Functional Modulation of Gene Expression by Ultraconserved Long Non-coding RNA TUC338 during Growth of Human Hepatocellular Carcinoma

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SUMMARY

TUC338 is an ultraconserved long non-coding RNA that contributes to transformed cell growth in hepatocellular carcinoma (HCC). Genomic regions of TUC338 occupancy were enriched in unique or known binding motifs homologous to the tumor suppressors Pax6 and p53. Genes involved in cell proliferation were enriched within a 9-kb range of TUC338-binding sites. TUC338 RNA-based purification was used to isolate chromatin for mass spectrometry, and the plasminogen activator inhibitor-1 RNA-binding protein (PAI-RBP1) was identified as a TUC338 RNA-binding partner. The PAI-RBP1 target gene plasminogen activator inhibitor-1 (PAI-1) itself could also be post-transcriptionally regulated by TUC338. Thus modulation of transformed cell growth by TUC338 may involve binding to PAI-RBP1 as well as to sequence-defined cis-binding sites to modulate gene expression. These findings suggest that ultraconserved RNAs such as TUC338 can function in a manner analogous to transcription factors to modulate cell proliferation and transformed cell growth in HCC.

Graphical Abstract

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DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and one table and can be found with this article online at https://doi.org/10.1016/j.isci.2018.03.004.

AUTHOR CONTRIBUTIONS

H.-J.W. and T.P. conceived the experiments and wrote the article. H.-J.W., I.K.Y., and K.T. conducted the experiments and M.P.W. performed data analysis. A.F. provided expertise and feedback. T.P. provided support.
INTRODUCTION

A large portion of the non-protein coding regions of the human genome has been shown to be actively transcribed into RNA molecules (Costa, 2010; Ponting and Belgard, 2010). A subgroup of this transcribed non-coding RNA genome consists of long non-coding RNA, i.e., with nucleotide sequences >200 bp, which include ultraconserved elements that are fully conserved across human and rodent genomes (Bejerano et al., 2004; Calin et al., 2007). The molecular function of these transcribed ultraconserved RNAs (ucRNAs) is unknown. Although many long non-coding RNAs lack primary sequence conservation across species, ucRNAs are highly conserved, suggesting an important evolutionarily conserved biological function for transcribed ucRNAs (Katzman et al., 2007). Indeed, ucRNA expression has been shown to occur in a tissue-specific manner, and aberrant expression of ucRNAs has been noted in several types of cancers, such as chronic lymphocytic leukemias, colorectal carcinoma, and hepatocellular carcinomas (HCCs) (Braconi et al., 2011; Calin et al., 2007; Sana et al., 2012). Scaruffi et al. also discovered 28 ucRNAs associated with good outcome in patients diagnosed with metastatic neuroblastoma (Scaruffi et al., 2009). Given these findings, some ucRNAs may have functional roles that are of relevance to cancer diagnosis, prognosis, or treatment response.

HCC is the major primary liver cancer and the third leading cause of cancer-related death worldwide. We have previously identified a striking expression of uc.338 in HCC and cloned the transcript encoding this ultraconserved element as a long non-coding RNA, TUC338. Modulation of TUC338 expression resulted in altered expression of several genes and transformed cell growth in human and mouse hepatocytes (Braconi et al., 2011). Understanding the molecular basis of these actions may provide new insights into the molecular functions of the transcribed ucRNA as well as identify therapeutic targets for HCC. Thus the aim of this study was to understand the mechanisms by which TUC338...
could regulate the expression of genes that could contribute to transformed cell growth in HCC.

A number of long non-coding RNAs (lncRNAs) have been revealed to associate with nuclear factors to form long non-coding RNA-ribonucleoproteins (RNPs) that may function as transcriptional activators or repressors for gene expression. In addition, it is believed that long non-coding RNAs are able to recruit the chromatin-modifying complex to specific genomic loci. Findings from these previous studies of long non-coding RNAs raise the possibility that TUC338 may function in HCC by associating with its protein partners and/or selective regions of the genome to mediate its biological effects. Thus we performed chromatin isolation by RNA purification followed by mass spectrometry and genomic analysis to identify TUC338-binding proteins and TUC338 occupancy sites throughout the genome.

