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Tissue, Cell Type, and Breast Cancer Stage-Specific Expression of a TGF-β Inducible Early Transcription Factor Gene

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Abstract This laboratory has previously identified a novel TGF-β inducible early gene (TIEG) in human osteoblasts [Subramaniam et al. (1995): Nucleic Acids Res 23:4907–4912]. Using TIEG specific polyclonal antibody and immunoprecipitation methods in normal human fetal osteoblast cells (hFOB cells), we have now demonstrated that TIEG encodes a 72-kDa protein whose levels are transiently increased at as early as 2 h of TGF-β treatment. Polarized confocal microscopic analysis of hFOB cells shows a nuclear localized TIEG protein in untreated cells under the conditions described under Methods. Interestingly, the levels of TIEG protein in the nuclei increase when the cells are treated with TGF-β_1 for 2 h. In contrast, similar analyses of untreated human keratinocytes show a cytoplasmic localized TIEG protein that appears to be translocated to the nucleus after H_2O_2 treatment. Additional immunohistochemical studies have demonstrated that TIEG protein is expressed in epithelial cells of the placenta, breast, and pancreas, as well as in osteoblast cells of bone and selected other cells of the bone marrow and cerebellum with some cells showing a cytoplasmic localization and others a nuclear localization. All cells of the kidney display negative staining for this protein. Interestingly, a stage specific expression of TIEG protein is found in a dozen breast cancer biopsies, using immunohistochemistry. The cells in normal breast epithelium displays a high expression of TIEG protein, those in the in situ carcinoma display less than one-half of the levels, and those in the invasive carcinoma show a complete absence of the TIEG protein. TIEG has been localized to chromosome 8q22.2 locus, the same locus as the genes involved in osteopetrosis and acute myeloid leukemia and close to the c-*myc* gene locus and a locus of high polymorphism in cancer biopsies. The correlation between the levels of TIEG protein and the stage of breast cancer, its prime location in human chromosome 8q22.2, and past studies with pancreatic carcinoma, suggests that TIEG may play a role in tumor suppressor gene activities, apoptosis, or some other regulatory function of cell cycle regulation. J. Cell. Biochem. 68:226–236, 1998.

Key words: TGF-β; transcription factor; rapid regulation; tumor suppressor; osteoblasts; immunohistochemistry; breast cancer stage

The transcription factors are categorized into different classes, depending on their structural motifs. One such structural motif gaining much recent attention is the "zinc finger"-containing transcription factors. The zinc finger-containing motifs were first identified in xenopus transcription factor TFIIIA [Miller et al., 1985]. The well-characterized zinc finger-containing transcription factor genes are SP-1 [Mermod et al., 1989], Wilms' tumor [Call et al., 1990; Gessler et al., 1990], Egr-1 [Sukhatme et al., 1988], steroid hormone receptor family [Evans, 1988], and Zif 268 [Christy et al., 1988]. These zinc finger proteins have been shown to be involved

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in the transcription of other genes. The c-Krox gene, a mouse homologue of the *Drosophila* Krüppel segmentation gene, is known to regulate the α1(I) collagen gene [Galera et al., 1994]. The NGFI-C, a nerve growth factor-induced early response, gene is induced within minutes after the nerve growth factor treatment in PC12 cells [Crosby et al., 1991]. This gene is also induced in brain after a metrazol-induced seizure.

Another member of the zinc finger transcription factor gene, growth factor independence-1 (Gfi-1), is activated by provirus integration in T-cell lymphoma cell lines selected for interleukin independence in culture [Zweidler-McKay et al., 1996]. The zinc finger binding protein of the serum response element in the c-*fos* gene (SRE-ZBP) is another member of the zinc finger transcription factor family and might play an important role in the positive and negative regulation of this gene [Attar and Gilman, 1992]. One member of the Krox gene family, Krox-20, is a growth factor-induced early gene known to be involved in the G_0/G_1 transition of the cell cycle [Chavrier et al., 1988]. Mouse Krox-20 gene knockout studies have demonstrated that the animals develop skeletal abnormalities in the newly formed bone and a drastic reduction of calcified trabeculae and severe porosity and hind brain abnormalities [Levi et al., 1996].

Recent studies in our laboratory have identified a novel TGF-β inducible early gene (TIEG) in human osteoblasts [Subramaniam et al., 1995]. This gene encodes a 480-amino acid protein and contains three zinc finger motifs at the C-terminal end. The zinc finger region of this gene shows homology to known three zinc finger containing transcription factor family of genes, e.g., SP-1, BTEB, and Wilms' tumor. This laboratory has reported that the mRNA steady state level for this gene is induced as early as 30 min after TGF-β treatment in human osteoblasts, with a maximal induction at 1–2 h [Subramaniam et al., 1995]. This induction displays a growth factor specificity in osteoblasts, with TGF-β, BMP-2, and EGF as the major inducers, while other growth factors and cytokines show a minimal effect. The induction of TIEG mRNA levels occurs at the level of transcription and is independent of protein synthesis. The mRNA steady-state levels are shown to be high in several whole human organs (heart, liver, muscle, placenta, pancreas) but low in others (kidney, brain, lung). In this paper, we use a new PAb to the TIEG protein for immunohistochemistry, immunoprecipitation, and immunofluorescence confocal microscopy in order to determine basal expression of TIEG in specific human tissues and cell types, the size of the TIEG protein, its intracellular location, and the chromosome location of the gene.

METHODS

Preparation of Anti-TIEG Polyclonal Antibody

Since the primary structure of TIEG protein is known, the amino acids spanning 134–154 were synthesized in our institutional Protein Core Facility. The peptides were KLH conjugated and sent to Cocalico Biologicals (Reamstown, PA) to synthesize a polyclonal antibody (TIEG-228). Epitope purification of TIEG antibody was performed. In brief, PAb TIEG-228 was centrifuged at 3,000*g* for 30 min at 4°C. To the supernatant was added an equal volume of saturated ammonium sulfate in phosphate-buffered saline (PBS), pH 8.0, stirred for 6 h at 4°C. The solution was centrifuged at 3,000*g* for 30 min, the pellet was resuspended in TBS and dialyzed for 18 h with several changes of TBS buffer. The dialyzed protein was allowed to bind to a TIEG peptide-coupled Sepharose 4B column. The IgG was eluted from the column using 3.0 M sodium thiocyanate, 20 mM Tris, pH 7.6, 1.0 M sodium chloride. The eluate was dialyzed against several changes of 50 mM ammonium bicarbonate for 18–20 h at 4°C. The dialyzed antibody was lyophilized and reconstituted in PBS. The purified antibody was used in the immunoprecipitation and immunohistochemical studies.

