

Mitogenic Insulin Receptor-A Is Overexpressed in Human Hepatocellular Carcinoma due to EGFR-Mediated Dysregulation of RNA Splicing Factors

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Abstract

Insulin receptor (IR) exists as two isoforms resulting from the alternative splicing of IR pre-mRNA. IR-B promotes the metabolic effects of insulin, whereas IR-A rather signals proliferative effects. IR-B is predominantly expressed in the adult liver. Here, we show that the alternative splicing of IR pre-mRNA is dysregulated in a panel of 85 human hepatocellular carcinoma (HCC) while being normal in adjacent nontumor liver tissue. An IR-B to IR-A switch is frequently observed in HCC tumors regardless of tumor etiology. Using pharmacologic and siRNA approaches, we show that the autocrine or paracrine activation of the EGF receptor (EGFR)/mitogen-activated protein/extracellular signal-regulated kinase pathway increases the IR-A:IR-B ratio in HCC cell lines, but not in normal hepatocytes, by upregulating the expression of the splicing factors CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF. In HCC tumors, there is a significant correlation between the expression of IR-A and that of splicing factors. Dysregulation of IR pre-mRNA splicing was confirmed in a chemically induced model of HCC in rat but not in regenerating livers after partial hepatectomy. This study identifies a mechanism responsible for the generation of mitogenic IR-A and provides a novel interplay between IR and EGFR pathways in HCC. Increased expression of IR-A during neoplastic transformation of hepatocytes could mediate some of the adverse effects of hyperinsulinemia on HCC. *Cancer Res*; 73(13); 3974–86. ©2013 AACR.

Introduction

Recent epidemiologic data showing that hyperinsulinemia is a risk factor for a variety of malignancies has renewed the interest for studies examining insulin receptor (IR) signaling in cancer development (1–3). Dysregulations of IR expression and/or downstream signaling have been reported in cancers (4, 5). Hepatocellular carcinoma (HCC), which is the most common primary liver tumor, is no exception. Indeed, elevated insulin levels in serum have been shown to be predictive of HCC recurrence in hepatitis C virus (HCV) and hepatitis B virus (HBV) carriers (6, 7). Furthermore, the use of exogenous insulin or of insulin secretagogues might increase the risk of HCC

among diabetics (8, 9). IR expression was found to be slightly higher in a small collection of HCC relative to the adjacent nontumor liver tissue (10). Moreover, aberrant expressions of IRS-1 and IRS-2, which are IR cytosolic substrates occur in HCC (11, 12).

IR is a heterotetrameric $\alpha_2\beta_2$ receptor with structural and functional homologies with insulin-like growth factor type I receptor (IGF-IR). IR is expressed as 2 isoforms, IR-A and IR-B, which originate from the alternative splicing of exon 11 in the IR pre-mRNA (IR-A^{-Ex11}; IR-B^{+Ex11}) and differ from 12 amino acids located at the carboxyl terminus of the extracellular α -subunit. Exon 11 skipping is developmentally regulated and IR-A is predominantly expressed in embryo and fetal tissues (13). This isoform becomes less expressed in adult differentiated tissues especially in target tissues of insulin, such as the liver, which expresses nearly exclusively IR-B. The structural feature of IR-A isoform confers specific functional properties in terms of ligand affinity. Indeed, IR-A is a high-affinity receptor not only for insulin but also for IGF-II, a fetal growth peptide produced by the liver, which is overexpressed in HCC tumors and cell lines (14). IR-A also displays a faster internalization and recycling kinetics and a higher propensity to signal proliferation and survival compared with IR-B (15). IR-A is overexpressed in tumors from different tissue origin including breast, colon, and lung (4) and in a variety of cancer cell lines including the hepatoblastoma-derived HepG2 cell line (16).

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The recognition of exons and introns during pre-mRNA splicing relies on regulatory sequence elements located within exons and flanking introns that are recognized by splicing factors. Oncogenic signals involving receptor tyrosine kinases (RTK), Ras, phosphoinositide 3-kinase (PI3K), AKT, and *c-jun*-NH2-kinases (JNK) may promote aberrant mRNA splicing in cancer cells by regulating the expression, the localization, and/or the phosphorylation of splicing factors (17, 18). Using IR minigenes transiently transfected in myoblasts, HepG2, or HEK293 cells, splicing factors belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP), SR, and CELF/bruno-like families have been identified as regulators of IR pre-mRNA exon 11 inclusion (16, 19–21). The contribution of these splicing factors in the regulation of endogenous IR pre-mRNA splicing has not been yet explored in cancer cells. In addition, the signal pathways and molecular mechanisms that regulate the function of these splicing factors are currently unknown in the context of IR-A alternative splicing.

In the present study, we have examined the status of IR-A in a large panel of human HCC tumors. The observation that IR-A is markedly upregulated to the detriment of IR-B in malignant lesions but not in adjacent nontumor liver tissues led us to investigate the underlying mechanism. We identified a new mechanism by which activation of the EGF receptor (EGFR) pathway triggers aberrant IR alternative splicing and subsequent IR-A overexpression in HCC cells through the upregulation of the splicing factors CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF. Dysregulation of IR pre-mRNA splicing was confirmed in chemically induced HCC in rat but not in regenerating livers after partial hepatectomy.

Materials and Methods

Patients and liver tissue specimens

Two independent collections of HCC tumors and adjacent nontumoral liver tissues were used in this study. Samples were collected from patients with HCC undergoing curative liver resection or liver transplantation at the Saint-Antoine Hospital (tissue collection #1, $n = 42$; tissue collection #2, $n = 43$) in accordance with the French laws and regulations. Patient characteristics are recapitulated in Supplementary Table S1. Histologically normal liver tissue (control liver samples) was obtained from 11 patients undergoing partial hepatectomy for the treatment of metastases or benign tumor. Tissue samples were flash-frozen in liquid nitrogen and stored at -80°C until analyses.

Cell culture and treatments

HepG2, Hep3B, and HuH7 cells were obtained from the American Type Culture Collection. HuH6 and peritoneal lymphocytes (PLC)/PRF5 cells were provided by Dr. Christine Perret (Institut Cochin, Paris, France) and were characterized for β -catenin^{G34V} (HuH6), axin-1 ^{Δ Ex4} (PLC/PRF5) and p53^{R249S} (PLC/PRF5) mutations. Cell lines were cultured as previously reported (22). Primary cultures of human hepatocytes were established as previously described (23). Cells were incubated with pharmacologic inhibitors described in Supplementary Table S2.

Liver carcinogenesis and regeneration in rats

Liver carcinogenesis was induced in rats by intraperitoneal injection of diethylnitrosamine (DEN) according to previously established protocol (24). Liver regeneration was induced in rats by two-third liver hepatectomy using a conventional procedure (25). Animals were sacrificed one, 3, or 10 days after hepatectomy. Animals were maintained in accordance with the French guidelines for the humane treatment and care of laboratory animals.

DNA synthesis

Cells were incubated for 17 hours with or without 10 nmol/L insulin or IGF-II and [methyl-³H] thymidine (1 $\mu\text{Ci}/\text{well}$) was added for the last 3 hours of the incubation period. The amount of radioactivity incorporated into DNA was determined as previously described (26).

Real-time cell impedance analysis

Proliferation was monitored using xCELLigence System (Roche Applied Science), which measures in real-time electrical impedance and provides quantitative information on cell adherence. Cells were incubated in 0.3% FBS overnight and 10 nmol/L insulin or IGF-II was added. Cell Index slope (a parameter calculated as a relative change in measured electrical impedance) was measured using the RTCA Software 1.2.