RESULTS

Chromatin-Binding Sites of TUC338

In order for long non-coding RNA to modulate gene expression, access to chromatin is required. We postulated that binding to DNA would be important for TUC338 function and sought to identify and analyze the genomic binding sites of TUC338 RNA. Two non-overlapping sets of biotinylated TUC338 RNA-binding probes were used as affinity reagents for chromatin precipitation from HepG2 cells (see Table S1). This was followed by high-throughput sequencing to identify DNA bound to the pooled TUC338 RNA probes in each of the two pooled probe sets (see Transparent Methods). We analyzed data that were obtained only from both probe sets and excluded all data obtained from any one probe set alone. This approach avoids any potential effects of direct or indirect off-target hybridization. TUC338 RNA chromatin immunoprecipitation (IP) peaks could be distinguished from nonspecific background based on the strength of the TUC338 RNA IP signals. We excluded sharp peaks of <600 bp because these could be artifacts. Because the probe sets do not show any overlap, artificial identification of peaks with motifs homologous to those of probe sets is also avoided. Examination of RNA occupancy regions using model-based analysis of ChIP-seq (MACS) (Zhang et al., 2008) identified 2,469 high-TUC338-occupancy regions in the genome. These TUC338-binding regions were identified in all chromosomes, with the greatest number being in chromosome 2 (Figure 1). These data indicate that there are several TUC338 RNA occupancy sites and that these are focal and non-randomly distributed across the genome.

Sequence Specificity of Binding Sites

These observations suggest that TUC338 may bind to specific sequences. To understand how TUC338 may be targeted to specific regions in the chromatin and to determine the role of potential sequence-specific binding sites in targeting TUC338 complexes, we analyzed the sequence of the 2,469 TUC338-binding sites by Multiple EM for Motif Elicitation (MEME) (Machanick and Bailey, 2011).
Motif analysis revealed that 110 TUC338-binding sites were enriched for a motif \( p = 1.1 \times 10^{-13} \) that is homologous to the p53-binding motif (Figure 1B). To examine the potential effect of TUC338 on p53 binding at these sites, we analyzed p53 activation in response to TUC338 knockdown using small interfering RNA (siRNA) to TUC338 and observed a 31% ± 6% increase in p53 activity. In addition, other transcription-factor-binding motifs were also identified. For example, 107 TUC338-binding sites were enriched for a motif \( p = 5.6 \times 10^{-19} \) that is homologous to the Pax6-binding motif (Figure 1C). In addition, other unique TUC338 binding motifs were also identified (Figure 1D). The motifs lacked homology with the ultraconserved sequence. Consequently, genomic binding interactions may be unrelated to factors that determine sequence ultraconservation.

Analysis of cis-Genes Associated with TUC338-Binding Sites

We next examined the genomic locations of all TUC338-binding regions to identify neighboring genes that could be cis-regulated by TUC338. A total of 3,306 potential target genes were identified within 1,000 kb of the 2,469 TUC338-binding sites. Of these potential targets, 404 were located within 9 kb of gene loci, and gene ontology analysis showed highly significant enrichment of TUC338-associated gene loci involved in cell proliferation (Table 1), distinct from those genes located at a further distance (Table 2). Of the 18 genes associated with cell proliferation, 9 have been functionally linked to HCC: E2F1 (Chen et al., 2012), BOP1 (Chung et al., 2011), CYR61 (Feng et al., 2008), EGFR (Huether et al., 2005), ERCC1 (Fautrel et al., 2005), FOXC1 (Xia et al., 2012), OSM (Liang et al., 2012), SHH (Chen et al., 2010), and ZEB2 (Cai et al., 2012) (Table 3). Enrichment was also noted for genes involved in Ras signal transduction, tetrahydrobiopterin and pteridine metabolic process, exocrine system development, and actin filament organization. Potential long-range interactions between target genes and TUC338-binding sites were also analyzed for the 2,979 genes identified between 9 kb and 1 Mb of TUC338-binding sites. Gene ontology analysis of this gene set revealed significant enrichment of genes involved in cell proliferation as well as cell morphogenesis and differentiation, neuron differentiation, cell motion, and DNA-dependent transcriptional regulation (Table 2). Experimentally, modulation of TUC338 using siRNA in HCC cells alters the expression of several genes. Using Affymetrix GeneChip mRNA analysis, we identified 611 genes that are altered in expression by greater than 2-log-fold in response to TUC338 knockdown. TUC338-binding sites were identified within 1,000 kbp of the gene loci for 89 of these genes, indicating the potential for their direct regulation by TUC338. Functional annotation analysis of these 89 genes using DAVID identified that the most highly significant enrichment occurred for genes involved in biological processes related to cell proliferation. TUC338 has been shown to modulate HCC cell proliferation. Thus these data suggest that cis-regulation of expression of genes involved in aberrant cell proliferation could represent a mechanism by which TUC338 contributes to tumor growth in HCC.