In Vitro Translation of TIEG Protein

The coding region for TIEG cDNA was polymerase chain reaction (PCR) amplified using a specific 5′ and 3′ primers to the cDNA. The 5′-primer was incorporated with a T7 phage promoter sequence, so that when transcribed, this should generate a sense transcript. The in vitro transcription, capping of RNA, and in vitro translation of TIEG protein was performed using the procedure as described in protocols and application guide from Promega (Madison, WI). In brief, the transcription reaction contained the following: 5× transcription buffer, 100 mM DTT, ribonuclease inhibitor, 10 mM rNTP mix, 5 mM mG(5′)PPP(5′)G, DNA

template (1 mg/ml), and T7 RNA polymerase. The transcription reaction was performed at 37°C for 1 h. Following the reaction, RQ1 RNase free DNase was added to the reaction mixture and incubated for an additional 15 min at 37°C. The reaction mixture containing RNA was phenol : chloroform extracted and ethanol precipitated. For in vitro translation of TIEG protein, the reaction mixture contained nuclease-treated rabbit reticulocyte lysate (Promega), capped TIEG sense RNA transcript (0.3 µg), 1 mM amino acid mixture (minus methionine), ribonuclease inhibitor, and [^{35}S]methionine (10 mCi/ml). The reaction mixture was incubated at 30°C for 2 h. After the incubation period, a portion of the lysate was separated by 10% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was fluorographed and exposed to an x-ray film.

Immunoprecipitation

Human fetal osteoblast-like (hFOB) cells were routinely maintained in DMEM + F12 containing 10% (v/v) fetal bovine serum (FBS) media and 300 µg/ml geneticin. The cells were then plated on 100-mm culture dishes and allowed to grow to 90% confluency. At this stage, the cells were washed and incubated in 0.25% (w/v) bovine serum albumin (BSA) containing media for 48 h. After this incubation period, the media was changed to DMEM without methionine, and the cells were treated with either vehicle [(0.25% (w/v) BSA in PBS)] or TGF-β_1 (2 ng/ml) for 2 h in the presence of 150 µCi/ml of [^{35}S]methionine. The treated cells were lysed in 0.5 ml of lysis buffer (0.5% (v/v) SDS, 0.05 M Tris–HCl, pH 8.0, and 1 mM DTT), transferred to an Eppendorf tube and boiled for 5 min. The cell lysate was diluted with 4 vol of RIPA correction buffer and centrifuged for 90 min at 26,000*g*. The affinity purified PAb TIEG-228 (4 µg) was added to aliquots of the supernatants containing equivalent CPM and incubated on a rocker at 4°C for 2–4 h. Following this incubation, 60 µl of a 50% (w/v) protein A–Sepharose bead slurry (Pharmacia) was added and the mixture was incubated at 4°C for 2 h. The immunocomplex was washed 4 times with RIPA buffer [(PBS, 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS)]. Finally, the beads were resuspended in 80 µl of SDS sample buffer, boiled to dissociate the TIEG protein and the supernatant was loaded onto a 5–15% gradient SDS-polyacrylamide gel electrophoresis (PAGE). For in vitro translated TIEG protein, the translated reticulocyte lysate was made up to 1 ml in RIPA buffer and immunoprecipitated using PAb TIEG-228 as described above.

Immunohistochemical Analysis

Immunoperoxidase staining for TIEG protein in formalin-fixed paraffin-embedded tissue sections was performed using the following labeled streptavidin-biotin method: 6-µm sections were mounted on silanized glass slides, deparaffinized, and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2. Sections were immersed in 10 mM sodium citrate buffer, pH 6.0, and subjected to heat-induced antigen retrieval. Sections were then treated with 10% (v/v) normal goat serum for 10 min to block nonspecific protein binding. PAb TIEG-228 (1 µg/ml) was applied, and tissue sections were incubated for 1 h at room temperature. After brief rinsing, the sections were treated with biotinylated antirabbit IgG for 30 min at room temperature, rinsed, and then incubated with peroxidase-labeled streptavidin for 30 min at room temperature. After a brief washing, sections were incubated with aminoethylcarbazole (AEC) and H_2O_2 for 15 min. Sections were lightly counterstained with hematoxylin and coverslipped with aqueous mounting media (Kaiser's glycerol jelly). Negative controls included non-immune rabbit IgG in place of specific antibody and antibody preabsorbed with the immunizing peptide.

Immunofluorescence Scanning Confocal Microscopy

Cells grown on Permonex chamber slides (Nunc, Naperville, IL) were fixed for 10 min in ice-cold acetone. Subsequently, sections were incubated with PAb TIEG-228 (1 mg/ml) in a humidifier chamber for 30 min at room temperature. Cells were extensively rinsed with PBS and were incubated with both the secondary antibody and propidium iodide (2 mg/ml) for 30 min at room temperature. A goat antirabbit antibody, conjugated with dichlorotriacinyl aminofluorescein (DTAF) (Jackson ImmunoResearch, West Grove, PA), was used at a dilution of 1 : 200. Following extensive washing with PBS, the slides were mounted in glycerol solution containing paraphenylenediamine and analysed with a laser scanning confocal microscope (LSM 310, Carl Zeiss, Oberkochen, Germany)

equipped with an argon/krypton laser tuned to an excitation wavelength of 488 nm. An emission filter of 530 nm was used in front of the photomultiplier tube. The objective lens was a Zeiss plan-neofluar 40×, oil immersion. Images were digitized and stored at 512 × 512-pixel resolution.

Chromosomal Localization of TIEG

Human lymphocytes were stimulated with phytohemagglutinin M and cultured for 72 h at 37°C. The cells were dropped onto slides, to optimize chromosome spreading and morphology. The genomic DNA probe (12 kb) was labeled with biotin-UTP by the nick-translation technique (Gibco BRL). The labeled DNA probe was used to hybridize to normal human male metaphase with or without G banding. Following harvest, the slides were aged in 90°C oven for 8 min. Aged slides were treated with 2× SSC at 37°C for 1 h and dehydrated in 70%, 85%, and 100% (v/v) ethanol. Chromosomal DNA was denatured in 70% (v/v) formamide at 70°C for 1 min, and the probe DNA was denatured in hybridization solution [50% (v/v) formamide, 10% (v/v) dextran sulfate in 2× SSC] in a 70°C waterbath for 5 minutes. Following denaturation, the preparation was dehydrated in 70%, 85%, and 100% (v/v) ethanol and air dried. Probe DNA was hybridized to each slide for 16 hours at 37°C in a humidifier chamber. The biotin was detected by FITC/avidin, following post-hybridization washes (2× SSC at 70°C and rinsed in PBS). The preparation was counterstained with 100 ng/ml DAPI in antifade mounting media. The analysis and photography were performed either by the Vysis Smart Capture or by Oncor Imaging System involving digitized images from a fluorescent microscope. The counterstain was pseudo-colored red, in some cases, to enhance the contrast between the counterstain and the signal.

