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TGF β Signaling Confers Sorafenib Resistance via Induction of Multiple RTKs in Hepatocellular Carcinoma Cells

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Abstract

Transforming growth factor β (TGF β) is a multifunctional cytokine which is importantly implicated in hepatocarcinogenesis. The current study provides novel evidence that TGF β upregulates the expression of multiple receptor tyrosine kinases (RTKs), including IGF1R, EGFR, PDGFR, and FGFR1 in human hepatocellular carcinoma (HCC) cells. This, in turn, sensitized HCC cells to individual cognate RTK ligands, leading to cell survival. Our data showed that the TGF β -mediated increase in growth factor sensitivity led to evasion of apoptosis induced by the mitogen-activated protein kinase inhibitor, sorafenib. Conversely, we observed that inhibition of the TGF β signaling pathway by LY2157299, a TGF β RI kinase inhibitor, enhanced sorafenib-induced apoptosis, in vitro. Our findings disclose an important interplay between TGF β and RTK signaling pathways, which is critical for hepatocellular cancer cell survival and resistance to therapy.

Keywords

TGF β ; sorafenib; RTKs; IGF1R; Akt; HCC

INTRODUCTION

Transforming growth factor β (TGF β) is a secreted protein that regulates a wide variety of cellular processes including apoptosis and proliferation. The TGF β signal is propagated through its type I and II receptors to activate intracellular messengers, SMAD2 and SMAD3. Upon phosphorylation, SMAD2 and SMAD3 form heterotrimeric complexes with SMAD4, translocate to the nucleus and coordinate expression of target genes [1]. Noncanonical TGF β signaling includes activation of the phosphoinositide-3-kinase (PI3K) [2] and the mitogen-activated protein kinase (MAPK) pathways [3]. The response to TGF β signaling can differ dramatically depending on cell type [4]. For example, TGF β triggers proliferation of quiescent fibroblasts [5], while functioning as a tumor suppressor, inhibiting division [6], and inducing apoptosis in hepatocytes [7]. Following the transformation of hepatocytes to hepatic tumor cells, however, TGF β can lose its growth inhibitory and proapoptotic effects,

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and instead cause cells to undergo the epithelial mesenchymal transition (EMT) [8], and increase cell capacity for colony formation [9], both processes intricately involved in metastasis. Coulouarn et al. [10] have shown that hepatocytes exhibit “early” and “late” gene expression patterns depending on length of exposure to TGF β , and that HCC patients with “late” signatures have poorer prognosis [10]. Other studies show that TGF β can worsen prognosis by preventing tumor cell death induced by cytotoxic chemotherapies [11,12].

Hepatocellular carcinoma (HCC) is the fifth most common cancer type worldwide [13] and has a 5 yr survival rate of merely 12% [14]. The only available systemic therapy for advanced HCC is sorafenib, a multikinase inhibitor of RAF, vascular endothelial growth factor receptor (VEGFR), and platelet derived growth factor receptor (PDGFR) [15]. While sorafenib has been shown to improve survival, patients only gain an average of 3 months of survival before the tumor develops resistance to the drug and patients relapse [16]. This resistance is associated with an increase in PI3K-AKT activity [17], a pathway known to promote cell survival [18,19]. The PI3K-AKT pathway is activated by a variety of receptor tyrosine kinases (RTKs) following receptor-cognate ligand binding. Thus, RTKs have been implicated in the progression of many tumor types, including HCC. For this reason, many ongoing HCC clinical trials are investigating the efficacy of targeting RTKs, including platelet-derived growth factor β receptor (PDGF β R), epithelial growth factor receptor (EGFR), insulin like growth factor receptor 1 (IGF1R), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and hepatocyte growth factor receptor (c-MET) [20]. In the current study, we implicate multiple RTKs as effectors of a TGF β -mediated circumvention of sorafenib-induced apoptosis.