Identification of PAI-RBP1 as a TUC338-Binding Protein

Binding at target sites can occur either via direct hybridization or in association with other factors such as proteins or other RNAs. None of the binding sites that were identified by our analyses showed homology to TUC338, making it unlikely that TUC338 RNA binding occurred by direct hybridization. Thus we sought to identify proteins that would bind to
TUC338. As with studies to identify DNA-binding sites, two independent pools of biotinylated oligonucleotide probes that were complementary to separate regions of TUC338 without any overlapping sequences were hybridized to TUC338 RNA in HepG2 cell lysates. The proteins that were pulled down were then separated and analyzed by nano-high-pressure liquid chromatography (HPLC)-electrospray tandem mass spectrometry. Potential protein binding partners to TUC338 RNA were identified as proteins that were pulled down by both independent probe sets. The plasminogen activator inhibitor-1 RNA-binding protein (PAI-RBP1) isoform 2 was identified as a TUC338 RNA-binding protein. To verify these findings, we performed RNA immunoprecipitation using monoclonal PAI-RBP1 antibody (Figure 2A) and confirmed that PAI-RBP1 is a TUC338 RNA-binding protein. PAI-RBP1 was initially identified as an RNA-binding protein that could post-transcriptionally regulate plasminogen activator inhibitor-1 (PAI-1) expression (Heaton et al., 2001, 2003). Indeed, enrichment of PAI-1 mRNA (Figure 2B, upper panel) was noted with PAI-RBP1 antibody compared with control IgG antibody pull-down. Furthermore, enrichment of TUC338 was also observed using the PAI-RBP1 antibody but not the IgG antibody (Figure 2B, bottom panel). These data show that PAI-RBP1 can bind to PAI-1 mRNA as well as to TUC338 RNA.

**Aberrant Expression of PAI-RBP1 in Malignant Hepatocytes Regulated by TUC338**

To examine the biological correlates of TUC338 binding, we next evaluated PAI-RBP1 mRNA expression in malignant (HepG2 and Huh7) and non-malignant (HH) human hepatocytes. We found that PAI-RBP1 mRNA expression was increased in the malignant hepatocytes (Figure 3), similar to the increased expression of TUC338 observed in malignant hepatocytes (Braconi et al., 2011; Calin et al., 2007; Sana et al., 2012). The relationship between expression of PAI-RBP1 and TUC338 was further studied. Knockdown of TUC338 using siRNA in HepG2 cells decreased both PAI-RBP1 mRNA and protein (Figure 3). The knockdown efficiency of TUC338 was confirmed by real-time PCR. Thus PAI-RBP1 expression is positively regulated by TUC338. Moreover, knockdown of PAI-RBP1 using siRNA resulted in a reduction in TUC338 RNA expression. However, knockdown of PAI-RBP1 did not alter the effect of the siRNA to TUC338 on p53 activation. Because PAI-RBP1 is a PAI-1-binding protein, we next examined the effect of TUC338 on the regulation of PAI-1 expression. PAI-1 mRNA and protein expression were examined following siRNA-mediated TUC338 knockdown in HepG2 cells. Compared with cells transfected with control non-targeting (NT) siRNA, a reduction in PAI-1 mRNA as well as in secreted PAI-1 protein was noted in cells transfected with siRNA to TUC338 (Figures 3E and 3F).