Western blotting, antibody array, and ELISA

Antibodies are described in Supplementary Table S3. Blot quantifications were conducted by scanning densitometry using ImageJ software (NIH Image). To evaluate splicing factor phosphorylation, cell lysates were incubated with alkaline phosphatase (1 U/ μg protein) for 30 minutes at 37°C and analyzed by Western blot analysis on 4% to 12% polyacrylamide gradient gels. The phosphorylation status of 42 RTKs was determined by Proteome Profiler array (R&D Systems). Total amounts of IR were quantified in liver tissue extracts by ELISA (R&D Systems).

RNA interference

The expression of specific mRNA was downregulated using a mixture of 4 siRNAs (ON-TARGETplus SMARTpool; Dharmacon). Control siRNA was a pool from Dharmacon (siGENOME nontargeting siRNA pool). Cells were cultured for 24 hours to 40% confluency, transfected with 100 nmol/L siRNA using Lipofectamine 2000 (Life Technologies SAS) for 5 hours, and harvested 72 hours later for mRNA and protein analyses.

Transient plasmid transfections

Cells were transfected with 2.5 μg of a plasmid coding for a dominant-negative RAF-1 mutant (RAF-C4; ref. 27) or of a control empty plasmid together with 0.5 μg of p β gal-Control (Clontech Laboratories, Inc.) by using Lipofectamine 2000.

RNA isolation and analysis of gene expression

Total RNA was extracted from cell cultures using RNeasy Mini Kits (Qiagen). For tissues, a preliminary RNA extraction step was conducted using TRIzol Reagent (Life Technologies). Quantitative measurements of transcripts were conducted by

real-time PCR (RT-PCR) on a LightCycler 480 instrument (Roche) using SYBR Green chemistry and specific primers (Supplementary Table S4). For each sample, gene expression was normalized to that of hypoxanthine phosphoribosyltransferase mRNA content and was expressed relatively to the same calibrator. The relative quantity of each target gene was determined from replicate samples using the formula $2^{-\Delta\Delta C_t}$. Qualitative evaluation of IR isoform expression was conducted by RT-PCR analysis using primers for the flanking exons 10 and 12 (Supplementary Table S4) and analyzed on 2% agarose gels.

Immunofluorescence analysis

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde, blocked with 1% bovine serum albumin and 10% FBS in PBS followed by overnight incubation with a 1:50 dilution of the primary antibody in PBS at 4°C. Cells were then incubated with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse or mouse anti-goat antibody in PBS for 1 hour at room temperature. The slides were counterstained with 4',6-diamidino-2-phenylindole for nuclei detection. Fluorescence was visualized using an immunofluorescence microscope (Leica Microsystems) with a DFC300 FX digital camera.

Statistical analysis

Results are given as mean \pm SEM. Data were analyzed using Prism 5.0 software (GraphPad Software Inc.). Expression data obtained from human and rat liver tissue specimens were compared using Mann-Whitney *U* test (unpaired data) and Wilcoxon test (paired data). Correlations between IR-A and splicing factor mRNA expression in human liver tissue samples were conducted using Spearman rank correlation coefficient. Data obtained from *in vitro* experiments in cell lines were compared using the unpaired Student *t* test. Statistical tests were 2-sided. *P* value of less than 0.05 was considered significant.

Results

IR is upregulated and aberrantly spliced in human HCC tumors

We examined the expression level of IR in HCC tumors (T) and paired surrounding nontumor liver tissues (NT) from 42 patients (tissue collection #1) as well as in healthy livers from 11 subjects (Cont). Quantitative real-time PCR (qRT-PCR) experiments were conducted with a pair of primers that amplifies the 2 IR isoform mRNAs (total IR). Total IR mRNA expression was similar between nontumor liver tissues and healthy livers, whereas it was significantly higher in HCC tumors compared with matched nontumor tissues (Fig. 1A). Similar data were obtained using an independent collection of 43 paired T/NT samples (tissue collection #2; Supplementary Fig. S1A). In paired T/NT samples available for protein analysis ($n = 27$, collection #1), Western blotting (Fig. 1B) and ELISA (Fig. 1C) confirmed IR overexpression at the protein level in HCC tumors.

Then, we analyzed the expression of IR-B (+Ex11) and IR-A (−Ex11) isoforms by PCR only because there are no discrim-

inative antibodies. Standard PCR experiments using primers for the flanking exons 10 and 12 combined with agarose gel electrophoresis showed that IR-A mRNA was detected in tumors but not in adjacent nontumor tissues, whereas IR-B mRNA expression was decreased in tumors in comparison with adjacent nontumor tissues (Fig. 1D). These findings were supported by quantitative PCR (qPCR) using primer pairs specific for each isoform. As shown in Fig. 1E (tissue collection #1) and in Supplementary Fig. S1B (tissue collection #2), IR-A mRNA was expressed at significantly higher levels in HCC tumors than in nontumoral surrounding livers and healthy liver tissues. The relative expression ratio of IR-A to IR-B (IR-A:IR-B) was also significantly higher in tumors compared with adjacent nontumor tissues, indicating increased alternative splicing of IR mRNA (Fig. 1F and Supplementary Fig. S1C). Supplementary Fig. S1D recapitulates the fold inductions of total IR, IR-B, IR-A, and IR-A:IR-B ratio in each of the 85 HCC samples compared with the matched adjacent liver tissue. In tissue collection #1, IR-A mRNA transcripts and IR-A:IR-B ratio were increased by 5.21 ± 0.68 and 3.89 ± 0.70 folds in HCC specimens, respectively, whereas IR-B and total IR mRNA were increased by only 2.02 ± 0.20 and 2.00 ± 0.27 folds, respectively. Tumor induction of IR-A splicing was observed regardless of the etiology of the liver disease and the presence or absence of significant fibrosis/cirrhosis in the peritumoral liver (Supplementary Fig. S1E).

HCC cell lines express the IR-A isoform and proliferate in response to insulin and IGF-II

We tested 5 human HCC cell lines, all of which overexpressed IR in comparison with normal primary human hepatocytes (Fig. 2A). Phospho-RTK array analysis revealed that after treatment with 10% FBS, IR was, together with IGF-IR and EGFR, the most intensively phosphorylated RTK in HCC cells (Fig. 2B). Insulin (Fig. 2C) and IGF-II (Fig. 2D) promoted proliferation in HCC cells but not in hepatocytes as evaluated by the incorporation of [³H]thymidine into DNA and by real-time monitoring of cell index based on impedance variations. Standard PCR experiments clearly evidenced the coexpression of the 2 IR isoforms in HCC cell lines, whereas normal hepatocytes predominantly expressed IR-B (Fig. 2E). Quantitative PCR experiments confirmed that the IR-A:IR-B ratio was significantly increased in HCC cell lines, specially in HuH6 and PLC/PRF5 cells (Fig. 2F).

Activation of EGFR promotes IR-A alternative splicing through the MEK pathway in human HCC cell lines but not in normal hepatocytes

The EGFR pathway plays a major role in HCC development and is frequently overactivated in HCC (24, 28). Consistently, EGFR is activated in HCC cell lines (Fig. 2B). With the objective to identify whether this pathway promoted IR alternative splicing in HCC cells, HuH6 and PLC/PRF5 cells were incubated with a panel of specific kinase inhibitors to block EGFR and the downstream signaling molecules mitogen-activated protein/extracellular signal-regulated kinase (MEK), PI3K, AKT, mTOR, JNK, and STAT3. As shown in Fig. 3A and Supplementary Fig. S2A, EGFR and MEK inhibitors significantly

