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Original Article

The aryl hydrocarbon receptor agonist benzo(a)pyrene reactivates LINE-1 in HepG2 cells through canonical TGF-β1 signaling: implications in hepatocellular carcinogenesis

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Abstract: Long interspersed nuclear element-1 (L1) is a genetic element that mobilizes throughout the mammalian genome via retrotransposition and damages host DNA via mutational insertions, chromosomal rearrangements, and reprogramming of gene expression. The cellular mechanisms responsible for aberrant L1 expression during cancer pathogenesis are unclear. Previously, we have shown that L1 reactivation in several human cell lines is dependent upon the activation of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor member of the PAS superfamily of proteins. We also showed that ectopic expression of L1 reprograms the HepG2 genome leading to epithelial-to-mesenchymal transition (EMT). Here we present evidence that reactivation of L1 and modulation of EMT in HepG2 cells by the AhR ligand benzo(a)pyrene (BaP) is effected through the canonical TGF-β1 signaling pathway. BaP increased TGF-β1 mRNA, SMAD2 phosphorylation and decreased expression of E-Cadherin. The functional relevance of these interactions and the involvement of TGFBR1/ALK5 and SMAD2/3 were confirmed by siRNA interference. Furthermore, expression of L1-encoded ORF1p was positively correlated with the activation of TGF-β1 signaling in human hepatocarcinoma samples at various stages of malignant progression. These results indicate that ligand-mediated AhR activation regulates L1 via canonical TGF-β1 signaling and raise important questions about the molecular etiology of human hepatocarcinomas.

Keywords: Long interspersed nuclear element-1, benzo(a)pyrene, AhR, TGF-β1, SMAD

Introduction

Long interspersed nuclear element-1 (LINE-1 or L1) is a repetitive DNA sequence that mobilizes via a "copy and paste" retrotransposition mechanism. A functional L1 element in humans is ~6 kb in length and consists of a 5' untranslated region (UTR) with promoter activity, two open reading frames, and a terminal 3' UTR [1, 2]. L1 encodes two proteins; ORF1p, a 40 kDa protein with nucleic acid binding activity and ORF2p, a 150 kDa protein with endonuclease and reverse transcriptase activities [3]. A complete L1 retrotransposition cycle consists of transcription of L1 RNA, export into the cytoplasm, translation of ORF1 and ORF2, association of L1 RNA with ORF1 and ORF2 proteins to form ribonucleoprotein (RNP) particles, import into the nucleus, reverse transcription, and integration into new genomic locations [4].

L1 retrotransposition is tightly regulated in somatic tissues via genetic and epigenetic mechanisms [5-7]. However, aberrant ORF1p expression and new somatic L1 insertions have been detected in several epithelial cancers [8-11]. L1 retrotransposition is highly mutagenic and may promote cancer through induction of aberrant splicing, exon skipping, and genome rearrangements that change gene expression and promote genome instability [3, 4]. Our group has shown that L1 reactivation exerts retrotransposition-dependent and retrotransposition-independent functions that may be critical to neoplastic transformation and cancer progression [12, 13]. Ectopic expression of wild type or a mutant L1 protein incapable of retrotransposition modulates the expression of a large number of genetic targets involved in cancer through changes in cellular adhesion, inflammation and metabolism [12, 13]. L1 also

disrupts epithelial cell differentiation programs and induces epithelial-to-mesenchymal transition (EMT) [13], a process that facilitates metastasis by promoting cell motility and conferring transformed cells the ability to invade [14].

A master promoter of EMT is transforming growth factor-β1 (TGF-β1) [15], a potent immunosuppressive and pro-inflammatory cytokine [16]. TGF-β1 plays a dual role in carcinogenesis, having the ability to inhibit proliferation of cancer cells or to activate migration and invasiveness through EMT [15] or neoangiogenesis [17]. TGF-β1 signaling is mediated through a complex that includes type I and two type II transmembrane receptors with serine/threonine kinase activity. Type II receptors phosphorylate type I components, while type 1 receptors transduce the signal from the cell membrane to the nucleus canonically through a complex of SMAD proteins or alternatively, non-canonically, through other signaling pathways e.g. Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK) [18]. Interestingly, *in silico* analysis of the L1 regulatory genetic network [12], and biological validation in HepG2 cells [13], showed that some of genetic targets of L1 are also targets of TGF-β1 (*e.g.* CCL2, VCAM, CXCL1) [19-21]. These data suggested that L1 activation and TGF-β1 signaling in hepatoma cells may be cooperative and important in hepatocarcinogenesis.

The regulatory networks involved in L1 reactivation during cell transformation and cancer progression are not clear. We have previously shown that L1 reactivation by the environmental carcinogen BaP is mediated through binding to the aryl hydrocarbon receptor (AhR). AhR is a ligand-activated transcription factor ubiquitously distributed that translocates from the cytosol to the nucleus after ligation by polycyclic aromatic hydrocarbons. Ligand-bound AhR forms a heterodimer with the AhR nuclear translocator (ARNT) and binds to a specific sequence to regulate gene expression. The hallmark response of AhR activation is the transcriptional activation of cytochrome P4501A1 (CYP1A1) in hepatic parenchymal cells [22].

