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Astrocyte Elevated Gene (*AEG*)-1: recent insights into a novel gene involved in tumor progression, metastasis and neurodegeneration

Luni Emdad[1,2], **Devanand Sarkar**[1], **Zao-Zhong Su**[1], **Seok-Geun Lee**[1], **Dong-chul Kang**[4], **Jeffrey N. Bruce**[2], **David J. Volsky**[3,5], and **Paul B. Fisher**[1,2,3]

1*Department of Urology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, College of Physicians and Surgeons, New York, NY*

2*Department of Neurosurgery, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, College of Physicians and Surgeons, New York, NY*

3*Department of Pathology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, College of Physicians and Surgeons, New York, NY*

4*Ilsong Institute of Life Science, Hallym University, Republic of Korea*

5*St. Luke's Roosevelt Medical Center, New York, NY*

Abstract

Tumor progression and metastasis are complex processes involving intricate interplay among multiple gene products. Astrocyte Elevated Gene (*AEG*)-1 was cloned as an HIV-1- and tumor necrosis factor α (TNF-α)-inducible transcript in primary human fetal astrocytes by a rapid subtraction hybridization approach. *AEG*-1 downregulates the expression of the glutamate transporter EAAT2, thus it is implicated in glutamate-induced excitotoxic damage to neurons as evident in HIV-associated neurodegeneration. Interestingly, *AEG*-1 expression is elevated in subsets of breast cancer, glioblastoma multiforme and melanoma cells and *AEG*-1 cooperates with Ha-*ras* to augment the transformed phenotype of normal immortal cells. Moreover, *AEG*-1 is overexpressed in >95% of human malignant glioma samples when compared with normal human brain. Overexpression of *AEG*-1 increases and siRNA inhibition of *AEG*-1 decreases migration and invasion of human glioma cells, respectively. *AEG*-1 contains a lung-homing domain facilitating breast tumor metastasis to lungs. These findings indicate that *AEG*-1 might play a pivotal role in the pathogenesis, progression and metastasis of diverse cancers. Our recent observations indicate that *AEG*-1 exerts its effects by activating the NF-κB pathway and *AEG*-1 is a downstream target of Ha-*ras* and plays an important role in Ha-*ras*-mediated tumorigenesis. These provocative findings are intensifying interest in *AEG*-1 as a crucial regulator of tumor progression and metastasis and as a potential mediator of neurodegeneration. In this review, we discuss the cloning, structure and function (s) of *AEG*-1 and provide recent insights into the diverse actions and intriguing properties of this molecule.

Corresponding Author: Dr. Paul B. Fisher Professor Departments of Pathology and Urology Columbia University Medical Center College of Physicians and Surgeons 630 West 168th Street New York, NY 10032 Tel: (212) 305-3642; -3441; Fax: (212) 305- 8177; E-mail pbf1@columbia.edu

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1. Introduction

Cancer arises from a stepwise accumulation of genetic alterations that drive the progressive transformation of normal human cells ultimately culminating in highly malignant derivatives. This multi-step process results in alterations in cellular physiology that underlie malignant cell growth, e.g., self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion from apoptosis, unlimited replicative potential, and aberrant angiogenesis, as well as tissue invasion and metastasis (Fidler & Ellis, 1994;Hahn & Weinberg, 2002). The initiation and progression of solid tumors is in part dependent on a variety of signaling pathways, as are the processes that allow for invasion and metastasis. Cellular motility and extracellular matrix degradation are two major determinants of cancer cell invasion. Invasion through basement membrane from solid tumors to blood vessels and then to the secondary sites involves secretion of chemokines that help in tumor cell motility in a defined direction and proteolytic enzymes involved in extracellular matrix degradation, by the host as well as tumor cells (Liotta & Kohn, 2001).

Metastases arise following the spread of cancer cells from a primary site with the formation of new tumors in a distant organ. On the molecular level, the metastatic phenotype is orchestrated by the expression of homing receptors with associated signaling molecules, their ligands, and extracellular matrix-degrading proteases (Kurschat & Mauch, 2000). Metastasis consists of a series of sequential steps that include shedding of cells from a primary tumor into the circulation, survival of the cells in the circulation, arrest in a new organ, extravasations into the surrounding tissue, initiation and maintenance of growth, and vascularization of the metastatic tumor (Fig. 1). This process involves coordination of diverse signal-transduction pathways that allow cancer cells to proliferate, remodel their surrounding environment, invade to a distant site and reestablish the tumor. The precise molecular events leading to acquisition of the metastatic phenotype remain largely ambiguous.

We recently reported the cloning and functional characterization of an HIV-inducible gene, astrocyte elevated gene-1 (*AEG*-1), which is induced in primary human fetal astrocytes (PHFA) infected with HIV-1 or treated with recombinant HIV-1 envelope glycoprotein (gp120), or tumor necrosis factor-α (TNF-α) (Kang et al., 2005;Su et al., 2003a;Su et al., 2002). *AEG*-1 is a downstream target molecule of Ha-*ras* and c-*myc* mediating their tumor promoting effects (Lee et al., 2006). Intriguingly, *AEG-1* induces increased anchorage-independent growth and invasiveness of tumor cells and increased expression of adhesion molecules by activating the NF-κB pathway, and *AEG-1* can physically interact with the p65 subunit of NF-κB and modulate its function in the nucleus (Emdad et al., 2006). Moreover, *AEG-1* expression is elevated in diverse neoplastic conditions; it cooperates with Ha-*ras* to promote transformation; and its overexpression augments invasion of transformed cells demonstrating its functional involvement in Ha-*ras*-mediated tumorigenesis (Kang et al., 2005,Emdad et al., 2006;Lee et al., 2006). This review explores the multiple functions of *AEG*-1 including effects on oncogenic signaling pathways that are linked to an invasive, metastatic phenotype as well as neurodegeneration associated with brain tumor development and progression and HIV-1 infection.

2. Cloning of *AEG*-1

HIV-1 infection can impact on CNS functions resulting in neurodegeneration and HIV-1 associated dementia (HAD) (Lipton & Gendelman, 1995). The pathogenic events triggered by HIV-1 in the brain ultimately result in neuronal loss and CNS dysfunction. Neurons are rarely infected with HIV-1 *in vivo* and most evidence suggests that macrophages and microglial cells are the primary target cells for productive HIV-1 infection in the CNS (Lipton & Gendelman, 1995). Astrocytes also can be infected with HIV-1 *in vitro* and *in vivo*, although with lower efficiency than T cells and macrophages (Brack-Werner, 1999). There is general agreement, however, that HIV-1 can persist in astrocytes for prolonged periods in a low productive, non-cytolytic state, from which it can be induced by physiologic stimuli, such as TNF-α (Shahabuddin et al., 1992;Tornatore et al., 1991).

HIV-1 infection results in downregulation of the glutamate transporter, especially the astrocyte-specific Excitatory Amino Acid Transporter-2 (EAAT2), in astrocytes (Wang et al., 2003). Lack of EAAT2 precludes removal of glutamate from synaptic clefts and the accumulation of glutamate induces excitotoxicity and damage to neurons (Choi, 1988). Treatment of astrocytes with gp120, an HIV-1 envelope protein, alters cell physiology (Benos et al., 1994a;Benos et al., 1994b), including downregulation of glutamate transport (Wang et al., 2003), which is essential for normal neuronal functions, as indicated by increased D-aspartate efflux from astrocytes. Impairment of glutamate transport is also observed after incubation of human astrocytes with TNF-α (Fine et al., 1996) or co-cultivation with T cells infected with human T cell leukemia virus type I (HTLV-I) (Szymocha et al., 2000), and similar defects are found in feline astrocytes after infection with feline immunodeficiency virus (FIV) (Yu et al., 1998). More recent studies indicate that ligation of the HIV-1 coreceptor CXCR4 on astrocytes, by either stromal cell-derived factor 1 (SDF-1) or gp120 can stimulate a novel signaling pathway that involves Ca^{2+}-dependent release of glutamate (Sharma & Vijayaraghavan, 2001) in a process including activation of the CXCR4 receptor, an autocrine/paracrine TNF-α-dependent signaling, and prostaglandin production (Bezzi et al., 2001). These results suggest that HIV-1, gp120, and other neuropathogenic agents can alter specific signaling pathways in astrocytes in a way that may impair important physiological functions of these cells in neuronal signal transmission and response to brain injury.

Based on the profound alterations occurring in astrocyte physiology following HIV-1 infection, or treatment with gp120 and TNF-α, we assumed that modifications in gene expression would occur that correlate with and may be causative of these functional changes. Using a robust and selective rapid subtraction hybridization approach (RaSH) method developed in our laboratory (Boukerche et al., 2007;Jiang et al., 2000), we have addressed these possibilities (Su et al., 2002;Su et al., 2003b). Limited amounts of RNA and technical complexities are factors that can prevent the efficient use of classical subtraction hybridization approaches for identifying differentially expressed genes. The RaSH (Boukerche et al., 2007;Jiang et al., 2000) approach was able to ameliorate these problems in gene identification and cloning when employing subtraction hybridization. RaSH consists of library construction from double-stranded cDNAs that are enzymatically digested into small fragments, ligated to adapters and PCR amplified followed by incubation of tester and driver PCR fragments (Boukerche et al., 2007;Jiang et al., 2000). A schematic of this approach employed in early passage primary human fetal astrocytes (PHFA) and HIV-1 infection is outlined in Fig. 2. RaSH has been successfully used for identifying known and novel differentially expressed cDNAs, including genes related to terminal differentiation of cancer cells (Jiang et al., 2000;Kang et al., 2001), genes displaying elevated expression as a function of acquisition of enhanced metastatic ability (Bouckerche et al., 2004), genes displaying differential expression in HIV-1 resistant versus sensitive human T-cell clones (Simm et al., 2001), genes upregulated as a function of infection of PHFA with HIV-1 or treatment with gp120 (astrocyte elevated genes, *AEGs*) and genes downregulated as

a function of infection of PHFA with HIV-1 or treatment with gp120 (astrocyte suppressed genes, *ASGs*) (Kang et al., 2005;Su et al., 2003b;Su et al., 2002).

