1 **TITLE: IGF2 is up-regulated by epigenetic mechanisms in hepatocellular**

2 **carcinoma and is an actionable oncogene in experimental models**

3 **SHORT-TITLE : IGF2 as key target for HCC therapy**

4

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56 **Abbreviation list:**

57 AKT: protein kinase B

58 Akt1:   gene encoding for AKT

59 BrdU: Bromodeoxyuridine

60 CD31:   cluster of differentiation 31

61 EPCAM: Epithelial cell adhesion molecule

62 FC: fold change

63 GEMM: genetically-engineered mouse model

64 GSEA: Gene Set Enrichment Analysis

65 IGF: insulin-like growth factor

66	IGF1/2:	insulin-like growth factor 1 and 2
67	IGF2:	insulin-like growth factor 2
68	INSR:	insulin receptor
69	INSR-A:	insulin receptor, isoform A
70	INSR-B:	insulin receptor, isoform B
71	IPA:	Ingenuity Pathway Analysis
72	KRT19:	Keratin, type I cytoskeletal 19
73	LOI:	loss of imprinting
74	mAb:	monoclonal antibody
75	miRNA:	micro-RNA
76	P1, P2, P3 and P4:	promoters 1, 2, 3 and 4
77	SALL4:	Sal-like protein 4
78	sh:	short hairpin
79	TKI:	tyrosine kinase inhibitors
80	VEGFA:	Vascular endothelial growth factor A

81

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88   **Transcript-Profiling:** Microarray data were deposited in Gene Expression Omnibus

89   database with the accession numbers GSE63898, GSE56588, GSE74618 and GSE85274.

Abstract

Background & Aims: Effective treatments are urgently needed for hepatocellular carcinoma (HCC), which is usually diagnosed at advanced stages. Signaling via the insulin-like growth factor (IGF) pathway is aberrantly activated in HCC by IGF2 overexpression. We aimed to elucidate the mechanism of IGF2 overexpression and its oncogenic activities, and evaluate the anti-tumor effects of reducing IGF2 signaling.

Methods: We obtained 228 HCC samples from patients who underwent liver resection, 168 paired non-tumor adjacent cirrhotic liver samples, and 10 non-tumor liver tissues from patients undergoing resection for hepatic hemangioma. We analyzed gene expression, micro RNA (miRNA), and DNA methylation profiles for all samples, focusing on genes in the IGF signaling pathway. IGF2 was expressed in SNU449 and PLC5 HCC cells and knocked down with small hairpin RNAs in Hep3B and Huh7 cell lines. We analyzed these cells for proliferation, apoptosis, migration, and colony formation. We performed studies of mice engineered to express Myc and Akt1 in liver, which develop liver tumors, with or without hepatic expression of Igf2. Mice with xenograft tumors grown from HCC cells were administered with a monoclonal antibody against IGF1 and IGF2 (BI 836845), along with sorafenib; tumor growth was measured and tissues were analyzed by immunohistochemistry and immunoblots.

Results: Levels of IGF2 mRNA and protein were increased more than 20-fold in 15% of human HCC tissues, compared with non-tumor liver tissues. Methylation at the fetal promoters of *IGF2* was reduced in the HCC samples and cell lines that overexpressed IGF2, compared with those that did not overexpress IGF2 and non-tumor tissues. Tumors that overexpressed IGF2 had gene expression patterns significantly associated with hepatic

progenitor cell features, stellate cell activation, NOTCH signaling and an aggressive phenotype (*P*<.0001). In mice engineered to express *Myc* and *Akt1* in liver, co-expression of *Igf2* accelerated formation of liver tumors, compared to mice with livers expressing only *Myc* and *Akt1*, and shortened survival times (*P*=.02). The antibody BI 836845 blocked phosphorylation of IGF1 receptor (IGF1R) in HCC cell lines and reduced their proliferation and colony formation. In mice with xenograft tumors, injection of BI 836845, with or without sorafenib, slowed tumor growth and increased survival times compared to vehicle or sorafenib alone. BI 836845 inhibited phosphorylation of IGF1R and AKT and reduced decreased tumor vascularization, compared with vehicle.

Conclusions: A large proportion of HCC samples were found to overexpress IGF2, via demethylation of its fetal promoter. Overexpression of IGF2 accelerates formation of liver tumors in mice with hepatic expression of MYC and AKT1, via activation of IGF1R signaling. An antibody against IGF1 and IGF2 slows growth of xenograft tumors and increases survival of these mice.

Keywords: mouse model, hepatocarcinogenesis, IGF receptor, epigenetic modification

Background

Liver cancer is the second cause of cancer-related death worldwide and has an incidence of 850,000 new cases per year, thus representing a major public health problem[1]. Hepatocellular carcinoma (HCC) is the most common type of liver cancer ranking as the 16th cause of death globally [1]. Although surveillance programs for high-risk patients have been implemented during the past decade, most patients are still diagnosed at advanced stages. The only approved systemic therapy for these patients is sorafenib, which extends survival from 8 to 11 months [2]. Seven additional targeted therapies tested in phase III trials in first and second line have failed to improve survival [3,4]. Among the reasons for these unsatisfactory results are the suboptimal understanding of the HCC critical drivers and the lack of biomarker-based studies [3,4]. Thus, the identification of novel molecular targets and therapies is an unmet medical need in HCC [3].

Over the past decade, comprehensive sequencing efforts have established the landscape of gene mutations, chromosomal aberrations and epigenetic alterations that characterize different cancer types, including HCC[5–9]. The oncogenic consequences of various structural alterations have been extensively validated [3,5]; however, the role of genes altered by epigenetic mechanisms (epi-drivers [9]) has not been fully elucidated in HCC [3,7,10]. Similar to other cancers [11,12], the identification of actionable epi-drivers could provide novel treatment options for the clinical management of this malignancy.

The IGF (insulin-like growth factor) signaling is frequently altered in HCC and constitutes a promising therapeutic target [13,14]. Earlier studies pointed to IGF1R (insulin-like growth factor 1 receptor) as a potential oncogene [13]; however, subsequent clinical trials blocking this

150   target by means of IGF1R monoclonal antibodies (mAb) or IGF1R/INSR (insulin receptor)

151   tyrosine kinase inhibitors (TKI) failed to demonstrate beneficial outcomes [15]. Alternative

152   therapeutic strategies to inhibit IGF-pathway activation have been designed. For example,

153   BI 836845 is a mAb that abrogates IGF signaling and the pro-proliferative isoform INSR-A

154   by neutralizing IGF1 and 2 without affecting insulin metabolic functions through INSR-B

155   [16]. IGF2 is highly overexpressed in HCC [13,14], and could be a potential driver in

156   hepatocarcinogenesis. *IGF2* is a paternally imprinted growth factor regulated by four

157   different promoters. In the human liver, *IGF2* is monoallelically expressed during fetal

158   stages from three promoters (P2, P3 and P4) and in adults from both alleles of promoter P1

159   [17]. The upregulation of *IGF2* observed in HCC can be partially explained by the

160   reactivation of *IGF2* transcription from the fetal-specific promoters [13]. However, further

161   studies are needed to elucidate the precise mechanisms of deregulation of *IGF2* in HCC,

162   the specific contribution of *IGF2* overexpression to HCC development and its potential as a

163   therapeutic target.

164   In this study, we describe an epigenetic mechanism responsible for the reactivation of *IGF2*

165   fetal promoters in HCC and the association of these tumors with hepatic-progenitor cell

166   features. Moreover, we define the contribution of *IGF2* to the development of

167   hepatocarcinogenesis in genetically engineered mouse models (GEMM), and demonstrate

168   the *in vitro* and *in vivo* antioncogenic efficacy of IGF1/2-mAb through the reduction of cell

169   proliferation and angiogenesis. Taken together, we identify an actionable epi-driver in HCC

170   and a subset of patients that could benefit from anti-IGF2 therapy.

Materials and Methods

Human tissue samples

Human samples were collected following Institutional Review Board (Hospital Clinic de Barcelona) and patient written informed consent. The study included samples from the Heptromic Consortium used in previous studies [7]: 228 HCC tumor samples from patients who underwent liver resection, 168 paired non-tumor liver adjacent cirrhotic tissue samples and 10 normal liver samples obtained from patients undergoing resection for hepatic hemangioma. Samples were collected from three institutions of the HCC Genomic Consortium (IRCCS Istituto Nazionale Tumori-Milan, Hospital Clínic-Barcelona and Mount Sinai-New York). **Table S1** presents the main clinico-pathological features of the patients included in the study.

Chemically-induced mouse model of HCC

All protocols involving animals were approved by the Institutional Animal Use and Care Committee from the University of Barcelona. The induction of hepatic fibrosis and HCC was based on a recently established model [18], and recapitulates the physiologic, histological and molecular features of fibrosis, cirrhosis and HCC. Briefly, 15-days-old C57BL/6 male mice (*n* = 10) received a single intraperitoneal injection of the hepatocarcinogen diethylnitrosamine (DEN) (25 mg/kg dissolved in 0.9% of sodium chloride). At 4 weeks of age, mice received a weekly intraperitoneal administration of the hepatotoxin carbon tetrachloride (CCl_4) diluted in corn oil at a dosage 0.5 µl/g for 11-14 weeks. Control mice (*n* = 9) received the vehicles of DEN and CCl_4. Livers were removed from the mice at 18 weeks of age and stored at -80 ºC for RNA extraction.

Genetically engineered mosaic mouse models

Hydrodynamic tail vein injection was described recently [19–21]. Mice (four to six weeks old) received 25 μg of transposon plasmids and 5 μg of transposase (Sleeping Beauty 13; SB13). *SB13*, transposons encoding for *Myc* and *Akt1* and empty transposon plasmids were previously described [19,21]. The *Igf2* transposon plasmid was generated by PCR cloning, the *Igf2* cDNA was obtained from Open Biosystems, while p19$^{Arf-/-}$ mice (male C57BL/6 background) were provided by Scott W. Lowe (Memorial Sloan Kettering Cancer Center, New York, New York, USA). These mice were generated by Charles Sherr (St. Jude Children's Research Hospital, Memphis, Tennessee, USA). C57BL/6 wildtype male mice were obtained from Harlan (Rossdorf, Germany). DNA for hydrodynamic tail vein injection was generated using the Qiagen EndoFreeMaxi Kit. The DNA was diluted in 0.9% NaCl and injected at a volume of 10% of mouse body weight.

Human HCC subcutaneous xenograft mouse model

Ten million Hep3B cells were subcutaneously injected in a ratio 1:1 with matrigel (Corning, Christiansburg, VA) into the right flank of NOD/SCID 6-8 weeks old female mice (Harlan). Tumor size was measured three times per week using a hand calliper and tumor volume was calculated using the following equation: (width^2 x length)/2. Animals were randomized once tumor volume reached 100-200 mm^3 into four arms: a) sorafenib (n = 12), b) IGF1/2-mAb (n = 12), c) combination (sorafenib + IGF1/2-mAb, n = 13) and d) vehicle (drug vehicles, n = 8). Mice randomization was performed using the Random number generator module from Graph Pad software (San Diego, USA). Sorafenib was dissolved in ethanol 95%/cremophor/sterile water (12.5:12.5:75) and administered orally at

215   15 mg/kg/day dosage [22]. The IGF1/2-mAb (BI 836845) was injected once weekly

216   intraperitoneally at 200 mg/kg according to provider recommendations [16]. When tumors

217   reached 2000 mm^3, animals were sacrificed following institutional ethical guidelines. For

218   survival analysis, mice were censored at the time of sacrifice according to IACUC

219   guidelines. Tumors were rapidly extracted and formalin-fixed for immunohistochemical

220   analysis or stored at -80 ℃ for molecular analysis. Toxicity was monitored according to

221   weight loss three times per week. Weight loss greater than 15% was considered as a sign of

222   toxicity and these mice were sacrificed and excluded from the analysis.

223   **Statistical analysis**

224   Statistical analyses were performed using SPSS version 20 (SPSS Inc., IL, USA) or

225   GraphPad Prism software (San Diego, USA). $p < 0.05$ was considered significant. Values are

226   presented as mean ± s.d. When data sets met normal distribution criteria, we used Student's

227   two-sided t-test analysis (for two-group comparisons) and one-way ANOVA analysis (if

228   more than two groups were compared). We used Bonferroni test as a post hoc test. If data

229   did not meet normal distribution criteria, we used Mann-Whitney test (for two-group

230   comparisons) and Kruskal-Wallis test (if more than two groups were compared). We used

231   Dunn's test as a post hoc test. *In vitro* experiments were repeated independently at least

232   three times, using technical triplicates. Variances were similar between groups in all

233   experiments, as determined by the F test using GraphPad Prism. For GSEA module

234   analysis, significance was corrected for multiple tests (FDR<0.05). The sample size in each

235   *in vivo* experiment was based on our previous studies on such experiment [13,19–21]. Mice that

236   were sacrificed due to treatment-related toxicity were excluded from the survival analyses.

237   The investigators were not blinded to allocation during experiments and analyses.

238 **Genomic profiling, Methylome profiling and data analysis**

239 See the Supplementary Materials and Methods section.

240 **Cell lines, plasmids and reagents, Decitabine demethylation treatment, *In vitro***

241 **functional cell assays, IGF2 immunostaining, Ligand mediated IGF pathway**

242 **activation, Reverse transcription (RT) Polymerase Chain Reaction (PCR) and**

243 **quantitative RT-PCR**

244 See the Supplementary Materials and Methods section.

245 **Metabolic toxicity in the HCC subcutaneous xenograft mouse model, Tumor**

246 **xenograft molecular characterization and *Igf2* and *H19* expression analysis in mouse**

247 **models of HCC**

248 See the Supplementary Materials and Methods section.

249

Results

IGF2 overexpression in HCC is triggered by epigenetic mechanisms

We and others have previously demonstrated that IGF2 is overexpressed in human HCC [13,14]. In this study, we conducted an integrative oncogenomic analysis of 228 human HCCs to elucidate the underlying mechanism of *IGF2* enhanced expression. *IGF2* transcriptional and protein levels were significantly higher compared with matched surrounding cirrhotic and non-tumor liver tissues. IGF2 was mainly expressed by hepatocytes, though bile duct epithelial cells also expressed it to a lesser extent **(Figure 1A and Supplementary Figure 1).** *IGF2* overexpression above 20-fold was observed in 15% (34/228) of the samples, among which 24 samples showed upregulation above 100-fold. Interestingly, the expression of *INSR-A* isoform, a pro-proliferative receptor with high affinity for IGF2, was upregulated in 38% (86/228) of HCCs, being significantly higher in *IGF2*-overexpressing samples **(Supplementary Figure 2).** None of these events was significantly associated with any clinic-pathological characteristics.

We previously reported that *IGF2* overexpression in HCC is associated with the reactivation of its fetal promoters [13], but the mechanism responsible for this reactivation has not been described yet. As *IGF2* is an imprinted gene, we hypothesized that epigenetic deregulation could be the underlying cause. The methylation status of the *IGF2-H19* imprinting control region (ICR1) and the *IGF2* promoters was evaluated in human HCCs with low ($n = 173$; FC<20) or high ($n = 27$; FC$\geq$20) *IGF2* levels and in 10 healthy liver samples. We observed hypomethylation in both alleles of the strongest fetal promoters (P3-P4) and increased methylation of the adult promoter P1 in samples overexpressing *IGF2*

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272 compared to low-*IGF2* and healthy liver samples **(Figure 1B and C, and Supplementary**

273 **Figure 3A-C).** This fetal promoter hypomethylation was associated with increased

274 expression of transcripts from P3**,** while increased methylation of P1 was associated with a

275 severe downregulation in P1-derived transcripts **(Supplementary Figure 3D and E).** *IGF2*

276 overexpression was also associated to hypomethylation of the *IGF2/H19* ICR1 and to

277 increased *H19* expression **(Figure 1C and Supplementary Figure 3A-C and F)**.

