenter injecting cells was unaware of the genotypes. As shown in Figure.28, H-RasV12-transformed cells formed faster tumors than H-RasV12/siNGEF cells at 30 days after injection. Representative pictures of the lung tumors both macroscopically and microscopically after hematoxylin and eosin (H&E) stained (Figure.28). These results suggest that NGEF expression is required for oncogenic H-Ras-mediated in vivo metastasis (Figure.28).

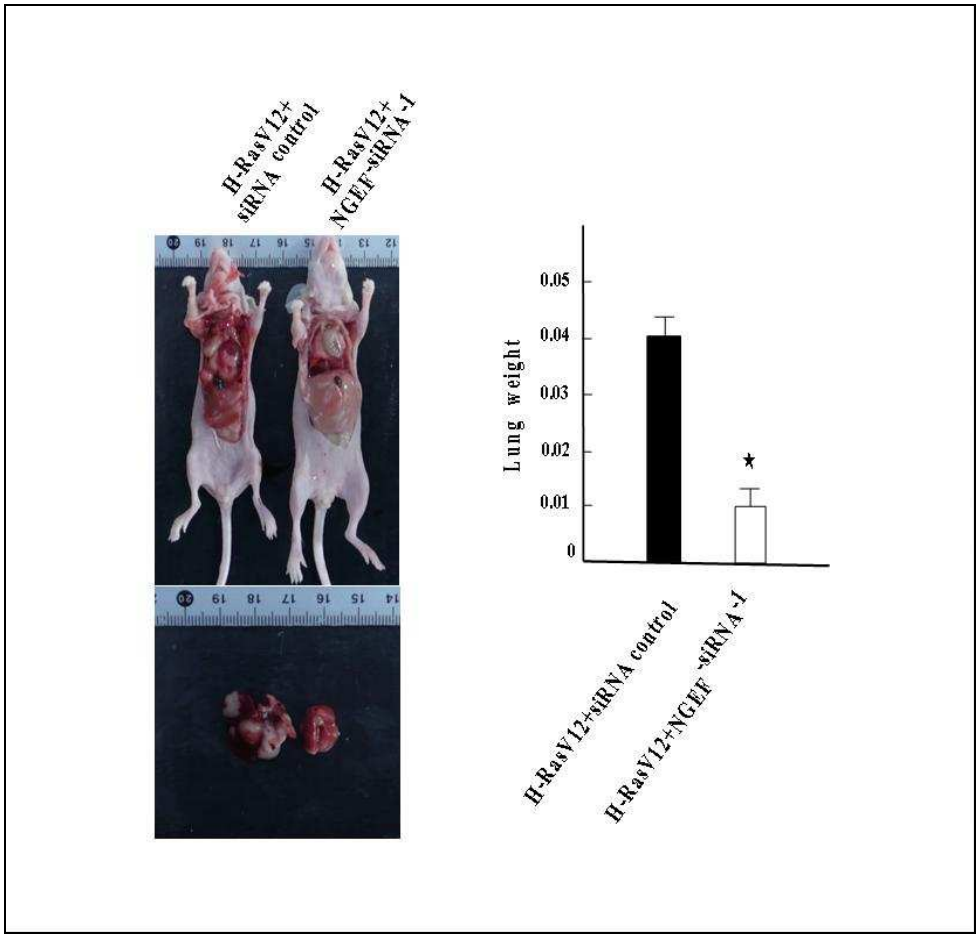


**Fig.28. Animal metastasis assay**

Representative macroscopic and microscopic images are shown. Control siRNA- and NGEF siRNA- transfected H-RasV12-transformed cells were injected (i.v.) to the nude mice via tail vein ($2\times10^6$ cells per mouse). Lungs at 30 days after injection were excised, weighted, and the lung/body weight ratio calculated. The mean ±S.E. of four mice is shown.

# 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.10-11). 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.12). 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.12-13). 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.14). These result suggest that NGEF increase of Rho, Rac1 and Cdc42 activity in NIH3T3 and oncogenic H-Ras-transforming NIH3T3 cells.

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.16, Fig.18-19). 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.16, Fig18-19). 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.20-23). In addition, ability of *in vitro* cellular migration, invasion and angiogenesis of NIH3T3 cells was significantly induced by NGEF overexpression (Fig.20-23). 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.22). 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.22). 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.23). 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.20~23).

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, animal survival, animal angiogenesis and animal metastasis. The ability of animal tumor growth, animal survival, animal angiogenesis and animal metastasis of NIH3T3 cells with NGEF overexpressing was increased (Fig.24-28), 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.

