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The Basic Helix-Loop-Helix/Leucine Zipper Transcription Factor USF2 Integrates Serum-Induced PAI-1 Expression and Keratinocyte Growth

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ABSTRACT

Plasminogen activator inhibitor type-1 (PAI-1), a major regulator of the plasmin-dependent pericellular proteolytic cascade, is prominently expressed during the tissue response to injury although the factors that impact PAI-1 induction and their role in the repair process are unclear. Kinetic modeling using established biomarkers of cell cycle transit (c-MYC; cyclin D_1; cyclin A) in synchronized human (HaCaT) keratinocytes, and previous cytometric assessments, indicated that PAI-1 transcription occurred early after serum-stimulation of quiescent (G_0) cells and prior to G1 entry. It was established previously that differential residence of USF family members (USF1→USF2 switch) at the PE2 region E box (CACGTG) characterized the $G_0 \rightarrow G_1$ transition period and the transcriptional status of the PAI-1 gene. A consensus PE2 E box motif (5′-CACGTG-3′) at nucleotides −566 to −561 was required for USF/E box interactions and serum-dependent PAI-1 transcription. Site-directed CG → AT substitution at the two central nucleotides inhibited formation of USF/probe complexes and PAI-1 promoter-driven reporter expression. A dominant-negative USF (A-USF) construct or double-stranded PE2 "decoy" attenuated serum- and TGF-β1-stimulated PAI-1 synthesis. Tet-Off induction of an A-USF insert reduced both PAI-1 and PAI-2 transcripts while increasing the fraction of Ki-67$^+$ cells. Conversely, overexpression of USF2 or adenoviral-delivery of a PAI-1 vector inhibited HaCaT colony expansion indicating that the USF1 → USF2 transition and subsequent PAI-1 transcription are critical events in the epithelial go-or-grow response. Collectively, these data suggest that USF2, and its target gene PAI-1, regulate serum-stimulated keratinocyte growth, and likely the cadence of cell cycle progression in replicatively competent cells as part of the injury repair program. J. Cell. Biochem. 115: 1840–1847, 2014.

KEY WORDS: PAI-1; USF; TRANSCRIPTION; KERATINOCYTES; GENE REGULATION; EXPRESSION PROFILING; SERPINE1

Upstream stimulatory factor-1 and -2 (USF1/2) are members of the conserved basic helix-loop-helix/leucine zipper (bHLH-LZ) MYC family of E box-binding transcription factors [Littlewood and Evan, 1995; Corre and Gailbert, 2005]. Multiple signaling networks influence USF1/2 function largely through site-specific phosphorylation; dimer composition and recruited co-factors dictate target gene expression and growth control [Gailbert et al., 2001; Qi et al., 2006; Pawlus et al., 2013]. The cell cycle-related anti-proliferative functions of USF1/2, moreover, are context-dependent and involve specific mitogen-activated (p38, PKC, PKA) and cyclin-dependent (CDK1, CDK4) kinases as well as the APC, p27, BRCA2, p53, and TGF-β1 tumor suppressor pathways [Luo and Sawadogo, 1996; Qyang et al., 1999; Jaiswal and Naravan, 2001; Corre and Gailbert, 2005; Jung et al., 2007; Kim et al., 2008]. Inhibition of cell proliferation by USF family members likely requires down-regulation of cyclin B_1 and CDK1, c-MYC suppression and p27 and p53 induction [Corre and Gailbert, 2005; Jung et al., 2007; Kim et al., 2008].

USF1/2 regulate growth state-dependent transcription of the plasminogen activator inhibitor-1 (PAI-1, SERPINE1) gene [Qi et al., 2006], a major TGF-β1 and p53 target [Akiyoshi et al., 2001; Allen et al., 2005; Kortlever et al., 2006; Freytag et al., 2010].

Abbreviations: USF, upstream stimulatory factor; PAI-1, plasminogen activator inhibitor-1; TGF-β1, transforming growth factor-β1.

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Physiologically, PAI-1 modulates the pericellular plasmin-generating cascade and is a prominent member of the serum-induced "wound-response" transcriptome [Iyer et al., 1999; Qi et al., 2008]. In immortalized keratinocytes, PAI-1 is required for TGF-β1-stimulated planar migration and Matrigel barrier invasion perhaps by LRP1-dependent engagement of the Jak/Stat pathway [Degryse et al., 2004; Providence et al., 2008; Freytag et al., 2010]. Matricellular (i.e., anchored) PAI-1, in fact, promotes a mesenchymal-to-amoeboid transition with activation of signaling pathways required for efficient 3-D "stromal" migration [Cartier-Michaud et al., 2012]. The consistent association of PAI-1 expression with the global program of tissue injury [Iyer et al., 1999; Providence and Higgins, 2004; Fitsialos et al., 2007] suggests that this SERPIN integrates cycles of cell-to-substrate adhesion/dis-adhesion with repair "scaffold" remodeling to meet the requirements for effective cellular migration [Czekay et al., 2011]. The specific phenotypic impact of PAI-1, however, likely depends on a balance of its intracellular (caspase-3 inhibition) and extracellular (protease inhibitory, receptor signaling, suppression of CUB domain-containing protein 1 cleavage by plasmin) activities that may be both cell type- and stimulus-specific [Law et al., 2013].

