

## Concurrent Down-regulation of Egr-1 and Gelsolin in the Majority of Human Breast Cancer Cells

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**Abstract.** *A growing body of evidence suggests that early growth response-1 (Egr-1), a transcription factor, may function as a tumor suppressor. The aim of this study was to gain more evidence to support the role of Egr-1 in the suppression of cancer cell growth and to examine the potential correlation between Egr-1 and gelsolin. Materials and Methods: Histochemical staining coupled with breast cancer tissue arrays were used to examine the expression levels of Egr-1 and gelsolin. Reporter assays and gel shift were used to study the transcriptional activity of Egr-1 on the regulation of gelsolin. Results: Our data showed that most normal mammary tissues expressed high levels of Egr-1, while the majority of breast cancer tissues expressed very small amounts of Egr-1. The expression pattern of Egr-1 in human breast cancer tissues was highly correlated with gelsolin expression. Induction of Egr-1 by serum stimulation accompanied the increase of gelsolin expression. In cells lacking the induction of Egr-1 in response to serum stimulation, gelsolin expression remained unchanged. Furthermore, gelsolin promoter activity was profoundly reduced in Egr-1 null mouse embryonic fibroblasts compared to Egr-1 wild-type mouse embryonic fibroblasts. Gel shift experiments indicated that Egr-1 can directly bind to the gelsolin promoter. Conclusion: Our results suggest that Egr-1 may be an important breast cancer marker and that an as yet uncharacterized pathway involved in Egr-1 and gelsolin expression exists which leads to breast cancer cell development.*

Early growth response (Egr-1) is a DNA-binding transcription factor structurally related to the tumor suppressor gene Wilm's tumor gene (WT1) (1). Egr-1 expression is strongly

and rapidly induced in response to a wide spectrum of stimuli, such as serum, growth factors, UV, gamma-radiation, X-ray radiation and stress (1). Upon induction, Egr-1 binds to GC-rich consensus DNA sequences in the promoter regions of numerous genes and up-regulates or down-regulates their expression (2-4). Egr-1 may have diverse biological functions, including macrophage differentiation, neurite outgrowth, wound healing and apoptosis (5-8). Expression of Egr-1 has also been linked to a variety of diseases such as atherosclerosis, arthritis, post lung transplantation thrombosis, severe pulmonary emphysema and infertility.

Egr-1 contains DNA-binding domains, activation domains and inhibitory domains. The activity of Egr-1 is regulated by phosphorylation (9, 10), redox (11) and Egr-1 binding proteins (12, 13). Both phosphorylation (9) and redox (11) can modulate Egr-1 DNA-binding activity. Two Egr-1 binding proteins, NGF1-A binding protein 1 (NAB1) and NGF1-A binding protein 2 (NAB2) can mediate the inhibitory effect of Egr-1 on the regulation of gene expression (12, 13).

A number of studies suggest that Egr-1 can function as a tumor suppressor gene. Egr-1 can suppress the growth of a number of tumor cells, including human fibrosarcoma (14), breast cancer (14-16), glioblastoma (14, 17-19), neuroblastoma (20), hepatoma (21), esophageal cancer (22, 23), leiomyoma (24) and leukemia (25). We have previously demonstrated that Egr-1 expression was profoundly reduced in human breast cancer cells (15). To further extend this result, we examined the Egr-1 expression in human breast cancer tissues using immunohistochemical staining and examined its relationship to gelsolin expression.

### Materials and Methods

*Human breast cancer tissues.* Breast cancer tissues were obtained from the following different sources: Clinomics Biosciences (Frederick, MD, USA), NIH/NCI tissue bank and First Affiliated Hospital Sun Yat-sen University, China. Only the pathology and clinical diagnosis data were available to us.

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**Immunohistochemical staining.** Immunohistochemical staining was performed as previously described (26). Tissue sections were stained with anti-Egr-1 antibody (1:1,000 dilution) overnight at 4°C. The ABC peroxidase method of staining was then employed as described by the manufacturers (ABC; Vector Laboratories, Burlingame, CA, USA). The slides were reacted with the substrate H<sub>2</sub>O<sub>2</sub> in conjugation with diaminobenzidine. Moderate to strong expression was considered when more than 25% of cells were stained positively, while weak to negative expression when fewer than 25% of cells were stained positively. Differences of Egr-1 expression between normal and cancer tissues were statistically analyzed using Mann-Whitney test.

