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Early Growth Response-1 Suppresses Human Fibrosarcoma Cell Invasion and Angiogenesis

RUOCHUN HUANG[1], SHIYONG LI[2], WEIMIN YANG[1], LIPAI CHEN[1], CHEN YAO[3] and RUO-PAN HUANG[1]

[1]Department of Gynecology and Obstetrics and [2]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.;
[3]Department of Thyroid and Vascular Surgery, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

Abstract. *The development of malignant tumors is the result of sequential genetic and epigenetic lesions that lead to alterations in a number of gene expressions, which are primarily controlled by transcription factors. A growing body of evidence suggests that early growth response-1 (Egr-1), a transcription factor, may function as a tumor suppressor. Here, the possible role of Egr-1 in the suppression of tumor cell invasion, angiogenesis and metastasis was investigated. Expression of Egr-1 significantly reduced the invasion of human fibrosarcoma cells through matrigel. Mouse embryonic fibroblasts, from Egr-1 knockout mice, also showed increased invasion through matrigel compared with MEFs from wild-type mice. Conditioned medium from Egr-1-transfected cells compared with control transfected cells also reduced proliferation, invasion through matrigel and tube formation of human umbilical cord vein endothelial cells and human microvascular endothelial cells. In addition, Egr-1-transfected cells inhibited vessel formation in mouse skin plug assays. To study the possible molecular mechanisms responsible for this function, the expression of multiple cytokines, chemokines, growth factors and angiogenic factors were examined by using human cytokine antibody array technology it was observed that tissue inhibitor of metalloproteinase-2 (TIMP-2) expression was up-regulated in Egr-1-transfected cells. Addition of Egr-1-transfected cell conditioned medium and TIMP-2 recombinant protein suppressed fibrosarcoma cell invasion. In summary, it was shown that Egr-1 may have novel functions in the suppression of tumor cell invasion and angiogenesis, while TIMP-2 may be involved in the suppression of tumor cell invasion and angiogenesis in Egr-1-transfected cells.*

Correspondence to: Ruo-Pan Huang, MD, Ph.D., Department of Gynecology and Obstetrics, Emory University School of Medicine, 1639 Pierce Drive, Rm. 4219, Atlanta, GA 30322, U.S.A. Tel: (404)712-9673, Fax: (404)727-8615, e-mail: rhuang@emory.edu

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Egr-1 (72), also known as Zif268 (17), NGFIA (58), Krox24 (16) and Tis8 (49), was identified virtually simultaneously by several groups of researchers in the late 1980s. Four related genes, including the tumor suppressor gene WT-1, make up a highly conserved family. As its name indicates, Egr-1 is rapidly and strongly induced in response to a wide spectrum of stimuli including serum, growth factors, UV radiation, gamma-radiation, cytokines, stresses, phorbol esters and vascular injury (52) and has been implicated in a variety of cellular processes including growth, differentiation, apoptosis, neurite outgrowth and wound healing (26, 42, 63, 70). Knockout of Egr-1 in mice leads to female infertility due to a defect in luteinizing hormone-β (LH-β) production (47). Egr-1 is a nuclear phosphoprotein with three tandem-repeat zinc finger domains which can bind to the consensus binding sequence: 5' GCGG/AGGGCG3' (GC-rich element or GCE) (17, 62) or 5'-TCCTCCTCCTCCTC3' (77). Besides the DNA-binding domain, Egr-1 also contains a number of activation domains and an inhibitory domain (27, 64). The inhibitory effect is mediated through interaction with the NGFI-A (Egr-1)-binding proteins, NAB1 and NAB2 (65). NAB1 is widely expressed at low levels in many cells. In contrast, NAB2 only accumulates in certain tissues and is regulated in a more restricted manner. The expression of NAB2 is induced in a delayed fashion by many stimuli that also induce Egr-1. Thus, NAB2 may function as a negative regulator to control Egr-1 activity in a feedback loop (57).

Since we first demonstrated that Egr-1 may function as a tumor suppressor gene in human fibrosarcoma (40), a number of studies also described the tumor suppressive activity of Egr-1 in breast cancer (37, 40), glioblastoma (13, 15, 40), hepatoma (29), esophageal cancer (81, 83) and leiomyoma (69). In this study, we found that Egr-1 can also suppress tumor cell invasion and angiogenesis. Furthermore, the anti-invasion and anti-angiogenesis activity of Egr-1 is mediated through the up-regulation of the tissue inhibitor of mettalloproteinase-2 (TIMP-2).

Materials and Methods

Cell culture. H4E9, H4E3, H4E4 are different clones stably-transfected with the Egr-1 expression vector and H4N were the control cells. They were originally derived from human fibrosocoma cells H4 (40). The cells were maintained in DMEM containing 10% fetal calf serum (FCS). Primary human umbilical cord vein endothelial cells (HUVECs) were isolated and cultured (71) to study angiogenic activity, as described in our previous study (40). Human microvascular epithelium cells (HMECs) were obtained from the Emory core facility. The HMECs were maintained in MCDB131 medium including 20% human serum, 10 ng/ml epidermal growth factor (EGF), 100 μg/ml penicillin and streptomycin, 292 μg/ml L-glutamine, 1 μg/ml hydrocortisone and 250 μg/ml cAMP. The mouse embryonic fibroblasts (Egr-$1^{+/+}$ and Egr-$1^{-/-}$) were a gift from Dr. Eileen Adamson (The Burnham Institute, La Jolla, CA, USA) and maintained in DMEM containing 10% FCS.

Invasion assays. Invasion assays were performed, as described our previous publication (51). Briefly, the cells were seeded into 12-well polycarbonate filter (12-mm pore size) Transwell (Corning-Costar, Coming, NY, USA) coated with 60 μl matrigel (BD PharMingen, San Diego, CA, USA) diluted with an equal volume of serum-free DMEM. After 24-h incubation, cells that had migrated to the lower side of the filter were fixed in 4% formaldehyde and stained with Giemsa solution (Sigma, St. Louis, MI, USA). The number of matrigel-invading cells was counted under a microscope. Each experiment was carried out at least three times.