METHODS

Cell Culture

Human hepatocellular carcinoma cell lines, PLC/PRF/5, Hep3B, and Huh7 were not authenticated or tested for contamination but were purchased from ATCC, Manassas, VA, and JCRB Cell Bank, Japan, and experiments were performed at early passage numbers. Cells were cultured at 37°C at 5% CO₂, in high glucose DMEM (Sigma–Aldrich, St. Louis, MS) with 10% heat-inactivated FBS and 1% Antibiotic-Antimycotic solution (Life Technologies, Carlsbad, CA). Wildtype cells were serum starved beginning 24h prior to treatment and cells remained in serum free media throughout the duration of each treatment. Stable shIGF1R and shScrambled expressing cells were made by transfection with either pGFP-V-RS-shScrambled or pGFP-V-RS-shIGF1R plasmids, using Lipofectamine and PLUS Reagent (Life Technologies). Cells were then treated with 0.5 μ g/mL puromycin (Life Technologies) for 3wk, at which point the surviving cells were considered to be stable knockdowns and puromycin was removed. Stable pGFP-V-RS-shScrambled and pGFP-V-RS-shIGF1R cells were then treated in the presence of 10% FBS. Recombinant human IGF1, IGF2, PDGF β , FGF1, and EGF were purchased from R&D Systems, Minneapolis, MN. Recombinant human TGF β 1 was purchased from Cell Signaling Technologies, Danvers, MA. Sorafenib and LY2157299 were purchased from Cayman Chemicals, Ann Arbor, MI, and were dissolved in DMSO (Sigma–Aldrich).

Cell Viability Assays

Cell viability was determined by crystal violet staining. PLC/PRF/5 cells were seeded at 2×10^5 cells per well in 6 well dishes. Cells were then serum starved for 24 h, then treated for 48 h with 5 ng/mL TGF β in serum-free media, followed by 5 μ M sorafenib in serum free media for 24 h. Cells were then stained with crystal violet solution.

ChIP

PLC/PRF/5 cells were grown to 75% confluence, serum starved for 24 h, then treated with or without 5 ng/mL TGF β for 1 h. ChIP was performed according to the manufacturer's protocol, using a kit from Cell Signaling Technologies. Rabbit IgG and SMAD2/3 antibodies (Cell Signaling Technologies) were used to pull down cross-linked DNA. Immunoprecipitated DNA was amplified and analyzed via qRT-PCR using gene promoter-specific primers.

qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies). Purified RNA was then reverse transcribed into cDNA using SuperScript II (Life Technologies), primed with OligodT (12–18) primers. Gene expression was assayed by qRT-PCR using the SsoAdvanced Universal SYBR green Mastermix (Bio-Rad, Hercules, CA). A cDNA standard curve was run for each primer set and sample expression was interpolated onto the curve, then normalized to GAPDH expression levels. Primer sequences are listed in Table I.

Western Blotting

Cellular protein was extracted in a 1% NP40 cell lysis buffer with protease (Complete Mini; Hoffman-La Roche, Basel, Switzerland) and phosphatase (PhosSTOP; Hoffman-La Roche) inhibitors. Protein concentrations were determined via Bradford assay (Bio-Rad, Hercules, CA), and samples were diluted in 2 $\times$ Laemmli buffer with β -Mercaptoethanol (Sigma–Aldrich). Protein levels were measured using specific antibodies: (pSMAD2; catalogue number: 3108, pSMAD3; 9520, SMAD2/3; 8685, IGF1R; 9750, EGFR; 4267, c-Met; 8198, pIGF1R; 3021, AKT; 4685, pAKT; 4060, MCL1; 4572, Caspase 7; 9494, PARP; 9532 (Cell Signaling, Danvers, MA), and diluted 1:500 in inblocking buffer (5% nonfat milk in PBST), followed by 1:5000 IRDye680 anti-mouse and/or IRDye800 anti-rabbit secondary antibodies (Li-Cor, Lincoln, NE) in blocking buffer. Our β -Actin antibody was purchased from Sigma–Aldrich and β -Actin (catalogue number: A2228) was used for normalization of protein levels. Bands were quantified using ImageJ software [21].

Plasmids

The shScrambled and shIGF1R plasmids were constructed by inserting double stranded oligos into pGFP-V-RS plasmids (OriGene, Rockville, MD). Oligos were purchased from IDT (Coralville, Iowa). The oligo sequences were taken from the RNAi consortium(The Broad Institute, Cambridge, MA; IGF1R: 5'-GCTGT ACGTCTTCCATAGAACTCGAGTTTCTATGGAAGA CGTACAGCTTTTT-3', GFP: 5'-GACCACCCTGACCT ACGGCGTCTCGAGACGCCGTAGGTCAGGGTGGT CTTTTT-3').

