1 **AICAR and Compound C negatively modulate HCC-induced primary human**

2 **hepatic stellate cell activation in vitro**

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16

17 **Figure number:** 5 Figures, 6 supplementary figures

18 **Word count:** 9366

19 **Running title: HCC-stellate cells paracrine cross-talk**

20

21 **Key words:** Hepatocellular carcinoma (HCC), tumour-stromal interactions, liver fibrosis,

22 adenosine monophosphate-activated kinase (AMPK), Hepatic Stellate Cells (HSC), AICAR,

23 Compound C, TCGA, LICA-FR

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34   **List of abbreviations:**

35   **hHSC** human hepatic stellate cell
36   **AMPK** adenosine monophosphate-activated kinase
37   **AMPKα1** AMP-activated protein kinase subunit α1
38   **AMPKα2** AMP-activated protein kinase subunit α2
39   **p-AMPK** phosphorylated AMPK
40   **AMPKα1α2-null** AMP-activated protein kinase subunit α1α2 – deficient
41   **MEF** mouse embryonic fibroblasts
42   **Wt** wild type
43   **BrdU** 5-bromo-2-deoxyuridine
44   **MTS** 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
45   tetrazolium inner salt
46   **qPCR** quantitative polymerase chain reaction
47   **MEF** mouse embryo fibroblasts
48   **CM** Complete medium
49   **SFM** Serum free medium
50   **4E-BP1** eukaryotic translation initiation factor 4E-binding protein 1
51   **AICAR** 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranosid
52   **CC** Compound C
53   **BrdU** bromodeoxyuridine
54   **CCL** CC motif chemokine ligand
55   **ECM** extracellular matrix
56   **ELISA** enzyme linked immunosorbent assay
57   **GAPDH** glyceraldehyde 3-phosphate dehydrogenase
58   **IL** interleukin
59   **LKB1** liver kinase B1, also called STK11 serine/threonine kinase 11
60   **LOX** lysyl oxidase
61   **mTOR** mammalian target of rapamycin
62   **TIMP** tissue inhibitor of matrix metalloproteinase
63   **GLUL** Glutamate-Ammonia Ligase
64   **LGR5** Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
65   **LAMA3** Laminin Subunit Alpha 3
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80   Conflict of interest

81   "The funders had no role in the design of the study; in the collection, analyses, or

82   interpretation of data; in the writing of the manuscript, or in the decision to publish the

83   results". The authors declare no conflict of interest with regards to the present manuscript.

84   Giuseppe Mazza, and L.L. are now full time employees at Engitix Ltd., and Giuseppe Mazza,

85   L.L., M.P., and K.R. own shares in Engitix Therapeutics Ltd., M.P., T.V.L, A.H. and K.R. receive

86   consultancies from Engitix Ltd.

87

88   Authors contributions:

89   KR designed the study; KB conducted experiments; KR performed HSC isolation; GM, KB,

90   and LL performed HSC culture; AH, TVL performed ICH, LG and SC performed bioinformatics;

91   BV provided MEFs; JZR provided HCC cell lines; KB, KR and MP analysed data; KB and KR

92   wrote the manuscript; KR and MP critically revised the manuscript; all authors have edited

93   the paper and approved the final manuscript.

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Abstract

101

102 Tumour stroma and microenvironment have been shown to affect hepatocellular carcinoma

103 (HCC) growth, with activated hepatic stellate cells (HSC) as a major contributor in this

104 process. Recent evidence suggests that the energy sensor adenosine monophosphate-

105 activated kinase (AMPK) may mediate a series of essential processes during carcinogenesis

106 and HCC progression. Here, we investigated the effect of different HCC cell lines with known

107 *TP53* or *CTNBB1* mutations on primary human HSC activation, proliferation and AMPK

108 activation. We show that conditioned media obtained from multiple HCC cell lines

109 differently modulate human hHSC proliferation and hHSC AMPK activity in a paracrine

110 manner. Pharmacological treatment of hHSC with AICAR and Compound C inhibited the

111 HCC-induced proliferation/activation of hHSC through AMPK-dependent and AMPK-

112 independent mechanisms, which was further confirmed using mouse embryonic fibroblasts

113 (MEFs) deficient of both catalytic AMPKα isoforms (AMPK$^{\alpha1/\alpha2-/-}$) and wild type (wt) MEF.

114 Both compounds induced S-phase cell-cycle arrest and, in addition, AICAR inhibited the

115 mTORC1 pathway by inhibiting phosphorylation of 4E-BP1 and S6 in hHSC and wt MEF.

116 Datamining of the Cancer Genome Atlas (TCGA) and the Liver Cancer (LICA-FR) showed that

117 AMPKα1 (*PRKAA1*) and AMPKα2 (*PRKAA2*) expression differed depending on the mutation

118 (*TP53* or *CTNNB1*), tumour grading and G1-G6 classification, reflecting the heterogeneity in

119 human HCC. Overall, we provide evidence that AMPK modulating pharmacological agents

120 negatively modulate HCC-induced hHSC activation and may therefore provide a novel

121 approach to target the mutual, tumour-promoting interactions between hHSC and HCC.

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132 **NEW & NOTEWORTHY**

133 HCC is marked by genetic heterogeneity and activated hepatic stellate cells (HSC) are

134 considered key players during HCC development. The paracrine effect of different HCC cell

135 lines on the activation of primary hHSC was accompanied by differential AMPK activation

136 depending on the HCC line used. Pharmacological treatment inhibited the HCC-induced

137 hHSC activation through AMPK-dependent and AMPK-independent mechanisms. This

138 heterogenic effect on HCC-induced AMPK activation was confirmed by datamining TCGA

139 and LICA-FR databases.

140

141 **Introduction**

142 Hepatocellular carcinoma (HCC) is the most common primary liver tumour and one of the

143 leading causes for cancer deaths (18). More than 80% of HCC develop on the background of

144 liver cirrhosis following long-standing liver injury most commonly caused by chronic

145 infection with hepatitis B or C viruses, excess alcohol consumption, or non-alcoholic

146 steatohepatitis (37). In these clinical conditions, chronic liver damage is characterized by the

147 activation of hepatic stellate cells (HSC), which represent the main cellular effectors of

148 hepatic fibrosis and consequent progression to liver cirrhosis (42). Activation of HSC is

149 characterized by the transition from a quiescent pericyte to a myofibroblast-like cell with

150 increased proliferation, migration, contraction and expression of pro-fibrogenic factors, pro-

151 inflammatory cytokines and extracellular matrix (ECM) components, resulting in the

152 formation of a scar tissue (48, 57). There is growing evidence that the tumour

153 microenvironment, in particular the bidirectional cross-talk between HCC and HSC, is a

154 crucial factor for HCC onset and progression (10) as activated HSC enhance HCC cell

155 proliferation, migration and tumour growth (2) by fostering the pro-inflammatory and pro-

156 angiogenic microenvironment of the tumour (16).

