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Galectin-1 Triggers Epithelial-Mesenchymal Transition in Human Hepatocellular Carcinoma Cells

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Galectin-1 (Gal1), a β-galactoside-binding protein abundantly expressed in tumor microenvironments, is associated with the development of metastasis in hepatocellular carcinomas (HCC). However, the precise roles of Gal1 in HCC cell invasiveness and dissemination are uncertain. Here, we investigated whether Gal1 mediate epithelial-mesenchymal transition (EMT) in HCC cells, a key process during cancer progression. We used the well-differentiated and low invasive HepG2 cells and performed 'gain-of-function' and 'loss-function' experiments by transfecting cells with Gal1 cDNA constructs or by siRNA strategies, respectively. Epithelial and mesenchymal markers expression, changes in apico-basal polarity, independent-anchorage growth, and activation of specific signaling pathways were studied using Western blot, fluorescence microscopy, soft-agar assays, and FOP/TOP flash reporter system. Gal1 up-regulation in HepG2 cells induced down-regulation of the adherens junction protein E-cadherin and increased expression of the transcription factor Snail, one of the main inducers of EMT in HCC. Enhanced Gal1 expression facilitated the transition from epithelial cell morphology towards a fibroblastoid phenotype and favored up-regulation of the mesenchymal marker vimentin in HCC cells. Cells overexpressing Gal1 showed enhanced anchorage-independent growth and loss of apico-basal polarity. Remarkably, Gal1 promoted Akt activation, β-catenin nuclear translocation, TCF4/LEF1 transcriptional activity and increased cyclin D1 and c-Myc expression, suggesting activation of the Wnt pathway. Furthermore, Gal1 overexpression induced E-cadherin downregulation through a PI3K/Akt-dependent mechanism. Our results provide the first evidence of a role of Gal1 as an inducer of EMT in HCC cells, with critical implications in HCC metastasis.

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Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third cause of cancer-related death annually (Ferlay et al., 2010). Although the management of this disease has improved during the past decade, the incidence still matches mortality, reflecting the poor prognosis of HCC (de Lope et al., 2012). Recurrence, metastasis, and development of new primary tumors are the most common causes of mortality among patients with HCC. The approval of molecular therapies including sorafenib, a multikinase inhibitor capable of restraining tumor progression (Villanueva et al., 2013), has underscored the importance of exploring the mechanisms underlying HCC cell invasiveness for the development of novel therapeutic agents.

Epithelial-mesenchymal transition (EMT) is a critical event in developmental and tissue remodeling processes (Thiery et al., 2009). In addition, EMT represents a key step during cancer progression, which involves changes in cell-cell and cell-matrix interactions, cell motility and anchorage allowing tissue epithelial cancers to invade and metastasize (Nieto, 2011). Hallmarks of EMT include the functional loss of the adherens junction protein E-cadherin, which expression is frequently repressed by the Snail, ZEB and Twist family of transcription factors (Sanchez-Tillo et al., 2012). Additionally, other features of EMT involve the development of a fibroblastoid phenotype, the up-regulation of mesenchymal markers and the loss of apico-basal polarity (Nieto, 2011).

Accumulating evidence suggests that EMT plays a pivotal role in the dissemination of malignant hepatocytes during HCC

Abbreviations: BC, bile canaliculi; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; Gal1, galectin-1; HCC, hepatocellular carcinoma; MRP2, multidrug resistance associated-protein 2; PI3K, phosphatidylinositol 3-kinase; TCF4/LEF1, T-cell factor/lymphoid-enhancer factor.

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progression (van Zijl et al., 2009). It has been demonstrated that 58% of human HCC tumors have reduced levels of E-cadherin and diminished expression of β-, α- and p120-catenins associated with nuclear translocation of β-catenin. These events often correlate with capsular invasion, intrahepatic metastasis and poor prognosis (Zhai et al., 2008). Moreover, overexpression of Snail and/or Twist have been found to be associated with E-cadherin down-regulation, non-membranous expression of β-catenin, HCC tumor invasiveness, metastasis and worse prognosis (Yang et al., 2009).

The interplay between different stimuli and activation of diverse signaling pathways are critical for EMT induction. Evidence from both experimental models and human HCC revealed that EMT involves the recurrence of embryonic programs implicating transforming growth factor-β_1 (TGF-β_1) and Wnt/β-catenin signaling with collaboration of hepatic viruses (van Zijl et al., 2009; Firrincieli et al., 2010). Activation of Wnt/β-catenin pathway leads to nuclear translocation of β-catenin, where it interacts with the T-cell factor/lymphoid-enhancer factor (TCF4/LEF1) transcription factor that regulates cell cycle progression, differentiation, and motility. Further, β-catenin/TCF complex suppresses E-cadherin promoter activity. This pathway also induces EMT through the expression of target genes that negatively control the expression of E-cadherin, genes that regulate the mesenchymal phenotype and genes that encode ECM-modulating metalloproteinases. Moreover, Wnt signaling inhibits glycogen synthase kinase-3β (GSK-3β)-mediated Snail1 destabilization (Heuberger and Birchmeier, 2010).

Galectin-1 (Gal1) belongs to a family of carbohydrate-binding proteins with specific affinity to β-galactosides and a consensus sequence in the carbohydrate recognition domain (Yang et al., 2008). Gal1 expression is often a hallmark of malignant tumor progression as it is abundantly overexpressed in advanced stages of the disease (Demydenko and Berest, 2009). By acting extracellularly, Gal1 modulates cell adhesion, tumor growth, migration, angiogenesis, tumor-immune escape and metastasis (Elola et al., 2007; Ito et al., 2012). Intracellularly, Gal1 specifically interacts with H-Ras to mediate Ras membrane anchorage and cell transformation (Paz et al., 2001). Specifically, Gal1 is up-regulated in human HCC samples and in cell lines, including HepG2 and HuH-7 cells (Chung et al., 2002; Kondoh et al., 2003). Its overexpression promoted HuH-7 cell migration and invasion in vitro (Spano et al., 2010). Up-regulation of Gal1 in HCC tumors is often associated with tumor invasiveness, metastasis, tumor recurrence, and shortened survival, suggesting its potential value as a marker for predicting the poor prognosis of HCC (Wu et al., 2012; Bacigalupo et al., 2013). Interestingly, a dual role of endogenous Gal1 in liver inflammation and tumorigenesis has been recently dissected in the multidrug resistant (Mdr2) model of inflammation-induced hepatocarcinogenesis showing strain-dependent differences in Gal1 effects (Potikha et al., 2013). Recently, we demonstrated that Gal1 acts as a glycan- and integrin-dependent modulator of HepG2 cell adhesion and polarization. Moreover, Gal1-overexpressing HepG2 cells injected in nude mice promoted tumor growth and contributed to metastasis in tumor-associated draining lymph nodes (Espelt et al., 2011).

Given the role of Gal1 in HCC adhesion, migration and metastasis, we sought to investigate whether an increase in Gal1 expression could trigger EMT in well-differentiated and low invasive HCC cells. This is the first evidence implicating Gal1 in HCC EMT transition.

Materials and Methods

Materials

Bovine serum albumin (BSA), laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, poly-L-lysine, aprotinin, phenylmethylsulfonyl fluoride (PMSF), tetramethylrhodamineisothiocyanate (TRITC)-phalloidin, bisBenzimide H33258 (Hoescht), wortmannin, PD98059, low melting point agarose, and collagen type I, were purchased from Sigma–Aldrich Co. (St. Louis, MO). Fetal bovine serum was from Natocor (Villa Carlos Paz, Córdoba, Argentina). Dulbecco's modified Eagle medium (DMEM) and trypsin/EDTA solution, from Gibco were purchased from Invitrogen Corporation (Carlsbad, CA). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was from Fluka. A complete list of primary and secondary antibodies used for Western blot and immunodetection experiments can be found in Supplementary Table S1.