TCGA Analysis

Level 3 RNA-seqV2 expression data was downloaded from the TCGA database (<http://cancergenome.nih.gov/>). The data in the TCGA database was generated using the Illumina HiSeq 2000 platform, and annotated based on the UCSC hg19 gene standard track. RNA expression values were quantified using the RSEM algorithm [22]. Data from 269 HCC patients, downloaded on 06/01/15 using the TCGA-assembler R package [23], were analyzed using the built in correlation test function in the R statistical software package.

Animal Study

Four-week-old male athymic nude NOD.CB17-prkdc/SCID mice were purchased from Jackson Laboratory (Bar Harbor, ME). The tumor xenografts were established by inoculating 1.5×10^6 PLC/PRF/5 cells mixed with Matrigel into both flanks of mice. Treatments began 9 d after injections. LY2157299 (Selleckchem, Houston, TX) was dissolved in 50/50 Kollipher/95% EtOH solution and further diluted 1:4 in H₂O in advance of each daily treatment. Each animal was administered 25 mg/kg LY2157299 or vehicle, daily, via oral gavage. All animal studies were conducted according to the protocol approved by the Tulane Institutional Animal Care and Use Committee.

Statistics

Two-tailed Student's *t*-tests were performed using GraphPad Prism 5.0. Pearson, Spearman, and *P* values for correlations in TCGA datasets were performed using the R statistical package [24].

RESULTS

The Effect of TGF β on HCC Cell Apoptosis, in the Presence or Absence of Sorafenib

TGF β has an enigmatic role in HCC; it can either inhibit or promote tumor progression. To explore the dynamics of the cellular response to TGF β signaling, we treated PLC/PRF/5 cells with 5 ng/mL of TGF β 1 for various lengths of time, ranging from 0 to 48 h and measured the effector caspase, caspase 7 (CAS7), and PARP levels at each time point (Figure 1A–C). For the first 24 h, TGF β induced PARP and CAS7 cleavage. Interestingly, following 48 h of treatment, the TGF β -treated cells exhibited reduced levels of PARP and CAS7 cleavage. To determine whether TGF β could protect cells against the proapoptotic effect of sorafenib, the cells treated with or without TGF β at each time point were incubated with 5 μ M sorafenib for an additional 2 h period. We observed that at the 48 h time point, sorafenib was unable to efficiently induce PARP or CAS7 cleavage in TGF β pretreated cells (Figure 1A–C). These findings suggest that sorafenib is less cytotoxic in tumor cells with persistently active TGF β signaling. The dose of TGF β was critical for the cytoprotective effect, as 2.5 ng/mL TGF β was essential for the prevention of PARP and CAS7 cleavage in the presence or absence of sorafenib (Figure 1D). Accordingly, we observed that TGF β pretreatment increased the survival of PLC/PRF/5 cells treated with sorafenib (Figure 1E).

Sorafenib-Induced HCC Apoptosis Is Enhanced by the TGF β RI Inhibitor, LY2157299

The observed protective effect of TGF β against sorafenib-induced HCC cell apoptosis suggests that inhibition of the TGF β signaling pathway may be of therapeutic value for HCC. To test this possibility, we utilized LY2157299 (galunisertib), a small molecule TGF β RI kinase inhibitor currently being investigated in HCC clinical trials [25]. This inhibitor has shown promise in preclinical models and we confirm it was able to reduce tumor burden in immunocompromised mice (Figure S1). After verification that LY2157299 inhibits TGF β signaling in PLC/PRF/5 cells (Figure 2A), we treated the cells with LY2157299 in the presence or absence of TGF β for 48 h, followed by a 2 h incubation with sorafenib or vehicle control. Consistent with TGF β -mediated cell survival, we observed that inhibition of TGF β signaling by LY2157299 led to cell apoptosis, as reflected by increased CAS7 and PARP cleavage (Figure 2B). These results were similar but heightened in cells that were further treated with sorafenib (Figure 2B). As sorafenib targets RAF and inhibits the MAPK signaling pathway [15], we reasoned that other pro-survival molecule(s) may be activated by TGF β that confer resistance to sorafenib-induced apoptosis. Interestingly, we observed high levels of activated AKT in cells treated with TGF β (Figure 2B), which suggests that TGF β may activate AKT, rendering cells resistant to sorafenib-induced cytotoxicity. The latter assertion is further corroborated by the fact that TGF β is known to activate the PI3K-AKT cascade [26], a crucial signaling pathway for cell survival [27].