To avoid cloning genes displaying normal cell cycle fluctuations in astrocytes, RNAs were isolated and pooled from 6, 12 and 24 hrs and 3 and 7 days uninfected and infected cells and were used to produce temporal cDNA libraries for RaSH. By subtracting temporal cDNAs derived from uninfected PHFA from temporal cDNAs (Su et al., 2003b;Su et al., 2002) derived from HIV-1 infected cells, a series of genes displaying elevated expression in virus infected cells, astrocyte elevated genes (*AEGs*), were identified. Both known and novel *AEGs* (total of 15), at the time of initial identification and isolation, including *AEG*-1, were identified that displayed early or late expression kinetics following HIV-1 infection or treatment with gp120. The comparable pattern of gene expression changes following HIV-1 infection or gp120 treatment suggested that HIV-1 exposure of astrocytes, even in the absence of productive infection, could induce profound changes in cellular gene expression and physiology.

The full-length cDNA of the novel *AEG*-1 gene was obtained using a novel modified rapid amplification of cDNA ends (RACE) approach called C-ORF (complete open reading frame technology) and bioinformatics (Kang & Fisher, 2007;Kang & Fisher, 2005;Kang et al., 2005). This cloning has permitted a detailed analysis of the functional properties of this intriguing gene providing new insights into the processes of cancer development, progression to metastasis and neurodegeneration.

3. Structure and localization of *AEG*-1

The full-length *AEG*-1 cDNA consists of 3611-bp, excluding the poly-A tail (Kang et al., 2005). The open reading frame (ORF) from 220- to 1968-nts encodes a putative 582 amino acid protein with a calculated molecular mass of 64-kDa with a pI of 9.33. Genomic blast search demonstrated that the *AEG*-1 gene consists of 12 exons/11 introns and is located at 8q22 where cytogenetic analysis of human gliomas indicated recurrent amplifications. Protein motif analysis, such as SMART, predicted a single transmembrane domain at the N-terminus of the protein (amino acids 50-70) that includes putative dileucine repeats involved in protein trafficking, which was supported by three independent transmembrane protein prediction methods (PSORT II,TMpred, and HMMTOP). However, PSORT II and TMpred predicted *AEG*-1 as a type Ib protein (C-terminal inside), whereas TMHMM and TopPred 2 predicted a type II protein (C-terminal outside). Analysis of the amino acid sequence of *AEG*-1 further revealed the presence of putative, either monopartite or bipartite, nuclear localization signals (NLS) between amino acids 79 to 91, 432 to 451 and 561 to 580 (Fig. 3A) suggesting import into the nucleus. The existence of a cleavable signal peptide was not evident based on the motif analyses.

Intracellular localization of *AEG*-1 was examined in various cell types, using a chicken polyclonal anti-AEG-1 antibody and immunofluorescence microscopy. Fig. 3B shows endogenous *AEG*-1 staining in immortalized primary human fetal astrocytes (IM-PHFA), in which AEG-1 was detected at the perinuclear region and in endoplasmic reticulum (ER)-like structures, but not at the plasma membrane, and co-localized with the ER-specific protein calreticulin, but not with the mitochondrial marker Mito-Tracker. The observed ER localization of AEG-1 favors type Ib membrane topology of the AEG-1 protein. Our recent observations, however, reveal that TNF-α treatment, as well as forced overexpression of AEG-1, which upregulates AEG-1 expression, resulted in the localization of the protein both in the cytoplasm and in the nucleus in HeLa cells (Emdad et al., 2006). This finding is analogous to the nuclear receptor co-activator SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) that is located mainly in the cytoplasm, translocates from the cytoplasm to the nucleus on TNF-α activation (Wu et al., 2002).

A separate research group cloned mouse *AEG*-1 as metadherin from 4T1 mouse breast carcinoma cells and the cloning of the mouse (3D3) and rat (lyric) homologues of *AEG*-1 have been reported (Brown & Ruoslahti, 2004;Sutherland et al., 2004). Mouse AEG-1 protein, containing 579 amino acids, is 3 amino acids shorter than human AEG-1. There are discrepancies among the *AEG*-1/metadherin/3D3 reports with respect to its localization. According to Sutherland et al. (2004) Western blot analysis of HeLa cells revealed that most isoforms of 3D3/lyric were found predominantly in the nuclear pellet, although some higher molecular weight isoforms were also located in the cytoplasmic and soluble nuclear fractions. They confirmed the subcellular localization of endogenous 3D3/lyric by immunostaining various human and mouse cell lines, which showed perinuclear and cytoplasmic staining as well as some nuclear rim and general nuclear diffuse staining (Sutherland et al., 2004). Brown and Ruoslahti (2004) detected Metadherin staining throughout the cytoplasm in fixed and permeabilized 4T1 cells by using a rabbit antibody raised against the lung–homing domain of metadherin. Antibody-assisted FACS analysis suggests that metadherin has type II membrane topology (Brown & Ruoslahti, 2004). However, 3D3/lyric and AEG-1 suggest type Ib topology based on ER location, detected by immunofluorescent microscopy, and clusters of basic amino acids at the C-terminal juxtaposition of its transmembrane domain. Sequence motif analysis equivocally supports both topologies, depending on the algorithm employed. For that reason, the exact localization and membrane topology of *AEG*-1, 3D3/lyric and metadherin and the functional significance of this localization require further clarification.

4. Functional studies of *AEG*-1

4.1. *AEG*-1 in tumor progression and metastasis

The accumulation of mutations during carcinogenesis results in six essential alterations in cell physiology that collectively dictate malignant growth: self sufficiency in cell growth, insensitivity to growth-inhibitory signals, evasion of cell death, limitless replicative proliferation, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Most of these alterations affect cell signaling pathways, and the majority of oncogenes are integral components of cellular signaling circuits, which are up-regulated or constitutively activated in malignant cells. Recent studies highlight a potential role of *AEG*-1 in promoting tumor progression and metastasis (Brown & Ruoslahti, 2004;Emdad et al., 2006;Kang et al., 2005). The current data suggests that *AEG*-1 is capable of promoting several aspects of tumor progression and metastasis. *AEG*-1 regulates several hallmarks of metastatic cancers (Fidler, 1991), including aberrant proliferation, increased adhesion and invasiveness and resistance to apoptosis under certain stress-full condition. Elevated levels of *AEG*-1 are detected in subsets of breast cancer, glioblastoma multiforme and melanoma cells (Fig 4A) and ectopic overexpression of *AEG-1* promotes colony-forming ability of immortalized melanocytes and astrocytes in soft agar. *AEG*-1 also cooperates with Ha-*ras* to augment the transformed phenotype of normal immortal cells (Fig. 4B). Ectopic expression of *AEG*-1 promotes anchorage-independent growth of normal immortal human melanocytes (FM516-SV) that do not normally form colonies in soft agar. Although expression of the T24-Ha-*ras* oncogene in FM516-SV cells promotes a higher rate in agar growth, co-expression of *AEG*-1 and Ha-*ras* in FM516-SV cells results in a synergistic increase in colony formation in soft agar (Fig. 4B). Expression levels of Ha-*ras* positively correlate with the agar cloning efficiency of AEG-1/Ha-*ras* stable transfectants, suggesting a complementing role of AEG-1 with Ha-*ras* in development of the cancerous phenotype. A correlation of *AEG*-1 upregulation with enhanced soft agar colony forming ability is also evident in adult human astrocytes transformed by sequential expression of SV40 *T* antigen, hTERT and Ha-*ras* (THR) (Lee et al., 2006;Rich et al., 2001). Increased expression of *AEG*-1 in THR cells indirectly supports tumor-forming properties of *AEG*-1 in glial tumor cells. Of potential clinical relevance, significantly increased *AEG*-1 expression is detected in tissue samples by immunofluorescence studies and Western

blot analyses from diverse human brain tumors including highly invasive glioblastoma multiforme, anaplastic astrocytoma and aggressive meningiomas in comparison to that from normal human brain (unpublished observations).

Experiments have documented that *AEG*-1 increases anchorage-independent growth and invasiveness of HeLa cells by activating the NF-κB pathway (Emdad et al., 2006). Overexpression of *AEG*-1 increases and siRNA inhibition of *AEG*-1 decreases migration and invasion of human glioma cells, respectively. In addition, *AEG*-1 expression is significantly induced by oncogenic Ha-*ras* through the PI3K/AKT/GSK3β/c-Myc signaling pathway (Lee et al., 2006) and *AEG*-1 siRNA inhibits the colony formation activity of oncogenic Ha-*ras* (Fig. 4C). Rat lyric is an overexpressed gene in rat liver and colon tumors (Sutherland et al., 2004). Human Metadherin is overexpressed in cultured breast cancer cells and tissue sections from breast adenocarcinoma, and contains a lung-homing domain that facilitates metastasis of breast cancer cells to the lung. Antibodies reactive to the lung-homing domain of AEG-1/Metadherin and siRNA mediated knock down of AEG-1/Metadherin in breast cancer cells inhibiting experimental lung metastasis, indicating that tumor cell AEG-1/Metadherin mediates localization at the metastatic site (Brown & Ruoslahti, 2004). In preliminary studies, overexpression of AEG-1 in immortalized human melanocyte (FM516-SV) cells results in augmented adherence to sections of lung tissue (unpublished data).