Cell culture

The human HCC cell line HepG2 was cultured in DMEM containing 4.5 g/L glucose, supplemented with 10% v/v fetal bovine serum, 2 mM L-glutamine and antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C.

Preparation of recombinant human Gal1

Human recombinant Gal1 (rGal1) was expressed in *Escherichia coli* strain BL21 (DE3) (Couraud et al., 1989) transformed with the plasmid *pT7IML-1-Lgals1* (a generous gift from Dr. L. Baum, University of California, Los Angeles, CA). rGal1 was purified by affinity chromatography on a lactose-agarose column (Sigma–Aldrich Co.) under reducing conditions (2 mM dithiothreitol) to maintain maximal lectin activity, which was measured as described before (Troncoso et al., 2000). Purity was confirmed by SDS-PAGE, immunoblotting and mass spectrometry. To obtain an endotoxin-free sample, purified protein solution was then submitted to polymyxin B-agarose chromatography (Sigma–Aldrich Co.) (Anspach and Hilbeck, 1995; Liu et al., 1997) and lipopolysaccharide content (less than 0.25 endotoxin units/ml) was tested using a Gel Clot Limulus Test, Pyrotell, Associates of Cape Cod, Inc. (Palmouth, MA).

Transfections

Transfections to overexpress or knockdown Gal1 were performed as previously described (Espelt et al., 2011). Briefly, cells were transfected with pcDNA3.1-*Lgals1* or pcDNA3.1 expression vector (Invitrogen Corporation) alone as control, using Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer's instructions. Stable Gal1-overexpressing cells were selected by G418 resistance. Gal1 knockdown was performed with 80 nM nontargeting scrambled siRNA or a pool of three target-specific Gal1 siRNAs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Overexpression and silencing efficiencies were checked by Western blot.

Morphologic analysis

Cells were cultured in 24-well plates on sterilized 12 mm diameter glass coverslips coated with collagen type I (5 $\mu g/cm^2$). After 72 h, cells were photographed using a bright field inverted microscope.

Western blot analysis

Cells were homogenized in lysis buffer (100 mM Tris, pH 7.4, 1% v/v Triton X-100, 10 mM EDTA, 10 mM EGTA, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium vanadate, 2 mM PMSF and 0.012–0.034 TIU/ml aprotinin) at 4 °C. After 45 min, the cellular extracts were centrifuged for 10 min at 12,000 x g and supernatants were collected. For protein phosphorylation analysis, cells were cultured on 6-well plates for 24 h in serum-free media, and then scrapped with lysis buffer. To analyze the involvement of phosphatidylinositol 3-kinase (PI3K)

and the ERK1/2-specific mitogen-activated protein kinase (MAPK) kinase, cells were incubated for 72 h in the presence of the PI3K inhibitor wortmannin (1 μM) or MAPK inhibitor PD98059 (25 μM) in dimethyl sulfoxide (DMSO) and then homogenized as previously described. To evaluate the effect of TGF-β_1, cells were incubated with 5 ng/ml of recombinant TGF-β_1 in serum-free media for 48 h. Twenty-five micro gram protein per sample were separated by SDS-PAGE, and then transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with primary antibodies overnight at 4 °C followed by a 60 min-incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. A complete list of antibodies used can be found in Supplementary Table S1. Immunoreactive bands were detected by chemiluminiscence (Amersham ECL prime Western Blotting Detection Reagents, GE Healthcare, Uppsala, Sweden). Densitometric analysis of protein levels was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD; http://rbsweb.nih.gov/ij/).

Soft-agar colony formation assay

The anchorage-independent growth was determined by assaying colony formation in soft-agar (Cokakli et al., 2009). Briefly, 24-well plates were coated with 0.6% low melting point agarose (Sigma–Aldrich Co.) supplemented with DMEM. Cells were trypsinized, and resuspended in 0.4% low melting point agarose supplemented with DMEM. 2,500 cells per well were seeded on the base agar layer. Medium was added every 3 days. The number of colonies was counted and photographed after 3 weeks of incubation at 37 °C.

Determination of cell polarization

Cells were cultured in 24-well plates on sterilized 12 mm diameter glass coverslips coated with 0.1% w/v poly-L-lysine at a density of 50,000 cells per coverslip (30–40% confluence) for the indicated time. Cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% v/v Triton X-100 in PBS for 10 min. F-actin was stained with 2 μg/ml tetramethylrhodamineisothiocyanate (TRITC)-phalloidin for 20 min. Multidrug resistance associated-protein 2 (MRP2), was stained with anti-MRP2 antibody and the corresponding FITC-conjugated anti-IgG antibody (Supplementary Table S1). Nuclei were stained with 100 ng/ml Hoechst 33258 for 10 min. The degree of cell polarity was determined by counting the number of bile canaliculi (BC; identified by dense F-actin or MRP2 staining) per 100 cells (identified by fluorescently labeled nuclei). Ten fields per coverslip with more than 50 nuclei per field (two coverslips per treatment) were examined with a Nikon TE-200 epifluorescence inverted microscope.

Immunofluorescence

Cells cultured on poly-L-lysine-coated coverslips for 72 h were fixed with methanol for 10 min at −20 °C, and then incubated for 1 h in PBS-0.1% v/v Triton X-100 containing 1% w/v BSA to block nonspecific binding sites. Primary and secondary antibodies used are detailed in Supplementary Table S1. Nuclei were stained with 0.5 μg/ml 4',6'diamidino-2-phenylindole (DAPI). Slides were analyzed with a FluoView 1,000 confocal microscope (Olympus, Japan).

Quantitative analysis of β-catenin nuclear translocation was performed in Image Pro Plus software (version 5.1.). To determine nuclear translocation, the overlap coefficient according to Manders (R) between nuclear β-catenin and DAPI fluorescence was estimated after background correction, and an R value higher than 0.6 was considered positive nuclear colocalization (Zinchuk et al., 2007). At least three randomly selected fields per coverslip (two coverslips per treatment) were analyzed.

Luciferase reporter assay

To evaluate TCF transcriptional activity, HepG2-M and HepG2-Gal1 cells were cultured in six-well plates, grown to 80–90% confluence, and transiently transfected with TOP-flash and FOP-flash plasmids. The TOP-flash reporter gene construct contains four consensus TCF binding sites and a luciferase reporter gene (Korinek et al., 1997). The FOP-flash construct with a mutated TCF binding site was used as a negative control for measuring nonspecific activation of the reporter. All transfections were performed with Lipofectamine 2,000 (Invitrogen Corporation) according to the manufacturer's instructions using 0.7 μg of each plasmid. To normalize transfection efficiency, cells were co-transfected with 0.1 μg of β-galactoside expression vector. After 24 h, cells were harvested and lysed with 100 μl of lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 1% v/v Triton X-100, 10% v/v glycerol) for determination of luciferase activity. This activity was measured in relative light units using a luminometer and the Promega luciferase assay system. Relative luciferase activity is reported as fold induction after normalization for transfection efficiency. Each transfection was done in triplicate and three independent experiments were performed.

Statistical analyses

Data are expressed as the means ± s.e.m. and were analyzed using GraphPad Prism Software (GraphPad Software Inc, LaJolla, CA). Statistical analysis was performed using *t* test or one-way analysis variance, with the pertinent post-test. P values <0.05 were considered to be significant.

