ABSTRACT

Hypoxia occurs during development of cancers and is correlated with cancer progression. Hypoxia also induces epidermal growth factor receptor (EGFR) expression. The EGFR plays a vital role in cell growth, and its overexpression can lead to transformation. We sought to determine the regulator(s) of EGFR expression during hypoxia. We demonstrate that early growth response factor 1 (Egr-1), which is induced by hypoxia, can activate the basal transcriptional activity of the EGFR promoter. Egr-1 not only transactivates the EGFR promoter activity but also enhances endogenous EGFR expression. Using a series of EGFR promoter deletion mutants, we show that the region between −484 and −389, which contains a putative Egr-1 consensus motif, is crucial for EGFR transactivation by Egr-1. Electrophoretic mobility shift assays show that Egr-1 binds to the oligonucleotide containing this Egr-1 motif. Also, introduction of an antisense oligonucleotide for Egr-1 diminishes EGFR expression during hypoxia, indicating that the up-regulation of EGFR by hypoxia is mediated through Egr-1. Our results provide evidence that regulation of EGFR promoter activity by Egr-1 represents a mechanism for epidermal cell growth during hypoxia.

INTRODUCTION

Growth factor receptors are known to play an important role in normal cell proliferation and in neoplastic growth (1). The EGFR (2) is a Mr 170,000 membrane-spanning glycoprotein that mediates the biological signals for EGF, transforming growth factor α, and other ligands (2–4). EGFR is detected on many nonhematopoietic tissues and is frequently overexpressed in human tumors (5–9). Knockout of the EGFR can also lead to many defects in development (10). Because the EGFR plays a critical role in both normal and abnormal growth and development, it is important to have a clear understanding of the EGFR gene regulation. We initially characterized the EGFR promoter region as a GC-rich TATA-less complex with multiple transcription initiation sites and Sp1 binding sites (11–15). Several DNA-binding factors have been identified that interact with the promoter region including the WT1, EGFR receptor transcriptional repressor, TCC binding factor, EGFR transcription factor, EGFr-responsive DNA-binding protein, AP-1, AP-2, and p53 (16–23). These factors and others must mediate the response of the EGFR promoter region as a GC-rich TATA-less complex with multiple transcription initiation sites and Sp1 binding sites (11–15). Several DNA-binding factors have been identified that interact with the promoter region including the WT1, EGFR receptor transcriptional repressor, TCC binding factor, EGFR transcription factor, EGFr-responsive DNA-binding protein, AP-1, AP-2, and p53 (16–23). These factors and others must mediate the response of the EGFR promoter region to a variety of cellular stresses such as hypoxia exposure and UV or ionizing radiation, which have been shown to increase EGFR gene expression (24–27).

Regions of low oxygen concentration are thought to occur commonly during the development of cancers and are prognostically important for cancer therapy (28, 29). Many reports show that oxygen levels in cancers are significantly lower than those in normal tissues (30–36). It has also been shown that low level pO2 is correlated with prognosis, and tumor hypoxia can confer resistance to radiotherapy and some forms of chemotherapy (24, 28, 37). The occurrence of hypoxia promotes tumor aggressiveness and metastases (30–32). Thus, hypoxia is a specific stimulus for alteration of gene expression. Whereas hypoxia induces many stress-response genes such as AP-1, Ras, Src, HIF-1α, NFκB, p53, and Egr-1, it is still unclear what genes are involved in hypoxia-induced changes in cancers (38–43).

Egr-1 is a zinc finger transcription factor that belongs to a multifamily gene that includes Egr-2, Egr-3, Egr-4, and WT1 (44). The effects of Egr-1 are more likely to be evident in the response to stress rather than under homeostatic conditions. Consistent with this view, cell culture studies have shown Egr-1 to be involved in expression of a range of “inducible” genes associated with the host response, such as intercellular adhesion molecule-1, tumor necrosis factor-α, macrophage colony-stimulating factor, transforming growth factor β, PDGF A and B, and NFκB (45–50).

