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YAP activation is an early event and a potential therapeutic target in liver cancer development

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Key words: Hippo Pathway, HCC, preneoplastic stages, verteporfin, TCPOBOP

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Abbreviations: 2-AAF, 2-acetylaminofluorene; *Birc5*, survivin; *Ccnd1*, cyclin D1; *Ctgf*, Connective Tissue Growth Factor; *Gpc3*, glypican-3; HCC, hepatocellular carcinoma; DENA, diethylnitrosamine; GSTP, placental glutathione S-transferase; IHC, immunohistochemistry; KRT-19, cytokeratin-19; miRNA, microRNA; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; R-H model, Resistant-Hepatocyte model; *Spp1*, osteopontin; TCPOBOP: 1,4-bis(2-(3,5-dichloropyridyloxy)benzene; YAP, Yes-associated protein 1; VP: verteporfin

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ABSTRACT

Background and Aims: Although the growth suppressor Hippo pathway has been implicated in hepatocellular carcinoma (HCC) pathogenesis, it is unknown at which stage of hepatocarcinogenesis its dysregulation occurs. We investigated in early rat and human preneoplastic lesions whether overexpression of the transcriptional co-activator Yes-associated protein (YAP) is an early event.

Methods: The experimental model used is the Resistant-Hepatocyte (R-H) rat model. Gene expression was determined by qRT-PCR or immunohistochemistry. Forward genetic experiments were performed in human HCC cells and in murine oval cells.

Results All foci of preneoplastic hepatocytes generated in rats 4 weeks after diethylnitrosamine (DENA) treatment, displayed YAP accumulation. This was associated with down-regulation of the β-TRCP ligase, known to mediate YAP degradation, and of microRNA-375, targeting YAP. YAP accumulation was paralleled by up-regulation of its target genes. Increased YAP expression was also observed in early dysplastic nodules and adenomas in humans. Animal treatment with verteporfin (VP), which disrupts the formation of the YAP–TEAD complex, significantly reduced preneoplastic foci and oval cell proliferation. *In vitro* experiments confirmed that VP-mediated YAP inhibition impaired cell growth in HCC and oval cells; notably, oval cell transduction with wild type or active YAP conferred tumorigenic properties *in vitro* and *in vivo*.

Conclusions: These results suggest that i) YAP overexpression is an early event in rat and human liver tumorigenesis; ii) it is critical for the clonal expansion of carcinogen-initiated hepatocytes and oval cells, and, iii) VP-induced disruption of YAP-TEAD interaction may provide an important approach for the treatment of YAP-overexpressing cancers.

Key words: Hippo Pathway, HCC, preneoplastic stages, verteporfin, TCPOBOP, YAP

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INTRODUCTION

Hepatocellular carcinoma is the fifth most common neoplasm and the third leading cause of cancer-related mortality worldwide [1]. HCC carries a dismal prognosis, potentially curative surgery being possible only in the subset of patients diagnosed at the early stages of the disease. Although some responses are seen with conventional and targeted chemotherapies, their impact on overall survival is modest. For this reason, a more complete understanding of the molecular mechanisms involved in HCC development could have a significant influence on improving therapeutic strategies for this pathology [2].

Recently, landmark studies have implicated the Hippo signaling pathway in the pathogenesis of HCC [3-5]. First identified in *Drosophila*, this pathway has emerged as an evolutionarily conserved mechanism that restricts organ size in different species. In mammals, this organ size regulator comprises several tumor suppressors (Mst1/2, Sav1/WW45, Lats1/2 and Mob1), acting in a kinase cascade that culminates in the phosphorylation and inactivation of the transcriptional co-activator YAP. On the contrary, uninhibited YAP localizes in the nucleus where it serves as a co-activator for the TEA-domain family member (TEAD) group of DNA-binding transcription factors. The YAP/TEAD complex promotes proliferative and survival programs by inducing the expression of target genes [4-6]. Notably, both the transgenic overexpression of YAP in mice [3,7] and the liver-specific knockout of Mst1/2 or Sav1 [8-11] expanded the liver size and ultimately induced HCC, revealing a significant role for the Hippo pathway in regulating organ size and tumorigenesis in mammals. In humans, amplification of the chromosomal region containing the YAP gene (11q22) has been reported in several tumor types [12]. Consistent with these findings, the comprehensive survey of the most common solid cancer types revealed elevated YAP protein levels and nuclear localization in multiple human cancers, including HCC, cholangiocarcinoma (CC) and hepatoblastoma (HB) [5,13]. Furthermore, clinical studies showed that YAP was an independent prognostic marker for overall survival and disease-free survival for HCC patients and that it was associated with tumor differentiation [14].

Interestingly, the mammalian Hippo pathway is required to repress the activation of facultative adult liver stem cells, known as oval cells [15]. In normal adult liver, oval cells progenitors are localized in biliary ductules (canals of Hering) and are bipotential. Although hepatic oval cells are not commonly observed in physiological conditions, they can be isolated from liver following certain types of injury, such as treatments in which hepatocytes are unable to proliferate in response to toxic damage (e.g. treatment with 3,5-

diethoxycarbonyl-1,4-dihydrocollidine or choline-deficient diets and ethionine (16-18). Oval cell homeostasis is regulated by the Hippo pathway, as Mst1/2 or Sav1 ablation in mouse liver resulted in abundant oval cell accumulation [9,11].

A well-known protocol that induces a rapid expansion of the oval cell population is the Resistant-Hepatocyte (R-H) rat model [19]. In this model, the need to regain parenchymal cell mass after 2/3 partial hepatectomy (PH) leads to the rapid expansion of preneoplastic hepatocytes and oval cells, since proliferation of normal hepatocytes is completely inhibited by 2-acetylaminofluorene (2-AAF). Therefore, the advantage of this model is that it allows to investigate not only the evolution of the early preneoplastic lesions to fully developed HCCs, but also the expansion of oval cells.

Since the Hippo signaling pathway holds great promise in liver cancer therapy, but no information are available on the stage (early or late) at which it may play a critical role, the aim of the present study was to investigate the role of the Hippo-YAP pathway in the early stages of HCC development and in oval cell proliferation. Moreover, we also wanted to investigate if pharmacological YAP inhibition could impact on the hepatocarcinogenic process.

MATERIALS AND METHODS

Animals and Treatment

Guidelines for Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari and the Italian Ministry of Health. Male Fischer F-344 rats (100-125g) were purchased from Charles River (Milan, Italy).