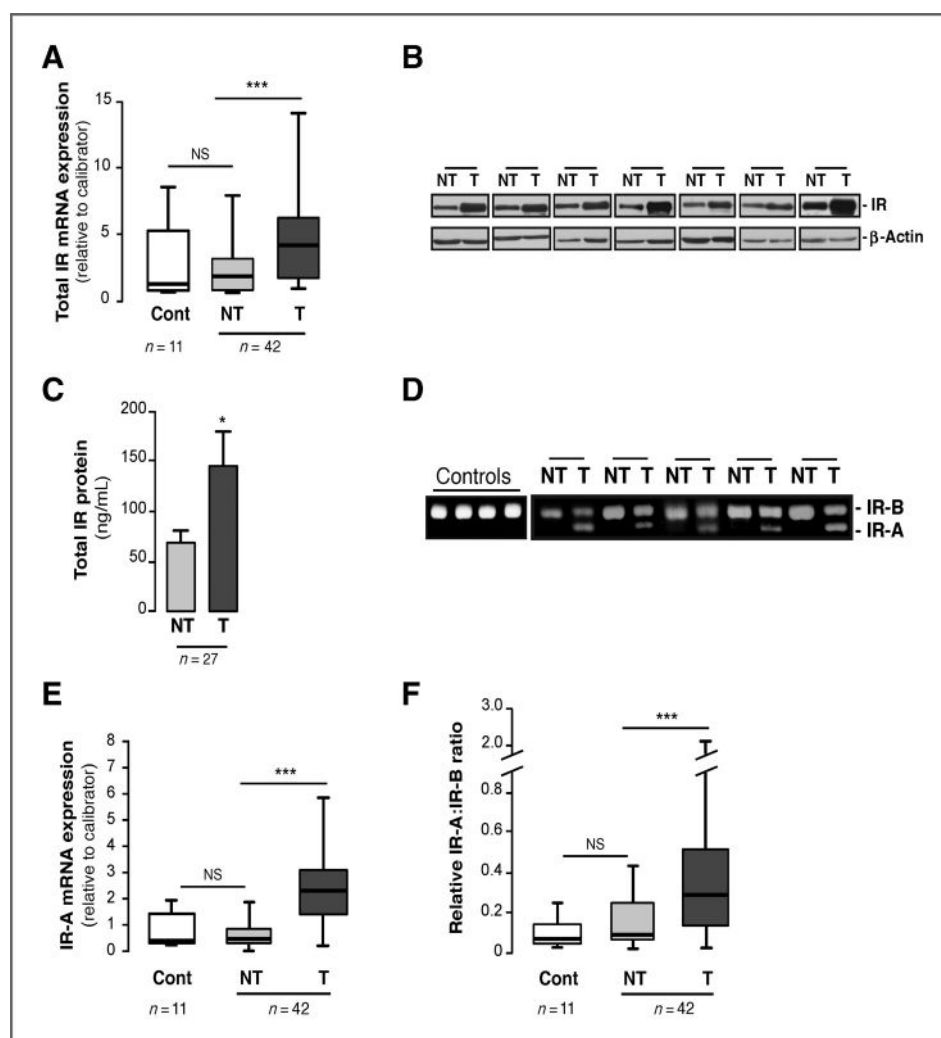


Figure 1. IR status in human HCC tumors. **A**, qPCR analysis of total IR mRNA expression in 42 human paired HCC (T)/nontumor (NT) liver tissue samples (tissue collection #1) and 11 healthy liver tissue samples (Cont). **B**, representative Western blot analysis showing IR overexpression in HCC. **C**, total IR protein levels measured by ELISA in 27 paired T/NT liver tissue samples. **D**, representative semiquantitative PCR analysis showing IR-A induction in HCC. **E**, qPCR evaluation of IR-A mRNA expression in T/NT liver tissue collection. **F**, relative IR-A:IR-B ratio in T/NT liver tissue collection. Statistical analysis: NT vs. Cont, Mann-Whitney test for unpaired values; *P* is not significant (NS); T vs. NT: Wilcoxon test for paired values. *, *P* < 0.05; ***, *P* < 0.001.

decreased the IR-A:IR-B ratio in these cells, without modifying the total levels of IR mRNA (data not shown). The JNK inhibitor induced a significant reduction in the IR-A:IR-B ratio in HuH6 but not in PLC/PRF5 cells. EGFR inhibitor gefitinib induced a time-dependent inhibition of IR-A mRNA splicing (Fig. 3B and Supplementary Fig. S2B). To confirm the role of EGFR activation in the promotion of IR-A expression, the expression of EGFR and of amphiregulin, an EGFR ligand actively secreted by HCC cells (29), was downregulated with specific siRNAs. Both EGFR (Fig. 3C) and amphiregulin (Fig. 3D) siRNAs induced a significant decrease in the IR-A:IR-B ratio in HuH6 cells. Similar results were obtained with PLC/PRF5, Hep3B and HuH7 cells (Supplementary Fig. S2C). The involvement of the MEK pathway in the regulation of IR mRNA splicing was confirmed by conducting a time-dependent kinetics with the MEK inhibitor U0126 (Fig. 3E and Supplementary Fig. S2D) and by transient

overexpression of a plasmid coding for a dominant-negative RAF-1 mutant (RAF-C4) in HuH6 and PLC/PRF5 cells (Fig. 3F). We also tested the effect of exogenously added amphiregulin, EGF, or HB-EGF on IR mRNA splicing. These experiments were conducted in Hep3B and HuH7 cells because the activation of EGFR with an exogenous ligand strongly stimulated the ERK pathway above basal in these cell lines, whereas it was less effective in HuH6 and PLC/PRF5 cells (Supplementary Fig. S3). In the latter 2 cell lines, the basal level of ERK phosphorylation was stimulated mainly through an EGFR-dependent pathway. EGFR ligands induced a significant increase of the IR-A:IR-B ratio in Hep3B and HuH7 cells but not in normal human hepatocytes (Fig. 3G). U0126 abolished the effect of amphiregulin on the IR-A:IR-B ratio (Fig. 3G), indicating that activated EGFR promotes IR-A alternative splicing through MEK activation in HCC cells.

