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Egr-1 and Serum Response Factor Are Involved in Growth Factors- and Serum-Mediated Induction of E2-EPF UCP Expression That Regulates the VHL-HIF Pathway

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ABSTRACT

E2-EPF ubiquitin carrier protein (UCP) has been shown to be highly expressed in common human cancers and target von Hippel-Lindau (VHL) for proteosomal degradation in cells, thereby stabilizing hypoxia-inducible factor (HIF)-1α. Here, we investigated cellular factors that regulate the expression of UCP gene. Promoter deletion assay identified binding sites for early growth response-1 (Egr-1) and serum response factor (SRF) in the UCP promoter. Hepatocyte or epidermal growth factor (EGF), or phorbol 12-myristate 13-acetate induced UCP expression following early induction of Egr-1 expression in HeLa cells. Serum increased mRNA and protein levels of SRF and UCP in the cell. By electrophoretic mobility shift and chromatin immunoprecipitation assays, sequence-specific DNA-binding of Egr-1 and SRF to the UCP promoter was detected in nuclear extracts from HeLa cells treated with EGF and serum, respectively. Overexpression of Egr-1 or SRF increased UCP expression. RNA interference-mediated depletion of endogenous Egr-1 or SRF impaired EGF- or serum-mediated induction of UCP expression, which was required for cancer cell proliferation. Systemic delivery of EGF into mice also increased UCP expression following early induction of Egr-1 expression in mouse liver. The induced UCP expression by the growth factors or serum increased HIF-1α protein level under non-hypoxic conditions, suggesting that the Egr-1/SRF-UCP-VHL pathway is in part responsible for the increased HIF-1α protein level in vitro and in vivo. Thus, growth factors and serum induce expression of Egr-1 and SRF, respectively, which in turn induces UCP expression that positively regulates cancer cell growth. J. Cell. Biochem. 105: 1117–1127, 2008.

KEY WORDS: GROWTH FACTORS; E2-EPF UCP; GENE EXPRESSION; Egr-1; SRF; VHL; HIF-1α

Human E2-EPF ubiquitin carrier protein (UCP) is a member of the E2 ubiquitin-conjugating enzyme family [Liu et al., 1996; Pickart, 2001]. UCP has been shown to ubiquitinate von Hippel-Lindau (VHL) tumor suppressor for proteosomal degradation [Jung et al., 2006]. VHL forms part of the E3 ubiquitin ligase complex [Iwai et al., 1999], which ubiquitinates hydroxylated hypoxia-inducible factor (HIF)-1α for proteosomal degradation [Ivan et al., 2001; Jaakkola et al., 2001]. HIF-1α forms the HIF-1 heterodimeric transcription factor with HIF-1β [Semenza, 2001]. HIF-1 increases expression of a number of hypoxia-inducible genes including the gene encoding vascular endothelial growth factor (VEGF) and associates with tumor growth and vascularization [Semenza, 2001; Pugh and Ratcliffe, 2003]. Thus, loss of VHL function, such as germ line mutations of VHL gene, results in constitutive activation of HIF-1α, and associates with a hereditary cancer syndrome characterized by the development of multiple tumors, such as renal cell carcinoma and hemangioblastomas [Kaelin, 2002]. These results suggest that UCP regulates the VHL-HIF pathway.

UCP has been shown to be highly expressed in common human cancers [Welsh et al., 2001; Wagner et al., 2004] and many cancer cell lines [Jung et al., 2006]. However, it is unknown how the expression of UCP gene is regulated in response to extracellular stimuli. Epidermal or hepatocyte growth factors (EGF or HGF), phorbol 12-myristate 13-acetate (PMA), or serum have been shown to increase the level of HIF-1α protein in cells under non-hypoxic conditions [Bilton and Booker, 2003] and associate with cell proliferation. These effects of the growth factors or serum on HIF-1α

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protein level and cell growth may be attributed to UCP expression. Therefore, we analyzed here the UCP promoter activity in response to cell proliferation signal and examined whether UCP expression is required for cell proliferation by the growth factors and serum. We also examined whether UCP expression associates with the growth factors-mediated increase of HIF-1α protein level under normoxia.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

HeLa cervical cancer and CAKI-1 renal carcinoma cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. We purchased EGF and HGF (R&D System), PMA (Sigma), and following antibodies: mouse anti-early growth response gene-1 and anti-Ser103-phosphorylated serum response factor (anti-Egr-1 and anti-phospho-SRF, Cell Signaling Technology), anti-VHL and anti-HIF-1α (BD Pharmingen), anti-β-actin (Sigma), and anti-SRF and anti-Tyr1173-phosphorylated-EGF receptor (anti-phospho-EGFR, Santa Cruz). The anti-UCP antibody was generated as reported previously [Jung et al., 2006].

PLASMIDS

A UCP promoter region encompassing 1,000 bp upstream from the transcriptional initiation site of the human UCP gene was PCR-amplified from HeLa genomic DNA. The sequence of the upstream primer was 5′-GATCCCAGTGCCCATTTGTC-3′; whereas that of the downstream primer was 5′-CGGCCGGGGCGGGGGGCCCAACTGCT-GCCGC-3′ (R2). Amplified PCR product was cloned into the *Sac*I–*Xho*I sites of pGL2 that contains a promoter-less firefly luciferase (luc) reporter gene (Promega). The resulting plasmid was named p-1000/luc. Four 5′-deletion reporter constructs of p-800/luc, p-700/luc, p-400/luc, and p-200/luc were generated from p-1000/luc by PCR amplifications using relevant primers. The p-200/SV40-luc reporter plasmid was constructed by inserting the *Sac*I–*Xho*I fragment of p-200/luc into pGL3 (Promega), in which the luc reporter gene is under the transcriptional control of SV40 minimal promoter. ΔEBS1 reporter plasmid, in which Egr-1 binding sequence 1 (EBS1; 5′-GCGGGCGG-3′) in the UCP promoter was deleted, was constructed from the p-200/SV40-luc by PCR amplification using the F1 primer 5′-AGGGCGTGGCCTTTATAAA-3′ and the R2 primer. ΔEBS2 reporter plasmid, in which EBS2 (5′-CGGGGCGG-3′) in the UCP promoter was deleted, was constructed as followings: p-200/SV40-luc was amplified by PCR using the F2 primer 5′-TGCCGGGCACCGGGTT-3′ and the R2 primer, and the F0 primer 5′-AGGGCGTGGCCTGCGGG-3′ and the R1 primer 5′-AACCCGGTGCCCGGCA-3′, respectively. Each amplified PCR product was mixed and amplified by PCR using the F0 and R2 primers. To construct ΔEBS1-2 reporter plasmid, in which both EBS1 and EBS2 were deleted, ΔEBS1 plasmid was PCR-amplified by using the F2 and R2 primers, and the F1 and R1 primers, respectively. Each amplified PCR product was mixed and PCR-amplified by using the F1 and R2 primers. To construct p-1000/-801 reporter plasmid, the UCP promoter region between -1000 to -801 that includes serum response element (SRE) was PCR-amplified from p-1000/luc by using the forward primer 5′-GATCCCAGTGCCCATTTGTC-3′ and the reverse primer 5′-ACTACATTTTTCTATGAG-3′. The resulting PCR product was inserted into the *Sac*I–*Xho*I sites of pGL3. ΔSRE reporter plasmid, in which the SRE (5′-TCACATAAGG-3′) was deleted, was constructed from p-1000/-801 by PCR amplification using the forward primer 5′-GATCCCAGTGCCCATTTGAAACTGA-3′ and the reverse primer 5′-ACTACATTTTTCTATGAG-3′. All the reporter plasmids constructed were confirmed by sequencing.