278 We confirmed the association between aberrant methylation of *IGF2* promoters and *IGF2*

279 overexpression in human HCC cell lines. Hep3B and Huh7 presented the highest *IGF2*

280 levels when compared to normal liver, while SNU449 and PLC5 had the lowest expression

281 **(Supplementary Figure 4A).** Overexpression of the ligand was associated with higher

282 activation of the pathway **(Supplementary Figure 4B).** The methylation status of CpGs

283 located at fetal and adult promoters was evaluated in these cell lines **(Supplementary**

284 **Figure 4C and D).** HCC cells overexpressing *IGF2* displayed a decreased proportion of

285 methylated CpGs in P3-P4 compared to HCC cell lines with low *IGF2* levels**.** This aberrant

286 methylation was associated with an increased expression of transcripts derived from fetal

287 promoters and abrogated expression of those derived from the adult promoter

288 **(Supplementary Figure 4E).** As expected, forced demethylation of *IGF2* promoters by

289 decitabine treatment in SNU449 and PLC5 led to *IGF2* overexpression due to promoter

290 reactivation **(Supplementary Figure 5A).**

291 Furthermore, several microRNAs (miRNAs) have been shown to be involved in IGF2

292 deregulation: miR-483-5p, an intronic miRNA expressed from the *IGF2* locus, has been

293 reported to increase fetal *IGF2* mRNA levels [23]. Accordingly, patients in our cohort

294 overexpressing *IGF2* displayed significantly overexpression of miR-483-5p when

295   compared with low-*IGF2* HCCs **(Figure 1D).** A direct link between miR-483 and *IGF2*

296   expression was established *in vitro*. SNU449 and PLC5 *IGF2* expression levels were

297   significantly increased upon transfection of miR-483 **(Supplementary Figure 5B and C).**

298   Overexpression of *IGF2* from fetal promoters was detected in 94% (32/34) of our HCC

299   cohort, and out of these, 66% (21/32) of cases presented aberrant methylation of fetal

300   promoters while 53% (17/32) overexpress miR-483-5p, pointing to epigenetic mechanisms

301   as the main cause of re-expression of fetal *IGF2* in HCC **(Figure 1E and Supplementary**

302   **Figure 6)**. Overall, these results suggest that DNA methylation deregulation is the

303   predominant cause of *IGF2* overexpression in human HCC samples, but alternative

304   epigenetic mechanisms, such as miRNA deregulation, could contribute to the reactivation

305   of fetal *IGF2*.

306   **IGF2 is an epi-driver in *in vivo* experimental models of HCC**

307   To evaluate the specific role of IGF2 in HCC we established a genetically engineered

308   mosaic mouse model in which transposable elements containing *c-Myc/Akt1* and *Igf2* were

309   delivered into the liver of wild-type mice through hydrodynamic tail vein injection **(Figure**

310   **2A)** [19–21]. All *Igf2*-injected mice showed increased *IGF2* expression and constitutive

311   activation of the IGF pathway, as indicated by higher phosphorylation of IGF1R and its

312   downstream protein AKT (**Figure 2B and C**). Mice overexpressing *Igf2* presented

313   significant reduction of survival in comparison to control mice due to accelerated tumor

314   progression **(Figure 2D and E)**. Importantly, all *Igf2*-overexpressing mice died 2.5 months

315   after plasmid delivery. Interestingly, liver-specific *Igf2* overexpression in knock-out mice

316   lacking the tumor suppressor p19[Arf] did not promote tumor initiation **(Supplementary**

Figure 7), suggesting that IGF2, although might not be a transforming oncogene *per se*, significantly accelerates HCC progression.

Further evidence on the role of IGF2 in HCC tumorigenesis was obtained from the analysis of *Igf2* expression of 11 HCC mouse models, including 10 available in GEO database and one generated in our laboratory with CCl_4 and diethylnitrosamine (DEN) [18]. *Igf2* (FC>2) was overexpressed in 10 models, with a prevalence ranging from 22-100% of the tumors. Importantly, overexpression of *Igf2* was coupled to *H19* overexpression (FC>2) in all cases **(Supplementary Table 6)**.

IGF2 overexpression increases HCC proliferation

To decipher the role of *IGF2* overexpression in enhancing hepatocarcinogenesis, the effect of altered *IGF2* expression was evaluated *in vitro* through colony formation, apoptosis and migration assays. *IGF2* knockdown using short hairpin RNAs was conducted in Hep3B and Huh7 cells (*Hep3B-shIGF2; Huh7-shIGF2*), which present high endogenous *IGF2* expression. On the other hand, *IGF2* ectopic overexpression was induced in SNU449 and PLC5 cells (*SNU449-IGF2;PLC5-IGF2*), characterized by very low endogenous *IGF2* levels **(Supplementary Figure 4A and B and Supplementary Figure 8A-C)**. Downregulation of *IGF2* levels by >60% (shIGF2#2 and #3) caused a significant decrease in colony number compared to controls **(Figure 3A, and Supplementary Figure 8D).** On the other hand, *SNU449-IGF2* and *PLC5-IGF2*, displayed a significant increase in the number of colonies **(Figure 3B, and Supplementary Figure 8D).** The colonies were also bigger in size, indicating an increase in cell proliferation. However, the rate of cell death and migration remained unaltered in both cases **(Supplementary Figure 9).** Altogether,

17

340 these results provide evidence that IGF2-driven tumorigenesis in HCC relies on the

341 increase in cell proliferation rather than antiapoptotic or enhanced migratory effects.

342 **High *IGF2* levels correlate with an undifferentiated and aggressive phenotype**

343 To shed light on the functional consequences of *IGF2* overexpression in HCC, we carried

344 out Gene Set Enrichment Analysis (GSEA), Ingenuity Pathway Analysis (IPA) and

345 Comparative Marker Selection (CMS) using expression data from human HCC patients,

346 GEMM overexpressing IGF2 and HCC cell lines with ectopic overexpression or silencing-

347 mediated downregulation of IGF2. We found that expression profiles from HCC patients

348 with up-regulated *IGF2* were significantly associated with three equivalent molecular

349 subclasses of HCC [5]: (a) the G1 molecular subclass [24], which has been reported to

350 overexpress paternally imprinted genes such as *IGF2*; (b) the Proliferation subclass [25],

351 characterized by IGF pathway activation; and (c) the S1-S2 proliferation subclasses [26]. In

352 addition, *IGF2*-overexpressing samples were associated with several hepatic progenitor

353 cell-like signatures [27,28], hepatic stellate cell activation**,** NOTCH signaling [29], genes up-

354 regulated in hepatoblastoma [30], and tumor invasiveness [31,32]. We also observed enrichment

355 of poor-prognosis liver-cancer signatures [33,34] and up-regulation of VEGFA targets[35,36]

356 **(Figure 1E and Table 1).** Furthermore, *IGF2* overexpression was associated with

357 significant upregulation of the hepatic progenitor-like markers *SALL4* [37,38] (mean FC 4.9 vs

358 1.4), *EPCAM* [27] (mean FC 11.7 vs 1.4) and *KRT19* [28] (mean FC 5.3 vs 0.8) and increased

359 alpha fetoprotein plasma levels (median 90 ng/dL vs 60 ng/dL) compared to low-*IGF2*

360 samples **(Supplementary Figure 10).** GEMM tumors overexpressing IGF2 shared the

361 main characteristics of human IGF2-overexpressing tumors, particularly a more

362 proliferative, undifferentiated and invasive tumor profile **(Supplementary Table 7).**

363   Consistently, target genes of IGF2 identified by comparing expression profiles of SNU449-

364   IGF2 and Hep3B-shIGF2 **(Supplementary Table 8),** were involved stem cell pluripotency,

365   cellular proliferation and survival **(Supplementary Table 9).** Taken together these data

366   suggest that the reexpression of fetal *IGF2* leads to HCCs with progenitor cell-like features

367   and aggressive phenotype.

368   **Antiproliferative effect of monoclonal antibodies against IGF ligands in human HCC**

369   **cell lines**

370   The marked overexpression of *IGF2* in HCC patients and the causal relationship between

371   IGF2 and accelerated tumor progression *in vivo* prompted us to assess the selective effect of

372   IGF2 blockage as a putative novel therapy for HCC. To this end, the effects of BI 836845, a

373   monoclonal antibody against IGF ligands (IGF1/2-mAb)[16], were first evaluated *in vitro*

374   using HCC cell lines **(Supplementary Figure 3A and B)**. A significant reduction in

375   colony number and colony size was observed in Hep3B and Huh7 cells when treated with

376   IGF1/2-mAb, but not in cells with low *IGF2* levels **(Figure 3C and D, and**

377   **Supplementary Figure 11A).** Consistently, the IGF1/2-mAb was able to significantly

378   reduce cell viability (>20%) in the two cell lines**.** BrdU incorporation showed that this

379   reduction was due to a decrease in the proliferation rate **(Supplementary Figure 11B, and**

380   **C).** In contrast, IGF1/2-mAb had no significant effect in cells with low *IGF2* levels

381   **(Supplementary Figure 11D and E).** No effects on apoptosis or migration were observed

382   **(Supplementary Figure 12A-C)**. These results suggest that IGF1/2-mAb is capable of

383   attenuating IGF2-dependent cell proliferation *in vitro*. To confirm that the antiproliferative

384   effects of the antibody were specific for IGF2-blocking as opposed to IGF1, *shIGF2- and*

385   *IGF2-transfected* cell lines were subjected to colony formation assay after IGF1/2-mAb

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386   treatment. In line with our preceding results, *Hep3B-shIGF2 and Huh7-shIGF2*, contrary to

387   wild-type cells, were not sensitive to antibody treatment **(Figure 3A).** Moreover, forced

388   expression of *IGF2* in SNU449 and PLC5 cells promoted an increase in colony formation

389   that was abolished upon IGF1/2-mAb treatment **(Figure 3B).** Remarkably, IGF1/2-mAb

390   completely blocked IGF2-dependent activation of IGF1R and INSR in HCC cell lines,

391   while it had no effect on insulin-mediated pathway activation assessed by phosphorylation

392   of INSR **(Figure 3E, and Supplementary Figure 12D).** Conversely, IGF1R inhibition

393   with the TKI linsitinib simultaneously impaired both IGF2 and insulin-mediated pathway

394   activation. These results suggest that IGF1/2-mAb-based treatments, by simultaneously

395   blocking IGF2-dependent activation of IGF1R and INSR-A without affecting the metabolic

396   pathway, might be more efficient than other IGF1R-targeted therapies and better tolerated

397   than TKI-based therapies.

398   **IGF2 inhibition delays tumor growth and improves survival *in vivo***

399   The antioncogenic efficacy of IGF1/2-mAb was evaluated using an Hep3B xenografted

400   mouse model. Animals received vehicle, sorafenib (the standard of care), IGF1/2-mAb, or a

401   combination of both. Strikingly, tumor growth was significantly lower in the IGF1/2-mAb

402   and in the combination arm compared to either vehicle or sorafenib alone **(Figure 4A)**.

403   These differences were more remarkable after 3 weeks, when tumor growth inhibition for

404   IGF1/2-mAb and the combination was maximum **(Figure 4B and C).** Furthermore, mice in

405   the IGF1/2-mAb- and combination arms exhibited significantly increased survival **(Figure**

406   **4D)** compared to both vehicle and sorafenib, respectively. Differences between single agent

407   IGF1/2-mAb and combined arms were not statistically significant at any time point.

408   Overall, all drugs were well tolerated, although three mice were sacrificed due to body

409   weight loss (one from the sorafenib arm (1/13) and two from the combination arm (2/13)).

410   No significant differences in blood glucose or glycosylated hemoglobin levels were

411   detected among arms further supporting the low toxicity related to this monoclonal

412   antibody **(Supplementary Figure 13 A-C)**.

413   Molecular characterization of the xenografted HCC tumors confirmed the antiproliferative

414   effect of IGF1/2-mAb seen *in vitro* and unraveled an additional anti-angiogenic

415   mechanism. Specifically, significant reduction of Ki-67 staining was observed in all groups

416   compared to vehicle. IGF1/2-mAb and the combination presented stronger reduction in

417   proliferation than sorafenib **(Figure 5A).** Consistently, IGF1/2-mAb inhibited

418   phosphorylation of IGF1R and AKT, two well-known molecular players in proliferation

419   **(Figure 5B).** Additionally, staining for the endothelial marker CD31 showed that both

420   sorafenib and IGF1/2-mAb decreased tumor vascularization compared to vehicle **(Figure**

421   **5C).** No apoptotic events were detected **(Supplementary Figure 13D).** These results

422   indicate that the capacity of IGF1/2-mAb to impair tumor growth *in vivo* is not only due to

423   its strong antiproliferative effect, but also to inhibition of angiogenesis. Taken together,

424   IGF2 inhibition *in vivo* displays superior efficacy than sorafenib, the only approved

425   targeted therapy for HCC, emphasizing the relevance of IGF2 as a novel and efficient drug

426   target for HCC.

Discussion

The landscape of driver genes and epigenetic alterations in cancer has been thoroughly reviewed[9]. Although epigenetic overexpression of different genes has been shown to be sufficient to drive formation of cancer *in vivo* (e.g. bladder cancer [39]), the biological relevance of any candidate epi-driver has not yet been confirmed in HCC [3,5–8]. Here we present *IGF2,* which is re-expressed through epigenetic mechanisms, as the first actionable validated epi-driver in HCC, and we propose monoclonal antibodies against this growth factor as a potential targeted therapy in a defined subset of HCC patients.

We detected a high *IGF2* overexpression in 15% of HCC patients associated with aberrant promoter methylation and miRNA deregulation. The fetal *IGF2*-promoter region was heavily demethylated, while the adult promoter P1 was repressed [40,41], and thus this aberrant methylation is proposed as the crucial mechanism leading to *IGF2* overexpression. Our data suggest that the standard model of *IGF2* LOI does not apply to HCC (similarly to other cancers [42–44]). LOI of *IGF2* is believed to occur through *IGF2/H19* ICR1 hypermethylation turning the monoallelic expression of *IGF2* into biallellic, and reducing expression of *H19* [45]. However, our data suggest that ICR1 is significantly hypomethylated in most HCC samples and *H19* is overall upregulated. In fact, *IGF2* overexpression in liver cancer cell lines is often associated to upregulation of *H19* [46,47]. The coupled expression of *Igf2* and *H19* that we observed in ten HCC mouse models suggests that these genes share an aberrant epigenetic regulatory mechanism occurring during hepatocarcinogenesis.

Imprinted genes such as *IGF2* are overexpressed in somatic stem cells [48,49]. We tested whether tumors with high levels of *IGF2* were associated with self-renewal. We unravel a

449   significant association between *IGF2*-overexpressing HCCs and hepatic progenitor cell-like

450   signatures and progenitor markers (SALL4 [37,38], EPCAM [27] and KRT19 [28]). Tumors with

451   progenitor cell-like origin are known to be more aggressive and undifferentiated [28], which

452   is consistent with the association of high-*IGF2* tumors with poor prognosis signatures. Our

453   experiments using mosaic GEMMs demonstrated that, although liver-specific *Igf2*

454   overexpression did not promote tumor initiation, when co-expressed with *Myc* and *Akt1*

455   oncogenes, it significantly enhanced HCC tumorigenesis and reduced survival. Further

456   analysis using HCC cells overexpressing or downregulating *IGF2* allowed us to define the

457   mechanism of action of IGF2-driven tumorigenesis, through increased proliferation.

458   The ability of this growth factor to accelerate tumor progression in a murine HCC model,

459   together with the overexpression of *IGF2* in 15% of HCC samples, suggests that direct

460   targeting of this ligand is an attractive therapeutic strategy for treatment of patients with

461   IGF2-dependent HCC tumors. BI 836845 (IGF1/2-mAb) is currently being tested in phase

462   I/II clinical trials in solid tumors (NCT02123823, NCT02204072). The mechanism of

463   action of IGF1/2-mAbs (BI 836845 or similar antibodies [50,51]) is distinct from IGF1R-

464   targeted antibodies, which to date have shown discouraging results in clinical studies [15].

465   Our results confirmed that BI 836845 offers the potential to inhibit IGF1R and INSR-A

466   activation without interfering with the insulin/INSR-B-dependent glucose metabolism. This

467   confers important advantages to previous attempts of blocking IGF signaling, since (a) the

468   expression of INSR-A variant is increased in HCC and represents a potential mechanism of

469   intrinsic resistance to IGF1R-targeted therapies [52] and (b) the inhibition of insulin signaling

470   *in vivo* can result in severe metabolic toxic effects such as hyperglycemia and systemic

471   infections [15].