Results

Gal1 overexpression in HepG2 cells triggers EMT

To investigate the involvement of Gal1 in EMT transition, we first investigated whether the expression levels of this lectin in HCC cells are associated with suppression of cadherin-mediated cell-cell adhesion, a critical event for initiation of the metastatic cascade. We used HepG2 cells, which represent a reliable model of differentiated HCC cells that express epithelial cell markers and retain the ability to polarize in vitro. We stably transfected HepG2 cells with Gal1 cDNA and selected two G418-resistant-clones, obtaining HepG2-Gal1 cells with approximately 2-fold (HepG2-Gal1A) and threefold (HepG2-Gal1B) higher expression of Gal1 with respect to nontransfected cells and cells transfected with empty vector (HepG2-M) (Fig. 1A). These cells also secreted higher levels of the lectin to the extracellular medium (Fig. 1B). Moreover, HepG2 cells transfected with Gal1 siRNA exhibited an approximately 70% decrease in Gal1 protein (Gal1 siRNA) 72 h after transfection when compared to cells transfected with scrambled siRNA (scrambled siRNA) (Fig. 1C).

By immunoblot analysis, we observed a significant down-regulation of E-cadherin expression both in HepG2-Gal1A (58 ± 8%) and HepG2-Gal1B (46 ± 6%) cells, respect to HepG2-M control cells (Fig. 1D). Therefore, the remaining experiments were conducted with the clone B, hereafter called HepG2-Gal1 cells. In contrast, knocking down Gal1 using siRNA strategies (Gal1 siRNA) significantly increased E-cadherin expression (119 ± 3%) with respect to control scrambled siRNA cells (Fig. 1D). Reduced expression of E-cadherin in HepG2-Gal1 cells was independent of the cell density and incubation time (Fig. 1E). To rule out the possibility that manipulation of Gal1 expression could alter β-actin levels, we evaluated β-tubulin expression, another protein used as a loading control. Positive or negative modulation of Gal1 expression did not alter β-tubulin or β-actin expression (Fig. S1). Immunocytochemistry and confocal microscopy revealed that in most control cells, E-cadherin was localized

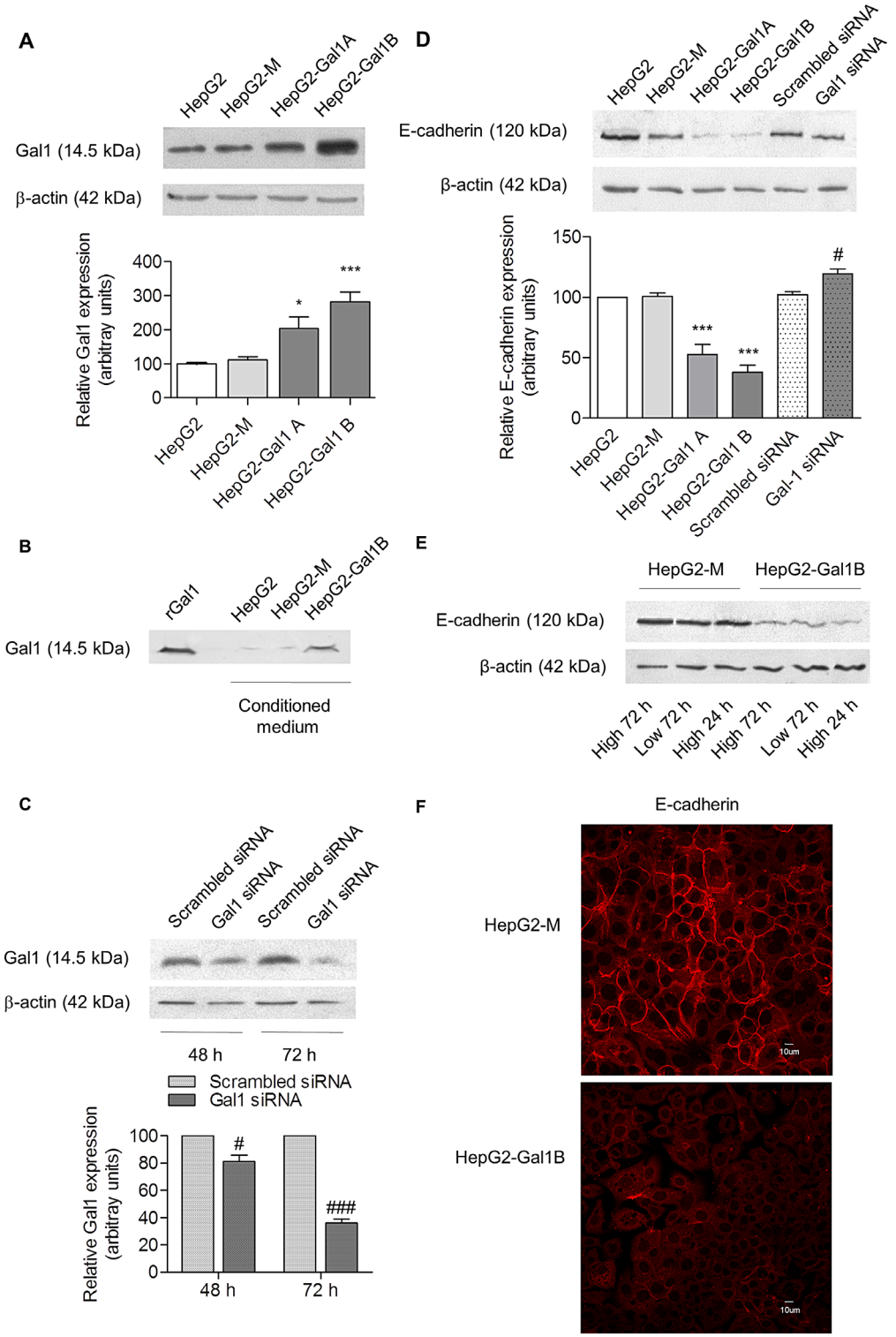

Fig. 1. Gal1 overexpression in HepG2 cells downregulates E-cadherin expression. A: Western blot and densitometric analysis showing Gal1 expression in non-transfected and transfected with pcDNA3.1-*Lgals1* (HepG2-Gal1A and B) or empty vector (HepG2-M) HepG2 cell lysates. (n = 5). B: Secretion of Gal1 to the extracellular medium. Western blot analysis of Gal1 was performed in serum-free conditioned medium of nontransfected and transfected cell cultures. rGal1: recombinant Gal1. (n = 3). C: Western blot analysis of Gal1 knocked down cells (Gal1 siRNA) 48 or 72 h after transfection. Scrambled siRNA: cells transfected with scrambled siRNA. (n = 3). D: Western blot and densitometric analysis showing E-cadherin expression in HepG2-M, HepG2-Gal1A and B, Scrambled siRNAand Gal1 siRNA cells; β-actin, loading control (n = 5). E: Western blot for E-cadherin in cells seeded on 6-well plates at low or high density (3 or 6 × 10^5 cell/well, respectively), cultured for 24 or 72 h. F: E-cadherin immunofluorescence (400x). Data are representative of three independent experiments. *$P < 0.05$, ***$P < 0.001$ respect to HepG2-M cells. #$P < 0.05$, ###$P < 0.001$ respect to scrambled siRNA cells.

mainly at the basolateral plasma membrane of adjacent cells as expected; whereas Gal1 overexpression led to an altered localization of E-cadherin (Fig. 1F).

E-cadherin turnover represents a crucial component of cell adhesion events. Catenin p120 binds to the juxtamembrane domain of E-cadherin and increases its stability at the cell membrane. As depletion of p120 leads to an immediate turnover of E-cadherin, we investigated whether Gal1 up-regulation affected p120 levels. Although different splice variants exists for catenin p120 (Nieto, 2011), no significant differences were observed in p120-catenin isoforms expression in HepG2 cells when Gal1 expression was positively or negatively regulated, suggesting that Gal1 does not affect E-cadherin turnover (Fig. 2A).