In this report, we examined the relationship between hypoxia, Egr-1, and the effect on EGFR transcription. We were able to show Egr-1-dependent transactivation of the EGFR promoter through transfection assays and Egr-1 binding to EGFR promoter fragments by electrophoretic mobility shift assays. Furthermore, we show that inhibition of Egr-1 expression under hypoxic condition prevents up-regulation of EGFR expression. The results detail a mechanism of hypoxia-induced activation of the EGFR gene.

MATERIALS AND METHODS

Cell Culture. The human osteosarcoma cell lines U-2OS and Saos-2 were maintained in McCoy’s 5A medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 15% FBS. The human epidermoid cancer cell line KB was maintained in modified Eagle medium (Life Technologies, Inc.) with 10% FBS. The human cervical cancer cell line HeLa was maintained in DMEM (Life Technologies, Inc.) with 10% FBS.

To mimic hypoxia conditions, U-2OS cells were plated at 2.5 × 105 cells in 30 mL of medium in 150-mm dishes and incubated overnight in 20% O2. Cells were exposed to 100 μM of DFX for 19 h. For hypoxic exposure, U-2OS cells were subjected to 1% oxygen in a water-jacketed CO2 incubator (NAPCO, Winchester, VA) at 37°C in a humidified atmosphere with 5% CO2 for indicated times. In experiments using transfected cells, each transfection was performed before treatment with DFX and exposure to hypoxia.

DNA Plasmids. Luciferase reporter constructs containing the EGFR promoter, pER1-luc, pER8-luc, pER9-luc, pER9A-luc, pER9C-luc, and pER10-luc, were prepared by ligation of the HindIII promoter fragments from EGFR-chloramphenicol acetyltransferase constructs into pGL3-Basic (Promega, Madison, WI; Ref. 22). The 3’ end of each of the following EGFR-luciferase constructs is located −16 relative to the EGFR-translational start site, whereas the 5’ end is the following positions: pER1-luc (−1109), pER8-luc (−849), pER9-luc (−388), pER9A-luc (−348), pER9C-luc (−292), and pER10-luc (−150). The TK minimal promoter luciferase construct was generated by subcloning of the EcoRI/HindIII TK promoter fragment from pRL-TK (Promega) into pGL3-Basic and removal of the EcoRI/BglII fragment of TK promoter. This results in the TK minimal promoter fragment. This construct was generated by subcloning of the EcoRI/HindIII TK promoter fragment from pRL-TK into pGL3-Basic and removal of the EcoRI/BglII fragment of TK promoter. This results in the TK minimal promoter fragment that binds to luciferase expression. The pERE848–293-luc was constructed by subcloning the EGFR promoter region between −388 and −293 into pGL3-Basic carrying the TK minimal promoter construct using the Xhol site. This site is located upstream of the TK promoter region. The pERE484–389-luc and pERE484–389GERmt-luc constructs were obtained by subcloning the TK minimal promoter region between −388 and −293 into pGL3-Basic carrying the TK minimal promoter using the Xhol site. These sites are located upstream of the TK promoter region. The pERE484–389-luc and pERE484–389GERmt-luc constructs were obtained by subcloning the TK minimal promoter region between −388 and −293 into pGL3-Basic carrying the TK minimal promoter using the XhoI site. These sites are located upstream of the TK promoter region. The pERE484–389-luc and pERE484–389GERmt-luc constructs were obtained by subcloning the TK minimal promoter region between −388 and −293 into pGL3-Basic carrying the TK minimal promoter using the XhoI site. These sites are located upstream of the TK promoter region. The pERE484–389-luc and pERE484–389GERmt-luc constructs were obtained by subcloning the TK minimal promoter region between −388 and −293 into pGL3-Basic carrying the TK minimal promoter using the XhoI site. These sites are located upstream of the TK promoter region. The pERE484–389-luc and pERE484–389GERmt-luc constructs were obtained by subcloning the TK minimal promoter region between −388 and −293 into pGL3-Basic carrying the TK minimal promoter using the XhoI site. These sites are located upstream of the TK promoter region.
have the EGFR promoter region between −484 and −389 with or without putative Egr-1 binding site mutation as an insert instead of the region between −388 and −293 (Fig. 6B). The Egr-1 expression plasmid pCB6Egr-1 was kindly provided by Dr. Levon Khachigian (University of New South Wales, Sydney Australia; Ref. 51). The CMV-Egr-1 expression plasmid was prepared by subcloning the Egr-1 cDNA into pcDNA3 (Invitrogen, Carlsbad, CA) using the EcoRI site.