RESULTS

The expression of the TIEG protein was determined in paraffin-embedded sections of human kidney, placenta, bone marrow, pancreas, cerebellum, and bone tissues (Figs. 1, 2). A polyclonal antibody against a TIEG peptide was developed and shown to be specific for TIEG by competitive binding of the TIEG protein and peptides. Using this antibody (Ab) in an immunoperoxidase procedure, a cytoplasmic or nuclear staining of selected cell types is shown in most tissues. The nonspecific staining is negligible and all positive staining is eliminated by omission of the primary antibody or by preabsorption of TIEG antibody with the immunizing peptide. In the kidney, there are no immunoreactive cells (Fig. 1A). However, in human placenta (Fig. 1B), an intense labeling of the cytoplasm of syncitiotrophoblast cells is observed with no staining of cytotrophoblast, endothelial, or red blood cells (RBCs). In bone marrow (Fig. 1C), TIEG protein is detected in nuclei and cytoplasm of osteoblasts, megakaryocytes, macrophages/monocytes, and possibly osteoclasts. Granulocytic cells and their precursors are negative as are erythroid precursors and RBCs. Macrophages associated with clusters of erythroid precursors, however, do stain positively. In human pancreas (Fig. 1D), intense cytoplasmic staining is observed in cells of islets of Langerhans and also occasionally in scattered cells of pancreatic acini. In the cerebellum (Fig. 1E), the cell nuclei in the white matter stain positively for TIEG protein, as do a layer of cells at the interface of the molecular and granular layers. Unlike other cell types that display only cytoplasmic labeling, these cells show nuclear positivity. Although the exact nature of the cell layer is unknown, they also stained positively for S100, but not for synaptophysin, suggesting a glial origin (Bergman glia). The Purkinje cells are negative for TIEG protein. Immunohistochemical analyses were also performed on decalcified human bone sections. As shown in Figure 2, the osteoblasts show an intense nuclear staining for TIEG protein, wheras the preosteoblasts and stromal cells show a weak or no staining, indicating the presence of high levels of TIEG protein in the mature osteoblasts.

To identify TIEG protein on SDS–PAGE, the sense RNA was in vitro translated in rabbit reticulocyte lysate in the presence of [^{35}S]-methonine and the protein analyzed on SDS–PAGE. As shown in Figure 3, a 57-kDa protein is observed only in the RNA-added lysate. The PAb TIEG-228 was then used to immunoprecipitate the in vitro translated TIEG protein, a 57-kDa protein is again detected (Fig. 3). The TGF-β_1 treatment of human osteoblasts was previously shown to maximally induce TIEG mRNA levels at 1–2 h of the growth factor treatment [Subramaniam et al., 1995]. Therefore, it was of interest to determine whether this increase in mRNA levels is also reflected by