There exists a cell-specific and context-dependent crosstalk between AhR and TGF-β1. Both AhR and TGF-β1 participate in the regulation of common cellular processes *e.g.* cell cycle progression, apoptosis, cell adhesion and interaction with extracellular matrix [23]. Several studies have shown that AhR can regulate TGF-β1 signaling, through deregulation of TGF-β1 secretion, modulation of TGF-β1 expression or down-regulation of the latency-associated protein (LTBP-1) expression [24-26]. TGF-β1 also regulates AhR expression and CYP1A1/1B1 enzyme activity in a cell/tissue specific manner [27-29]. Thus, different mechanisms have been proposed to explain AhR and TGF-β1 crosstalk in endothelium [30], regulatory T cells [31], Th17 cells [32] or dendritic cells [33]. The complexity of tissue-context specific mechanisms in the regulation of L1 by AhR/TGF-β1 crosstalk is the primary focus of the present investigation.

Materials and methods

Materials

BaP was purchased from Ultra Scientific (Kingstown, RI). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Monoclonal anti-GAPDH, and horseradish peroxidase (HRP) linked anti-mouse IgG antibodies were from Santa Cruz Biotech (Dallas, TX). Rabbit anti-AhR (13790), anti-E-cadherin (24E10), anti-vimentin (D21H3), anti-SMAD2 (5339), anti-phospho-SMAD2 (3108), anti-SMAD2/3 (8685), anti-TGFBR1 (3712), and horseradish peroxidase (HRP) linked anti-rabbit IgG antibodies were from Cell Signaling Technology (Beverly, MA). Protein lysates from: normal limits liver tissue (male, case ID. CU0000001489, Cat No. CP565754), staging I hepatocellular liver carcinoma tissue (male, case ID. CU0000012132, Cat No. CP641361), staging II hepatocellular liver carcinoma tissue (male, case ID. CU0000005407, Cat No. CP19427), staging IIIA hepatocellular liver carcinoma tissue (male, case ID. CU000001197, Cat No. CP607175), and staging IV hepatocellular liver carcinoma tissue (male, case ID. CU0000013002, Cat No. CP532216) were from OriGene (Rockville, MD). Pathological staging of tissue samples followed established guidelines. DMSO was from American Type Culture Collection (ATCC).

Polyclonal anti-human ORF1p antibody

A custom made polyclonal antibody produced by New England Peptide Inc. was diluted

Table 1. Oligonucleotides sequences used in this study

Sequence	Description
5'-CCA AGTTGGAAAACACTCTGC-3'	ORF1-Forward-RT-PCR
5'-TGTGGCGTTCTCTGTATTTCC-3'	ORF1-Reverse-RT-PCR
5'-TCGACACATACACTCTCCCAAG-3'	ORF2-Forward-RT-PCR
5'-TGGTCCTGGACTCTTTTTGG-3'	ORF2-Reverse-RT-PCR
5'-GGATACCAACTATTGCTTCAGCTCC-3'	TGF β1-Forward-RT-PCR
5'-AGGCTCCAAATATAGGGGCAGGGTC-3'	TGF β1-Reverse-RT-PCR
5'-GATCATCAGCAATGCCTCCT-3'	GAPDH-Forward-RT-PCR
5'-TGTGGTCATGAGTCCTTCCA-3'	GAPDH-Reverse-RT-PCR
5'-CGGAUGAAAUCCUGACGUAtt-3'	AHR-siRNA-Sense
5'-UACGUCAGGAUUUCAUCAGtt-3'	AHR-siRNA-Antisense
5'-CAGUUACUGUGGAAGGAAUtt-3'	TGFBR1-siRNA-Sense
5'-AUUCCUUCCACAGUAACUGTg-3'	TGFBR1-siRNA-Antisense
5'-GCUUCUCUGAACAAACCAGtt-3'	SMAD2-siRNA-Sense
5'-CUGGUUUGUUCAGAGAAGCtg-3'	SMAD2-siRNA-Antisense
5'-GGCCCAGUGCAUAUGCAAUtt-3'	SMAD3-siRNA-Sense
5'-AUUGCAUAUGCACUGGGCCtc-3'	SMAD3-siRNA-Antisense

1:1000 and used in all experiments. The specificity of the antibody was validated in Western blot experiments against L1 ORF1p expressed from plasmid constructs or following neutralization with antigenic peptide.

Cell culture and treatments

The HepG2 hepatocellular carcinoma cell line was purchased from the American Type Culture Collection (ATCC). Cell line was confirmed to be absent of mycoplasma contamination (MycoAlert; Lonza). Verification of this cell line was performed by short tandem repeat (STR) using reference databases from ATCC (Genetics Core, University of Arizona, AZ). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), Thermo Fisher Scientific (Grand Island, NY), supplemented with 62.5 μg/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific) in a humidified incubator at 37°C with 5% CO_2. HepG2 cells were plated one day before treatments. Cultures were challenged with BaP dissolved in DMSO or an equivalent DMSO (0.5%) at ~50% confluence. For TGF-β1 treatments, HepG2 cells were washed once with serum-free medium and then challenged with 10 ng/ml TGF-β1. For biochemical analyses, cells were lysed with buffer containing 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L Tris-HCl, 0.25% deoxycholic acid, 1% IGEPAL CA-630 (pH 7.5), supplemented with protease and phosphatase inhibitor cocktails (EMD Millipore) for 5 min at 4°C and then cleared by centrifugation at 16,000 × g for 10 minutes at 4°C. All protein concentrations were determined using the bicinchoninic acid assay (Thermo Fisher Scientific).