PAI-1 transcription is an early event in serum-stimulated quiescent (G_0) keratinocytes. Expression occurs prior to G_1 entry and involves a USF subtype switch (USF1 → USF2) at the PE1/PE2 site E box motifs (5′-CACGTG-3′) in the PF1 region (nucleotides −794 to −532) of the PAI-1 promoter [Qi et al., 2006]. The typical rapid decline in PAI-1 mRNA levels several hours prior to the onset of DNA synthesis [Mu et al., 1998; Qi et al., 2006], however, suggests that PAI-1 may have a negative influence on cell growth. PAI-1 knockdown, in fact, leads to escape from both senescence-associated proliferative arrest and TGF-β1-induced cytostasis in primary fibroblasts and human (HaCaT) keratinocytes [Kortlever et al., 2006, 2008]. Certain growth suppressive genes, particularly those responsive to the USF family of anti-proliferative bHLH-LZ transcription factors, may actually function as key regulators of the "go or grow" proliferation/migration dichotomy. The current paper addresses this issue; the evidence implicates USF2 as an integrator of human keratinocyte growth and PAI-1 gene control.

MATERIALS AND METHODS

CELL LINES AND EXPRESSION VECTORS

Human HaCaT keratinocytes were cultured as described [Qi et al., 2006]. A HA-tagged dominant-negative USF construct (A-USF) [Krylov et al., 1997; Qyang et al., 1999] was cloned in the *Mlu* I/*Eco*R V site of pBl and co-transfected with pTRE2 (for Tet-Off induction). Mock transfectants (empty vector + pTRE2) served as controls. Insert expression after oxytetracycline removal was confirmed by RT-PCR and Western blotting. Full-length human PAI-1 cDNA, excised from pcDNA3.1 using 5′-*Kpn*I and 3′-*Not*I, was cloned into AdTrack-CMV to produce recombinant adenovirus [Law et al., 2013]. The pCMV-USF2 vector was detailed previously (4). Regions of the human PAI-1 promoter were PCR-amplified using primers to add 5′ *Sac*I and 3′ *Xho*I restriction sites. PCR products were gel-purified, ligated into TOPO TA and cloned into the *Sac*I/*Xho*I site of the pGL3 luciferase expression vector to create a full length (nucleotides −806 to +72) construct [Allen et al., 2005]. The dinucleotide substitution CACGTG → CAATTG in the PE1 and PE2 region E boxes was created by site-directed mutagenesis. HaCaT cells were co-transfected with individual reporter constructs and a SV40-driven β-galactosidase-expressing plasmid (for signal normalization) using Lipofectamine, FBS-deprived for 1 day then stimulated with serum or TGF-β1 for 8 h prior to extraction and reporter analysis.

MICROARRAY ANALYSES

Transcript profiling utilized Affymetrix Human Genome U133 Plus 2.0 arrays [Qi et al., 2008] and Affymetrix GCOS software; probe signal outputs (pivot tables) were imported into GeneSpring v6.1. Expression profiles of empty vector versus A-USF transfectants after oxytetracycline removal used the PAHS-028A human tumor metastasis and PAHS-012A human apoptosis RT^2 PCR arrays (SABiosciences). Expression levels of selected genes ($2^{\Delta\Delta Ct}$ based-fold change calculations), normalized to housekeeping controls, was determined by real-time PCR used a MyiQ Cycler system (Bio-Rad, Hercules, CA).

NORTHERN BLOTTING AND RT-PCR

Cellular RNA was denatured at 55°C for 15 min in 1× MOPS, 6.5% formaldehyde and 50% formamide, size-fractionated on 1% agarose/formaldehyde gels using 1× MOPS, transferred with 10× SSC to positively charged nylon membranes and UV crosslinked. ^{32}P-labeled cDNA probes to PAI-1, uPA, or GAPD were hybridized overnight at 42°C in 50% formamide, 2.5× Denhardt's solution, 1% SDS, 100 μg/ml sheared/denatured salmon sperm DNA, 5× SSC, 10% dextran sulfate and washed three times with 0.1× SSC/0.1× SDS for 15 min each at 42°C then at 55°C prior to exposure to film. For RT-PCR, total RNA was isolated with Qiagen RNeasy mini-columns (Qiagen, Valencia, CA) and first strand cDNA synthesized by addition of MMLV RNase H^+ iScript reverse transcriptase (BioRad, Hercules, CA) to a mixture of 2–10 μg RNA and oligo(dT)/random primers. Forward and reverse primer sets for selected genes are described in Table I.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Keratinocytes were disrupted in cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM EGTA, 0.5 mM PMSF, 0.6% NP-40), nuclei collected at 15,000*g* for 1 min, lysed on ice for 30 min (in 20 mM HEPES, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and nuclear extracts clarified at 15,000*g* for 5 min. The ^{32}P-end-labeled double-stranded PAI-1 promoter PE2 E box region probe: 5′-CCAAGTCC-TAGACAGACAAAACCTAGACAATCACGTGGCTGGCTGC-3′ was incubated with 2–10 μg nuclear extract protein in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 1 μg/ml poly dI:dC, 10 mM Tris–HCl, pH 7.5) at room temperature for 20 min followed by an additional 30 min incubation (where indicated) with antibodies to USF2, MAX, TEF3 or E2F1 (Santa Cruz Biotechnology) and complexes separated on 4% polyacrylamide gels in TBE buffer. PE2 sequence requirements for PE2 region probe binding were determined using wild-type and mutant PE2 competing constructs and mobility shift assay as detailed [Qi et al., 2006].