**Transfections and Luciferase Assays.** Each cell line was seeded at 5 × 10^5 cells/35-mm dish and incubated overnight at 37°C in a 5% CO₂ atmosphere. For each transfection, 0.5–3 μg of empty vector and/or expression vector along with 0.1–0.3 μg of promoter-lucerase DNA were mixed in 0.2 ml of Opti-MEM (Life Technologies, Inc.) and a precipitate formed using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s recommendations. Cells were washed with Opti-MEM, and complexes were applied to the cells. After transfection (24 h), cells were harvested and extracts prepared with lucerase cell lysis buffer (PharMingen, San Diego, CA). Luciferase activity was measured in extracts from triplicate samples using the lucerase assay kit (PharMingen).

**AS and Transfections.** To inhibit the expression of endogenous Egr-1, we prepared a HPLC-purified phosphorothioate AS and, as a control, AS according to the sequence of the Egr-1 gene (51). The sequences of the AS and SE Egr-1 were 5′-tcGCGGCGGCGGCAGTACT-3′ and 5′-AgTGGTCCCCCGCCGC-3′, respectively (lowercase letters indicates phosphorothioate nucleotides). Each oligonucleotide was synthesized commercially (Sigma Chemical Co. Genosys, Woodlands, TX). The AS or SE (0.6 μm) was complexed with LipofectAMINE 2000 reagent (Life Technologies, Inc.) and applied to cells. Cells were then treated with DFX or exposed to hypoxia conditions. After transfection (24 h), cells were harvested and lysates prepared for Western blot analysis.

**Western Blot Analysis.** U-2OS cells were seeded at 2.5 × 10⁶ cells/150-mm dish, incubated overnight at 37°C, and transfected with 15 μg of either empty vector (pcDNA3) or constructs expressing Egr-1 using the LipofectAMINE 2000 method for 24 h as described above. Subsequently, cells were treated with DFX or exposed to hypoxia conditions. Cells were harvested and lysed on ice for 30 min in lysis buffer [10 mM Tris (pH 8.0), 1 mM EDTA, 500 mM NaCl, 10% glycerol, 0.5% NP40, 0.1 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT], containing complete protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). The lysate was subjected to centrifugation at 14,000 rpm for 15 min and the soluble fraction subjected to centrifugation at 200,000 g for 1 h. After washing twice in 2× SSC buffer [1× SSC containing 0.05% SDS at room temperature (22°C)] were subjected to Northern blot analysis. After electrophoresis in 3-morpholinepropanesulfonic acid buffer [1× SSC] were loaded onto a 5% nondenaturing polyacrylamide gel and subjected to electro- phoresis at 200 V for 50 min. The protein was transferred onto a polyvinylidene difluoride membrane and probed with anti-EGFR antibodies (1005; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Egr-1 antibody (588; Santa Cruz Biotechnology), anti-Sp1 antibody (PEP2; Santa Cruz Biotechnology), and actin antibody (C4; Boehringer Mannheim). The same blot was probed after stripping the membrane with the different antibodies. Each protein was detected by horseradish peroxidase-conjugated secondary antibody coupled with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Sweden, Uppsala). Each band intensity was normalized by the intensity of the actin band.

**Northern Blot Analysis.** U-2OS cells were seeded and treated as described for Western blot analysis. Transfected cells were harvested, and total cellular RNA was isolated using TRIzol reagent (Life Technologies, Inc.) and quantified by A260/A280 measurement using an Ultraspec 3000 (Amersham Pharmacia Biotech). Total RNA samples (20 μg) were subjected to Northern blot analysis. After electrophoresis in 3-morpholinepropanesulfonic acid buffer [0.1 M MOPS (pH 7.0), 40 mM sodium acetate 5 mM EDTA (pH 8.0)], the RNAs were transferred in 10 × SSC buffer [1× SSC = 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)] by capillary action to a nylon membrane. The RNAs were fixed to the nylon membrane by UV light exposure with a UV Strata linker (Stratagene, La Jolla, CA). The membrane was hybridized with random primed 32P-labeled probes in ExpressHyb hybridization solution (Clontech) according to manufacturer’s recommendations. After hybridization at 65°C, the membrane was washed twice in 2× SSC containing 0.5% SDS at room temperature and then washed twice at 50°C using 0.1× SSC containing 0.1% SDS. The filters were autoradiographed with Kodak XAR film for 24–72 h at −80°C. The signals obtained from the Northern blots were normalized to the signal for β-actin.