In vitro angiogenesis assays. In vitro angiogenesis was assessed using three different assays, which have been described in our previous paper (50).

Migration assay. The migration assay was performed using 12-well Transwell culture plates inserted with polycarbonate filters. HUVECs ($1x10^6$) were placed on the upper chambers precoated with 60 μl matrigel. The bottom plates were fed with conditioned media prepared from H4E9 and H4N. After 24 h, the matrigel was removed from the plate. The cells that had migrated through the matrigel were stained and quantitated, as described above.

Collagen tube formation. Three-dimensional collagen gel plates (12 plates) were prepared by adding 0.8 ml of chilled rat-tail collagen into each well and adjusting to neutral pH with $NaHCO_3$. The collagen was allowed to solidify at 37˚C for 30 min. $1x10^5$ HUVEC cells per well were plated on the collagen gel in M199 media containing 15% (vol/vol) fetal bovine serum (FBS) and endothelial cell growth factor. When the cells were confluent, the media were replaced by conditioned media from the H4E9 and H4N cells. The wells were monitored and photographs were taken 24 h after the addition of the reagents. The experiments were repeated three times and the results were viewed by two independent researchers.

Cell proliferation. Cell proliferation was assayed with a cell titer 96 non-radioactive cell proliferation assay kit (MTT assay), according to the manufacturer's instructions (Promega, Madison, WI, USA), which have been described previously in our publications (33, 38). Briefly, cells were plated in triplicate in 96-well plates for a given period of time, before being treated with 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h. Overnight incubation with lysis buffer at room temperature followed. The optical density (OD) at 570 mm was then determined and the values were expressed as relative viable cell numbers. The experiments were repeated at least twice and as triplicate assays.

Matrigel plug assays. Matrigel plug assays (56) were used to evaluate the angiogenic potential of the conditioned media prepared from H4E9 and H4N (40). Briefly, the mice received subcutaneous injections of 350 μl Matrigel together with 30 μl of the conditioned media (concentrated 10-fold). Matrigel alone served as the control. Fibroblast growth factor (FGF)-2 was used as a positive control. After 5 days, the under-surface of the skin was exposed and photographed for evaluation of angiogenesis.

Immunohistochemistry. Skin tissue sections were made from formalin-fixed and paraffin-embedded samples. Immunohistochemistry was performed as described elsewhere (37). Briefly, tissue sections were incubated with anti-CD31 (Santa Cruz, Biotechnology, Santa Cruz, CA, USA), at a dilution of 1:1,000 overnight at 4˚C. The ABC peroxidase method of staining was then employed, as described by the manufacturer's (ABC, Vector Laboratories, Burlingame, CA, USA). The slides were reacted with the substrate H_2O_2 in conjugation with diaminobenzidine.

Cytokine antibody arrays. The cytokine antibody arrays kit (human cytokine antibody arrays V) was a gift from RayBiotech, INC (Norcross, GA, USA). This human cytokine antibody array system can simultaneously detect the expression of 79 cytokines. The list of cytokines can be found on the RayBiotech company website (www.raybiotech.com). Cytokine antibody array membranes were blocked with blocking buffer for 30 min. The membranes were then incubated with about 1 ml of conditioned media prepared from H4E9 (high Egr-1-expressing cells) or H4N (low Egr-1-expressing cells) for 2 h after normalization with equal amounts of protein. Following extensive washing, 2 ml of diluted biotin-conjugated antibodies was added to the membranes and the mixture was incubated on a shaker for 2 h. After a thorough wash, the membranes were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 1 h. Finally, an extensive wash was carried out. The signals were visualized using the chemiluminescence detection system and quantitated with the UVP Chemiluminescent imaging system (Upland, CA, USA). Signals from blank spots were used as background. The final spot intensities were the original intensities subtracted from the background. The data were also normalized to the positive controls and negative controls in the individual membranes.

Western blot. A Western blot assay was conducted, as described in Huang *et al.* (36). Cell extracts containing equal amounts of protein were analyzed by SDS-PAGE and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). The membranes were then probed with corresponding antibodies. Signals were detected with an enhanced chemiluminescence (ECL) system (Amersham Corp., Amersham, UK).

Enzyme-linked immunosorbent assay (ELISA). The TIMP-2 levels in the conditioned media were confirmed with the TIMP-2 ELISA kit, a gift from RayBiotech, INC. The minimal detectable amount of TIMP-2 is less than 75 pg/ml. The experiment was performed according to the manufacturer's manual.