One of the main inducers of EMT in HCC is Snail, a transcription factor which acts as an E-cadherin repressor. Remarkably, Gal1 overexpression resulted in a considerable increase of Snail expression (140 ± 7%), whereas silencing of this lectin induced a significant decrease in its expression (38 ± 12%) (Fig. 2B).

Since a hallmark of EMT is the up-regulation of mesenchymal markers, we further analyzed vimentin expression, an intermediate filament involved in cytoskeleton remodeling, which defines a mesenchymal phenotype. We found a significant increase in vimentin expression in HepG2-Gal1 cells (163 ± 12%), whereas Gal1-silenced HepG2 cells showed diminished expression of this mesenchymal marker (Fig. 2C). By immunocytochemistry, we showed vimentin expression in HepG2-Gal1 cells (Fig. 2D). Moreover, when cultured on type I collagen, the major component of the hepatocyte ECM, HepG2-Gal1 cells became flat, spindle-shaped, and appeared loosely associated (Fig. 2E). Thus, increased Gal1 expression in well-differentiated HepG2 cells promotes EMT as shown by a shift towards down-regulation of the epithelial molecule E-cadherin and up-regulation of the mesenchymal marker vimentin, augmented expression of the Snail transcription factor and changes from epithelial cell morphology towards a fibroblastoid phenotype.

Up-regulation of Gal1 promotes HepG2 cell resistance to anoikis and loss of apico-basal polarity

Cells undergoing EMT display a higher metastatic potential, in part by acquiring resistance to anoikis (Onder et al., 2008). This resistance mechanism has been shown to promote metastasis as tumor cells can disseminate through the bloodstream. We next performed soft-agar colony formation assays to examine whether Gal1 up-regulation in HCC cells confers resistance to anoikis. We observed a significant increase in the number of colonies growing in soft-agar in HepG2-Gal1 cells compared to control-transfected cells (18 ± 2 vs. 6 ± 2 colonies/well) (Fig. 3A).

The plasma membrane of hepatocytes is separated by tight junctions in sinusoidal (basolateral) and canalicular (apical) domains. When cultured in vitro, HepG2 cells acquire a polarized phenotype characterized by the appearance of apical bile pseudocanaliculi (BC) between adjacent cells in a time-dependent manner. We previously observed that as a consequence of favoring HepG2 cell adhesion, exogenously added recombinant (rGal1) accelerated BC development, reaching similar values as control cells after 72 h (Espelt et al., 2011). However, during EMT hepatocytes lose their apico-basal polarity. Thus, we asked whether Gal1 overexpression interfered with HCC cell polarity after prolonged incubation times. Similarly to HepG2 cells treated with rGal1, HepG2-Gal1 cells showed accelerated BC development up to 24–48 h after plating with respect to HepG2-M cells (data not shown). Nevertheless, a decrease in the percentage of polarization, determined as the number of TRITC-phalloidin-stained BC/100 cells was observed after 72 h (72 ± 7%). Diminished polarization was maintained even after 96 h (81 ± 4%) (Fig. 3B). By immunostaining a bile canalicular specific protein, multidrug resistance associated-protein 2 (MRP2), we observed a similar pattern of reduction in the percentage of polarized HepG2-Gal1 cells after 72 h (66 ± 2%) (Fig. 3C).

As proteins localized in tight junctions are also altered during EMT, we next evaluated the expression of the zona-occludens protein ZO-1, which often serves as a tight junction prototypical marker. We found that expression of this protein was diminished in HepG2-Gal1 cells after 72 h (52 ± 12%). On the other hand, when Gal1 was silenced in HepG2 cells, ZO-1 expression was not significantly altered with respect to scrambled-transfected cells (107 ± 13 vs. 95 ± 5) (Fig. 3D).

These results demonstrate that an increase in Gal1 expression in HepG2 cells favors anchorage-independent growth. We can also conclude that HepG2 cells cultured for prolonged time periods develop less apical bile canaliculi, and show diminished expression of the tight junction marker ZO-1. Thus, resistance to anoikis and loss of apico-basal polarity, other hallmarks of EMT, are associated with Gal1 overexpression in HepG2 cells.

Gal1 expression in HepG2 cells alters β-catenin protein expression, localization and signaling

Besides its function in maintaining intercellular contacts through adherens complex with β-catenin, E-cadherin has an important role in the transmission of the Wnt pathway. Accumulating evidence demonstrate that canonical Wnt signaling and cadherin-mediated cell adhesion depend on the same pool of β-catenin (Heuberger and Birchmeier 2010). As a loss of E-cadherin-mediated cell adhesion induces β-catenin release and signaling, we explored whether changes in Gal1 expression in HepG2 cells altered β-catenin protein expression, localization, and/or signaling. An activating mutation of the Wingless/Wnt/β-catenin proliferation pathway is found in HepG2 cells, where a large deletion (amino acids 25–140) in the β-catenin (*CTNNB1*) gene removes the glycogen synthase kinase-3β (GSK-3β) regulatory site (de La Coste et al., 1998). Thus, HepG2 cells, heterozygous for the β-catenin gene mutation, co-express both the β-catenin wild-type (WT) isoform (92 kDa) and the deleted protein (75 kDa). We observed that overexpression of Gal1 in HepG2 cells significantly decreased β-catenin WT isoform (73 ± 7%), whereas silencing its expression did not significantly altered the expression of this β-catenin isoform (113 ± 7%) (Fig. 4A). Of note, no changes were observed in truncated β-catenin isoform related to modifications in Gal1 expression.

Carboxy-terminal phosphorylation of β-catenin at Ser^{552} residue mediated by Akt changes the adhesiveness to cadherin and promotes nuclear localization (Fang et al., 2007), resulting in transcription activation of specific target genes of the Wnt pathway, such as c-Myc and cyclin D1 (Rimerman et al., 2000). Interestingly, HepG2-Gal1 cells showed an increase in phosphorylation at Ser^{552} residue of the truncated β-catenin isoform (150 ± 18%), while no significant changes were observed when Gal1 expression was down-regulated (siRNA Gal1 97 ± 12% vs scrambled siRNA 109 ± 8%) (Fig. 4B). By immunocytochemistry and confocal microscopy we confirmed that Gal1 overexpression increased β-catenin translocation and/or accumulation in the nucleus of HepG2 cells with respect to HepG2-M control cells (Fig. 4C). We analyzed β-catenin fluorescence that colocalized with DAPI-stained nuclei determining the overlap fluorescence coefficient according to Manders (R), for example, R1 = 0.9, R2 = 0.36, R3 = 0.77, and R4 = 0.89 (Fig. 4C). We found a significant increase in the percentage of HepG2-Gal1 cells showing β-catenin expression in the nucleus with respect to control cells (59 ± 7 vs. 32 ± 12%).