157 The energy-sensing enzyme adenosine monophosphate-activated kinase (AMPK) is a highly

158 sensitive safeguard that responds to changes in ATP production and promotes catabolic

159 pathways while inhibiting anabolic pathways when activated (41). Importantly, AMPK has

160 been implicated in tumour development and progression as AMPK regulates the cell cycle

161 by stabilizing p53 and p27 (32, 52). Thus, AMPK inhibits cell growth, metabolism and

162 proliferation through inhibition of the mTOR pathway (20). Further evidence for AMPK as a

163 tumour suppressor is originating from clinical and experimental data showing that

164 pharmacological AMPK modulators inhibit cancer development and progression (34, 53).

165 Indeed, the incidence of HCC is lower in diabetic patients treated with metformin which

166 induces AMPK activity (13). Metformin has also been shown to inhibit angiogenesis in an

167 HCC – HSC cell line *in vitro* co-culture model, supporting an inhibitory role for AMPK in

168 tumour-stromal interactions (45). In addition, AICAR, another well-established AMPK

169 activator, supresses HCC cell proliferation and inhibits tumour growth in murine HCC models

170 *in vivo* (14). Of note, pharmacological activation of AMPK has been shown to reverse HSC

171 activation *in vitro* and AICAR and metformin inhibits PDGF-induced HSC proliferation (1, 9).

172   Nevertheless, there is limited evidence for a role of AMPK in the tumour-stromal interaction

173   between HCC cells and primary human HSC, as well as for the possible working mechanisms

174   of AMPK in such cross-talk. Accordingly, the present study was designed to provide further

175   evidence on the involvement of this pathway in the cross-talk between HCC and its stroma.

176   As HCC is known to be a highly heterogeneous cancer (8, 17) several HCC cell lines with

177   different types of mutations were investigated in this study.

178   The results of the study show that activation of AMPK is involved in HCC tumour-stromal

179   interaction and suggests AMPK as a potential target for novel treatments for the overall

180   stromal derangement typical of chronic liver disease and the associated development of

181   HCC.

182

183   **Material and Methods**

184

185   **Patients and tumours**

186   The expression levels of AMPKα1 (*PRKAA1*) and AMPKα2 (*PRKAA2*) were assessed by RNA-

187   seq in two independent datasets, including 160 HCC samples from LICA-FR cohort previously

188   described, and 339 HCC samples from the TCGA (The Cancer Genome Atlas) cohort. Patients

189   with co-occurring *TP53* and *CTNNB1* mutations were excluded from the analysis. At least

190   two liver pathologists used multiple slides of the same tumour to establish the Edmondson–

191   Steiner grades in both cohorts, as previously described (23). Furthermore, the expression of

192   AMPKα1 (*PRKAA1*) and AMPKα2 (*PRKAA2*) was investigated in relation to the G1-G6

193   classification (5). Detailed clinical characteristics for both datasets are provided in

194   Supplemental Table S1 (supplemental material for this article is available online at

195   https://figshare.com/s/6953f837617d681232d7 and DOI 10.5522/04/12562631.

196

197   **Immunohistochemistry**

198   Immunostaining of AMPKα1 subunit was performed as previously described on formalin

199   fixed paraffin-embedded human liver sections from histologically normal tissue retrieved

200   from sites remote to colorectal liver metastasis, cirrhotic liver without HCC and HCC in the

201   context of a cirrhotic liver obtained from the Royal Free Hospital histopathology archives

202   (ethics 07/Q0501/50) (39). Liver sections were de-paraffinized and hydrated through

203 xylenes and ethanol. Antigen retrieval was achieved by microwaving the section at 640 W

204 for 20 minutes in 1L of pH9.0 Tris EDTA buffer. The slides were soaked in TBS with 0.04%

205 Tween-20 (Sigma) for 5 minutes and blocked for endogenous peroxidase and non-specific

206 binding of secondary antibodies using reagents from the Novolink kit (Leica), 5 minutes

207 each. AMPKα1 antibody (Abcam ab32047) was incubated for 1 hour at room temperature.

208 The primary antibody was then detected using Novolink kit reagents, post primary 30

209 minutes, polymer 30 minutes and DAB 5 minutes. The slides were then counterstained with

210 Mayer's haematoxylin. All sections were dehydrated, cleared in xylene, mounted with DPX

211 (Leica biosystems), and observed using a Zeiss Axioskop 40. Images were captured with an

212 Axiocam IcC5 using Zeiss Axiovision (version 4.8.2).

213

214 **Human HSC isolation**

215 Human HSC (hHSC) were isolated from wedge sections of human liver tissue, obtained from

216 patients undergoing liver surgery at the Royal Free Hospital after giving informed consent

217 (NC2015.020 (B-ERC-RF)), as described before (38, 49). Briefly, 10g of total human liver

218 tissue was digested with 0.01% Collagenase type IV (Sigma Aldrich), 0.05% Pronase

219 (Calbiochem) and 0.001% DNase I (Sigma Aldrich). The homogenate was filtered through a

220 100μm cell strainer (BD Falcon) and the flow-through was centrifuged at 50 x g for 2

221 minutes at 4°C to remove hepatocytes. After washing the supernatant, gradient

222 centrifugation was performed at 1400 x g for 17 minutes using a 11.5% Optiprep gradient

223 (Sigma Aldrich). Finally, the interface was collected and washed. The obtained hHSC were

224 cultured in Iscove's Modified Dulbecco's Medium supplemented with 20% foetal bovine

225 serum (FBS), 2 mM/l glutamine, nonessential amino acids 1x, 1.0 mM/l sodium pyruvate,

226 antibiotic-antimycotic 1x (all GIBCO), referred to as complete HSC medium hereinafter.

227 Experiments were performed on cells between passage 3 and 8 employing at least three

228 different cell preparations/donors for all experiments.

229

230 **Cell lines**

231 HepG2 and PLC/PRF/5 were purchased from American Type Culture Collection (ATCC®) and

232 cultured in Minimum Essential Medium α supplemented with 10% FBS, nonessential amino

233 acids 1x, 1.0 mM/l sodium pyruvate, Antibiotic-Antimycotic 1x. HCC cells lines SNU398,

234 Mahlavu, Huh-6 and Huh-7 cells were kindly provided by Prof Jessica Zucman-Rossi and

235 identity of the cell lines was confirmed by sequencing (11). Cells were cultured in Dulbecco's

236 modified Eagle Medium, supplemented with 10% FBS and Penicillin/Streptomycin. All cell

237 cultures are frequently analysed for mycoplasma using an in-house qPCR assay as previously

238 described (58).