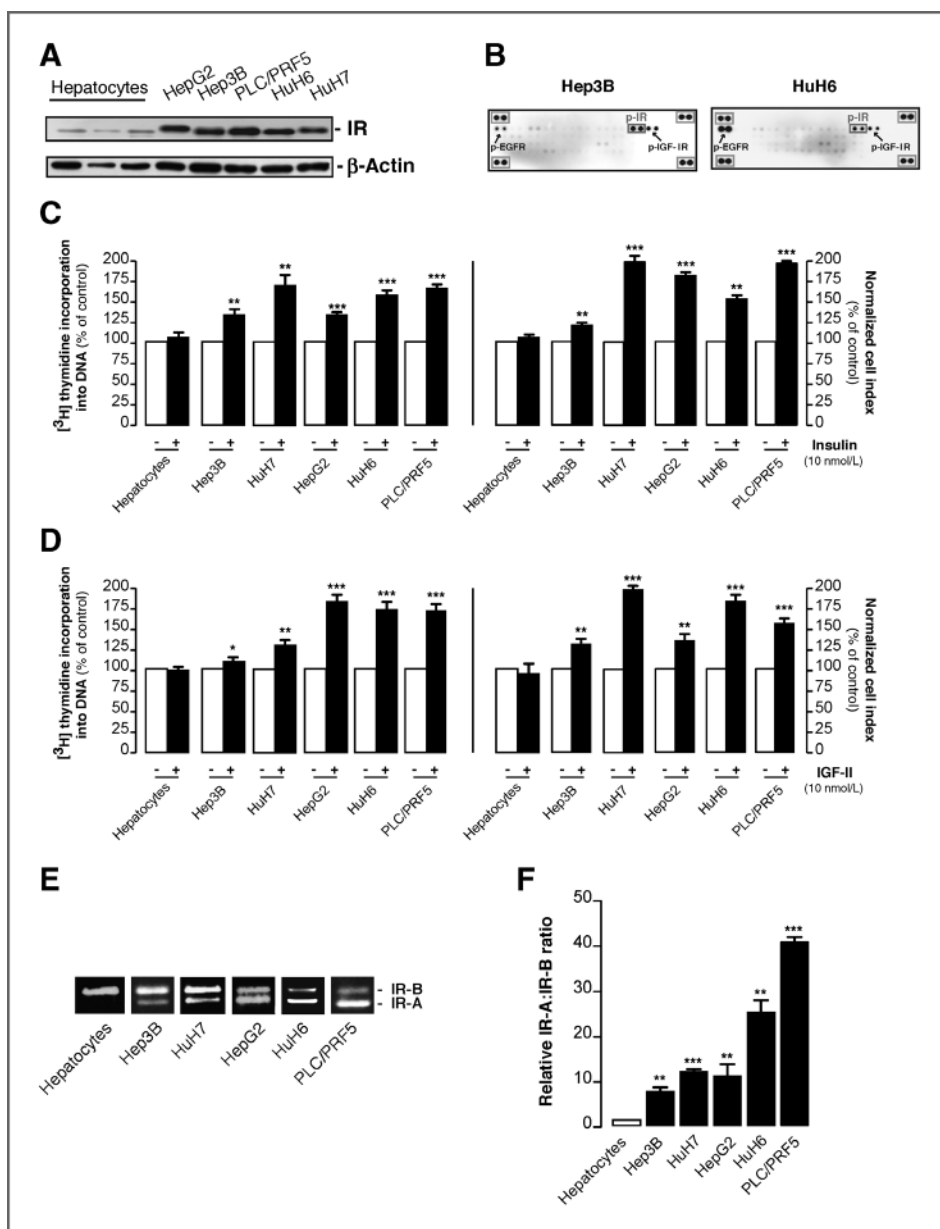


Figure 2. IR status in human HCC cell lines. **A**, representative blot of 3 independent experiments showing IR expression in normal human hepatocytes and HCC cell lines. **B**, RTK profiler array showing IR, IGF-IR, and EGFR tyrosine phosphorylation in Hep3B and HuH6 cells cultured in complete medium for 24 hours. Corner boxes delineate control spots. **C**, proliferation of hepatocytes and HCC cells in response to insulin evaluated by $[^3\text{H}]$ thymidine incorporation into DNA (left) and by real-time impedance measurements (right). **D**, proliferation of hepatocytes and HCC cells in response to IGF-II using the same techniques. **E**, representative image of 3 independent experiments showing IR-A and IR-B mRNA isoforms in normal human hepatocytes and HCC cell lines by semiquantitative PCR and agarose gel electrophoresis. **F**, IR-A:IR-B ratio in HCC cell lines relative to normal hepatocytes. Statistical analysis, $n = 3-6$ in duplicate; Student t test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

EGFR controls the expression of different splicing factors that promote IR-A alternative splicing in HCC cells

To characterize the mechanisms whereby EGFR promoted the emergence of IR-A in HCC cells, we first examined the expression of splicing factors from CELF (CUG-BP1), hnRNP (H, F, and A1 isoforms) and SR (SF2/ASF, SRp20) families that were previously identified as regulators of IR exon 11 splicing using IR minigene transfection systems (16, 19–21). hnRNP2B1 was also investigated as it has been recently shown to be overexpressed in HCC (30). As shown in Fig. 4A, the protein levels of CUGBP1, hnRNPH, hnRNPA1, hnRNP2B1 and to a lesser extent of SF2/ASF were increased in HCC tumors compared with nontumoral liver tissues. hnRNP2B1 protein level remained unchanged and SRp20 was undetectable in these samples (data not shown). Analysis of

transcript expression by qPCR corroborated these findings (Fig. 4B). IR-A mRNA levels in tumors were significantly correlated with those of splicing factors (Supplementary Table S5). With the exception of hnRNP2B1, splicing factors were overexpressed in HCC cell lines compared with normal human hepatocytes (Fig. 4C).

We next used a siRNA strategy to examine the contributory role of splicing factors in IR-A mRNA expression in HCC cells (Fig. 4D). The siRNA against CUGBP1, hnRNPH, hnRNPA1, or hnRNP2B1 induced a significant decrease in the IR-A:IR-B ratio in the 4 HCC cell lines (Figs. 4E and 5A). The knockdown of SF2/ASF and of hnRNP2B1 had no effect on the IR-A:IR-B ratio in the PLC/PRF5 cell line, whereas it has a decreasing effect in the other 3 cell lines (Figs. 4E and 5A). In HuH7 cells, each siRNA inhibited the stimulatory effect of exogenously added