TABLE I. Primer Sets for RT-PCR Validation of Microarray Profiling

Primer	Sequence
Human beta-actin, forward primer	5′-GAGAAAATCTGGCACCAC-3′
Human beta-actin, reverse primer	5′-CTAGAAGCATTTGCGGTG-3′
Human uPAR, forward primer	5′-GGGAAGAAGGAGAAGAGC-3′
Human uPAR, reverse primer	5′-TTTCGGTTCGTGAGTGCC-3′
Human HSPC159, forward primer	5′-CCCACGACTGATAGTTCC-3′
Human HSPC159, reverse primer	5′-TGGTGATCTGGAGGTCTC-3′
Human MAP3K8, forward primer	5′-CACCTCATGAGACTCTCC-3′
Human MAP3K8, reverse primer	5′-GTTGCTAGGTTTAATATC-3′
Human EGR3, forward primer	5′-GGCTACAGAGAATGTAAT-3′
Human EGR3, reverse primer	5′-GAATGCCTTGATGGTCTC-3′
Human dual specificity phosphatase 1, forward primer	5′-CAAAGGAGGATACGAAGC-3′
Human dual specificity phosphatase 1, reverse primer	5′-GATGGAGTCTATGAAGTC-3′
Human 18S rRNA, forward primer	5′-TTCAAAGCAGGCCCGAGC-3′
Human 18S rRNA, reverse primer	5′-CGTCTTCTCAGCGCTCCG-3′

WESTERN BLOTTING

Trypsin-released cells (at the indicated time points after serum re-introduction) were collected by centrifugation, lysed in 4% SDS (in Ca^{2+}/Mg^{2+}-free PBS) and extracts boiled for 5 min. Following electrophoretic separation (of 30 μg of protein) and transfer to nitrocellulose, membranes were blocked in 5% milk in 0.05% Triton X-100/PBS, incubated overnight with antibodies to the HA tag, PAI-1 or c-MYC in 5% milk in 0.05% Triton X-100/PBS, washed three times in 0.05% Triton X-100/PBS and incubated with appropriate secondary antibodies. Immunoreactive proteins were visualized with ECL reagent. Membranes were stripped and reprobed with ERK2 antibodies (Santa Cruz Biotechnology) to assess loading levels.

RESULTS

KINETICS OF SERUM-INDUCED PAI-1 EXPRESSION

The 2–6 h window after serum-stimulation, based on c-MYC and cyclin D_1 induction (Figs. 1A–C) as well as prior cytometric staging [Qi et al., 2006], appears to be a commitment point in the entry of quiescent HaCaT keratinocytes into a cycling G_1 state. Microarray analysis of serum-starved and 2 h FBS-"activated" HaCaT cells served, therefore, to define the transcriptional signature of the early $G_0 \rightarrow G_1$ transition [Qi et al., 2008]. Prominently up-regulated transcripts included those encoding proteins involved in the initial growth response (EGR1–4), transcription (Myc, Fos, Jun, KLF4, AT3, p300/pTAF) and ECM remodeling (uPA, uPAR, tPA, SERPINE1 [PAI-1], SERPINB1 [leukocyte elastase inhibitor], SERPINB2 [PAI-2], CTGF, MMP-2, MMP-12, CYR61) (Fig. 2A). Rank ordering of microarray, northern blot and RT-PCR data indicated that PAI-1 and the uPA receptor (PLAUR) (Fig. 2A–C) partitioned to the most significantly elevated group. PAI-1 transcripts, both the 3.0- and 2.2-kb species, were low to undetectable in quiescent HaCaT cells, peaked approximately 2 h after serum-addition (Fig. 2D) which correlated with residence in an early activated G_0 substate and declined prior to synchronous entry into S phase [Qi et al., 2006]. PAI-1 protein synthesis closely followed induction of PAI-1 mRNA (Fig. 2E). uPA levels increased (by 12-fold) as well although maximal expression occurred several hours later than PAI-1 mRNA (Fig. 2D).

INTERFERENCE WITH USF-DNA BINDING IMPACTS PAI-1 EXPRESSION

The PE2 CACGTG hexanucleotide motif resides three nucleotides downstream of a trio of SMAD-binding elements located at positions −595 to −569 upstream of the transcription start site [Dimova and Kietzmann, 2006] (Fig. 3A). Mutation of the central nucleotides (CG → AT) (Fig. 3A), which ablates binding of USF to PE2 region probes [Qi et al., 2006], established the importance of an intact PE2 E box consensus sequence in serum-stimulated PAI-1 promoter-driven reporter transcription (Fig. 3B). Mobility shift data indicated that PE2 probe recognition by USF2 is constitutively present and active regardless of cellular growth state (Figs. 3C and D). Prior chromatin immunoprecipitation analyses established that the growth factor-responsive PE2 E box sites in the PAI-1 gene are, indeed, USF target sequences in vivo and that serum-stimulated PAI-1 expression reflected a dynamic USF1 → USF2 switch at this CACGTG site [Qi et al., 2006]. Expression control by USF family members, therefore, is distinct from simple motif recognition [Samoylenko et al., 2001; Dimova and Kietzmann, 2006; Qi et al., 2006]. Mobility shift assessments, moreover, confirmed that constructs that retained an intact E box site functioned as effective competitors while those lacking the CACGTG motif or containing the transcription-attenuating CG → AT mutation (as in Figs. 3A and B) failed to block complex formation (Fig. 3E). Based on these collective findings, it was important to determine if disruption of USF function would specifically affect induced PAI-1 expression. Two approaches were selected to evaluate this possibility. Initially, keratinocytes were transfected with a dominant-negative A-USF construct in which replacement of the wild-type DNA-binding domain with acidic residues (Fig. 3F) inhibited formation of endogenous USF/DNA complexes [Furbass et al., 2010] with the PE2 (human) and HRE-2 (rat) PAI-1 target probes [Gailbert et al., 2001; Kutz et al., 2006]. A-USF expression significantly attenuated both serum- and TGF-β-induced PAI-1 levels (Fig. 3G). Secondly, using the identified sequence restraints for reporter activation (Figs. 3A and B) and DNA binding [Qi et al., 2006], a double-stranded 45-bp PE2 DNA construct was designed based on the previously identified requirements for an intact CACGTG motif for probe recognition by USF [Allen et al., 2005]. Transfection of these double-stranded USF-