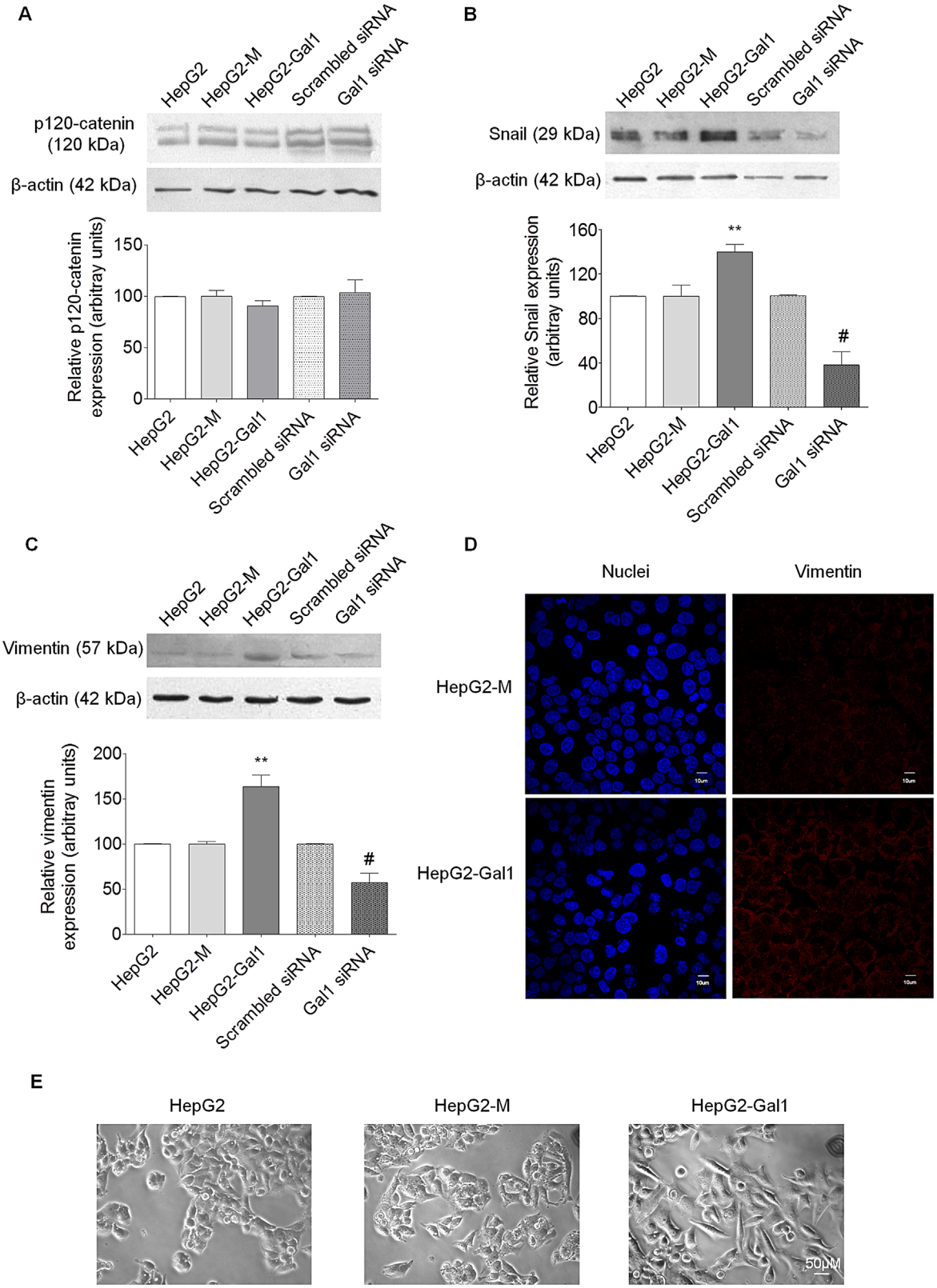

Fig. 2. Gal1 up-regulation in HepG2 cells increases Snail and vimentin levels. Western blot and densitometric analysis relative to β-actin expression in non-transfected and transfected HepG2 cells for A: p120-catenin, B: Snail and C: vimentin (n = 3). D: Vimentin immunofluorescence (600x). Nuclei were stained with DAPI. E: Bright field microphotographs showing cells cultured on type I collagen (200x). Photographs are representative of two independent experiments. **$P < 0.01$ respect to HepG2-M cells. #$P < 0.05$ respect to scrambled siRNA cells.

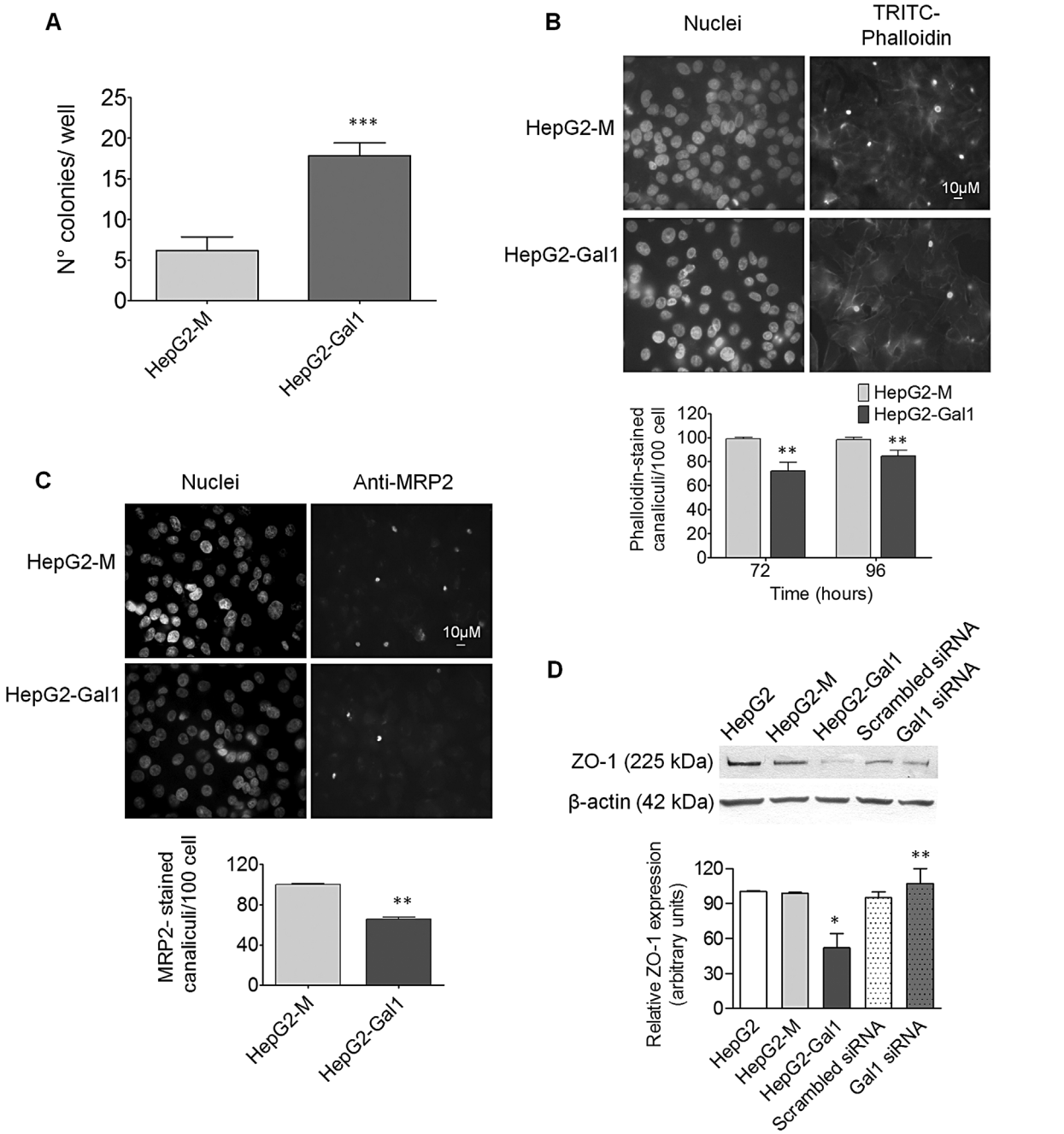

Fig. 3. Overexpression of Gal1 induces HepG2 cell resistance to anoikis and loss of apico-basal polarity. A: Anchorage-independent growth was evaluated by soft-agar assay after culturing cells in 24-well plates for 3 weeks. Bile canaliculi were identified by B: dense F-actin staining with TRITC-phalloidin or C: by MRP2 immunostaining. Nuclei were stained with Hoechst 33258 and polarized cell morphology was visualized by fluorescence microscopy (400x). Cell polarization was quantified determining the number of phalloidin- or MRP2-positive canaliculi per 100 cells (identified by fluorescently labeled nuclei) ± SEM of duplicate determinations of three independent experiments. D: Western blot and densitometric analysis showing ZO-1 expression in non-transfected and transfected cells (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ respect to HepG2-M cells.