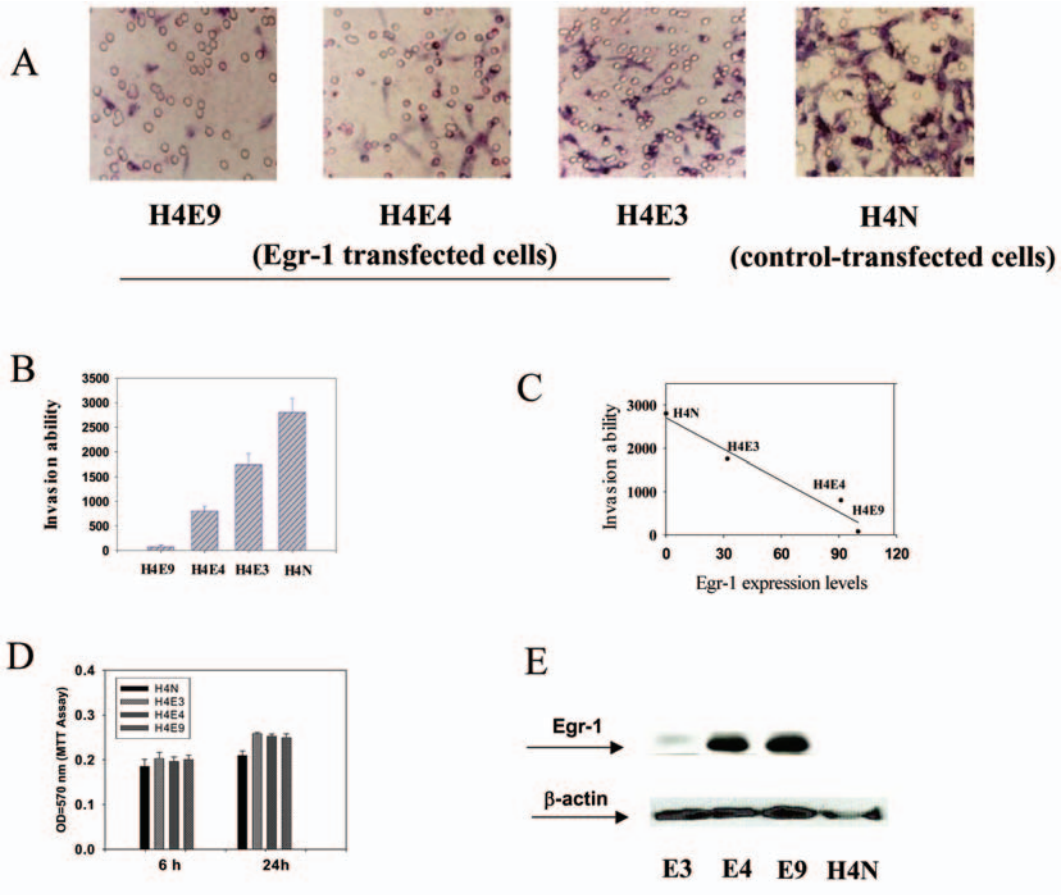

Figure 1. *The expression of Egr-1 inhibited the invasion of human fibrosarcoma cells. Cells stably-expressing Egr-1 (H4E3, H4E4 and H4E9) and control cells (H4N) ($5x10^5$) were seeded at a density of $5x10^5$ cells on the top of Biocoat matrigel invasion chambers and incubated at 37°C in 5% CO_2 for 24 h. Then the matrigel was removed and the cells that had invaded through the matrigel were stained with Giemsa. (A) shows raw images. (B) shows the quantitative result (cells traveled through matrix gel). (C) plots the invasion level as a function of Egr-1 expression. In (D), $1x10^3$ cells were seeded into 96-well plates and the MTT assay was performed at 6 h and 24 h after seeding. (E) The expression levels of Egr-1 in different cell lines were determined by Western blot. Fifty mg of cell lysates prepared from different cell lines were separated in 7.5% SDS page. After being transferred, the presence of Egr-1 and beta-actin were determined using specific antibodies.*

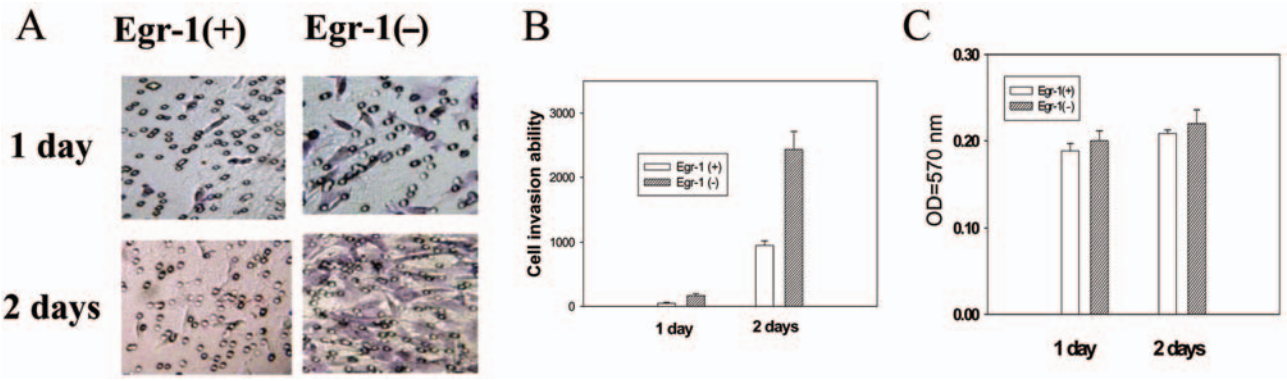

Figure 2. *Egr-1 knockout mouse embryonic fibroblasts (MEFs) demonstrated increased invasion through matrigel. The ability of Egr-1 null MEF and Egr-1 wild-type MEF invasion was examined using 12-well Transwell culture plate inserts coated with matrigel. The cells ($1x10^6$) were plated on the top of matrigel. After 1 and 2 days, the matrigel was removed from plate and the cells that had migrated through the matrigel were stained (A) and quantitated (B). At the same time, cell proliferation was determined using the MTT assay (C).*

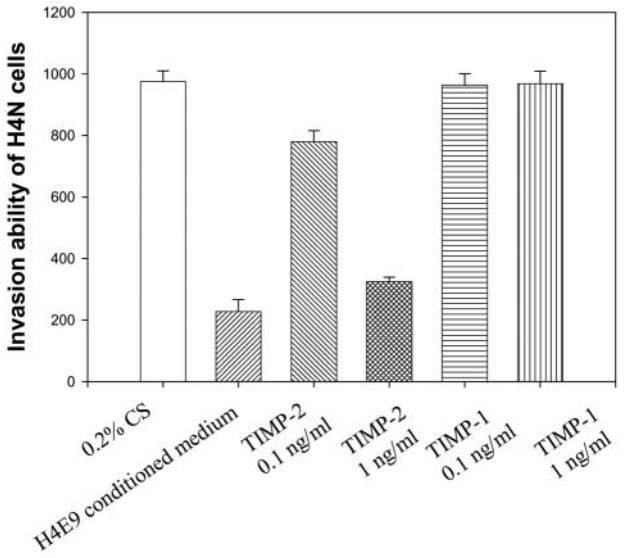

Figure 3. *H4E9 conditioned medium and the recombinant protein TIMP-2 inhibited fibrosarcoma cell invasion. The H4N cells (control-transfected H4 cells) were assayed for their invasion ability using the matrigel chamber as a model system in the presence of H4E9 conditioned medium or medium alone (0.2% calf serum (cs)) or medium plus recombinant protein TIMP-2.*

Animal model. Two different cell lines (H4E9, stably-transfected Egr-1 clone and H4N, vector-transfected clone) were used. Single cell suspensions ($2x10^6$) were injected into the tail vein of female nude mice (6 to 10 weeks old). Three weeks later, the mice were sacrificed and the lungs, livers and kidneys were dissected out.