**Cell culture.** Mouse embryonic fibroblasts (Egr-1<sup>+/+</sup> and Egr-1<sup>-/-</sup>) were a gift from Dr. Eileen Adamson (The Burnham Institute, La Jolla, CA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Human breast cancer cell lines were in our collection, originally obtained either from Dr. D. Salomon (NCI, Bethesda, MD, USA; ZR751, MCF-7, T47D, BT-20) or the American Tissue Culture Collection (MDA-MB-157, SK-BR-3, MDA-MB-468, MDA-MB-231, HS-578t, BT-474, BT-549). Cells were cultured as described previously (13). Cells were cultured with DMEM containing 10% FCS.

**Western blotting analysis.** The Western blot assay was conducted as described by Huang *et al.* (27). Cell extracts containing equal amounts of protein were analyzed by SDS-PAGE and 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).

**Luciferase assays.** Activation of the gelsolin promoter by Egr-1 was determined by luciferase assays (Promega, Madison, WI, USA). A vector containing a 2,524 bp gelsolin promoter (pGSNLuc1) was a gift from Dr. Asch (28). Gelsolin full promoter vector (pGSNLuc1), or control vector (pGL3), and 0.5 µg of internal control (pRL-TK) were transfected into Egr-1 null mouse embryo fibroblasts and wild-type mouse embryo fibroblasts by the calcium phosphate precipitation method. The medium was changed 16 hours after transfection. Cells were grown in fresh medium for another 24 hours. Transfected cells were then harvested and luciferase activity was measured according to the manufacturer's instructions (Promega). Luciferase activity was then normalized with the internal reference control (pRL-TK).

**Gel shift assays.** All oligonucleotides including the perfect Egr-1 binding site (5-GAT CTC GCG GGG GCG AGG GGGATC [BioTEG-Q]-3), gelsolin promoter Egr-1 putative binding site (5-CGG CTC CCG CCC GCG CCC TGC CC [BioTEG-Q]-3) and mutant binding site (5-CGG CTC CaG CCC GaG CCC TGC CC [BioTEG-Q]-3) were synthesized by Santa Cruz Biotechnology (Santa Cruz, CA, USA). A total of 10 µg of nuclear extract was incubated in a final volume of 20 µL containing 20 mmol/L Hepes (pH 7.5), 70 mmol/L KCl, 0.5 mmol/L EDTA (pH 8.0), 5 mmol/L MgCl<sub>2</sub>, 100 µmol/L ZnCl<sub>2</sub>, 10% glycerol, 1 mmol/L dithiothreitol, 250 µg/ml sperm NDA and 1 µg polydeoxyinosinic-deoxycytidylic acid at room temperature for 5 min. A total of 1 pmol of biotin end-labeled oligonucleotide corresponding to Egr-1 and gelsolin

response element were added and incubated for an additional 30 min. Competition was performed with the addition of unlabeled oligonucleotides at a 50-fold molar excess and two mutant oligonucleotide with C-A substitution in the gelsolin binding site. To assess specificity, 2 µl anti-Egr-1 antibody (12) and the same amount of non-specific IgG were added to the appropriate sample after the probe addition. The reaction mixture was loaded directly onto a 5% polyacrylamide gel in 0.5X Tris-borate-EDTA buffer and analyzed at 150 V for 1 h, and the gel was transferred to nylon membrane at 300 mA 90 min then UV-crosslinked. The biotin end-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and signals were detected with an enhanced chemiluminescence (ECL) system.

**RT-PCR.** Semi-quantitative RT-PCR was used to determine the mRNA levels of Egr-1 and gelsolin in Egr-1 wild-type and null cells (29). The PCR primers used were as follows: gelsolin, sense primer: 5'-GGTGCAGAGACTCTTCCAGG-3', antisense primer: 5'-CTGTTGGAACCACACCACTG-3'; Egr-1, sense: 5'-ATCCCC GACTACCTGTTTCC-3', antisense: 5'-CCGCAAGTGGATCTT GGTAT-3'; β-actin, sense: 5'-TCGTGGGCGCTCTAGGCAC-3', antisense: 5'-TGGCCTTAGGGTTCAGGGGG-3'. All primers were purchased from Molecular Research Laboratories (Herndon, VA, USA). Total RNA (2 µg) was used for cDNA synthesis and PCR amplification according to the accompanying manual from Qiagen (Valencia, CA, USA). The expected amplified fragments for each gene were then checked by DNA agarose gel. β-Actin was amplified in tandem as an internal control. The sizes of amplified genes were 507 bp (*Egr-1*), 141 bp (*gelsolin*) and 274 (*β-actin*) respectively.