472   BI 836845 blocked IGF2-mediated proliferation and displayed significant antitumor

473   activity. We expanded the preclinical evidence on this drug, which to date was restricted to

474   colon carcinoma and Ewing's sarcoma xenograft models [16]. Unsurprisingly, the well-

475   known antiangiogenic effects of sorafenib [2] were also observed with IGF1/2-mAb. IGF

476   signaling is involved in VEGFA production under hypoxic conditions. Hypoxia inducible

477   factors upregulate *IGF2* which in turn promotes *VEGFA* expression leading to angiogenesis

478   [53,54]. Moreover, it has been reported that blockage of *IGF2* expression causes

479   downregulation of *VEGFA* and inhibits growth in HCC cells [55]. This was further supported

480   by our GSEA and IPA analysis, where those samples with increased *IGF2* expression

481   presented strong association with VEGF activation. Altogether, these results suggest that

482   IGF2 might play a role in regulating tumor angiogenesis in HCC, consistent with the

483   reduced vascularization observed in the xenograft model. Since tumor cells secreting IGF2

484   are able to circumvent the antiangiogenic effects of IGF1R-targeted antibodies by signaling

485   through the INSR-A [56], the antioncogenic effects of IGF1/2-mAbs are in part due to its

486   ability to neutralize IGF2/IGF1R/INSR-A-mediated angiogenic activity [51,57].

487   Intriguingly, no synergic effects were found in mice treated with a combination of IGF1/2-

488   mAb and sorafenib, suggesting a high dependency of Hep3B tumors on IGF2. This

489   suggests that the subset of HCC patients overexpressing *IGF2* may benefit from a precision

490   medicine approach using IGF1/2-mAbs. One of the limitations of the current study is that

491   we proposed a cut-off to define overexpression of IGF2 levels (>20 fold), but we do not

492   have enough information on what is the ideal biomarker to be tested in early clinical trials.

493   Thus, whether the biomarker for enrichment of HCC-subset is based upon transcriptional

494   expression of IGF2 or based on other means, such as immunohistochemistry, needs to be

495   further explored.

496   After sorafenib approval, all molecular targeted therapies failed to provide survival

497   advantages [4]. In order to overcome this problem, more effective drugs targeting all comers

498   should be tested in clinical trials. Alternatively, proof of concept trials [3] based on

499   biomarkers able to recognize HCC subpopulations might be critical for precision medicine.

500   Herein, we propose a new concept to be explored and tested in the clinical setting capable

501   to benefit a subgroup of patients overexpressing the epi-driver IGF2.

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648   Author names in bold designate shared co-first authors.

649

Acknowledgments

Figure Legends

655 **Figure 1.** *IGF2* **overexpression in HCC is driven by epigenetic mechanisms. (A)** *IGF2*

656 levels determined by quantitative RT-PCR in healthy liver samples ($n = 10$), adjacent non-

657 tumor (NT) tissue ($n = 47$) and HCC tumors ($n = 228$). Dots represent the expression value

658 of each individual sample and the line is the mean value of each group. Overexpression of

659 *IGF2* was defined as >20-fold. Statistical significance between groups is calculated by

660 Kruskal-Wallis with Dunn's multiple comparison test. **(B)** Schematic representation of the

661 methylation pattern in *IGF2* adult promoter (blue) and fetal promoters (green). **(C)**

662 Methylation levels measured by methylome array in CpGs of the maternal allele located

663 within adult promoter (P1), fetal promoters (P3-P4) and the *IGF2/H19* imprinting locus

664 (ICR1) in 200 HCC samples expressing low (blue; $n = 173$) or high (red; $n = 27$) *IGF2*

665 levels. Dots represent the mean value in each CpG and bars the SD between samples. Fold

666 change is normalized to 1 (mean expression value in healthy liver). **(D)** Expression analysis

667 of miR-483-5p in HCC patients with low (blue; $n = 190$) or high (red; $n = 28$) *IGF2* levels.

668 Error bars are mean ± SD. Statistical significance between groups is calculated by two-

669 sided t-test. **(E)** Schematic representation of epigenetic deregulations affecting each of the

670 34 *IGF2*-overexpressing tumors in our cohort and its integration with the current molecular

671 classification of HCC [24–26]. Statistical significance is calculated by χ^2 test.

672 **Figure 2. IGF2 contributes to HCC progression in a transposon-based mouse model.**

673 **(A)** Schematic representation of transposable elements encoding *Myc/Akt1* and *Igf2*. Caggs,

674 CAGGS promoter; IR/DR, inverted repeats and direct repeats; IRES, internal ribosome

675 entry site. **(B)** *Igf2* expression levels measured by qRT-PCR in tumors of mice upon

676 intrahepatic delivery of *Myc/Akt1*- and either an *Igf2* expressing transposon ($n = 7$) or a

677 transposon without any gene (Control) ($n = 6$). Fold change is normalized to 1 (mean

678 expression value in healthy livers of control mice). Dots represent the expression value of

679 each individual sample and the line is the mean value of each group. Statistical significance

680 between groups is calculated by Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001. **(C)**

681 Western blot analysis of murine livers upon delivery of corresponding constructs

682 (representative pictures; $n = 4$). Intrahepatic delivery of *Igf2* induces overexpression of

683 IGF2 and the consequent phosphorylation (activation) of IGF1R and its downstream target

684 AKT. **(D)** Survival analysis (Kaplan-Meier) of mice upon intrahepatic delivery of *Myc/Akt1*

685 and either an *Igf2* expressing transposon *(n = 7)* or a transposon without gene expression

686 (Control) ($n = 6$). For survival analysis, mice were censored at the time of sacrifice

687 according to IACUC guidelines. Statistical significance was calculated using a log-rank

688 test. **(E)** Representative images and H&E (20x magnification) staining of mice livers upon

689 intrahepatic delivery of *Myc/Akt1* and either an *Igf2* expressing transposon or a transposon

690 without a gene (Control).

691 **Figure 3. IGF2 blockage by shIGF2 and IGF1/2-mAb on HCC cell lines impairs cell**

692 **proliferation. (A)** Quantification of colony formation assay using Hep3B or Huh7 cell

693 lines stably transfected with shMock or shIGF2#2, and treated with the IGF1/2-mAb. Error

694 bars are mean ± SD. corresponding to ≥3 experiments in triplicate. Statistical significance

695 between groups is calculated by one-way ANOVA with post hoc Bonferroni test in all

696 panels. *p<0.05, **p<0.01, ***p<0.001 (vs. non-treated sh-Mock) or #p<0.05, ##p<0.001,

697 ###p<0.0001 (vs. shMock treated with IGF1/2-mAb). **(B)** Quantification of colony

698 formation assay using SNU449 or PLC5 cell lines stably transfected a Control vector or an

699 IGF2-overexpression vector, and treated with the IGF1/2-mAb. *p<0.05, **p<0.01,

700   ***p<0.001 (vs. non-treated Control) or #p<0.05, ##p<0.001, ###p<0.0001 (vs. Control

701   treated with IGF1/2-mAb). **(C)** Quantification of colony formation assay using Hep3B or

702   Huh7 cell lines treated with different concentrations of IGF1/2-mAb. **(D)** Quantification of

703   colony formation assay using SNU449 or PLC5 cell lines treated with different

704   concentrations of IGF1/2-mAb.   *p<0.05, **p<0.01, ***p<0.001 (vs Non-treated). **(E)**

705   Representative Western Blot analysis of Hep3B (high *IGF2*) cell lysates stimulated by

706   IGF2 or Insulin and treated with IGF1R TKI or IGF1/2-mAb for 15 min. Tubulin was used

707   as a loading control.

708   **Figure 4. IGF1/2-mAb and its combination with sorafenib delay tumor growth and**

709   **improve survival in subcutaneous xenografted HCC tumors. (A)** Tumor growth in mice

710   treated with sorafenib (*n* = 13), IGF1/2-mAb (*n* = 12), a combination of sorafenib and

711   IGF1/2-mAb (*n* = 13) or vehicle (*n* = 8). Error bars are mean ± SD. Statistical significance

712   between groups is calculated by one-way ANOVA with post hoc Bonferroni test. **(B)**

713   Representative picture of tumors in each group after 21 days of treatment. **(C)** Percentage

714   of tumor growth inhibition (TGI) of sorafenib (*n* = 13), IGF1/2-mAb (*n* = 12) and

715   combination (*n* = 13) compared to vehicle (*n* = 8) after 7, 14 or 21 days of treatment**.**

716   Statistical significance between sorafenib and other treatment groups is calculated by one-

717   way ANOVA with post hoc Bonferroni test.   *p<0.05, **p<0.01, ***p<0.001. **(D)** Survival

718   analysis (Kaplan-Meier) of mice upon treatment with sorafenib (*n* = 13), IGF1/2-mAb (*n* =

719   12), a combination of sorafenib and IGF1/2-mAb (*n* = 13) or vehicle (*n* = 8). Statistical

720   significance between groups is calculated by log-rank test.

721   **Figure 5. IGF1/2-mAb delays tumor growth by inhibiting proliferation and**

722   **angiogenesis. (A)** *Left*: Quantification of Ki-67 positive stained nuclei in 10 fields (40x

723 magnification). Bars are mean ± SD. (*n* = 5 mice/group). Statistical significance between

724 vehicle and treated groups is calculated by one-way ANOVA with post hoc Bonferroni test.

725 *p<0.05, **p<0.01, ***p<0.001. *Right*: Representative pictures of Ki-67 staining. **(B)** *Left*:

726 Quantification of the percentage of CD31$^+$ area stained in 10 fields (20x magnification)

727 relative to vehicle. Bars are mean ± SD. (*n* = 5 mice/group). Statistical significance

728 between vehicle and treated groups is calculated by one-way ANOVA with post hoc

729 Bonferroni test.   *p<0.05, **p<0.01, ***p<0.001. *Right*: Representative pictures of CD31

730 staining. **(C)** Representative Western Blot analysis of tumor lysates from vehicle- (*n* = 3),

731 sorafenib- (*n* = 3), IGF1/2-mAb- (*n* = 3), and combination-treated mice (*n* = 3). Tubulin

732 was used as a loading control.

Table 1. Human HCCs displaying high *IGF2* levels are portrayed by an hepatic progenitor cell-like and aggressive phenotype. Association of gene signatures was evaluated using the GSEA Module from GenePattern. NES denotes Normalized Enrichment Score in gene-set enrichment analysis. A NES score higher than 1 indicates enrichment of the gene-set in high-IGF2 HCC. FDR, false discovery rate. The Broad Institute Gene Set enrichment analysis website (www.broad.mit.edu/gsea) provides detailed information about the computational method. Association of signaling pathways was evaluated using Ingenuity Pathway Analysis (www.ingenuity.com).

	Gene signatures associated to IGF2 overexpression in HCC (GSEA)				Signaling pathways associated to IGF2 overexpression in HCC (IPA)	
	Gene signature	p value	FDR	NES	Signaling pathway	p value
Hepatic progenitor cell-like	VILLANUEVA_LIVER_CANCER_CK19[28]	<0.0001	<0.0001	2.314	Human Embryonic Stem Cell Pluripotency	0.04
	YAMASHITA_LIVER_CANCER_WITH_EPCAM_UP[27]	<0.0001	0.005	1.857	Hepatic Stellate Cell Activation	0.02
	REACTOME_ACTIVATED_NOTCH1_SIGNAL_TO_THE_NUCLEUS	<0.0001	0.009	1.958	Notch signaling	0.03
	PID_NOTCH_PATHWAY	0.002	0.01	1.919		
	REACTOME_SIGNALING_BY_NOTCH1	0.002	0.03	1.788		
	KEGG_NOTCH_SIGNALING_PATHWAY	<0.0001	0.03	1.788		
	REACTOME_SIGNALING_BY_NOTCH	<0.0001	0.04	1.734		
	VILLANUEVA_NOTCH_SIGNALING[29]	<0.0001	0.01	1.811		
Hepatoblastoma	CAIRO_HEPATOBLASTOMA_UP[30]	<0.0001	0.01	1.672		
Poor prognosis	LEE_LIVER_CANCER_SURVIVAL_DN[34]	<0.0001	0.006	2.016		
	WOO_LIVER_CANCER_RECURRENCE_UP[33]	<0.0001	<0.0001	2.786		
Proliferation	BOYAULT_LIVER_CANCER_SUBCLASS_G1_UP[24]	<0.0001	<0.0001	3.201	Molecular mechanisms of cancer	0.01
	CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP[25]	<0.0001	<0.0001	2.751		
	HOSHIDA_LIVER_CANCER_SUBCLASS_S1[26]	<0.0001	0.004	2.058		
	HOSHIDA_LIVER_CANCER_SUBCLASS_S2[26]	<0.0001	0.004	2.056		
Invasiveness	ANASTASSIOU_CANCER_MESENCHYMAL_TRANSITION[31]	<0.0001	0.000	2.316	Epithelial Adherens Junction Signaling	0.03
	GOTZMANN_EPITHELIAL_TO_MESENCHYMAL_TRANSITION_UP[32]	<0.0001	0.004	2.056		
Angiogenesis	WESTON_VEGFA_TARGETS[35]	<0.0001	0.03	1.771	VEGF Signaling	0.03
	VEGF_A_UP.V1_UP[36]	<0.0001	0.02	1.635		

Figure 1

Figure 2

Figure 3

a

b

c

d

e

Figure 4

a

b

c

d

Figure 5

a

b

c

Supplementary Table 1. Clinical characteristics of HCC patients

Variable	Cohort (n=228)
Median age	66
Gender (male)	180 (79%)
Etiology	
Hepatitis C	103 (45%)
Hepatitis B	48 (21%)
Alcohol	33 (14%)
Others	38 (17%)
Child-Pugh score:	
A	222 (97%)
B	3 (1%)
Tumour size (cm)	
<2	28 (12%)
2-3	72 (32%)
>3	126 (55%)
Multiple nodules	
No	169 (74%)
Yes	57 (25%)
Micro-vascular invasion	
No	146 (64%)
Yes	67 (29%)
Satellites	
No	164 (72%)
Yes	63 (28%)
BCLC early stage (0-A)	197 (86%)
Degree of tumour differentiation	
Well	33 (14%)
Moderately	107 (47%)
Poor	44 (19%)
Bilirubin (≥ 1 mg/dL)	94 (41%)
Albumin (<3.5 g/L)	24 (11%)
Platelet count (<100,000/mm^3)	43 (19%)
AFP (>100 mg/dL)	53 (23%)
Events	
Recurrence	154 (66%)
Death	133 (58%)

Supplementary Table 2. Methylation probes located at *IGF2* promoters and IGF2-H19 ICR1

Target	Probe ID
IGF2 P1 promoter_maternal allele	cg10650127
IGF2 P1 promoter_maternal allele	cg03553386
IGF2 P1 promoter_maternal allele	cg27263998
IGF2 P1 promoter_maternal allele	cg01921126
IGF2 P1 promoter_paternal allele	cg08686462
IGF2 P1 promoter_paternal allele	cg27331871
IGF2 P1 promoter_paternal allele	cg25742037
IGF2 P3-P4_maternal allele	cg20895511
IGF2 P3-P4_maternal allele	cg16977706
IGF2 P3-P4_maternal allele	cg18087943
IGF2 P3-P4_maternal allele	cg14608156
IGF2 P3-P4_maternal allele	cg12773325
IGF2 P3-P4_maternal allele	cg12614029
IGF2 P3-P4_maternal allele	cg19002337
IGF2 P3-P4_maternal allele	cg24366657
IGF2 P3-P4_maternal allele	cg24917382
IGF2 P3-P4_maternal allele	cg16415340
IGF2 P3-P4_maternal allele	cg21667878
IGF2 P3-P4_maternal allele	cg11915650
IGF2 P3-P4_paternal allele	cg20766090
IGF2 P3-P4_paternal allele	cg02166532
IGF2 P3-P4_paternal allele	cg08162473
IGF2 P3-P4_paternal allele	cg03760951
IGF2 P3-P4_maternal allele	cg20339650
IGF2-H19 ICR1_maternal allele	cg06676088
IGF2-H19 ICR1_maternal allele	cg25763864
IGF2-H19 ICR1_maternal allele	cg09503234
IGF2-H19 ICR1_maternal allele	cg23889607
IGF2-H19 ICR1_maternal allele	cg02719427
IGF2-H19 ICR1_paternal allele	cg26913576
IGF2-H19 ICR1_paternal allele	cg00221747
IGF2-H19 ICR1_paternal allele	cg02045936

Supplementary Table 3. Primers for bisulfite conversion sequencing

Primer	Sequence 5'→3'
FW_Meth_P1	gctagcttggggaagaggtt
RV_Meth_P1	ctaggaggtgggggctatgt
FW_Meth_P3	ccgcctcctcttcatctacc
RV_Meth_P3	gaaggttgcgggagaaaga

Supplementary Table 4. Target sequences of shIGF2

Name	Target sequence
shIGF2#1	ccacaaaagctcagaaattgg
shIGF2#2	ggccattcggaacattggaca
shIGF2#3	tcctggagacgtactgtgcta
Mock shRNA	gcttcgcgccgtagtctta

Supplementary Table 5. List of Taqman probes used for quantitative RT-PCR

Target Gene	Taqman Assay ID or Sequence (Reference)
Human *IGF2*	Hs01005963_m1
Human *IGF2* P1derived	Hs01005962_m1
Human *IGF2* P3-derived	Hs00171254_m1
Human *INSR-A*	5'-TCCCCAGGCCATCT-3' [26]
Human *18S*	Hs99999901_s1
Human *H19*	Hs00262142_g1
Mouse *Igf2*	Mm00580426_g1
Mouse *H19*	Mm01156721_g1
Mouse *Rn18s*	Mm03928990_g1
Human miR-23b	002126
Human miR-483-5p	002338

Supplementary Table 6. *Igf2* and *H19* **expression in mouse models of HCC.** *Igf2* and *H19* mRNA levels were assessed by qRT-PCR in the chemically (DEN+CCl$_4$) induced mouse model and by microarray in 10 other mouse models of HCC with expression datasets available in GEO database (http://www.ncbi.nlm.nih.gov/geo/). FC was normalized to 1 using the mean expression value of the mouse healthy liver. A FC>2 was considered overexpression. Statistical significance between groups is calculated by Mann-Whitney test.