R-H Protocol: Rats injected intraperitoneally with diethylnitrosamine (DENA, 150 mg/kg body weight, Sigma-Aldrich, Milan, Italy) were subjected to the R-H model (for details, see Supporting Material). Animals were sacrificed 7 days after surgery (4 weeks after DENA) or 10 weeks and 14 months thereafter. The number of preneoplastic foci positive for the placental form of glutathione S-transferase (GSTP) was determined as previously described [20]. To investigate the effect of verterporfin (VP) (CAS number: 129497-78-5; USP Reference Standards) on early preneoplastic foci and oval cells, F-344 rats were exposed to the R-H protocol. VP was administered intraperitoneally (100 mg/kg) 1 hour prior to- and 3 and 5 days after PH. Animals were sacrificed 7 days after surgery.

CMD protocol: Four week-old male F-344 rats were initiated with DENA (150 mg/kg) and 2 weeks later fed a choline-methionine deficient (CMD) [21] diet for 4 months. Preneoplastic lesions were detected by GSTP staining.

Effect of VP on TCPOBOP-induced mouse hepatomegaly: The effect of VP on TCPOBOP-induced hepatomegaly was investigated in CD-1 female mice (See Supp. Material).

Immunohistochemistry

Immediately after sacrifice, liver sections were fixed in 10% formalin or snap-frozen in liquid nitrogen. For details see Supp. Material.

Laser-capture Micro-dissection (LMD)

We microdissected 400 foci (1 week after PH), 20 nodules (10 weeks after initiation with DENA), and 9 HCCs (14 months), and random areas of surrounding GSTP-negative hepatocytes. Oval cells were also microdissected 1 week after PH. Microdissection was performed according to [22] (for details see Supp. Material).

qRT-PCR analysis

RNA was retro-transcribed using the High Capacity Kit (Life Technology). Analysis of YAP, β-TRCP, *Birc-5, Ccnd1, c-myc*, *Gpc3* and *Spp1* was performed using specific TaqMan probes (Life Technology) and GAPDH as endogenous control.

Cell lines

Human HCC cell lines, MLP29 mouse oval cells and RH rat cell lines were cultured as described in Supp. Material. Transduction of cells was performed as described in [23].

Clinical features and histopathological diagnosis

For clinical features and histopathological diagnosis of 28 liver samples, see Table 1, Supp. Table 1 and Supp. Material.

Statistical Analysis. Comparison between treated and control group was performed by Anova and student's *t*-test Instant Software.

RESULTS

YAP accumulates in early preneoplastic lesions and oval cells.

To investigate whether derangement of the Hippo pathway is an early event in hepatocarcinogenesis, we used the Resistant-Hepatocyte (R-H) rat model [19]. This model is characterized by a synchronous expansion of carcinogen-initiated cells that can be easily identified by preneoplastic markers as early as 7 days after PH. Unlike normal

hepatocytes, preneoplastic hepatocytes and oval cells, are capable to divide after 2/3 PH, in the presence of the cytostatic environment generated by 2-AAF. This experimental model thus offers the advantage of studying the carcinogenic process from its very initial steps. As seen in **Fig. 1A,** immunohistochemistry of more than 300 foci, performed on serial liver sections 7 days after PH, showed that all the hepatocyte foci positive for the preneoplastic marker GSTP, were also homogeneously positive for YAP and its phosphorylated form (pYAP). Thus, YAP represents an extremely sensitive marker of preneoplastic stages. As already described in the original work (19), a massive expansion of oval cells (identified by their morphological feature and by positivity to GGT, KRT-19 and GSTP, data not shown) was observed. Notably, while YAP positivity was also detected in the vast majority of proliferating oval cells, an almost negligible YAP staining could be observed in the surrounding hepatocytes (**Fig. 1B**). YAP staining was also observed at later stages, namely 10 weeks (a time when nodules shared histomorphological characteristic with human dysplastic nodules) [24], (**Fig. 1C**) and 14 months (fully malignant HCCs, and lung metastases, **Fig. 1D**). Cholangiocarcinomas (CCs) detected 14 months after initiation were also strongly positive for YAP (**Supp. Fig. 1**). Unlike very early stages, characterized by a homogenous positivity of all preneoplastic cells, YAP staining in dysplastic nodules was not uniformly distributed (**Fig. 1C**). Notably, while YAP staining was mainly limited to the cytoplasm of early preneoplastic and dysplastic hepatocytes and of oval cells, a clear nuclear staining was evident only in HCCs and CCs (**Fig. 1D; Supp. Fig. 1).**

YAP cytoplasmic accumulation is associated to down-regulation of β-TRCP.

To investigate whether YAP protein overexpression was paralleled by augmented mRNA levels, we performed qRT-PCR analyses on microdissected early preneoplastic foci and HCCs. As shown in **Fig. 1E,** no increase of YAP mRNA was detected at any of the time points examined. Since the increase of the YAP protein was not mediated by transcriptional mechanisms, we wondered if the observed increased level of YAP protein could be due to impaired protein degradation. Indeed, preneoplastic foci exhibited a marked down-regulation of the β-TRCP E3 ligase, known to mediate YAP degradation [25] **(Fig. 1F)**.

miR-375 is downregulated in YAP-overexpressing preneoplastic nodules and HCCs.

MiR-375, that is down-regulated in human HCCs [26] and in a YAP overexpressing mouse model of liver chemical carcinogenesis [27], regulates YAP expression and impairs HCC cells tumorigenic properties [26]. To investigate whether miR-375 down-regulation parallels YAP increase in early lesions, we performed a RT-PCR in 10 dysplastic nodules. MiR-375 was significantly down-regulated in preneoplastic lesions ($P < 0.01$) (**Fig. 1G**), suggesting that it can contribute to YAP increase at early stages of liver tumorigenesis. Notably, down-regulation of miR-375 was maintained in HCCs as well, suggesting its relevant role in HCC development (**Fig. 1H**).

YAP target genes are up-regulated in very early preneoplastic foci and in oval cells.