An NF-κB gene array revealed that ectopic overexpression of *AEG*-1 results in marked upregulation NF-κB-responsive cell adhesion molecules (ICAM-2 and I-CAM-3, selectin E, selectin L, and selectin P ligand), TLR4 and TLR5, and cytokines such as IL-8 (Fig. 4D), uncovering a potential mechanism of *AEG*-1-mediated enhanced lung metastasis of breast cancer cells and increased adhesion of immortalized melanocytes to lung endothelial cells. Taken together, these findings indicate that in both human and rat; *AEG*-1 is involved in promoting tumor progression and metastasis.

Although a definitive link between HIV infection and malignant glioma is lacking, one report indicates that in a series of 8 HIV positive patients with brain malignancies, five had glioblastoma multiforme. Glioblastoma developed in these five patients >6-years after diagnosis of HIV infection suggesting that HIV-infected patients may be at risk for intracranial neoplasms other than primary central nervous system lymphoma (Blumenthal et al., 1999). In addition, an interesting connection between these two apparently independent pathological conditions is found in elevated expression of matrix metalloproteinase (MMPs). HAD associates with increased MMP-2, 7 and 9 levels which are also observed in highly metastasizing and rapidly expanding tumors and HIV-1 Tat-associated neuropathy is inhibited by MMP inhibitors (TIMP) (Conant et al., 1999;Johnson et al., 2001;Sporer et al., 1998). Accordingly, in addition to downmodulation of EAAT2 expression that results in glutamate excitotoxicity, the elevated expression of *AEG*-1 in HIV-infected astrocytes might contribute to HIV-1-associated neuropathy by up-regulating MMPs that are essential in tumor expansion. Indeed, our initial experiments suggest that downregulation of *AEG*-1 by siRNA targeting *AEG*-1, significantly inhibits MMP-2 and MMP-9 promoter activity in rat glioma cells (unpublished observation). Thus, the elevated expression of AEG-1 in HIV-infected astrocytes might contribute to HIV-1-associated neuropathy and malignant transformation of astrocytes.

4.2. *AEG*-1 and the NF-κB pathway

Cancer is a hyperproliferative disorder that results from tumor initiation and promotion, which in certain contexts can lead to acquisition of metastastic potential by an evolving tumor cell. The transcription factor nuclear factor kappa B (NF-κB) can affect oncogenesis by virtue of its capacity to regulate the expression of a plethora of genes that modulate apoptosis and cell survival as well as proliferation, inflammation, cell adhesion, tumor metastasis and angiogenesis (Baldwin, 2001a;Baldwin, 2001b;Kabrun & Enrietto, 1994). NF-κB regulates

the expression of various molecules such as cell adhesion proteins, matrix metalloproteinase, cycloxygenase 2, inducible nitric oxide synthase, chemokines, and inflammatory cytokines, all of which promote tumor cell invasion and angiogenesis (Baldwin, 1996;Mettouchi et al., 1997). The ability of NF-κB to suppress apoptosis and to induce expression of proto-oncogenes such as c-*myc* and cyclin D1, which directly stimulate proliferation, suggests that NF-κB participates in many aspects of oncogenesis (Guttridge et al., 1999;Hinz et al., 1999). The earliest evidence for a role for NF-κB in oncogenic transformation was derived from the fact that v-Rel, a highly oncogenic retroviral homologue of c-Rel, causes carcinogenesis in avian lymphoid cells (Gilmore et al., 1996). Later studies suggested that v-Rel also has the capacity to transform mammalian cells *in vivo* (Carrasco et al., 1996). Transgenic mice expressing v-Rel under the control of the T-cell specific lck promoter develop T-cell lymphomas. Inhibition of NF-κB by overexpression of a degradation-resistant IκBα delays the development of T-cell lymphomas and prolongs the survival of v-Rel transgenic mice (Carrasco et al., 1996). Indeed, constitutive NF-κB activity has been observed in several different tumor cell types, including T- or B-lymphocyte leukemia, lymphoma, myeloma, melanoma, prostate, colon, breast, thyroid, pancreas, glioma, and head and neck squamous cell carcinoma cell lines, as well as in samples obtained from cancer patients, and the inhibition of NF-κB abrogates tumor cell proliferation (Bargou et al., 1997;Bours et al., 1994;Devalaraja et al., 1999;Gilmore et al., 1996;Mori et al., 1999;Mukhopadhyay et al., 2001;Visconti et al., 1997;Wang et al., 1999).

Our recent observations indicate that, *AEG*-1 facilitates IκBα degradation, resulting in an increase in NF-κB DNA binding activity and NF-κB promoter activity in reporter assays (Fig. 5A, and 5B). Additionally we show that, inhibition of NF-κB by IκBα-mt32 (using an adenovirus expressing the mt32IκBα superrepressor, which inhibits IκBα degradation and subsequent NF-κB nuclear translocation) significantly inhibits *AEG*-1-induced augmentation in soft agar colony formation and invasion of HeLa cells (Fig. 5C and 5D). *AEG*-1 can physically interact with the p65 component of NF-κB and modulate its function in the nucleus (Emdad et al., 2006). Taken together these data indicate that *AEG*-1 is decisively involved as a positive regulator of NF-κB–induced gene expression, and this activation provides a molecular basis for the ability of *AEG*-1 to promote anchorage-independent growth and invasion, prominent contributors to cancer progression.

4.3. *AEG*-1 and Ha-*ras*

The aberrant activation of *RAS* proteins has been implicated in facilitating virtually all aspects of the malignant phenotype of the cancer cell, including cellular proliferation, transformation, invasion and metastasis (Malumbres & Barbacid, 2001). Ras interacts with and regulates multiple downstream effectors that stimulate diverse cytoplasmic signaling activities (Feig & Buchsbaum, 2002;Shields et al., 2000). The most widely studied effectors of Ras signaling are the Raf serine/threonine kinases (c-Raf-1, A-Raf, B-Raf) (Chong et al., 2003). Ras promotes Raf association with the plasma membrane, where other events facilitate Raf activation. Raf then phosphorylates and activates the MEK1 and MEK2 dual specificity kinases. MEK1/2 are kinases for ERK1/2 mitogen-activated protein kinases (MAPKs). Activated MAPKs translocate to the nucleus where they regulate gene expression by modulating transcription factors, including those of the Ets family (Chambers & Tuck, 1993;Seth et al., 1992;Yordy & Muise-Helmericks, 2000).

Another commonly studied signal transduction route downstream of oncogenic Ras activation is the PI3K pathway (Rodriguez-Viciana et al., 1994;Rodriguez-Viciana et al., 1997). This kinase phosphorylates the signaling molecule phosphatidylinositol 4,5-biphosphate to form phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 can then activate the serine/threonine kinase Akt/PKB. Ras-dependent stimulation of Akt activation leads to upregulation of the

transcription factor NF-κB, and can increase cell survival, perhaps by blunting apoptotic signaling (Mayo et al., 1997).

Besides classical protein kinase cascades as Ras effector pathways in tumorigenesis, recent publications point towards an important synergistic participation of Rho GTPases, especially Rac1, RhoA and cdc42, in the cellular transformation of epithelial cells by oncogenic Ras (Sahai et al., 2001;Shields et al., 2000). Rho GTPases are mainly involved in regulating actin cytoskeleton organization and cell cycle progression and gene transcription (Etienne-Manneville & Hall, 2002;Ridley, 2001). Ras signaling also involves Rac/JNK and Rac/p38 MAPK pathways, both of which appear to be involved in cell stress responses, growth inhibition, and apoptotic signaling (Coso et al., 1995;Minden et al., 1995;Xia et al., 1995). Activation of Ras signaling pathways is essential for cells to leave a quiescent state and pass through the G_1 phase of the cell cycle (Peeper et al., 1997). Under normal conditions the action of Ras and other members of the Ras pathway are stringently regulated during the cell cycle and under different growth conditions (Macaluso et al., 2002).

Amplification of *ras* proto-oncogenes and activating mutations that lead to the expression of constitutively active Ras proteins are observed in approximately 30% of all human cancers (Bos, 1989;Flotho et al., 1999;Stirewalt et al., 2001). In a tumor cell, the oncogenic activation of *ras* is a consequence of point mutations that either impair GTPase activity or enhance GTP binding affinity, resulting in a highly active proliferative signal (White et al., 1995). The effects of Ras on proliferation and tumorigenesis have been documented in immortal cell lines (Chang et al., 2003). However, antiproliferative responses of oncogenic Ras have also been observed in nontransformed cells including primary rat Schwann cells and primary fibroblast cells of human and murine origins (Bar-Sagi & Feramisco, 1985;Benito et al., 1991;Hirakawa & Ruley, 1988;Malumbres et al., 2000). Consequently, aberrant Ras signaling represents a nodal pathway regulating tumor cell growth and provides a potential target for cancer therapy.

Our recent study indicates that *AEG*-1 is a downstream target gene of Ha-*ras* and Ha-*ras*-mediated *AEG*-1 induction is regulated mainly at the transcriptional level rather than by modulating protein stability. Transfection with *AEG*-1 siRNA results in a significant reduction in colony formation of THR cells (Fig. 4C), indicating that *AEG*-1 plays an essential role as a downstream target gene in oncogenic Ha-*ras*-mediated proliferation and transforming activities. We further documented that Ha-*ras* mediates *AEG*-1 induction through the PI3K/GSK3β/c-Myc signaling pathway (Lee et al., 2006). Accordingly, Myc, but not USF-1, protein levels were higher in THR cells than in THV cells and LY294002, a PI3K inhibitor, reduced Myc protein levels in THR cells suggesting a correlation between GSK3β phosphorylation by Ha-*ras*-mediated AKT activation and Myc expression in THR cells (Lee et al., 2006). Reduction of *AEG*-1 expression by LY294002 was confirmed at the protein level as well as the transcription level. siRNA targeting c-*myc* significantly decreased *AEG*-1 protein expression further supporting a functional role of c-*myc* in Ha-*ras*-mediated *AEG*-1 expression.