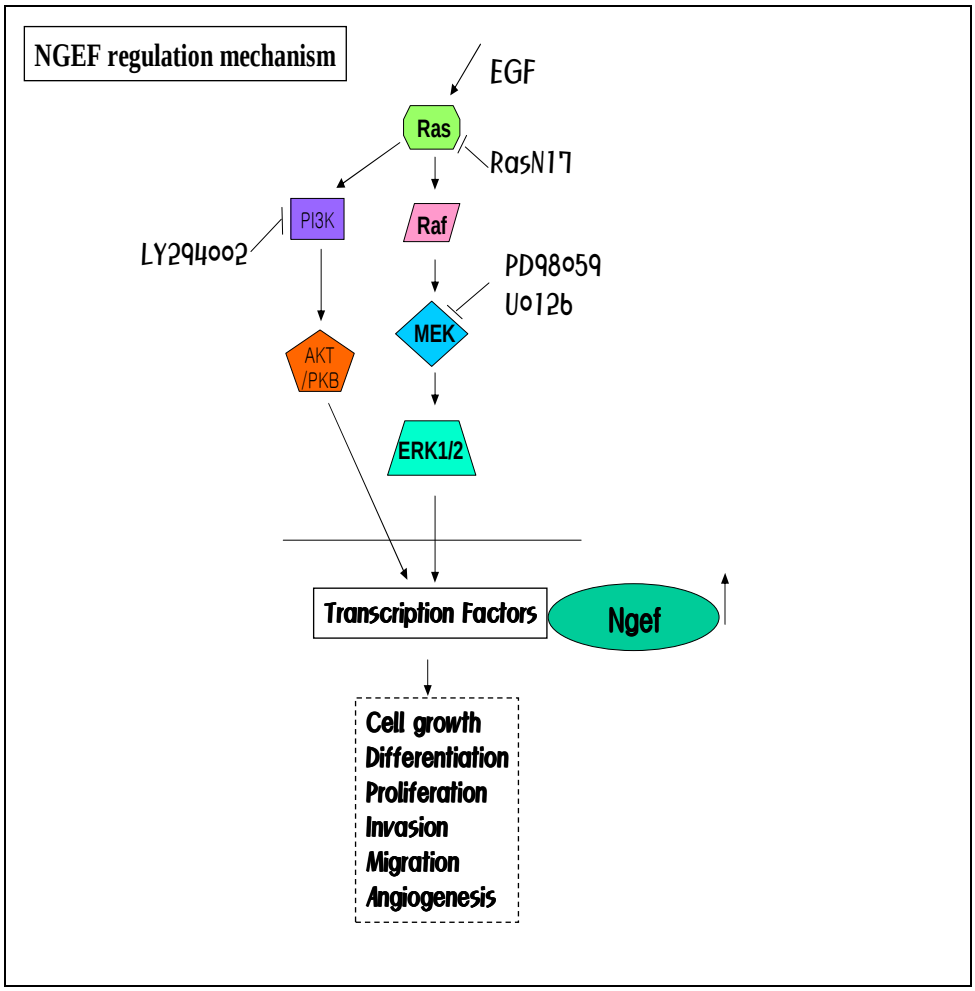


**Fig.29. Schematic representation of NGEF-regulation mechanism by induced oncogenic H-RasV12**

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# <국문초록>

## NGEF의 발암효과에 관한 연구

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활성화된 Ras 종양유전자는 다양한 포유류 세포들을 형질전환 할 수 있고 악성 사람 종양들의 많은 개체의 성장에 관련되어있다. Ras 유전자에 의해 유도된 종양 발달의 작용은 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 mRNA전사와 단백질의 발현 정도가 감소하였고 ERK와 PI3K 억제제들을 처리함에 Oncogenic H-RasV-induced NGEF의 발현은 현저히 억제되었다. 게다가 NGEF의 과 발현은 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 도입 시킨 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 성장, 신생혈관형성 그리고 전이를 현저히 감소하는 것으로 나타났고 반면에 control siRNA 형질도입은 감소하지 않았다. 더욱이 H-RasV12-transformed 세포들에서 NGEF의 억제는 동물 생존 능력의 비율을 증가시킴을 이끌었다. 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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| 논문제목 | 한글 : NGEF의 발암효과에 관한 연구<br>영문 : The study for the oncogenic effect of NGEF | | | | |

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 ( )**

2008 년 2 월 일

저작자: 김 홍 범(서명 또는 인)

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