Immunoblotting

Total cell lysates were resolved by SDS-Tris PAGE and transferred onto polyvinylidine fluoride membranes (Thermo Fisher Scientific) in Tris-glycine buffer containing 20% methanol. Proteins were detected by immunoblotting. Where indicated, membranes were stripped of bound antibodies using 62.5 mmol/L Tris-HCl (pH 6.7), 100 mmol/L 2-mercaptoethanol, and 2% SDS for 30 minutes at 60°C and reprobed as detailed in figure legends.

RT-PCR

Total RNA was isolated using the RNeasy Plus Kit (Qiagen) and 2 μg RNA digested with TurboDNase-I (Thermo Fisher Scientific). DNAse digested RNA (1 μg) was employed for cDNA synthesis using high-capacity cDNA reverse transcription Kit (Thermo Fisher Scientific). The resulting cDNAs (50 ng) were used as templates for qRT-PCR to analyze mRNA expression using *Power* SYBR® Green PCR Master Mix and primers for L1-ORF1, L1-ORF2, TGF-β1 and GAPDH. The specific sequences are indicated in **Table 1**. Fold changes were determined by comparing the ΔCT value of each product normalized to GAPDH as an internal control.

RNA interference

Small interfering RNA (siRNA) duplex sequences were chemically synthesized and annealed by Thermo Fisher Scientific. The siRNA duplexes for AhR (ID#s1200), SMAD2 (ID#115715), SMAD3 (ID#107877), and TGFBR1 (ID#s22939) sequences are indicated in **Table 1**. BLAST analysis showed no homology to any sequence in the Human Genome Database, other than the intended target. The scrambled siRNAs