We synthesized the Egr-1 specific siRNA (5′-GGACAAGAAAG-CAGACAAA-3′) and SRF specific siRNA (5′-TGAGTGC-CACTGGCTTTGA-3′) oligonucleotides, and cloned them into the *Hin*dIII–*Bgl*II sites of pSuper vector to construct pEgr-1-siRNA and pSRF-siRNA. The pCon-siRNA construct expressing a scrambled control siRNA was previously described [Jung et al., 2006]. To construct Flag-tagged Egr-1 and SRF expression vectors (pF-Egr-1 and pF-SRF), coding regions for Egr-1 and SRF were PCR-amplified from a HeLa cDNA library by using the relevant primers. The *Eco*RI–*Xho*I fragment of amplified Egr-1 cDNA and the *Hin*dIII–*Bam*HI fragment of amplified SRF cDNA were cloned into pFlag-CMV2, respectively.

WESTERN BLOT ANALYSIS

Cells were lysed in a buffer (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide). Cleared cell lysates were resolved by SDS–PAGE. Proteins on the gels were transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% skim milk in TBS-T buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.05% Tween-20) for 1 h, and then incubated with relevant antibodies for 16 h at 4°C. Membranes were washed with TBS-T buffer and incubated for 1 h at RT with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG at a dilution of 1:10,000. Protein bands were visualized using enhanced chemiluminescence. Frozen mouse liver tissues were lysed in a lysis buffer (Intron) and immunoblotted as described above.

LUCIFERASE REPORTER ASSAY

Cells were transfected with reporter plasmids by a standard calcium–phosphate method. Transfection efficiency was adjusted by co-transfection of a Renilla co-reporter plasmid, phRL-SV40. Firefly and Renilla luciferase assays were performed by using assay kits under the recommended conditions by the supplier (Promega). Hypoxia-response element (HRE)-reporter assay was performed as previously described [Jung et al., 2006].

NORTHERN BLOT ANALYSIS

Total RNA was extracted from cells by using an RNeasy Mini kit (Qiagen). RNA was quantified by using a UV spectrophotometer, subjected to electrophoresis in 1% agarose gel/2.2 M formaldehyde in MOPS buffer, and transferred onto nitrocellulose membranes. As probes, we used the *Not*I–*Bam*HI fragment of UCP cDNA, the *Eco*RI–*Sac*I fragment of Egr-1 cDNA, and VHL and HIF-1α cDNA fragments amplified by PCR using the relevant primers. Probes were labeled with [α-^{32}P]dCTP using a labeling kit (RPN 1633, Amersham). Blots were pre-hybridized at 55°C for 2 h in a pre-hybridization buffer (50% formamide, 5 × SSC, 5 × Denhart's solution, 1% SDS, 100 μg/ml salmon sperm DNA). Hybridization was performed by the addition of labeled probes at 55°C for 16 h. Blots were washed with a

solution (0.1 × SSC, 0.1% SDS) at 55°C and exposed to a PhosphorImager (BAS 1500, Kodak).

RT-PCR

Total RNA from cells was subjected to RT-PCR with the primers 5′-TGCCGGGCAGGCGGGTT-3′ and 5′-AACCCGCCGTGCCCGGCA-3′ for Egr-1, 5′-ATGAACTCCAACGTGGAGAA-3′ and 5′-CTACAG-CCGCCGCAGCGC-3′ for UCP, 5′-ATGCCCCGGAGGGCGGAG-3′ and 5′-TCAATCTCCCATCCGTTG-3′ for VHL, 5′-AGAAAAAGATAAGT-TCTGAACGTCG-3′ and 5′-ATTTCCTCATGGTCACATGGATG-3′ for HIF-1α, and 5′-ATGGGGAAGGTGAAGGTCGG-3′ and 5′-TGGTTC-ACACCCATGACGAA-3′ for GAPDH. Frozen mouse liver tissues were lysed in a Trizol lysis buffer. Total RNA was extracted from the lysates by using an RNA extraction kit (Intron) and subjected to RT-PCR with the primers 5′-CCAACACTGACATTTTTCCTGA-3′ and 5′-CTGAAGTTACGCATGCAGATTC-3′ for mouse Egr-1, 5′-ACCCA-CCTGATGGCATTAAA-3′ and 5′-TGGATTTCTGTGAGCAGACG-3′ for mouse UCP, 5′-CTCAGGTCATCTTCTGCAACC-3′ and 5′-TCCT-CTTCCAGGTGCTGACT-3′ for mouse VHL, and 5′-AAAAACAGA-GACGAAGGACA-3′ and 5′-TGCTAAATCGGAGGGTATTA-3′ for mouse HIF-1α.

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

HeLa cells were suspended in a buffer (10 mM HEPES [pH 7.5], 1.5 mM $MgCl_2$, 10 mM NaCl, 0.25% NP-40), allowed to swell on ice for 10 min, and centrifuged for 1 min at 10,000*g*. Pellets were suspended in an ice-cold buffer (20 mM HEPES [pH 7.5], 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.25% NP-40), and incubated on ice for 20 min for high salt extraction. Supernatants were obtained by centrifugation at 10,000*g* for 2 min and used for EMSA. We synthesized and annealed sense and antisense oligonucleotides of EBS1 (5′-GGATCCAGCGGGCG-GAGCGGGCGGAGCGGGCGGA-3′) and SRE (5′-GGATGTTCACA-TAAGGACCTCACATAAGGACCTCACATAAGGTCT-3′) on the basis of the Egr-1 and SRF binding sequences in the UCP promoter. Annealed duplex oligonucleotides were end-labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Takara) and used as probes. Nuclear extracts were incubated with probes in a binding buffer (10 mM Tris–HCl [pH 7.5], 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 25 μg/ml poly[dI−dC], and 1 mg/ml salmon sperm DNA) at RT for 30 min. For competition experiment, nuclear extracts were pre-incubated with unlabeled consensus oligonucleotide probes containing EBS (5′-GGATCCAGCGGGGGCGAGCGG-GGGCGAGCGGGGGCGA-3′), SRE (5′-GGATGTCCATATTAGGAC-CCCA TATTAGGACCCCATATTAGGTCT-3′), or interferon response element (IRE, 5′-GGAAGCGAAAATGAAATTGACGAAAATGAAA-TTGACGAAAATGAAATTCT-3′) at RT for 15 min, and further incubated with ^{32}P-labeled probes for 15 min. Reaction products were separated by 6% native PAGE in a buffer containing 0.5 × Tris–borate–EDTA at RT. Gels were dried and exposed to a PhosphorImager.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

HeLa cells were incubated in 1% formaldehyde at 37°C for 10 min to cross-link DNA and proteins, and then sonicated in a lysis buffer (50 mM Tris–HCl [pH 8.1], 1% SDS, 10 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The DNA/protein complex was immunoprecipitated with relevant antibodies, and genomic DNA was recovered by phenol/chloroform extraction. Identification of the captured UCP genomic fragment was performed by PCR analysis using the primers 5′-AGGGCGTGGCCTGCGG-GCGG-3′ and 5′-CTGCTGCCGCTGCGGCCCTG-3′ for Egr-1 and the primers 5′-CATTTGTCACATAAGGAAAC-3′ and 5′-ACTACATTTTT-CTATGAG-3′ for SRF.