PI3K-AKT signals control several growth-regulatory transcription factors. Two prominent examples are the forkhead box (FOXO) protein and NF-κB. Other transcriptional regulators whose activities are affected by PI3K-AKT signaling include MIZ-1, p53, AP-1, c-Myc, β-catenin and HIF1α (Bader et al., 2005). The exact roles of these proteins during PI3K-mediated oncogenesis are currently unknown, but they have all been linked to oncogenic transformation. AKT phosphorylates and thereby inactivates the cell-cycle inhibitor MIZ1 and also suppresses p53 activity by a mechanism that involves MDM2. By contrast, the activity of AP-1, c-Myc and β-catenin are increased by AKT. These targets are negatively controlled by GSK3β, which is inactivated by AKT-mediated phosphorylation (Bader et al., 2005). *In vitro* studies demonstrated that GSK3β could phosphorylate serine residues 38, 42, and 62, and threonine residue 58 within the transactivation domain of Myc, that are considered important for

regulation of Myc transcriptional activity *in vivo* (Grimes & Jope, 2001;Henriksson et al., 1993;Pulverer et al., 1994;Saksela et al., 1992). Phosphorylation of Thr-58 and Ser-62 by GSK3β negatively regulate Myc function, resulting in decreased cellular growth and transformation. In human neuroblastoma SH-SY5Y cells, inhibition of GSK3β by lithium causes increased Myc nuclear protein levels (Grimes & Jope, 2001). Our recent study confirms that PI3K signaling in THR cells phosphorylates AKT and GSK3β, which activates c-Myc, but not USF-1, resulting in increased *AEG*-1 expression (Lee et al., 2006). We also observed that *AEG*-1 expression was mildly elevated by treatment with the MEK inhibitor PD98059 and siRNA of MEK2. Recent studies demonstrate that blockade of ERK1/2 with PD98059 cause induction of specific genes, such as IL-6, CYP1A1 and NF-κB, even though other downstream Ras signaling pathways induce their activity (de Haij et al., 2005;Janssen-Heininger et al., 1999;Shin et al., 2005).

The complex relationships among diverse extracellular conditions and cellular responses to a distinct stimulus have only recently begun to be elucidated at the mechanistic level. It will be very interesting to delineate the potential cross-talk relationship between the PI3K and MEK signaling pathways in regulating Ha-*ras*-mediated *AEG*-1 expression. Our findings elucidate a novel mechanism of Ha-*ras*-mediated tumorigenesis and delineate a crucial role of *AEG*-1 in promoting cancer development and/or maintenance (Lee et al., 2006).

4.4. *AEG*-1- and HIV-induced neurodegeneration

Glutamate is the main excitatory amino acid transmitter in the mammalian central nervous system and is probably involved in most aspects of normal brain function including cognition, memory and learning (Fonnum, 1984;Headley & Grillner, 1990). Glutamate acts at ionotropic and metabotropic receptors, the primary ionotropic receptor classes being NMDA and AMPA/kainite (Pitt et al., 2000). Extracellular glutamate levels are tightly regulated by transporters; there are distinct classes of transporters on neurons and on astrocytes but the bulk of glutamate uptake appears to be mediated by astrocytes (Anderson & Swanson, 2000). Recent research suggests that astrocytes play a critical role in neuronal signal transmission by enhancing synaptic activity and strength, increasing the number of synapses, and modulating neuronal activity (Beattie et al., 2002;Oliet et al., 2001;Ullian et al., 2001). Excessive glutamate exposure is toxic to neurons, probably resulting in large part from glutamate-triggered Ca^{2+} entry (Choi, 1988). Because glutamate is not metabolized in the extracellular environment, the maintenance of normal glutamatergic neurotransmission or the prevention of glutamate-induced neurodegenerative disorders depend on the presence of active glutamate transport systems in glial cells and neurons (Perego et al., 2000).

Five different isoforms of glutamate transporters (excitatory amino acid carriers) have now been identified and cloned: GLAST (EAAT1), GLT-1 (EAAT2), EAAC-1 (EAAT3), EAAT4, and EAAT5 (Arriza et al., 1994;Fairman et al., 1995;Kanai & Hediger, 1992;Pines et al., 1992;Storck et al., 1992). Immunolocalization studies have revealed that EAAT2 and EAAT1 are primarily expressed in astroglial cells, whereas EAAC1 and EAAT4 are present primarily in neurons (Chaudhry et al., 1995;Fairman et al., 1995;Furuta et al., 1997;Lehre & Danbolt, 1998;Pines et al., 1992). Tanaka et al have demonstrated that the astroglial EAAT2 transporter is responsible for the majority of the clearance of glutamate from neuronal synapses in the central nervous system (Tanaka et al., 1997). Impaired glutamate uptake by glial cells can result in cell death from excessive levels of glutamate and overstimulation of glutamate receptors (Choi, 1988). Indeed, downregulation of EAAT2 followed by accumulation of glutamate in the ECF and neuronal death have been documented in a wide variety of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, several forms of epilepsy, ischemia/stroke, HIV-1-associated dementia (HAD), traumatic brain injury and hepatic encephalopathy (Choi, 1988;Doble, 1999;Kaul et al., 2001;Masliah et al., 1996). The

importance of regulating glutamate transport is further underscored by the observation that mice lacking EAAT2 develop progressive neurodegeneration and epilepsy as a result of aberrant glutamate homeostasis (Rothstein et al., 1996;Tanaka et al., 1997).

Dementia is a major complication of HIV-1 infection (Lipton & Gendelman, 1995;Navia & Price, 1987). The incidence of HAD declined after the introduction of highly active antiretroviral therapies, but it still reaches 10% (Sacktor et al., 2001). The pathological hallmarks of HAD include neuronal loss, reactive gliosis, and white matter pallor (Budka, 1991;Everall et al., 1991;Sharer et al., 1986). HIV-1 infects infiltrating macrophages, microglial cells, and astrocytes in the brain but it is rarely found in neurons (Koenig et al., 1986;Saito et al., 1994;Wiley et al., 1986), suggesting indirect viral action in neuronal cell loss. HIV-1 infection of PHFA results in downregulation of both EAAT2 mRNA and protein and also glutamate uptake in these cells (Wang et al., 2003). In a similar manner, treatment with TNF-α, which might be secreted from HIV-infected macrophages, downregulates both EAAT2 and EAAT1 mRNA and protein and also glutamate uptake in PHFA suggesting an involvement of glutamate excitotoxicity in the pathogenesis of HAD (Su et al., 2003c;Wang et al., 2003).

Neoplastic transformation of human astrocytes causes malignant gliomas, which are often associated with seizures and neuronal necrosis (Ye et al., 1999). It has been shown that Na^+-dependent glutamate uptake in glioma cell lines derived from human tumors is up to 100-fold lower than in astrocytes (Ye et al., 1999). Immunohistochemical studies document very low expression levels of EAAT2 both in glioma cell lines as well as in brain tissues from glioblastoma patients (Ye et al., 1999). This leads to excessive glutamate release from glioma cells in peritumoral brain tissue that may contribute to tumor-associated necrosis and possibly to seizures.

Since it has an enormous role in maintaining normal brain function, numerous studies have been performed to understand the regulatory mechanisms underlying the expression pattern of EAAT2 (Collier, 1998;Gegelashvili et al., 1997;Miralles et al., 2001;Mitrovic et al., 1998;Munch et al., 2000;Palmada et al., 2002;Robinson, 1998;Vandenberg et al., 1997;Zelenaia et al., 2000). Expressions of both the mRNA and protein have been shown to be downregulated by TNF-α and upregulated by TGFα, EGF and cAMP (Gegelashvili et al., 1997;Schlag et al., 1998;Zelenaia et al., 2000). However, how the gene was transcriptionally controlled had not been elucidated because of lack of information about the genomic organization of the gene. Previous attempts to identify the promoter region of the EAAT2 gene were not fruitful because of the presence of an extremely large intron (approximately 100kb) that separates exon 1 from exon 2 and incomplete sequence information for exon 1. We successfully cloned the EAAT2 promoter (EAAT2-promoter) using a sequential progressive genomic scanning (SPGS) approach (Su et al., 2003c). The EAAT2 promoter functions primarily in astrocytes (PHFA) and its activity is modulated by those agents that regulate expression of EAAT2 mRNA and protein (Su et al., 2003c).

Sitcheran et al. (2005) have shown that both TNF-α-mediated repression and EGF-mediated activation of EAAT2 expression require NF-κB, though they utilize distinct signaling pathways. TNF-α induces the classical IκBα degradation pathway to trigger NF-κB nuclear translocation and DNA binding to repress EAAT2 expression. In contrast, EGF activates EAAT2 expression independently of IκBα degradation and NF-κB nuclear translocation. HIV-1 infection downregulates EAAT2-promoter activity (Su et al., 2003c). TNF-α plays an important role in the pathogenesis of HAD. Exogenous TNF-α inhibits glutamate uptake through a reduction in Vmax for glutamate transport and activation of endogenous TNF-α by ligation of CXCR4 on astrocytes induces glutamate release (Bezzi et al., 2001). However, Wang et al. (2003) recently observed no TNF-α production following HIV-1 infection or gp120 treatment of PHFA under conditions where EAAT2 function and transcription are inhibited.

TNF-α requires 24 hr to achieve maximal inhibition of glutamate uptake and it affects both EAAT1 and EAAT2 expression. HIV-1, in contrast, achieves maximal inhibition 6 hr after infection and its effects were mediated predominantly by changes in EAAT2 expression.