**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

**Down-regulation of Egr-1 expression in human breast cancer tissues.** We have previously reported that the expression of Egr-1 is reduced in human breast cancer cell lines and breast cancer frozen tissue sections (15). To investigate whether reduced expression of Egr-1 in cell lines reflects the patient's tissues and correlates with disease states, we performed immunohistochemical staining to examine the expression of Egr-1 in human breast cancer tissues.

Tissue arrays were first used to examine Egr-1 expression in 62 normal breast mammary tissues and 124 breast cancer tissues. All 62 normal mammary tissues expressed moderate to strong Egr-1 levels, while among 124 ductal carcinoma, 93 did not express a detectable amount of Egr-1 or expressed very low amounts of Egr-1 as shown in Figure 1. Statistical analysis indicated a high significance (*p*-value <0.001) of the difference between Egr-1 expression in normal mammary tissues and cancer tissues as shown in Table I.

One potential concern when using tissue arrays is the lack of overall representation. To avoid this problem, we used gross tissue sections to examine the expression of Egr-1 breast cancer. As shown in Table I, among normal mammary

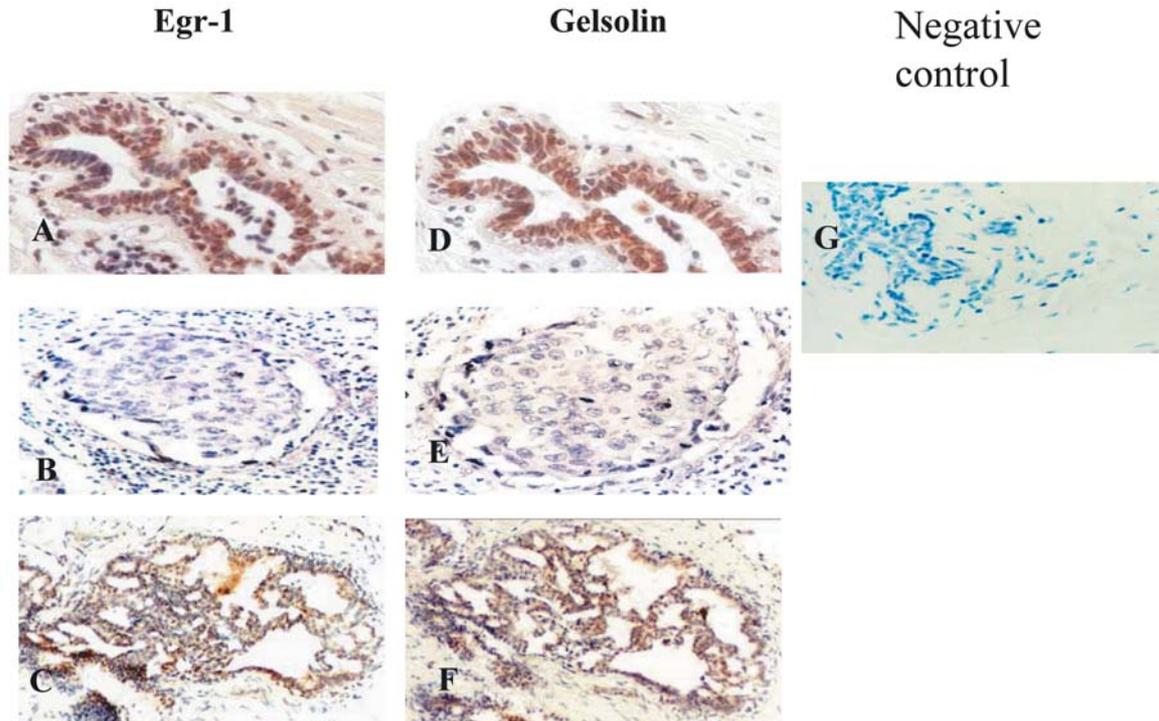


Figure 1. Immunohistochemical analysis of human breast cancer tissues with anti-Egr-1 and anti-gelsolin antibodies. Sections of formalin-fixed, paraffin-embedded tissues were stained with anti-Egr-1 antibody (A, B and C) or anti-gelsolin antibody (D, E and F) or preimmunoserum (G). A, D and G: benign tissue; B, C, E and F: breast carcinoma. Magnification: A and D x400; B, C, E, F and G x100.

tissues, 12 accumulated moderate to strong levels of Egr-1 and 3 had weak to negative Egr-1. Among 25 cancer tissues, only one expressed moderate to strong Egr-1. The difference again was highly significant with a  $p$ -value  $<0.001$ .