As shown by IHC **(Fig. 1),** YAP was overexpressed in the cytoplasm of highly proliferating cells (preneoplastic hepatocytes and oval cells) and barely detectable in the nuclei. Since it is possible that a relatively small increase of nuclear YAP cannot be detectable by IHC (similar to what observed for β-catenin in mouse liver adenomas where, in spite of activating mutations of the gene, cytoplasmic, but not nuclear staining was reported [28], we directly investigated YAP transcriptional activation by measuring the expression of its target genes. Microarray analysis of YAP target genes performed in microdissected foci generated 7 days after PH showed that most of the previously identified YAP target genes [29] were up-regulated in early lesions compared to the surrounding non-preneoplastic liver **(Supp. Fig. 2A).** qRT-PCR performed to validate some of these genes and to investigate the expression of other YAP target genes not present in the array is shown in **Supp. Fig. 2B.** A high expression of YAP-target genes was observed in microdissected oval cells **(Supp. Fig. 2C).**

YAP accumulation at early stages of hepatocarcinogenesis is not restricted to the R-H model.

To investigate whether YAP accumulation is an event unique to the R-H model of hepatocarcinogenesis, we studied a different rat model consisting of a choline-devoid methionine-deficient diet [21], and characterized by extensive fatty liver. IHC analysis showed a strong YAP accumulation in preneoplastic lesions **(Supp. Fig. 3),** suggesting that an early increase of YAP is a common event in multistage hepatocarcinogenesis.

The TEAD-YAP binding disruptor Verterporfin inhibits the growth of preneoplastic foci and oval cell proliferation.

Formation of complexes between YAP and the TEAD/TEF transcription factors is mandatory for unleashing YAP oncogenic activity [30]. Treatment with the porphyrin verteporfin (VP) disrupts the formation of the YAP/TEAD complex, thus impairing the transcription of YAP target genes [29]. To investigate whether YAP co-transcriptional activation is required for the clonal expansion of DENA-initiated hepatocytes, we administered VP to rats exposed to the R-H protocol. VP caused a significant reduction of the number of GSTP-positive preneoplastic foci (111.8 vs. 79.6/cm^2, $P<0.05$) and of their average size (0.035 vs. 0.023 mm^2; $P<0.01$) **(Fig. 2A,B).**

Resection of 2/3 of the liver in the presence of the cytostatic environment generated by 2-AAF causes a massive expansion of oval cells, expressing markers specific for biliary epithelial cells (for example KRT-19). While oval cells observed in DENA-AAF-PH-treated animals strongly expressed KTR-19**,** KRT-19 immunoreactivity in VP treated rats was restricted to bile ductular epithelial cells, thus demonstrating the complete inhibitory effect of VP on infiltration of liver parenchyma by oval cells (**Fig. 2C).** This suggests that YAP-TEAD interaction is an absolute requirement for the proliferation of this progenitor cell compartment.

VP-mediated YAP inhibition impairs viability of HCCs and oval cells.

To further investigate the effect of VP, *in vitro* experiments were performed on Huh7 HCC cells transduced with YAP (**Supp. Fig. 4A**) or the empty vector. As shown in **Fig. 2D,** VP impaired the growth of control Huh7 cells, while a much weaker effect was observed on cells transduced with YAP, suggesting that high levels of YAP protect cells from VP. VP also impaired both the viability and the colony forming ability of rat tumorigenic cells derived from HCCs generated by the R-H model (**Supp. Fig. 4B-C**).

The effect of VP was evaluated in oval cells as well. To mimic the physiological stimuli present in a damaged liver, we treated mouse oval cells (MLP29), with Hepatocyte Growth Factor (HGF), known to stimulate their growth. As shown in **Fig. 2E**, VP inhibited HGF-induced proliferation.

YAP overexpression confers tumorigenic and metastatic properties to oval cells.

The Hippo signaling pathway plays a pivotal role in regulating oval cells, putative liver cancer progenitor cells [15]. As oval cells in the livers of R-H rats are positive for YAP (**Fig 1B**), we investigated the effect of YAP overexpression in MLP29 cells. Cells were transduced with the empty vector (MLP29 pRRL2), wild type YAP (MLP29 YAP wt) or a

YAP active form mutated in Serines 127 and 381 (MLP YAP SS); this form is preferentially translocated into the nucleus and is more stable [31] **(Supp. Fig. 4D)**. As shown in **Fig. 3A,** YAP WT overexpressing MLP29 cells acquired the ability to grow in anchorage-independent conditions; the effect was significantly stronger in cells overexpressing active YAP (YAP SS).

To test their tumorigenic ability, we subcutaneously inoculated MLP29 cells in nude mice. Tumors generated by MLP29 YAP WT and MLP YAP SS cells were bigger than those of MLP29 control cells (**Fig. 3B).** Moreover, mice inoculated with MLP29 YAP WT or MLP29 YAP SS presented several and large lung metastases **(Fig. 3C)**; the number and the size of metastases was significantly higher in mice inoculated with MLP29 YAP SS cells **(Fig 3D)**. All together these data suggest that YAP overexpression promotes the tumorigenic and metastatic properties of oval cells.

VP inhibits nuclear receptor-mediated hepatomegaly.

A recent study on transgenic mice reported that while YAP-TEAD interaction is dispensable for the achievement of normal liver size, it is required for organ overgrowth [29]. To examine whether this mechanism is operating also in genetically unmodified animals, we induced liver hyperplasia by the powerful liver mitogen TCPOBOP, a ligand of the constitutive androstane receptor (CAR). In agreement with our previous reports [27], a single treatment with TCPOBOP induced hepatomegaly within 3 days, which was associated with a striking increase of hepatocyte proliferation (**Supp. Fig. 5A-C).** Nuclear translocation of YAP was often observed in hepatocytes from the hyperplastic liver. (**Supp. Fig. 5D).** Treatment with VP reduced TCPOBOP-induced hepatomegaly, strongly impaired hepatocyte proliferation (**Supp. Fig. 5A-C),** and decreased the number of hepatocyte nuclei positively stained for YAP (**Supp. Fig. 5D**). Similarly, the expression of the YAP target Birc-5 was poorly induced after TCPOBOP+VP treatment versus TCPOBOP alone, indicating decreased activation of YAP **(Supp. Fig. 6).**

YAP accumulation in human hepatic tumorigenesis

To investigate at which stage YAP accumulation takes place in human hepatic tumorigenesis, we examined its expression in early dysplastic nodules (**Table 1**). YAP immunoreactivity was negligible in the cirrhotic parenchyma, but visible in early dysplastic lesions, as isolated cells or sporadic clusters **(Fig. 4A-C).** YAP immunoreactivity increased from early HCC to advanced HCC, the highest expression being in poorly differentiated

HCC **(Supp. Fig. 7A-C)**. Cholangiocarcinomas (**Supp. Table 1, Supp. Fig. 7D-F**) showed cytoplasmic and nuclear immunoreactivity, regardless tumor differentiation. YAP levels were not associated with clinical parameters such as viral infections or alfa-fetoprotein, but rather with HCC grade (**Supp. Fig. 7A-C).** However, the small number of investigated cases did not allow drawing definitive conclusions.