**RESULTS**

**Hypoxia Induces Egr-1 and EGFR Expression.** Hypoxia has been reported to up-regulate both Egr-1 and EGFR expression (24, 43). To analyze whether there is a correlation between the two, we examined expression of the two genes in U-2OS cells in response to 1% O₂. Whole cell extracts prepared from control cells and cells exposed to 1% O₂ for 8 h were subjected to Western blot analysis. As shown in Fig. 1A, both Egr-1 and EGFR expression were increased significantly by this condition. To determine whether low pO₂ regulates Egr-1 and EGFR gene expression by way of an iron (heme)-containing protein, U-2OS cells were cultured at 20% O₂ in the presence of the iron chelator DFX, which interferes with binding of molecular oxygen to heme proteins, thus mimicking hypoxia. Treatment of cells with 100 μM of DFX for 19 h resulted in an increase of both Egr-1 and EGFR expression similar to that observed with cells maintained at 1% O₂ (Fig. 1A). As a control, Sp1 expression level was measured and did not change by hypoxia or DFX treatment (Fig. 1A).

To examine whether induction of EGFR expression by hypoxia is mediated via a transcriptional mechanism, U-2OS cells were transfected with pER1-luc and exposed to 1% O₂ for 12 h. Whole cell
Egr-1 INDUCES EGFR GENE EXPRESSION

Egr-1 Induces the Endogenous EGFR Expression.

Several previous results prompted us to examine whether the endogenous EGFR gene is regulated by Egr-1. U-2OS cells were transfected with pcDNA3-Egr-1 or pcDNA3. Total RNA and whole cell extracts were isolated 24 h after transfection and subjected to Northern blot and Western blot analysis, respectively. EGFR mRNA and protein level were induced by Egr-1 (Fig. 3, A and B). These results indicate that Egr-1 induces EGFR expression through direct interaction with the EGFR regulatory region.

Egr-1 Interacts with WT1 Sites in the EGFR Promoter.

To determine whether Egr-1 has direct interaction with the WT1 binding sites derived from the EGFR promoter, EMSAs were performed. Two oligonucleotides corresponding to nucleotides −368 to −339 (A-box) and −327 to −298 (B-box) of the EGFR promoter, which contain each WT1 binding site, were incubated with Egr-1 prepared by in vitro transcription/translation and subjected to electrophoresis.

A DNA-protein complex was formed when either A-box or B-box was incubated with Egr-1–programmed rabbit reticulocyte lysate (Fig. 4A, shift) but not with unprogrammed lysate (data not shown). These complexes were specifically retarded by anti-Egr-1 antibody (588; Santa Cruz Biotechnology; Fig. 4A, supershift). The addition of a 30-fold molar excess of cold Egr-1 consensus oligonucleotide (Santa Cruz Biotechnology) that contains two Egr-1 motifs markedly reduced each binding (Fig. 4A). To additionally substantiate an Egr-1 binding to these oligonucleotides, we performed competition assays using end-labeled Egr-1 consensus oligonucleotide and cold A-box or B-box oligonucleotides.

Egr-1 Induces the Endogenous EGFR Expression. The EGFR promoter contains two WT1 binding sites, one of which was shown to bind Egr-1 (16, 52). We examined whether Egr-1 directly regulated the EGFR gene by cotransfection assays with the EGFR promoter. Initially, the full-length proximal promoter construct, pER1-luc, was cotransfected into U-2OS, Saos-2, HeLa, and KB cells with the Egr-1 expression plasmid or empty vector. In all of the cell lines, Egr-1 increased EGFR promoter activity 2–5-fold (Fig. 2). Additional cotransfection experiments were performed with increasing amounts of Egr-1 and resulted in a dose-dependent activation of the EGFR promoter by Egr-1 in U-2OS and KB cells (Fig. 2).