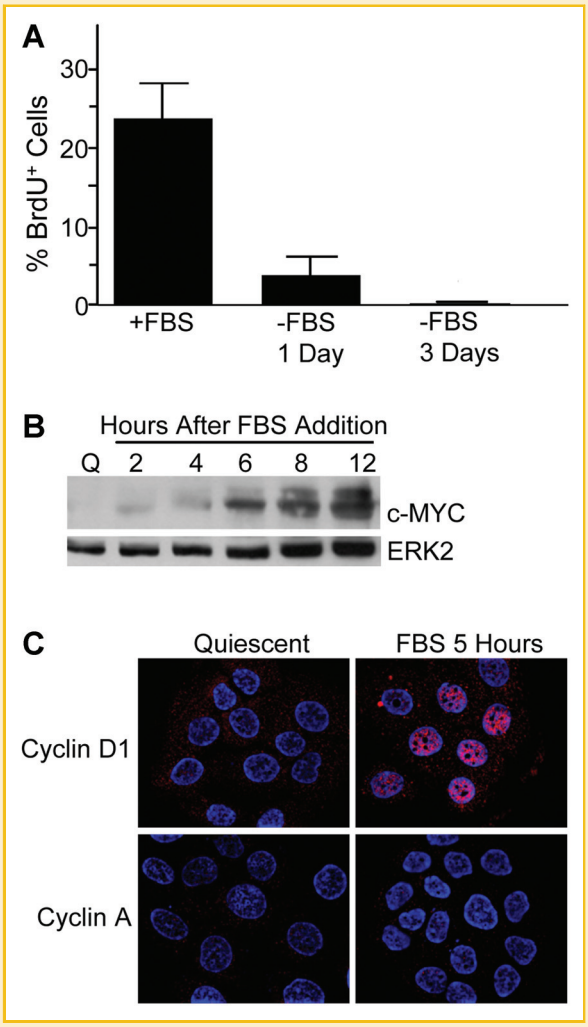

Fig. 1. Kinetics of c-MYC and cyclin D1 induction following serum-stimulation of quiescent HaCaT keratinocytes. The fraction of DNA-synthesizing (i.e., % $BrdU^+$) cells in non-synchronous proliferating HaCaT cultures approximated 24% of the total population (A). The switch to FBS-free medium resulted in a rapid decrease in S phase cells (to 3.8% within 24 h) which further declined to just 0.2% by day 3 of serum-deprivation. Data plotted in (A) represents the mean ± SD of the % $BrdU^+$ HaCaT keratinocytes in 20, randomly selected, fields for each culture condition. c-MYC was not detectable in quiescent (Q) HaCaT cells but was evident as early as 2 h post-serum addition; by 6 h, c-MYC expression was significantly elevated and subsequently increased upon progression through G_1 (B). Serum re-introduction induced expression and nuclear accumulation of the G_1 cyclin D1, but not the S phase cyclin A, within 5 h after initial stimulation (C).

binding, "decoys" (Fig. 3C) effectively reduced both serum- and TGF-β1-induced PAI-1 transcript levels in HaCaT keratinocytes (Fig. 3H).

INDUCIBLE A-USF EXPRESSION ATTENUATES PAI-1 EXPRESSION AND STIMULATES HUMAN KERATINOCYTE PROLIFERATION

Although USF1/2 regulate the expression of genes that impact cell growth [Corre and Gailbert, 2005], the actual cause-effect relationship between specific genomic outputs and the proliferative program is largely unknown. Initial experiments confirmed that transfection of a wild-type USF2 expression vector reduced colony-forming efficiency of HaCaT cells by approximately 80% (Fig. 4A). To more specifically implicate USF in proliferative control, a DOX-dependent A-USF expression system was developed. Keratinocytes were engineered to express an HA-tagged dominant-negative A-USF construct (DN-USF) in an inducible Tet-OFF system. Insert-containing clones were selected based on RT-PCR and HA-tag immunoreactivity following oxytetracycline removal (Fig. 4B, insert). Drug removal and subsequent A-USF induction significantly increased cell number (Fig. 4B), the fraction of Ki-67^+ keratinocytes (Figs. 4C and D) and Ki-67 transcripts while suppressing both PAI-1 and PAI-2 mRNA levels (Fig. 4E). Adenoviral-mediated PAI-1 overexpression, moreover, dramatically inhibited keratinocyte colony expansion (Fig. 4F) mimicing the results of USF2 transfection (Fig. 4A).