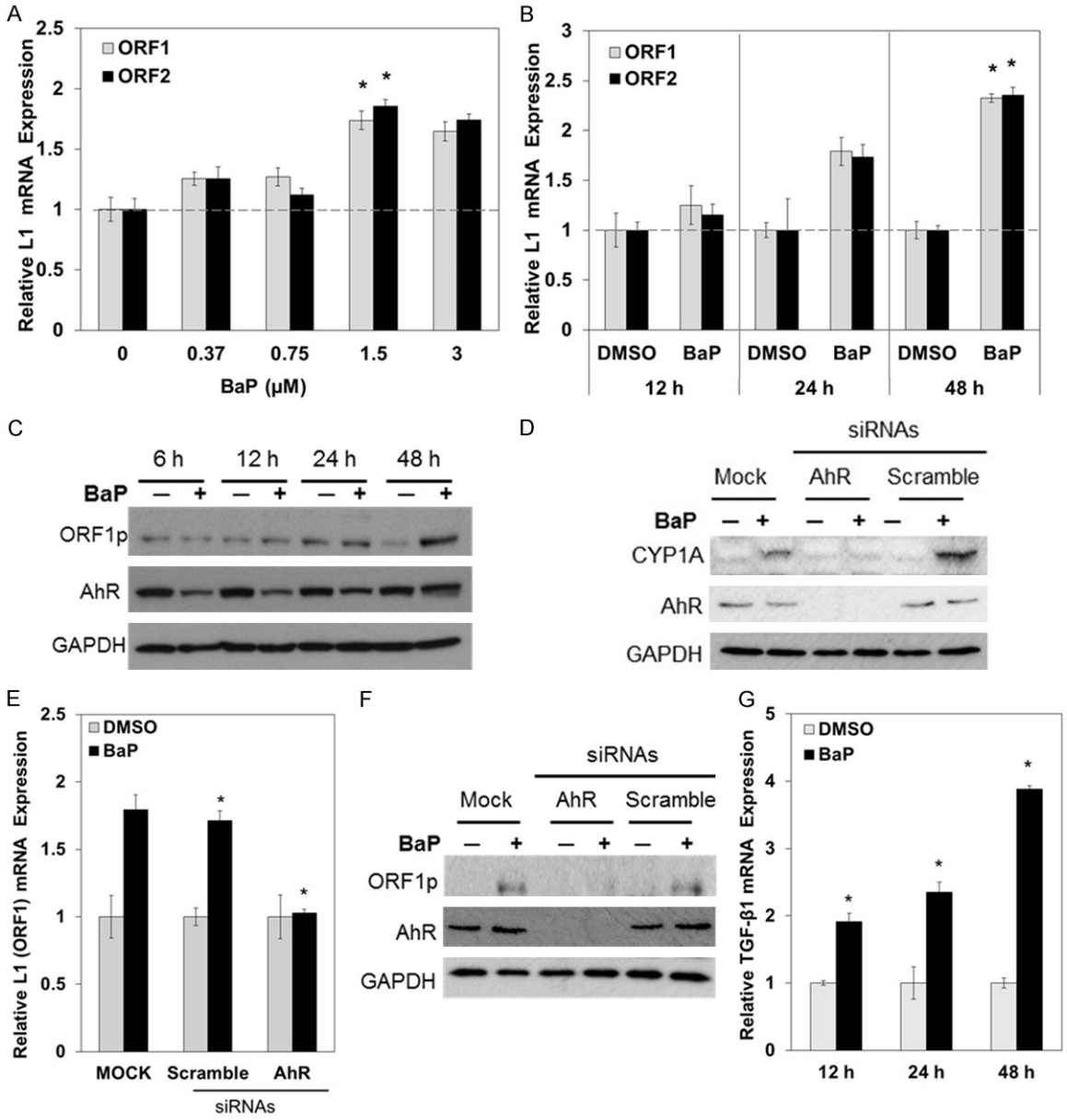

Figure 1. Induction of L1 and TGF-β1 expression by BaP is dependent upon AhR signaling. HepG2 cells were stimulated with: (A) different concentrations of BaP or an equivalent volume of DMSO for 24 h, or (B) 1.5 μM BaP or DMSO (0.5%) for different periods of times. Total RNA was isolated, and 1 μg of RNA subjected to cDNA synthesis. Samples were analyzed via RT-PCR using specific primers for human L1 ORF1 and ORF2, or GAPDH. (C) Whole cell lysates from cells stimulated with 1.5 μM BaP or DMSO (0.5%) for different periods of times were analyzed by immunoblotting for L1 protein (ORF1p), AhR and GAPDH (loading control). Cells were transfected without siRNA (mock), with AhR target-specific siRNA, or a control siRNA (scramble). Transfected cells were stimulated with 1.5 μM BaP or DMSO for 24 and 48 h. (D) Whole cell lysates from cells stimulated for 24 h were analyzed by immunoblotting for CYP1A1, AhR, or GAPDH antibodies. Cells stimulated for 48 h were analyzed via (E) RT-PCR using specific primers for human L1 ORF1 or GAPDH, or (F) immunoblotting for L1 protein (ORF1p), AhR and GAPDH (loading control). (G) Cells were treated with 1.5 mM BaP or an equivalent DMSO volume for different periods of time and analyzed via RT-PCR using human specific primers for TGF-β1 and GAPDH. Expression is presented relative to untreated cells. Points represent mean of triple samples with SE. Data representative at least two independent experiments.

used were Silencer® Negative Control #1 siRNA (AM4635) and Silencer® Select Negative Control #2 siRNA (4390847). The siRNAs were transfected using Lipofectamine™ RNAiMAX

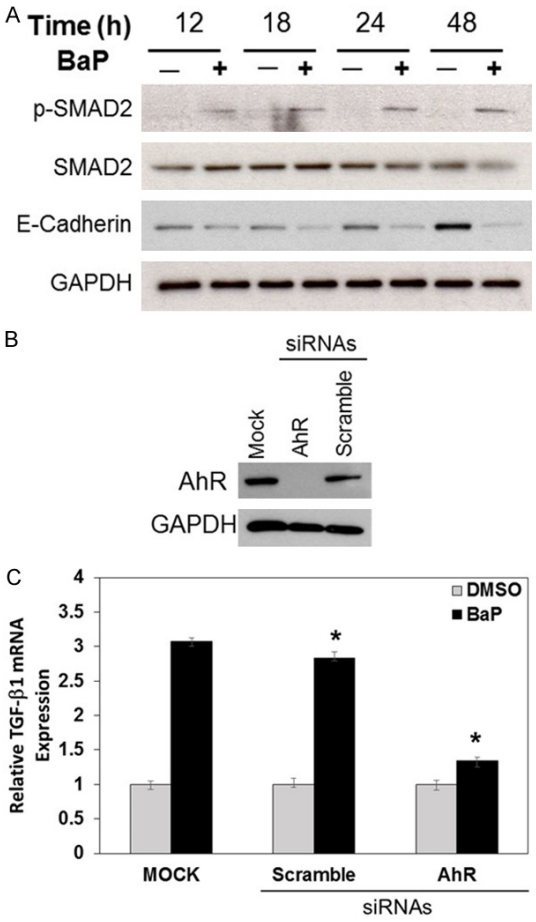

Figure 2. Activation of TGF-β1 signaling by BaP in coupled to AhR. Cells were treated with 1.5 mM BaP or an equivalent volume of DMSO for different periods of time. A. Whole cell lysates were analyzed by immunoblotting for phospho-SMAD2, total SMAD2, E-Cadherin, and GAPDH (loading control). Cells were transfected without siRNA (mock), with AhR target-specific siRNA, or a control siRNA (scramble). B. Transfected cells were lysed and whole cell lysates analyzed by immunoblotting for AhR or GAPDH antibodies (loading control) to confirm target knockdown. C. Transfected cells were treated with 1.5 mM BaP or DMSO for 48 h. Total RNA was isolated and subjected to cDNA synthesis. Samples were analyzed via RT-PCR using human specific primers for TGF-β1 and GAPDH. Expression is presented relative to untreated cells. Points represent the mean of triple samples with SE. Data are representative of at least two independent experiments.

(Thermo Fisher Scientific), according to the manufacturer's directions.

Statistical analysis

Experimental replicates were independent and performed on separate days. Comparisons were done between treated and control groups by ANOVA followed by Tukey's post hoc analysis.