To further confirm that Gal1 overexpression induces β-catenin signaling and hyperactivation of the Wnt pathway, we evaluated TCF transcriptional activity by transiently transfecting HepG2-M and HepG2-Gal1 cells with TOP-flash and FOP-flash constructs. When performing the luciferase reporter assay with TOP-flash plasmid, we observed that the relative luciferase activity in HepG2-Gal1 cells significantly increased (2.3-fold) with respect to HepG2-M cells (Fig. 4D). Worth mentioning is that no luciferase activity was detected when both HepG2-M and HepG2-Gal1 cells were transfected with FOP-flash construct (negative control).

Finally, in line with these findings, we sought to investigate if Gal1 overexpression could induce the expression of specific target genes of the Wnt pathway. Interestingly, HepG2-Gal1

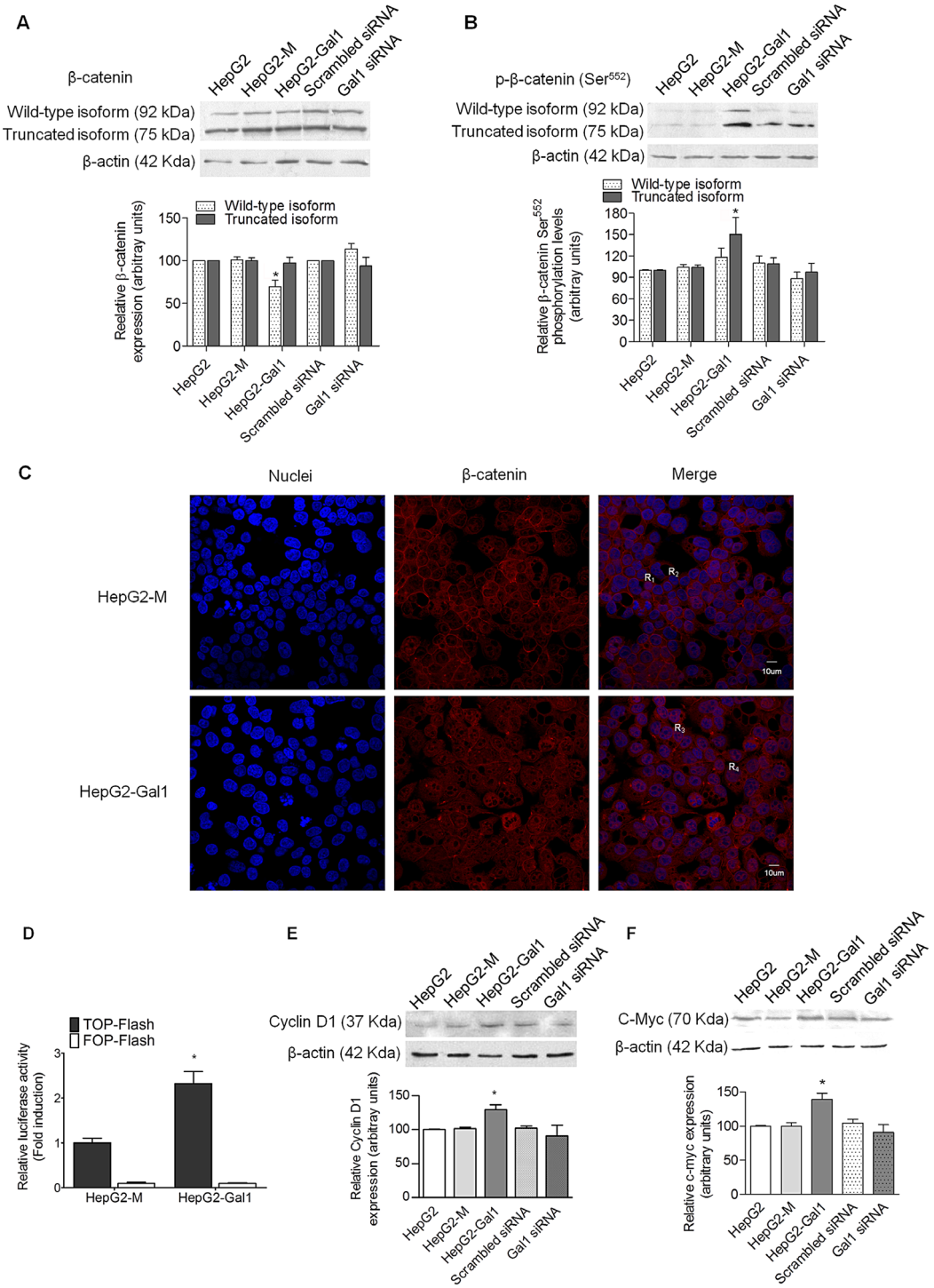

Fig. 4. Effect of Gal1 on β-catenin expression, Akt-dependent phosphorylation, localization and signaling. Western blot and densitometric analysis relative to β-actin expression for A: wild-type and truncated β-catenin isoforms (n = 4) and B: β-catenin phosphorylation at Ser^{552} relative to total β-catenin (n = 3) in non-transfected and transfected cells. C: β-catenin nuclear translocation is visualized by confocal microscopy (600x). Nuclei were stained with DAPI. Photographs are representative of two independent experiments. R indicates the overlap coefficient according to Manders. As examples, $R_1 = 0.9$, $R_2 = 0.36$, $R_3 = 0.77$, $R_4 = 0.89$. D: HepG2-M and HepG2-Gal1 cells were cotransfected with reporter plasmids TOP-flash or FOP-flash, and β-galactosidase codifying plasmid as internal control for 24 h. Relative luciferase activities of TOP-flash and FOP-flash were normalized to the internal control. Data represent mean ± S.E.M. from three experiments performed in triplicates. Western blot and densitometric analysis for cyclin D1 (E) and c-Myc (F) (n = 3) relative to β-actin expression. *$P < 0.05$ respect to HepG2-M cells.

cells showed elevated levels of cyclin D1 (129 ± 7%) and c-Myc (139 ± 9%) with respect to HepG2-M cells (Fig. 4E, F). On the contrary, downregulation of Gal1 expression resulted in no significant reduction in c-Myc and cyclin D1 expression respect to scrambled siRNA cells (91 ± 9% and 90 ± 16%, respectively). Thus, an increase in Gal1 expression favors the translocation of β-catenin to the nucleus of HepG2 cells, leads to an increase of TCF transcriptional activity and up-regulates expression of the Wnt pathway target genes, cyclin D1 and c-Myc, suggesting hyperactivation of the Wnt signaling pathway.

Gal1 overexpression induces E-cadherin downregulation in HepG2 cells through a PI3K/Akt-dependent mechanism

As Gal1-overexpressing HepG2 cells showed an increase in β-catenin phosphorylation at Ser^{552} residue which is known to be mediated by Akt, we sought to investigate if up-regulated expression of this galectin could promote Akt activation. Insulin-treated hepatocytes were used as a positive control for Akt phosphorylation. We observed a 1.88-fold increase in Akt phosphorylation at Thr^{308} residue in HepG2-Gal1 cells with respect to control HepG2-M cells (Fig. 5A), suggesting that Gal1 overexpression leads to constitutive Akt activation. Besides, we analyzed Akt phosphorylation in Gal1-silenced HepG2 cells. We found no significant differences in phosphorylation of Akt in cells transfected with Gal1 siRNA as compared to scrambled-transfected cells (112 ± 13 vs. 144 ± 11%) (Fig. 5A).