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Identification of an Additional Egr-1 cis-acting Element in the EGFR Promoter. Because the binding of Egr-1 to the A-box and B-box was of low affinity, we examined the EGFR promoter for additional Egr-1 response elements. Reporter constructs containing EGFR promoter deletions were cotransfected into U-2OS cells with the Egr-1 expression plasmid, and luciferase assays were performed after 24 h. As shown in Fig. 5, pER1-luc containing −1109 to −16, pER6-luc containing −771 to −16, and pER8-luc containing −484 to −16 were activated to the similar extents by Egr-1, whereas pER9-luc containing −389 to −16, pER9C-luc containing −292 to −16, and pER10-luc containing −150 to −16 were much less activated (Fig. 5).

To confirm whether the EGFR promoter regions between −389 and −293 and between −484 and −389 are responsible for Egr-1 transactivation, we generated luciferase constructs that have these regions upstream of a minimal TK promoter. Cotransfection assays were performed using these reporter constructs, pER389–293-luc and pER484–389-luc, respectively (Fig. 6, A and B). In U-2OS cells, cotransfection of pER484–389-luc in conjunction with the pcDNA3 or pcDNA3-Egr-1 revealed that Egr-1 activated pER484–389-luc > 2.5-fold but not pER389–293-luc (Fig. 6).

These results indicate that the EGFR promoter region between −484 and −389 appears crucial for Egr-1 transactivation. Computer analysis of this region revealed one putative Egr-1 consensus binding site, GCGGGGGCC, located between −433 and −425. We prepared a reporter construct, pER484–389Egrmt-luc, where the putative Egr-1 site is mutated by replacement of GGG with TAT. Egr-1 was unable to increase the activity of this reporter construct in cotransfection assays (Fig. 6B). Taken together, the putative Egr-1 binding site in the EGFR promoter (between −484 and −389) is essential for the regulation of EGFR by Egr-1.

We next examined Egr-1 binding to this region. EMSAs were performed with EGFR/EgrRE, an oligonucleotide corresponding to nucleotides −439 to −418 of the EGFR promoter, and Egr-1 prepared by in vitro transcription/translation. A DNA-protein complex was formed when the end-labeled EGFR/EgrRE was incubated with Egr-1 (Fig. 7A, shift) but not with control lysate (data not shown; Fig. 7A). This complex was supershifted by anti-Egr-1 antibody (Fig. 7A, supershift). The addition of a 30-fold molar excess of the Egr-1 consensus oligonucleotide was used. The shifted and supershifted bands as well as the labeled probe (free) are indicated.

B-box as competitors. Whereas both unlabeled A-box and B-box competed the Egr-1 binding to the wild-type probe when present in the reaction at a 200-fold molar excess, the cold Egr-1 consensus oligonucleotide competed more efficiently at a 50-fold molar excess (Fig. 4B). These results confirm that Egr-1 binds to both A-box and B-box, and indicate that the affinity of Egr-1 binding to the A-box or B-box is lower than to the consensus Egr-1 sequence. Additionally, we performed EMSAs with nuclear extract from K562 cells treated with phorbol 12-myristate 13-acetate (Santa Cruz Biotechnology), which is known to induce Egr-1 expression. Egr-1 from nuclear extract interacted with both A-box and B-box based on competition and supershift assays (data not shown).
excess of cold Egr-1 consensus oligonucleotide markedly reduced this binding (Fig. 7A). Although these results suggest that the Egr-1 interacts directly to EGFREgrRE, the binding appears to be of lower affinity relative to the Egr-1 consensus-binding site. To additionally confirm the binding of Egr-1 to EGFREgrRE, we performed competition assays using an end-labeled Egr-1 consensus oligonucleotide and unlabeled EGFREgrRE. EGFREgrRE efficiently competed for Egr-1 binding to the wild-type binding site when present in the reaction at a 200-fold molar excess; the cold Egr-1 consensus oligonucleotide was more efficiently at 50-fold molar excess (Fig. 7B). These results confirm that Egr-1 binds to EGFREgrRE and suggest that the affinity of Egr-1 to EGFREgrRE is lower. We also performed EMSA with nuclear extracts from K562 cells treated with phorbol 12-myristate 13-acetate. Egr-1 from the nuclear extracts interacted with EGFREgrRE based on supershift assays (data not shown).