Mouse model	GEO DataSet	% of tumors overexpressing *Igf2* (FC>2)	Mean *Igf2* FC (vs healthy liver)	p-value	% of tumors overexpressing *H19* (FC>2)	Mean *H19* FC (vs healthy liver)	p-value
Chemically induced by DEN+CCl$_4$	Unpublished data	30% (3/10)	106.9	ns	80% (8/10)	84.0	ns
Notch constitutive activation	GSE33486	80% (4/5)	26.8	0.02	80% (4/5)	43.5	0.02
Txnip KO	GSE2127	78% (7/9)	6.0	0.03	100% (9/9)	40.1	0.006
Pdgf-c transgenic	GSE31431	60% (3/5)	2.3	0.02	60% (3/5)	2.3	ns
HBsAg induced HCC	GSE15251	100% (2/2)	110.8	0.02	NA	NA	NA
HBsAg + Aflatoxin B induced HCC	GSE54054	22% (2/9)	3.2	0.02	78% (7/9)	20.8	0.0006
Chemically induced by B6C3F1	GSE26538	67% (4/6)	42.3	ns	100% (6/6)	242.5	0.002
Triple KO (RB, p130, p107)	GSE19004	80 % (4/5)	123.7	ns	100% (5/5)	587.6	0.02
Mdr2-KO + Partial hepatectomy	GSE61422	33% (2/6)	3.8	ns	50% (3/6)	3.0	ns
WHV/*c-myc* transgenic	GSE39401	NA (mix of five tumors)	104.5	NA	NA (mix of five tumors)	601.5	NA
Iqgap2 KO	GSE46646	0% (0/3)	0.4	ns	0% (0/3)	0.5	0.02

Supplementary Table 7. HCCs from mouse injected with *Myc+Akt+Igf2* reproduce the genetic features of human tumors overexpressing IGF2. Association of gene signatures was evaluated using the GSEA Module from GenePattern. Association of signaling pathways was evaluated using Ingenuity Pathway Analysis (www.ingenuity.com)

	Gene signatures associated to IGF2 overexpression in HCC (GSEA)				Signaling pathways associated to IGF2 overexpression in HCC (IPA)	
	Gene signature	**p value**	**FDR**	**NES**	**Signaling pathway**	**p value**
Hepatic progenitor cell-like/ Undifferentiated cancer	VILLANUEVA_LIVER_CANCER_CK19[29]	<0.0001	<0.0001	2.439	Embryonic Development	<0.0001
	YAMASHITA_LIVER_CANCER_WITH_EPCAM_UP[28]	<0.0001	<0.0001	2.332		
	RHODES_UNDIFFERENTIATED_CANCER	<0.0001	<0.0001	2.539		
	ZHANG_BREAST_CANCER_PROGENITORS_UP	<0.0001	<0.0001	2.288		
	BHATTACHARYA_EMBRYONIC_STEM_CELL	<0.0001	<0.0001	2.258		
Hepatoblastoma/ Embryonic liver	CAIRO_LIVER_DEVELOPMENT_UP[31]	0.002	0.04	1.590		
	CAIRO_HEPATOBLASTOMA_UP[31]	<0.0001	0.02	1.663		
Poor prognosis	LEE_LIVER_CANCER_SURVIVAL_DN[35]	<0.0001	<0.0001	2.762		
Proliferation	BOYAULT_LIVER_CANCER_SUBCLASS_G123_UP[25]	<0.0001	0.005	1.809	Cellular growth and proliferation	<0.0001
	CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP[26]	<0.0001	<0.0001	2.580		
	HOSHIDA_LIVER_CANCER_SUBCLASS_S1[27]	<0.0001	0.001	1.914		
	HOSHIDA_LIVER_CANCER_SUBCLASS_S2[27]	0.002	0.02	1.696		
	REACTOME_DNA_REPLICATION	<0.0001	<0.0001	2.708	Cell cycle	<0.0001
	REACTOME_CELL_CYCLE_MITOTIC	<0.0001	<0.0001	2.655		
	REACTOME_CELL_CYCLE_CHECKPOINTS	<0.0001	<0.0001	2.322		
Invasiveness	SARRIO_EPITHELIAL_MESENCHYMAL_TRANSITION_UP	<0.0001	<0.0001	2.552		
	GOTZMANN_EPITHELIAL_TO_MESENCHYMAL_TRANSITION_UP[33]	<0.0001	0.007	1.782		
	JECHLINGER_EPITHELIAL_TO_MESENCHYMAL_TRANSITION_UP	0.005	0.03	1.614		
	ALONSO_METASTASIS_EMT_UP	<0.0001	0.003	1.855		
	KAPOSI_LIVER_CANCER_MET_UP	0.02	0.02	1.687		
	ROESSLER_LIVER_CANCER_METASTASIS_UP	<0.0001	0.03	1.631		
	WANG_TUMOR_INVASIVENESS_UP	<0.0001	<0.0001	2.205		
Angiogenesis	ABE_VEGFA_TARGETS_2HR	<0.0001	<0.05	1.549		

Supplementary Table 8. List of genes potentially regulated by IGF2 in HCC cell lines.
Comparative Marker Selection Module from GenePattern was applied to compare the genetic profile of Hep3B-shIGF2 vs Hep3B-shMock and SNU449-IGF2 vs SNU449-Control. Only genes with FDR<0.05, FC>2 and common to both conditions were selected.
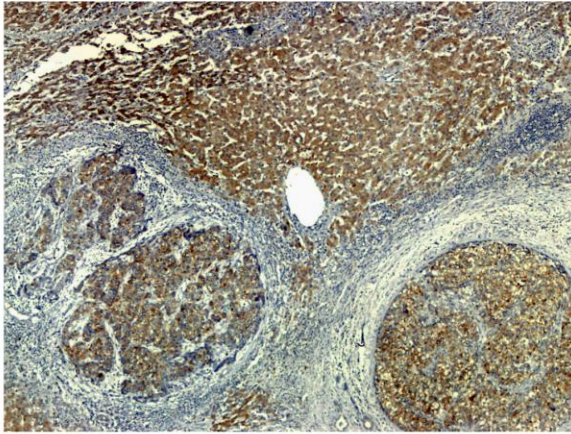
ANPEP	MIS18A	ACP2	FEZ2	PBX3
TAF9B	PAPSS1	ARHGEF38	CDCA5	ADAM15
NUP54	CDK10	FAM46B	CDCA7	ARL6IP1
NUP107	EXOG	SMCO4	USP7	FYTTD1
PRKAG2	MED20	RAE1	EED	PGLYRP4
CENPN	QSOX1	NDUFAF3	C7orf49	LIG3
SDC1	TDG	FGD5	GLG1	FAU
DBNL	DENR	RFC3	TMEM14A	MMAB
SART3	DZIP3	XXYLT1	CDCA4	TBCD
RPN2	NFKBIA	MRPL49	HACD3	MOV10
ESRP2	MCM2	RFC5	SNRPF	NKX3-1
PDIA4	PLAUR	TPMT	ARMC5	HS3ST1
LYSMD1	TTC3P1	PTPN1	PACSIN3	ZNF572
CMTM6	ATXN7L2	KDELC1	SGK2	FADD
ABCF1	IFI30	BATF	ALDH18A1	CXXC4
LSM6	GID8	C6orf89	COL5A2	TLDC1
GALNT2	FUOM	CCNE1	PSRC1	GRHL1
EFEMP1	CYP2R1	OS9	UBE2D3	EXOSC9
TOR1AIP1	CREG1	ARL2BP	EZH2	PIGS
NOP56	ZNF764	NAP1L3	RNF146	PRMT3
ALDH1A3	BACE2	PIK3CD	POGK	CASP3
STK26	WDR77	ANTXR2	MPDU1	MPHOSPH9
TROAP	PRCP	OAZ2	MSL2	PDSS2
SLC44A1	GINS1	PLEKHG6	ARF5	APIP
THOC6	SIX4	TMEM132E	COX19	DGKZ
CFLAR	E2F7	SOAT1	SENP1	MEST
ANXA10	RBM15B	CDYL	TCAIM	USP13
PSMD2	DOLK	SCAMP3	MYBL2	LARS2
OTUD7B	SCAF4	ARHGEF26	S100A16	CAPRIN2
SPATA2	RNMTL1	CPXM2	FTSJ2	SCPEP1
NFYA	C2CD5	FLII	NME4	POU3F3
CPM	HLA-F	TRA2B	FAM133A	CDK4
SFXN2	TACC3	PPP1R1A	FAM111B	GLYCTK
UNG	SSR2	RNF219	GLIPR1	LHFP
SLC38A3	ARMC8	CENPW	SRRT	TMEM256
CXCL1	EXT2	PRELP	CANT1	VTA1
MPP5	TAF10	SGK1	PCOLCE2	RIC8A

Supplementary Table 9. Top Canonical Pathways regulated by IGF2

| Canonical Pathway	p-value
Mammalian embryonic stem cell pluripotency	1.37E-03
Cellular growth and proliferation	2.16E-03
Cell death and survival	2.21E-03
IGF-1 Signaling	6.09E-04