The results suggest that inactivation of Egr-1 may be involved in the progression of breast cancer and reduced Egr-1 expression may be a valuable biomarker for diagnosis and prognosis of breast cancer.

*Correlation of Egr-1 expression and gelsolin expression in human breast cancer tissues.* Our cDNA microarray data indicated that gelsolin is up-regulated in Egr-1 transfected cells (30) and immunohistochemical staining suggested that Egr-1 expression was profoundly reduced in human breast cancer tissues. It has been demonstrated that expression of gelsolin is significantly reduced in human breast cancer (31) and that this gene functions as both a tumor and metastasis suppressor gene (31, 32). To investigate whether Egr-1 can regulate gelsolin expression, we determined the expression of gelsolin in human breast cancer tissues by tissue arrays. As shown in Table II, all 62 normal mammary tissues showed immunoreactivity with both Egr-1 and gelsolin. Among 124 human breast cancer tissues, 23 showed strong to moderate immunoreactivity with both Egr-1 and gelsolin,

86 showed weak to negative immunoreactivity with both Egr-1 and gelsolin, 8 showed immunoreactivity with Egr-1, but not gelsolin, while 7 showed immunoreactivity with gelsolin but not Egr-1. The expression correlation between Egr-1 and gelsolin was 0.99 with a  $p$ -value  $<0.001$ .

We also took advantage of two types of breast cancer cell lines based on the Egr-1 expression in response to stimuli. In ZR75-1 and T47D cells, no Egr-1 expression was detected even in response to serum stimulation. In other breast cancer cells such as MDA-MB-231 and MCF-7, expression of Egr-1 is strongly induced by serum. Thus, if Egr-1 can regulate gelsolin expression, gelsolin should not be expressed in ZR75-1 cells and T47D cells in response to serum but in MDA-MB 231 and MCF-7 cells, gelsolin expression will be induced by serum. Indeed, as shown in Figure 2, gelsolin was only expressed in both MDA-MB-231 and MCF-7 cells in response to serum but not in ZR75-1 and T47D, suggesting that Egr-1 may regulate gelsolin expression.

These data suggest that the expression of Egr-1 and gelsolin are closely correlated. Our results suggest that Egr-1 may be an important breast cancer marker and that there exists an as yet uncharacterized pathway involved in Egr-1 and gelsolin expression, which leads to breast cancer cell progression.

Table I. *Egr-1* expression in breast cancer and normal mammary tissues.

Methods	Pathology	Expression levels*			P-value	
		No. of cases	Moderate to strong	Weak to negative		
Gross section	Normal	15	12	3	<0.001	
	Cancer	25	1	24		
Tissue arrays	Normal	62	62		<0.001	
	Cancer	Ductal	124	31		93
		Lobular	6	3		3
		Cribriform	2	2		
		Mucinous	1	1		
Medullary	1		1			
Frozen section	Normal	3	3		3	
	Cancer	3				
Western blot	Normal	4	4		6	
	Cancer	9	3			

\*The expression levels were determined by percentage of positive staining: moderate to strong (more 25% of cells stained positively) and weak to negative (<25% of cells stained positively).

*Regulation of gelsolin expression by Egr-1.* Since Egr-1 is a transcription factor, the correlation of expression between Egr-1 and gelsolin suggests that Egr-1 may regulate gelsolin expression. To investigate whether Egr-1 can directly regulate gelsolin expression, we performed several additional experiments.

First we examined Egr-1 and gelsolin expression in Egr-1 null mouse embryo fibroblasts and wild-type mouse embryo fibroblasts before and after serum stimulation. As shown in Figure 3, serum stimulation strongly induced Egr-1 mRNA in wild-type cells, accompanying induction of gelsolin expression as determined by RT-PCR. Again, this observation further supported the hypothesis that Egr-1 may regulate gelsolin expression.

Then we examined gelsolin promoter activity in both Egr-1 null mouse embryo fibroblasts and wild-type mouse embryo fibroblasts. As shown in Figure 4, the gelsolin promoter activity was strongly induced in Egr-1 wild-type cells compared with Egr-1 null cells.

To demonstrate that Egr-1 binds to the sequence within the gelsolin promoter, the putative Egr-1 binding site was used in gel shift assays. As shown in Figure 5, the Egr-1 protein was able to bind to the gelsolin Egr-1 putative binding site (CGG CTC CCG CCC GCG CCC TGC CC), but not with the mutant Egr-1 putative binding sites (CGG CTC CaG CCC GaG CCC TGC CC). The binding could be competed for with the unlabeled Egr-1 consensus motif (GCGGGGGCG). Furthermore, non-Egr-1-expressing bacterial extract did not bind to the gelsolin. In addition, the specific DNA-protein complex was

Table II. *Egr-1* and gelsolin expression in breast cancer tissues.