Disruption of EGFR Expression by an AS of Egr-1. An AS has been used previously to inhibit Egr-1 expression (51). Thus, we used AS to examine whether hypoxia-induced EGFR expression is mediated by up-regulation of Egr-1. When U-2OS cells were transfected with AS and cells exposed to hypoxia or treated with DFX, AS down-regulated the EGFR expression by >50% and 60%, respectively, as compared with that in SE-transfected cells (Fig. 8). As a control, the level of Sp1 was monitored and found to be unaltered by AS (Fig. 8). The data indicate that hypoxia-induced EGFR expression is attributable to the enhanced activity of Egr-1 on the EGFR promoter. Similar results were obtained using Saos-2 and H1299 cells.

DISCUSSION

Hypoxia is recognized as a specific stimulus for gene expression. The critical genes modulated by hypoxia include transcription factors, growth factors, and oncoproteins such as AP-1, HIF-1α, NFXB, p53, Egr-1, vascular endothelial growth factor, EGFR, erythropoietin, and Ras (38-43, 53). The effect of these gene products seems to counteract the detrimental effects of low oxygen: angiogenic factors attract new vasculature to increase oxygenation, and oncoproteins give hypoxic tumor cells a growth advantage (54). A correlation between low-level PO2 in tumors, as measured by oxygen electrodes, and poor treatment outcome was
types of cancers, such as glioblastomas, ovarian, cervical, and kidney formation (1). High levels of the EGFR have been detected in many hypoxia acts to produce a more aggressive phenotype. Immortal evidence appear to be converging to suggest that tumor tumor hypoxia is related to tumor progression. Clinical and experimental evidence suggest that tumor hypoxia leads to the differences in expression. Because of activation and increased proliferative rates. This suggests the possibility that the increase in the Egr-1 and EGFR levels as a result of hypoxia exposure may be conducive to malignant transformation.

The role of various factors in mediating responses to hypoxia is also complicated. We have demonstrated the ability of Egr-1 to up-regulate EGFR expression and provided the correlation with Egr-1 induction via hypoxic conditions. Are additional known hypoxic factors such as HIF-1 involved? We specifically examined the role of HIF-1 in regulation of the EGFR gene. We could not shown any change in EGFR expression in response to increased HIF-1 expression (data not shown). AP-1 has also been implicated in essential contributions of hypoxia-mediated activation of gene expression as well as EGFR gene regulation (22, 38). AP-1 may play a compensatory role in mediating EGFR up-regulation by hypoxia, but the primary correlation that we detect is with up-regulation of Egr-1.

EGFR expression in solid tumors is varied as is it in most cancers (7, 8). In osteosarcomas, expression of both EGFR and platelet-derived growth factor receptor has been examined by immunohistochemical staining (68). EGFR was detected 81% of the time, whereas EGF (51%), PDGF (49%), and platelet-derived growth factor receptor (38%) were detected less frequently. In 30% of the primary tumors both receptors and their ligands were present. This suggests that there must be reasons, perhaps changes in hypoxia-induced factors, which lead to the differences in expression.

In conclusion, we demonstrate that EGFR expression is up-regulated after induction of Egr-1 during exposure of hypoxia in human tumor cells and that Egr-1 directly induces EGFR transcription. Given that low oxygen level and EGFR activation are crucial for tumor development and progression, this function of Egr-1 may represent an important mechanism of tumor growth mediated by EGFR during hypoxia. The unraveling of the complexity of EGFR induction by hypoxia will require additional studies involving cells lacking the factors that are thought to have a role.

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This article is dedicated to the memory of Dr. Francis T. (Frank) Kenney of Oak Ridge, Tennessee. A 32 year biochemist/cancer biologist at Oak Ridge National Laboratory and graduate advisor, mentor, and friend to A.C.J., who died of lung cancer on December 10, 2001.

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Early Growth Response-1 Gene Mediates Up-Regulation of Epidermal Growth Factor Receptor Expression during Hypoxia

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