239 **Mouse embryonic fibroblasts**

240 Simian virus 40 large T antigen immortalized mouse embryonic fibroblasts (MEFs) were

241 kindly provided by Dr Benoit Viollet. AMPKα1/α2$^{-/-}$ and wt MEFs were obtained from 10.5

242 day postcoitum embryos, the genotype was confirmed by PCR and immunoblot analysis.

243 MEFs from the second or third culture passage were immortalized by introducing the SV 40

244 large T antigen using the pSV-Ori- vector (30). MEFs were cultured in Dulbecco's Modified

245 Eagle Medium supplemented with 10% FBS, 1mM sodium pyruvate and Antibiotic-

246 Antimycotic 1x (all GIBCO).

247

248 **Preparation of conditioned medium**

249 To obtain conditioned medium 3 x 10^6 HepG2 or PLC/PRF/5 cells, or 0.6 x 10^6 hHSC were

250 cultured in a cell culture dish (100 x 22mm) in complete HCC or hHSC medium. After 24h,

251 cells were washed twice with HBSS and incubated in serum-free medium for 48h.

252 Conditioned medium was collected and centrifuged at 247 x g, 7 min, 21°C. The supernatant

253 was used immediately, or stored at -20°C, until used for experiments.

254

255 **Treatment of cells**

256 *Treatment of hHSC with conditioned medium of HepG2 or PLC/PRF/5 cells*

257 Human HSC were plated on a cell culture dish (0.3 x 10^6/6 well or 0.006 x 10^6/ 96 well) in

258 complete HSC medium. After 24 hours, cells were washed with HBSS (Gibco) and serum-

259 starved for 24 hours. Subsequently cells were treated with conditioned medium of HepG2

260 or PLC/PRF/5 cells for 24 hours.

261 *Treatment of HepG2 and PLC/PRF/5 cells with conditioned medium of hHSC*

262 HepG2 or PLC/PRF/5 cells were plated on a cell culture dish (0.006 x 10^6/ 96 well) in

263 complete HCC medium. After 24 hours, cells were washed with HBSS (Gibco) and serum-

264 starved for 24 hours, and cells were treated with conditioned medium of different primary

265 hHSC preparations subsequently.

266 *Treatment of cells with pharmacological compounds*

267 Human HSC were treated with different concentrations of AICAR (0.25-4mM) reconstituted

268 in water, or Compound C (2.5-40μM) reconstituted to 10mM in DMSO. Cells were plated in

269 complete medium and serum-starved for 24 hours prior to treatment.

270

271 **BrdU incorporation assay**

272 Cell proliferation was quantified by BrdU Cell Proliferation ELISA kit (Roche) and as

273 described before (4, 38). Human HSC (10^3) were plated in complete medium in a 96 well

274 plate in quadruplicates. Cells were washed and serum-starved for 24 hours prior to

275 treatment. BrdU labelling solution was added in parallel with treatment, BrdU ELISA was

276 developed according to the manufacturer's protocol after 24 hours treatment and

277 absorbance was detected with Fluostar Omega Plate Reader (BMG labtech).

278

279 **MTS assay**

280 To determine metabolic activity of cells, CellTiter 96® Aqueous One Solution Cell

281 Proliferation Assay kit (Promega) was used according to the manufacturer's protocol and as

282 previously described (39). Briefly, hHSC (10^3) were plated in complete medium in a 96 well

283 plate in quadruplicates and were cultured and treated as described above. Following 24

284 hours treatment, MTS was added for 2 hours and absorbance was measured with Fluostar

285 Omega Plate Reader (BMG labtech).

286

287 **Cell cycle analysis**

288 For cell cycle analysis, 0.3 x 10^6 HSC were plated on a 6 well plate in complete medium.

289 After 24 hours, cells were washed and cultured in serum-free medium for 24 hours,

290 followed by treatment. Cells were trypsinised after 24 hours of treatment, washed and fixed

291 with ice-cold 70% ethanol, for 30 minutes at 4°C. Cells were washed twice before incubation

292 with propidium iodide (50μg/ml, Promega) for 10 minutes at room temperature. Propidium

293 iodide accumulation was analysed with BD LSR Fortessa (5L SORP) using BD FACSDiva

294 software (version 6.2) and analysed with FlowJo version 10.0.

295

296 **Cell death enzyme linked immunosorbent assay (ELISA)**

297 To analyse apoptosis and necrosis in hHSC, Cell Death Detection ELISAPlus (Roche) was used

298 according to the manufacturer's protocol. Cells were treated in triplicates. Following 24

299 hours treatment, supernatants were removed and cells were lysed. Nucleosomes were

300 quantified in cell lysates (apoptosis) and supernatants (necrosis) photometrically.

301 Absorbance at 420nm was measured with Fluostar Omega Plate Reader (BMG labtech).

302

303 **Western blot analysis**

304 Protein isolation and western blot analysis was performed as previously described (4, 38,

305 39). To obtain total protein cell lysates, cells were washed and lysed with radio

306 immunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl pH 7.6, 150mM NaCl, 5mM EDTA,

307 1% NP-40 (nonyl phenoxypolyethoxylethanol), 1mM phenylmethylsulfonyl fluoride (PMSF),

308 1X Protease Inhibitors Mix, 1mM Na3VO4 and 1mM NaF). Cell lysates were sonicated with

309 the "Ultrasonic Processor" (Sonics, Vibra Cell). Protein quantification was carried out using

310 Micro BCATM Protein Assay Kit, (Thermo Scientific) according to the manufacturer's

311 protocol. SDS gel electrophoresis was performed with 25µg of protein lysate were loaded on

312 10% or 12% acrylamide gels. Primary antibodies were incubated overnight at 4°C or for 1

313 hour at room temperature. After washing, specific horseradish peroxidase coupled

314 secondary antibodies were applied for 1 hour at room temperature and SuperSignal® West

315 Pico Chemiluminescent Substrate (Thermo Scientific) was used to develop signals. For

316 following antibody incubations, antibodies were stripped with RestoreTM PLUS Western

317 Blot Stripping Buffer (Thermo Scientific). To verify equal loading of samples, expression of

318 the house-keeping proteins β-actin or tubulin was detected. All primary and secondary

319 antibodies are listed in supplemental table S2 and antibody specificity was validated by

320 performing western blot analysis.