We also examined neoplastic progression in the so-called healthy liver, whereby hepatocellular adenomas are associated or not with HCC development (**Table 1, Fig. 4).** YAP immunoreactivity was seen in different varieties of adenomas such as the β-catenin-activated (**Fig. 4D-F**), steatotic (or HNF-α mutated) **(Fig. 4G-I)**, and telangiectatic/inflammatory **(Fig. 4L-N)** subtypes, with abrupt disappearance in the adjacent non-neoplastic liver. YAP expression varied from 10 to 80% of neoplastic cells, and the pattern of staining was mostly cytoplasmic, diffuse or granular (**Fig. 4C,F,I,N)**, with occasional nuclear accumulation (**Fig. 4F).** In HCC arising in the context of an adenoma (**Table 1, case 16 & 17)**, the number of YAP immunoreactive cells was not different from that of the adjacent adenoma, but they showed increased YAP expression as compared to HCCs taking place in the cirrhosis-dysplasia-HCC sequence (**Supp. Table 1, cases 1 to 7**). YAP immunoreactivity was not seen in hyperplastic/non-neoplastic hepatocellular lesions such as focal nodular hyperplasia (**Table 1, case 18**).

DISCUSSION

Recent studies, both in *Drosophila* and mammals, have implicated the Hippo signaling pathway as a potent regulator of organ size and tissue homeostasis. Indeed, studies from different groups employing genetically modified animals showed that the overexpression of YAP and the combined *Mst1/2* deficiency lead to massive liver overgrowth and development of HCC [7-8]. Notably, Mst1/2 or Sav ablation in mouse liver originated mixed HCC and cholangiocarcinomas and resulted also in oval cell accumulation [9,11]. Later on, more strength to the critical role of dysregulation of the Hippo pathway in liver cancer development has come from the finding of its widespread involvement in human cancer [13], including HCC and CC, suggesting that this pathway might be an attractive therapeutic target.

Our study demonstrates that the expression of YAP is increased in very early preneoplastic hepatocyte foci in two non-transgenic models of hepatocarcinogenesis. These results strongly support the notion that the inactivation of the Hippo pathway allows genetically damaged cells to evade the intrinsic size-control mechanisms that normally

maintain tissue homeostasis, thus favouring their progression to malignancy. Moreover, we also present data confirming the involvement of YAP in liver overgrowth in non-genetically modified animals. As shown, in fact, hepatomegaly induced by activation of the nuclear hormone receptor CAR was associated with accumulation of YAP, and VP-mediated YAP inhibition resulted in severe reduction of liver overgrowth and cell proliferation.

Notably, the increased accumulation of YAP in preneoplastic lesions was associated to a concomitant down-regulation of the β-TRCP E3 ligase - which mediates YAP degradation - and of miR-375- known to target YAP. MiRNAs have recently emerged as important modulators of gene expression in cancer, including human HCC [32]. Down-regulation of miR-375 has been observed in human and mouse HCC [26,27] Although the inverse relationship between miR-375 and YAP found in the present study is only correlative, based on previous works showing that miR-375 does indeed target YAP and impairs HCC cell growth, the early dysregulation of this miR herein reported suggests its critical role not only for HCC progression, but also for the onset of this tumor.

Unexpectedly, immunohistochemistry showed YAP accumulation in the cytoplasm but not in the nuclei of the actively proliferating preneoplastic and dysplastic hepatocytes. Indeed, its phosphorylation in Serine 127 and subsequent cytoplasmic accumulation should reflect the activation of the Hippo pathway and the inability of YAP to translocate into the nucleus and activate transcription of its target genes. However, the expression of many YAP target genes was strongly induced in early preneoplastic hepatocytes, indicating the active co-transcriptional activity of YAP. The observation that VP, a disruptor of TEAD-YAP binding, was able to inhibit the *in vivo* expansion of initiated cells demonstrates that, in spite of its main cytoplasmic accumulation, YAP is transcriptionally active in preneoplastic hepatocytes.

Another interesting observation was that, in the R-H model, YAP accumulates in the cytoplasm of facultative adult liver stem cells, known as oval cells, as well; these cells, together with preneoplastic hepatocytes, are indeed capable to proliferate after PH in the presence of the mitoinhibitory effect exerted by 2-AAF. Even if previous work in transgenic animals has shown that the mammalian Hippo pathway is required to repress the activation of oval cells [15, 9,11], whether YAP is critical for the survival/expansion of this cell subset in non-transgenic models remains elusive. Our finding that i) VP completely inhibits the expansion of oval cells *in vivo*, ii) VP inhibits proliferation of the mouse MLP29

oval cells *in vitro*, and, iii) transduction of MLP29 cells with wild type or active YAP confers tumorigenic capacity *in vivo*, demonstrates that YAP is critical for the expansion of this cell population and its progression to cancer.

Our results also show that low- and high-grade dysplastic nodules, considered as the earliest stages analyzable in humans, and which display strict similarities to the 10-week nodules seen in the rat model [24], exhibit YAP in the cytoplasm, although at quite low levels. Moreover, a novel and interesting finding was the massive YAP accumulation in adenomas, arising in an otherwise healthy liver where YAP overexpression was found to occur independently from β-catenin mutations or the histological type. The latter findings are in disagreement with a previous work (33) revealing weak/absent staining for YAP in hepatocellular adenomas; however, they are in accordance with the work of Anakk et al (34) who also found YAP staining in adenomas. The reason for these discrepancies are not obvious and require further investigation. Why YAP is expressed at such high levels in human adenomas is unclear. However, since these tumors arise in a quiescent liver, it can be hypothesized that dysregulation of the Hippo pathway is a key event through which initiated/mutated cells can escape the growth suppressive signals controlling the size of the organ, thus clonally expanding to develop a tumoral mass.

In conclusion, our present data demonstrate that YAP overexpression and Hippo pathway dysregulation are early events in rat and human hepatocarcinogenesis, Moreover, the finding that impairment of TEAD-YAP binding inhibits the growth of preneoplastic hepatocytes and abolishes proliferation of oval cells suggests a causal relationship between YAP activation and liver tumor development, independently of their origin. All together, these results provide further evidence to the notion that pathways governing tissue overgrowth should be deeply explored as potential therapeutic targets in human HCC.