A

HCC

HCC

Cirrhotic adjacent tissue

Non-cirrhotic adjacent tissue

B

(3) fibroblast

(1) Hepatocyte

(2) Endothelial cell

(4) limphocyte

(5) Bile duct epithelial cell
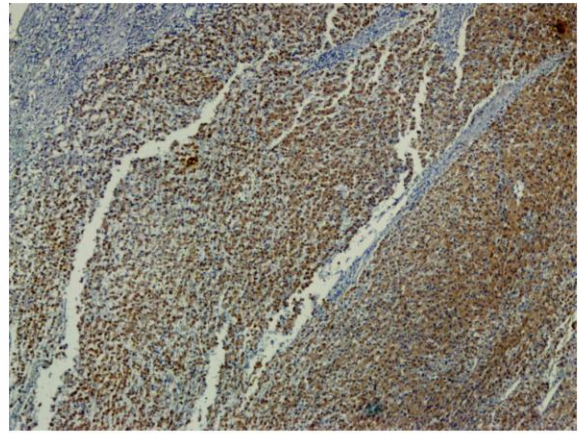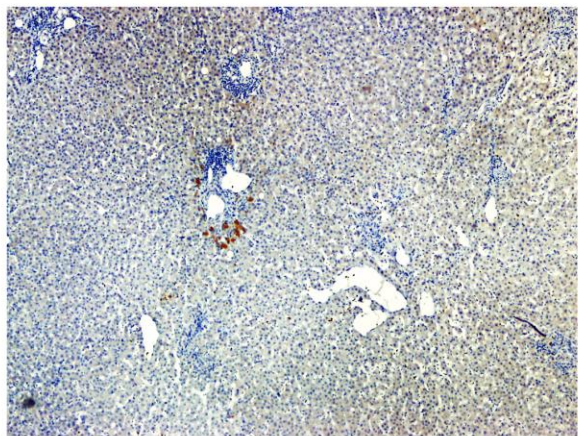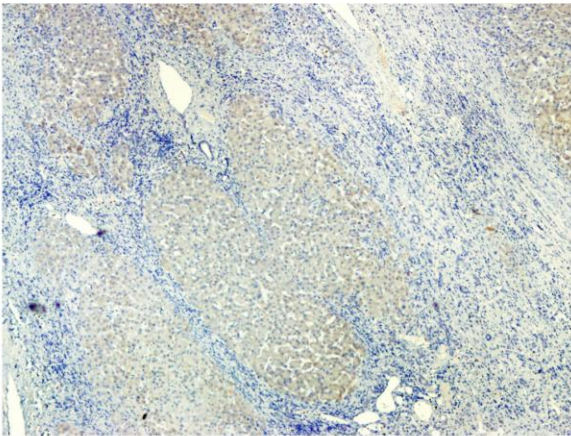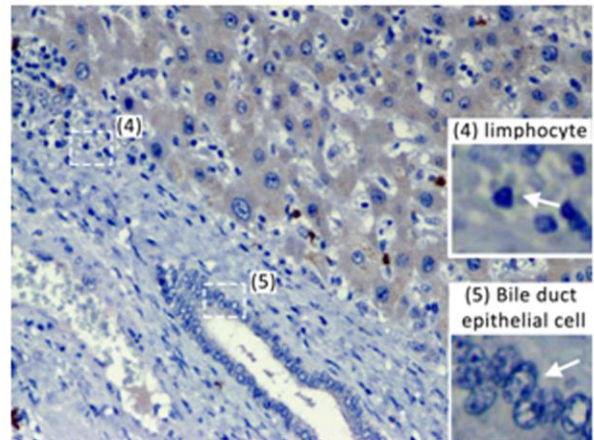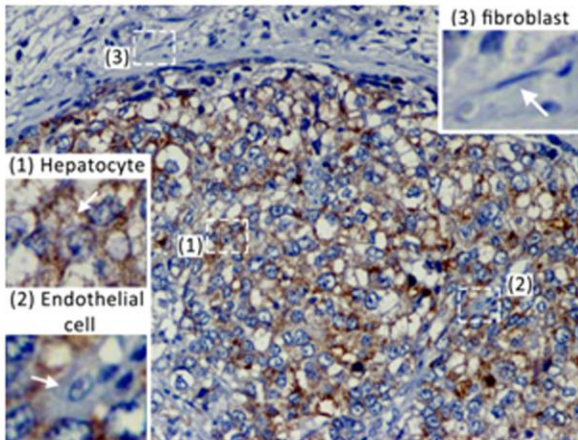
Supplementary Figure 1. IGF2 immunostaining in HCC human tumors. (A) Representative images of IGF2 protein levels in tumor and adjacent cirrhotic and non-cirrhotic tissue. **(B)** Cell types producing IGF2 in HCC tumors.
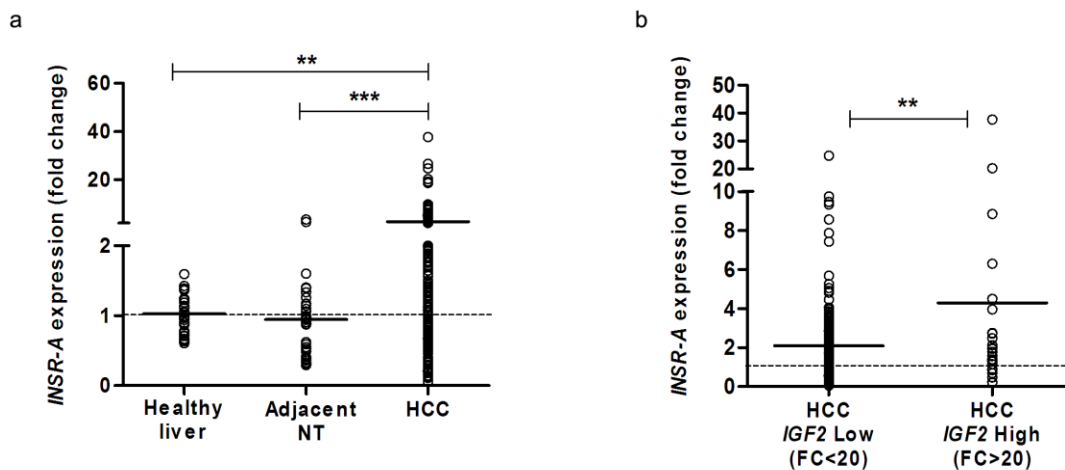
Supplementary Figure 2. Overexpression of *INSR-A* in HCC. (A) *INSR-A* isoform levels determined by quantitative RT-PCR in healthy liver samples ($n = 10$), adjacent non-tumor (NT) tissue ($n = 47$) and HCC tumors ($n = 228$). Dots represent the expression value of each individual sample and the line is the mean value of each group. Fold change is normalized to 1 (mean expression value in healthy liver). Overexpression of *INSR-A* was defined as >2-fold. Statistical significance between groups is calculated by Kruskal-Wallis with Dunn's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001. **(B)** *INSR-A* isoform levels determined by quantitative RT-PCR in HCC samples with low ($n = 194$) or high ($n = 34$) *IGF2* expression. Dots represent the expression value of each individual sample and the line is the mean value of each group. Fold change is normalized to 1 (mean expression value in healthy liver). Statistical significance between groups is calculated by Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001.
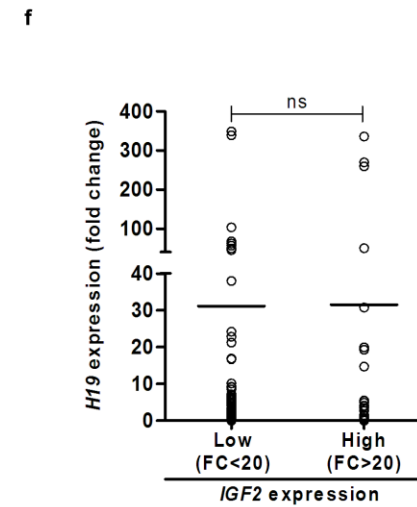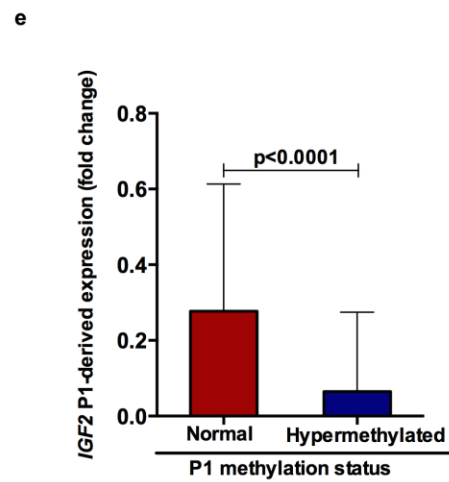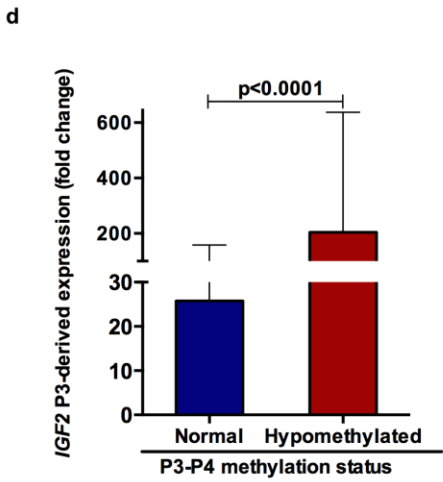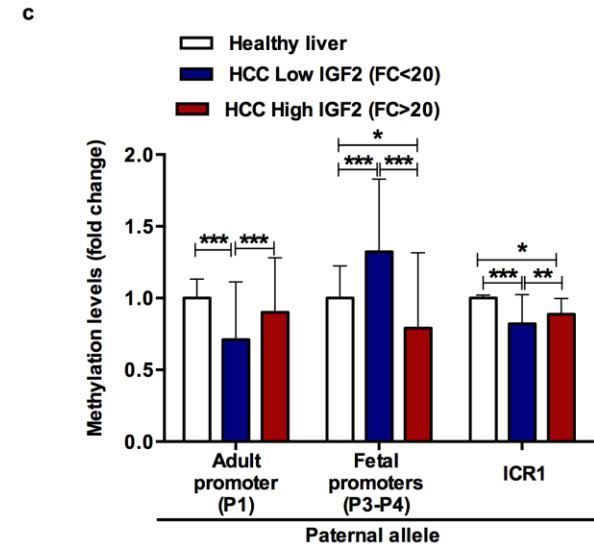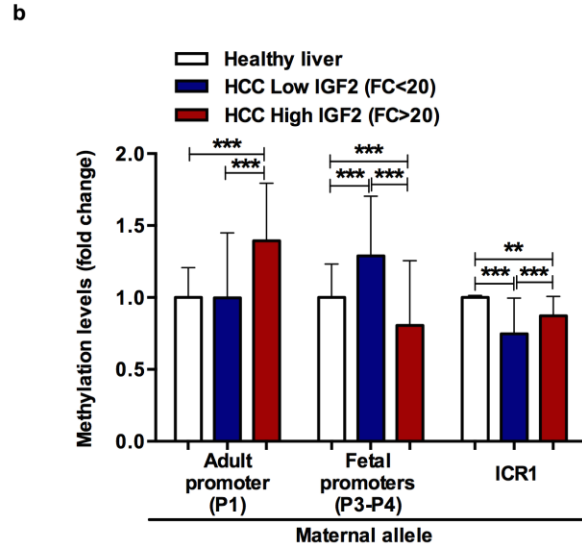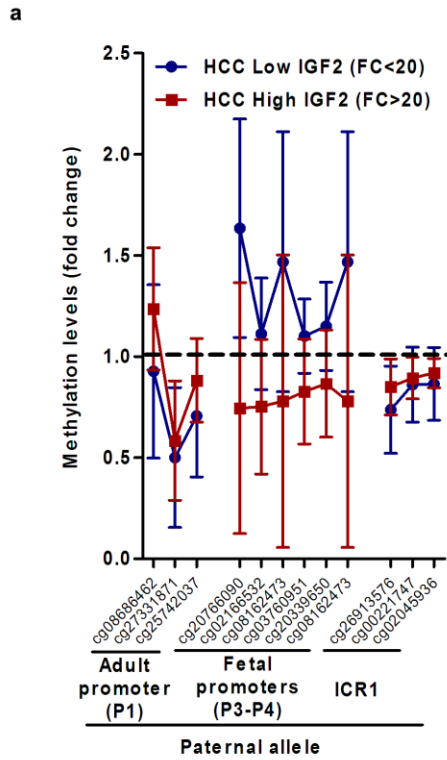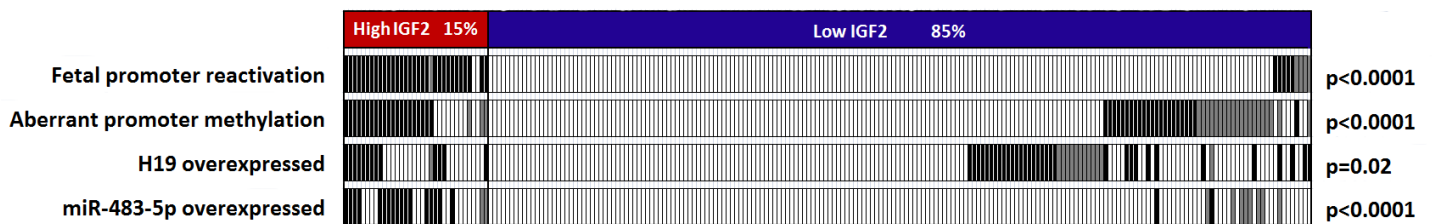
Supplementary Figure 3. **Aberrant methylation pattern in *IGF2* promoters is associated with *IGF2* overexpression in human HCC samples.** **(A)** Methylation levels measured by methylome array in CpGs of the paternal allele located within adult promoter (P1), fetal promoters (P3-P4) and the *IGF2/H19* imprinting locus (ICR1) in 200 HCC samples expressing low (blue; $n = 173$) or high (red; $n = 27$) *IGF2* levels. Dots represent the mean value in each CpG and bars the SD. between samples. Fold change is normalized to 1 (mean expression value in healthy liver). **(B)** Methylation levels measured by methylome array in CpGs located within adult promoter (P1), fetal promoters (P3-P4) and the *IGF2/H19* imprinting locus (ICR1) of the maternal allele in 10 healthy liver samples and 200 HCC samples expressing low ($n = 173$) or high ($n = 27$) *IGF2* levels. Fold change is normalized to 1 (mean expression value in healthy liver). Statistical significance between groups is calculated by Kruskal-Wallis with Dunn's multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(C)** Methylation levels measured by methylome array in CpGs located within adult promoter (P1), fetal promoters (P3-P4) and the *IGF2/H19* imprinting locus (ICR1) of the paternal allele in 10 healthy liver samples and 200 HCC samples expressing low ($n = 173$) or high ($n = 27$) *IGF2* levels. Fold change is normalized to 1 (mean expression value in healthy liver). Statistical significance between groups is calculated by Kruskal-Wallis with Dunn's multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(D)** *IGF2* expression levels derived from fetal promoter P3 measured by quantitative RT-PCR in HCC samples with hypomethylation or normal methylation in fetal promoters (P3-P4). Fold change is normalized to 1 (mean expression value in healthy liver). Error bars are mean ± SD in all panels. Statistical significance between groups is calculated Mann-Whitney test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(E)** *IGF2* expression levels derived from adult promoter P1 measured by quantitative RT-PCR in HCC samples with normal methylation

or hypermethylation in P1. Fold change is normalized to 1 (mean expression value in healthy liver). Error bars are mean ± SD. in all panels. Statistical significance between groups is calculated Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001. **(F)** *H19* levels determined by quantitative RT-PCR in HCC tumors and classified in low (*n* = 194) and high (*n* = 34) *IGF2* expression. Dots represent the expression value of each individual sample and the line is the mean value of each group. Fold change is normalized to 1 (mean expression value in healthy liver). Statistical significance between groups is calculated by Mann-Whitney test. ns; non-significant.
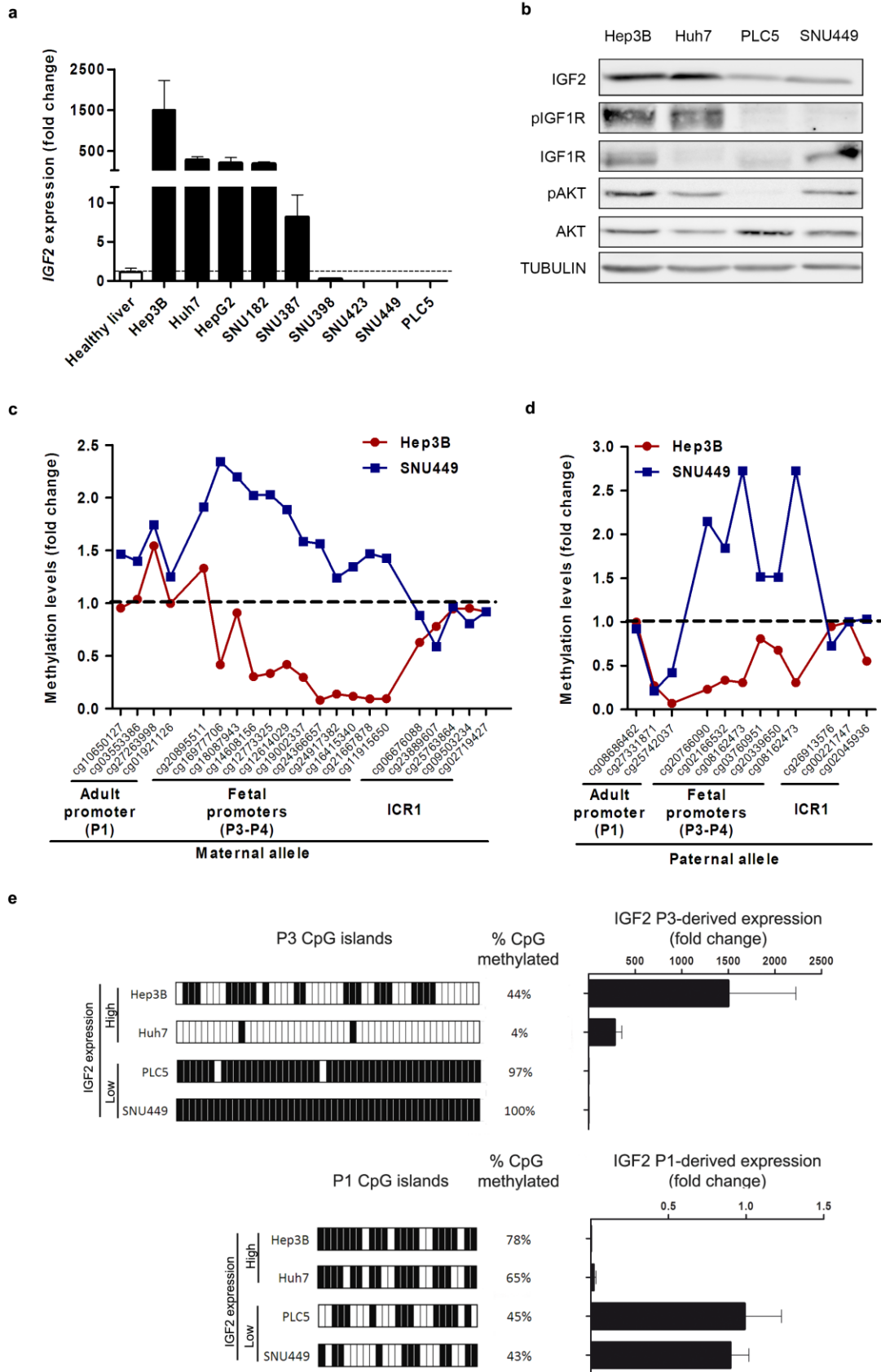
Supplementary Figure 4. Aberrant methylation pattern in *IGF2* promoters is associated with *IGF2* overexpression in HCC cell lines. (A) *IGF2* levels determined by quantitative RT-PCR in different human HCC cell lines. Fold change is normalized to 1 (mean expression value in healthy liver). Error bars are mean ± SD. corresponding to ≥3 experiments in triplicate. **(B)** Representative Western Blot analysis of IGF2 and consequent IGF1R and downstream pathway activation in HCC cell lines with high (Hep3B and Huh7) or low (PLC5 and SNU449) IGF2 expression. Tubulin was used as a loading control. **(C)** Methylation levels measured by methylome array in CpGs of the maternal allele located within adult promoter (P1), fetal promoters (P3-P4) and the *IGF2/H19* imprinting locus (ICR1) in Hep3B (red; high *IGF2*) or SNU449 (blue; low *IGF2*) cells. Dots represent the mean value in each CpG. Fold change is normalized to 1 (mean expression value in healthy liver). **(D)** Methylation levels measured by methylome array in CpGs of the paternal allele located within adult promoter (P1), fetal promoters (P3-P4) and the *IGF2/H19* imprinting locus (ICR1) in Hep3B (red; high *IGF2*) or SNU449 (blue; low *IGF2*) cells. Dots represent the mean value in each CpG. Fold change is normalized to 1 (mean expression value in healthy liver). **(E)** *Left panel:* Methylated (black) and unmethylated (white) CpGs in P3 fetal promoter and P1 adult promoter in HCC cell lines with high (Hep3B, Huh7) and low (SNU449, PLC5) *IGF2* levels. Methylation status of CpGs was analyzed by bisulfite sequencing. *Right panel: IGF2* expression derived from P3 or P1 promoters determined by quantitative RT-PCR in human HCC cell lines. Error bars are mean ± SD. corresponding to ≥3 experiments in triplicate.
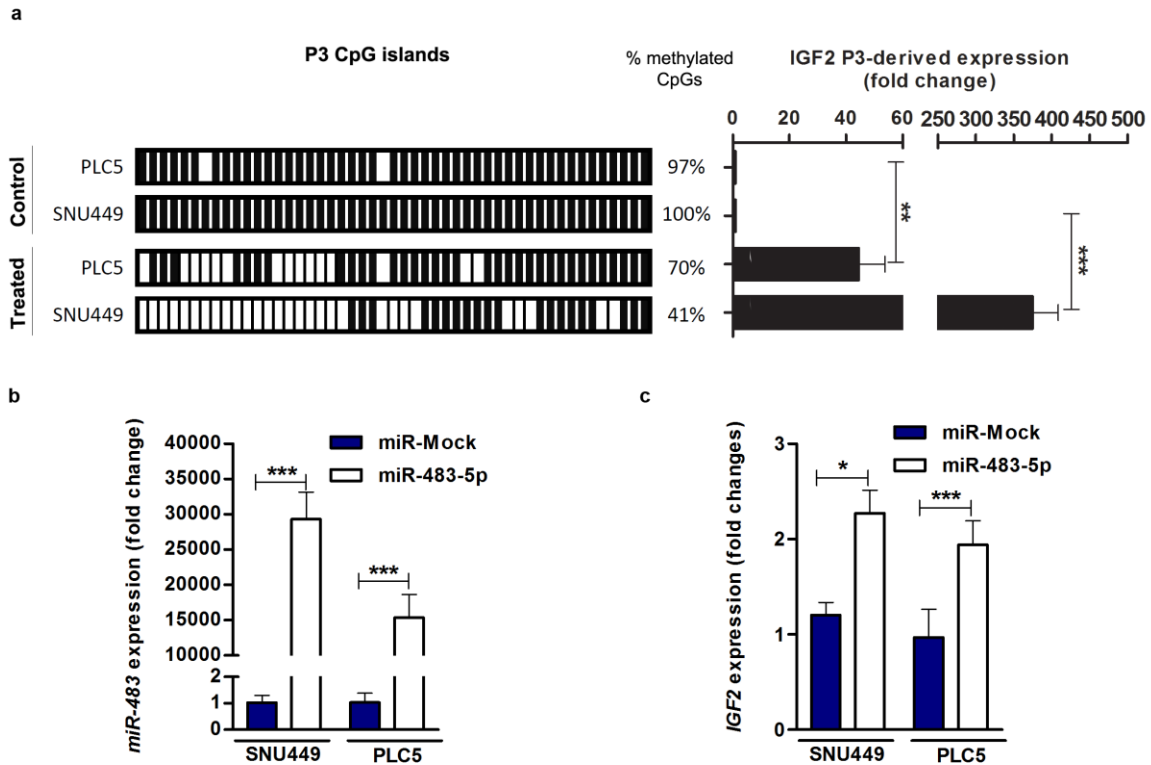
Supplementary Figure 5. Demethylation of *IGF2* fetal promoters leads to their reactivation and *IGF2* overexpression in HCC cell lines. **(A)** *Left panel:* Methylated (black) and unmethylated (white) CpGs in P3 fetal promoter in HCC cell lines with low *IGF2* levels (SNU449, PLC5) treated or not with 10 μmol/L of the demethylating agent decitabine. Methylation status of CpGs was analyzed by bisulfite sequencing. *Right panel: IGF2* expression derived from P3 promoter determined by quantitative RT-PCR in human HCC cell lines with low *IGF2* levels (SNU449, PLC5) treated or not with 10 μmol/L of the demethylating agent decitabine. **(B)** Expression of *miR-483-5p* measured by quantitative RT-PCR in SNU449 (left) or PLC5 (right) cells transfected with Mock miRNA or miR-483-5p. Fold change is normalized to 1 (mean expression value in the wild-type cell line). **(C)** Expression of *IGF2* measured by quantitative RT-PCR in SNU449 (left) or PLC5 (right) cells transfected with Mock miRNA or miR-483-5p. Fold change is normalized to 1 (mean expression value in the wild-type cell line).