MOUSE EXPERIMENT

Female Balb/c mice, 4–5 weeks of age, were purchased from Harlan Sprague–Dawley (Indianapolis, USA), and housed in the animal facilities of KRIBB with free access to food and water. Animals were treated humanely in accordance with the Korea National Institute of Health Guidelines. Murine EGF (100 μg/kg) was injected into mice intravenously. We excised and immediately froze livers from mice in liquid nitrogen.

RESULTS

IDENTIFICATION OF CIS-ACTING ELEMENTS IN THE UCP PROMOTER THAT MEDIATE INDUCIBILITY BY EGF, HGF, PMA, OR SERUM

We constructed p-1000/luc reporter plasmid harboring an upstream regulatory region of the UCP gene and 5′-deletion constructs of the p-1000/luc to identify cis-acting elements in the UCP promoter (Fig. 1A). The reporter constructs were transfected into HeLa cells and the reporter activities were analyzed (Fig. 1B). The p-800/luc, p-700/luc, p-400/luc, and p-200/luc constructs exhibited comparable reporter activities, but they were much higher than the pGL2 reporter activity, suggesting that a cis-element is located at the region between −200 to +1, but not between −800 to −200. We found two Egr-1 binding sequences, EBS1 and EBS2, in the region between −200 to +1 (Figs. 1A and 6A). The reporter activity of the p-800/luc construct was about twofold less than that of the p-1000/luc construct (Fig. 1B), suggestive of the presence of a cis-element at the region between -1000 and -801. Serum response element was found in the region between -1000 and -801 (Figs. 1A and 6A). To test functionality of these cis-elements, we constructed pGL3-based reporter constructs, in which EBS1, EBS2, both EBS1 and EBS2, or SRE in the UCP promoter were deleted or not (Fig. 1A). HeLa cells were transfected with the reporter constructs indicated in Figure 1C, incubated in serum-free media, and then treated with EGF, HGF, PMA, or serum at the indicated concentrations and luciferase assay was performed. EGF, HGF, or PMA increased the reporter activity of p-200/SV40-luc in a dose-dependent manner, but this increased reporter activity was markedly impaired in the order of ΔEBS1–2, ΔEBS1, and ΔEBS2 constructs (Fig. 1C). These results suggest that the EBS1 plays a major role in activation of the UCP promoter by the growth factors. Serum increased the reporter activity of p-1000/-801 in a dose-dependent manner, but deletion of the SRE abolished the reporter activity (Fig. 1C, ΔSRE), suggesting that the SRE is involved in serum-mediated activation of the UCP promoter.

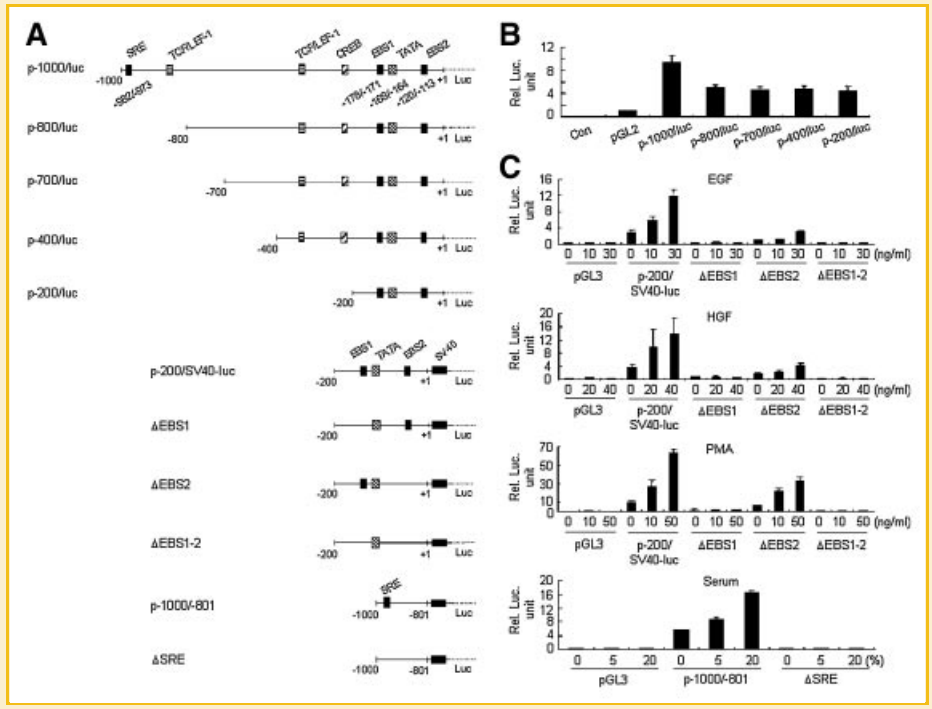

Fig. 1. Identification of cis-acting elements in the UCP promoter that mediate inducibility by EGF, HGF, PMA, or serum. A: Schematic illustration of the UCP promoter constructs cloned into upstream of pGL2- and pGL3-based firefly luciferase reporter plasmids. The 5′ boundaries of each reporter plasmid (the numbers indicated base pairs relative to the transcriptional initiation site of the UCP mRNA) were indicated to the left of each construct. '+1' indicates putative transcription initiation site of the UCP gene. SRE, EBS1, EBS2, and putative transcription factor binding sites are shown in the p-1000/luc schematic. TATA, TFIID binding site; TCF/LEF-1, T-cell factor/lymphoid enhancer factor; CREB, cyclic AMP response element-binding protein. B: HeLa cells were grown on 6-well plates, transfected with 1 μg of the indicated reporter constructs, incubated with DMEM containing 10% FBS for 16 h, and further incubated for 8 h. Luciferase assay was performed. Con, luciferase activity from HeLa cells without DNA transfection. C: HeLa cells were grown on 6-well plates, transfected with 1 μg of the indicated reporter constructs, and incubated for 16 h. These cells were then incubated in DMEM without FBS for 24 h, and treated or untreated with EGF, HGF, or FBS (serum) for 12 h, or PMA for 6 h, at the indicated concentrations. Luciferase assay was performed. Data in (B) and (C) are mean ± s.d. from three independent assays, each in duplicate.