Statistical analysis. The software SPSS 8.0 was used for statistical analysis. A *p*-value of less than 0.05 was considered as a significant difference.

Results

Suppression of human fibrosarcoma cell invasion by Egr-1. Our cDNA microarray survey indicated that Egr-1 could regulate the expression of a series of genes related to the actin cytoskeleton, which is important in maintaining cell shape and mobility (53). Therefore, Egr-1 may play a role in cell invasion. To test this, the cell invasion through matrigel, one of the indications of cell invasion, was examined. As shown in Figure 1, expression of Egr-1 significantly reduced the invasion of human fibrosarcoma cells through matrigel. The effect of Egr-1 on invasion was dose-dependent, strongly suggesting the involvement of Egr-1 in the inhibition of invasion of human fibrosarcoma cells. The cell invasion assays were performed at 24 h, when the cell number was not significantly different (Figure 1). Therefore, the suppression of cell invasion is independent of the Egr-1-mediated cell growth control.

To provide more evidence(s) that Egr-1 may play a role in the suppression of cell invasion, the ability of Egr-1 null embryonic fibroblasts and of Egr-1 wild-type embryonic fibroblasts to invade through matrigel was examined. As shown in Figure 2, genetic deletion of Egr-1 resulted in a remarkable increase of cell invasion through matrigel.

Since many soluble factors, such as growth factors, cytokines, angiogenic factors, matrix metalloproteinases (MMP) and tissue inhibitors of MMP (TIMP) play important roles in tumor cell invasion, angiogenesis and metastasis, the effects of conditioned media from Egr-1-transfected cells and control-transfected cells on tumor cell invasion were examined. As shown in Figure 3, the conditioned media from Egr-1-transfected cells significantly suppressed H4N cell invasion through matrigel, suggesting that soluble factor(s) present in the Egr-1-transfected cell conditioned media may be responsible for the Egr-1-mediated effect on tumor cell invasion.

Anti-angiogenesis by Egr-1. Next was to test for a role of Egr-1 in anti-angiogenesis. Three *in vitro* assays were initially performed to assess the effects of Egr-1 on the migration of endothelial cells, formation of new capillary tubes and the proliferation of endothelial cells. These three events are required in angiogenic responses.

As shown in Figure 4 A, many more HUVECs migrated through the matrigel using conditioned media from H4N compared to the high Egr-1-expressing cells (H4E9). Furthermore, the conditioned media from high Egr-1-expressing cells (H4E9) and low Egr-1-expressing cells (H4N) were used to test the tube formation of HUVECs, another indication of an angiogenic response *in vitro*. Significant number of HUVECs formed elongated tube-like structures within 24 h in response to the conditioned media from low Egr-1-expressing cells as compared to the high Egr-1-expressing cells (Figure 4 B). These findings further demonstrated that conditioned medium from low Egr-1-expressing cells significantly promoted an endothelial angiogenic response *in vitro*.

Finally, the HUVECs proliferated at a slow rate in conditioned media from high Egr-1-expressing cells (H4E9) in comparison to that from low Egr-1-expressing cells (H4N), as shown in Figure 4 C. These results suggested that the secreted factors from high Egr-1-expressing cells inhibited HUVEC survival and proliferation.

All three parameters suggested that conditioned medium from H4E9 cells inhibited angiogenesis-like events. Thus, it is suggested that factors secreted from H4E9 cells contributed to the inhibition of angiogenesis.

Human microvascular endothelium cells (HMECs) may be more relevant to new blood vessels, leading to examination of the effects of conditioned media from H4E9 and H4N on HMEC proliferation and invasion. As observed