**TUC338 Regulates PAI-1 Expression by Modulating the 3′ UTR of the 2.2-kb Transcript**

To examine the effects of TUC338 on PAI-1 expression, we first examined the PAI-1 promoter sequence and identified several putative TUC338-binding motifs in the 5′ UTR. However, PAI-1 was not identified among the genes that are candidates for direct regulation by TUC338 binding, suggesting that the regulation of PAI-1 expression did not occur via direct transcriptional activation. Two transcripts of human PAI-1 mRNA (3.2 and 2.2 kb) have been reported, and these differ only in the length of their 3′ UTR (Fattal and Billadello, 1993). We cloned the two 3′ UTRs of the 3.2- and 2.2-kb transcripts into pGL3
vector to generate the reporter constructs PAI-13U and PAI-13US, respectively (Figure 4A), and examined the response of 3′ UTR activation to TUC338 knockdown. Compared with control NT siRNA, the siRNA to TUC338 decreased PAI-13US and slightly increased PAI-13U activity (Figure 4B). Furthermore, PAI-13US activity was increased by the expression of full-length TUC338, further demonstrating the effect of TUC338 on the 3′ UTR of the 2.2-kb PAI-1 transcript (Figure 4C). These results suggest that TUC338 upregulates PAI-1 expression by stabilizing the 2.2-kb PAI-1 transcript.

Identification of Sites of Interaction between PAI-RBP1 and TUC338 RNA

We next sought to define the PAI-RBP1-interacting regions of the TUC338 RNA by electrophoretic mobility shift assay (EMSA) using HepG2 cell extracts and full-length or truncated TUC338 RNA fragments. First, the full-length TUC338 RNA was labeled with biotin at the 3′ end and incubated with binding buffer either alone or with HepG2 cell extracts (Figure 5A, lanes 1–4). A high-molecular-weight shift band was observed in the presence of cell extract. Binding increased with the concentration of full-length TUC338 RNA probes and was reduced by the addition of excess unlabeled TUC338 RNA (Figure 5A, lanes 3–6). Furthermore, the addition of PAI-RBP1 antibody confirmed the specificity of binding of TUC338 to PAI-RBP1 (Figure 5A, lane 7). RNA EMSA was then performed using five truncated biotin-labeled TUC338 RNA probes. After incubation with cell extract, strong binding was observed with TUC338/1–115, 116–237, 238–354, and 461–575 but not with TUC338/355–460 (Figure 5B). The specificity of binding was confirmed by using unlabeled homologous RNA oligonucleotides. In addition, all of the binding involved PAI-RBP1 protein as it was reduced by the addition of PAI-RBP1 antibody (Figure 5C). These results indicate that TUC338 associates with PAI-RBP1 through the regions nt 1–354 and nt 461–575 of TUC338.

PAI-RBP1 Modulates Transformed Cell Growth of Human Hepatocytes

TUC338 modulates transformed cell growth in hepatocytes. To further evaluate the contribution of the TUC338-binding protein PAI-RBP1 to biological effects, we next assessed the impact of PAI-RBP1 knockdown on cell proliferation in HepG2 cells. Knockdown efficiency of PAI-RBP1 was confirmed by real-time PCR (Figure 6A). HepG2 cell viability was unchanged by PAI-RBP1 knockdown compared with NT controls (Figure 6B). However, compared with control NT siRNA, siRNA to PAI-RBP1 decreased anchorage-independent cell growth of HepG2 HCC cells, assessed using two complementary assays to evaluate growth in soft agar (Figure 6C) as well as by a clonogenic growth assay (Figure 6D). These studies verify the contribution of PAI-RBP1 to transformed cell growth in human hepatocytes.

DISCUSSION

TUC338 is a long non-coding RNA that is capable of modulating gene expression and transformed cell growth in hepatocytes. Chromatin immunoprecipitation using pools of biotinylated probes with non-overlapping sequence complementarity to TUC338 RNA followed by genomic sequencing enabled us to localize specific loci at which TUC338 RNA can bind. We identified multiple genomic TUC338-binding sites; some of these are similar
to sites associated with known transcription factors. Because TUC338 RNA can bind to these sites, it is likely that these loci are involved in TUC338 function. There are some limitations of this approach. Different loci may be enriched with differing levels of efficiency, for example, if there is restricted access to the genomic site. Moreover, enrichment but not the stoichiometry of binding at a specific genomic locus is identified, and thus the molecular basis of the interaction between TUC338 and these loci is unknown.