321

322 **Quantitative real-time PCR (qPCR)**

323 RNA was isolated from hHSC using RNeasy mini Kit (Qiagen) according to the manufacturer's

324 protocol and as previously described (4, 38, 39). Purity and RNA concentration were

325 measured with Nanodrop spectrophotometer (Thermo Scientific) and cDNA was synthesized

326 with MultiScribe reverse transcriptase, random primers, deoxyribose nucleoside

327 triphosphate (dNTP) mix and RNase inhibitor (all Applied Biosystems). Gene expression was

328 measured via quantitative real time PCR (qPCR) using Taqman gene assays (supplemental

329 table S3) and 7500 Fast Real Time PCR System (all Applied Biosystems). To quantify gene

330   expression, the comparative CT method was used as described previously (4, 38, 39) using

331   Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

332

333   **Statistical analysis**

334   Data visualization and statistical analysis were performed using R software version 3.5.1 (R

335   Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org),

336   Microsoft Excel or Graph Pad Prism. Values are expressed as mean +/- standard deviation

337   (SD), or mean +/- 95% confidence interval as indicated in the figures. Statistical significance

338   was analysed using unpaired, non-parametric t-test, or ANOVA. Differences of mRNA

339   expression levels between groups were assessed using Wilcoxon signed-rank test to

340   compare two groups or Kruskall-Wallis test for post hoc analysis to compare more than two

341   groups. Spearman's rank-order correlation was used to test the association between

342   continuous variables. P-value< 0.05 was considered as significant.

343

344   **Results**

345

346   **HCC conditioned medium induces an activated phenotype and activation of the AMPK**

347   **pathway in hHSC.**

348   To investigate the interaction between primary hHSC and HCC cells, we incubated three

349   different hHSC preparations (i.e. cells obtained from three different donors) for 24 hours

350   with conditioned medium obtained from the hepatoblastoma cell line HepG2 (*TP53* gene

351   wild type cells with *CTNNB1* exon3 deletion activating ß-catenin), and the HCC cell line

352   PLC/PRF/5 (*TP53* mutation bearing cells with high levels of β-catenin protein expression) (7).

353   Both HCC cell lines belong to transcriptomic class 1 classification including the most

354   differentiated liver cancer cell lines with epithelial features (11). As shown in Figure 1A,

355   conditioned medium of PLC/PRF/5 and HepG2 cells induced a significant increase in

356   expression of several genes associated with hHSC activation, including the collagen

357   crosslinking enzyme lysyl oxidase (LOX) as well as the inflammatory genes IL-1β, IL-8 and

358   CCL2 in a cancer cell type specific manner. In contrast, 24 hours treatment with HCC cell

359   conditioned medium had no effect on TIMP1 and MMP2 expression, and resulted in

360   downregulation of collagen 1A1 and IL-6 gene expression (Figure 1A). Moreover, hHSC

361   proliferation was significantly increased following incubation with HepG2 conditioned

362 medium compared to serum free medium (Figure 1B), whereas hHSC proliferation remained

363 unchanged when treated with conditioned medium of PLC/PRF/5 cells. As HepG2 cells are

364 hepatoblastoma cells, further differences in the potential of HCC cells to induce hHSC

365 proliferation were confirmed employing conditioned medium of additional HCC cell lines.

366 Thus, HCC cell lines characterized by, amongst numerous other mutations (7, 19, 47),

367 various mutations in cell-cycle regulating genes, such as p53 (PLC/PRF/5, Huh-7, Mahlavu)

368 and β-catenin (HepG2, Huh-6, SNU398) were investigated. Similar to HepG2 cells,

369 conditioned medium of Huh-6 and Huh-7 cells induced hHSC proliferation *in vitro* (Figure

370 1C). In contrast, hHSC proliferation was unchanged following incubation with conditioned

371 medium of SNU398 or Mahlavu cells (Figure 1C), indicating that diverse HCC cell lines

372 differentially affect hHSC proliferation. Overall, these data further confirm that the

373 dysregulation of the cancer cell secretome plays a key role, not only in tumor

374 transformation/progression, but also affects the stromal cells in a HCC cell line specific

375 manner (6, 33, 62). It has been shown previously that cell proliferation is regulated via

376 AMPK and that activation of AMPK in HSC leads to inhibition of PDGF-BB-induced

377 proliferation (9, 41). AMPK activation is characterized by phosphorylation of AMPK at

378 threonine residue 172 of its catalytic subunit α (AMPK-Thr^{172}) whereas phosphorylation of

379 AMPK-Ser$^{485/491}$ results in inhibition of AMPK activity (21, 24) (Figure 1E). To test whether

380 activation of the AMPK pathway is associated with the induction of hHSC proliferation by

381 HCC cells, activation of AMPK and its upstream kinase LKB1 were analysed in hHSC after 24

382 hours incubation with HCC cell line conditioned medium. PLC/PRF/5 cell conditioned

383 medium induced a strong phosphorylation of AMPK at Thr^{172} and only mild phosphorylation

384 at AMPK-Ser$^{485/491}$ when compared to treatment with serum free medium (SFM), indicating

385 overall activation of AMPK (Figure 1D, Supplemental Figure S1A). In contrast, conditioned

386 medium of HepG2 cells induced a mild phosphorylation of AMPK-Thr^{172} associated with a

387 strong phosphorylation of AMPK-Ser$^{485/491}$, suggesting an overall inhibition of AMPK activity

388 (Figure 1D, Supplemental Figure S1A). Phosphorylation of the AMPK upstream kinase LKB1

389 that regulates phosphorylation of AMPK-Thr^{172} was strongly reduced after treatment with

390 the conditioned media of both HCC cell lines, compared to serum free medium (Figure 1D,

391 Supplemental Figure S1A), suggesting that phosphorylation of AMPK-Thr^{172} is regulated

392 differently in hHSC treated with conditioned media. Similar to conditioned medium of

393 PLC/PRF/5 cells, conditioned medium of the cancer cell lines SNU398 and Mahlavu induced

394   AMPK activation in hHSC, as suggested by the strong phosphorylation of AMPK-Thr^{172}, while

395   phosphorylation of AMPK-Thr^{172} was only mildly induced or absent following incubation

396   with Huh-6 and Huh-7 conditioned medium (Supplemental Figure S1B). Overall, these

397   results demonstrate the ability of HCC cells to induce hHSC activation, as well as activation

398   of the AMPK pathway in hHSC, with HCC cell line specific features.