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LEGEND TO FIGURES

Figure 1. YAP accumulates in early preneoplastic lesions and oval cells. F-344 rats submitted to the R-H protocol were sacrificed 7 days after PH (foci) or at 10 weeks (nodules) or 14 months (HCCs) from DENA treatment. **(A)** GSTP, YAP and phosphoYAP (pYAP) immunohistochemistry of serial sections through preneoplastic foci (x4). The analysis of the total number of YAP positive foci was performed on liver sections from 6 animals. An average of about 50 YAP-positive foci/liver section was counted for each liver slide. Similar results were obtained in 3 independent experimental protocols **(B)** YAP and pYAP positivity pattern in oval cells (x4). **(C)** GSTP, YAP and pYAP immunohistochemistry of serial sections of 10-week dysplastic nodules (x4). **(D)** nuclear YAP positivity in rat HCC, and intense YAP staining in lung metastases (x10). **(E)** YAP mRNA levels in early preneoplastic foci and HCC, NS: not statistically significant. **(F)** qRT-PCR analysis of β–TRCP expression in early preneoplastic foci, ***statistically significant for $P<0.001$ **(G,H)** qRT-PCR analysis of microRNA-375 expression in dysplastic nodules and HCCs. In all the

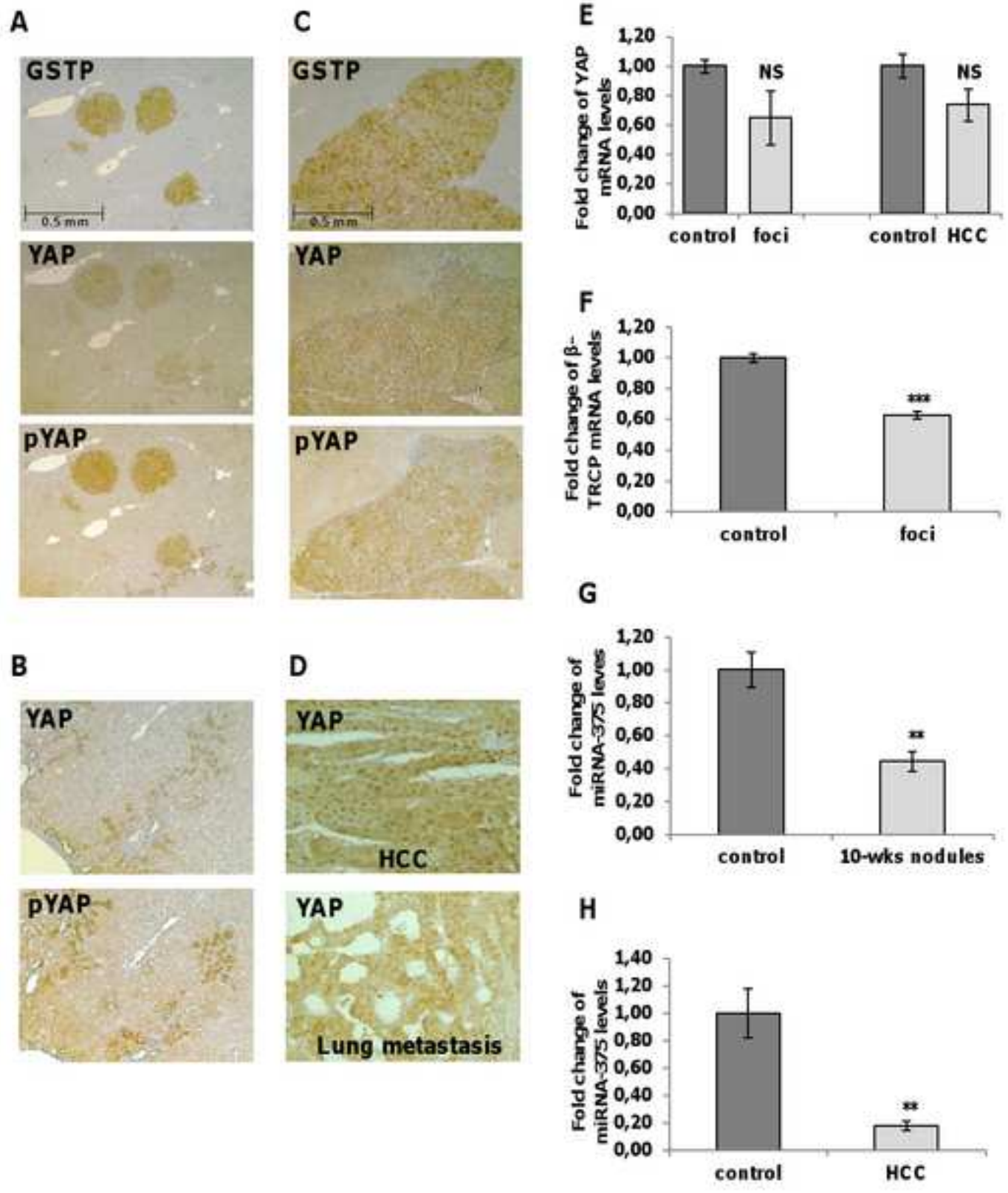
panels values are compared to controls (animal livers of the corresponding age) considered as 1, and expressed as mean ±.SEM. Statistically significant for **P<0.01.

Figure 2. Verteporfin (VP) administration reduces number and size of preneoplastic GSTP-positive foci, and impairs viability of HuH7 and MLP29 cells. Rats exposed to the R-H protocol were injected intraperitoneally with VP (100 mg/kg) 1 hour prior to- and 3 and 5 days after PH. Animals were sacrificed 7 days after PH. **(A)** GSTP immunohistochemistry of liver sections from rats with (+VP) or without (-VP) VP treatment (x4). **(B)** Effect of VP administration on the number of GSTP-positive foci, the mean GSTP-positive area and the percentage of the area occupied by GSTP-positive hepatocytes. Values are expressed as mean ±.SEM. Statistically significant for *P<0.05, **P<0.01 **(C)** KRT-19 immunohistochemistry staining illustrating a remarkable inhibition of oval cell proliferation in VP-treated rats; (x4 up) and (x20 down). **(D)** Effect of different doses of VP on viability of Huh7 cells. Huh7 transduced with empty vector (Huh7 pRRL2) or with YAP wt (Huh7 YAP WT) were treated with the indicated VP concentrations. Viability was evaluated 6 days after seeding. SD is indicated. Statistically significant for *P<0.05; **P<0.01;***P<0.001; **E)** Effect of VP on viability of MLP29 cells treated or nor with VP, in the absence or in the presence of 40 ng/ml of HGF. Viability was evaluated 3 days after seeding; SD is indicated. Statistically significant for *P< 0.05.