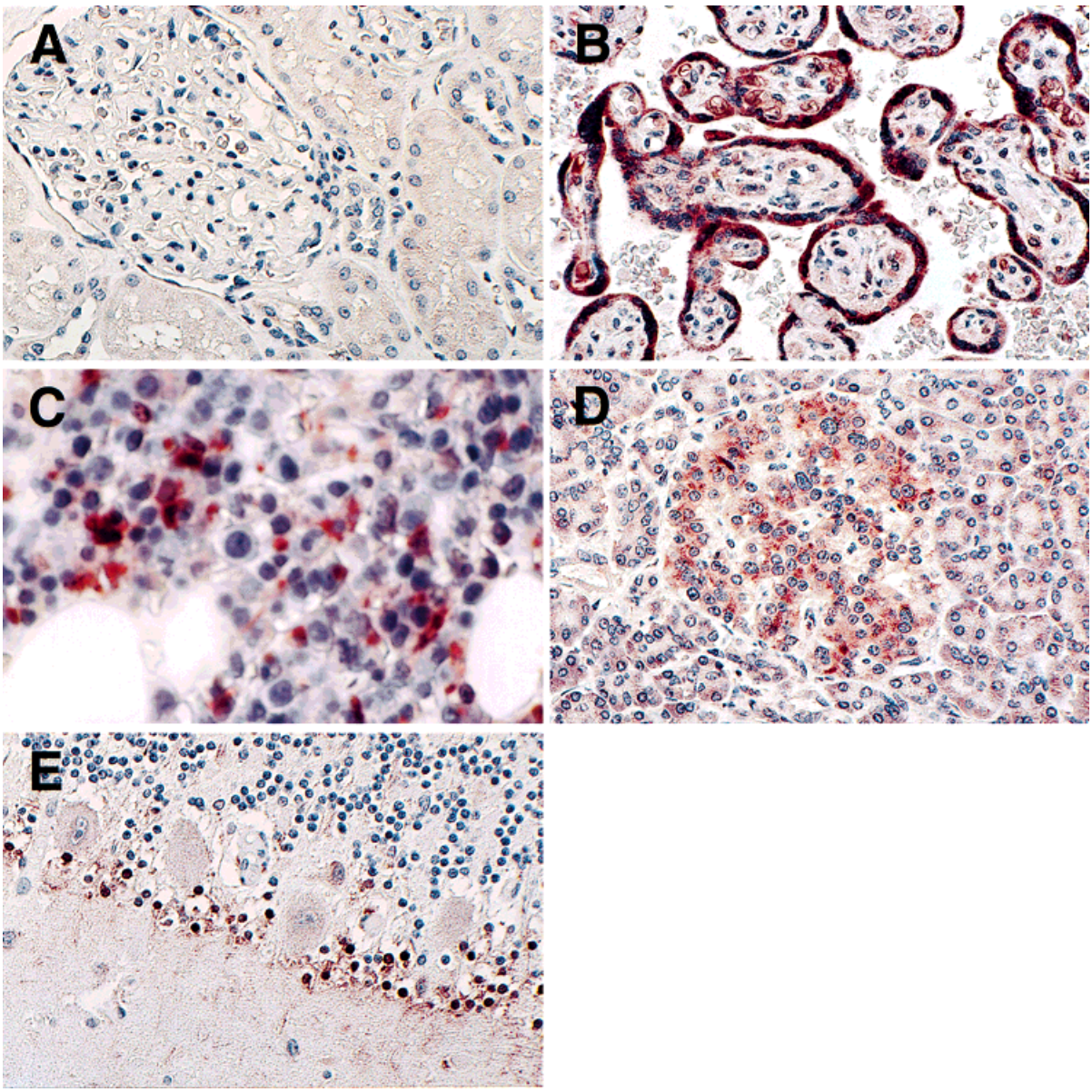

Fig. 1. Immunohistochemical staining of human tissues. Sections of kidney (**A**), placenta (**B**), bone marrow (**C**), pancreas (**D**), and cerebellum (**E**) were immunostained using TIEG polyclonal antibody; the slides were photographed. A, B, D, E: ×100; C: ×150.

an increase in TIEG protein. The hFOB cells were treated with TGF-β_1 for 2 h in the presence of [^{35}S]methonine and the cell lysates immunoprecipitated and analyzed as above on SDS–PAGE. As shown in Figure 4, the levels of the 57-kDa TIEG protein are shown to be induced by TGF-β treatment. Specifically, the 57-kDa TIEG protein is observed in the 2-h lysates of the TGF-β-treated cells, but not in the vehicle-treated cells. The antibody also precipitates other proteins in the vehicle and TGF-β-treated cells, but none of these displays a TGF-β regulation. It should be mentioned here that more detailed resolution of the [^{35}S]methionine labelled-TIEG protein using gradient PAGE more accurately determines a molecular mass of the TIEG protein at 72 kDa (data not shown).

The primary sequence of TIEG protein contains three zinc finger motifs at the C-terminal region which has homology in this region to other known transcription factors. Since most transcription factors are localized in the nucleus, and since the TIEG protein also contains a nuclear localization signal at amino

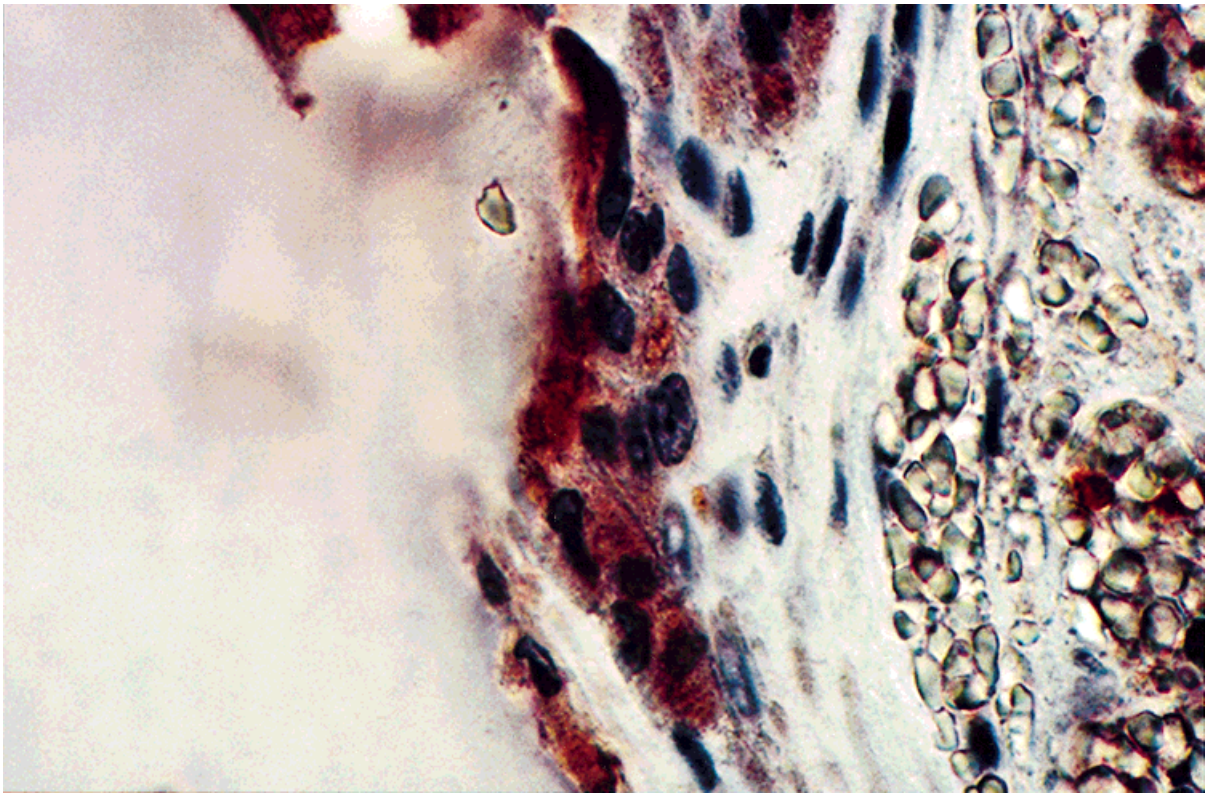

Fig. 2. Immunohistochemical staining of human bone. Immunohistochemical staining of human bone sections were performed as mentioned under Methods; the slides were photographed. ×200.

acids 186–198, immunofluorescence scanning confocal microscopy was performed to determine whether the newly synthesized, induced protein was localized in the nucleus of the hFOB cells both before and after TGF-β_1 treatment. The cells were serum starved and treated with TGF-β_1 for 2 h, fixed, and immunofluorescence analyses performed using a Zeiss laser scanning confocal microscope. As shown in Figure 5, the vehicle-treated cells show a diffuse nuclear staining for TIEG protein, while the TGF-β-treated cells show intensive nuclear staining. In contrast, in untreated human keratinocytes the TIEG protein is localized in the cytoplasm (Fig. 6). When these cells are treated with a TGF-β mimicking agent, H_2O_2 for 40 minutes, the TIEG protein appears to be translocated to the nuclei. Studies by Ohba et al. [1994] have shown that TGF-β induces H_2O_2 production, which in turn acts as a mediator for the TGF-β action in osteoblastic cells. These results demonstrate that there is not only an increase in synthesis of the TIEG protein, but also concurrent enhanced staining in and possible translocation of the TIEG protein to the nucleus after the growth factor treatment. As a preliminary step in assessing the biological functions of TIEG, we examined the expression of TIEG in various stages of breast carcinoma. A possible role of a gene in the cell cycle control and cancer is sometimes achieved by this approach. The neoplastic epithelial cells of an intraductal comedo carcinomas (Fig. 7B) display less intense cytoplasmic staining compared to the benign (normal) epithelium (Fig. 7A), while cells of an invasive ductal carcinomas are negative for TIEG protein expression (Fig. 7C). As shown in Figure 8, the TIEG maps to chromosome 8q22.2 locus. Future analyses of mutations in this locus (e.g., deletion, loss of heterozygosity) in breast cancer biopsies may provide more information regarding TIEG expression in different stages of the disease.