Egr-1	+	+	-	-	Total
Gelsolin	+	-	+	-	no. of cases
Normal tissues	62	0	0	0	62
Cancer tissues	23	8	7	86	124
Total	85	8	7	86	

Egr-1 was significantly related to gelsolin in breast cancer and normal mammary tissues by preliminary analysis of 62 normal and 125 cancer tissues (gross section and tissue microarrays)  $r=0.99, p<0.001$ .

supershifted by the addition of Egr-1 antibody, strongly suggesting that Egr-1 binds to the gelsolin promoter Egr-1 putative binding site.

## Discussion

Since we first reported that Egr-1 can suppress human tumor cell growth, growing evidence suggests that Egr-1 functions as a tumor suppressor gene (14). First, Egr-1 suppresses growth of a number of tumor cell types both *in vitro* (tissue culture) and *in vivo* (tumorigenicity assay in nude mice), including human fibrosarcoma, glioblastoma and breast cancer cell lines (14, 15). Other studies also demonstrated that Egr-1 can inhibit the growth of human glioblastoma (18, 19), neuroblastoma (20), hepatoma (21), esophageal cancer (22, 23), leiomyoma (24) and leukemia (25). Secondly, expression of Egr-1 was significantly reduced in a number of tumor cells such as fibrosarcoma (14), breast cancer (15), lung cancer (33), glioblastoma (15, 17-19, 46), esophageal cancer (22, 23), thyroid carcinoma (34) and leiomyoma (24). In addition, cancer cells often become resistant to apoptosis. Interestingly, Egr-1 plays an important role in thapsigargin-inducible apoptosis (35), UV-induced apoptosis (36), calyculin A-induced apoptosis (37) and promotion of apoptosis in human neuroblastoma cells (20). Egr-1 also inhibits angiogenesis (29, 38) which is important for tumor cell growth. A number of anticancer agents, such as genistein (39), COX inhibitor (40), curcumin (41) and trifluoperazine (19), function through modulation of Egr-1. Genetic evidence suggests that Egr-1 was deleted in malignant myeloid (42) and ER-negative human breast carcinoma (16). Furthermore, Egr-1 can regulate the coordinated expression of a set of genes, including *PTEN* (36), *TGF-β1*, *Bcl-2*, *fibronectin (FN)* and *plasminogen activator inhibitor-1 (PAI-1)* (43), all of which are important to normal growth control (44). Lastly, growing evidence suggests that many zinc finger proteins function as tumor suppressors or as negative regulators of cell proliferation. Our finding that expression of Egr-1 was significantly