Results

Ligand activation of AhR regulates L1 and TGF-β1 in HepG2 cells

We have previously shown that ligand-dependent activation of AhR by BaP reactivates L1 transcriptionally in several cell lines [34], and that ectopic expression of L1 mediates EMT in HepG2 cells [13]. Given that TGF β1 is a master inducer of EMT [15], and that crosstalk between AhR and TGF-β1 is cell-specific and context-dependent [23], studies were conducted to evaluate the role of AhR and TGF-β1 signaling in the regulation of L1 expression. First, we analyzed L1 induction profiles in HepG2 cells challenged with various concentrations of BaP for various times. Expression of L1 mRNAs and protein ORF1p was analyzed by qRT-PCR and immunoblotting, respectively. Peak induction of L1 ORF1 and ORF2 mRNAs by BaP was seen in cells treated with 1.5 μM for 24 h (**Figure 1A**). The induction response exhibited long latencies, as L1 mRNA continued to accumulate for up to 48 h (**Figure 1B**). Longer exposures to the carcinogen were associated with progressive losses of cell density and appearance of stress granules and vacuolization (not shown). Immunoblotting analysis showed that BaP challenge transiently downregulated AhR (**Figure 1C**), a response mediated by ubiquitination and subsequent degradation of the activated protein [35]. The expression of L1 protein (ORF-1p) was detected after 48 h, coincident with the restoration of AhR levels (**Figure 1C**).

Ligand-bound AhR activation is known to induce the expression of metabolic enzymatic activities encoded by cytochrome P450 (CYP) genes, such as CYP1A1 [36]. To confirm that AhR activation mediated the regulation of L1 by BaP, genetic knockdown of AhR protein was achieved by transfection of AhR-specific siRNA into HepG2 cells. Immunoblotting confirmed a >90% reduction in protein levels compared to controls (**Figure 1D** and **1F**). BaP challenge for 24 h induced CYP1A1 expression (**Figure 1D**), confirming functional integrity of the AhR signaling cascade. CYP1A1 expression was not detected in vehicle-treated control cells. Genetic knockdown of AhR blocked CYP1A1 expression (**Figure 1D**) and L1 mRNA and protein expression (**Figure 1E** and **1F**) following of

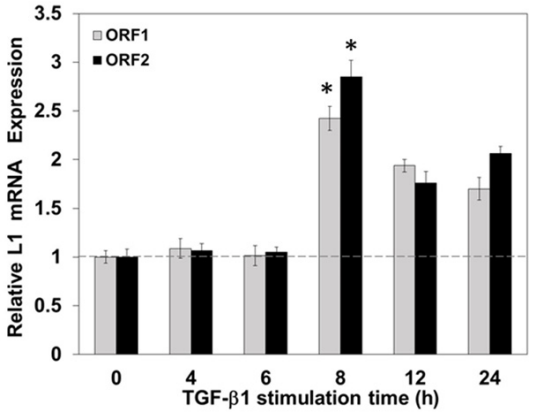

Figure 3. TGF-β1 induces L1 mRNA expression in HepG2 cells. Cells were stimulated for different times by addition of 10 ng/ml TGF-β1. Total RNA was isolated, and 1 μg of RNA subjected to cDNA synthesis. Samples were analyzed via RT-PCR using specific primers for human L1 ORF-1 and ORF-2 or GAPDH. Expression is presented relative to untreated cells. Points represent the mean of triple samples with SE. Data are representative of at least two independent experiments.

BaP challenge compared with controls. Together, these findings identified AhR as an essential molecular target involved in the regulation of L1 expression by BaP.

Next, we determined whether AhR activation by BaP regulates the expression of TGF-β1 in HepG2 cells. Cells were challenged with 1.5 μM BaP for 12, 24 and 48 h, and the expression of TGF-β1 mRNA analyzed by qRT-PCR. TGF-β1 mRNA levels were increased by BaP after 12 h and peaked after 48 h (**Figure 1G**). Of note, induction of TGF-β1 mRNA by BaP preceded the induction of L1 proteins (compare **Figure 1C** and **1G**), pointing to the possibility that the reactivation of L1 by BaP is dependent upon regulation of TGF-β1 signaling.

Functional crosstalk between AhR and TGF-β1 signaling in the regulation of L1

We next examined whether induction of TGF-β1 by BaP is coupled to the activation of TGF-β1 signaling by analyzing downstream targets of TGF-β1 signaling, activation of SMAD2 and modulation of EMT markers. Immunoblotting showed that BaP challenge for up to 48 h increased the phosphorylation of SMAD2 and downregulated the expression of E-Cadherin compared to untreated DMSO controls in HepG2 cells (**Figure 2A**). The expression of vimentin was not changed by BaP challenge (data not shown). To determine if AhR was involved in the regulation of TGF-β1 mRNA expression by BaP, AhR expression was inhibited by transfection of specific siRNA into HepG2 cells (**Figure 2B**). Genetic knockdown of AhR (**Figure 2B**) caused a substantial decrease in TGF-β1 mRNA expression following BaP challenge (**Figure 2C**), and established AhR as an essential molecular target in the regulation of TGF-β1 mRNA by BaP.