399

400   **AICAR and Compound C inhibit hHSC activation induced by HCC cells**

401   We next investigated the effect of the pharmacological agents AICAR (15, 54) and

402   Compound C (1), known to affect AMPK signalling. We first assessed the effect of both

403   pharmacological agents on AMPK activation/phosphorylation in hHSC, as well as their effect

404   on metabolic activity, proliferation and hHSC gene expression. We observed an increase in

405   AMPK phosphorylation at Thr^{172} in hHSC treated with AICAR whereas p-AMPK-Thr^{172} was

406   absent in hHSC exposed to Compound C (Figure 2A). Further, we did not observe changes in

407   phosphorylation of AMPK-Ser$^{485/491}$ following treatment with AICAR or Compound C

408   compared to the untreated control (Figure 2A), suggesting activation of AMPK through

409   AICAR but not Compound C. The metabolic activity and proliferation in hHSC were

410   significantly inhibited by both pharmacological agents in a dose-dependent manner

411   indicating that both AICAR and Compound C can inhibit hHSC activation (Figure 2B − 2C)

412   without inducing cell death as assessed by cell death ELISA (Figure 2D).

413   We next investigated the effect of both pharmacological agents on HCC-induced hHSC

414   activation by treating hHSC with conditioned medium of HepG2 or PLC/PRF/5 cells and

415   AICAR or Compound C in parallel. As demonstrated in Figure 2E, treatment with AICAR was

416   sufficient to abrogate the induction of inflammatory gene expression by HepG2 and

417   PLC/PRF/5 conditioned medium in hHSC, while expression of LOX was unchanged. This

418   effect was less prominent in hHSC exposed to Compound C (Supplemental Figure S2A).

419   Moreover, both AICAR and Compound C were able to reverse HepG2 induced proliferation

420   in hHSC (Figure 2F). Similarly, proliferation of HepG2 and PLC/PRF/5 cells treated with

421   conditioned medium of hHSC was inhibited following treatment with AICAR and Compound

422   C (Supplemental Figure S2B and S2C). These data indicate that both AICAR and Compound C

423   inhibit the cross-talk between HCC and hHSC and *vice versa*. Next, protein expression

424   analysis was performed and showed that AICAR could induce p-AMPK-Thr^{172} expression in

425   hHSC treated with conditioned medium of PLC/PRF/5 and HepG2 cells. These effects were

426   not observed when hHSC were exposed to Compound C in combination with HCC

427   conditioned medium (Figure 2F), indicating that the inhibitory effect of Compound C might

428   be AMPK-independent. Taken together, these data show that both AICAR and Compound C

429   are potent inhibitors of hHSC activation and are able to reverse HCC-induced hHSC

430   proliferation and activation *in vitro*.

431

432   **AICAR and Compound C inhibit MEF proliferation in an AMPK independent manner**

433   Although AMPK is known as a crucial regulator of cell proliferation, pharmacological AMPK

434   modulating agents have been shown to act both in an AMPK-dependent and -independent

435   manner (34, 35, 46, 59, 60). Likewise, the previous set of data suggested that inhibition of

436   hHSC proliferation and metabolic activity by AICAR and Compound C (Figure 2) may be

437   driven by both AMPK-dependent and AMPK-independent signals. To further test this

438   hypothesis, we employed mouse embryonic fibroblasts (MEFs) deficient of both existing

439   isotypes of the catalytic AMPK isoform α (AMPK$^{\alpha1/\alpha2-/-}$ i.e. DKO) and wild type (wt) MEFs

440   (30). Cells were treated with AICAR or Compound C for 24 hours and a significant, dose-

441   dependent reduction of metabolic activity was observed in wt MEFs as well as in AMPK$^{\alpha1/\alpha2-}$

442    $^{/-}$ MEFs (Figure 3A, 3B). Moreover, AICAR and Compound C showed a trend to inhibit cell

443   proliferation in wt MEFs and AMPK$^{\alpha1/\alpha2-/-}$ MEFs (Figure 3C, 3D).

444

445   **AICAR and Compound C regulate hHSC cell proliferation through AMPK-dependent and**

446   **AMPK-independent mechanisms**

447   We next aimed at unravelling the pathways and mechanisms through which AICAR and

448   Compound C inhibited proliferation of hHSC and MEFs and their dependency on AMPK.

449   First, cell cycle analysis was performed on hHSC treated with 1mM AICAR or 10µM

450   Compound C for 24 hours. As shown in Figure 4A, both AICAR and Compound C induced cell

451   cycle arrest in hHSC in the S-phase. When analysing the cell cycle in MEFs treated with

452   AICAR or Compound C, we surprisingly observed inhibition of the cell cycle in both wt and

453   AMPK$^{\alpha1/\alpha2-/-}$ MEFs, with AICAR inhibiting the cell cycle in the S-phase and Compound C in the

454   G1-phase, respectively (Figure 4B). These data suggest that both Compound C and AICAR

455   can induce cell cycle arrest independently of AMPK.

456 Next, the mammalian target of rapamycin (mTOR), a critical regulator of cell metabolism

457 and proliferation, was investigated to test another signalling pathway by which AICAR and

458 Compound C may inhibit cell proliferation. Although mTOR is downstream of AMPK (20), the

459 pathway can alternatively be regulated through various other pathways (51). The mTOR

460 complex 1 (mTORC1) mediates its effects through phosphorylation of its downstream

461 targets eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the p70

462 ribosomal S6 kinase (S6) (31). Thus, hHSC were treated with 1mM AICAR or 10μM

463 Compound C for 24 hours and phosphorylation of S6 and 4E-BP1 proteins was assessed.

464 Figure 4C demonstrates that phosphorylation of 4E-BP1 was mildly reduced, whereas

465 phosphorylation of S6 was abrogated following treatment with AICAR, but not upon

466 Compound C exposure (Supplemental Figure S3A). These data show that AICAR, but not

467 Compound C, inhibits cell proliferation through mTORC1 inhibition in hHSC. To further test

468 whether AICAR inhibited mTORC1 in an AMPK-dependent manner, wt MEFs and AMPK$^{\alpha 1/\alpha 2-}$

469 $^{/-}$ MEFs were treated with AICAR for 24 hours. As shown in Figure 4D, phosphorylation of 4E-

470 BP1 was slightly reduced and phosphorylation of S6 was abrogated in AICAR treated wt

471 MEFs. In contrast, such reduction of 4E-BP1 and S6 phosphorylation did not occur in

472 AMPK$^{\alpha 1/\alpha 2-/-}$ MEFs (Figure 4D, Supplemental Figure S3A), indicating that AICAR inhibits

473 mTORC1 in wt MEFs, as well as in hHSC, in an AMPK-dependent manner. The data further

474 show that Compound C only mildly reduced phosphorylation of 4E-BP1 in wt MEFs, and that

475 S6 phosphorylation was unchanged in wt MEFs and AMPK$^{\alpha 1/\alpha 2-/-}$ MEFs, clearly indicating that

476 Compound C does not affect the mTORC1 pathway in MEFs or hHSC (Figure 4D). Overall,

477 these data show that both AICAR and Compound C inhibit cell proliferation through

478 different pathways involving both AMPK-dependent and independent mechanisms.