In prior studies, we have observed an increase in nuclear TUC338 in human HCC, raising the possibility that TUC338 may be involved in chromatin remodeling to facilitate gene expression. The greatest number of TUC338-binding sites occurs on chromosome 2, and two of the most significantly enriched sites of TUC338 occupancy are homologous to the binding sites of Pax6 and p53. p53 can bind to DNA as a tetramer, in a sequence-specific manner, and can regulate gene transcription (Vazquez et al., 2008). Owing to the high similarity between the binding motifs of TUC338 and p53, competition for binding to DNA could occur between TUC338 and p53. Thus the increased expression of TUC338 could result in sequestration of p53 from DNA binding at these sites, functionally silencing the p53 target genes. Similar effects could also occur at other motifs such as Pax6. In addition to its role in development and cell differentiation, Pax6 transcriptionally regulates the genes involved in cell growth and apoptosis (Mayes et al., 2006; Shyr et al., 2010) and can act as a tumor suppressor. Thus a potential mechanism of action of TUC338 could involve interference with binding of tumor suppressors to target DNA with suppression of downstream transcription. Similar to the mechanisms reported for regulatory properties of other long non-coding RNAs (Rinn and Chang, 2012), we speculate that TUC338 could act as a guide to route transcription factors to specific genomic loci, which in turn activate or represses gene expression, or alternatively as a scaffold for the interaction between specific binding proteins and molecular complexes, resulting in chromatin remodeling.

The lack of sequence homology to TUC338 among the enriched occupancy sites indicates that direct binding through sequence-based hybridization is unlikely and supports the involvement of additional factors such as RNA-binding proteins or other RNAs that could serve to recruit and facilitate TUC338 RNA binding at these enriched target sites. To identify TUC338 RNA-binding proteins, a similar approach was used in which TUC338 RNA was enriched and co-purified proteins were identified by mass spectrometry. This approach to identify binding proteins is the converse of an RNA immunoprecipitation study in which proteins are first pulled down and RNA-binding partners identified. Proteomic analysis identified PAI-RBP1 as an RNA-binding protein that could bind to TUC338 and contribute to transformed cell growth. PAI-RBP1 contributes to post-transcriptional regulation of PAI-1 mRNA by binding to PAI-1 mRNA 3′ UTR, and we further demonstrated that TUC338 can regulate PAI-1 mRNA through its 3′ UTR. The lack of any complementary sequence between PAI-1 3′ UTR and TUC338 RNA supports the involvement of RNA-binding proteins in post-transcriptional regulation instead of direct complementary binding of TUC338 to PAI-1 3′ UTR.

Further indirect evidence that TUC338 can interact with RNA-binding proteins is provided by the results of the EMSA studies. PAI-RBP1 can bind to rat PAI-1 mRNA 3′ UTR at the cyclic nucleotide responsive sequence (CRS), which comprises A- and U-rich sequences
AUUUA and UUAUUUAUU motifs are targets of RNA-binding proteins, and we identified several A- and U-rich regions in the TUC338 sequence. Use of truncated TUC338 RNA probes and EMSA revealed that the only region unable to form protein complexes (between nucleotides 355 and 460) also lacks A-rich, U-rich, and AUUUA sequences.

Biological roles for PAI-RBP1 and PAI-1 in tumor progression have been postulated. PAI-RBP1 expression is increased in several different cancers, such as ovarian, prostate, and lung cancer, and, moreover, correlates with advanced stage of ovarian cancer (Koensgen et al., 2007; Sun et al., 2012). Likewise, an increased expression of PAI-1 has also been shown in many solid tumors, including HCC, and is associated with poor patient prognosis (Berger, 2002; Koensgen et al., 2007; Zhou et al., 2000). In addition to post-transcriptional regulation, PAI-RBP1 has a nuclear function associated with chromatin remodeling based on its interaction with proteins such as chromodomain helicase DNA-binding protein 3 (CHD3) (Lemos et al., 2003). Although CHD3 was not identified as a TUC338-binding protein in our studies, it remains possible that the complex of TUC338 and PAI-RBP1 could be involved in gene regulation through chromatin remodeling.

These results showing the pervasive genomic nature of TUC338 RNA occupancy emphasize a broad role in gene regulation. More specifically, there is evidence that direct physical interactions between TUC338 RNA and PAI-RBP1 resulting in post-transcriptional regulation of PAI-1 mRNA could contribute, in part, to the oncogenic effects of enhanced TUC338 expression that is observed in HCC. These observations provide new insights and will generate new hypotheses into understanding the dynamic mechanisms by which the ucRNA TUC338 modulates gene expression, which will be helpful to understand the effects of this long non-coding RNA in tumorigenesis.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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TUC338 can modulate cell proliferation by sequence-specific genomic binding.

TUC338 binds to motifs homologous to those of the tumor suppressors Pax6 and p53.