We recently discovered that infection of PHFA with HIV-1, treatment with gp120 or TNF-α resulted in elevated *AEG*-1 expression and decreased EAAT2 expression (Kang et al., 2005;Su et al., 2003a). This inverse correlation of *AEG*-1 and EAAT2 expression by HIV infection and *AEG*-1 downregulation of the EAAT2 promoter indicate a potential contribution of *AEG*-1 to the generation of HAD. The direct effect of *AEG*-1 expression on EAAT2 promoter activity was analyzed by transient co-transfection of an *AEG*-1 expression vector with a luciferase reporter linked to the EAAT2-promoter (Fig. 6A). Ectopic over-expression of *AEG*-1 specifically inhibited EAAT2 promoter activity (~3.5 fold) in PHFA, while EAAT1 promoter activity was not affected by *AEG*-1. *AEG*-1 downregulates the EAAT2 promoter activity in IM-PHFA (h-TERT immortalized PHFA) and to a similar extent in PHFA, but not in H4 astroglioma cells (Fig. 6B). In contrast, expression of *PTEN* that suppresses the PI–3K signaling pathway, effectively inhibited EAAT2-promoter activity in all three-cell types (Fig. 6B). Activation of the PI–3K–Akt signaling pathway was required for upregulation of EAAT2 promoter activity by EGF and cAMP (Su et al., 2003c). In this context, high EAAT2 expression in H4 cells might result from constitutive activation of the PI–3K–Akt pathway (Knobbe et al., 2002). Taken together, *AEG*-1 expression induced after 3 and 7 days following HIV-1 infection or treatment with TNF-α or gp120 (Su et al., 2002) in PHFA may directly contribute to EAAT2 downregulation through a PI3K–Akt-independent pathway. Analysis of *AEG*-1 expression in other neurodegenerative diseases will provide a global perspective of the role of *AEG*-1 in the molecular pathogenesis of these disorders.

In the glioma model, promotion of tumor formation might be ascribed to *AEG*-1-mediated EAAT2 downmodulation. Growth of the glioma mass is restricted by limited intracranial space and neighboring tissue. Glutamate excitotoxicity to neighboring neurons resulting from active release of glutamate or by reduction of glutamate transporter activity and subsequent glutamate uptake is known to promote malignant glioma pathogenesis (Sontheimer, 2003;Ye et al., 1999). In fact, EAAT2 promoter activity in glioma cells, except H4, is lower by an order of magnitude than in PHFA and several-fold lower than in IM-PHFA (Kang et al., 2005) and our recent evidence indicates that *AEG*-1 expression is significantly upregulated in malignant glioma cells as well as in tumor samples (unpublished data). It is conceivable that an impairment of glutamate transporter regulation by *AEG*-1 may contribute to distinct biological properties of astrocytic tumors such as edema, necrosis and tumor-related seizures.

4.5. *AEG*-1 promoter analysis

Genomic BLAST search indicates that human *AEG*-1 (accession no. AF411226) consists of 12 exons and 11 introns that extend over a region of ~82-kb and is located at chromosome 8q22.1. We isolated the 5′ upstream region of *AEG*-1, employing a PCR technique, using an anti-sense primer in exon 1 of the *AEG*-1 gene and a sense primer in the 5′ genomic sequence with genomic DNA isolated from primary human fetal astrocytes as a template. The resulting 2,759-bp fragment was cloned into the luciferase reporter plasmid pGL3-Basic (pGL3-AEG promoter). Employing primer extension analysis using an antisense oligonucleotide derived from the first exon of *AEG*-1, four transcription initiation sites were identified (Lee et al., 2006). Bioinformatics analysis revealed that the human *AEG*-1 promoter (−2710/+49) lacks consensus TATA and CAAT boxes, but contains multiple Sp1 binding motifs and high GC content. The presence of multiple transcription initiation sites, multiple Sp1 sites and high GC content has also been recognized in many other TATA-less promoters. Ha-*ras* overexpression results in an ~4-fold increase in human *AEG*-1 promoter activity (Lee et al., 2006). Similarly, *AEG*-1 promoter activity is significantly higher in Ha-*ras*-stably transfected cells such as THR

and CREF-*ras* cells, indicating that the *AEG*-1 promoter has a significant transcriptional response to the activated Ha-*ras* pathway (Lee et al., 2006).

To determine the intracellular signaling pathway by which Ha-*ras* induces *AEG-1* promoter activity, a PI3K inhibitor LY294002 and a MEK inhibitor PD98059 were used. The addition of LY294002 but not PD98059, significantly attenuated Ha-*ras*-mediated *AEG*-1 promoter activation in THR cells with little change in basal *AEG*-1 promoter activity in THV cells (Lee et al., 2006). *PTEN* is a phosphatase that selectively dephosphorylates the 3 positions of both phophatidylinositol-3,4,5-triphosphate and phophatidylinositol-4,5-bisphosphate, antagonizing the diverse downstream signaling effector pathways activated by PI3K-derived phospholipids (Bader et al., 2005;Sulis & Parsons, 2003). Co-transfection of a *PTEN* expression plasmid with pGL3-AEG1-promoter also significantly attenuated Ha-*ras*-induced *AEG-1* promoter activity in THR cells without affecting the basal promoter activity in THV cells (Lee et al., 2006). As observed with PD98059, siRNA for MEK1 and MEK2 showed no inhibitory effect on Ha-*ras*-induced *AEG*-1 promoter activity, indicating that the PI3K signaling pathway is involved in Ha-*ras*-mediated *AEG*-1 promoter activation.

We also determined in more detail the *cis*-elements in the *AEG*-1 promoter essential for response to Ha-*ras*. Serial deletions from −2710-bp to −459-bp showed an ∼3-fold increase in promoter activity in both THV and THR cell lines, indicating the presence of negative transcriptional control elements in this region (−2710/−459) (Lee et al., 2006). Deletion from −459 to −301-bp resulted in a 90% loss of basal as well as Ha-*ras*-induced promoter activity in THV and THR cells, suggesting that transcription factors binding to this region (−459/−301) are capable of regulating both basal as well as Ha-*ras*-induced *AEG*-1 promoter activity.

Further experiments identified the transcription factors binding to the −459/−301 region as critical elements in regulating Ha-*ras*-induced *AEG*-1 promoter activity. Employing electrophoretic mobility shift assays we demonstrated that the region −356/−302 of the *AEG*-1 promoter binds to Ha-*ras*-activated transcription factors. This region contains several putative transcription factor binding sites, including two E-box elements to which the bHLH proteins such as c-Myc and USF-1 bind. Indeed, activation of two E-box elements in the *AEG*-1 promoter by increased c-Myc binding was shown to be critical for the Ha-*ras*-mediated *AEG*-1 induction (Lee et al., 2006).

A number of genes involved in tumor progression and metastasis, such as osteopontin, cdc2, connexin 43 and MMP-9, show augmented expression by the cooperative action of Ras and c-Myc (Born et al., 1994;Carystinos et al., 2003;Denhardt et al., 2003;Himelstein et al., 1997;Leone et al., 1997). However, as yet no study has demonstrated that the induction of a *ras*-responsive gene is mediated by direct binding of c-Myc to the gene promoter. In these contexts, our studies add a new dimension to the molecular circuitry in the *ras-c-myc* axis. We also confirmed that the human *AEG*-1 promoter has both positive (459/−302) and negative regulatory regions (−738/−460). Although the positive regulatory region contains several putative transcription factor binding sites critical for basal promoter activity, such as Sp1, E-box element, CREB and Ets-2 (Ghosh et al., 2005;Kim et al., 2003;O'Leary & Kasper, 2000;Ye et al., 1993), our data indicate that two E-box elements in this region are functional and important for both basal and *ras*-induced promoter activity. The negative regulatory region has putative RAR-α and YY1 binding sites that have been shown to act predominantly as a repressor of transcription (Gordon et al., 2006;Schule et al., 1991;Sucharov et al., 2004). Current studies are in progress to elucidate the involvement of these transcription factors in mediating transcriptional repression of *AEG-1*.

5. Conclusion and future perspectives

An enhanced understanding of the regulators of common signaling pathways involved in tumor progression and metastasis will pave the way for the development of more potent and selective inhibitors of neoplasia. This should be a useful adjunct to conventional therapies, potentially permitting interference with tumor progression at several nodal points in this process. This review highlights recent studies on *AEG*-1, which strongly support a central role of *AEG*-1 in tumor progression and metastasis through the regulation of highly integrated signaling events (Fig. 7). There are many questions that remain regarding the cellular function(s) of *AEG*-1 and how it influences metastatic progression. Although *AEG*-1 interacts with the p65 component of NF-κB, it remains to be determined how this interaction affects the function of interacting partners and whether this interaction is relevant to tumor progression. Further studies are also necessary to determine if *AEG*-1 alters oncogenic activation of specific signaling pathways that results in differential expression of functionally relevant proteins. By identifying and characterizing the interaction of *AEG*-1 with its effectors, we can expand our understanding of how this molecule mechanistically engages cells to adopt a more progressive, and consequently, a more metastatic phenotype. Other important areas worth exploring include defining the role of *AEG*-1 in the pathogenesis of neurodegenerative diseases as well as HAD and the mechanism by which *AEG*-1 modulates EAAT2 promoter activity. Understanding the detailed molecular mechanism(s) of *AEG*-1 function and regulation will help clarify its role in the process of tumor progression and facilitate development of potential therapeutic strategies to impede not only tumor development, but also metastasis. Moreover, based on the *ras*-responsive nature of the *AEG*-1 promoter, constructs containing this region could provide useful vectors to selectively deliver tumor suppressor genes, such as p53 or *mda*-7/IL-24 (Fisher, 2005;Gupta et al., 2006;Lebedeva et al., 2003), for gene therapy of cancers containing activated Ras oncogenes.