DISCUSSION

As outlined in the introduction, TGF-β is both an autocrine and a paracrine growth factor for many cell types. In bone tissue, TGF-β is produced by both osteoblasts and osteoclasts and is stored in large quantities in the bone

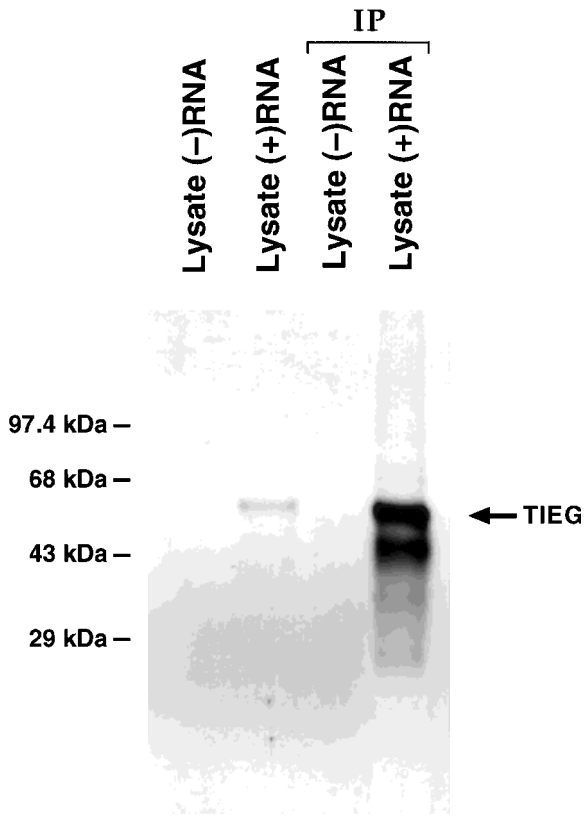

Fig. 3. Immunoprecipitation of the in vitro synthesized TIEG protein. The coding sequence of TIEG cDNA was PCR amplified and transcribed to generate the sense transcript. The sense RNA was in vitro translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. A portion (5 µl) was directly loaded onto the gel, with the remainder immunoprecipitated using TIEG-228 PAb. The immunoprecipitate was separated on a 10% SDS–PAGE, fluorographed, and exposed to X-ray film. Lysate (−) RNA, no RNA was added; IP-immunoprecipitate of (−) and (+) RNA added lysates.

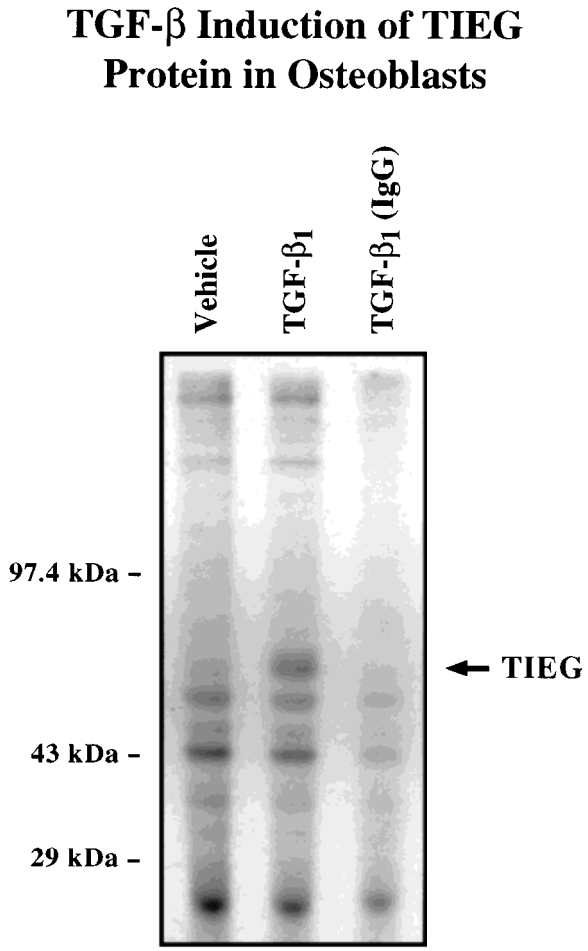

Fig. 4. Induction of TIEG protein levels in FOB cells by TGF-β. Human fetal osteoblastic cells were serum starved for 48 h and then treated with TGF-β (2 ng/ml) and vehicle (0.25% BSA in PBS) for 2 h in the presence of [^{35}S]methionine. The cells were lysed and immunoprecipitated using PAb TIEG-228 or normal rabbit IgG. The immunoprecipitates of vehicle and TGF-β-treated samples were separated on a 10% SDS–PAGE, fluorographed and exposed for X-ray film.

matrix, where it is believed to play an important role in the coupling of bone resorption to bone formation. The TIEG was originally identified in human osteoblasts as a TGF-β-inducible early gene and its presence in osteoblasts might indicate that this protein plays an important role in TGF-β signaling and osteoblast growth and differentiation [Subramaniam et al., 1995]. This paper characterizes the TIEG protein with respect to size, cell-specific expression, and intracellular distribution. The in vitro cDNA translates and the immunoprecipitated both identify a 57-kDa TIEG protein. A more refined gradient SDS–PAGE system indicates a MW of 72 kDa. The additional hFOB proteins immunoprecipitated with TIEG are of interest, even though their levels are not regulated by TGF-β. The appearance of these proteins could be due to nonspecific precipitation along with the antibody protein complex or to specific interactions of the antibody or of TIEG protein with other cellular proteins.