DISCUSSION

The PE2 E box is 5′-flanked by three SMAD-binding sites and a minimum of two SMAD-recognition (AGAC) sequences is necessary for the maximal response of the PAI-1 gene to TGF-β1, at least in cells in which TFE3 is a major E box-binding *trans*-activator [Hua et al., 1999]. The two proximal AGAC motifs, however, are not required for USF occupancy of a PAI-1 PE2 region E box target since non-SMAD site E box constructs or SMAD site-deleted or mutated constructs effectively compete for USF-PE2 probe complex formation [Allen et al., 2005; Qi et al., 2006; this paper]. Successful competing sequences include the 18-bp region of the rat PAI-1 proximal promoter containing the USF/HIF-1α-binding HRE-2 E box (5′-TACACA**CACGTG**TCCCAG-3′) and a 23-bp consensus E box construct with no PAI-1 flanking homologies (5′-CACCCGGT-**CACGTG**GCCTACACC-3′) but not the 23-bp central nucleotide E box mutant (5′-CACCCGGT**CAATTG**GCCTACACC-3′) [Qi et al., 2006; this paper]. Use of separate PE1/PE2 region probes confirmed that nuclear USF1/2 binding to PE1/PE2 probe targets in serum-stimulated cells was independent of growth state; transcriptional activation of the PAI-1 gene, moreover, reflects a USF1 → USF2 switch at both the PE1 and PE2 E box sites [Qi et al., 2006]. USF1 → USF2 dimer replacement at the critical PE2 E box motif and induced PAI-1 expression occurs early after cellular "activation." Collectively, these findings as well as the luciferase reporter data (Figs. 3A and B) highlight the importance of an intact PE2 region E box in PAI-1 gene control.

While a consensus E box motif at the PE2 site is both necessary and sufficient for USF binding, the role of co-factors in PAI-1 gene control reflects whether transcription is TGF-β1- or serum-initiated. pSMAD2/3 involvement, at the PE2 region 5′ SMAD sites, for example, is stimulus-dependent as inhibition of SMAD3 phosphorylation with SIS3 effectively attenuates PAI-1 expression in response to TGF-β1 but not serum [Samarakoon et al., 2013]. Similarly, Yin Yang 1 (YY1) represses some SMAD-dependent transcriptional targets (the early-response PAI-1, Id1 genes) but not others (p15, p21, c-myc). YY1, in fact, inhibits SMAD occupancy of the PAI-1 promoter likely via Interactions with the N-terminal MAD homology domain in SMAD4 and SMAD2/3 with suppression dependent on the number of SMAD-binding elements in the specific