479

480 **AMPK gene expression is overexpressed in a subset of HCC patients.**

481 In order to further investigate possible changes in AMPK gene expression during the process

482 of HCC we analysed the gene expression level of both isotypes of the catalytic AMPK α

483 isoforms i.e. AMPKα1 (*PRKAA1)* and AMPKα2 (*PRKAA2)* in 2 independent cohorts of HCC

484 tissue i.e. the TCGA and LICA-FR (3). In both cohorts, samples were excluded when carrying

485 both the *TP53* and *CTNNB1* gene mutations. The main difference between both cohorts

486 concerns aetiology, i.e. the TCGA cohort is enriched by HBV patients and contains a lower

487   amount of patients featuring the CTNNB1 mutation rate. In contrast, there is no significant

488   difference in the METAVIR fibrosis score between the two cohorts (Supplemental Table S1).

489   A correlation analysis was performed and showed that *PRKAA1* and *PRKAA2* expression

490   levels are not associated (Figure 5A, LICA-FR: R= -0.101, P value=ns and TCGA: R=-0.026, P

491   value=ns). Next, *PRKAA1* and *PRKAA2* expression was analysed and no significant correlation

492   was found in tumours carrying the *TP53* mutation (Supplemental Figure S4). In contrast, a

493   significant association was demonstrated between *PRKAA1* and *CTNNB1* mutation in the

494   TCGA dataset but not in LICA-FR, whereas a significant association was demonstrated

495   between *PRKAA2* expression and tumours carrying the *CTNNB1* gene mutation in both

496   cohorts (Figure 5B). Importantly, a positive correlation was observed between *PRKAA2*

497   expression and *CTNNB1* target genes such as Glutamate-Ammonia Ligase (GLUL), Leucine

498   Rich Repeat Containing G Protein-Coupled Receptor 5 (LGR5) and Laminin Subunit Alpha 3

499   (LAMA3) in both datasets confirming that *PRKAA2* is a potential CTNNB1 target gene (Figure

500   5C).

501   Furthermore, both *PRKAA1* and *PRKAA2* expression were compared in both datasets by

502   using Edmondson grading (I-II and III-IV) and the previous described unsupervised

503   transcriptome analysis (5) which classifies human HCC tumours in 6 subgroups (G1 to G6).

504   *PRKAA1* gene expression showed no significant correlation with different degrees in

505   Edmondson grading (I-II and III-IV) (Supplemental Figure S5A and S5E) and G1 *versus* G6

506   subgroups (Supplemental Figure S5B and S5F) in both datasets. In contrast, *PRKAA2*

507   expression showed significant differences between grade I-II *versus* III-IV (P=0.011, LICA-FR

508   dataset, Supplemental Figure S5C), whereas no significant correlation was found in the

509   TCGA dataset. Notably, in both datasets a high *PRKAA2* expression was found in G5-G6

510   subgroups in line with its association with *CTNNB1* gene mutations (Supplemental Figure

511   S5D and S5H).

512   Furthermore, IHC for the detection of AMPKα1 was performed on formalin fixed paraffin-

513   embedded human liver sections from histologically normal liver, cirrhotic liver without HCC

514   and HCC in the context of a cirrhotic liver (Supplemental Figure S6). AMPKα1 weakly stained

515   the hepatocyte membranes in both normal liver tissue and cirrhotic liver tissue without HCC

516   (Supplemental Figure S6A and S6B). Similar localization was observed in cirrhotic tissue

517   surrounding the HCC lesion. AMPKα1 staining showed a stronger membrane staining with

518   some cytoplasm staining in the HCC tumour tissue of patient with grade 1 (Supplemental

519   Figure S6C). Further, the tumour tissue of grade 2 classified patient showed a strong

520   cytoplasmic staining for AMPKα1, which appeared even stronger in the HCC tissue classified

521   as grade 3 (Supplemental Figure S6D and S6E).

522   Overall, these data, although reflecting the heterogeneity in human HCC (37), suggest an

523   important role for AMPK modulation for the development of novel therapeutic strategies

524   against HCC.

525

526

527

528   **Discussion**

529   HCC is one of the leading causes for cancer death worldwide (18, 27). In spite of the recent

530   progress in treatment options for HCC with the introduction of new multi-kinase inhibitors

531   and immune-therapy (36), the identification of new therapeutic targets, more widely

532   reflecting HCC biology, represents a current crucial effort in Oncology.

533   The tumour microenvironment plays a crucial role in HCC development and progression (10,

534   37), as over 80% of HCC develop on the background of liver fibrosis and cirrhosis (56). In this

535   context, genetic alterations and deregulation of signalling pathways are the result of chronic

536   hepatocellular necrosis, inflammation, oxidative stress and a dysregulated extracellular

537   matrix (ECM) deposition which further favours cancer development (10, 22). Moreover, in a

538   previous study we identified, by proteomic analysis, specific enriched proteins in the human

539   cirrhotic ECM in comparison to healthy ECM proteins. Culturing the cells in a stiffer cirrhotic

540   3D microenvironment demonstrated the unique up-regulation in genes related to epithelial

541   to mesenchymal transition (EMT) and TGFβ signalling. Further demonstrating that the

542   inherent features of the human cirrhotic liver ECM are key pro-carcinogenic components in

543   HCC (40). One of the key hallmarks of the development of liver fibrosis is the activation of

544   HSC (42). Recent evidence showed that the bi-directional cross-talk between activated HSC

545   and HCC cells promotes HCC cell proliferation and tumour growth (2, 16), in addition to

546   favouring a pro-inflammatory and pro-fibrogenic microenvironment (16). Here, we

547   investigated the potential paracrine effect of different HCC cell lines to activate primary

548   human HSC and the possible implication of AMPK in the HCC-induced activation of hHSC. As