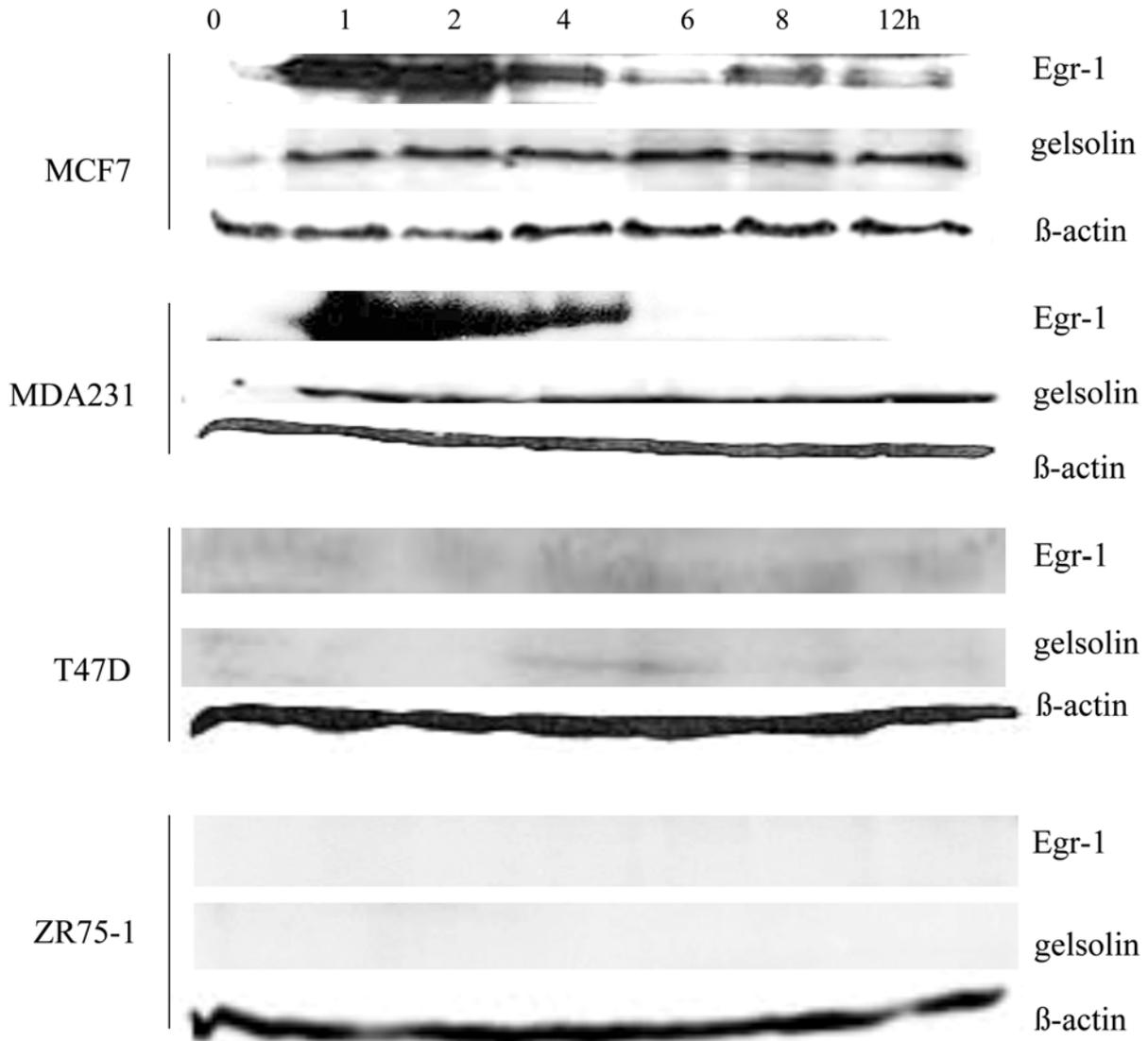


Figure 2. Time-course of expression of Egr-1 and gelsolin in human breast cancer cell lines stimulated by serum. Different human breast cancer cell lines were seeded at  $2 \times 10^5$  cells per dish. After 2 days, complete cell culture media were replaced with 0.2% FCS media for overnight. Cells were then stimulated with 20% FCS for different times. The expression levels of Egr-1 and gelsolin were analyzed by Western blot. Beta-actin was used as control for loading normalization.

reduced in human breast cancer cells in this report further support the role of Egr-1 as a tumor suppressor gene.

Interestingly, our data show that both Egr-1 and gelsolin were often deregulated simultaneously. Since Egr-1 is transcription factor, it is possible that Egr-1 may regulate gelsolin expression. Some experimental evidence supports this notion. Expression of Egr-1 and gelsolin was highly correlated in human breast cancer tissues as demonstrated in this study. cDNA microarray data indicate that Egr-1 can up-regulate gelsolin expression (30). Furthermore, serum stimulation only induced gelsolin expression in cells which Egr-1 expression is

induced by serum. It has been reported that gelsolin promoter activity is much higher in normal mammary cells (184A1N4) than in breast cancer cells (T47D and MCF-7) (28), consistent with our finding that Egr-1 was highly expressed in 184A1N4 cells compared with T47D and MCF-7 cells. In addition, Egr-1 can stimulate gelsolin promoter activity and directly bind to the gelsolin promoter. Furthermore, no induction of gelsolin expression in Egr-1 null embryonic fibroblasts was observed in contrast to Egr-1 wild-type fibroblasts. All these observations suggest that gelsolin is an important target of Egr-1 in human breast cancer cells.

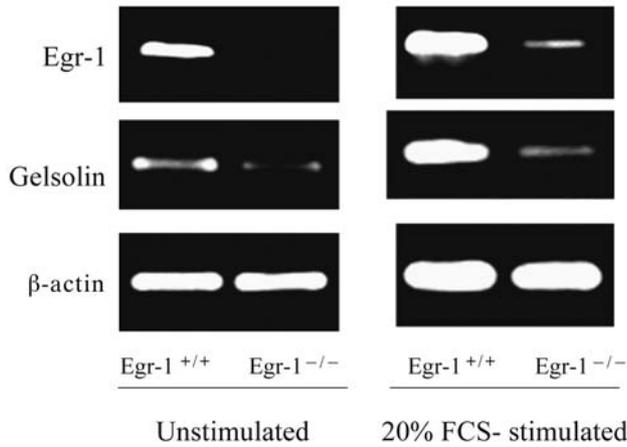


Figure 3. Analysis of gelsolin expression in *Egr-1* null cells by RT-PCR. Two  $\mu\text{g}$  of total RNA prepared from unstimulated and 20% FCS-stimulated cells were subjected to RT-PCR analysis as described in Materials and Methods.