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Abbreviations

AEG-1, astrocyte elevated gene-1; AKT, protein kinase B; bHLH, basic-helix-loop-helix; C-ORF, complete open reading frame; CXCR4, chemokine (C-X-C motif) receptor 4; EAAT2 and EAAT1, excitatory amino acid transporter 1 and 2, respectively; EGF, epidermal growth factor; GSK-3β, glycogen synthase kinase 3 beta; HIF1α, hypoxia-inducible factor 1 α subunit; HIV-1, human immunodeficiency virus-1; HAD, HIV-associated dementia; IM-PHFA, hTERT (human telomerase) immortalized primary human fetal astrocyte; MIZ1, Msx-interacting-zinc finger; MMP, matrix metalloproteinase; ORF, open reading frame; PHFA, primary human fetal astrocyte; PI3K, phosphoinositide kinase-3; PTEN, phosphatase and tensin homolog; RAR-α, retinoic acid receptor –α; RaSH, rapid subtraction hybridization; THV, human adult astrocytes immortalized by SV40 T/t Ag and hTERT; THR, stable overexpression of Ha-*ras* in THV cells; TNF-α, tumor necrosis factor-α; USF-1, upstream stimulating factor-1; YY1, YY1 transcription factor.

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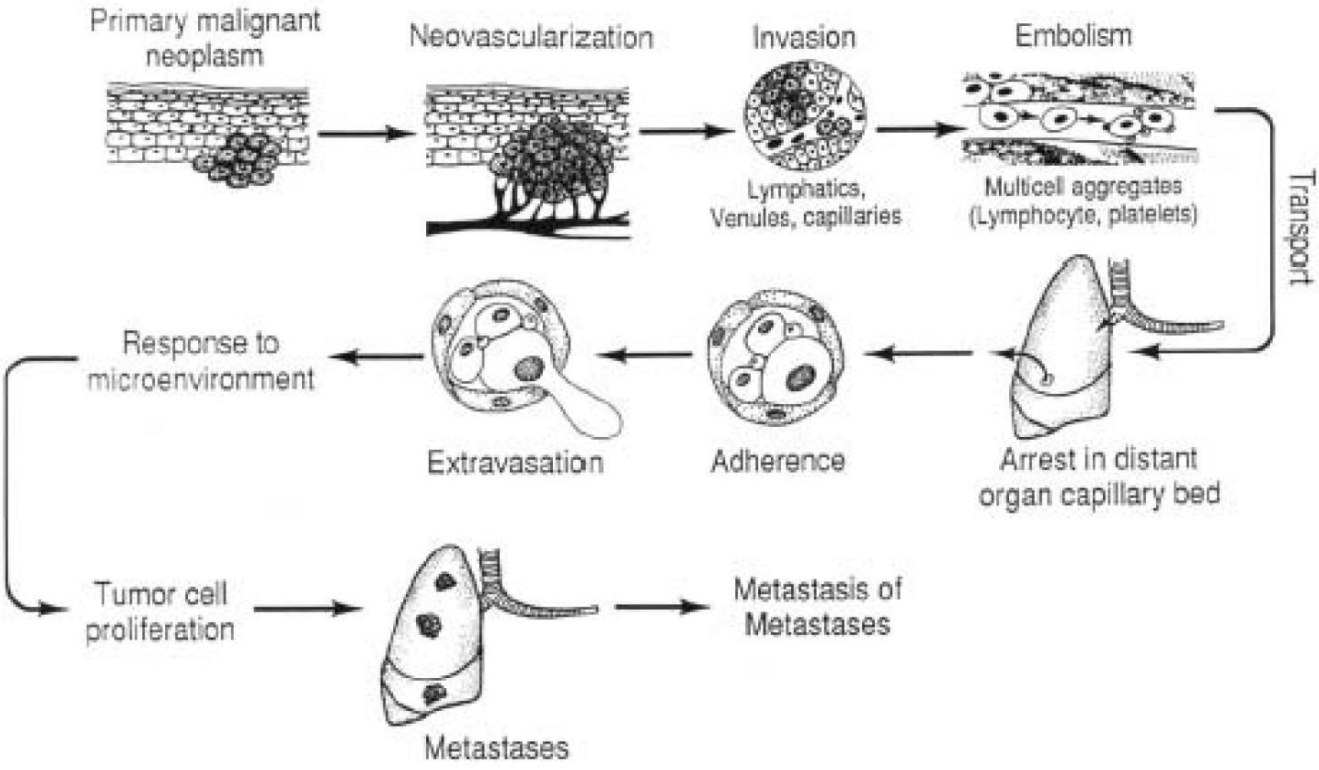

Fig 1.

A schematic representation of the metastatic process. (reproduced, by permission of the publisher, from Fidler & Ellis, 1994).

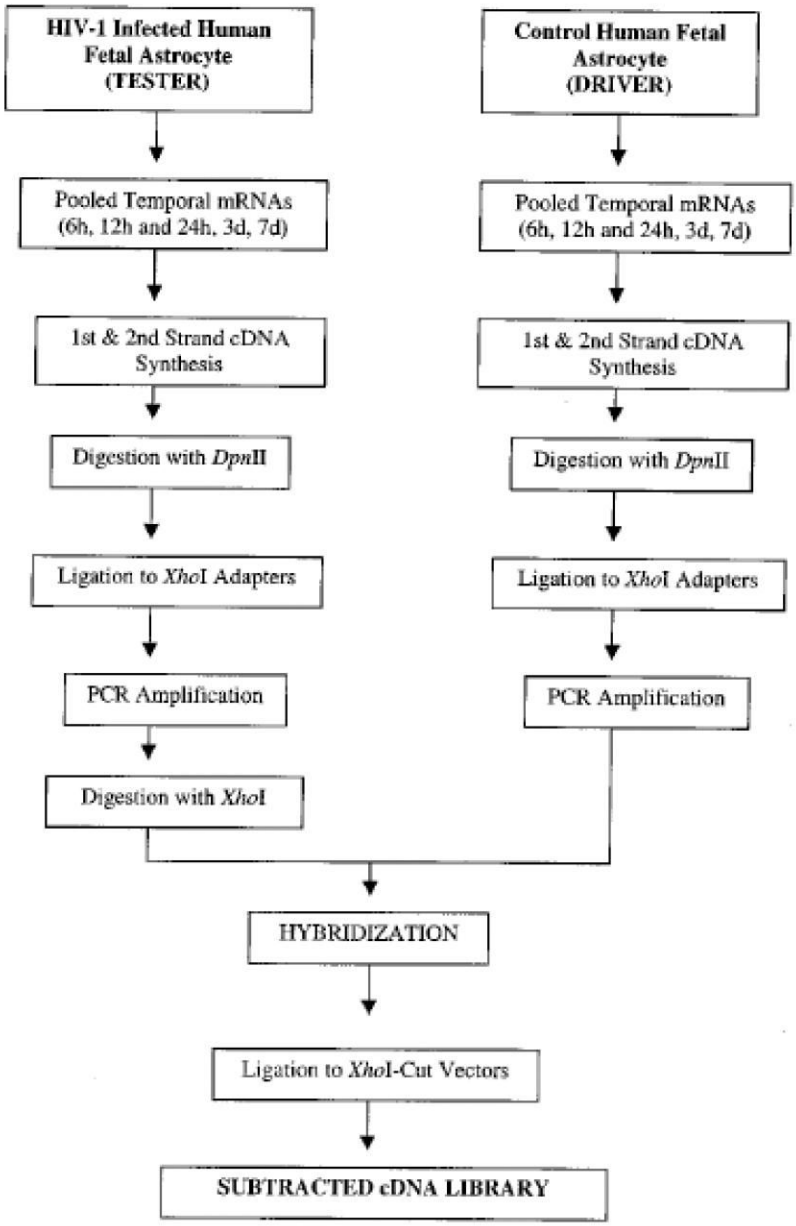

Fig 2.
A schematic representation of the RaSH approach. For details, please refer to Su et al (2003).

A.

Table 1. AEG-1 contains nuclear localization signal

Protein	NLS sequence*
AEG-1	^{561}**K**SETSWESPKQI**KKKKK**ARR^{580}
SIMPL †	**K**VFITFEVKGKE**KKKK**HL^{259}
Nucleoplasmin	**R**PAATKKAGQA**KKKK**LDK^{174}
Human CBP80	**RR**RHSDENDGGQPH**KRRK**^{20}
Bipartite NLS consensus	**K/R K/R** (10-12 residues), 3 **K/R** in 5 residues
AEG-1	^{432}**K**GEGALPTGKS**KKKKKKKKK** ^{451}
SIMPL	**K**VFITFEVKGKE**KKKK**HL^{259}
SV40 T-ag ‡	P**KKKRK**V^{132}
NF-κB p65	HRIEE**KRKR**TYE^{340}
Monopartite NLS consensus	**K R/KX R/K**

Abbreviation: NLS, nuclear localization signal.
*Single-letter amino acid code is used. Bold letters indicate the basic residues of the monopartite and bipartite nuclear localization signals.
†Pelle-like kinase.
‡SV40 T antigen.

B.

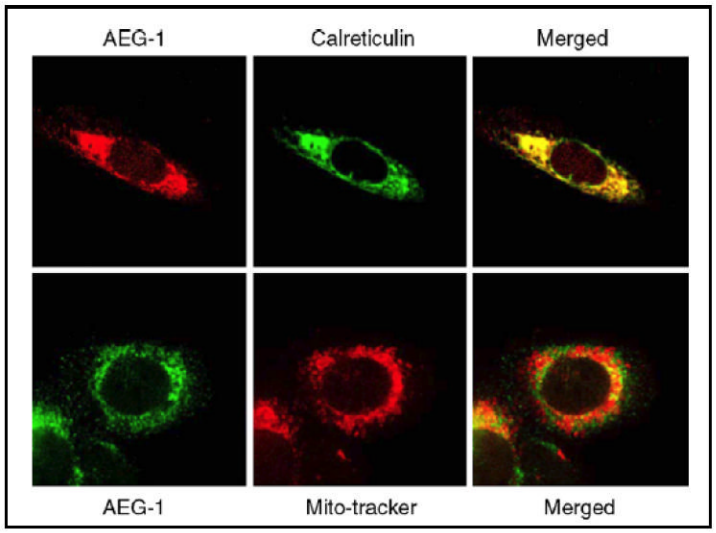

Fig 3.