TGF β Treatment Leads to Increased RTK Levels

We observed that AKT phosphorylation peaked following 48 h of TGF β treatment (see below, Figure 6A), whereas activation of canonical TGF β signaling proteins, SMAD2 and SMAD3, peak at 1 h in PLC/PRF/5 cells [28]. The substantial time lag between TGF β treatment and AKT phosphorylation suggests that TGF β may influence AKT activity through regulation of other signaling molecules. Given that PDGF β R is a known TGF β regulated gene that is functionally upstream of AKT [29,30], we measured the expression levels of PDGF β R, as well as several other RTKs currently being targeted for HCC therapy in clinical trials. We observed that TGF β treatment led to an increase in the mRNA expression of PDGF β R, FGFR1, EGFR, and IGF1R, but not c-MET in PLC/PRF/5 cells (Figure 3A), Hep3B cells (Figure S2), and Huh7 cells (see below, Figure 8A). While both PDGF β R and FGFR1 proteins were undetectable, even following TGF β treatment (data not shown), EGFR and IGF1R proteins were upregulated by TGF β (Figure 3B). Next, we performed chromatin immunoprecipitation (ChIP) assay to determine SMAD2/3 association with the promoter regions of the RTKs. Our data showed that TGF β treatment significantly increased SMAD2/3 binding to the promoter regions of FGFR1 (Figure 4A), PDGF β R (Figure 4B), and IGF1R (Figure 4C), suggesting SMAD2/3-mediated transcription for induction of these RTKs. While TGF β treatment slightly increased SMAD2/3 association with the EGFR and c-MET promoters, the effect was not statistically significant.

We next analyzed HCC patient RNA-SeqV2 datasets available from The Cancer Genome Atlas (TCGA; <http://cancergenome.nih.gov/>). Notably, of the RTKs upregulated by TGF β , FGFR1, IGF1R, and PDGF β R are positively correlated with TGF β 1 in patient tumors (Figure 5A–E). These findings provide important clinical evidence in support of the interaction between TGF β and receptor tyrosine kinases pathways in human HCC.

TGF β Treatment Promotes HCC Cell Survival by Sensitizing Cell Response to IGF1R Ligands

Because FGFR1 and PDGFR protein levels were undetectable in PLC/PRF/5 cells, and EGFR did not correlate with TGF β in patient samples, we next focused on the IGF signaling pathway in the subsequent experiments. We observed that TGF β treatment increased IGF1R protein levels in a time dependent manner (Figure 6A, B). To determine whether the cells with elevated IGF1R levels show enhanced sensitivity to the IGF1R ligands (IGF1 and IGF2), we further treated TGF β -incubated cells with IGF1 or IGF2. Specifically, the cells with or without TGF β treatment for different time periods were incubated with IGF1 or IGF2 for an additional 30 min and AKT phosphorylation was measured. We observed that TGF β treatment sensitized the cells to IGF1 and IGF2-induced AKT phosphorylation (Figure 6A–D). The enhanced sensitivity followed the upregulation of IGF1R protein and was dependent on IGF1 or IGF2 as evidenced by lack of IGF1R phosphorylation following 48 h TGF β treatment alone. Interestingly, the most dramatic increase in IGF1R protein levels and IGF sensitivity took place between the 24 and 48 h time points following TGF β treatment, which is concurrent with the time points that TGF β switched from a proapoptotic to a prosurvival signaling molecule (Figure 1A–C). To determine whether IGF signaling was essential for the antiapoptotic effect of TGF β , we stably knocked down IGF1R using a shRNA construct (Figure 7A, C). As expected, the cells with shRNA knockdown of IGF1R were no longer responsive to IGF (Figure 7C, D). This was further evidenced by an approximately fivefold increase in the expression of phosphoenol pyruvate carboxykinase (PEPCK; Figure 7B), a gene normally repressed by IGF signaling [31]. Accordingly, we observed that treatment of IGF1R knockdown cells with TGF β led to apoptosis (Figure 7E). These findings suggest that IGF1R is necessary for the TGF β -mediated prosurvival effect.