EGF, HGF, OR PMA INDUCES UCP EXPRESSION FOLLOWING EARLY INDUCTION OF Egr-1 EXPRESSION

The Egr-1 has been shown to be immediately induced by a variety of stimuli including growth factors, and to function as a transcription factor that couples short-term changes in the extracellular milieu to long-term changes in gene expression [Thiel and Cibelli, 2002]. To test whether Egr-1 indeed associates with UCP expression that regulates the VHL-HIF pathway, we treated serum-starved cells with EGF, HGF, or PMA, and examined time-dependent alterations of mRNA and protein levels of Egr-1, UCP, VHL, or HIF-1α by Northern and Western blot analyses (Fig. 2). Egr-1 mRNA level peaked at 1 h and afterward declined rapidly, while UCP mRNA level began to increase at 1 h and increased by 16 h (Fig. 2A, EGF). The level of Egr-1 protein peaked at 1 h and afterward declined, while the level of UCP protein began to increase at 3 h and boosted by 16 h (Fig. 2B, EGF). HGF or PMA also increased mRNA levels of Egr-1 and subsequently UCP (Fig. 2A), and increased the levels of Egr-1 and UCP proteins (Fig. 2B). The induction of UCP expression following Egr-1 expression was also detected in EGF-treated CAKI-1 cells (Fig. 2C,D), in which VHL but not UCP is highly expressed [Jung et al., 2006]. EGF treatment did not greatly affect transcript levels of VHL and HIF-1α in both HeLa and CAKI-1 cells (Fig. 2A,C).

SERUM INDUCES EXPRESSION OF SRF AND UCP AT BOTH mRNA AND PROTEIN LEVELS

Serum-stimulation has been shown to induce serum response factor that binds to SRE in the cell [Treisman, 1986]. To examine the involvement of SRF in the expression of UCP gene, we stimulated serum-starved HeLa cells with 5% or 20% FBS, and determined mRNA and protein levels of SRF, UCP, VHL, or HIF-1α by RT-PCR and Western blot analyses. Serum induced expression of SRF and UCP at both mRNA and protein levels, but did not much affect mRNA levels of VHL and HIF-1α (Fig. 3A). EGF, HGF, PMA, or serum increased the levels of UCP protein in cells, decreased VHL protein levels, and increased HIF-1α protein levels (Figs. 2B,D and 3B). The HRE-reporter activity was increased in proportion to increased HIF-1α levels after EGF treatment, suggesting that accumulated HIF-1α is biologically active (Fig. 2B, EGF).

Egr-1 AND SRF PROTEINS BIND SEQUENCE-SPECIFICALLY TO THE UCP PROMOTER

To test the binding of Egr-1 or SRF to the UCP promoter, we synthesized EBS1 and SRE oligonucleotides, which were ^{32}P-labeled and used as probes in EMSA. Nuclear extracts were prepared from HeLa cells treated or untreated with PMA, EGF, or HGF, and

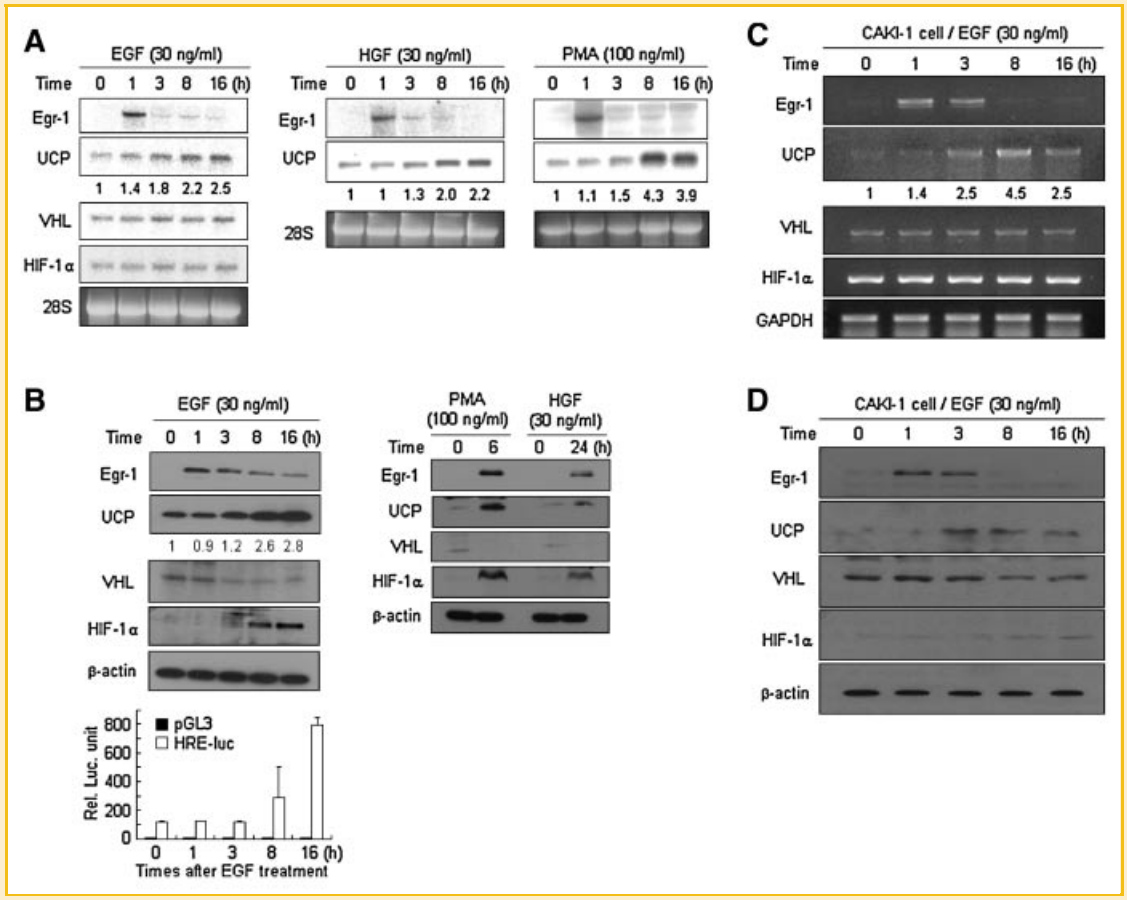

Fig. 2. EGF, HGF, or PMA induces UCP expression following early induction of Egr-1 expression. A: Serum-starved HeLa cells were treated with EGF, HGF, or PMA at the indicated concentrations, and harvested at the indicated times. Total RNA was prepared and mRNA levels of the indicated genes were analyzed by Northern blot hybridization. The panels of 28S show the ethidium bromide-stained photograph of the gel before transfer to demonstrate equal loading of RNA. B: Serum-starved HeLa cells were treated with EGF, HGF, or PMA under the same conditions as described in (A), and harvested at the indicated times. Cell lysates were immunoblotted with the indicated antibodies or subjected to HRE-report assay. The reporter assay was performed three times, each in duplicate. C,D: Serum-starved CAKI-1 cells were treated with EGF and harvested at the indicated times. Cells were split into two fractions for preparation of total RNA and cell lysates. C: mRNA levels of the indicated genes were analyzed by RT-PCR. D: Cleared cell lysates were immunoblotted with the indicated antibodies. mRNA and protein levels of UCP were quantified by densitometry and relatively expressed.

incubated with the ^{32}P-labeled EBS1 probe. The incubation mixtures were subjected to EMSA. The Egr-1/EBS1 DNA complex was detected in nuclear extracts from cells treated with PMA, EGF, or HGF (Fig. 4A). The specificity of the formed complex was examined with nuclear extracts from HeLa cells treated or untreated with EGF by cold competition assay. The Egr-1/EBS1 DNA complex formed was specifically abrogated in the presence of 50-M excess of unlabeled consensus EBS oligonucleotide (Fig. 4B). Nuclear extracts were prepared from HeLa cells stimulated with or without serum, and incubated with the ^{32}P-labeled SRE probe. The reaction products were analyzed by EMSA. The SRF/SRE DNA complex was detected in nuclear extract from cells stimulated with serum, and the complex formed was specifically abolished in the presence of 50-M excess of unlabeled consensus SRE oligonucleotide (Fig. 4C).