Plasminogen activator inhibitor-1 mRNA binding protein is a TUC338-binding protein.

TUC338 can regulate PAI-RBP1 target gene plasminogen activator inhibitor-1.
Figure 1. Genomic Binding Sites of TUC338
(A) Chromosome distribution of TUC338-binding sites derived from 2,469 true TUC338 peaks identified by sequencing of TUC338 RNA-bound chromatin.
(B and C) Homology of an enriched binding site motif with (B) p53 and (C) Pax 6 motifs from the Jaspar core database.
(D) Unique TUC338-binding site motif.
Figure 2. Association of TUC338 with PAI-RBP1 and PAI-1 RNA

RNA immunoprecipitation was performed using a monoclonal PAI-RBP1 antibody (Ab) or mouse IgG (IgG) to immunoprecipitate PAI-RBP1 and RNA complex from HepG2 cell lysates.

(A) Western blot was performed using anti-PAI-RBP1 antibody.

(B) TUC338 and PAI-1 were amplified from RNA immunoprecipitates by RT-PCR using TUC338- and PAI-1-specific primers.
Figure 3. TUC338 Positively Regulates PAI-RBP1 and PAI-1 Expression

(A) PAI-RBP1 mRNA level in non-malignant (HH) or malignant (HepG2, HUH-7) hepatocytes was assessed by reverse transcription followed by SYBR green real-time PCR and normalized by GAPDH. Values are mean ± SE (n = 3, *p < 0.01).

(B–E) HepG2 cells were transfected with non-targeting (NT) siRNA or siRNA against TUC338. The RNA expression levels of PAI-RBP1 (B) and TUC338 (C) were measured by SYBR green real-time PCR and normalized to GAPDH. (D) The protein expression of PAI-RBP1 and β-actin was determined by western blot. Values are mean ± SE of the fold over NT (n = 3, *p < 0.01). (E) The mRNA expression level of PAI-1 was measured by SYBR green real-time PCR and normalized to GAPDH.

(F) PAI-1 protein secreted in the media was determined by western blot. Values are mean ± SE of the fold over NT (n = 3, *p < 0.01).
Figure 4. TUC338 Regulates the Activity of 3′ UTR of 2.2 kb PAI-1 mRNA

(A) PAI-1 3′ UTR (solid black box) was cloned into pGL3-control vector downstream of the firefly luciferase gene. PAI-13U contains the 3′ UTR of 3.2 kb PAI-1 mRNA. PAI-13US contains the 3′ UTR of 2.2 kb PAI-1 mRNA.

(B) After 24-hr transfection of siRNAs against non-targeting (NT) or TUC338, HepG2 cells were co-transfected with the *Renilla* luciferase reporter vector and the firefly luciferase reporter construct (PAI-13U or PAI-13US). After another 24 hr, luciferase assay was performed.

(C) HepG2 cells were co-transfected with PAI-13US construct, the *Renilla* luciferase reporter vector, and pcDNA3.1 empty vector or pcDNA3.1 containing full-length TUC338 (p-tuc338). Luciferase assay was performed at 24 hr post transfection. The firefly luciferase activities were normalized to the *Renilla* luciferase activities. Values are mean ± SE of the fold over NT or pcDNA3.1 empty vector (n = 3, *p < 0.05).
Figure 5. *In Vitro* Interaction of TUC338 and PAI-RBP1 Protein by RNA EMSA

(A) Full-length TUC338 RNA labeled at the 3′ end was used as the RNA probe (Biotin-TUC338) and incubated with cell lysate extracted from HepG2 cells. Full-length TUC338 RNA and PAI-RBP1 antibody served as competitors. The signal was quantified by NIH Image software.

(B and C) Five truncated Biotin-TUC338 (1–115, 116–237, 238–354, 355–460, 461–575) were used as RNA probes. The unlabeled truncated RNA homologous to the labeled truncated RNA was used as a specific competitor (Homologous TUC338).
Figure 6. PAI-RBP1 RNAi Affects Anchorage-Independent Growth

HepG2 cells were transfected with non-targeting (NT) or PAI-RBP1 siRNA.

(A) The RNA expression levels of TUC338 were measured by SYBR green real-time PCR and normalized to GAPDH.

(B) At 24 hr post transfection, cells were plated in 96-well plates. Cell proliferation was assessed at indicated time points.