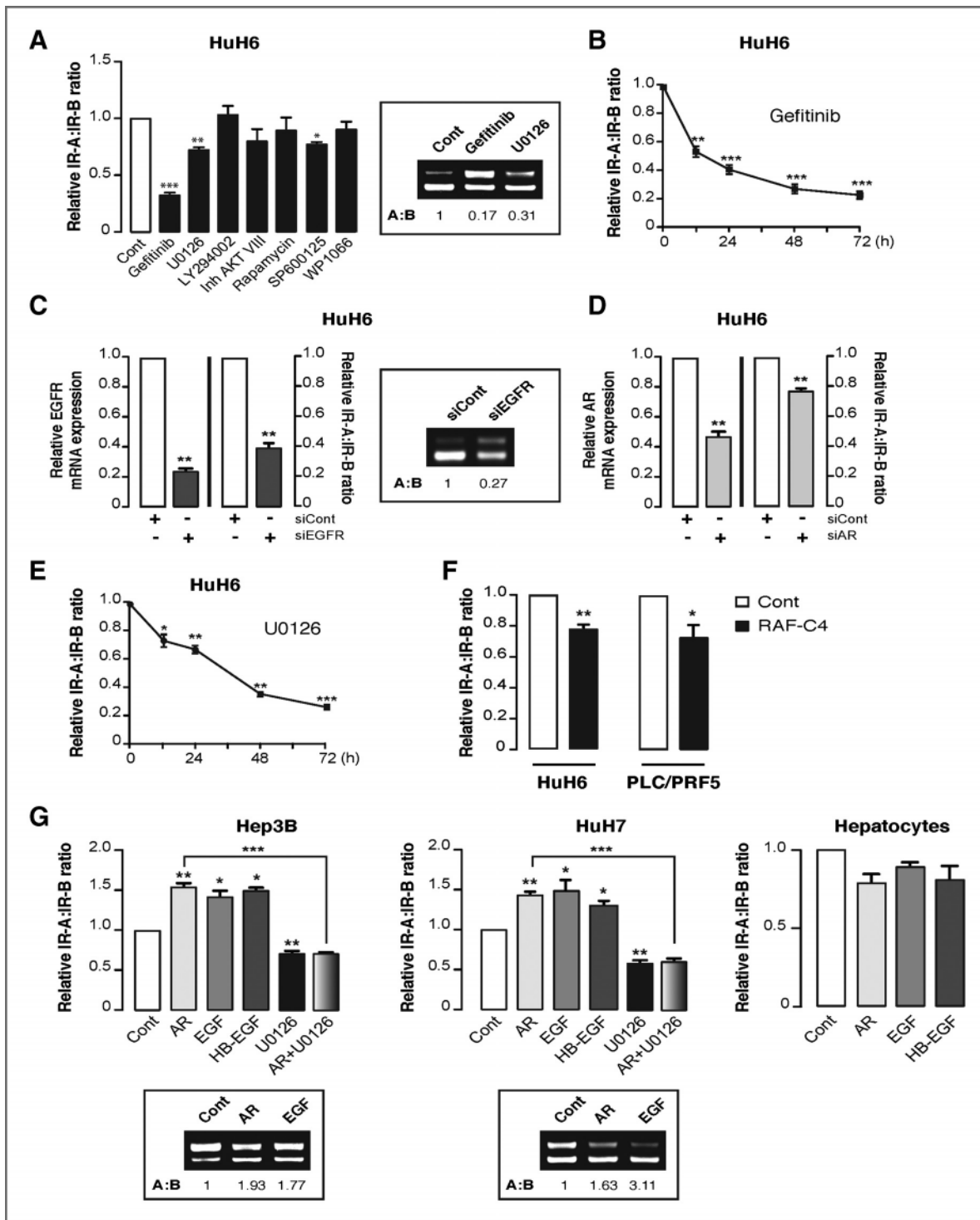
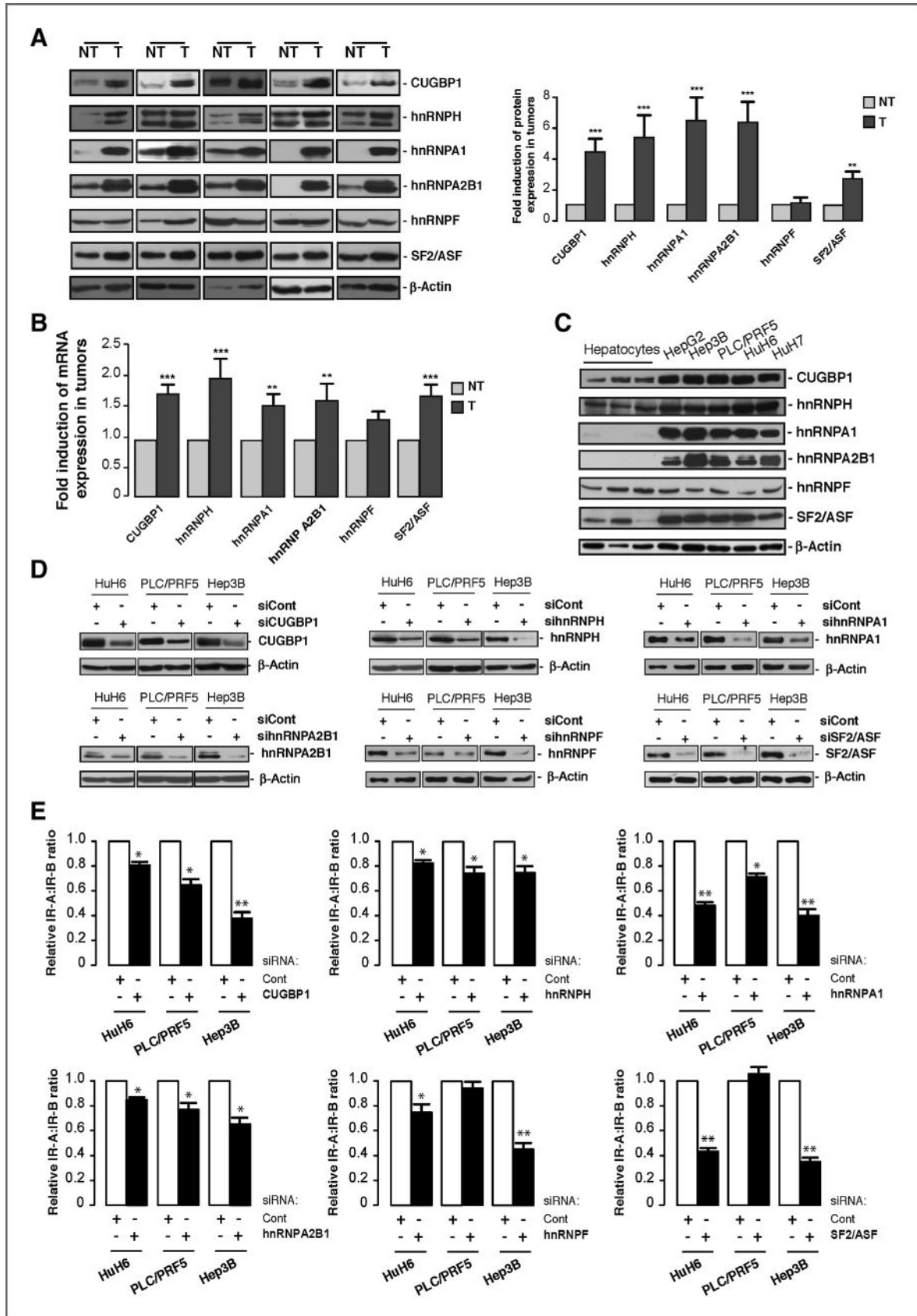


Figure 3. EGFR-dependent signaling increases IR-A mRNA splicing in HCC cells. A, HuH6 cells were treated for 24 hours with inhibitors against EGFR (gefitinib, 2.5 μmol/L), MEK (U0126, 5 μmol/L), PI3K (LY294002, 10 μmol/L), AKT (Inh AKT VIII, 5 μmol/L), mTOR (rapamycin, 1 μmol/L), JNK (SP600125, 5 μmol/L), or STAT3 (WP1066, 1 μmol/L). The relative IR-A:IR-B ratio was evaluated by quantitative (left) and semi-quantitative (right) PCR. B, effect of treatment duration with gefitinib (2.5 μmol/L) on the IR-A:IR-B ratio in HuH6 cells. C, HuH6 cells were transiently transfected with 100 nmol/L of control siRNA (siCont) or EGFR siRNA (siEGFR). After 72 hours, the IR-A:IR-B ratio was analyzed by quantitative (left) and semi-quantitative (right) PCR. D, HuH6 cells were transiently transfected with 100 nmol/L of siCont or amphiregulin siRNA (siAR) and the IR-A:IR-B ratio were analyzed by qPCR. E, effect of treatment duration with U0126 (5 μmol/L) on the IR-A:IR-B ratio in HuH6 cells. F, HuH6 and PLC/PRF5 cells were transiently transfected with 2.5 μg of a plasmid coding a dominant-negative RAF-1 mutant (RAF-C4) or of a control empty plasmid for 72 hours. The IR-A:IR-B ratio was analyzed using qPCR. G, Hep3B, HuH7, and hepatocytes were treated for 24 hours with EGFR ligands (AR, EGF, or HB-EGF at 50 ng/mL) in the presence or absence of the MEK inhibitor U0126 (5 μmol/L) and the IR-A:IR-B ratio was analyzed by quantitative (top) and semi-quantitative (bottom) PCR. Statistical analysis, $n = 3$ in duplicate; Student t test; *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.



EGFR ligands on the IR-A:IR-B ratio (Fig. 5A), indicating that these splicing factors play a contributory role in the promotion of IR-A expression by EGFR.

We next examined whether EGFR signaling could regulate splicing factor function through modifications of expression, subcellular localization, and/or phosphorylation state. The blockade of EGFR signaling with EGFR siRNA or gefitinib decreased CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF at both mRNA (Fig. 5B) and protein (Fig. 5C) levels in HuH6 cells, whereas hnRNPF levels remained unchanged. With the exception of hnRNPA1, similar results were obtained in HuH7 cells (Supplementary Fig. S4A). Reciprocally, in these cells, EGFR ligands increased the expression levels of CUGBP1, hnRNPH, hnRNPA2B1, and SF2/ASF but not of hnRNPA1 and hnRNPF (Supplementary Fig. S4B). Immunofluorescence experiments showed that splicing factors were mainly expressed in the nuclear compartment in HCC cells, which is fully compatible with their function as promoter of mRNA alternative splicing (Supplementary Fig. S5). The blockade of EGFR signaling with gefitinib or U0126 did not affect their subcellular localization. The phosphorylation status of splicing factors was then analyzed by comparing the electrophoretic migration profiles in the presence or absence of alkaline phosphatase. Treatment with alkaline phosphatase alone caused a marked shift in the migration of CUGBP1, hnRNPH, and SF2/ASF but not of hnRNPA1, hnRNPA2B1, and hnRNPF in control cells, indicating that only CUGBP1, hnRNPH, and SF2/ASF were phosphorylated in HCC cells (Fig. 5D). The inactivation of the EGFR pathway with EGFR siRNA or gefitinib did not modify the electrophoretic mobility patterns obtained with or without alkaline phosphatase (Fig. 5D), indicating that EGFR activation did not modify splicing factor phosphorylation in these cells.