The immunohistochemistry shows a marked cell type specificity for the TIEG protein, both in expression and cytoplasmic/nuclear localization. The TIEG protein is present in placental syncitiotrophoblastic cells which are probably target cells for TGF-β and other hormones (e.g., EGF). The same applies to the pancreas wherein TIEG protein is present only in the islets of Langerhans, which are glucagon, somatostatin, and insulin hormone-producing cells. Whether TIEG plays a role in mediating the functions of

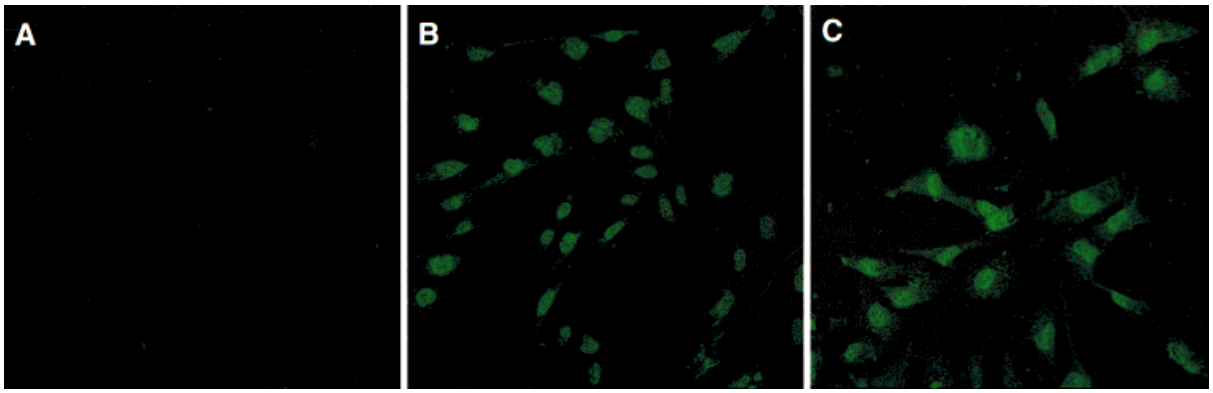

Fig. 5. Fluorescence laser scanning confocal microscopy of FOB cells for TIEG protein: Effects of TGF-β treatment. Human fetal osteoblastic cells were grown on 8-well chamber slides. The cells were serum starved for 48 h and then treated with TGF-β (2 ng/ml) and vehicle (0.25% BSA in PBS) for 2 h. The cells were fixed and immunostained with TIEG polyclonal antibody and fluorescence tagged antirabbit antibody. The images were obtained on a Zeiss laser scanning confocal microscope (LSM 310) equipped with an argon/krypton laser tuned to an excitation wave length of 488 nM. An emission filter of 530 ± 30 nM was used in front of the photomultiplier tube. **A:** Stained with rabbit IGg. **B:** Vehicle treated. **C:** Treated with TGF-β for 2 h. The objective lens was Zeiss-plan neoblur 40×.

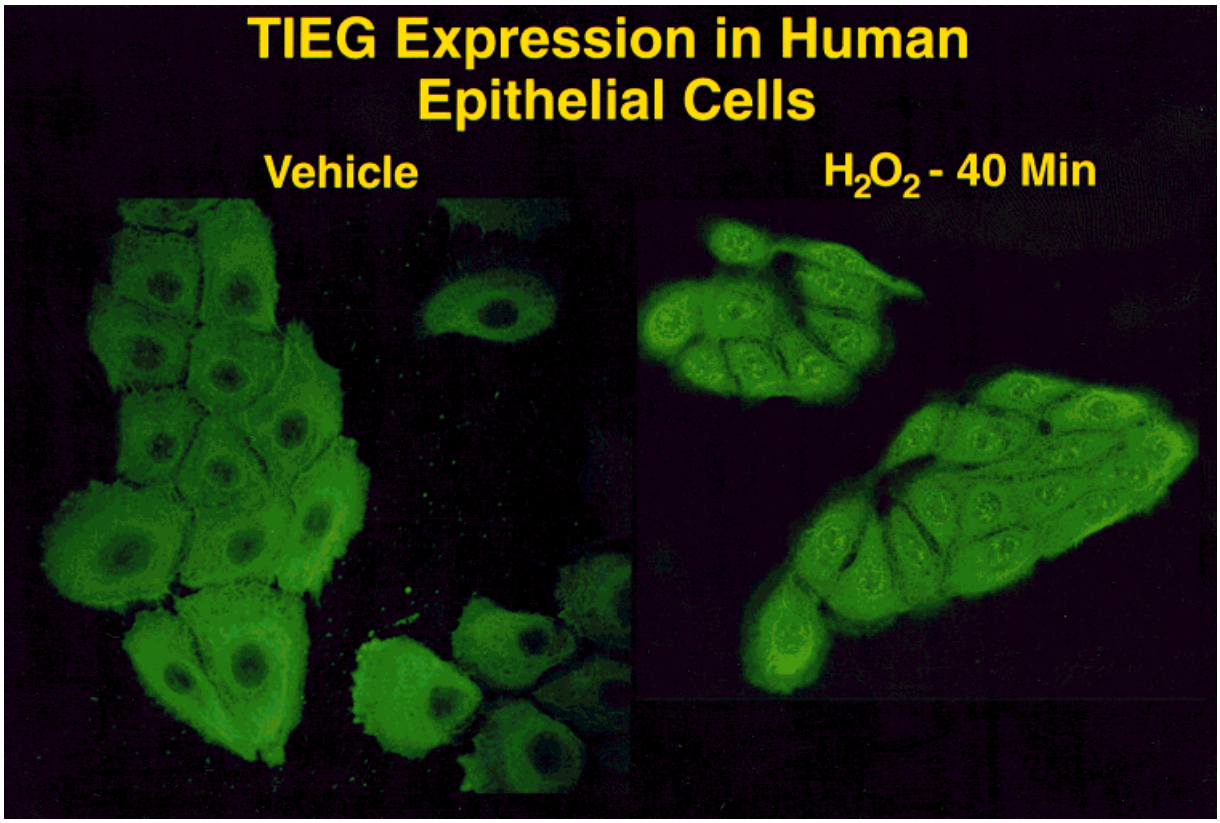

Fig. 6. Localization of TIEG in human keratinocytes. Human keratinocytes were isolated from neonatal foreskin and the cells were cultured in complete, serum-free MCDB 153 medium. Cells were treated with vehicle (**A**) or with 100 μM H_2O_2 (**B**) for 40 min and subsequently fixed and stained with TIEG PAb 228, as described under Methods. Pictures were taken with a laser scanning confocal icroscope.