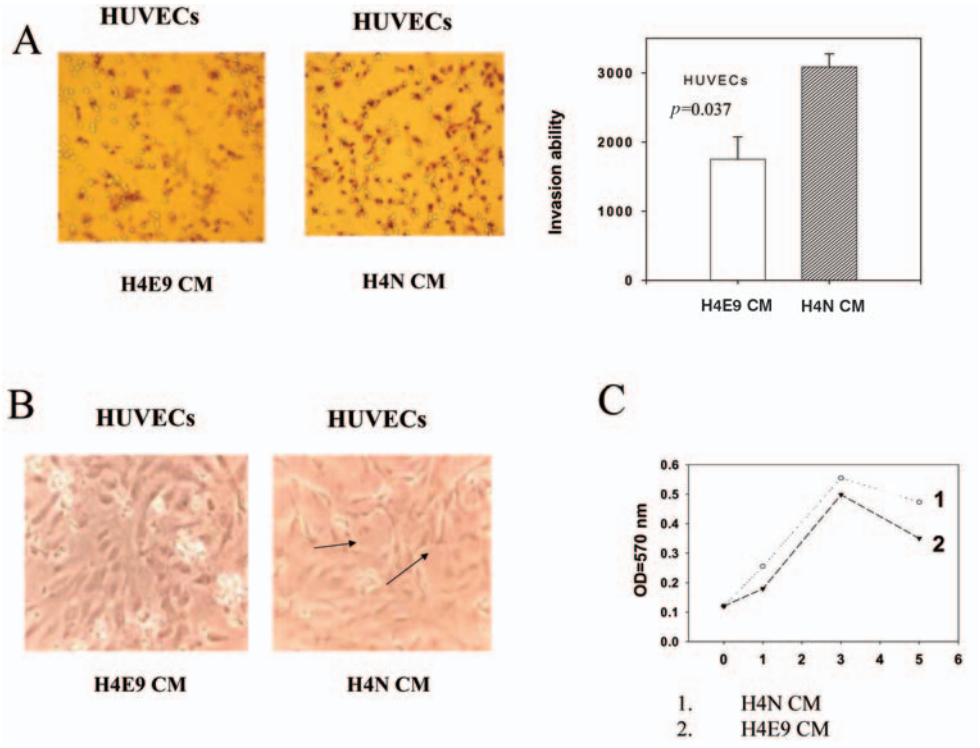

Figure 4. *Expression of Egr-1 in human fibrosarcoma cells inhibited angiogenesis using HUVEC in vitro assays. (A). Migration assay. Migration assay was done by using 12-well Transwell culture plate inserts coated with matrigel. HUVECs (1x10^6) were plated on the top of the matrigel. The bottom plates were fed with conditioned media prepared from H4E9 (H4E9 CM) or H4N cells (H4N CM). After 2 days, the matrigel was removed from the plate. Cells that had migrated through the matrigel were stained and quantitated. (B). Tube formation assays. Three-dimensional collagen gel plates were prepared by adding 0.8 ml of chilled rat tail collagen into each well and adjusting to neutral pH with $NaHCO_3$. Collagen was allowed to solidify at 37˚C for 30 min. HUVEC cells (1x10^5 per well) were plated on collagen gel in M199 medium containing 15% (vol/vol) FBS and endothelial cell growth factor. When the cells were confluent, the medium was replaced with conditioned medium from H4E9 and H4N cells. The wells were monitored and photographs were taken 24 h after the addition of the reagents. The experiments were repeated three times and the representative results are shown. (C). Cell proliferation assay. HUVEC cells (1x10^4) were plated in 96-well plates with half complete medium plus half conditioned medium from either H4E9 cells or H4N cells. MTT assays were performed at day 1, day 3 and day 5.*

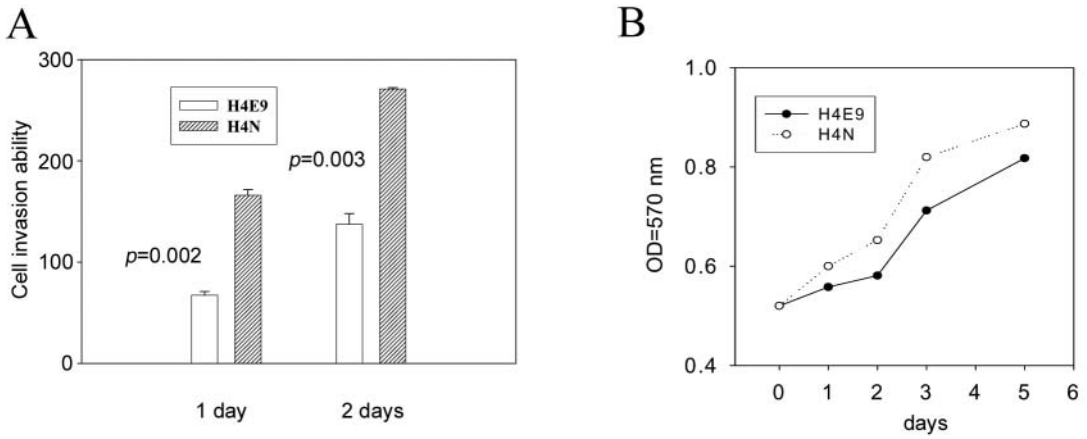

Figure 5. *Expression of Egr-1 in human fibrosarcoma cells inhibited angiogenesis using HMEC in vitro assays. (A). Migration assay. HMEC (5x10^5) cells were plated on the top of matrigel. The bottom plates were fed with conditioned media prepared from H4E9 or H4N cells. After 2 days, the matrigel was removed from the plate. Cells that had migrated through the matrigel were stained and quantitated. (B). Cell proliferation assay. 2,000 HMEC cells were plated in 96-well plate with half complete medium plus half conditioned medium from either H4E9 cells or H4N cells. MTT assays were performed at day 1, day 2, day 3 and day 5.*

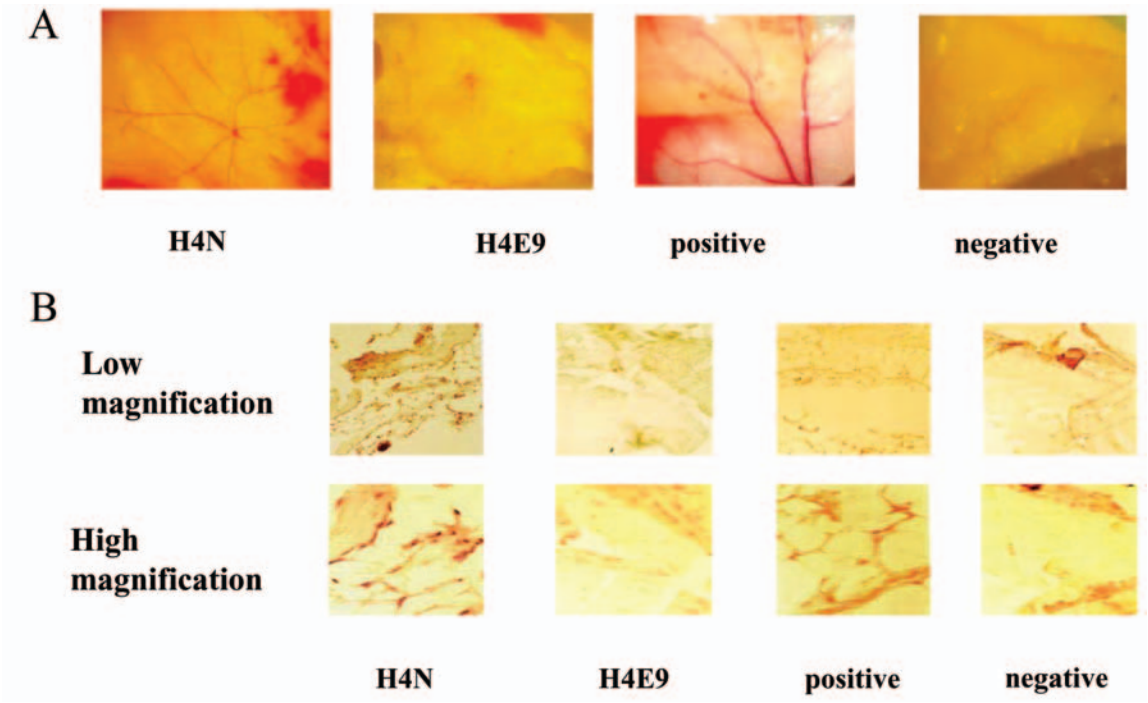

Figure 6. *In vivo angiogenesis assay. (A). Thirty ml of 10-fold concentrated conditioned media from H4E9, H4N, bFGF or mock were injected into mice (4 mice/group). On day 5 after injection, the mice were sacrificed and the vessel formation under the skin was observed. (B). Representative results are shown in the top panel. Immunohistochemical staining data using antibody against CD31 are shown in the middle and low panels.*