We next examined whether Egr-1 and SRF bound to the regions between −190 and −21 (170 bp) and between −988 and −801 (188 bp) in the UCP promoter in living cells, respectively. To this end, HeLa cells were treated or untreated with EGF or serum, and ChIP assay was performed as described in Materials and Methods Section. The UCP genomic fragment captured by anti-Egr-1 or anti-SRF antibodies was detected by PCR amplification using the specific primers located at each end of the 170 bp containing EBS1 and EBS2 or the 188 bp containing SRE. The UCP promoter was efficiently recovered from Egr-1 immunoprecipitate from EGF-treated cells (Fig. 4D, lane 4), but not from untreated cells (lane 3) or from immunoprecipitates of non-specific IgG (lanes 1 and 2). The PCR product amplified from the Egr-1 immunoprecipitate (Fig. 4D, lane 4) migrated at the same position as PCR products directly amplified from cross-linked chromatin or p-1000/luc DNA (lanes 5 and 6). The PCR product amplified from SRF immunoprecipitate from serum-stimulated cells (Fig. 4D, lane 11) migrated at the same position as PCR products directly amplified from the cross-linked chromatin or p-1000/luc DNA (lanes 12 and 13). No amplification was found from non-specific IgG immunoprecipitates (Fig. 4D, lanes 8 and 9) or SRF immunoprecipitate from unstimulated cells (lane 10). Collectively, the results suggest that Egr-1 and SRF bind sequence-specifically to

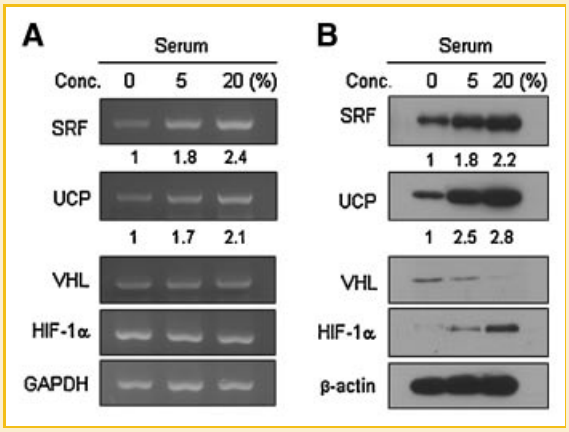

Fig. 3. Serum stimulation induces expression of SRF and UCP at both mRNA and protein levels. HeLa cells were serum-starved for 72 h, and were stimulated with 5% or 20% serum for 2 h for total RNA preparation, or for 12 h for cell lysates preparation. A: mRNA levels of the indicated genes were analyzed by RT-PCR. B: Cell lysates were immunoblotted with the indicated antibodies. The mRNA and protein levels of SRF and UCP were quantified by densitometry and relatively expressed.

the UCP promoter in the cells treated with EGF and serum, respectively.

Egr-1 AND SRF ARE INVOLVED IN EGF- AND SERUM-MEDIATED INDUCTION OF UCP EXPRESSION, RESPECTIVELY

We transfected Egr-1 or SRF expression vectors into HeLa cells, and examined whether forced expression of Egr-1 or SRF induced the expression of UCP gene. Expression of Egr-1 or SRF increased the level of UCP protein in a dose-dependent manner (Fig. 5A). We next examined whether depletion of endogenous Egr-1 or SRF decreased the level of UCP protein in cells. Expression of Egr-1-siRNA abrogated EGF-mediated induction of Egr-1 expression (Fig. 5B, lanes 2 and 4), suggestive of functionality of the Egr-1-siRNA expression vector. EGF treatment increased the level of UCP protein by 2.9-fold (Fig. 5B, lanes 1 and 2), but increased the UCP protein level by 1.4-fold when Egr-1 was depleted (lanes 3 and 4). Similar results were observed in a HeLa cell line constitutively expressing Egr-1-siRNA (data not shown). Serum-mediated induction of SRF expression was abolished by expression of SRF-siRNA (Fig. 5B, lanes 6 and 8), suggesting that the SRF-siRNA expression vector depletes endogenous SRF efficiently. Serum increased the level of UCP protein by 1.5-fold, but did not affect UCP protein level when SRF was depleted (Fig. 5B, lanes 5–8). Protein levels of VHL and HIF-1α reflected alterations in the levels of UCP protein (Fig. 5A,B).

UCP EXPRESSION IS REQUIRED FOR CANCER CELL GROWTH BY GROWTH FACTORS OR SERUM

To test whether the induced UCP expression is essential for cancer cell proliferation, HeLa cells with or without UCP-siRNA or Con-siRNA expression vectors were serum-starved, and incubated in medium with or without EGF, HGF, or serum. Viable cells were counted at the indicated times (Fig. 5C). EGF and HGF increased the growth rates of serum-starved cells by 1.7- and 1.5-fold, respectively. Serum robustly increased the growth rate of the cell by 3.1-fold, presumably because serum contains multiple factors for cell proliferation. UCP-siRNA expression vector markedly inhibited EGF- and HGF-mediated cell proliferation by 81% and 79% at day 6 after seeding, respectively, and moderately inhibited serum-mediated cell proliferation by 55% (Fig. 5C). The results suggest that UCP expression is required for the growth factors- or serum-mediated cell proliferation and UCP may differentially regulate cell proliferation signal.