Localization of *AEG*-1. A. Putative nuclear localization signals (NLS) in *AEG*-1. B. Subcellular localization of *AEG*-1 in IM-PHFA. Calreticulin and Mito-Tracker were used for specific ER and mitochondria markers, respectively. Reproduced, by permission of the publishers, from Emdad et al (2006), and Kang et al (2005), respectively.

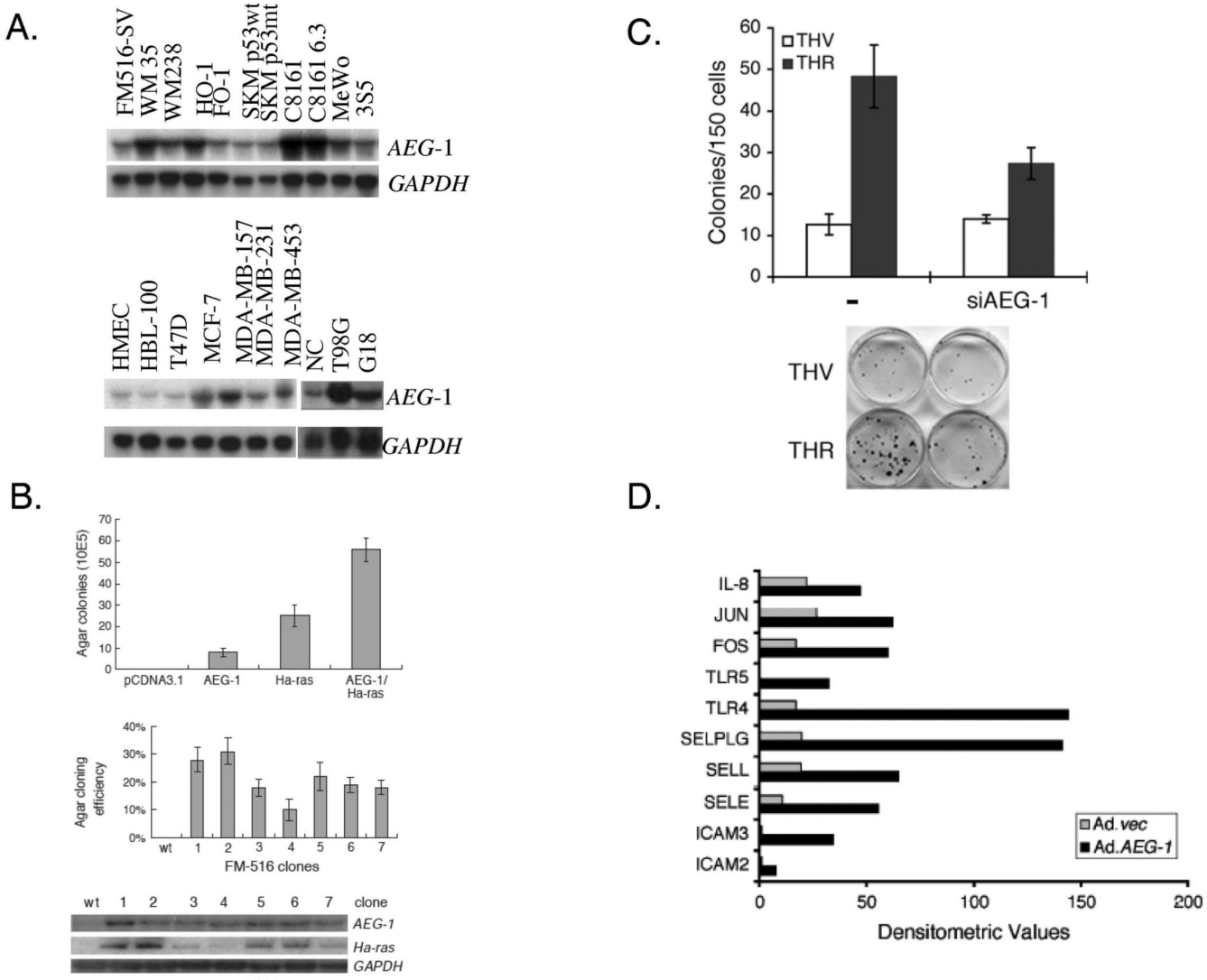

Fig 4.

AEG-1 in tumor progression and metastasis. A. *AEG*-1 expression in various cancer cell lines. Northern blots of the indicated RNA samples were probed with *AEG*-1 and *GAPDH* probes. FM516-SV: SV40-immortalized normal human melanocyte; WM35: radial growth phase primary human melanoma; WM238, HO-1, FO-1, SKM p53wt, SKM p53mut, C8161, C8161 6.3, MeWo, 3S5: metastatic and variant human melanoma cells; HMEC: early passage human mammary epithelial cells; HBL-100: immortal normal human breast epithelial cell line; T47D, MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-453: human breast carcinoma cell lines; NC, normal cerebellum cell line; T98G, G18: human malignant glioma cell line. B. Effect on soft agar colony formation of FM516-SV cells following expression of *AEG*-1 and Ha-*ras*, alone or in combination. Upper panel, colony forming ability of FM516-SV cells transiently transfected with the indicated expression vectors. Data shown is average ± S.D. of three independent transfections. Middle panel, colony forming ability of FM516-SV cells stably transfected with *AEG*-1 and Ha-*ras*. Data shown is average ± S.D. of three independent transfections. Lower panel, expression of *AEG*-1 and Ha-*ras* mRNA in stable transfectants determined by Northern blot analysis. C. *AEG-1* is required for Ha-*ras*-mediated colony formation. THV and THR cells were transfected with control or *AEG-1* siRNA using the Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. After 2

days, the cells were trypsinized and counted, and 150 cells were plated in 6-cm dishes. Colonies of ≥ 50 cells were scored after 10 days. The data are represented as mean ± SD and represent three independent experiments in triplicate. D. Ad.*AEG*-1 infection upregulates NF-κB downstream genes. The expression levels of NF-κB signaling pathway genes after Ad.*vec* and Ad.*AEG-1* infection were analyzed in total RNA using the Human NF-κB signaling gene array (SuperArray Bioscience Corporation) according to the manufacturer's protocol. Graphical representation of expression levels of each gene obtained by densitometric analysis. Reproduced, by permission of the publishers, from Kang et al (2005),Lee et al (2006), and Emdad et al (2006), respectively.

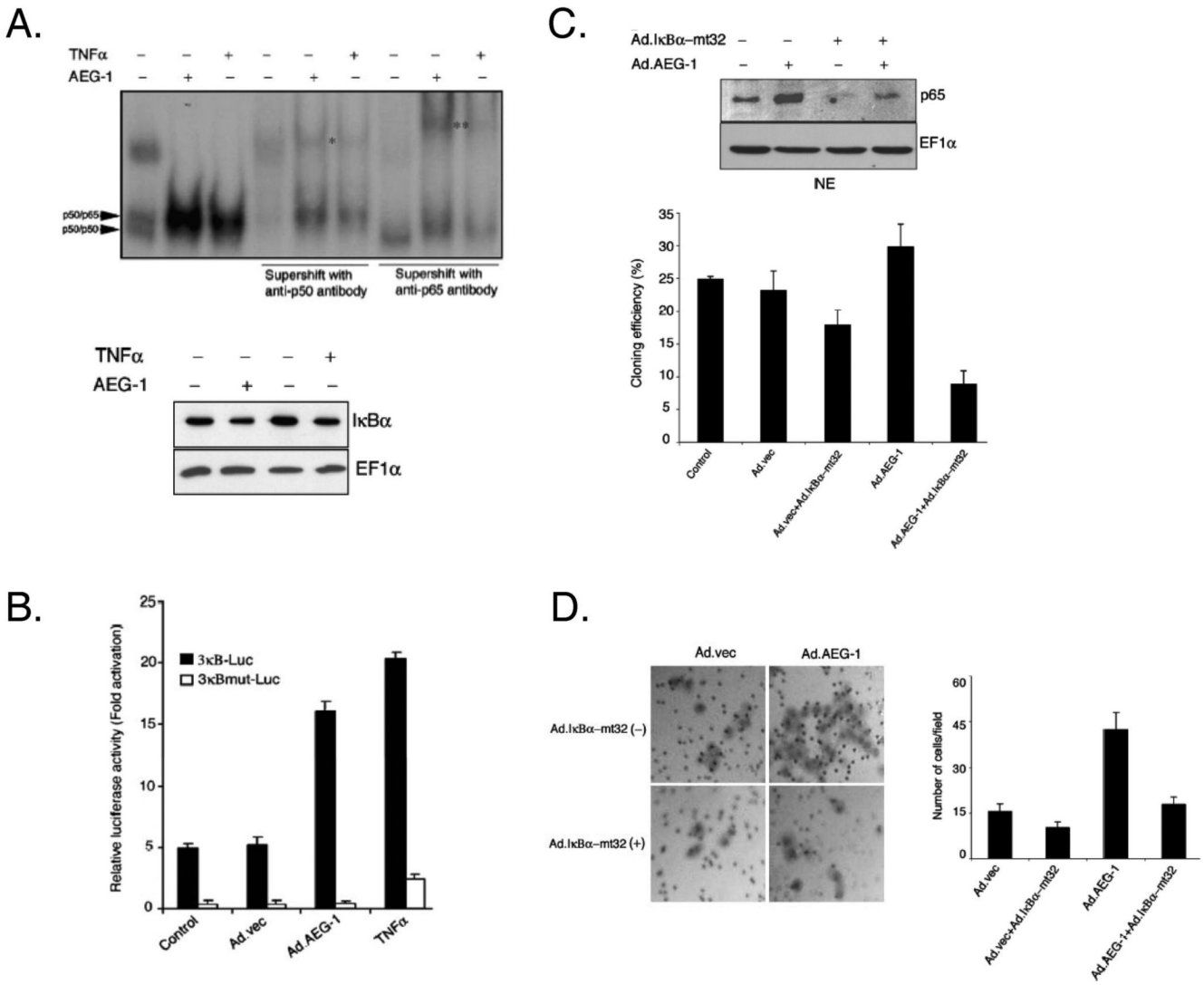

Fig 5.