IR mRNA splicing is altered during liver carcinogenesis but not during liver regeneration in rat models

Finally, we took advantage of 2 experimental models developed in rats to gain further insights into the relationship between IR-A expression and hepatocellular proliferation under pathologic conditions. First, we investigated the rat model of HCC and cirrhosis induced by weekly intraperitoneal injections of DEN during 16 weeks that we previously established (24). qPCR experiments showed that the IR-A:IR-B ratio was significantly increased in tumors compared with the cirrhotic matched liver tissues, whereas total IR mRNA levels remained unchanged in this model (Fig. 6A). The expression of some splicing factors involved in IR-A splicing was significantly increased in tumors (Fig. 6B). To determine whether IR mRNA splicing was altered during nontumoral hepatocellular proliferation, we analyzed IR-A:IR-B ratio and total IR mRNA levels

in rat liver 1, 3, and 10 days after a two-third hepatectomy. In this model, maximal hepatocyte proliferation occurs 1 day after hepatectomy. No significant change in IR-A:IR-B ratio and total IR mRNA was observed during rat liver regeneration (Fig. 6C). The analysis of splicing factor expression showed that CUGBP1, hnRNPH, and hnRNPA2/B1 levels remained constant during the liver regenerative process, whereas a transient induction of hnRNPA1, hnRNPF, and SF2/ASF levels was observed (Fig. 6D). This might suggest that hnRNPA1, hnRNPF, and SF2/ASF have other targets and/or functions during liver regeneration.

Discussion

In the vast majority of HCC tumors that we tested, IR-A expression was strongly increased as compared with healthy liver or adjacent nontumoral liver tissue, suggesting that IR-A is a hallmark of HCC. Experiments conducted in rats support these conclusions, because the induction of IR-A expression occurred in DEN-induced HCC tumors but not in proliferating hepatocytes from regenerating livers. Increased IR-A splicing was observed in human HCC tumors irrespective of etiology, suggesting that IR-A emergence is a general molecular mechanism of liver carcinogenesis.

Hyperinsulinemia is a major determinant for HCC. Hyperinsulinemia is favored by chronic liver diseases as a result of liver dysfunction and inflammation and decreased hepatic insulin clearance. Elevated insulin levels are predictive of HCC recurrence in HCV and HBV carriers (6, 7). Hyperinsulinemia is also a common feature of type II diabetes and obesity, which are independent risk factors for HCC (31). Recent clinical studies examining the impact of diabetes treatments on HCC incidence show that exogenous insulin and insulin secretagogues tend to increase HCC risk among diabetics (8, 9).

The underlying mechanisms that may account for the development of HCC in the presence of hyperinsulinemia are probably multiple (32). Our findings support the hypothesis that hyperinsulinemia has growth-promoting effects by directly stimulating IR-A activity on liver cancer cells. Because of their structural homology, IR-A may heterodimerize and form hybrids with IR-B and IGF-IR. Heterodimer formation will depend upon the relative expression of each hemireceptor. In accordance with previous studies (33), IGF-IR was expressed in a limited number of HCC tumors (24%). Thus, it is reasonable to assume that HCC tumors express more frequently a mixture of IR-A/IR-A and IR-A/IR-B dimers. Both dimers can bind insulin with high affinity and stimulate cell proliferation and survival (34). Receptor dimers engaging IR-A can also bind the mitogen IGF-II with a high affinity. IGF-II is overexpressed in HCC notably as a result of loss of promoter-specific imprinting and reactivation of fetal promoters (14). Consistently, IGF-II

Figure 4. Splicing factors from CELF, hnRNP, and SR families are overexpressed in HCC cells and favor IR-A mRNA expression. A, representative blots (left) and quantifications (right) showing splicing factor protein levels in HCC (T) and surrounding nontumor tissue (NT) from 27 paired liver specimens (collection #1). B, qPCR analysis of splicing factor mRNA levels in 42 paired T/NT liver tissues (collection #1). C, representative analysis ($n = 2$) of splicing factor protein levels in normal human hepatocytes and HCC cell lines by Western blotting. D, HuH6, PLC/PRF5, and Hep3B cells were transiently transfected with 100 nmol/L of specific siRNA against CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, hnRNPF, or SF2/ASF or with a siCont for 72 hours and protein levels were examined by Western blotting. E, using qPCR, the IR-A:IR-B ratio was analyzed in cells transiently transfected with control siRNA or with siRNA targeting splicing factor mRNA. Statistical analysis, $n = 3$; Student *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