L1 reactivation has been implicated in malignant transformation and cancer progression [3, 13, 37]. Given the ability of BaP to regulate TGF-β1 mRNA and downstream signaling, we next determined whether TGF-β1 induces L1 mRNA expression. HepG2 cells were treated with 10 ng/ml TGF-β1 in serum-free media for 4 to 24 h (**Figure 3**). The expression of L1 mRNAs was measured by qRT-PCR. TGF-β1 induced ORF-1 and ORF-2 L1 mRNAs, with peak induction levels seen 8 h post treatment (**Figure 3**), and preservation of the response for up to 24 h. These results indicate that TGF-β1 signaling may be a key cellular pathway for regulation of L1 expression in HepG2 cells.

BaP-regulated expression of L1 is effected through TGF-β1 signaling

Having confirmed the involvement of ligand-activated AhR signaling in the regulation of TGF-β1, our next objective was to determine the role of TGF-β1 signaling in the regulation of L1 expression. To this end, we investigated whether downstream targets of TGF-β1 signaling including, TGFBR1, SMAD2 and SMAD3, are required for BaP-induced L1 mRNA expression. Expression of each of these molecules was inhibited by transfection of corresponding siRNAs into HepG2 cells. Immunoblotting confirmed a >80% reduction in the expression of these proteins compared to controls (**Figure 4A**). Inhibition of TGFBR1 expression markedly decreased BaP-induced L1 mRNA expression (**Figure 4B**). Furthermore, inhibition of SMAD2 and SMAD3 was associated with complete ablation of the L1 response, as well as a slight reduction in baseline L1 mRNA levels (**Figure 4B**). Thus, ligand-activated L1 expression requires activation of the canonical TGF-β1 signaling pathway.

Expression of proteins involved in the L1, TGF-β1 and EMT signaling pathways in hepatocellular carcinoma

To further evaluate the relevance of our findings in human hepatocarcinogenesis, we next

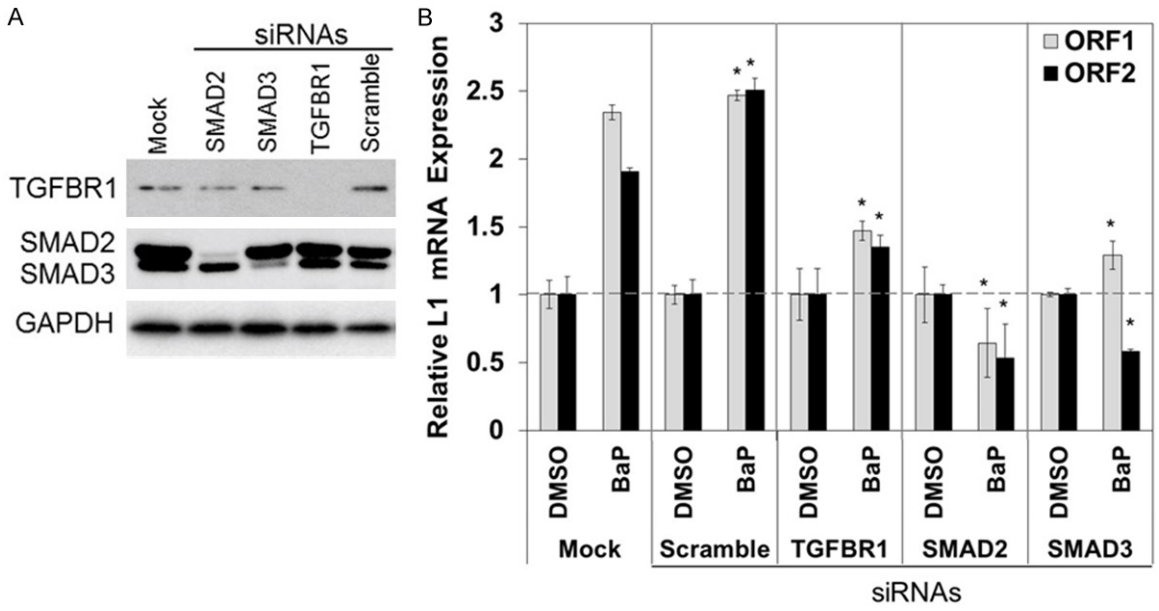

Figure 4. TGFR1, SMAD2 and SMAD3 are required for L1 mRNA expression. Cells were transfected without siRNA (mock), with target-specific siRNAs to SMAD2, SMAD3, and TGFBR1, or a control siRNA (scramble). A. Untreated transfected cells were lysed and whole cell lysates analyzed by immunoblotting for TGFBR1, SMAD2/3 or GAPDH antibodies (loading control) to confirm target knockdown. B. Transfected cells were treated with 1.5 mM BaP or DMSO for 48 h. Total RNA was isolated, and 1 μg of RNA subjected to cDNA synthesis. Samples were analyzed via RT-PCR using human specific primers for L1 ORF1 and ORF2, or GAPDH. Expression is presented relative to untreated cells. Points represent mean of triple samples with SE. Data are representative at least two independent experiments.

determined if the expression of L1 ORF1p correlates with the expression of proteins involved in TGF-β1 signaling pathway during staged progression of liver malignancy. The expression of proteins involved in EMT was also examined. Proteins from human liver hepatocellular carcinoma, Stages I through IV and normal tissue, were analyzed by immunoblotting. Normal liver presented high expression of E-Cadherin, while the expression of ORF1p was undetectable (**Figure 5A**). ORF-1p was detected in liver tumor stages I, II and to some extent at stage IV (**Figure 5A**). All human hepatocellular carcinoma samples presented an EMT phenotype characterized by low to no detectable expression of E-cadherin, and high expression of the mesenchymal marker, vimentin (**Figure 5A**) compared to normal liver. A marked increase in phosphorylation of SMAD2 was observed in stages I, II and IV compared with normal and tumor stage III (**Figure 5B**). The phosphorylation of SMAD2 showed strong overlap with that of ORF-1 (compare **Figure 5A** and **5B**). These results suggest deficits in TGF-β1 signaling in human hepatocellular carcinoma and a role of L1 proteins in disease progression.