Error bars are mean ± SD. corresponding to ≥3 experiments in triplicate. Statistical significance between groups is calculated by two-sided t-test. *p<0.05, **p<0.01, ***p<0.001.


Supplementary Figure 6. Epigenetic deregulations affecting the 228 tumors in our cohort. Overexpression of *IGF2* from fetal promoters, as well as aberrant promoter methylation, and H19 and miR-483-5p overexpression, were all significantly associated with IGF2 overexpression (p<0.0001 in all cases, except for H19 which was p=0.02). Statistical significance is calculated by χ^2 test.
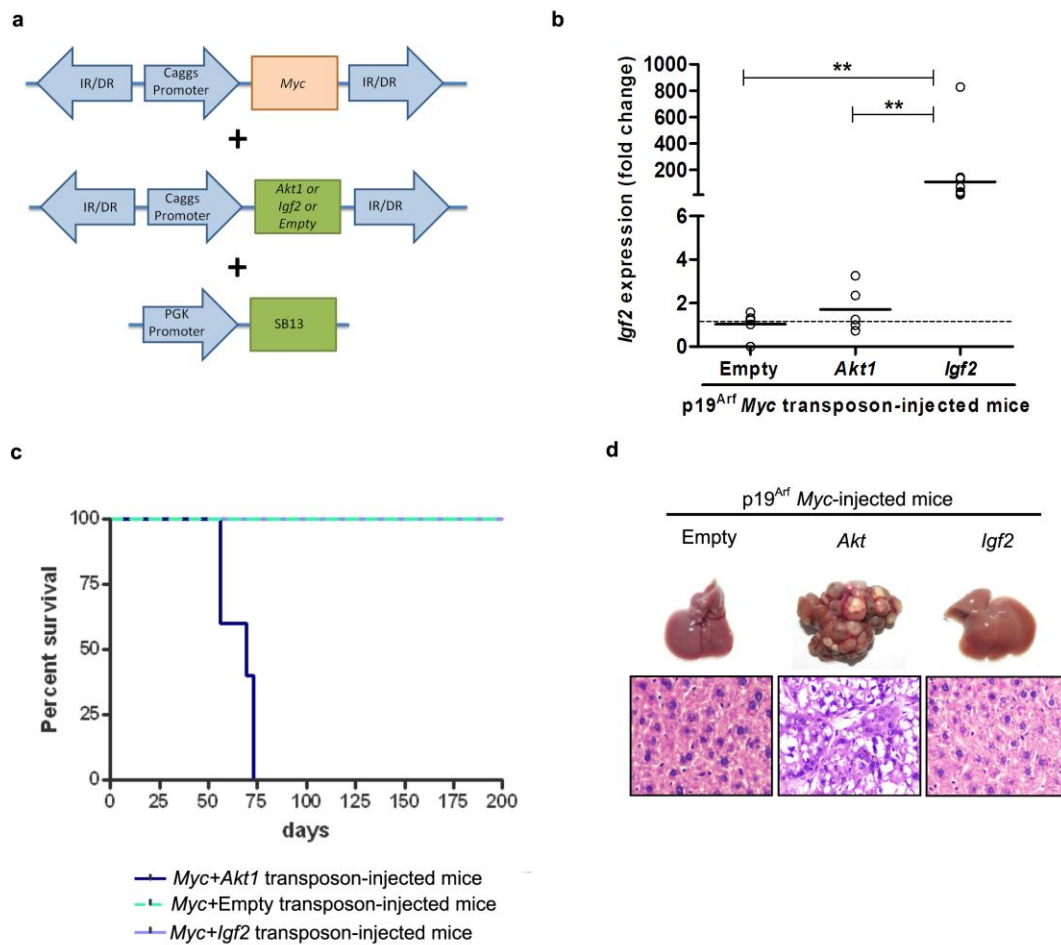
Supplementary Figure 7. *Igf2* **overexpression alone is not able to initiate hepatocarcinogenesis in** *in vivo* **models.** **(A)** Schematic representation of transposable elements encoding *Myc* and *Igf2, Akt1* or an Empty vector. Caggs, CAGGS promoter; IR/DR, inverted repeats and direct repeats; IRES, internal ribosome entry site. **(B)** *Igf2* expression levels measured by qRT-PCR in livers upon intrahepatic delivery of *Myc*+Empty vector (Negative Control; $n = 5$), *Myc*+*Akt1* (Positive control; $n = 6$) and *Myc*+*Igf2* ($n = 6$). Dots represent the expression value of each individual sample and line is the mean value of each group. Fold change is normalized to 1 (mean expression value in

livers of Negative control-transposon injected mice). Statistical significance between groups is calculated by Kruskal-Wallis with Dunn's multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(C)** Survival analysis (Kaplan-Meier) of p19$^{Arf-/-}$ mice upon intrahepatic delivery of *Myc*+Empty vector (Negative Control; $n = 5$), *Myc+Akt1* (Positive control; $n = 6$) and *Myc+Igf2* constructs ($n = 6$). For survival analysis, mice were censored at the time of sacrifice according to IACUC guidelines. Statistical significance between groups is calculated by log-Rank test. **(D)** Representative images and H&E staining of intrahepatic tumor burden 200 days after delivery of *Myc*+Empty vector, *Myc+Akt* or *Myc+Igf2* into livers of p19$^{Arf-/-}$ mice.
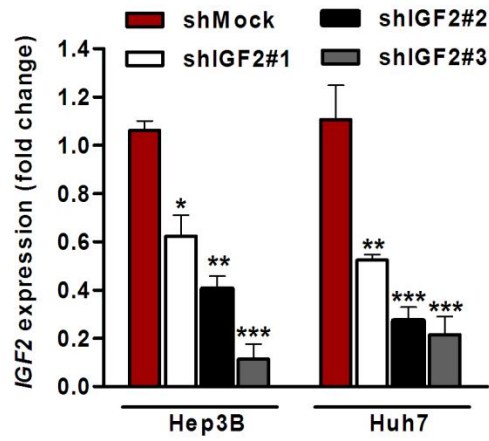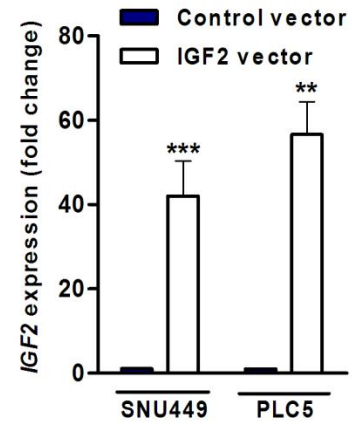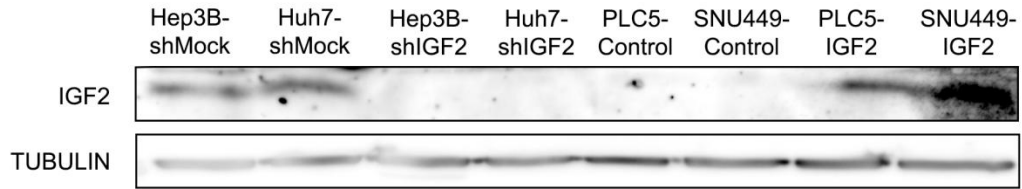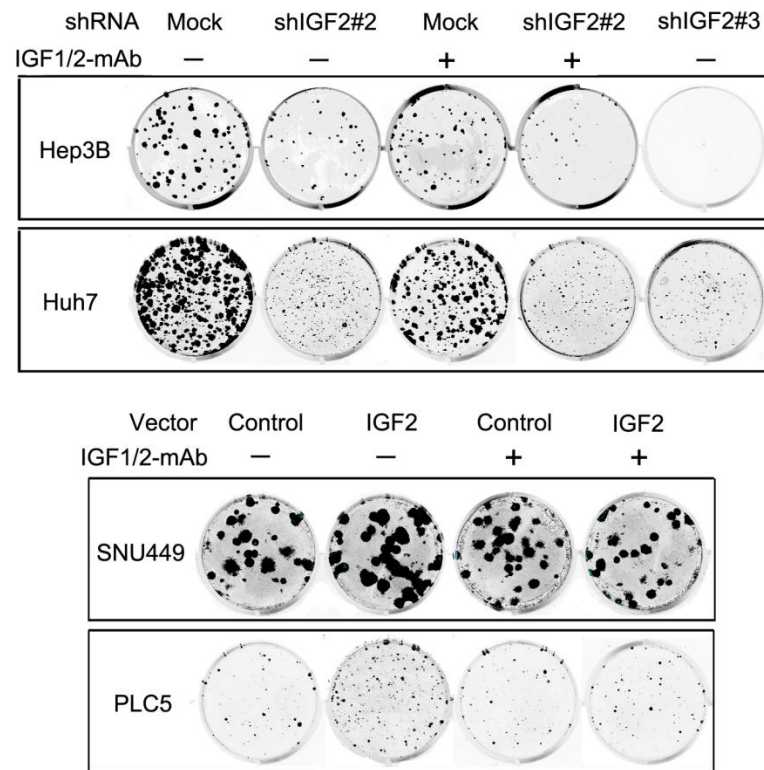
Supplementary Figure 8. Effects of IGF2 on HCC cell proliferation. (A) Expression of *IGF2* measured by quantitative RT-PCR in Hep3B (left) or Huh7 (right) cells stably transfected with Mock shRNA or three different shIGF2**.** Fold change is normalized to 1 (mean expression value in the wild-type cell line). Statistical significance between groups is calculated by one-way ANOVA with post-hoc Bonferroni test. *p<0.05, **p<0.01, ***p<0.001. **(B)** Expression of *IGF2* measured by quantitative RT-PCR in SNU449 (left) or PLC5 (right) cells stably transfected with a Control vector or an IGF2-overexpression vector. Fold change is normalized to 1 (mean expression value in the wild-type cell line). Statistical significance between groups is calculated by one-way ANOVA with post-hoc Bonferroni test. *p<0.05, **p<0.01, ***p<0.001. **(C)** Representative Western Blot analysis of IGF2 in cells stably transfected with Mock shRNA or a shIGF2 (Hep3B and Huh7) and cells stably transfected with a Control vector or an IGF2-overexpression vector (PLC5 and SNU449). Tubulin was used as a loading control. **(D)** Representative image of a colony formation assay (crystal violet staining) using Hep3B or Huh7 cell lines stably transfected with Mock shRNA or two different shIGF2, and treated with the IGF1/2-mAb *(upper panel)* or SNU449 or Huh7 cell lines stably transfected with a Control vector or a IGF2-overexpression construct, and treated with IGF1/2-mAb *(lower panel).*
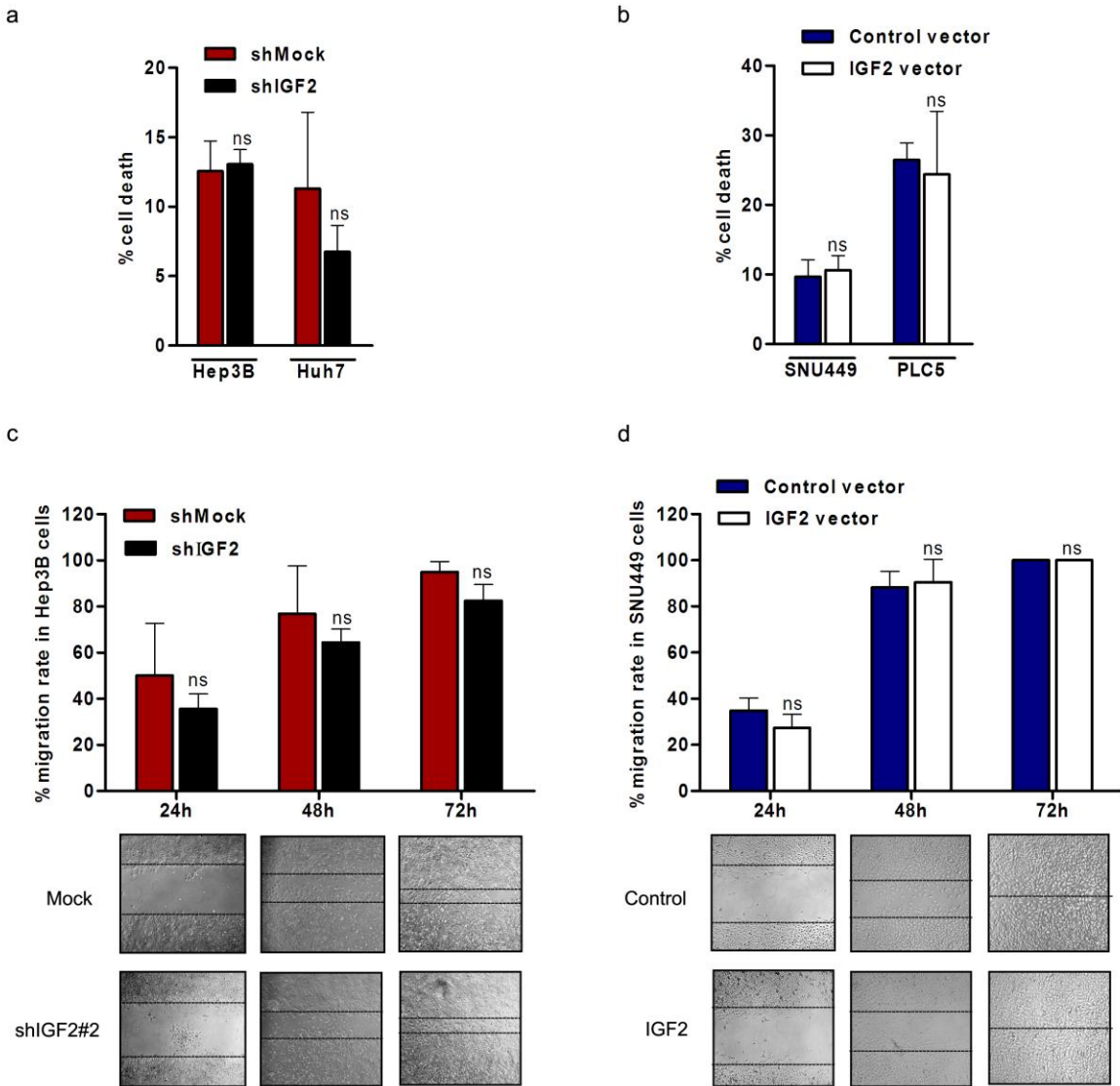
Supplementary Figure 9. IGF2 has no effect in cell death and migration of HCC cells.