549   HCC is known to be a highly heterogeneous cancer (8, 17) several HCC cell lines were

550   investigated in this study. These cell lines are characterized by different mutations in cell-
551   cycle regulating genes such as p53 (PLC/PRF/5, Huh-7, Mahlavu) and β-catenin (HepG2,
552   Huh-6, SNU398) (7, 19, 47) and express a characteristic secretome (6, 33, 62). Besides
553   exerting different effects on hHSC proliferation and gene expression, HCC conditioned
554   media differentially activated the AMPK pathway in hHSC, again emphasizing the complexity
555   of HCC heterogeneity by possibly affecting the stromal compartment through AMPK
556   induction. Indeed, when datamining 2 independent cohorts of HCC tissues i.e. the TCGA and
557   LICA-FR we showed no significant correlation between tumours carrying the *TP53* mutation
558   and both catalytic AMPKα isoforms, whereas a significant correlation was found between
559   tumours carrying the *CTNNB1* gene mutation and the AMPKα isoforms. It is known that
560   *CTNNB1* and *TP53* mutations are affecting 25-30% of HCC patients and both mutations are
561   defined by different subgroups in HCC and correlate with better or worse patient outcome,
562   respectively (5, 8, 11). Another layer of complexity may emerge knowing that in certain
563   cancer cell lines and primary tumours a correlation has been demonstrated between
564   *PRKAA1* gene copy number and mRNA expression suggesting that gene amplification does
565   lead to increased expression, whereas the frequency of alterations in the *PRKAA2* gene in
566   cancer is lower overall (12, 29). Moreover, recent studies have shown that the liver
567   microenvironment may play a crucial role in NAFLD/NASH towards HCC progression. Such
568   changes in HCC incidence are affected by obesity, type 2 diabetes, and NAFLD, which is the
569   most common liver disease and marked by aberrant AMPK activity (43, 65). Indeed, in a
570   previous study an unbiased transcriptomic and ingenuity pathway analysis revealed no
571   AMPK-related pathway enrichment in NAFLD patients. Nevertheless, a list of AMPK related
572   genes, unbiasedly generated by means of STRING analysis and UCSC Genome Browser data
573   mining tool goldenPath, showed to be significantly affected in the RNA sequence data of
574   patients with NAFLD *versus* healthy controls. Several AMPK subunits such as *PRKAB1*,
575   *PRKAB2*, *PRKAG1* were significantly downregulated in NAFLD patients. Most strikingly, a
576   perturbation was found in many AMPK pathway genes such as TBC1D1, SLC2A4/GLUT4,
577   AKT1/2, and genes involved in the regulation of lipid metabolism and
578   activation/phosphorylation of AMPK such as Sirt3 and TSC2 in NAFLD patients (64).
579   AMPKα1 is widely expressed across tissues, whereas AMPKα2 is more restricted in its tissue-
580   and intracellular distribution (29, 50). Both AMPKα isoforms are expressed in human healthy
581   and diseased liver tissue (12, 44, 64). Furthermore, we demonstrated that primary hHSC

582 express more isoform AMPKα1 than AMPKα2 (39). Besides changes of AMPKα1/α2 gene

583 expression in HCC, posttranslational modifications, such as the phosphorylation of AMPK

584 are important in the development/progression of HCC as was demonstrate by Jiang et al,

585 that the risk of HCC occurrence was significantly higher in patients with a low expression of

586 p-AMPK (28). In this study, we demonstrate that HCC-induced activation of hHSC can involve

587 AMPK and its phosphorylation. Importantly, the catalytic AMPK subunit α can be

588 phosphorylated at different phosphorylation sites (61). While phosphorylation at Thr[172]

589 activates AMPK (55), phosphorylation at Ser[485/491] inhibits its activity and favours

590 dephosphorylation at Thr-172 (24, 26). We demonstrate that, depending on the HCC cell

591 line used, either phosphorylation of AMPK-Ser[485/491] or phosphorylation of AMPK-Thr[172] can

592 be achieved by HCC cells. This further resulted in differences observed in hHSC proliferation.

593 In this study, we also provide new evidence for the possible working mechanisms of

594 pharmacological agents known as AMPK activators and inhibitors in the cross-talk between

595 hHSC and HCC. Here, we demonstrate that the AMPK activator AICAR inhibits both the

596 proliferation and the pro-fibrogenic and pro-inflammatory phenotype of primary human

597 HSC in a dose-dependent manner under basal conditions as well as after exposure to HCC

598 conditioned medium. Indeed, pharmacologically induced AMPK activation has been shown

599 to exert anti-HCC properties by inhibiting proliferation through AICAR (14, 32) and the

600 incidence of several tumour types, including HCC, has been shown to be lower in patients

601 treated with the AMPK activator and oral anti-diabetic drug metformin (13). Our data

602 reinforce the concept that AMPK activation may be beneficial for tumour-stromal

603 interactions in HCC, as it can target both hHSC and HCC cells. Therefore, our data add new

604 evidence about pharmacological AMPK activators and their effect on HCC-induced

605 proliferation in primary hHSC.

606 Next, we demonstrate in more detail an AMPK-dependent but also AMPK-independent

607 working mechanisms of Compound C, a known AMPK inhibitor, which inhibits proliferation

608 and induces cancer cell death (25, 34, 63). Treatment with Compound C showed significant,

609 dose-dependent anti-proliferative effects on hHSC to the same extent as the AMPK activator

610 AICAR without affecting the inhibitory phosphorylation of AMPK-Ser[485/491], suggesting

611 AMPK-independent effects. Indeed, recent evidence suggests that Compound C exerts

612 AMPK-independent anti-proliferative effects in different cancers, by inducing apoptosis,

613 inhibiting Akt and the mTOR signalling pathway, as well as induction of cell cycle arrest (1,

614   34). Unlike in cancer cells (34, 63), Compound C did not induce apoptosis in primary human

615   HSC and did not modulate mTOR signalling pathway. However, we show that Compound C

616   induces cell cycle arrest in the S-phase in hHSC, as well as in the G1-phase in MEFs and

617   independently of AMPK. Moreover, we show that treatment with AICAR inhibits the

618   mTORC1 pathway in primary hHSC, as well as in wt MEFs in an AMPK-dependent manner.

619   Overall, our data show that AICAR and Compound C inhibit hHSC proliferation through

620   different mechanisms, some of which are AMPK-dependent.

621   This study clearly demonstrates the existence of a cross-talk between human HCC cells and

622   primary human HSC which affects hHSC activation, as well as activation of the AMPK

623   pathway in hHSC, pointing towards a role for AMPK in tumour stromal interactions in HCC

624   development. Moreover, the data show that pharmacological AMPK activation of the anti-

625   proliferative pharmacological compounds AICAR and Compound C could represent a novel

626   approach for anti-cancer and anti-fibrotic therapy.