EGF INDUCES UCP EXPRESSION FOLLOWING EARLY INDUCTION OF Egr-1 EXPRESSION IN VIVO

A comparison of the UCP promoter sequence from human genome with that from mouse genome revealed that the Egr-1 and SRF binding sites are conserved in the human and mouse UCP promoters (Fig. 6A). Putative binding sites for TCF/LEF1 and CREB transcription factors in the human UCP promoter appeared not to be functional under the experimental conditions (Fig. 1 and data not shown). The systemic delivery of EGF into mice has been shown to increase phosphorylations of the EGR receptor and extracellular signal related kinases in the liver, and also to increase Egr-1 mRNA and protein levels in hepatocytes [Ruff-Jamison et al., 1993; Boylan and Gruppuso, 1998; Liu et al., 2000; Tsai et al., 2001]. EGF has been shown to phosphorylate SRF in cells [Rivera et al., 1993]. These findings led us to examine the possibility whether EGF induces UCP expression in mouse liver. To this end, we injected murine EGF into mice via the tail vein, and examined time-dependent alterations of mRNA and protein levels of the molecules indicated in Figure 6B,C in the liver. EGF injection resulted in early induction of Egr-1 expression at both mRNA and protein levels, and phosphorylations of EGF receptor and SRF (Fig. 6B,C), suggesting that the EGF signal pathway is activated in the liver. UCP mRNA level began to increase at 3 h and increased by 8 h, but the levels of VHL and HIF-1α mRNAs were not greatly changed (Fig. 6B). After EGF injection, the levels of Egr-1 and SRF proteins peaked at 1 and 2 h, respectively, and the level of UCP protein subsequently began to increase at 3 h (Fig. 6C). The levels of UCP protein appeared to partially reflect time-dependent alterations of UCP mRNA levels (Fig. 6B,C). This may represent the presence of a mode by which UCP protein level is regulated at post-translational step; however, this requires further clarification. The increase of UCP expression resulted in a decrease of VHL and an increase of HIF-1α at protein levels (Fig. 6C). Collectively, these results support an in vivo presence of the Egr-1/SRF-UCP-VHL-HIF pathway.

DISCUSSION

UCP may be considered as a promising target for cancer therapy, as UCP is highly expressed in human cancer tissues compared with normal tissues [Welsh et al., 2001; Wagner et al., 2004], and promotes tumor growth [Jung et al., 2006]. Identifying cellular factors that regulate the expression of UCP gene may help understand why UCP is highly expressed in human cancers and thereby underscore its potential as a therapeutic target. Therefore,

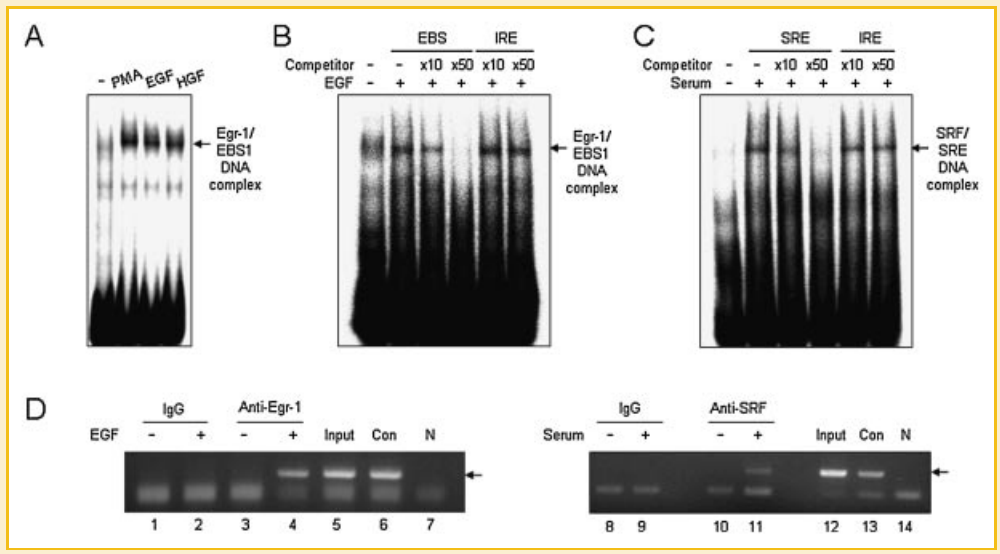

Fig. 4. Egr-1 and SRF transcription factors bind sequence-specifically to the UCP promoter. A: HeLa cells were serum-starved for 24 h, and treated or untreated with 100 ng/ml PMA, 30 ng/ml EGF, or 30 ng/ml HGF for 1 h, and nuclear extracts were prepared. Nuclear extracts (10 μg of each) were incubated with ^{32}P-labeled EBS1 oligonucleotide probe. The reaction products were resolved by native PAGE and autoradiographed. B: Nuclear extracts were prepared from HeLa cells treated or untreated with 30 ng/ml EGF for 1 h. Nuclear extracts (10 μg of each) were pre-incubated with or without unlabeled oligonucleotide probes containing consensus EBS or IRE at the indicated molar excess, and then incubated with the ^{32}P-labeled EBS1 probe. The reaction products were resolved by native PAGE and autoradiographed. C: Serum-starved HeLa cells were stimulated or not with 20% FBS for 2 h and nuclear extracts were prepared. Ten micrograms of nuclear extracts were pre-incubated with or without unlabeled consensus SRE or IRE oligonucleotide probes at the indicated molar excess, and then incubated with ^{32}P-labeled SRE oligonucleotide probe. The reaction products were resolved by native PAGE and autoradiographed. D: Serum-starved HeLa cells were stimulated or not with 30 ng/ml EGF for 1 h or 20% FBS for 2 h. Following formaldehyde cross-linking, chromatin complexes were immuoprecipitated by a non-specific rabbit serum (IgG) (lanes 1, 2, 8, and 9), anti-Egr-1 antibody (lanes 3 and 4), or anti-SRF antibody (lanes 10 and 11). Genomic DNA fragments recovered by anti-Egr-1 or anti-SRF antibodies were amplified by PCR using the primers described in Materials and Methods Section. Input (lanes 5 and 12) and Con (lanes 6 and 13) represent DNA fragments (indicated by arrows) directly amplified by PCR using the relevant primers from cross-linked chromatin or p-1000/luc. N (lanes 7 and 14), PCR amplification without DNA as a negative control. Representative images of agarose gel electrophoresis of PCR products are shown.

we analyzed here the UCP promoter activity and identified Egr-1 and SRF transcription factors responsible for the induction of UCP expression by the growth factors and serum, respectively. Furthermore, our findings provide a molecular basis that the Egr-1/SRF-UCP-VHL pathway is in part responsible for the growth factors-mediated increase of HIF-1α protein level in non-hypoxic conditions.

Deletion of Egr-1 binding sites abrogated the UCP promoter activation by the growth factors (Fig. 1C). The treatment of HeLa cells with EGF, HGF, or PMA resulted in the increase of UCP mRNA and protein levels following Egr-1 expression (Fig. 2A,B). EGF-mediated induction of Egr-1 expression and subsequent UCP expression were also detected in CAKI-1 cells (Fig. 2C,D) and mouse liver (Fig. 6B,C). The induced Egr-1 protein bound sequence-specifically to the UCP promoter in vitro and in living cells (Fig. 4B,D). Egr-1 overexpression increased the level of UCP protein in the cell (Fig. 5A). EGF-mediated induction of UCP expression was suppressed by transfection of Egr-1-siRNA expression vector (Fig. 5B). Serum has been shown to activate the Egr-1 promoter containing five SREs [Sakamoto et al., 1991]. Therefore, serum-mediated increase of UCP mRNA level (Fig. 3) may be attributed to Egr-1 induced by serum. However, serum stimulation increased mRNA and protein levels of SRF in the cell (Fig. 3), and resulted in the SRE-specific binding of serum-induced SRF to the UCP promoter in vitro and in living cells (Fig. 4C,D). SRF overexpression increased the level of UCP protein (Fig. 5A) and serum-mediated induction of UCP expression was abolished by depletion of endogenous SRF (Fig. 5B). Phosphorylated SRF was detected in the liver of mice treated with EGF (Fig. 6C). Thus, these results suggest that Egr-1 and SRF induce the expression of UCP gene independently and directly, although Egr-1 and SRF may also mutually induce UCP expression.