Gelsolin is an important cytoskeleton-regulated factor. It can directly bind to actin, through which regulation of actin activity may control cell mobility and thereby inhibit tumor

growth and metastasis (45). Expression of gelsolin is frequently reduced in human cancer tissues, particularly in human breast cancer tissues (31). It is interesting to see whether down-regulation of gelsolin may be responsible for at least part of the tumor suppressive activity of *Egr-1*.

Five different approaches have been used to examine the *Egr-1* levels in breast cancer cells and tissues: Frozen tissue sections, paraffin tissue sections, tissue arrays, Western blot and Northern blot. Our results suggest that expression of *Egr-1* was profoundly reduced in human breast cancer cells and tissues. The results are summarized in Tables I and II. Reduced *Egr-1* expression had been observed in human breast cancer cell lines, breast cancer tissues, transformed mouse cells and DMBA-induced rat breast cancer tissues. Thus, a decrease of *Egr-1* expression may play an important role in the development of breast cancer and serve as a biomarker. The molecular mechanism responsible for the down-regulation of *Egr-1* in human breast cancer cells remains to be defined. Reduced expression of *Egr-1* mRNA in human breast cancer cells and tissues suggests transcriptional regulation. Southern blots did not reveal detectable changes, suggesting that gene deletion is not the cause for such low levels of *Egr-1* in breast cancer cells. DNA

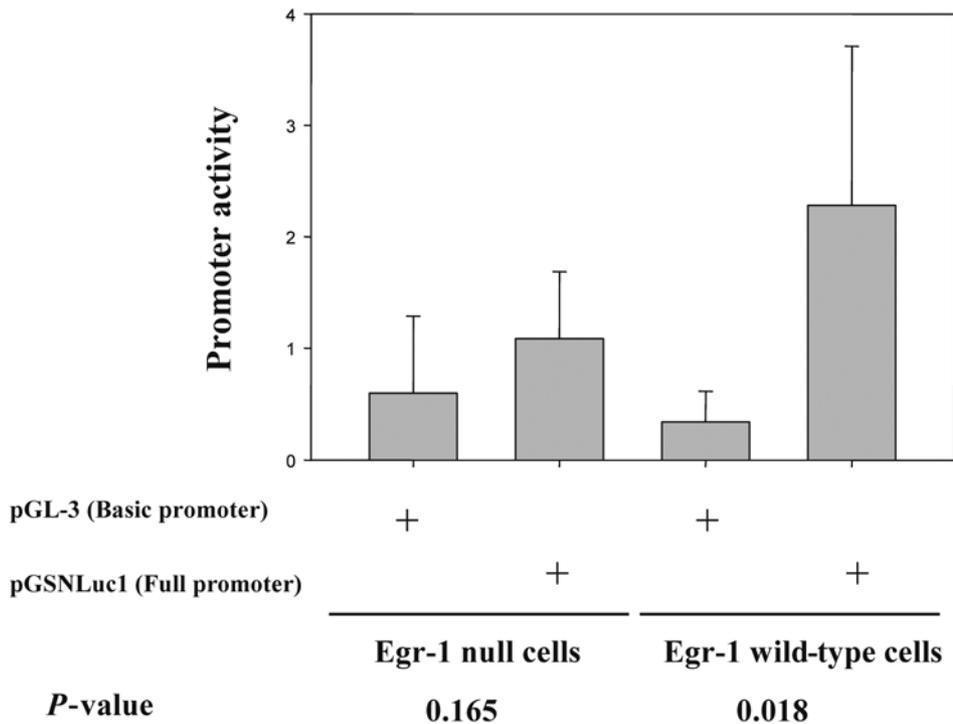


Figure 4. Stimulation of gelsolin promoter activity in *Egr-1* wild-type cells. Two  $\mu\text{g}$  of gelsolin promoter vector (*pGSNLuc1*) and control vector (*pGL-3*) were transfected together with internal control vector (*pRL-TK*) into *Egr-1* null cells and *Egr-1* wild-type cells. The medium was changed 16 hours after transfection. Cells were grown in fresh medium for another 24 hours. Transfected cells were then harvested and luciferase activity was measured according to manufacturer's instruction (Promega). Luciferase activity was then normalized with internal reference control (*pRL-TK*).