AEG-1 and NF-κB pathway. A. *AEG*-1 activates the NF-κB pathway. Upper panel, HeLa cells were infected with Ad.*AEG-1* (50 pfu/cell) or treated with TNF-[unk] (10 ng/ml) and 2 days later NF-κB binding activity was analyzed in the nuclear extracts of the cells by EMSA. Supershift analysis was performed with the indicated antibodies. *indicates a supershifted band by anti-p50 antibody; ** indicates a supershifted band by anti-p65 antibody. Lower panel, Ad.*AEG*-1 infection activates NF-κB by IκB[unk] degradation. HeLa cells were infected with Ad.*AEG*-1 (50 pfu/cell) or treated with TNF-[unk](10 ng/ml). The expressions of the indicated proteins were analyzed in cytoplasmic extracts by Western blot analysis 2 days later. B. *AEG*-1 activates NF-κB promoter activity. HeLa cells were infected with Ad.*AEG*-1 (50 pfu/cell) or treated with TNF-α (10 ng/ml) and 12 hr later were transfected with the indicated plasmids using Lipofectamine 2000. Luciferase assays were performed 48 hr after transfection using a Luciferase Reporter Gene Assay kit according to the manufacturer's protocol. The β-galactosidase activity was determined using the Galacto-Light Plus kit. Luciferase activity was normalized by β-galactosidase activity, and the data presented is the fold-activation relative to pGL3-Basic from triplicate determinations. C. Inhibition of NF-κB blocks *AEG*-1-induced increased anchorage independent growth. Upper panel, HeLa cells were either uninfected or

infected with Ad.*AEG*-1 or Ad.IκBα–mt32 either alone or in combination at an m.o.i. of 50 pfu/cell for each Ad. Two days later the level of p65 was determined in the nuclear extracts by Western blot analysis. Lower panel, HeLa cells were infected as in the upper panel. Twenty-four hr later, cells (1×10^5) were replated in 0.4% agar on 0.8% base agar. Two weeks later, colonies ≥ 50 cells were counted under a dissection microscope. Data represents mean ± SD of triplicate plates in three independent experiments. D. Inhibition of NF-κB blocks *AEG*-1-induced enhanced matrigel invasion. Left panel, HeLa cells were infected as in Fig. 4C. Twenty-four hr later, cells (2.5×10^4) were seeded onto the upper chamber of a matrigel invasion chamber system in the absence of serum. Forty-eight hr after seeding the filters were fixed, stained and photographed. Right panel, graphical representation of the invasion assay. Data represents mean ± SD of three independent experiments. Reproduced, by permission of the publisher, from Emdad et al (2006).

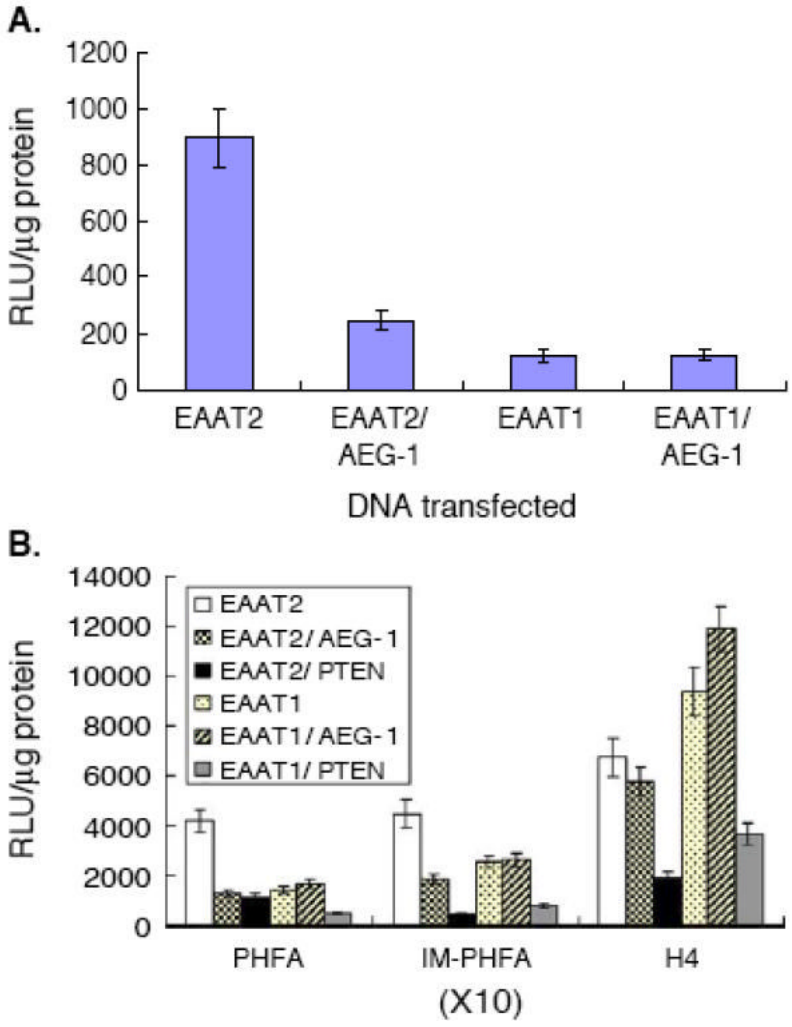

Fig 6.

Effect of *AEG*-1 expression on EAAT2 promoter activity. A. A luciferase reporter vector containing the complete EAAT2 or EAAT1 promoter was co-transfected with either pcDNA3.1 or pcDNA3.1-*AEG*-1-HA. Two days later, cells were harvested and luciferase activity of the protein extracts was measured. Data shown is representative of multiple experiments, which varied by ≤ 15%. Results presented ± S.D. from two independent experiments. B. A luciferase reporter vector containing the complete EAAT2 or EAAT1 promoter was co-transfected with the indicated expression vectors. Samples were obtained and analyzed as above. Data shown is representative of multiple experiments, which varied by ≤ 15%. Results presented ± S.D. from two independent experiments. Reproduced, by permission of the publisher, from Kang et al (2005).

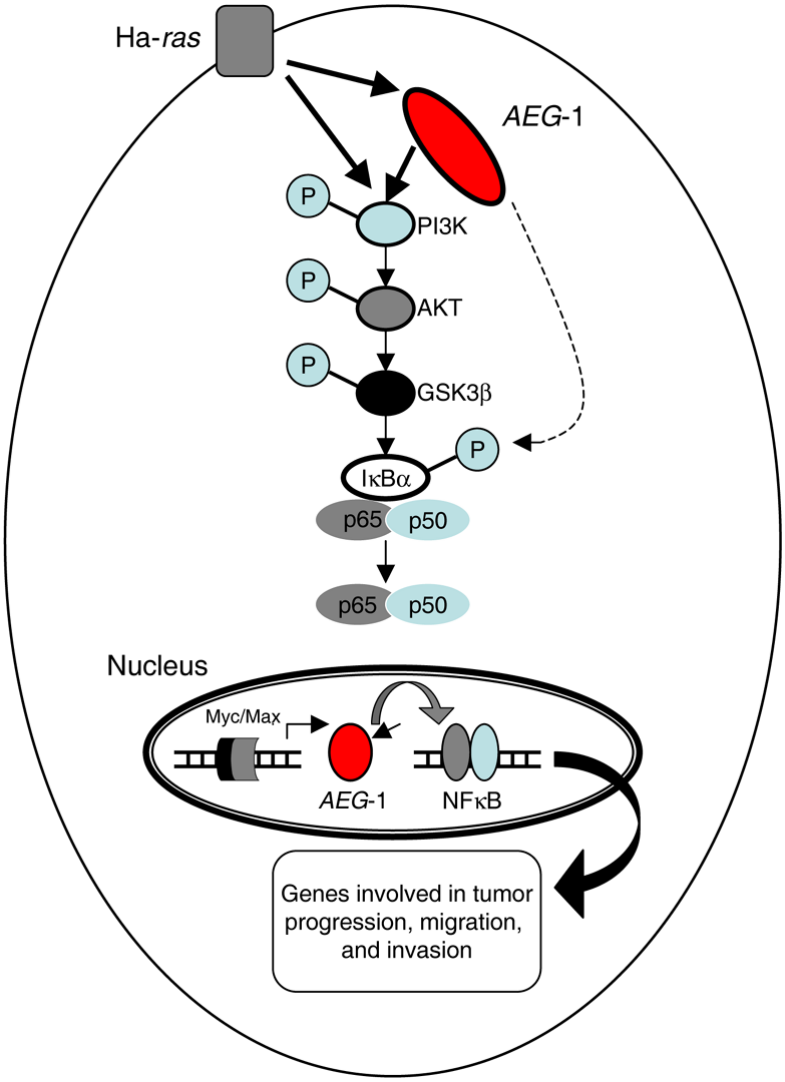

Fig 7.

Hypothetical model of signal transduction pathways involved in Ha-*ras*-mediated *AEG*-1 induction. Ha-*ras* activates the PI3K signaling cascade resulting in increased binding of Myc-Max to the *AEG*-1 promoter and augments *AEG*-1 expression. *AEG*-1 activates the NF-κB pathway that regulates expression of genes involved in migration and invasion and thus plays a crucial role in Ha-*ras*-mediated tumor progression.