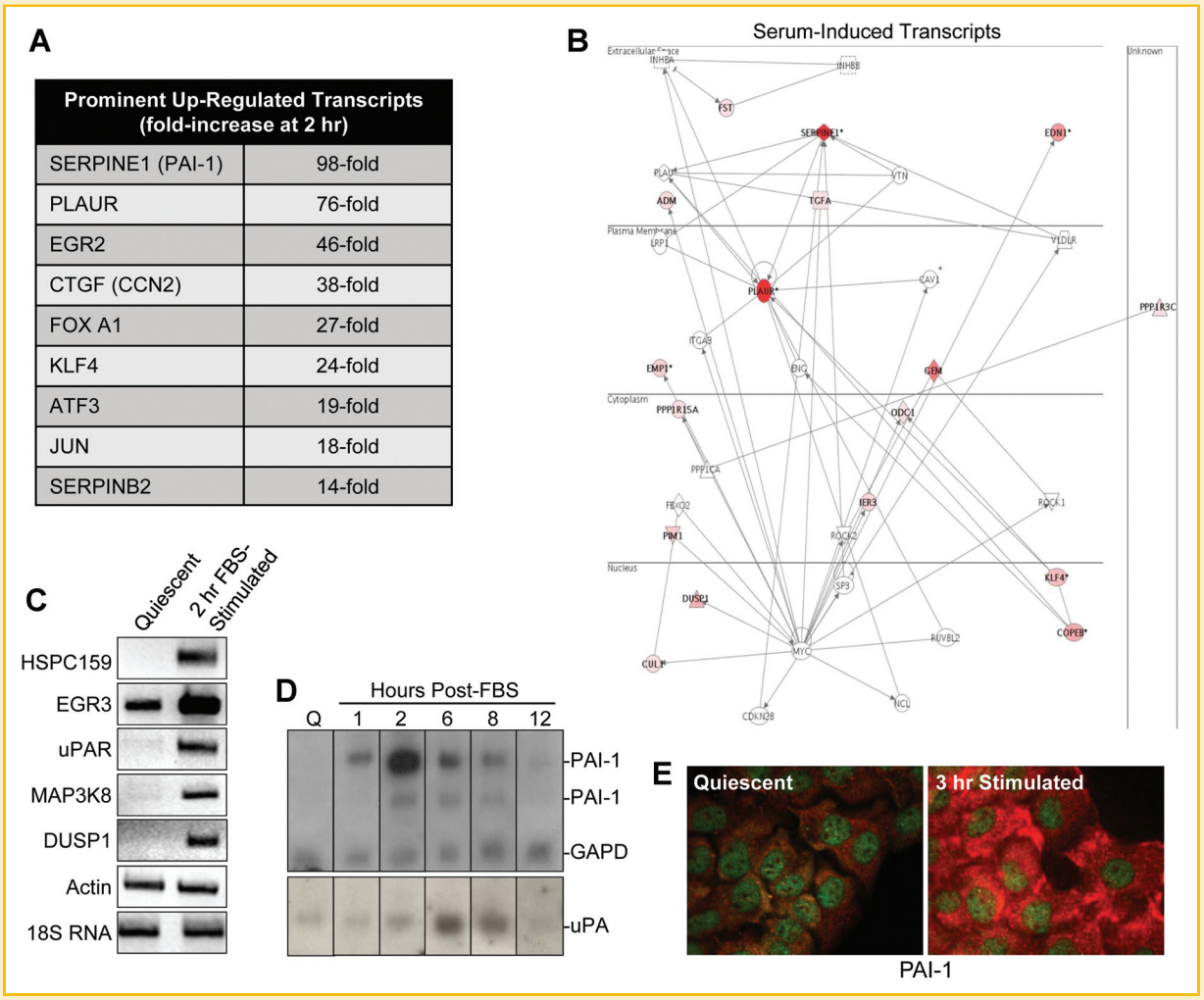

Fig. 2. Expression profiling of serum-responsive genes. Microarray analysis identified several significantly up-regulated transcripts within 2 h after serum addition (A). The clustergram (B) illustrates functional and locational groupings for proteins encoded by several highly-expressed (red to orange shading), relative to more moderately-stimulated, genes (map constructed with Ingenuity Pathways software). A complete list of 79 genes that are >10-fold serum-increased in HaCaT cells is available [Qi et al., 2008]. RT-PCR confirmed induction of several transcripts including HSPC159, EGR3, uPAR, MAP3K8 and DUSP1 (C). PAI-1 mRNAs (both the 3.0 and 2.2 kb species) are low to undetectable in quiescent (Q) HaCaT keratinocytes and induced within 1–2 h of serum re-introduction (D). uPA transcripts, while also elevated at the 2 h time point, were not highly expressed until 6 h post-FBS stimulation. Induction of PAI-1 mRNA was closely followed by a marked increase in immunocytochemically detectable PAI-1 protein (E). Collectively, these data indicate that three important modulators of the pericellular plasmin generating system (uPA, uPAR, and the pathway negative regulator PAI-1) are prominent members of the early response to serum-stimulation.

target gene [Kurisaki et al., 2003]. Importantly, USF2 appears to contribute to C/EBPβ target gene expression by interacting with YY1, either via the repressive domain or by blocking formation of YY1/co-regulator complexes to antagonize YY1 [Viart et al., 2013]. Interactions between USF2 and YY1 release YY1 inhibition stimulating C/EBPβ transactivation of target genes [Viart et al., 2013]. Other mechanisms have implications with regard to cell growth control. USF1/2 interact with components of the basal transcription complex [Garcia-Sanz et al., 2013] and guide recruitment of the hSET1A histone methylase complex to chromatin as part of lineage-specific cell differentiation [Deng et al., 2013]. Recent findings suggest, moreover, that USF2 is required for induction of HIF2α responsive genes (including PAI-1) under hypoxic conditions likely involving recruitment of the histone acetylases p300 and CBP [Palmeri et al., 2002]. While introduction of a USF2 expression vector alone stimulated PAI-1 promoter activity, co-transfection of a construct encoding a stabilized form of HIF2α along with the USF2 vector potentiated PAI-1 transcription more than either alone [Pawlus et al., 2013].

PAI-1 transcriptional activation has important phenotypic consequences. Several SERPINS (SERPINE1 [PAI-1], SERPINB1, SERPINB2) are prominent members of the "tissue repair" transcriptome functioning in the integrated control of focalized matrix restructuring, cell-to-substrate adhesion/detachment, migration and proliferation [Iyer et al., 1999; Chan et al., 2001; Palmeri et al., 2002; Dayem et al., 2003; Degryse et al., 2004; Providence and Higgins, 2004; Rossignol et al., 2004; Wang et al., 2005; Fitsialos et al., 2007; Providence et al., 2008; Qi et al., 2008; Ebrahimian