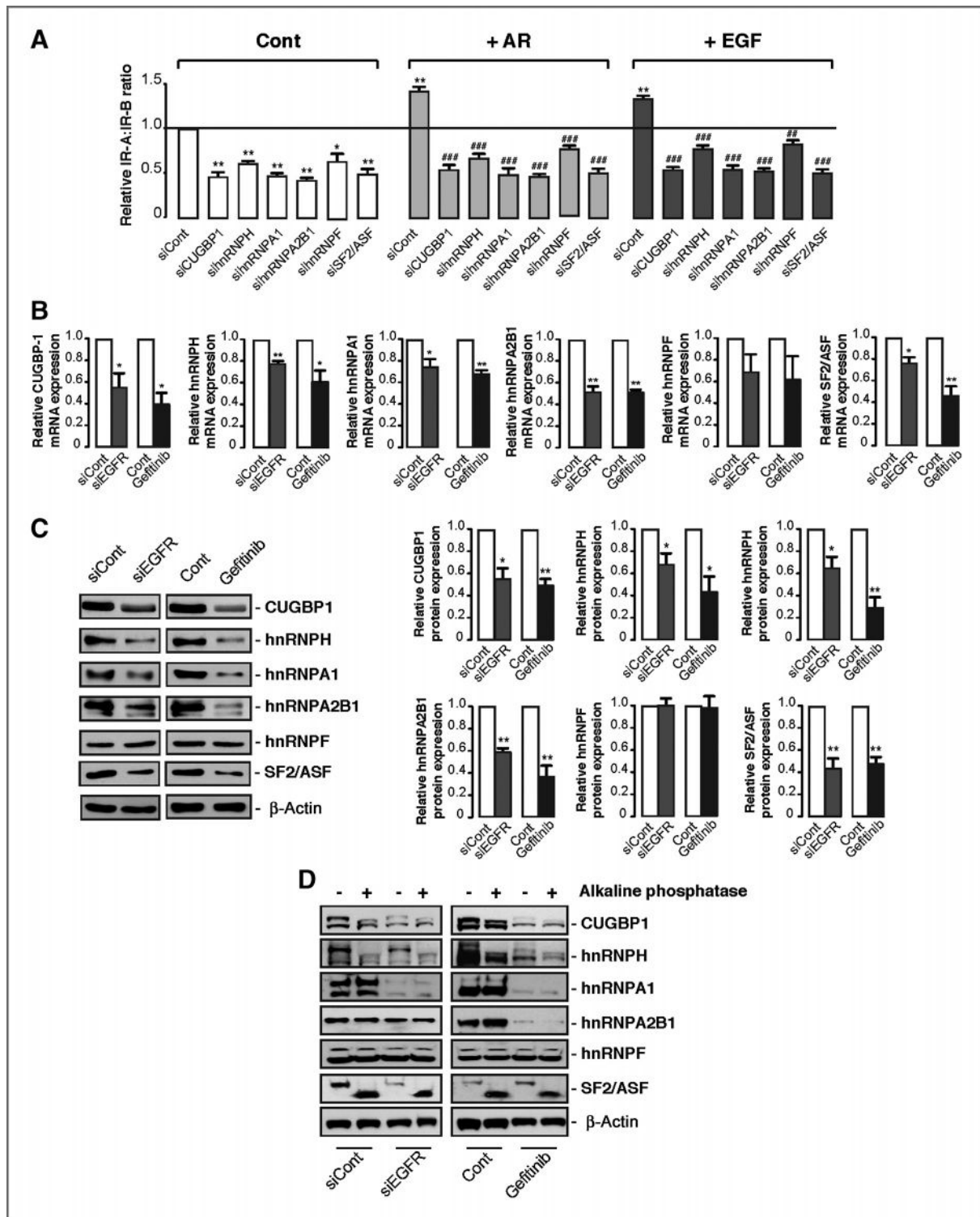
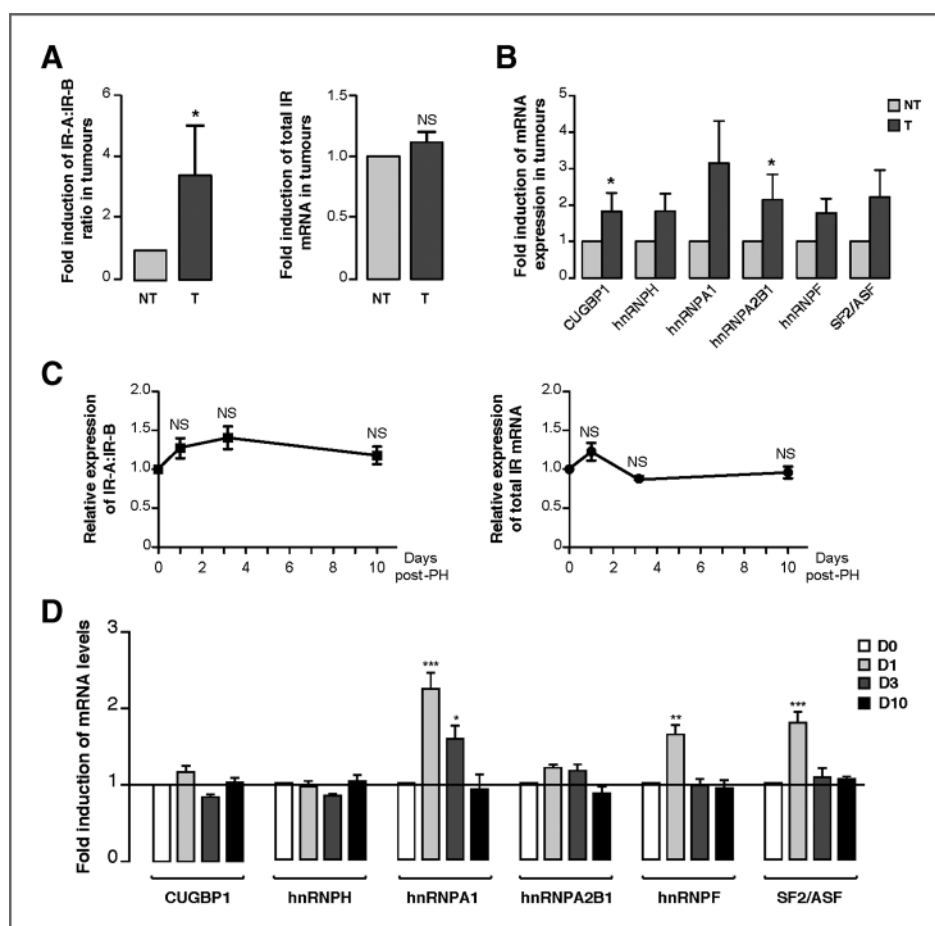


Figure 5. Splicing factors are involved in the stimulatory effect of EGFR ligands on IR-A expression and are under the control of EGFR signaling. **A**, HuH7 cells were transiently transfected with 100 nmol/L of specific siRNA against CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, hnRNPF, or SF2/ASF or with a siCont for 72 hours. For the last 24 hours, cells were incubated with or without EGFR ligands (50 ng/mL) and the IR-A:IR-B ratio was analyzed by qPCR. **B**, HuH6 cells were transiently transfected with 100 nmol/L of EGFR siRNA or control siRNA for 72 hours or treated with or without gefitinib (2.5 μ mol/L). The expression of splicing factors was analyzed by qPCR. **C**, representative blots of 3 independent experiments showing downregulation of CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF protein levels upon inhibition of EGFR signaling with EGFR siRNA or gefitinib in HuH6 cells (left) and blot quantifications (right). **D**, HuH6 cells were transiently transfected with 100 nmol/L of EGFR or control siRNA for 72 hours or treated with or without gefitinib (2.5 μ mol/L). Cell extracts were then treated or not with alkaline phosphatase and analyzed by Western blotting. Statistical analysis, $n = 3$; Student t test; *, $P < 0.05$; **, $P < 0.01$ compared with controls; ###, $P < 0.001$ compared with cells stimulated with an EGFR ligand.

Figure 6. Alteration of IR mRNA splicing during liver carcinogenesis but not during liver regeneration in rat. A, the IR-A:IR-B ratio (left) and the total IR mRNA content (right) were evaluated by qPCR in 5 paired HCC (T)/nontumor (NT) liver tissue samples obtained from rats weekly intraperitoneally injected with DEN during 12 weeks. B, qPCR analysis of splicing factor mRNA levels in rat T/NT liver tissues. C, the relative IR-A:IR-B ratio (left) and the total IR mRNA (right) content were evaluated by qPCR in liver tissue samples from rats 1 day ($n = 5$), 3 days ($n = 4$), and 10 days ($n = 6$) after partial hepatectomy (PH) and compared with baselines (day 0). D, qPCR analysis of splicing factor mRNA levels in rat regenerating liver tissues. Statistical analysis, Wilcoxon test for paired values, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.



mRNA expression was increased in 17% of IR-A expressing HCC in our collection. Therefore, receptor dimers engaging IR-A can also support HCC lesions to prosper in a high-IGF-II environment.

Increasing evidence indicates that alternative splicing is subject to regulation by extracellular stimuli, such as ligands for RTKs. During the preparation of this article, Zhou and colleagues have reported that massive reprogramming of alternative splicing is induced upon EGF treatment in HEK293T cells (18). In these cells, EGF-induced mRNA splicing was efficiently blocked with PI3K/AKT inhibitors, whereas inhibition of other pathways (such as the MEK pathway) had much lesser effects. Here, we show that the stimulation of EGFR activity with exogenous ligands or with autocrine amphiregulin loop enhances IR-A mRNA splicing through MEK activation in HCC cells. In full agreement with these results, the 2 cell lines that exhibit a high basal level of ERK phosphorylation controlled by EGFR, namely HuH6 and PLC/PRF5 cells, have the highest ratio of IR-A:IR-B. The finding that the EGFR/MEK pathway promotes aberrant IR-A splicing in HCC cells makes logical sense in relation to cellular transformation as this pathway is frequently dysregulated in human HCC (28) and participates in liver carcinogenesis in animal models (24, 35). Consistently, high serum levels of EGF are associated with increased HCC risk in patients with cirrhosis (36, 37). EGFR

ligands are overproduced by HCC tumors and also by cells from tumor environment such as cirrhotic hepatocytes, hepatic fibroblasts, and inflammatory cells (29, 38–40). Taken together, these data suggest that EGFR activation of IR-A splicing in HCC cells may result not only from autocrine but also from paracrine stimulatory loops involving EGFR ligands and that tumor environment could play a prominent role in this process. Of particular note, while normal hepatocytes are known to be responsive to EGFR ligands with respect to proliferation (41), EGFR ligands could not promote the IR-B to IR-A shift in these cells. These data suggest that the intracellular mechanisms coupling EGFR stimulation to IR-A production are specific of transformed hepatocytes.