(A) Percentage of cell death measured by FACS in Hep3B (left) or Huh7 (right) cells stably transfected with Mock shRNA or a shIGF2. **(B)** Percentage of cell death measured by FACS in SNU449 (left) or PLC5 (right) cells stably transfected with a Control vector or an IGF2-overexpression construct. **(C)** *Upper panel*: Migration rates in Hep3B-Mock and Hep3B-shIGF2 are represented as the percentage of the initial wound surface covered by cells 24, 48 and 72h after injury. *Lower panel*: representative images of each time point of

the migration assay. **(D)** *Upper panel*: Migration rates in SNU449-Control vector and SNU449-IGF2 24, 48 and 72h after injury. *Lower panel*: representative images of each time point of the experiment. Error bars are mean ± SD. corresponding to ≥3 experiments in triplicate. Statistical significance between groups is calculated by two-sided t-test. ns, non-significant.
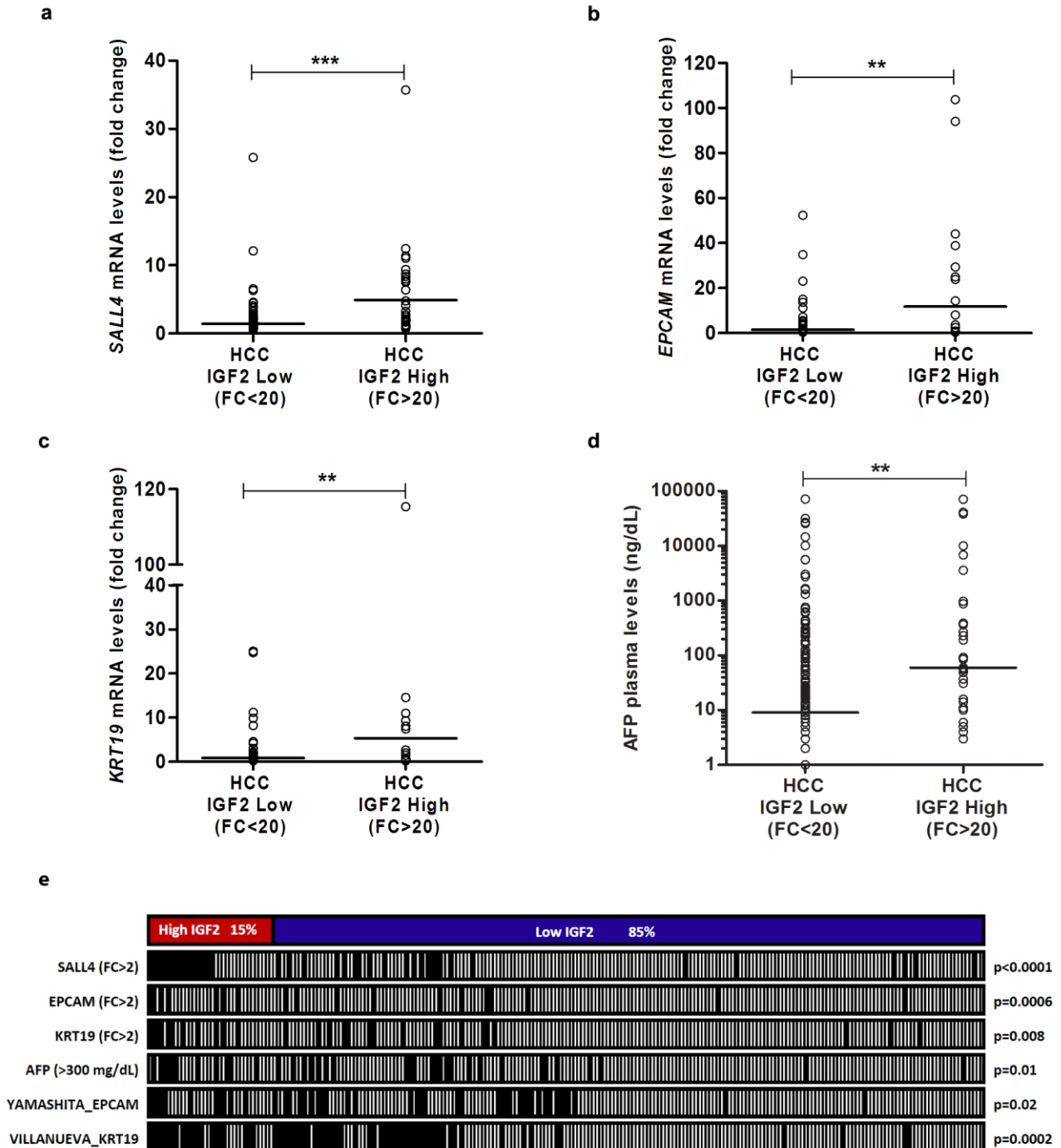
Supplementary Figure 10. Upregulation of hepatic progenitor cell markers and increased plasma AFP levels are associated with *IGF2* re-expression in HCC. (A) *SALL4*, **(B)** *EPCAM* and **(C)** *KRT19* mRNA levels were determined by expression array and **(D)** Alpha fetoprotein levels (AFP) were detected in plasma of HCC patients with low

($n = 194$) and high ($n = 34$) *IGF2* tumor expression. Dots represent the expression value of each individual sample and the line is the mean value of each group. Fold change is normalized to 1 (mean expression value in healthy liver). Statistical significance between groups is calculated by Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001. **(E)** Schematic representation of the overexpression (defined as >2 fold) of hepatic progenitor markers SALL4, EPCAM and KRT19; high plasma levels of alpha fetoprotein (AFP; defined as >300 ng/dL); and genomic signatures of EPCAM[24] and KRT19[25] markers associated with IGF2 reactivation. Statistical significance is calculated by χ^2 test.
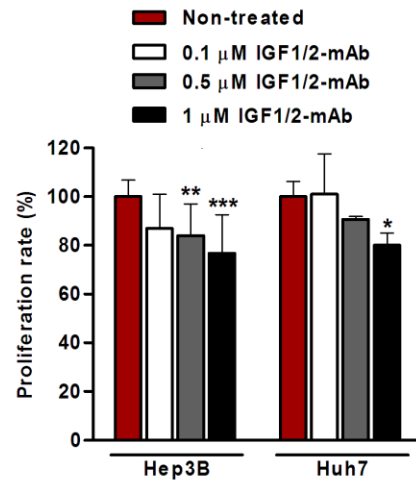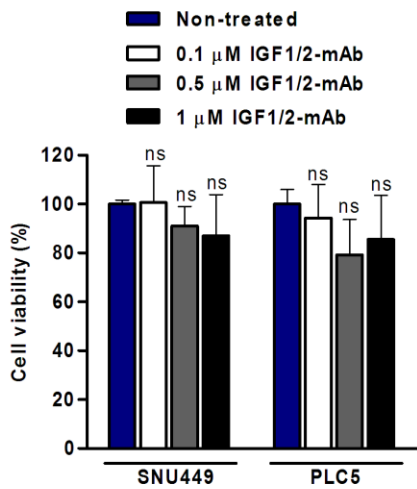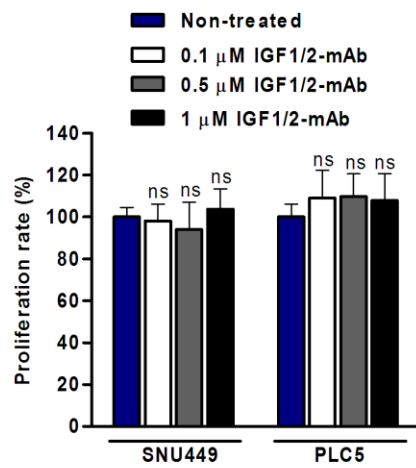
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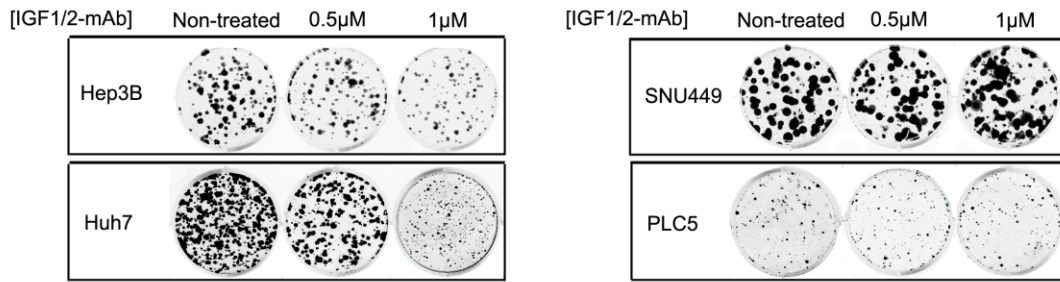
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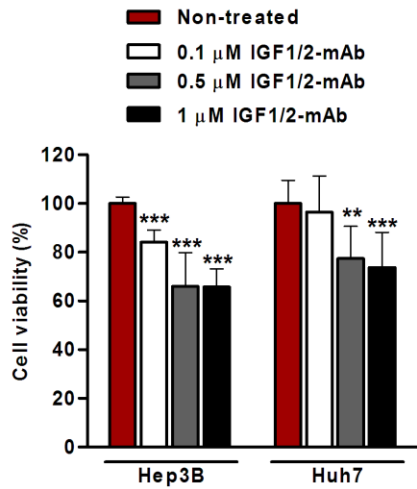
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Supplementary Figure 11. Anti-proliferative effects of IGF1/2-mAb in HCC cell lines overexpressing IGF2. (A) Representative image of a colony formation assay (crystal violet staining) using Hep3B and Huh7 cell lines (high IGF2) or SNU449 and PLC5 (low IGF2) cell lines treated with different concentrations of IGF1/2-mAb. **(B)** Cell viability of Hep3B and Huh7 cell lines (high IGF2) treated with IGF1/2-mAb for 48h at different concentrations. **(C)** Cell proliferation rate of Hep3B and Huh7 cell lines (high IGF2) treated with IGF1/2-mAb for 48h at different concentrations. **(D)** Cell viability of SNU449 and PLC5 cell lines (low IGF2) treated with IGF1/2-mAb for 48h at different concentrations. **(E)** Cell proliferation rate of SNU449 and PLC5 cell lines (low IGF2) treated with IGF1/2-mAb for 48h at different concentrations. Error bars are the percentage normalized to non-treated control ± SD. corresponding to ≥3 experiments in triplicate. Statistical significance between groups is calculated by one-way ANOVA with post hoc Bonferroni test. *p<0.05, **p<0.01, ***p<0.001, ns; non-significant.
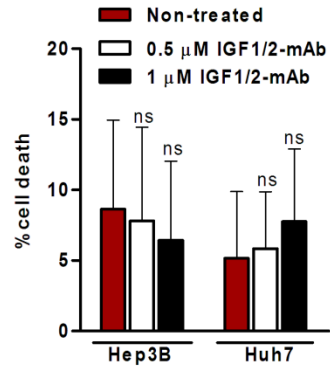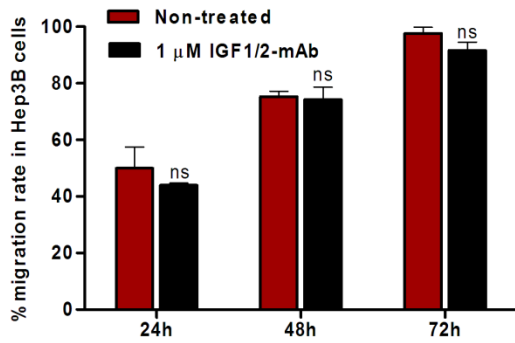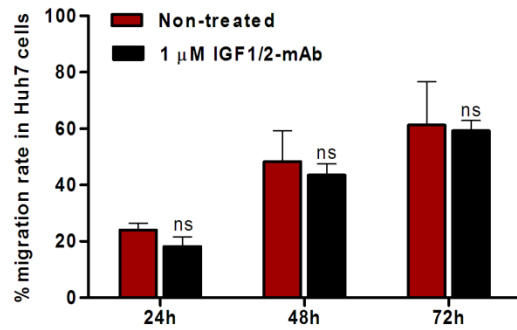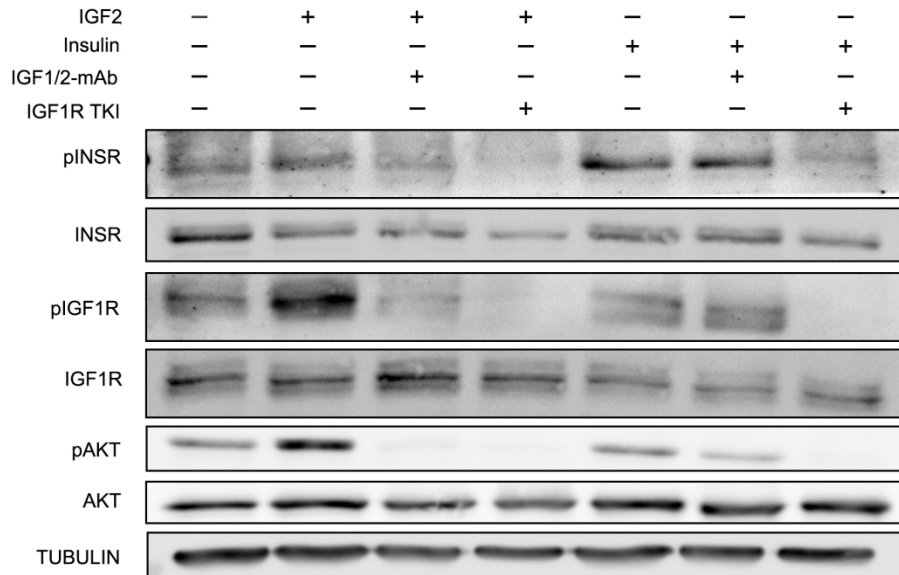
Supplementary Figure 12. IGF1/2-mAb has no effects in cell death and migration of HCC cells. (A) Percentage of cell death measured by FACS in Hep3B (left) or Huh7 (right) cells treated with 1 µmol/L of IGF1/2-mAb. **(B)** *Upper panel*: Migration rates in Hep3B treated or not with 1 µmol/L of IGF1/2-mAb are represented as the percentage of the initial wound surface covered by cells 24, 48 and 72h after injury. *Lower panel*: representative images of each time point of the migration assay. **(C)** *Upper panel*: Migration rates in Huh7 treated or not with 1 µmol/L of IGF1/2-mAb are represented as the percentage of the initial wound surface covered by cells 24, 48 and 72h after injury. *Lower panel*: representative images of each time point of the migration assay. Error bars are mean ± SD. corresponding to ≥3 experiments in triplicate. Statistical significance between groups is calculated by two-sided t-test. ns, non-significant. **(D)** Representative Western Blot analysis of Huh7 (high *IGF2*) cells stimulated by IGF2 or Insulin and treated with IGF1R TKI or IGF1/2-mAb for 15 min. Tubulin was used as a loading control.
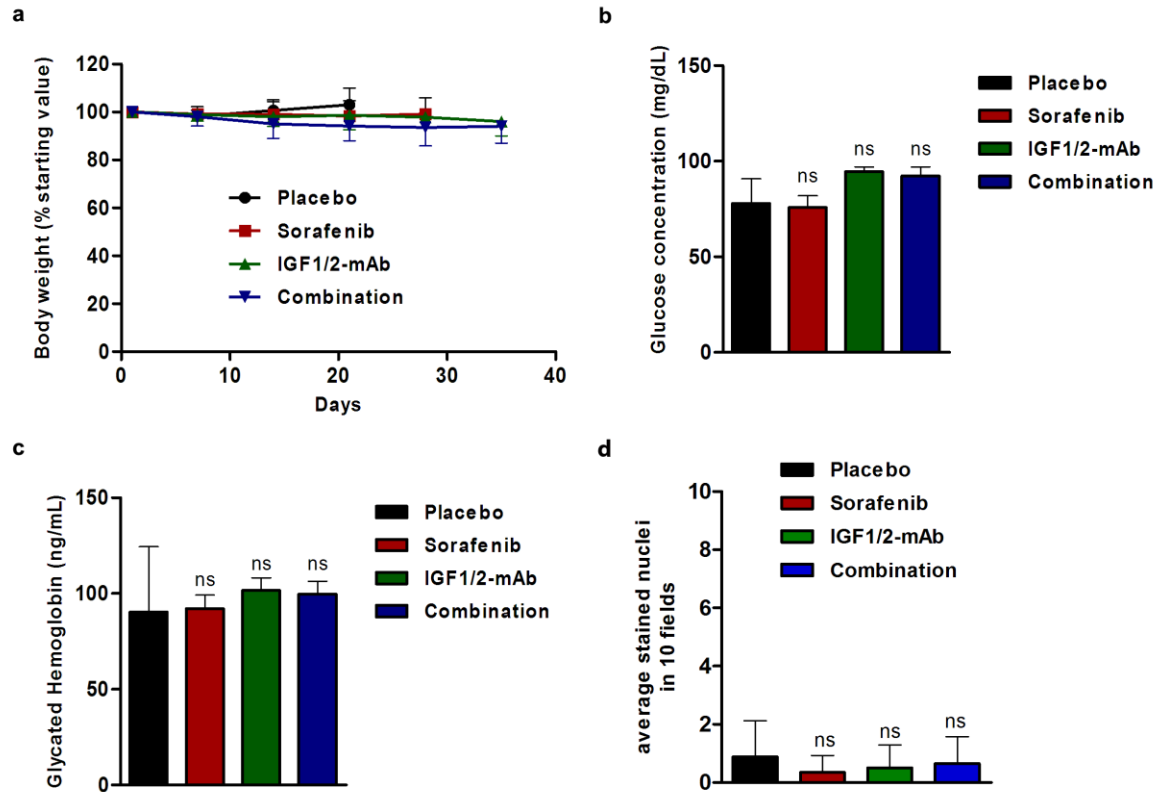
Supplementary Figure 13. IGF1/2-mAb and its combination with sorafenib are well tolerated in HCC xenograft model. (A) Mean mice body weight in each treatment group over time. **(B)** Glucose levels on tail vein blood measured after three weeks of treatment. **(C)** Concentration of glycated hemoglobin in whole blood samples assessed before sacrifice. **(D)** Quantification of apoptosis events by TUNEL assay in 10 fields (40x magnification). Statistical significance between vehicle ($n = 8$), sorafenib ($n = 13$), IGF1/2-mAb ($n = 12$) and combination ($n = 13$) is calculated by one-way ANOVA with post hoc Bonferroni test. ns, non-significant.