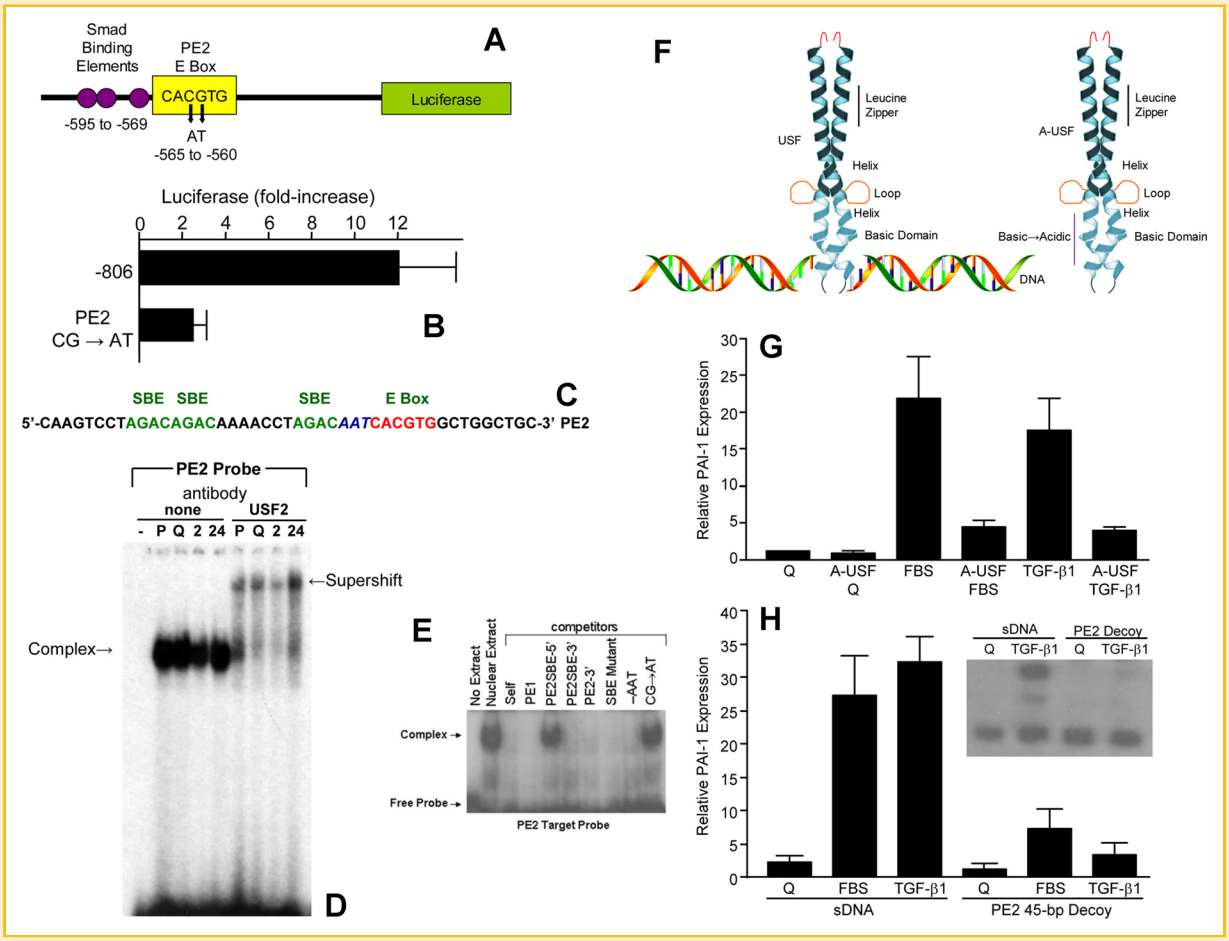

Fig. 3. The PE2 region E box is an important USF2-binding, PAI-1 expression-regulating motif, in HaCaT cells. Position of the PE2 region E box motif (including the introduced CG → AT mutations) and the three SMAD-binding AGAC sites just upstream of the PE2 E box are indicated (A). Luciferase activity for cells transfected with the wild-type and CG → AT mutated PE2 E box reporter constructs, or one lacking PAI-1 promoter sequences (basal; pGL3 only = 1.0), were determined after a 12 h serum exposure (B). Normalization was to a co-transfected SV40 promoter-β-galactosidase expression vector. Data plotted (B) is the mean ± SD of three independent experiments and expressed as fold-increase relative to basal luciferase signal. Mobility shift assays using a double-stranded ^{32}P-labeled PE2 construct (C; only top strand is illustrated) were incubated with nuclear extracts from proliferating (P), quiescent (Q) and 2 as well as 24 h (2, 24) serum-stimulated HaCaT cells. Positions of the original protein-probe complex and the USF antibody-induced supershift are indicated with arrowheads (D); (−) = absence of nuclear extract; none = no IgG added. Antibodies (2 μg) to MAX, TFE3, or E2F1 did not produce supershifts (not shown). The PE2 sequence requirements for PE2 region probe binding were determined using the topographic map in (C) to design wild-type and mutant PE2 competing constructs for mobility shift assay (E). Nuclear extracts were incubated with ^{32}P-labeled PE2 probe in the presence or absence of a 100-fold molar excess of the following unlabeled competing DNAs: Self (wild-type 45-mer PE2 sequence), PE1 (upstream region; only homology to PE2 is a consensus E box), PE2SBE-3′ (proximal SBE + AAT spacer + E box), PE2-3′ (AAT + E box), SBE mutant (45-mer PE2 sequence with all three SBEs mutated), -AAT (45-mer PE2 sequence with the AAT spacer deleted) and non-competing constructs: PE2SBE-5′ (2 5′-SBEs), CA → AT mutant (45-mer PE2 sequence with the 2 central E box nucleotides mutated [Qi et al., 2006; for details as to sequences used for mobility shift assay]. E: Adapted from [Qi et al., 2008]. To determine if interference with USF-PE2 site recognition had an effect on PAI-1 induction. Keratinocytes were transfected with the dominant-negative (A-USF) USF construct (F), cultured under quiescence (Q) conditions then stimulated with FBS (10% final concentration) or TGF-β1 (1 ng/ml) for 5 h prior to extraction for Western analysis of cellular PAI-1 protein. A-USF expression significantly attenuated (by approximately 80%) PAI-1 induction in response to both serum and TGF-β1 (G). Transfection of HaCaT cells with a double-stranded USF-binding 45-bp PE2 region decoy construct (C) markedly decreased the levels of both serum- and TGF-β1-induced PAI-1 transcripts (H). Insert (in H) is a representative northern blot. Graphed data (G,H) are the mean ± SD of three independent experiments. sDNA = sheared, double-stranded, control DNA; SBE = SMAD-binding elements.

et al., 2012]. PAI-1 effectively limits pericellular plasmin generation to maintain a supporting "scaffold" for cell movement [Czekay et al., 2011] while also regulating urokinase-dependent growth factor activation attenuating, thereby, the associated proliferative response [Kortlever et al., 2006]. Indeed, PAI-1 is highly-expressed in cells undergoing replicative senescence [Mu et al., 1998; Kortlever et al., 2006] and certain USF target "growth arrest-associated" genes (i.e., p16^{INK4a}, PAI-1) may function in the wound repair program to inhibit proliferation while promoting migration. Perhaps not unrelated is the finding that most genes activated by USF family members typify differentiated, largely non-cycling, cells [Liang et al., 2009]. The USF1 → USF2 transition at the PAI-1 PE2 E box, and subsequent PAI-1 transcription, appears to be a critical aspect of the go-or-grow response during epidermal wound healing. PAI-1 may regulate the temporal cadence of cell cycle progression in replicatively-competent cells as part of the injury repair program.