The involvement of EGFR in the control of alternative splicing remains largely uninvestigated in cancer in general and in HCC in particular. A recent study has revealed that the activation of EGFR promoted the splicing of the oncogenic variant $\Delta\text{Ex}2p73$, a dominant-negative inhibitor of p53/p73 (42). Our study identifies IR-A isoform as a novel target of EGFR splicing function in HCC. Our findings may also have broader implications given that the activation of EGFR is increased in other tumors such as colon carcinoma. Consistently, preliminary data from ours show that the blockage of EGFR signaling markedly reduced the IR-A:IR-B ratio in SW480 and LoVo colon cancer cell lines (Supplementary Fig. S6).

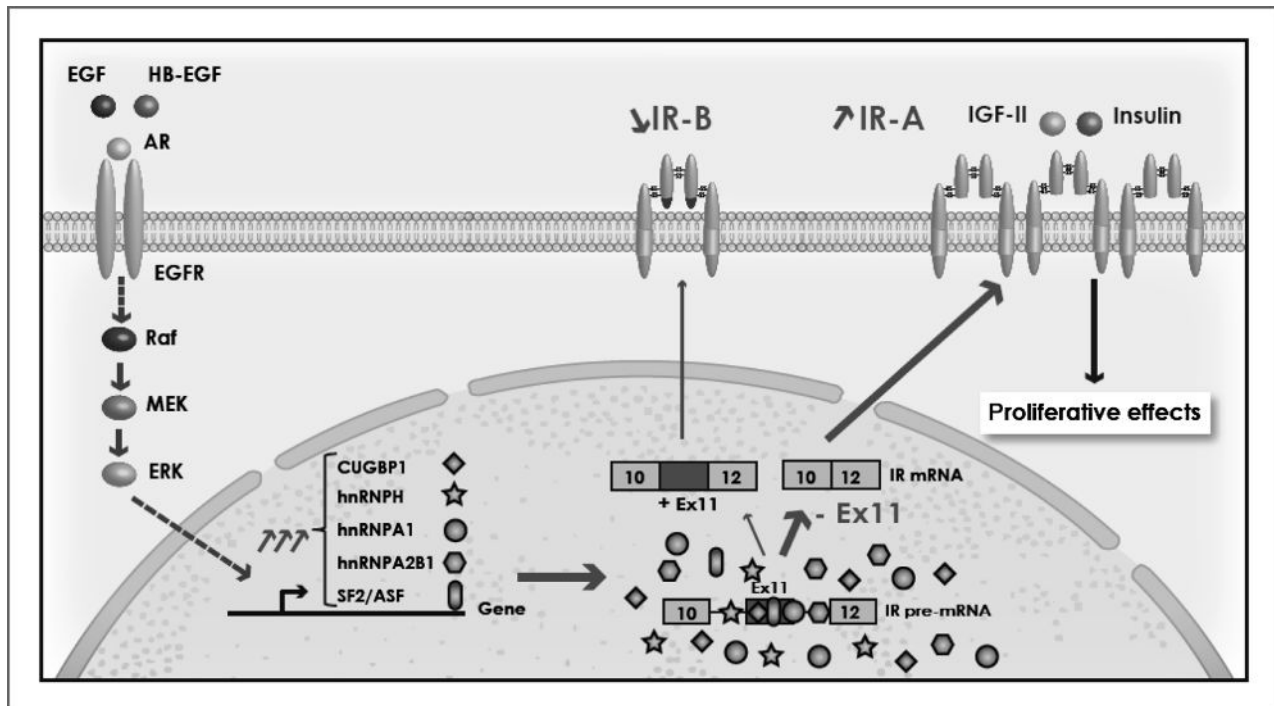


Figure 7. Model for the regulation of IR pre-mRNA alternative splicing by EGFR in HCC cells. Upon ligand binding, EGFR activation triggers an intracellular signaling cascade, implicating MEK activation. This results in the stimulation of transcription of genes coding for different splicing factors, namely CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF. The hnRNPF expression is not controlled by EGFR-dependent pathway. The interaction of splicing factors with IR pre-mRNA promotes IR exon 11 alternative splicing. As a consequence, the expression of IR-A isoform is increased to the detriment of IR-B, which allows the transmission of proliferative signals in response to insulin and IGF-II.

Studies conducted with IR minigene constructs have shown that CUGBP1, hnRNPH, and hnRNPA1 bind to exonic and intronic splicing silencer elements, which affects the selection of splice sites and favors IR exon 11 skipping (19–21). The overexpression of these splicing factors significantly alters the expression of IR splice variants in disease. Thus, in myotonic dystrophy type 1, the overexpression of CUGBP1 and hnRNPH is responsible for aberrant IR-A splicing in myoblasts (19, 20). So far, CUGBP1, hnRNPH, and hnRNPA1 have been found to be overexpressed in HCC (43–45). Our data support these findings and highlight the contribution of CUGBP1, hnRNPH, and hnRNPA1 in the promotion of IR-A splicing in HCC cells. Our data also identify hnRNPA2B1 as a new player promoting the exclusion of exon 11 from the IR pre-mRNA. The correlations we report between the expression of splicing factors and that of IR-A in HCC tumors support the role of abnormally expressed splicing factors in the production of IR-A in HCC.

SRp20 was undetectable in human HCC tumors. Recently, SRp20 has been identified as crucial for hepatocyte differentiation and metabolic function in mice with hepatocyte-specific deletion (46). SRp20 also antagonizes CUGBP1-mediated repression of exon 11 inclusion in minigene experiments and it seems that the SRp20:CUGBP1 relative ratio dictates the degree of exon inclusion (16). In this context, the loss of expression of SRp20 might be a characteristic of the dysfunction and transformation of hepatocytes and could

promote the activity of CUGBP1, which is overexpressed in HCC.

We observed that the siRNA-mediated knockdown of SF2/ASF and hnRNPF led to different effects on endogenous IR mRNA splicing, depending on the HCC cell lines. Both siRNA were without effect in PLC/PRF5 cells while they induced a marked decrease of the IR-A:IR-B ratio in the 3 other cell lines. In apparent contradiction with these data, siRNA against SF2/ASF or hnRNPF increased the IR-A:IR-B ratio in HepG2 cells transfected with a IR minigene (16, 21). These discrepancies may reflect differences in the experimental approaches (endogenous transcript vs. minigene). Indeed, in the same study, the downregulation of hnRNPF was without effect on endogenous IR-A:IR-B ratio in HepG2 cells (21). In other cell contexts, SF2/ASF has been identified as an oncoprotein (47) and as a promoter of exon skipping (48–50). Therefore, it is not excluded that SF2/ASF may favour exclusion of IR exon 11 and thus promote the expression of IR-A in HCC.

To date, knowledge of the mechanisms leading to changes in splicing factor concentrations is limited and the contribution of EGFR-dependent pathways in these processes remains unexplored. Our data reveal that, except hnRNPF, EGFR activation mediates CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF upregulation at mRNA and protein levels in HCC cells. The blockade of EGFR signaling affected neither their phosphorylation profile nor their subcellular localization indicating that EGFR predominantly regulates splicing factor

expression at a transcriptional level. The detailed mechanisms through which EGFR stimulates splicing factor expression remain to be studied.

In conclusion, the present study shows that the dysregulation of the alternative splicing of IR toward a proliferative phenotype is a frequent event in HCC. The underlying mechanism has been elucidated and connects the activation of EGFR to the regulation of a variety of splicing factors (Fig. 7). These findings expand our knowledge of the oncogenic mechanisms activated by EGFR in HCC and provide mechanistic support to the hypothesis that hyperinsulinemia participates to the development of HCC. They also provide a strong molecular rationale to ongoing clinical trials that target IR/IGF-IR RTK in HCC and open new strategies in modulating IR-A splicing therapeutically.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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