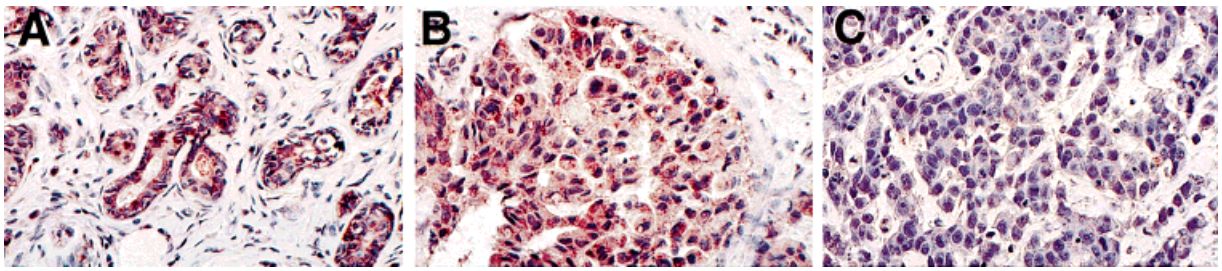

Fig. 7. Immunohistochemical staining of normal and neoplastic human breast epithelium. Sections of normal (benign), Comedo carcinoma, and invasive ductal carcinoma of human breast tissues were immunostained with TIEG polyclonal antibody and the slides were photographed. **A:** Benign breast tissue. **B:** Comedo carcinoma. **C:** Invasive ductal carcinoma of breast. ×100.

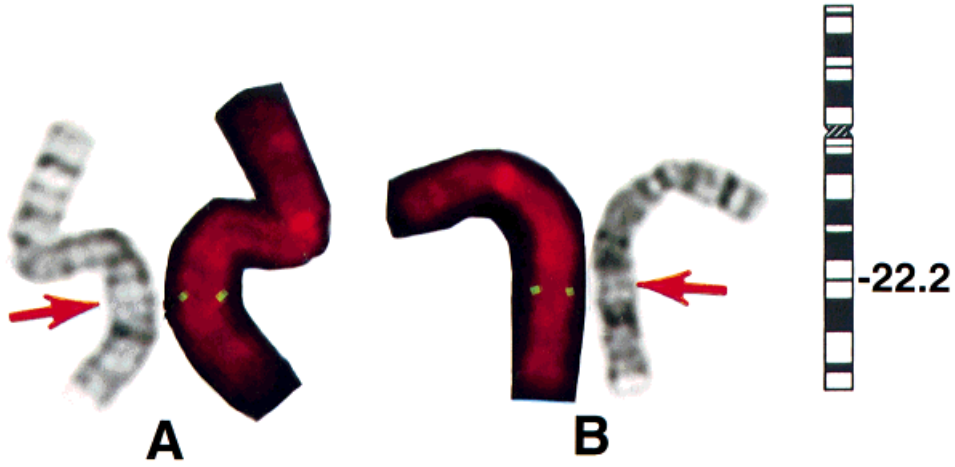

Fig. 8. Chromosomal localization of TIEG. Human lymphocytes were stimulated with phytohemaglutinin M and cultured for 72 h at 37°C. The cells suspension was dropped onto slides, to optimize chromosome spreading and morphology. In situ hybridization was performed sequentially after G-banding, using biotin-labeled TIEG cDNA and ONCOR's hybrizol. The hybridized signals were photographed using ONCOR's true color system. The arrows show the hybridized signal at 8q22.2. **A, B:** Replicates.

these hormones, or whether these cells are TGF-β target cells, is unknown. A specific strong, staining in the cerebellum is found in a single layer of what appears to be Bergman glial cells. The weaker staining of the cells in the white matter may or may not represent the presence of TIEG protein. The kidney shows negative staining for TIEG protein. It is interesting that the positive, epithelial staining is similar in the two reproductive tissues, breast and uterine endometrium (data not shown). The epithelial cells stain positively while surrounding stromal cells are negative. Immunohistochemical staining for TIEG protein in human tissues exhibited both cytoplasmic and nuclear staining, depending on the cell type. Immunofluorescence confocal microscopy supports this finding in the untreated cultured human cells wherein the osteoblasts show a nuclear staining and the keratinocytes a cytoplasmic staining. The different local cell environments in the human tissues might also play a role wherein some might have been exposed to TGF-β or its family members enhancing the nuclear binding of TIEG.

Breast cancer cells produce TGF-β and their growth is inhibited by this growth factor [Valverius et al., 1989; Knabbe et al., 1987]. More recently, TGF-β has been implicated in the antiestrogen-induced apoptosis of breast cancer cells [Chen et al., 1996b]. Thus, if TIEG is a key participant in the TGF-β action on breast cells, it might be involved in the primary pathway of TGF-β inhibition of cell proliferation. Support that TIEG may be involved in the cell cycle with properties of a tumor suppressor arises from recent studies in pancreatic carcinoma cells, TGF-β induces TIEG expression and inhibits cell proliferation. Interestingly, TIEG overexpression in these cells was shown to induce

apototic mediated growth inhibition [Tachibana et al., 1997]. In studies presented here, reduced TIEG protein levels were found in the in situ breast carcinomas and an absence of TIEG protein was found in invasive carcinomas. Although these preliminary observations certainly require more study with a significant number of samples, a pattern is evolving wherein TGF-β induces TIEG protein levels, in turn inhibiting proliferation. The TGF-β does inhibit cell proliferation in pancreatic carcinoma cells.

The breast cancer genes, *BRCA-1* and *-2,* are zinc finger proteins and are known to be involved in the inherited susceptibility of breast cancer. These two genes display high penetrance with *BRCA-1* exhibiting properties of the tumor suppressor gene. Over expression of the BRCA genes inhibits and underexpression encourages tumor growth. The BRCA genes have homology domains and properties of a granin family of proteins, which are a family of acidic proteins that bind calcium and participate in the regulated secretory pathway [Jensen et al., 1996; Holt et al., 1996]. There is speculation that the BRCAs contain productive tumor suppressive peptides cleaved from the secreted parent BRCA proteins to act on cell membrane receptors. However, more recent studies support that BRCA proteins are nuclear proteins. BRCA-1 is represented by two variants, which are cell cycle-regulated nuclear proteins that function as nuclear tyrosine phosphoproteins that associate with cell cycle regulators to inhibit cell proliferation [Wang et al., 1997; Ruffner and Verma, 1997; Thomas et al., 1997; Chen et al., 1996a] and may directly regulate gene transcription [Monteiro et al., 1996]. Thus, both the BRCA and TIEG proteins are nuclear localized proteins with implications as inhibitors of cell proliferation.