TGF β Primes Huh7 Cells to Various RTK Activating Stimuli

Due to undetectable levels of PDGFR and FGFR1 in PLC/PRF/5 cells, these cells provided a simplistic system to study the consequences of TGF β -mediated sensitization to one family of RTK ligands (IGF1/2). However, HCCs are characteristically heterogeneous and the array of overexpressed RTKs can differ greatly from patient to patient. To further determine whether sensitization of RTKs by TGF β may be a generalized mechanism in HCC cells, we utilized a different HCC cell line, Huh7. Huh7 cells express threefold higher basal PDGFR and sevenfold higher basal FGFR1 as compared to PLC/PRF/5 cells (data not shown), along with comparable IGF1R and EGFR. We observed that treatment of Huh7 cells with TGF β for 48h significantly increased IGF1R, EGFR, PDGFR, and FGFR1 mRNA levels (Figure 8A). In these cells, the elevation of these RTK levels sensitized the cells to IGF, PDGFR, and FGF1, as reflected by enhanced AKT phosphorylation (Figure 8B). Taken together, our findings suggest that TGF β can upregulate the expression of multiple RTKs, leading to enhanced AKT activation by cognate ligands in HCC cells (illustrated in Figure 8C).

DISCUSSION

While TGF β has been shown to prevent early stage tumorigenesis, following tumor initiation, TGF β can act in many ways to promote tumor progression. As an example, TGF β has been extensively studied for its role in the epithelial mesenchymal transition [32], its

impact on cancer stemness and tumor initiating cells [33], and its subsequent promotion of metastasis [34]. TGF β has also been implicated in promoting sorafenib resistance through CD44 as a mesenchymal-like phenotype [35]. In the current study, we present a novel mechanism by which TGF β contributes to hepatocellular cancer progression by priming cells to survival signals, allowing cells to evade apoptosis, even in the presence of the chemotherapeutic agent, sorafenib. Our gene expression profile analysis shows a strong association between TGF β 1 and several key RTKs, including IGF1R, PDGF β R, and FGFR1, in human liver tumor tissue. Given the progressive activation of the TGF β signaling pathway during the hepatocarcinogenic process, the abundance and diversity of RTKs induced by TGF β may represent an important mechanism by which HCC tumors progress and circumvent sorafenib induced cell death.

During the multistage carcinogenic processes, upregulation of RTKs provide tumor cells the ability to survive and proliferate in various microenvironments rich in RTK ligands [36]. While inhibiting specific RTKs has achieved therapeutic efficacy against a number of human cancers, such a strategy has been largely disappointing for HCC. To date, several HCC clinical trials targeting RTKs, such as those targeting IGF1R [37], EGFR [38], or FGFR [39] have led to unsatisfactory results, leaving sorafenib as the only FDA approved systemic therapy for HCC patients. However, many HCC patients develop resistance to sorafenib, and the exact mechanisms for tumor cell resistance to this multikinase inhibitor are not fully understood. In the present study, we provide novel evidence that TGF β induces the expression of several RTKs, in turn priming HCC cells to respond to a variety of different RTK ligands, a mechanism that may play an important role in the development of sorafenib resistance.

Sorafenib resistance in HCC has been attributed to overactive IGF1R [40] and EGFR [41] signaling pathways. In our system, we show that expression levels of IGF1R and EGFR, as well as PDGF β R and FGFR1, are upregulated by TGF β . Our findings suggest a potential role for TGF β -regulated RTKs in HCC resistance to sorafenib therapy. The implication of our findings are further corroborated by the observations that the levels of TGF β are increased in HCC patients [42] and that even higher levels of TGF β are found in patients that do not respond to sorafenib treatment [43]. A recent study has shown a synergistic anti-tumor effect between sorafenib and LY2157299 in an ex vivo model of HCC [44], although the mechanism for such synergism was not clearly defined. Preclinical studies of the effect of another TGF β signaling pathway inhibitor has demonstrated efficacy in reducing HCC tumor burden by inhibiting tumor cell-stroma cross-talk [45]. In the current study we show that blocking TGF β signaling by LY2157299 prior to sorafenib treatment was able to effectively prevent AKT activation and induce apoptosis, in vitro. Our findings provide important mechanistic explanation and justify targeting the TGF β signaling pathway in conjunction with other anti-tumor therapies (such as sorafenib and RTK inhibitors) for more effective treatment of human HCC.