Supplementary Materials and Methods

Genomic profiling and data analysis

All samples used for the genomic profiling were fresh-frozen. RNA and DNA were extracted as previously described[7]. Human and mouse transcriptomic profiling were conducted using the Human Genome U219 Array Plate (Affymetrix, Santa Clara, CA) and the MouseWG-6 v2.0 (Illumina, San Diego, CA), respectively. mRNA levels were additionally measured by quantitative RT-PCR. miRNA profiling of 218 samples was conducted using the GeneChip miRNA 2.0 Array (Affymetrix).

Processing of transcriptome data (i.e., normalization, background correction, and filtering) was conducted as previously reported.[25] Genes and miRNAs differentially expressed in HCC tumors with high levels of *IGF2* (>20-fold, FDR<0.05 in human samples; >2-fold, FDR<0.05 in cell lines) were identified through the Comparative Marker Selection module of Gene Pattern (www.broadinstitute.org), and later submitted to Ingenuity Pathway Analysis (IPA) (www.ingenuity.com). To provide further biological insight on samples with high *IGF2* levels, we used the Nearest Template Prediction (NTP) and Gene Set Enrichment Analysis (GSEA) modules of Gene Pattern. All gene signatures analyzed were already reported in the Molecular Signature Database (www.broadinstitute.org/gsea/msigdb). Microarray data were deposited in Gene Expression Omnibus database with the accession numbers GSE63898, GSE56588, GSE74618 and GSE85274.

Methylome profiling

Methylome profiling and data analysis from the human cohort and HCC cell lines was performed by using the Illumina Methylation platform 450K as previously described.[7] To study the differential methylation in *IGF2* between HCC and normal liver tissue, we used array probes located at the fetal (P3-P4) and adult (P1) promoters of the *IGF2* gene and the ICR1 (CTCF-binding site) according to Ensembl Genome Browser (Ensembl.org). Methylation probes located within the ICR1 and *IGF2* promoters are listed in the **Table S2.** Hypomethylation and hypermethylation were defined as the mean fold-change compared to healthy liver samples ± 2 SD.

Additionally, DNA methylation in HCC cell lines was evaluated through bisulfite conversion sequencing. 500 ng of DNA were converted using the Epitect® Fast DNA Bisulfite Kit (Qiagen, Valencia, CA), following the manufacturer's recommendations. Primer sets for PCR amplification and sequencing are summarized in **Table S3.** PCRs were performed under standard conditions, PCR products were sequenced by Sanger (Beckman Coulter Inc, Brea, CA) and results were analyzed using the Mutation Surveyor® software (SoftGenetics, State College, PA).

Decitabine demethylation treatment

PLC5 and SNU449 cell lines were treated with 10 μmol/L of decitabine (Sigma, St. Louis, MO) for 96h adding fresh drug daily. After 96h, DNA and RNA were extracted as previously described[7] and methylation status of IGF2 promoters and mRNA IGF2 expression was assessed.

Cell lines, plasmids and reagents

Hep3B, HepG2, SNU182, SNU387, SNU423, SNU398, SNU449 and PLC5 cell lines were obtained from the ATCC, while the Huh7 cell line was purchased from the Japanese Collection of Research Bioresources. Cell lines were regularly confirmed to be mycoplasma free using EZ-PCR kit (Biological Industries, Kibbutz Beit Haemek, Israel). HCC cell lines were cultured in DMEM or RPMI (ThermoFisher, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). SNU449 and PLC5 cell lines stably expressing *IGF2* were generated by transfecting 5 µg of EX-Z6323-M14 vector containing human *IGF2* ORF (NM_001127598.1) and a G418-selection cassette (GeneCopoeiaTM, Rockville, USA). The Hep3B and Huh7 cell lines were stably transfected with 5 µg of psi-H1 vector containing the shIGF2#1,2 or 3 **(Table S4),** a Puromycin-selection cassette and a eGFP reporter gene (GeneCopoeiaTM). Cells were transfected using Lipofectamine 3000® (ThermoFisher), and 48h after transfection cells were selected with G418 (1 mg/ml, G418 disulfate salt (Sigma) or Puromycin (1 µg/ml, Puromycin dihydrochloride (Sigma)). BI836845 (IGF1/2-mAb) humanized monoclonal antibody was provided by Boehringer Ingelheim (Viena, AT), whereas sorafenib and linsitinib (IGF1R TKI) were purchased from LC Laboratories (Woburn, MA, USA) and BioVision (Milpitas, CA, USA), respectively.

miRNAs transfection

SNU449 and PLC5 cell lines expressing miR-483-5p were generated by transfecting 100nM of miRIDIAN microRNA Human hsa-miR-483-5p mimic or miRIDIAN microRNA Mimic Negative Control #1 (Dharmacon). Cells were transfected using

DharmaFECT Transfection Reagent (Dharmacon), and 72 hours after transfection RNA was extracted using the miRNeasy mini kit (Qiagen).

cDNA was synthesized from 5ng of total RNA in 15 μl reaction using miRNA-specific primers and the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. High Capacity cDNA reverse Transcription kit (Applied biosystems) was used to synthesize cDNA for gene expression assays. For relative miRNA quantification, TaqMan® Gene Expression Assays were used following the manufacturer's instructions (Applied Biosystems). TaqMan® probes are listed in **Table S5**. miR-23b was chosen as the endogenous reference miRNA.

Reverse transcription Polymerase Chain Reaction (PCR) and quantitative RT-PCR

Total RNA was extracted either from cells collected at 80% confluence or from tissue, using the RNeasy Mini Kit (Qiagen). 1μg of RNA was retrotranscribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). For relative mRNA quantification, TaqMan® Gene Expression Assays were used following the manufacturer's instructions (Applied Biosystems). TaqMan® probes are listed in **Table S5**. Ribosomal RNA (*18S*) was chosen as the endogenous reference gene. Specific methods and TaqMan probes used for *INSR-A* gene expression assays were described in detail in Huang *et al. (58)*.

In vitro **functional cell assays**

For the cell viability assay, cells were seeded in 96-well plates and incubated with increasing concentrations (0 μmol/L, 0.1 μmol/L, 0.5 μmol/L or 1 μmol/L) of the IGF1/2-

mAb for 48 h in humidified atmosphere at 37°C and 5% CO_2. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye uptake using the CellTiter 96® Cell Proliferation Kit (Promega, Madison, WI) following the manufacturer's instructions. To assess proliferation, BrdU (bromodeoxyuridine) incorporation into newly synthesized DNA was measured by BrdU Cell Proliferation Assay Kit (Cell Signaling, Danvers, MA). For the colony formation assay, 200-1000 cells/well were seeded in 6-well plates and incubated for 2 weeks in the presence of 0 µM, 0.5 µM or 1 µM of IGF1/2-mAb. Thereafter, cells were stained with 0.5% Crystal Violet (Sigma) and the number of colonies and colony size was measured using Odyssey CLx Infrared Imaging System (Li-Cor, Lincoln, NE). To determine cell death, cells were seeded in 10 cm Petri dishes and maintained in FBS-free media for 24 h. Cells were treated either with 0 µM or 1 µM of IGF1/2-mAb. 48 h after treatment, cells were collected and fixed overnight at -20 ℃ using ice-cold 70%-ethanol, and stained with propidium iodide. Cell death was evaluated by determining the subG0 population on a BD FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Migration was assessed using the wound healing assay in cells treated with 0 µM or 1 µM of IGF1/2-mAb in the presence of mitomycin C (Sigma). Cell migration rate was evaluated at several time points by measuring the percentage of the initial wound area covered by cells over time on microscope images (ImageJ software).

IGF2 immunostaining

IGF2 immunohistochemistry was done on 5-µm sections of FFPE blocks using the anti-IGF2 antibody from Abcam (ab9574) Antigen retrieval was performed in citrate buffer using a microwave oven. After antigen retrieval, samples were incubated with peroxidase and blocked with Antibody Diluent containing Background Reducing Component (Dako,

Golstrup, Denmark). Sections were incubated O/N at 4°C with anti-IGF2 antibodies (1:400). EnVisionTM+ System-HRP (DAB) was applied as secondary antibody (Dako, Golstrup, Denmark). Samples were counterstained with hematoxylin.

Ligand mediated IGF pathway activation

Cells were seeded in 10 cm Petri dishes and incubated with FBS-free media overnight. IGF pathway activation was done by treating cells with 100 nM of IGF2 or 60 ng/ml of insulin (Prepotech, Rocky Hill, NJ) and inhibition, by applying 1 µM of IGF1/2-mAb (BI 836845) or 35 nM of IGF1R TKI (linsitinib) for 15min. IGF2 expression and pathway activation were analyzed by Western Blot. In short, cells were lysed in lysis buffer (50 mM Tris pH=7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.25 mM EDTA, 1% Sodium deoxycholate) containing protease and phosphatase inhibitors. 60 µg of protein were resolved by electrophoresis and transferred to nitrocellulose membranes (ThermoFisher). Membranes were incubated overnight at 4 ºC with IGF2 (ab9574), phosphoINSR(#3023), INSR(#3025), phosphoIGF1R (#6113), IGF1R (#3027), phosphoAKT (#9271) and AKT (#9272) antibodies (Cell Signaling), followed by incubation with HRP-linked secondary antibodies (Agilent Technologies, Santa Clara, CA). The HRP signal was used as a surrogate protein and was quantified through LAS4000 imaging and ImageGauge.v4 software (Fujifilm, Tokyo, Japan).

Metabolic toxicity in the HCC subcutaneous xenograft mouse model

Blood glucose levels were assessed after three weeks of treatment by using the ACCU-CHEK Sensor Confort test strips on tail vein blood (Roche, Basel, Switzerland). To evaluate glycated hemoglobin (hemoglobin A1c) whole blood samples were collected after

cardiac puncture immediately prior to euthanasia. GHbA1c ELISA Kit (Neobiolab, Woburn, MA) was used to measure the concentration of glycated hemoglobin.

Tumor xenograft molecular characterization

To evaluate cell proliferation and microvessel density, 4 μm sections of paraffin-embedded tumors were immunostained with Ki-67 and CD31 antibodies (Agilent Technologies). Ki-67 staining was quantified as the number of Ki-67 immunopositive cells divided by the total number of cells per field. Apoptosis was evaluated using the DeadEnd™ Colorimetric TUNEL System (Promega, Wisconsin, WI). Number of cells positively stained in the TUNEL assay was scored in ten microscopic fields. Microvessel density was determined by measuring the area of CD31 positive blood vessels. In all cases 10 randomly selected fields were quantified (40x and 20x, respectively). Images were produced using a Axioskop-2 microscope (Zeiss, Oberkochen, Germany). Phosphorylation of IGF1R, AKT and ERK was assessed by Western Blot. *Vegfa* gene expression was measured by TaqMan® Gene Expression Assays (Applied Biosystems).

***Igf2* and *H19* expression analysis in mouse models of HCC**

Expression of *Igf2* and *H19* was assessed by qRT-PCR in tumors from our chemically-induced mouse model of HCC and by microarray in 10 expression datasets from different mouse models of HCC publicly available in Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/): GSE33486, GSE2127, GSE31431, GSE15251, GSE26538, GSE39401, GSE19004, GSE46646, GSE54054, GSE61422. A FC>2 compared to healthy liver tissue in control mice was considered as overexpression for both genes.