(C) At 24 hr post transfection, cells were plated in 96-well plates with soft agar. After 1 week, anchorage-independent growth was assessed fluorometrically.

(D) At 24 hr post transfection, cells were plated in 24-well plates with soft agar. After 4 weeks, cell colonies were stained with Giemsa and counted.

Values are mean ± SE of the fold over NT (n = 3, *p < 0.01, **p < 0.005).
Table 1
Enriched Genes within Short Range (9 kb) of TUC338-Binding Sites

Gene ontology analysis was performed for biological processes using DAVID. The top five ontologies are listed. FDR, false discovery rate.

| Count | Gene ID | Gene Description | Count | p Value | FDR   |
|-------|---------|-------------------|-------|---------|-------|
| 1     | GO:0046578 | regulation of Ras protein signal transduction | 12 | 0.005403 | 8.8404 |
| 2     | GO:0006729 | tetrahydrobiopterin biosynthetic process | 3  | 0.006402 | 10.3927 |
| 3     | GO:0035272 | exocrine system development | 4  | 0.007187 | 11.5937 |
| 4     | GO:0046146 | tetrahydrobiopterin metabolic process | 3  | 0.008838 | 14.0722 |
| 5     | GO:0050679 | positive regulation of epithelial cell proliferation | 5  | 0.010974 | 17.1819 |
Table 2

Enriched Genes within Long Range (9–1000 kb) of TUC338-Binding Sites

Gene ontology analysis was performed for biological processes using DAVID. The top five ontologies are listed. FDR, false discovery rate.

|   | Count | p Value       | FDR       |
|---|-------|---------------|-----------|
| 1 | GO:0000902-cell morphogenesis | 109 | 4.19 × 10⁻¹¹ | 7.94 × 10⁻⁸ |
| 2 | GO:0000904-cell morphogenesis involved in differentiation | 80 | 6.37× 10⁻¹⁰ | 1.21 × 10⁻⁶ |
| 3 | GO:0030182-neuron differentiation | 123 | 9.72× 10⁻¹⁰ | 1.84 × 10⁻⁶ |
| 4 | GO:0006928-cell motion | 130 | 1.86 × 10⁻⁹ | 3.52 × 10⁻⁶ |
| 5 | GO:0035295-tube development | 73 | 2.16 × 10⁻⁹ | 4.09 × 10⁻⁶ |
Table 3
Enriched Genes within a Short Range of TUC338-Binding Sites and Associated with Cell Proliferation in Hepatocellular Cancer

| Gene Symbol | Gene Name                                      | Reported Roles in HCC                                                                 |
|-------------|-----------------------------------------------|-------------------------------------------------------------------------------------|
| E2F1        | E2F transcription factor 1                    | Associated with worse outcomes in patients with HCC                                  |
| MXD1        | MAX dimerization protein 1                    |                                                                                     |
| STIL        | SCL/TAL1 interrupting locus                   |                                                                                     |
| SH2D2A      | SH2 domain protein 2A                         |                                                                                     |
| BOP1        | Block of proliferation 1                      | Oncogene role in HCC invasiveness and metastasis                                    |
| CCKBR       | Cholecystokinin B receptor                    |                                                                                     |
| CYR61       | Cysteine-rich, angiogenic inducer, 61         |                                                                                     |
| DAB2        | Disabled homolog 2, mitogen-responsive phosphoprotein |                                                                                   |
| EGFR        | Epidermal growth factor receptor              | Predictive marker for HCC metastasis and recurrence                                 |
| EMP2        | Epithelial membrane protein 2                 |                                                                                     |
| ERCC1       | Excision repair cross-complementing rodent repair deficiency, complementation group 1 | Overexpression is associated with liver fibrogenesis and HCC                       |
| FOXC1       | Forkhead box C1                               | Overexpression promotes metastasis in HCC                                            |
| GFI1B       | Growth-factor-independent 1B transcription repressor |                                                                                   |
| LAMA5       | Laminin, alpha 5                              |                                                                                     |
| OSM         | Oncostatin M                                  | Overexpression is associated with HCC development                                    |
| PES1        | Pescadillo homolog 1, containing BRCT domain |                                                                                     |
| SHH         | Sonic hedgehog homolog                        | Overexpression is associated with size and invasion of HCC                          |
| ZEB2        | Zinc finger E-box binding homeobox 2          | Tumor suppressor role in HCC                                                       |