Figure 3. Effect of transduction of MLP29 cells with WT or active YAP on colony forming ability and tumorigenic properties. (A) MLP29 cells infected with (i) empty vector (MLP pRRL2), (ii) YAP wt (MLP YAP WT) or (iii) active YAP (MLP YAP SS), were grown in agar for 3 weeks. The number of viable cells was quantified with the Alamar Blue dye. Representative pictures are shown. SD is indicated. Statistically significant for*P<0.05; **P<0.01. (**B)** Mice were subcutaneously injected with MLP pRRL2, MLP YAP WT or MLP YAP SS. Mean tumor volume +SD was evaluated at the indicated times; statistically significant for *P<0.05; **P<0.01. **(C)** H&E staining of lung sections of mice subcutaneously injected with MLP pRRL2, MLP YAP WT or MLP YAP SS (x4). **(D)** (left graph) Percentage of the lung area covered by metastases calculated using the ImageJ software; (right graph) number of metastatic lesions/mice. These values have been calculated in 5 different slides for each mouse. Values are expressed as mean ±.SEM.. Statistically significant for *P<0.05; ***P<0.001;

Figure 4: YAP immunoreactivity in dysplastic nodules and in different subtypes of hepatocellular adenomas. **(A,B)** Low grade dysplastic nodule: (**A**) H&E staining; x10; **(B)**

YAP staining showing some immunoreactive cells with cytoplasmic staining immunoreactivity (x40); (**C)** High grade dysplastic nodule: YAP staining showing an increased number of YAP immunoreactive cells with granular cytoplasmic staining (x20) **(D-F)** Atypical and β-catenin mutated adenoma: **(D)** H&E staining, (x10); (**E)** β-catenin staining showing nuclear localization (x10) and (**F**) YAP staining showing diffuse cytoplasmic granular positivity (x20); **(G-I**) steatotic adenoma and surrounding parenchyma: **(G)** H&E staining, (X4); (**H**) YAP staining showing that YAP expression is restricted to the adenomatous component (x4); **(I)** higher magnification showing cytoplasmic and nuclear YAP immunostaining of the adenoma (x20); (**L-N)** telangiectatic adenoma: **(L-M)** H&E staining (x10 and x20); **(N)** YAP staining showing moderate, granular cytoplasmic YAP staining in the majority of tumor cells (x20).