A

	a	b	c	d	e	f	g	h	i	j	k
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
2	I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β	TNF-α	TNF-β	EGF	IGF-I	Ang
5	OSM	Tpo	VEGF	PDGF-B	Leptin	BDNF	BLC	Ckβ 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Osteoprotegerin	PARC	PIGF	TGF-β 2	TGF-β 3	TIMP-1	TIMP-2	Neg	Neg	pos

C

Cytokine	Ratio (H4E9/H4N)
IL-6	18.3
IL-8	1.4
FGF-4	0.2
NT-3	0.5
TIMP-1	4.6
TIMP-2	1.6

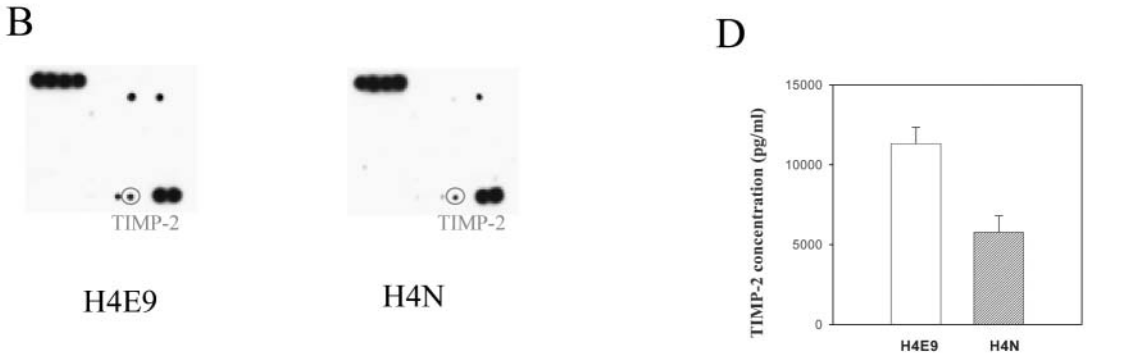

Figure 7. *Expression of Egr-1 up-regulated TIMP-2. (A). Location of cytokine antibody arrays spotted onto the membrane. (B). Representative of images of cytokine antibody array results. Conditioned media prepared from equal number of cells (H4E9 and H4N) were used to assay 79 cytokine expression levels. (C). Change of cytokine expression in Egr-1-transfected cells determined by cytokine antibody arrays. (D). Expression levels of TIMP-2 in H4E9 and H4N cells were determined by ELISA.*

in HUVECs, conditioned medium from H4E9 cells significantly reduced HMEC invasion (Figure 5 A) and proliferation (Figure 5 B) compared with conditioned medium from H4N.

To provide more direct evidence for the role of Egr-1 in anti-angiogenesis, an *in vivo* angiogenesis assay (plug assay) was also performed. The concentrated conditioned media (10-fold concentration) from H4E9 and H4N, together with matrigel, were injected subcutaneously into 8-week-old female BALB/c nude mice at sites near the abdominal midline. As shown in Figure 6 A, conditioned media from low Egr-1-expressing cells (H4N) stimulated more blood vessel formation compared with conditioned media from high Egr-1-expressing cells (H4E9). FGF-2 was used as a positive control, and media only were used as a negative control. Immunohistochemical staining (Figure 6 B) using CD31, a specific endothelial cell marker, further confirmed the blood vessel formation data.

Up-regulation of TIMP-2 in Egr-1-transfected cells. Our data suggested that the effects of Egr-1 in the suppression of tumor cell invasion and angiogenesis may be mediated by soluble factors. To search for possible target(s), the expression levels of a panel of cytokines, chemokines, angiogenic factors, growth factors, matrix metalloproteinases and tissue inhibitors of MMP were screened using the human cytokine antibody arrays. With this powerful technique, it was found that expression of Egr-1 led to alteration of the expression of several factors in Egr-1-transfected cells, as shown in Figure 7 B and C. Since TIMP-2 has been shown to be a critical factor involved in cell invasion, angiogenesis and metastasis (5) and Egr-1 can directly regulate TIMP-2 expression (4), we hypothesized that TIMP-2 may be involved in the suppression of human fibrosarcoma cell invasion and angiogenesis by Egr-1. The expression levels of TIMP-2 in H4E9 and H4N were further confirmed by ELISA (Figure 7 D). To provide more evidence(s) that Egr-1 regulates TIMP-2 expression, the TIMP-2 expression in Egr-1 null embryonic fibroblasts and Egr-1 wild-type embryonic fibroblasts was examined. As shown in Figure 8, TIMP-2 was significantly decreased in Egr-1 null embryonic fibroblast, as determined by Western blot analysis.

Involvement of up-regulation of TIMP-2 in Egr-1-mediated anti-invasion and anti-angiogenesis. The up-regulation of TIMP-2 in Egr-1-transfected cells suggests the potential involvement of TIMP-2 in the suppression of tumor cell invasion and anti-angiogenesis by Egr-1. To test this notion, H4N cell invasion in the presence of recombinant TIMP-2 was examined. As shown in Figure 3, addition of TIMP-2 inhibited H4N cell invasion in a dose-dependent manner. Interestingly enough, no inhibition of H4N cell invasion was observed on the addition of recombinant TIMP-1.