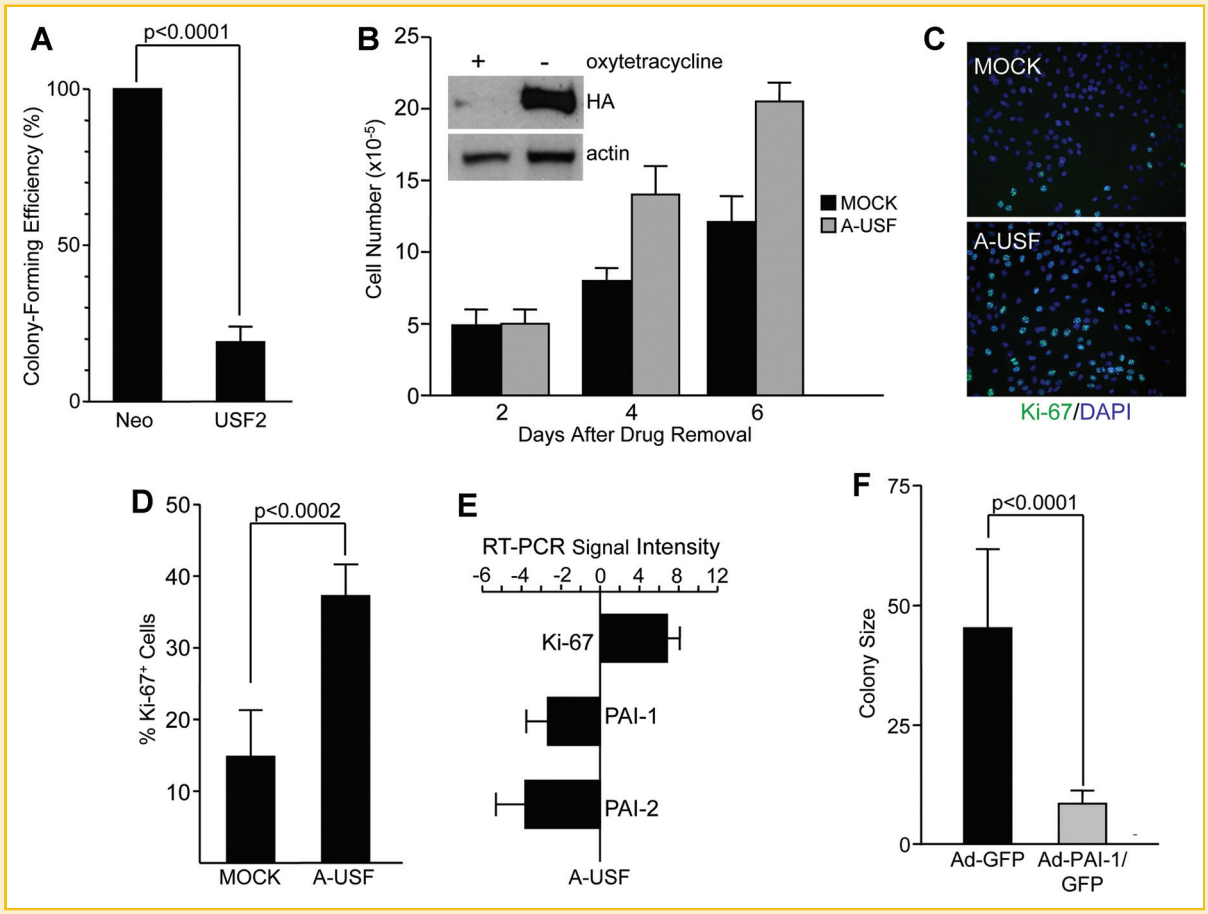

Fig. 4. Expression of pCMV-USF2, a DOX-responsive A-USF construct and adenoviral-mediated PAI-1 over-expression inhibit HaCaT cell proliferation. Transfection of pCMV-USF2 effectively attenuated HaCaT keratinocyte growth, relative to neomycin controls, as assessed by colony-forming efficiency in 10% FBS-containing medium (A). For HaCaT cells engineered to express an inducible A-USF insert upon DOX removal (confirmed by Western blot detection of the HA tag), a significant increase in growth was evident within 4 days after drug withdrawal compared to mock (empty vector + pTRE2) transfectants (B). Induction of A-USF increased the fraction of Ki-67$^+$ cells compared to empty vector controls (C,D). Tet-OFF A-USF expression augmented Ki-67 mRNA abundance approximately eightfold, relative to mock transfectants, while inhibiting expression of both PAI-1 and PAI-2 transcripts (E). Growth inhibition (i.e., reduced colony expansion) was evident in HaCaT populations infected with CMV-PAI-1/GFP encoding adenoviruses but not in keratinocytes infected with a control GFP-only adenovirus (F).

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