A
GSTP
0.5 mm
YAP
pYAP
C
GSTP
0.5 mm
YAP
pYAP
B
YAP
pYAP
D
YAP
HCC
YAP
Lung metastasis
E
Fold change of YAP mRNA levels
1,20
1,00
0,80
0,60
0,40
0,20
0,00
NS
NS
control
foci
control
HCC
F
Fold change of β-TRCP mRNA levels
1,20
1,00
0,80
0,60
0,40
0,20
0,00
***
control
foci
G
Fold change of miRNA-375 leves
1,20
1,00
0,80
0,60
0,40
0,20
0,00
**
control
10-wks nodules
H
Fold change of miRNA-375 levels
1,40
1,20
1,00
0,80
0,60
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0,20
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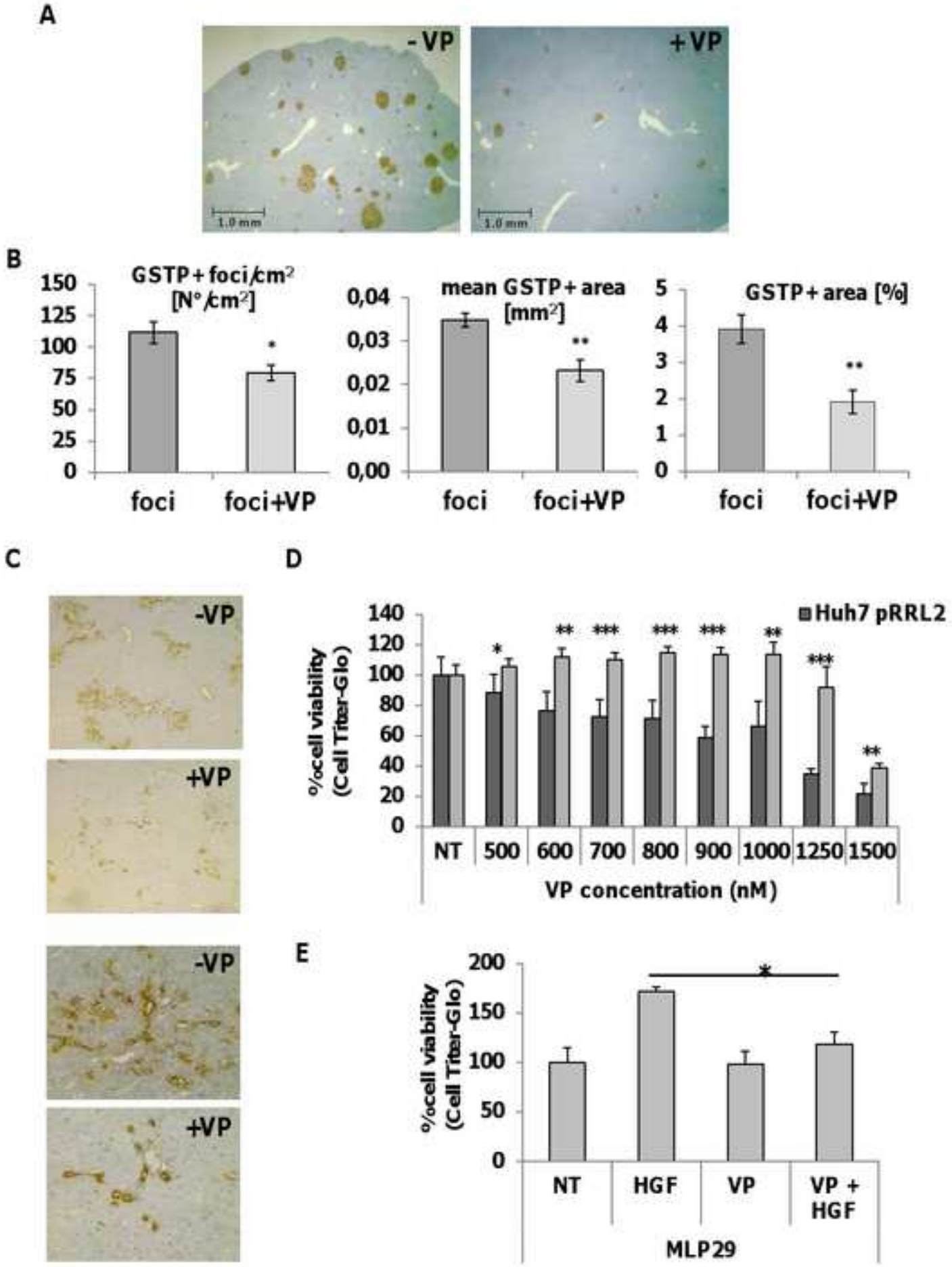
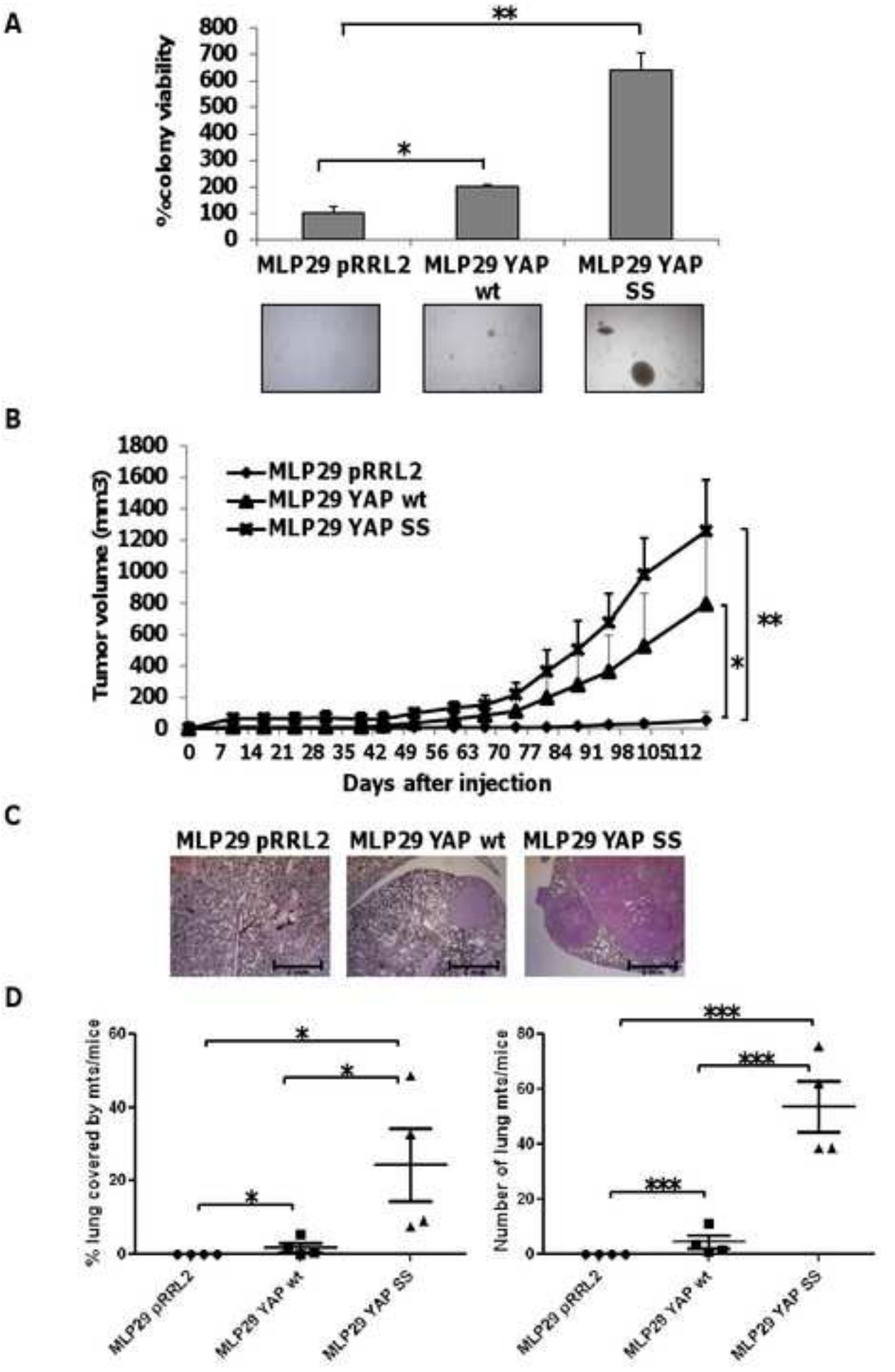
**
control
HCC


A
- VP
+ VP
1.0 mm
1.0 mm
B
GSTP + foci/cm²
[N°/cm²]
150
125
100
75
50
25
0
*
foci
foci+VP
mean GSTP + area
[mm²]
0,04
0,03
0,02
0,01
0,00
**
foci
foci+VP
GSTP + area [%]
5
4
3
2
1
0
**
foci
foci+VP
C
-VP
+VP
-VP
+VP
D
Huh7 pRRL2
%cell viability
(Cell Titer-Glo)
140
120
100
80
60
40
20
0
*
**
***
***
***
***
**
***
**
NT
500
600
700
800
900
1000
1250
1500
VP concentration (nM)
E
%cell viability
(Cell Titer-Glo)
200
150
100
50
0
*
NT
HGF
VP
VP +
HGF
MLP29


A
%colony viability
800
700
600
500
400
300
200
100
0
**
*
MLP29 pRRL2
MLP29 YAP wt
MLP29 YAP SS
B
Tumor volume (mm3)
1800
1600
1400
1200
1000
800
600
400
200
0
MLP29 pRRL2
MLP29 YAP wt
MLP29 YAP SS
**
*
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days after injection
C
MLP29 pRRL2
MLP29 YAP wt
MLP29 YAP SS
D
% lung covered by mts/mice
60
40
20
0
*
*
*
MLP29 pRRL2
MLP29 YAP wt
MLP29 YAP SS
Number of lung mts/mice
80
60
40
20
0
***
***
***
MLP29 pRRL2
MLP29 YAP wt
MLP29 YAP SS