UCP protein expression was detected in many tumor cell lines under normal culturing conditions (DMEM + 10% FBS, 37°C, and 5% CO_2) [Jung et al., 2006]. RNA interference-mediated depletion of endogenous Egr-1 and SRF appeared to negatively regulate only the EGF- and serum-mediated induction of UCP expression in HeLa cells, respectively (Fig. 5B). These findings suggest the presence of a mode by which UCP is constitutively expressed depending on cell lines. Egr-1 and Sp1 transcription factors have been shown to bind overlapping GC-rich binding sites in a number of promoters [Khachigian et al., 1995; Cui et al., 1996; Zhang et al., 2007]. Regulation of transcription by these two transcription factors is complex, with their synergistic interaction in some genes but mutual competition in other genes [Chen et al., 2006]. Sp family proteins regulate basal and constitutive expression of genes involved in multiple functions in both normal and cancerous tissues [Safe and Abdelrahim, 2005]. Endogenous SRF protein was detected in serum-starved HeLa cells (Fig. 5). Thus, SRF or Sp1 that may bind to the Egr-1 binding sites in the UCP promoter may affect expression of the UCP gene under normal culturing conditions. A variation of UCP

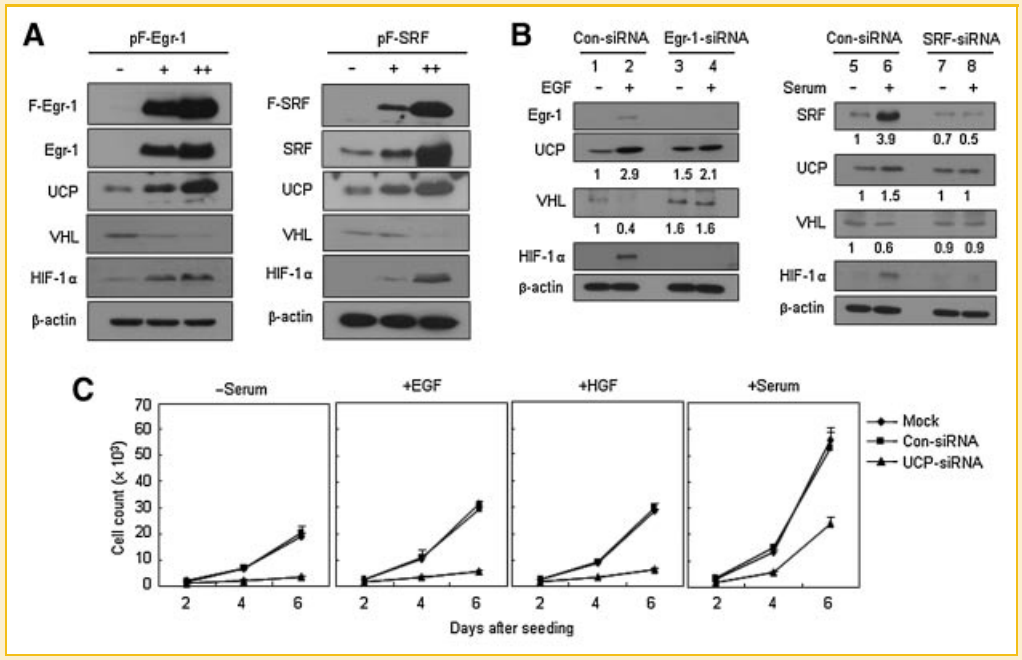

Fig. 5. Egr-1 and SRF are involved in EGF- and serum-mediated induction of UCP expression that is required for cancer cell proliferation. A: HeLa cells were mock-transfected or transfected with 5 or 15 μg DNA of the indicated expression vectors and incubated for 48 h. Cleared cell lysates were immunoblotted with the indicated antibodies. B: HeLa cells (3×10^6) were transfected with the indicated siRNA expression vectors (15 μg of each vector), incubated for 16 h, and further incubated in serum-free media for 24 h. These cells were treated or untreated with 30 ng/ml EGF for 24 h (lanes 1–4), or stimulated or not with 20% FBS for 12 h (lanes 5–8). Cell lysates were immunoblotted as indicated. The levels of SRF, UCP, and VHL proteins were quantified by densitometry and relatively expressed. C: HeLa cells were transfected with or without the indicated siRNA expression vectors, incubated for 16 h, and further incubated in serum-free medium for 48 h. These cells were seeded at a density of 500 cells per well in 6-well plates, incubated in medium with or without 30 ng/ml EGF or HGF, or 10% FBS, and harvested at the indicated times. We counted viable cells with a hemocytometer. Culture medium was changed at day 4 after seeding. We repeated the experiments once, each in duplicate. Data are mean ± s.d.

expression between cell lines may also result from a different combination of signaling molecules which are likely expressed at different levels depending on cell types. Thus, molecular mechanisms involved in constitutive UCP expression depending on the cell lines require further clarification; however, our findings clearly show functional involvement of Egr-1 and SRF in the growth factors- and serum-mediated induction of UCP expression, respectively.

Egr-1 has been shown to either promote or suppress cell proliferation, depending on the cellular context [Thiel and Cibelli, 2002]. Egr-1 positively regulates progression of prostate and skin cancers in mouse [Riggs et al., 2000; Abdulkadir et al., 2001], and growth of breast and bladder cell lines [Mitchell et al., 2004; Nutt et al., 2007]. Egr-1 is involved in HGF-mediated expression of fibronectin associated with metastasis [Gaggioli et al., 2004]. SRF has been shown to be activated by serum or growth factors, and to be involved in cellular processes, such as cell proliferation and wound healing [Treisman, 1986; Chai and Tarnawski, 2002]. SRF is increasingly expressed in advanced human liver cancers, and associates with tumor progression, specifically at the transition to an invasive metastatic stage of carcinogenesis [Psichari et al., 2002; Park et al., 2007]. Thus, this study provides additional evidence for the involvement of Egr-1 and SRF in cell growth and tumorigenesis, as these transcription factors positively regulate expression of UCP that degrades the VHL tumor suppressor.

It has been shown that HeLa cell proliferation is not affected by the reduction in UCP expression [Tedesco et al., 2007], which is different from our findings (Fig. 5C). The reason for this discrepancy is not clear. Presumably the discrepancy may result from a difference in RNA interference-mediated reduction levels of UCP or in duration to monitor UCP depletion effect on HeLa cell growth.