It is interesting to note that the tumor suppressor gene, p53 gene [Hurd et al., 1995], and the *BRCA-1* gene [Gudas et al., 1995; Weber, 1996], which are important in breast cancer metastasis and in apoptosis/cell death, are regulated by estrogen in breast cancer cells, the TIEG expression has also been recently shown to be estrogen regulated. The TIEG mapping to chromosome band 8q22.2 is also of interest, as this locus contains genes involved in bone disease (e.g., the renal tubular acidosis osteopetrosis syndrome), and cancer (e.g., acute myeloid leukemia). It is well established that osteopetrosis syndrome is related to a disorder in osteoclasts. The TIEG protein was identified in, and cloned from, osteoblasts. The biological functions of TIEG protein, and whether defects in TIEG are involved in the etiology of this bone disease or of cancer, remain to be determined. Further studies involving the under- or overexpression and mutations of this gene should help elucidate its biological function. In any event, the data presented here and in other papers support that TIEG is a novel gene implicated in cell cycle and tumor suppressor-like activities of bone cells and that it might serve as primary targets of TGF-β to inhibit/enhance cell proliferation, apoptosis, and gene expression in human osteoblasts and other cells.

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REFERENCES

Attar RM, Gilman MZ (1992): Expression cloning of a novel zinc finger protein that bines to the c-*fos* serum response element. Mol Cell Biol 12:2432–2443.

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Harber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE (1990): Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60:509–520.

Chavrier P, Zerial M, Lamaire P, Almendral J, Bravo R, Charnay P (1988): A gene encoding a protein with zinc fingers is activated during G_0/G_1 transition in cultured cells. EMBO J 7:29–35.

Chen CF, Li S, Chen Y, Chen PL, Sharp ZD, Lee WH (1996a): The nuclear localization sequences of the BRCA1 protein interact with the importin-alpha subunit of the nuclear transport signal receptor. J Biol Chem 271:32863–32868.

Chen H, Tritton TR, Kenny N, Absher M, Chiu JF (1996b): Tamoxifen induces TGF-β1 activity and apoptosis of human MCF-7 breast cancer cells in vitro. J Cell Biochem 61:9–17.

Christy BA, Lau LF, Nathan D (1988): A gene activated in mouse 3T3 cells by serum growth factor encodes a protein with "zinc finger" sequences. Proc Natl Acad Sci USA 85:7857–7861.

Crosby SD, Puetz JJ, Simburger KS, Fahrner TJ, Milbrandt J (1991): The early response gene NGF-1-C encodes zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element binding protein family. Mol Cell Biol 11:3835–3841.

Evans RM (1988): The steroid and thyroid hormone receptor superfamily. Science 240:889–895.

Galera P, Musso M, Ducy P, Karsenty G (1994): C-Krox, a transcriptional regulator of Type I collagen gene expression is preferentially expressed in skin. Proc Natl Acad Sci USA 91:9372–9376.

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GA (1990): Homozygous deletion in Willms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343:774–778.

Gudas J, Nguyen H, Li T, Cowan KH (1995): Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Res 55:4561–4565.

Holt JT, Thompson ME, Szabo C, Robinson-Benion C, Arteaga CL, King MC, Jensen RA (1996): Growth retardation and tumour inhibition by BRCA1. Nature Genet 12:298–302.

Hurd C, Khattree N, Alban P, Nag K, Jhanwar SC, Dinda S, Moudvil VK (1995): Hormonal regulation of the p53 tumor suppressor protein in T47D human breast carcinoma cell line. J Biol Chem 270:28507–28510.

Jensen RA, Thompson ME, Jetton TL, Szabo CI, van der Meer R, Helou B, Tronick SR, Page DL, King MC, Holt JT (1996): BRCA1 is secreted and exhibits properties of a granin. Nature Genetics 12:303–308.

Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB (1987): Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48:417–428.

Levi G, Topilko P, Maunoury SC, Lasagna M, Mantero S, Cancedda R, Charnay P (1996): Defective bone formation in Krox-20 mutant mice. Development 122:113–120.

Mermod N, O'Neill EA, Kelly TJ, Tjian R (1989): The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. Cell 58:741–753.

Miller J, Mclachlan AD, Klug A (1985): Repetitive zinc binding domains in the protein transcription factor IIIA from xenopus oocytes. EMBO J 4:1609–1614.

Monteiro AN, August A, Hanafusa H. (1996): Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc Natl Acad Sci USA 93:13595–13599.

Ohba M, Shibanuma M, Kuroki T, Nose K (1994): Production of hydrogen peroxide by transforming growth factor-β_1 and its involvement in induction of egr-1 in mouse osteoblastic cells. J Cell Biol 126:1079–1088.

Ruffner H, Verma IM (1997): BRCA1 is a cell cycle-regulated nuclear phosphoprotein. Proc Natl Acad Sci USA 94:7138–7143.

Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg TC (1995): Identification of a novel TGF-β regulated gene encoding a putative zinc finger protein in human osteoblasts. Nucleic Acids Res 23:4907–4912.

Sukhatme VP, Cao X, Chang LC, Morris CHT, Stamenkovich D, Ferreira PCP, Chohen DR, Edwards SA, Shows TB, Curran T, LeBean MM, Adamson E (1988): A zinc finger-encoding gene coregulated with c-*fos* during growth and differentiation, and after cellular depolarization. Cell 53:37–43.

Tachibana I, Imoto M, Adjei PN, Gores GJ, Subramaniam M, Spelsberg TC, Urrutia R (1997): Overexpression of the TGF-β regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. J Clin Invest 99:2365–2374.

Thomas JE, Smith M, Tonkinson JL, Rubinfeld B, Polakis P (1997): Induction of phosphorylation on BRCA1 during the cell cycle and after DNA damage. Cell Growth Diff 8:801–809.

Valverius EM, Walker-Jones D, Bates SE, Stampfer MR, Clark R, McCormick F, Dickson RB, Lippman ME (1989): Production of and responsiveness to transforming growth factor-β in normal and oncogene-transformed human mammary epithelial cells. Cancer Res 49:6269–6274.

Wang HC, Shao NS, Ding QM, Cui JQ, Reddy ESP, Rao VN (1997): Oncogene 15:143–157.

Weber BL (1996): Genetic testing for breast cancer. Science Med 3:12–21.

Zweidler-McKay PA, Grimes HL, Flubacher MM, Tsichlis PN (1996): Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. Mol Cell Biol 16:4024–4034.