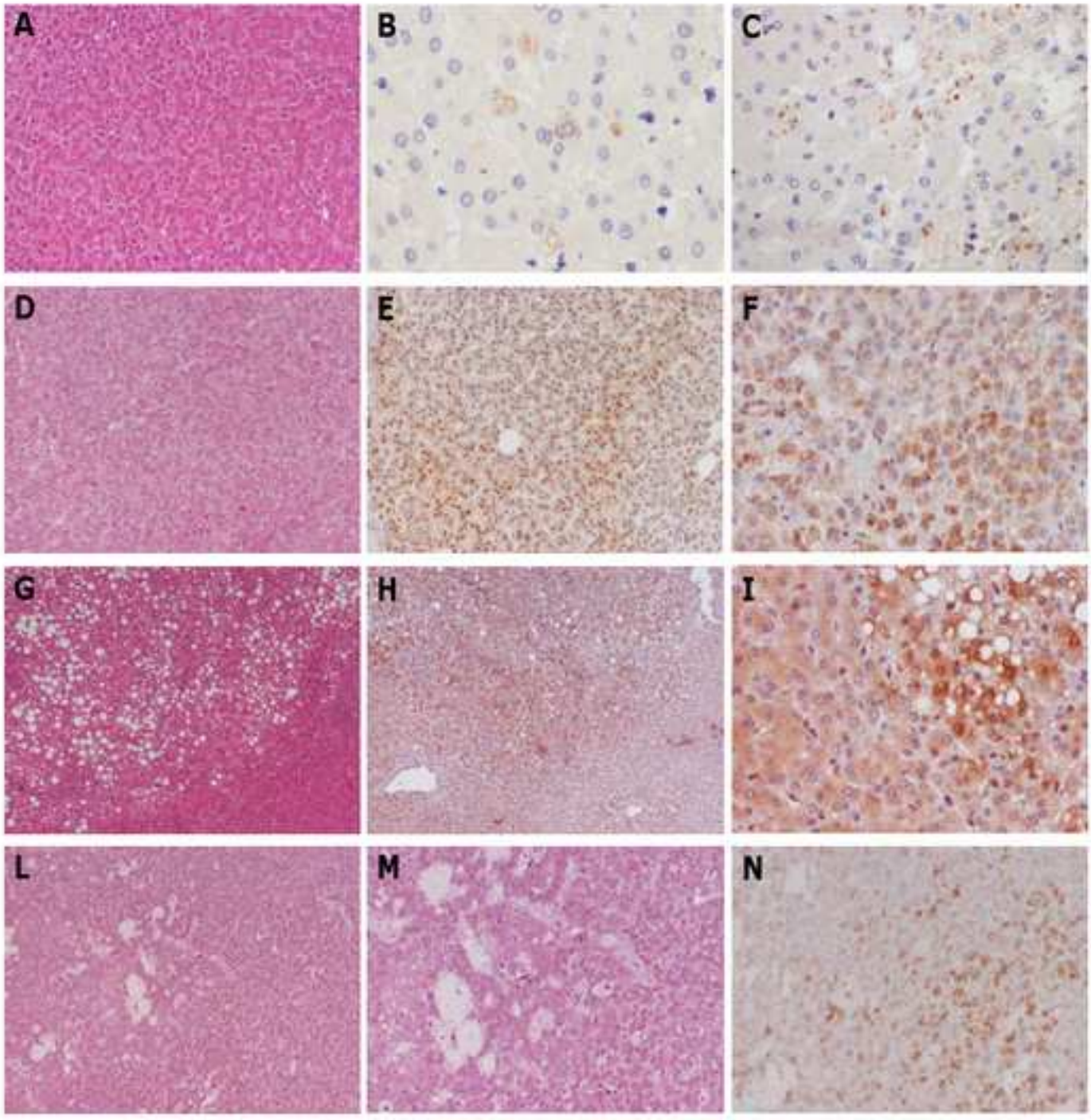

A
B
C
D
E
F
G
H
I
L
M
N

Table 1: YAP immunoreactivity in human dysplastic nodules and hepatocellular adenomas

Lesion	Clinical features	Histopathological diagnosis	YAP immunoreactivity
1	Male; 60 yrs; HCV related cirrhosis; 0.9 cm size	LGDN	10%,+, C
2	Male; 61 yrs; alcohol related cirrhosis; 1 cm size	LGDN	Negative
3	Male; 57 yrs; HCV and alcohol related cirrhosis; 3.0 cm size	LGDN	10%,+, C
4	Male; 64 yrs; HBV and alcohol related cirrhosis; 1.7 cm size	HGDN	10%, ++,C
5	Male; 67 yrs; HCV related cirrhosis; 1 cm size	HGDN	Negative
6	Male 74 yrs; metabolic cirrhosis, 1.2 cm size	HGDN	10%, ++, C
7	Female 33 yrs; HBV and HCV negative; multiple (> 10) liver lesions	Hepatocellular adenomatosis steatotic subtype	30%, ++, C
8			40%, ++, C
9			40%, ++, C
10			70%, ++, C
11			70%, +++, C (isolated N)
12	Female 38 yrs; HBV and HCV negative, Oral contraceptive assumption; Multiple (> 10) liver lesions	Hepatocellular adenomatosis steatotic subtype	80%,+++,C
13	Female 51 yrs; HBV and HCV-negative dyslipidemic syndrome; multiple (>10) liver lesions	Hepatocellular adenomatosis steatotic subtype	40%, +++,C
14	Male, 53 yrs, 8 cm size	Hepatocellular adenoma telangiectatic subtype	70%,+++,C (isolated N)
15	Female, 40 yrs, 7 cm size	Hepatocellular adenoma telangiectatic subtype	10%, ++, C (isolated N)
16	Male, 19 yrs; 12 cm size	HCC (G1) arising within a hepatocellular adenoma, telangiectatic and with β-catenin activation	70%, +++, C
17	Male, 26 yrs, 10 cm size	HCC (G1) arising within an atypical hepatocellular adenoma, with β-catenin activation	80%, +++, C
18	Female, 23 yrs, HBV and HCV negative (2 liver lesions) 5 and 1.5 cm size	Multiple focal Nodular Hyperplasia	Negative

LGDN: Low grade dysplastic nodules; HGDN: High grade dysplastic nodules
The number of YAP-positive cells is expressed as percentage (%);
C: cytoplasmic localization; N: nuclear localization.
The staining intensity is expressed as + (faint); ++ (moderate); +++ (strong)