In PLC/PRF/5 cells, where PDGFBR and FGFR1 were undetectable at the protein level, IGF1R was necessary for the TGF β mediated effect on sorafenib resistance. However, consistent with the functional redundancy in RTK signaling pathways [46], a significant clinical problem for RTK-targeted therapies is that inhibition of only one receptor often

leads to the upregulation of another similar RTK. This aspect is highlighted by a recent study demonstrated that inhibition of cell proliferation by targeting one particular RTK in a panel of 41 cell lines could be reversed by the activation of a separate RTK [47]. Our experimental findings in the current study suggest that TGF β -mediated induction of multiple RTKs may allow tumor cells to escape therapies that target individual RTKs.

In summary, the current study reveals an important interplay between TGF β and RTK signaling pathways which are crucial for hepatocellular cancer cell survival and resistance to therapy. Our findings provide important molecular basis for further clinical studies to assess the efficacy of targeting the TGF β signaling pathway to overcome sorafenib resistance and/or to develop new effective combination therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The effect of TGFβ on cell survival. PLC/PRF/5 cells were treated with TGFβ1 and/or sorafenib as indicated. (A–C) The cells were treated with 5 ng/mL TGFβ1 for up to 48 h, followed by 2 h of 5 μM sorafenib or vehicle, and lysates were obtained for immunoblotting (A) and quantified using ImageJ software (B, C). (D) Immunoblot of cells treated with 0, 0.25, 0.5, 1, 2.5, or 5 ng/mL TGFβ for 48 h then with 5 μM sorafenib or vehicle for 2 h. (E) Crystal violet stain of cells seeded at 2×10^5 cells per 3.5 cm well, treated with 5 ng/mL TGFβ for 48 h, then 5 μM sorafenib for 24 h.

Figure 2. TGFβR1 kinase inhibitor, LY2157299, enhances sorafenib-induced apoptosis in PLC/PRF/5 cells. (A) The cells were lysed and immunoblotted after incubation with either 5 μM LY2157299 or vehicle for 1 h, followed by 5 ng/mL TGFβ or vehicle for 1 h. (B) The cells were lysed and immunoblotted following treatment for 48 h with 5 μM LY2157299, vehicle, 5 ng/mL TGFβ or both LY2157299 and TGFβ for 48 h then with either 5 μM sorafenib or vehicle for 2 h.

Figure 3.

TGFβ increases the expression of several receptor tyrosine kinases. (A) mRNA expression in PLC/PRF/5 cells treated with 0–5 ng/mL TGFβ for 48 h. Data are presented as mean±SEM, $n=3$ biological replicates. (B) Protein levels of PLC/PRF/5 cells treated with 5 ng/mL TGFβ for 48 h.

Figure 4.

TGFβ treatment induces SMAD2/3 binding to PDGFβR, FGFR1, and IGF1R promoter regions. PLC/PRF/5 cells treated with or without 5 ng/mL TGFβ for 1 h were fixed and subjected to ChIP assay using a SMAD2/3 antibody (with rabbit IgG as control). The promoter regions of PDGFβR (A), FGFR1 (B), IGF1R (C), EGFR (D), and c-MET (E) were amplified and analyzed by qRT-PCR. Results were plotted as a percentage of input DNA from either control or TGFβ treated cells. Data are presented as mean±SEM. *N*=3 biological replicates, **P*<0.05; ***P*<0.01 by Student's *t*-test (compared to untreated cells).

Figure 5.