Discussion

L1 reactivation in somatic cells is highly mutagenic through a variety of genetic alternations including, gene deletions, inversions, and inter/intra-chromosome translocations. Not surprisingly, L1 reactivation has been linked to cancer initiation and progression [2-4, 6, 37]. Mammalian cells have developed numerous regulatory mechanisms targeting several steps of the L1 retrotransposition cycle [5, 6, 38], but at present these mechanisms remain poorly understood. In particular, the cell signaling cascades disrupted by chemical carcinogens and leading to aberrant L1 expression have not been systematically defined. The results of the present study established the convergence of two important signaling pathways in carcinogenesis in the regulation of L1 during hepatocarcinogenesis. Importantly, we show that the regulation of L1 by BaP AhR ligand is mediated via TGF-β1 signaling pathways acting through canonical SMAD-mediated mechanisms.

The TGF-β1 pathway has dual functions in carcinogenesis, serving both as a tumor suppressor and tumor promoter. As a tumor suppres-

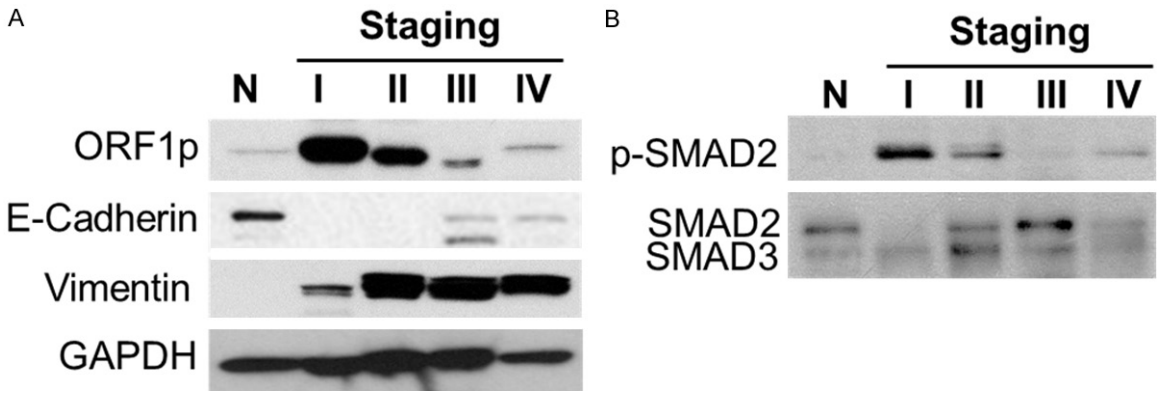

Figure 5. Expression of L1 proteins correlates with the activation of TGF-β1 signaling in liver tumors. Whole extracts of human liver hepatocellular carcinoma stages I, II, III, IV, and normal tissue were analyzed as follows: A. Immunoblotting for L1 protein (ORF1p), Vimentin, E-cadherin, and GAPDH (as loading control). B. Immunoblotting for phospho-SMAD2 and total SMAD2/3.

sor, TGF-β1 induces a cellular cytostatic and apoptotic program to restrain the growth of mammalian cells [14, 15, 18]. As a tumor promoter, TGF-β1 positively regulates cell growth, differentiation and migration in a cell- and context-specific manner [18]. The pro-tumorigenic effects of TGF β1 are known to involve EMT. Cells that undergo EMT increase their motility and acquire resistance to senescence and apoptosis, promoting cell invasion and tumor cell dissemination [15]. The finding that TGF-β1 increases the levels of endogenous L1 mRNA point to TGF-β1 signaling as one of the critical signaling cascades involved in L1 pathogenesis. The reciprocal nature of L1 and TGF-β1 signaling in the regulation of cancer initiation and progression remains elusive. However, L1 may represent an unsuspected regulator of the tumor promoter functions of TGF-β1 signaling. This hypothesis is supported by our previous research indicating that HepG2 cells expressing ectopic L1 (both wildtype and a reverse transcriptase-incompetent mutant) display an EMT phenotype, and that transdifferentiation involves interference with the expression of genes directly or indirectly regulated by TGF-β1. Of special interest in this regard are CXCL1 [19, 39, 40], periostin [41, 42], CCL2 [21, 43, 44], and VCAM-1 [20, 45, 46].