627

628

629 **Figure legends**

630

631 **Figure 1. HCC conditioned medium activates hHSC and affects the AMPK pathway in hHSC.**

632 (A) Gene expression and (B) proliferation response in hHSC following treatment with

633 conditioned medium of HepG2 or PLC/PRF/5 cells for 24h. (C) Proliferation of hHSC

634 following 24h treatment with different HCC conditioned media. (D) AMPK phosphorylation

635 profile expression in hHSC after exposure with HepG2 or PLC/PRF/5 conditioned medium for

636 24h. (E) Schematic of AMPK phosphorylation sites and AMPK activation/inhibition. (A) Data

637 represent mean +/- 95% confidence interval, *p = 0.05, pooled data of 3 independent

638 experiments, (B-D) Representative data of at least 3 independent experiments, data

639 represent mean +/- SD, **** $p<0.0001$, ns = not significant vs. SFM. CM = complete

640 medium, SFM = serum free medium.

641

642 **Figure 2: AICAR and Compound C reverse HCC-induced hHSC activation.**

643 (A) AMPK phosphorylation profile in hHSC treated with AICAR and Compound C. Human HSC

644 metabolic activity and proliferation following 24h treatment with (B) AICAR (0.25-4mM) or

645 (C) Compound C (2.5-40μM). (D) Quantification of nucleosome expression by ELISA in hHSC

646 following 24h treatment with AICAR or Compound C. (E) Gene expression, (F) proliferation

647 and protein expression in hHSC following 24h treatment with PLC/PRF/5 or HepG2

648 conditioned medium and AICAR (1mM) or Compound C (10μM). (B)-(D), (F) Data represent

649 mean +/- SD, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. SFM and [#]p< 0.05 vs. non-

650 AICAR treated control. (e) Data represent mean +/- 95% confidence interval, *p = 0.05 vs.

651 serum free medium, [#]p = 0.05 vs. non-AICAR treated control. (A) – (F) Representative data of

652 at least 3 independent experiments. CM = complete medium, SFM = serum free medium,

653 c.m. = conditioned medium, CC = Compound C.

654

655 **Figure 3: AICAR and Compound C inhibit hHSC metabolic activity and proliferation**

656 **independently of AMPK.**

657 (A-B) Metabolic activity and (C-D) proliferation in wt and AMPK$^{\alpha1/\alpha2-/-}$ (DKO) MEFs following

658 24h treatment with AICAR and Compound C. (A) – (D) Data represent mean +/- SD

659 ***p<0.001, ****p<0.0001 vs. SFM (wt), [##]p<0.01, [###]p<0.001, [####]p<0.0001 vs. SFM

660   (AMPK$^{\alpha1/\alpha2-/-}$), $^{+}$p<0.05, $^{++++}$p<0.0001 as indicated, representative data of at least 3

661   independent experiments. SFM = serum free medium, DKO = AMPK$^{\alpha1/\alpha2-/-}$

662

663   **Figure 4: AICAR and Compound C inhibit hHSC proliferation through various mechanisms.**

664   (A) Cell cycle analysis in hHSC following treatment with 1mM AICAR or 10μM Compound C

665   for 24h. (B) Cell cycle analysis in wt and AMPKα1/α2$^{-/-}$ MEFs following treatment with

666   0.5mM AICAR and 10μM Compound C for 24h. (C) Protein expression in hHSC following

667   treatment with 1mM AICAR or 10μM Compound C for 24h. (D) Protein expression in wt and

668   AMPKα1/α2$^{-/-}$ MEFs following 24h treatment with 0.25mM AICAR and 10μM Compound C.

669   (A) – (D) Representative data of at least 3 independent experiments. SFM = serum free

670   medium, CC = Compound C, CM = complete medium.

671

672   **Figure 5: Validation of AMPK gene expression in a subset of HCC patients.**

673   Gene expression levels of AMPKα1 (*PRKAA1)* and AMPKα2 (*PRKAA2)* were investigated in 2

674   independent cohorts of HCC (TCGA and LICA-FR). (A) Correlation analysis showed that

675   *PRKAA1* and *PRKAA2* expression levels are not associated (Spearman's R=-0.101 LICA-FR, P

676   value=ns and Spearman's R=-0.026, P value=ns in TCGA). (B) A significant association was

677   observed between *PRKAA1* and *CTNNB1* gene mutations in the TCGA cohort but not in the

678   LICA-FR dataset. A significant association was demonstrated between *PRKAA2* expression

679   and tumours carrying the *CTNNB1* gene mutation in both cohorts (Wilcoxon signed-rank

680   test). (C) A positive correlation was observed between *PRKAA2* expression and *CTNNB1*

681   target genes such as *GLUL*, *LGR5* and *LAMA3* in both datasets confirming that *PRKAA2* is a

682   potential CTNNB1 target gene (Spearman's rank-order correlation, p value <0.0001

683   (M=mutant, NM= non-mutant).

684

685

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898

A

B

C

D

conditioned medium

E

K. Böttcher et al., Figure 1

K. Böttcher et al., Figure 2

A metabolic activity

B metabolic activity

C proliferation

D proliferation

K. Böttcher et al., Figure 3

A

hHSC

SFM
G1% 72.8
S% 17.9
G2/M% 6.06

AICAR
G1% 59.5
S% 32.5
G2/M% 5.36

CC
G1% 65.5
S% 27.1
G2/M% 5.69

cell count

propidium iodide incorporation

B

wt MEF

CM
G1% 30.9
S% 57.0
G2/M% 9.84

AICAR
G1% 0.0
S% 98.4
G2/M% 0.0

CC
G1% 41.7
S% 49.2
G2/M% 8.54

AMPK$^{\alpha1/\alpha2 -/-}$ MEF

CM
G1% 39.3
S% 38.3
G2/M% 21.4

AICAR
G1% 0.0
S% 98.9
G2/M% 0.0

CC
G1% 47.7
S% 45.1
G2/M% 8.00

cell count

propidium iodide incorporation

C

hHSC

SFM AICAR CC

p4E-BP1 Thr 37/46
4E-BP1
tubulin

pS6 Ser235/236
S6
tubulin

D

wt MEF

SFM AICAR CC

AMPK$^{\alpha1/\alpha2 -/-}$ MEF

SFM AICAR CC

p4E-BP1 Thr 37/46
4E-BP1
tubulin

pS6 Ser235/236
S6
tubulin

K. Böttcher et al., Figure 4

K. Böttcher et al. Figure 5

HCC
cells

conditioned
medium

AICAR
Compound C

hepatic stellate
cells

activated
hepatic stellate cells

PLC/PRF/5

Thr^{172}

Ser$^{485/491}$

AMPK

Thr^{172}

P

P

Ser$^{485/491}$

AMPK

HepG2