Thirteen of 17 cell lines express HIF-1α protein constitutively under non-hypoxic conditions [Zhong et al., 2002]. A variety of growth factors and PMA have been shown to increase HIF-1α protein levels in non-hypoxic conditions by multiple mechanisms, such as activation of phosphatidyl-inositol-3-kinase (PI3K)/Akt or mitogen-activated protein kinase (MAPK) pathways [Semenza, 2001; Bilton and Booker, 2003]. Egr-1 expression has been shown to be regulated by different signaling molecules including PI3K, MAPK, and protein kinase C (PKC) in cells [Lo et al., 2001; Moorehead et al., 2003; Gaggioli et al., 2004; Worden et al., 2005]. PMA induces Egr-1 expression through the PKC-dependent or PKC-independent pathways [Wong et al., 2002; Malakooti et al., 2006]. We have not addressed here the signaling molecules directly involved in the increase of Egr-1 and SRF expression. However, previous findings and this study suggest that EGF, HGF, PMA, or serum likely activates PI3K, MAPK, and/or PKC pathways, resulting in the increased expression of UCP following the induction of Egr-1 or SRF expression. This increased UCP expression resulted in the decrease of VHL and the increase of HIF-1α at protein levels

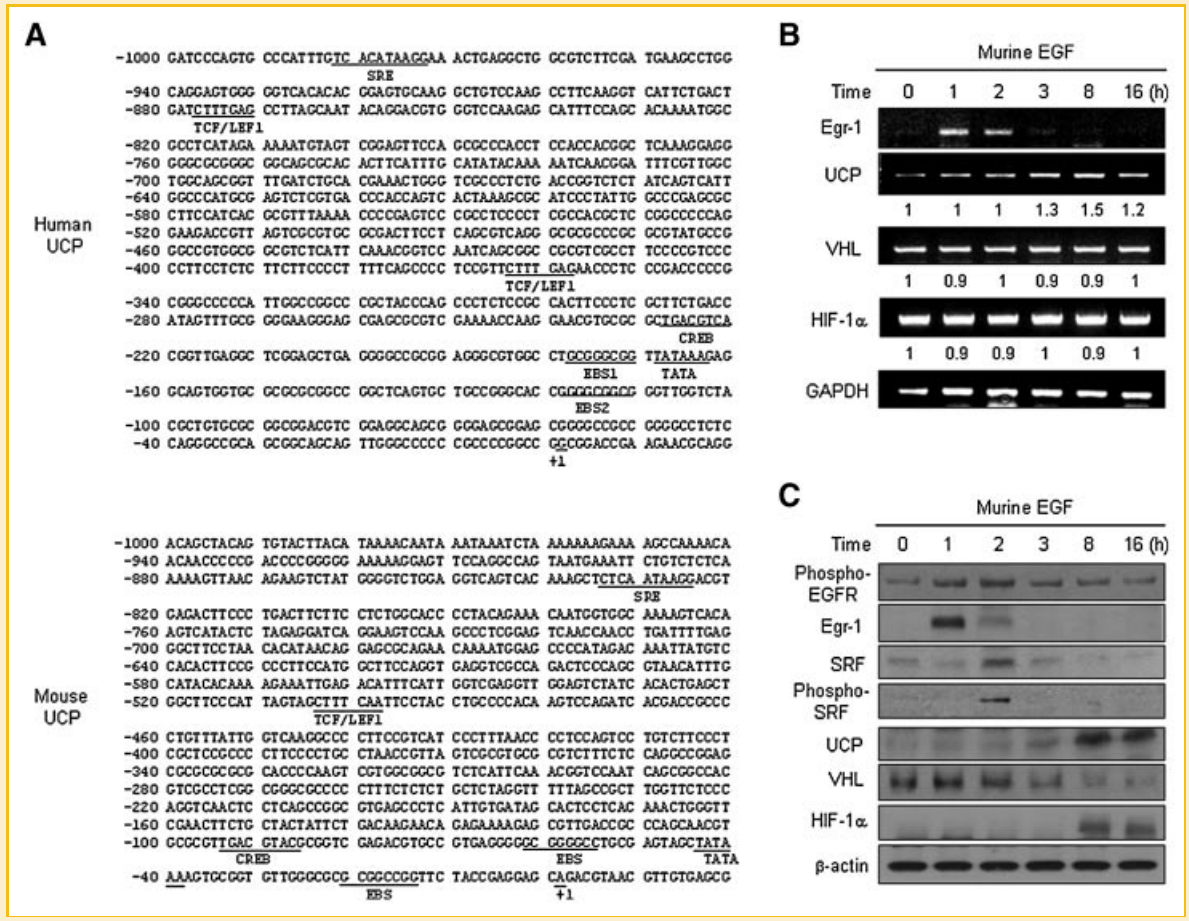

Fig. 6. EGF induces UCP expression following early induction of Egr-1 expression in vivo. A: The human and mouse UCP promoter sequences. Binding sites for SRF and Egr-1 and putative binding sites for TCF/LEF-1 and CREB transcription factors are shown. '+1' indicates putative transcription initiation sites. TATA, TFIID binding site. B,C: Murine EGF (100 μg/kg) was injected into mice intravenously. The liver was excised from mice at the indicated times. Total RNA and protein extracts were prepared from the excised liver. RT-PCR (B) and immunoblotting analyses (C) were performed as described in Materials and Methods Section. mRNA levels of UCP, VHL, and HIF-1α were quantified by densitometry and relatively expressed. Similar results were obtained from separate experiments. Phospho-EGFR, Tyr1173-phosphorylated-EGF receptor tyrosine kinase; phospho-SRF, Ser103-phosphorylated SRF.

(Figs. 2, 3, 5, and 6). Thus, these results suggest that growth factors or serum may increase the level of HIF-1α protein through the Egr-1/SRF-UCP-VHL pathway under normoxia.

What is the role of increased HIF-1α in cell proliferation under normoxia? HIF-1α has been shown to be involved in the induction of a number of genes encoding angiogenic factors, glucose transporter and glycolytic enzymes, and survival factors [Semenza, 2003]. Rapidly proliferating cells such as cancer cells accompany with increased metabolic demands, for which functionality of those HIF-1 target genes is likely essential. In this context, increased HIF-1α level may be beneficial for the cell under normoxia to adequately proliferate.

Egr-1 plays a key master regulatory role in multiple cardiovascular pathobiological processes [Khachigian, 2006]. Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth [Fahmy et al., 2003], and plays an important role in vascular recovery after occlusion [Lee et al., 2005]. However, it has remained unclear how the Egr-1 transcription factor associates with angiogenesis [Khachigian, 2006]. UCP expression increases the level of VEGF mRNA through the VHL-HIF pathway [Jung et al., 2006] and results in enhanced proliferation and tubule formation of human umbilical endothelial cell (data not shown). Our findings suggest that Egr-1 may in part promote angiogenesis through the UCP-VHL-HIF pathway.

In conclusion, we demonstrated here that growth factors and serum induce UCP expression through the induction of Egr-1 and SRF expression, respectively. The Egr-1/SRF-UCP-VHL pathway may be in part responsible for the growth factors-mediated increase of HIF-1α protein level in non-hypoxic conditions. The UCP expression is required for cancer cell proliferation by EGF, HGF, or serum, which may in part account for the high level of UCP expression in common human cancers.

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