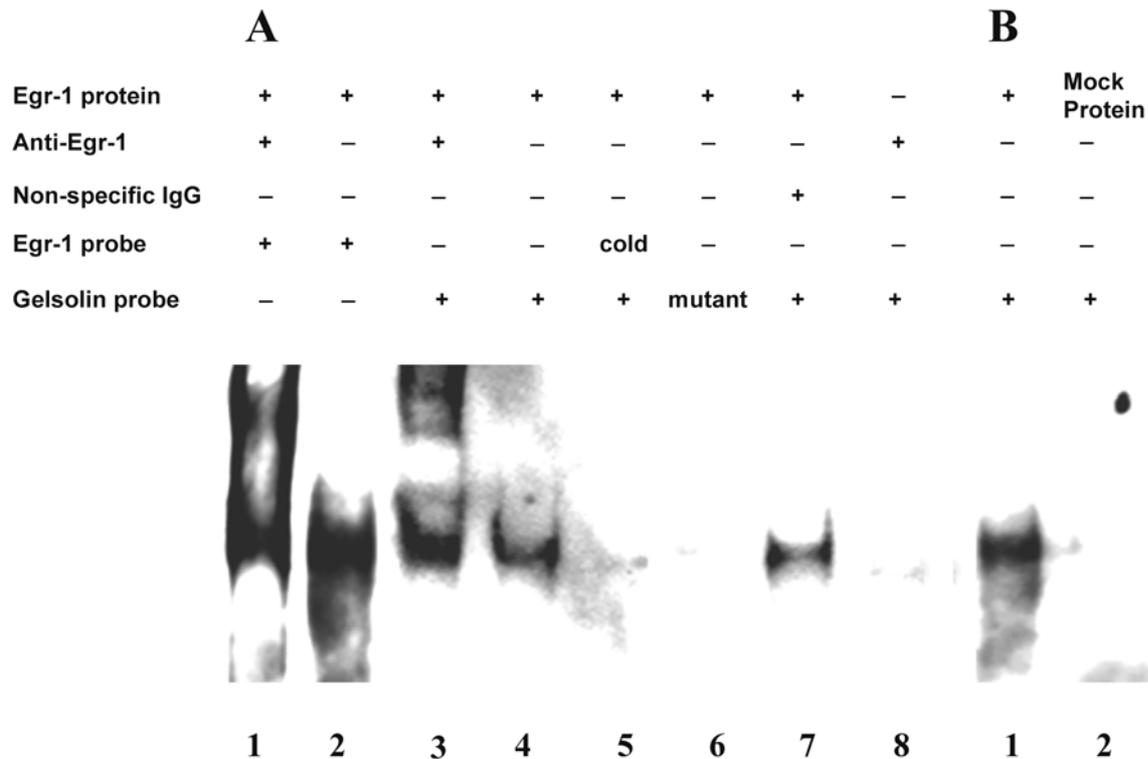


Figure 5. Gel shift analysis of candidate Egr-1 binding site in gelsolin promoter. An oligonucleotide derived from the gelsolin promoter region, a corresponding mutant oligonucleotide and a perfect Egr-1 binding site were labeled with biotin and used to test the DNA binding activity to the Egr-1 fusion protein. A, Egr-1 perfect binding site was used as reference (Lane 1 and lane 2). Addition of anti-Egr-1 led to supershift (Lane 1). Putative Egr-1 binding sequence derived from gelsolin promoter also bound to Egr-1 fusion protein (Lane 4) with supershift by anti-Egr-1 antibody (Lane 3). The complex was eliminated by addition of cold perfect Egr-1 binding site (Lane 5). Mutant gelsolin probe did not bind to Egr-1 fusion protein (Lane 6). Non-specific IgG did not result in supershift (Lane 7). Anti-Egr-1 antibody alone did not show any non-specific binding (Lane 8). B, Mock fusion protein failed to bind to putative Egr-1 binding site (Lane 2) in contrast to Egr-1 fusion protein (Lane 1).

sequencing did not reveal any change in the Egr-1 coding region and promoter region (data unpublished). Like many tumor suppressor genes, methylation may be the mechanism responsible for inactivation of Egr-1 in breast cancer tissues.

In summary, our results demonstrate widespread coincident deregulation of Egr-1 and gelsolin among human breast cancers and Egr-1 may regulate gelsolin expression. These observations suggest that a new pathway involved in down-regulation of Egr-1 and gelsolin may exist in human breast cancer cells.

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