AhR activity is altered by diverse environmental AhR ligands known to exert potent carcinogenic effects in humans, mainly, halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons, such as BaP [47]. AhR activation has been implicated in the transcriptional and post-transcriptional regulation of L1 [12, 34, 48-50]. However, not all AhR ligands induce L1 expression [34, 50-52], suggesting that L1 reactivation through AhR depends on additional cellular factors involved in determining the fate of cellular signaling. Such factors may dictate differential crosstalk between AhR and other cellular signaling pathways. In fact, BaP and DMBA, but not TCDD, reactivate L1, and this response depends on AhR activity [34, 48, 49, 51]. BaP and DMBA are intracellularly converted to genotoxic compounds by a ligand-bound AhR mechanism that induces the expression of metabolic enzymes encoded by CYP genes [36]. By contrast, TCDD is not genotoxic, suggesting that AhR activation and genotoxic stress may both be required to complete the reactivation cascade.

Our findings demonstrate that crosstalk between AhR and TGF-β1 signaling is crucial to L1 expression in HepG2 cells. We demonstrated that: 1) AhR activation by BaP induces the expression of TGF-β1 and activates TGF-β1/SMAD canonical signaling, and 2) AhR-mediated TGF-β1 signaling regulates L1 expression. L1 transcription is mainly regulated by its L1 5' UTR region internal promoter, which has been shown to be necessary and sufficient in directing reporter gene expression in transfected cells [53]. L1 5' UTR region contains binding domains for several transcription factors [38]. The Yin-Yang-1 (YY1), runt-domain transcription factor 3 (RUNX3), SRY-related HMG box containing (SOX), p53, and E2F bind L1 5' UTR region and have been proposed to regulate L1

promoter activity [6, 38]. Binding of these transcription factors to L1 5' UTR specific target sites may positively and negatively regulate transcription via direct control of the general transcription apparatus. TGF-β1 signaling may participate in the regulation of L1 by controlling the expression or the activity of L1 5' UTR binding transcription factors. TGF-β1 has been reported to: 1) upregulate YY1 expression [54], 2) regulate p53 activity by stimulating p53 phosphorylation and acetylation [55] or coordinating transcriptional and translational repression of p53 levels [56], and 3) regulate the transcriptional activity of E2F through suppression of pRb phosphorylation [57].

Depending on cellular context, TGF-β1 signaling has been involved in the regulation of epigenetic mechanisms including, DNA methylation, histone covalent modifications, nucleosome positioning, non-coding RNAs and other components that shape chromatin states to dictate gene expression [18]. L1 is extensively silenced via DNA methylation [5], and we have previously established that epigenetic control of mammalian L1 is mediated by recruitment of E2F/Rb via nucleosomal modifications and recruitment of histone deacetylases [6]. Moreover, we have demonstrated that BaP-mediated L1 transcription is regulated by enrichment of transcriptionally-active chromatin markers (histone H3 trimethylated at lysine 4 (H3K4Me3) and histone H3 acetylated at lysine 9 (H3K9Ac)), and reduced association of DNA methyltransferase-1 (DNMT1) with the L1 promoter [7], modifications tightly controlled by E2F/Rb [58]. Given the ability of SMAD proteins to regulate promoter activity, SMAD2 and 3 may in fact participate in epigenetic control of the L1 promoter. The transcriptional activity of signal-activated SMAD proteins involves close interactions with chromatin modifying complexes to remodel chromatin structure and/or access to cryptic loci secluded by repressive histone marks. SMAD proteins recruit the histone acetyl transferases (HATs) p300 and CBP leading to acetylation of Lys9, Lys14, Lys18 and Lys23 on histone H3 and stimulation of transcription [59, 60]. Moreover, it has been shown that the SMAD transcriptional complex recruits a base excision repair complex to remove repressive DNA methylation from a TGF-β1 target gene, suggesting that TGF-β1 signaling provides a remarkable example for signal-directed, locus specific DNA demethylation during gene activation [61]. Therefore, we speculate that regulation of L1 expression by TGF-β1 may be critically controlled by the context-specific regulation of the epigenetic landscape.

Endogenous L1 retrotransposition occurs both in germline and somatic cells of hepatocellular carcinoma (HCC) patients [9]. The most prevalent risk factors for HCC are infection by hepatitis B virus (HBV) and hepatitis C virus (HCV), followed by chronic alcoholism [62]. HBV and HCV can suppress host defense factors such as APOBEC proteins [63]. These are important relationships given that retrotransposition assays have highlighted APOBEC3A and APOBEC3A proteins as potent inhibitors of retrotransposon activity [64]. APOBEC3A can inhibit L1 retrotransposition by deamination of transiently exposed single-strand DNA arising during new L1 insertions into genomic DNA [65, 66]. Therefore, L1 activation in HCC has been suggested to be promoted by viral infection. As such, an attractive hypothesis to be tested in future studies is that BaP mediates L1 reactivation in HCC by induction of AhR-mediated TGF-β1 signaling. Although more evidence is needed to assert whether L1 reactivation reproducibly induces neoplastic transformation *in vivo*, L1 reactivation causes genetic aberrations in the HCC tumor cell genome that may be crucial in HCC pathogenesis [9].

In summary, evidence is presented here that crosstalk between the AhR and TGF-β1 signaling pathways is involved in the regulation of L1 retrotransposon in HepG2 cells. This interaction likely plays a central role in the progression of malignant phenotypes and the occurrence of human hepatocarcinogenesis.

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Disclosure of conflict of interest

None.

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