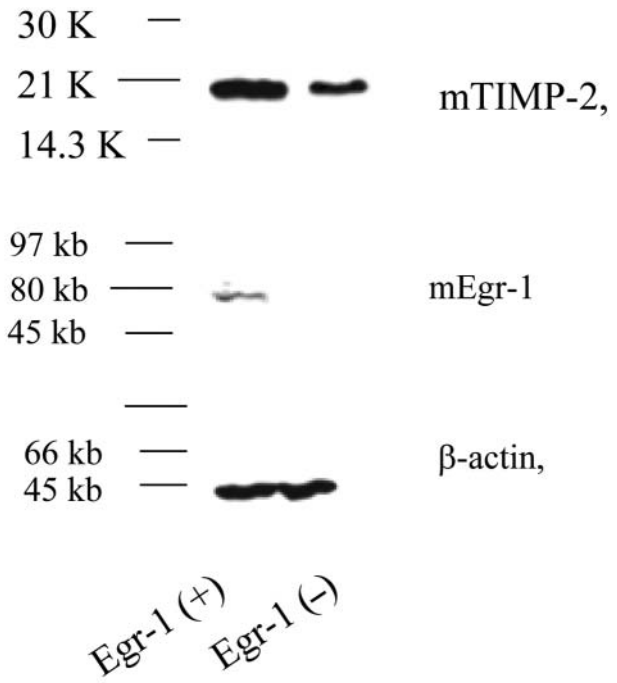

Figure 8. *Disruption of Egr-1 in vivo led to down-regulation of TIMP-2. Five-fold concentrated conditioned media (for TIMP-2) and cell lysates (for Egr-1 and β-actin) prepared from equal amount of cells were subjected to Western blot analysis using antibodies against TIMP-2, Egr-1 and β-actin, respectively.*

Anti-metastasis by Egr-1. As shown above, the expression of Egr-1 significantly reduced tumor cell growth, invasion and angiogenesis. Egr-1 also increases cell adhesion and can regulate a number of gene expressions whose activities are known to be involved in tumor metastasis. Therefore, it was reasonable to test whether Egr-1 could suppress tumor metastasis. As a first step, H4N (control-transfected) and H4E9 (Egr-1-transfected cells) were injected intravenously (*i.v.*) into the tail vein of BALB/c nu/nu mice. Twenty-one days later, the animals were sacrificed and metastatic nodules in the lungs were examined. The results showed that all three mice injected with H4N cells developed metastatic nodules in the lung (Figure 9 A) and the lungs were significantly heavier in H4N-injected mice than H4E9-injected mice ($p<0.05$, Table I). None of the three mice injected with H4E9 cells had metastatic nodules in the lungs. Immunohistochemical staining further confirmed this result (Figure 9 B). Although the number of mice was limited, the data suggested a potential role of Egr-1 in anti-metastasis.

Discussion

The development of malignant tumors is an extremely complex process during which cancer cells acquire multiple

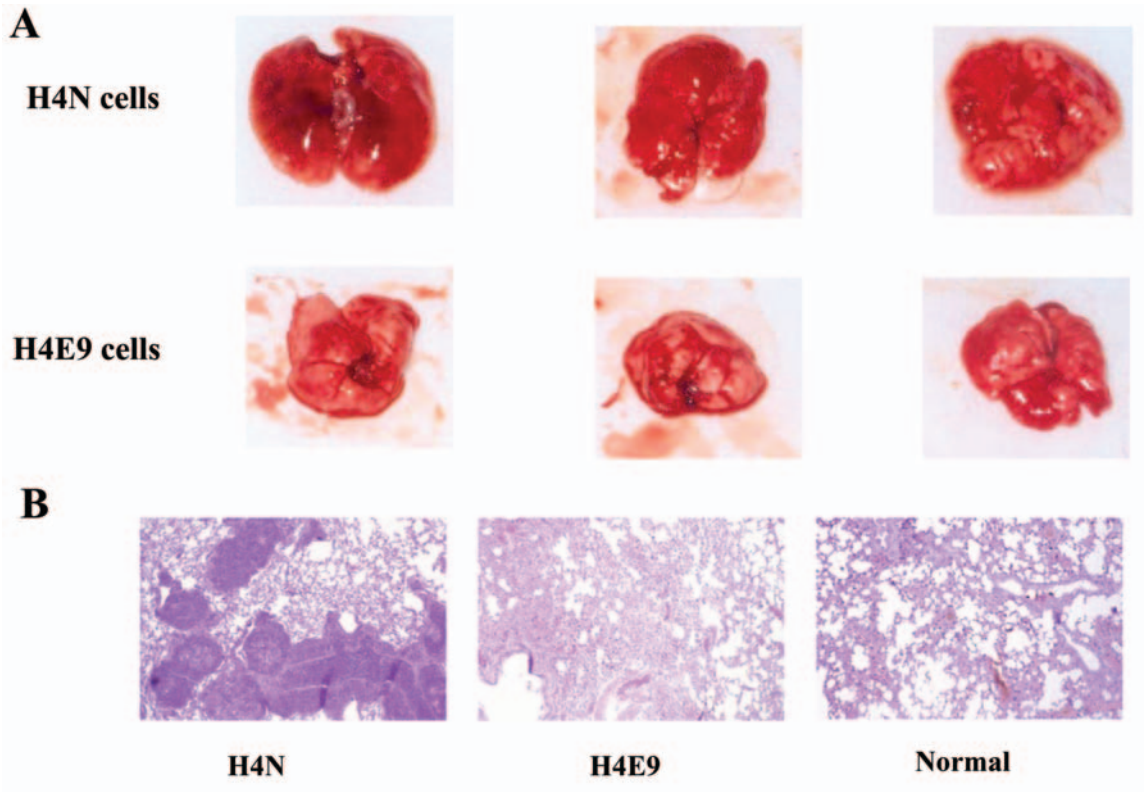

Figure 9. *Egr-1 inhibited metastasis. (A). Metastatic lung nodules in nude mice. The tail veins of mice (three for each cell line) were injected with 2x10^6 cells (H4N and H4E9). Three weeks later, the mice were sacrificed and the lungs were dissected out from the mice. (B). The lungs from mice injected with H4N and H4E9 or untreated were fixed in formalin. The sections were stained with H&E and observed under a microscope.*