Analysis of TCGA datasets reveal that TGFβ expression levels correlate with RTKs in human HCCs. RNASeqV2 mRNA expression data from tumors of HCC patients were extracted from The Cancer Genome Atlas (<http://cancergenome.nih.gov/>). Each data point represents a single patient tumor, with TGFβ1 and the listed RTK expression level represented on the y- and x-axis, respectively. Correlation analysis using the R software package was performed to test for association between paired samples. Pearson and Spearman correlation coefficients, and *P*-values are listed for each plot. Statistically significant ($P < 0.05$) correlation was found between TGFβ1 and FGFR1 (A), PDGFRβ (B), and IGF1R (C), but not between TGFβ1 and EGFR (D), or c-MET (E) mRNA expression levels.

Figure 6.

TGFβ increases cell sensitivity to IGF ligands by increasing IGF1R expression. PLC/PRF/5 cells were treated for up to 72 h with 5 ng/mL TGFβ, then followed with either control medium or 20 ng/mL IGF1 (A) or IGF2 (B) for 30 min. The cells were then lysed and the cellular proteins were subjected to immunoblotting. ImageJ densitometry analysis was performed to determine pAKT band density (normalized to total AKT).

Figure 7.

IGF1R is necessary for TGF β -mediated prevention of apoptosis. (A, B) mRNA was quantified from PLC/PRF/5 shScrambled and shIGF1R stable cell lines. Data are presented as mean $\pm$ SEM, with $N=3$ biological replicates, *** $P<0.001$, Student's t -test (compared to shScrambled). (C, D) Immunoblot and densitometry of lysates from shScrambled and shIGF1R PLC/PRF/5 cells treated with 0–100 ng/mL of IGF2 for 30 min. (E) Immunoblot of PLC/PRF/5 cell lysates after the cells were treated with 5 ng/mL TGF β or control medium for 48 h.

Figure 8.

TGFβ increases cell sensitivity to multiple growth factors in Huh7 cells. (A) mRNA expression of Huh7 cells treated with 5 ng/mL TGFβ for 48 h. Data are presented as mean ±SEM, N=3 biological replicates. (B) Immunoblot of Huh7 cell lysates after cells were treated with 5 ng/mL TGFβ for 48 h, then 20 ng/mL of IGF, EGF, PDGFβ, or FGF1 for 30 min. (C) Proposed model of TGFβ/RTKs/Akt signaling pathways for sorafenib resistance in HCC.

Table I

Sequences of Oligonucleotides Used for qRT-PCR and ChIP PCR

qRT-PCR	Sequence
PDGFbR FW	5' -TTCTCAGGCCACGATGAAAG-3'
PDGFbR REV	5' -GATCTTCAGCTCCGACATAAGG-3'
EGFR FW	5' -CAAGGAAGCCAAGCCAAATG-3'
EGFR REV	5' -CCGTGGTCATGCTCCAATAA-3'
c-MET FW	5' -CCACGGGACAACACAATACA-3'
c-MET REV	5' -TAAAGTGCCACCAGCCATAG-3'
FGFR1 FW	5' -CTTCCTGTCCAACTCCATCC-3'
FGFR1 REV	5' -CAGCAGGTGGCAGAAGTAAA-3'
IGF1R FW	5' -TCTCTCTGGGAATGGGTCGT-3'
IGF1R REV	5' -CCTCCCACGATCAACAGGAC-3'
PEPCK FW	5' -TAGCACCTCATCTGGGAATA-3'
PEPCK REV	5' -GTCTTTGTGGGAAGGTCTATGG-3'
ChIP	
PDGFbR FW	5' -GGAAAGGAGAGCCAGAGAATG-3'
PDGFbR REV	5' -TCCCAAAGTGCTGGGATTAC-3'
EGFR FW	5' -GGTGGACTTGCCAAAGGAATA-3'
EGFR REV	5' -AGGAGAAATGCCAGGGAAAC-3'
c-MET FW	5' -CAGGCCGCGTTGTTTATTAG-3'
c-MET REV	5' -GGAGAGTCACATAGCTGGTTAG-3'
FGFR1 FW	5' -CTCAACTCCCACTGCTTACTC-3'
FGFR1 REV	5' -GGAAGTGGACCCTGTAAATTCT-3'
IGF1R FW	5' -CTGGTCTCCGCAGCATTAT-3'
IGF1R REV	5' -CTAAGTCTCCGCGTTGTTT-3'