Next, we investigated if Akt phosphorylation is involved in EMT induced by Gal1 overexpression. As Akt is phosphorylated by phophatidylinositol 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1) pathway, we analyzed E-cadherin expression, by Western blot, in the presence of the PI3K inhibitor wortmannin (1 μM) in HepG2-M and HepG2-Gal1 cells. We confirmed that wortmannin effectively inhibited Akt phosphorylation in HepG2-Gal1 cells (Fig. 5B). Moreover, treatment with this pharmacological inhibitor completely prevented the decrease in E-cadherin expression observed in HepG2-Gal1 cells (108 ± 6 vs. 50 ± 5%) (Fig. 5B). Thus, augmented Gal1 expression induces E-cadherin downregulation in HepG2 cells through a mechanism involving PI3K-dependent Akt phosphorylation. As mitogen-activated protein kinase (MAPK) signaling pathway could also be involved in Gal1-induced EMT, we evaluated E-cadherin levels in the presence of PD98059 (25 μM). Decrease in E-cadherin expression induced by Gal1 was partially prevented after treatment of HepG2-Gal1 cells with the ERK1/2-specific MAPK inhibitor (73 ± 13%) (Fig. 5B). These findings suggest that Gal1-induced EMT in HepG2 cells involves, at least in part, modulation of the MAPK signaling pathway.

As mentioned above, by acting extracellularly, Gal1 can modulate cell adhesion, tumor growth, migration, angiogenesis, tumor-immune escape and metastasis (Elola et al., 2007; Ito et al., 2012). Nevertheless, Gal1 can also induce tumor cell transformation by acting intracellularly through binding to H-Ras to mediate Ras membrane anchorage (Paz et al., 2001). We previously observed that Gal1, being up-regulated in HepG2-Gal1 cells or added exogenously to HepG2 cell cultures, promoted cell-matrix adhesion (Espelt et al., 2011). On the other hand, the loss of HepG2 cell apico-basal polarity was observed only in HepG2-Gal1 cells. Thus, as these cells secrete high levels of the lectin to the conditioned medium (Fig. 1B), we aimed to evaluate if extracellular Gal1 was responsible for inducing E-cadherin downregulation, for instance, through binding to an extracellular ligand. We observed that when HepG2 cells where cultured in the presence of rGal1, E-cadherin expression was not altered (Fig. 5C). In conclusion, extracellular Gal1 (added in the form of recombinant protein or secreted by HepG2-Gal1 cells) could be involved in EMT processes like cell-matrix interactions and anchorage; however, it is not relevant for E-cadherin downregulation. Hence, high levels of intracellular Gal1 are responsible of inducing E-cadherin downregulation in HepG2 cells and triggering the PI3K/Akt signaling pathway.

TGF-β_1 increases Gal1 expression in HepG2 cells

It has been previously demonstrated that TGF-β_1 up-regulates Gal1 expression in metastatic mammary adenocarcinoma cells (Daroqui et al., 2007). Therefore, it was interesting to investigate if TGF-β_1, an inducer of EMT in HCC, could up-regulate Gal1 expression in HepG2 cells. We treated these cells with recombinant TGF-β_1 (5 ng/ml) for 48 h and analyzed Gal1 protein expression by Western blot. Notably, we found that TGF-β_1 increased Gal1 expression in cell lysates (174 ± 21%) with respect to untreated HepG2 cells (Fig. 6). These findings imply that TGF-β_1 and Gal1 may cooperate to induce EMT in HCC cells.

Discussion

Galectins have emerged as novel targets of anticancer therapy in a variety of tumors. Gal1, a member of this family, contributes to tumor progression by influencing tumor immunity, angiogenesis, tumor cell migration, and metastasis (Ito et al., 2012). Here we demonstrated that an augmented expression of Gal1 in HCC cells also induces EMT, an important process for tumor progression. Up-regulation of Gal1 in the well-differentiated HepG2 cells promoted a transition from epithelial cell morphology towards a fibroblastoid phenotype, an increment of the mesenchymal marker vimentin and loss of the adherens junction protein E-cadherin.

Disruption of the interactions between normal epithelial cells and the extracellular matrix or inappropriate anchorage can induce a type of programmed cell death called anoikis. Remarkably, resistance to anoikis has been shown to promote metastasis as tumor cells can disseminate through the bloodstream. Interestingly, by performing soft-agar colony formation assay, we determined that Gal1 up-regulation in HCC cells confers resistance to anoikis. In line with this finding, loss of E-cadherin was demonstrated to play a causal role in the acquisition of anoikis resistance (Derksen et al., 2006); and through this mechanism, loss of E-cadherin promotes metastasis (Onder et al., 2008).

Early alterations in epithelial cell polarity are a hallmark of epithelial cancers and contribute to their development as carcinomas in situ or to their progression to invasive adenocarcinomas. Accumulating evidence confirms that cancers hijack the machinery that integrates the epithelial polarity programs, resulting in a motile, invasive cell phenotype (Wodarz and Nathke, 2007; Tanos and Rodriguez-Boulan, 2008). In this study, we demonstrated that the loss of apico-basal polarity in HepG2 cells is associated with Gal1 up-regulation. We observed that HepG2 cells overexpressing Gal1 develop less apical bile canaliculi, and express reduced levels of ZO-1. Interestingly, the expression of the polarity protein Crumbs3, a transmembrane protein essential for generation of the apical membrane and tight junctions, is transcriptionally and post-translationally repressed by Snail in Madin-Darby Canine Kidney (MDCK) cells (Whiteman et al., 2008).

Our results also showed a positive correlation between Gal1 and Snail1 expression. Interestingly, in HCC, expression of either Snail1 or Twist1 is associated with shorter survival and their simultaneous expression has an additive negative