alterations, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, unlimited replicative potential, sustained angiogenesis, tissue invasion and metastasis (28). At the molecular levels, those acquired changes are the result of sequential genetic and epigenetic lesions that lead to the alteration of the expression of a number of genes, which are primarily controlled by transcription factors. Several well-known tumor suppressor genes, such as p53 and WT-1, are transcription factors. The identification and characterization of transcription factors that can potentially suppress tumor growth may eventually provide a rational basis for the development of new strategies for early detection and treatment of cancer. We have previously reported that Egr-1 can also suppress certain types of cancer growth (40). This conclusion was further supported by other evidence. The expression of Egr-1 is often decreased in tumor, such as fibrosarcoma (40), breast cancer (37), lung cancer (48), glioblastoma (12, 14, 40), esophageal cancer (80, 82) and uterine leiomyoma (68), compared to normal cells. Furthermore, restoration of Egr-1 expression in those cells often reverses the transformed phenotypes. Loss of apoptosis is another characteristic of cancer. Interestingly, the function of Egr-1 has been linked to thapsigargin-inducible apoptosis in the melanoma cell line A375-6C (61) and UV-induced apoptosis in fibroblast cells (76). The Egr-1 gene has been localized to chromosome 5q23-31 (72), a region often deleted in patients with therapy-related acute myelocytic leukemia (20, 45, 75) (30). Closer examination of 5q- syndromes indicated that the Egr-1 gene was deleted in all patients tested to date (46). Finally, Egr-1 can regulate a group of genes whose functions are involved in the regulation of cell growth control (52).

Table I. *Lung weight of mice injected with Egr-1-expressing cells.*

Injected cells	Lung weight (mg)	*p* value
H4N	290±36 (3 mice)	0.005 (H4N/H4E9)
H4E9	163±15 (3 mice)	0.009 (H4N/Normal)
Normal	180±20 (3 mice)	0.820 (H4E9/Normal)

In addition to the WT-1 gene, a well-characterized tumor suppressor gene, whose product recognizes the identical DNA-binding sequence as Egr-1 (54), lot1, has extended the evidence that this Egr-1 zinc-finger family functions as a tumor suppressor gene. This protein contains seven Egr-1

homologous zinc-finger motifs. The expression of this gene is lost or decreased in ovarian tumor cell lines (1). SK1 is another Egr-1 zinc-finger-containing protein, which can suppress transformation by the potent oncogenes Ha-ras, Galpha12 and Galpha13. Knockout of zinc-finger protein Mzf-1 resulted in lethal neoplasia (25). Another immediate early gene named PC3 also has been shown to have antiproliferative activity (59). Growing evidence suggests that many zinc-finger proteins function as tumor suppressors or as negative regulators of cell proliferation, including TIEG1 (73), TIEG2 (18), GKLF (67), Reguiem (24), ZAC (10, 11), PAG608 (41), MBT163 (79), HIC (19) and so on. Consistent with this data, we found that Egr-1 zinc-finger domains were capable of inhibiting the growth of human fibrosarcoma cells (40) and v-sis-transformed mouse NIH 3T3 (35). Expression of a fusion protein containing the Egr-1 DNA-binding domain in yeast significantly retarded their growth. Mutants resulting in loss of the DNA-binding activity reverted to normal yeast growth (78). Therefore, it seems that the zinc-finger domains have a critical role in the negative regulation of tumor cell growth in some, but not all, tissues.

To further extend our previous investigation, the role of Egr-1 in cell invasion and angiogensis was examined using human fibrosarcoma cells as a model system. Our data suggested that Egr-1 may play a role in anti-invasion and anti-angiogenesis. Preliminary data also suggested that Egr-1 may suppress cancer metastasis, meaning that Egr-1 can suppress cancer development in several stages.

The molecular mechanism involved in the suppression of tumor cell invasion and angiogenesis by Egr-1 is currently unclear. Since Egr-1 is a transcription factor, it is reasonable to examine the potential Egr-1 targets by human cytokine antibody arrays (32, 34, 39). Cytokine antibody arrays revealed that TIMP-2 was up-regulated in Egr-1-transfected cells. Egr-1 knockout mouse embryonic fibroblasts accumulated less TIMP-2. The addition of recombinant TIMP-2 to H4N cells (low Egr-1 expression) inhibited cell invasion. TIMP-2 is a matrix metalloproteinase inhibitor. The matrix metalloproteinases (MMPs) play key roles in the remodeling and turnover of the extracellular matrix (9). It is well known that MMPs are required at all stages of tumorigenesis, including tumor establishment and growth, neovascularization, intravasation, extravasation and metastasis (43, 55). Through inhibition of MMPs' activity, TIMP-2 made a significant contribution to the suppression of tumor cell metastasis (6, 23). It has been reported that Egr-1 can up-regulate TIMP-2 expression in immortalized synovial fibroblasts (3). Therefore, the anti-tumor invasion and anti-angiogenesis activity of Egr-1 may be mediated through the up-regulation of TIMP-2.

Since constant expression of Egr-1 led to the induction of its corepressor NAB2 (21) and NAB-2 has been shown to block angiogenesis (31), the suppression of angiogenesis by Egr-1 in our system may also have resulted from the induction of NAB2.

In spite of the active role of Egr-1 as a tumor suppressor, demonstrated in several types of tumors, Egr-1 has been reported to play an oncogenic role in certain tumors. Prostate cancer often accumulated high levels of Egr-1 compared with normal tissues (22, 74). Knockout of Egr-1 in transgenic mouse models of prostate significantly delayed the progression of the premalignant lesion to invasive carcinoma (2). Inhibition of Egr-1 expression by antisense oligonucleotide in prostate cancer cells significantly suppressed prostate cancer cell growth (7, 8). Egr-1 may also overexpress in later stage of gastric cancer (44) and Wilms' tumor (66). Clearly, the function of Egr-1 is dependent on the cell type. Different cells may express different Egr-1-associated factors, such as NAB-2. Egr-1 may have a distinct phosphorylation status in different cells and in response to different stimuli (38). Some cancer cells may contain mutated Egr-1. The location of Egr-1 may also have been different among cancer cells and normal cells (60). These differences may contribute to the parodoxical action of Egr-1 in the regulation of tumor cell growth.

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