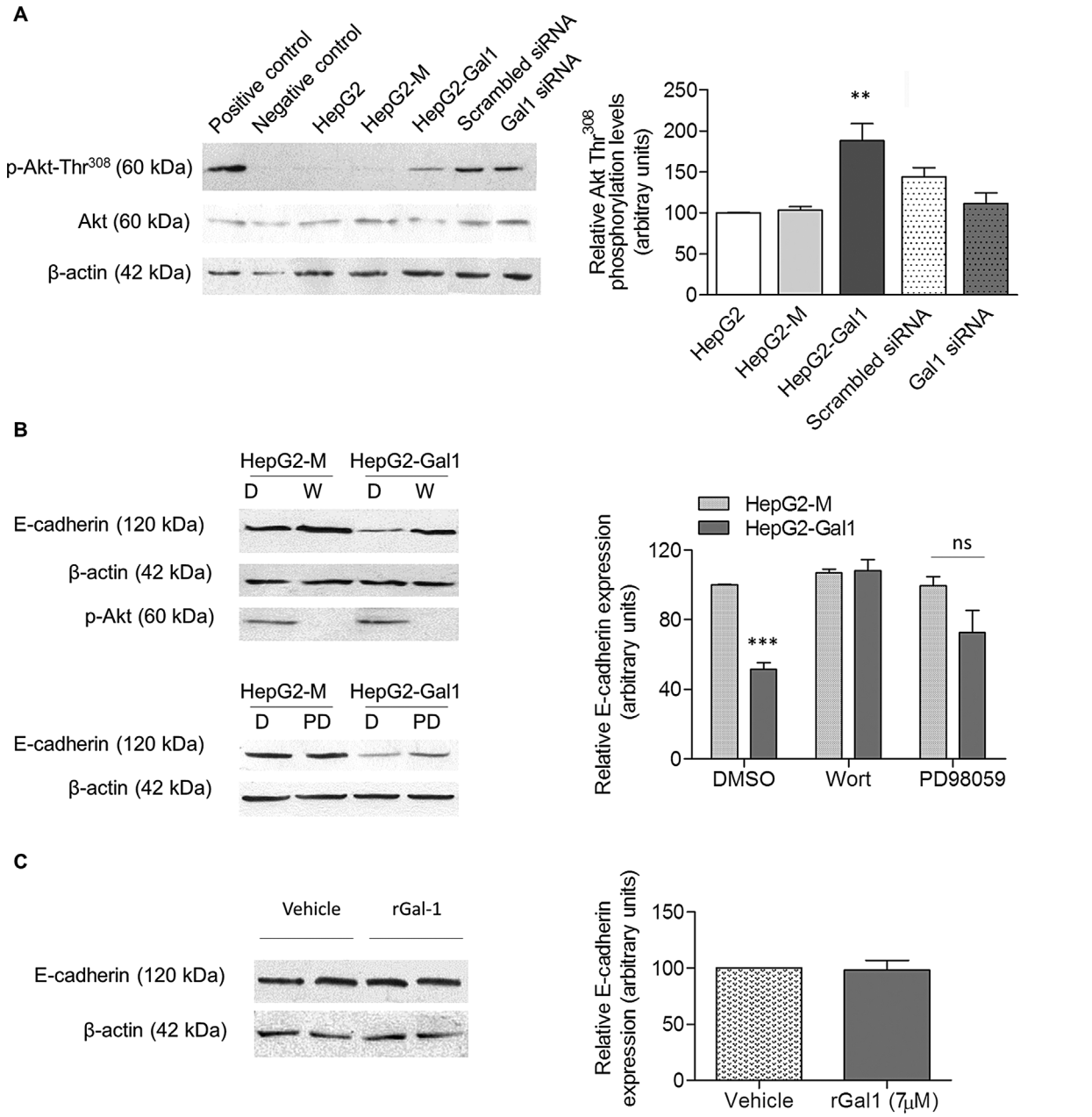

Fig. 5. Gal1 up-regulation induces E-cadherin downregulation in HepG2 cells through a mechanism involving PI3K-dependent Akt phosphorylation. A: Western blot and densitometric analysis in transfected and non-transfected cells for Akt phosphorylation at Thr^{308} relative to total Akt (n = 3) and β-actin expression. Negative control: homogenate of non-treated rat hepatocytes; Positive control: homogenate of insulin-treated rat hepatocytes. B: Western blot and densitometric analysis for E-cadherin relative to β-actin expression and Western blot for Akt phosphorylation at Thr^{308} in HepG2-M and HepG2-Gal1 cells treated with wortmannin 1 μM, PD98059 25 μM, or with DMSO as vehicle control (n = 3). C: Western blot and densitometric analysis for E-cadherin relative to β-actin expression in HepG2 cells treated with recombinant Gal1 or vehicle (PBS/dithiothreitol). *$P < 0.05$, ***$P < 0.001$ respect to HepG2-M cells, ns: non-significant.

effect, suggesting that these transcription factors play distinct but collaborative roles during cancer progression (Yang et al., 2009). Our findings add a gear to this circuit demonstrating that Gal1 modulates Snail-dependent E-cadherin expression in HCC cells.

Our results also revealed that the wild type β-catenin isoform is down-regulated in HepG2 cells overexpressing Gal1. Besides, we demonstrated that HepG2 cells overexpressing Gal1 show constitutive Akt activation, translocation of β-catenin to the nucleus, TCF4/LEF1 transcriptional activity and increased cyclin D1 and c-Myc expression, suggesting hyperactivation of the Wnt pathway. Moreover, a rise in intracellular expression of Gal1 induces E-cadherin downregulation in HepG2 cells through a PI3K/Akt-dependent mechanism. Interestingly, reduced expression of the E-cadherin/β-, γ- and α-catenins complex is frequently observed in HCC tumors (Zhai et al., 2008). In addition, squamous cell carcinoma lines expressing constitutively active

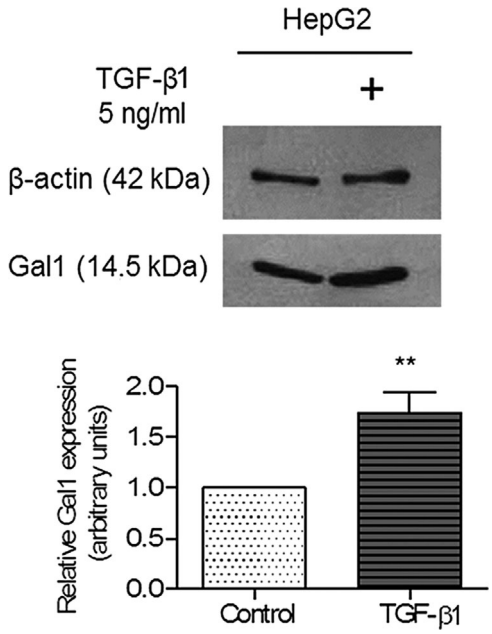

Fig. 6. TGF-β_1 increases Gal1 expression. Western blot and densitometric analysis for Gal1 expression relative to β-actin expression in HepG2 cells treated with TGF-β_1 (5ng/ml) for 48 h. **$P < 0.01$ respect to control HepG2 cells.

Akt underwent EMT, characterized by down-regulation of E-cadherin and β-catenin (Grille et al., 2003). Remarkably, alterations in the β-catenin (*CTNNB1*) gene such as those present in HepG2 cells, leading to hyperactivation of Wnt/β-catenin pathway, are frequently selected during liver carcinogenesis (de La Coste et al., 1998). Notably, these mutations, together with Gal1 up-regulation, may result in the development of more aggressive phenotypes.

Supporting the cross-talk between galectins and cadherins, it has been reported that silencing Gal1 expression in glioblastoma cells promotes the overexpression of cadherin-6 (Camby et al., 2005). Furthermore, another galectin, Gal3, binds directly to β-catenin and TCF4/LEF1 complex and induces Wnt target genes transcription (Shimura et al., 2005). Besides, protocadherin-24, a non-classical cadherin, when expressed in colon cancer cells, anchors Gal1 and Gal3 to the plasma membrane resulting in the inactivation of PI3K and suppression of β-catenin signaling (Ose et al., 2012). Collectively, these findings and our results demonstrate a strong relationship between galectins, cadherins and Wnt/β-catenin signaling during tumor progression.

Remarkably, we also observed that TGF-β_1 up-regulated Gal1 expression in HepG2 cells, suggesting that both molecules cooperate to induce EMT in HCC cells. Interestingly, it has been recently described that TGF-β_1 treatment of fibroblasts increases Gal1 via PI3K/Akt and p38 MAPK and accelerates fibrosis (Lim et al., 2014). Therefore, increased expression of Gal1 in HCC is associated with the development of metastasis, suggesting that, in addition to melanoma (Rubinstein et al., 2004), lung carcinoma (Wu et al., 2009; Croci et al., 2014), Kaposi's sarcoma (Croci et al., 2012), breast carcinoma, (Dalotto-Moreno et al., 2013) and prostate carcinoma (Laderach et al., 2013), HCC represents another tumor type amenable for Gal1 targeting. In conclusion, our results demonstrate that Gal1 is involved in the development of EMT in HCC cells and provide new insights which highlight Gal1 as an important therapeutic target to restrain HCC progression.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.