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Galectin-1 Controls the Proliferation and Migration of Liver Sinusoidal Endothelial Cells and Their Interaction With Hepatocarcinoma Cells

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Galectin-1 (Gal1), a β-galactoside-binding protein elevated in hepatocellular carcinoma (HCC), promotes epithelial-mesenchymal transition (EMT) and its expression correlates with HCC growth, invasiveness, and metastasis. During the early stages of HCC, transforming growth factor β_1 (TGF-β_1) acts as a tumor suppressor; however in advanced stages, HCC cells lose their cytostatic response to TGF-β_1 and undergo EMT. Here, we investigated the role of Gal1 on liver endothelial cell biology, and the interplay between Gal1 and TGF-β_1 in HCC progression. By Western blot and immunofluorescence, we analyzed Gal1 expression, secretion and localization in HepG2 and HuH-7 human HCC cells, and in SK-HEP-1 human liver sinusoidal endothelial cells (SECs). We used loss-of-function and gain-of-function experiments to down- or up-regulate Gal1 expression, respectively, in HepG2 cells. We cultured SK-HEP-1 cells with conditioned media from HCC cells secreting different levels of Gal1, and demonstrated that Gal1 derived from tumor hepatocytes induced its own expression in SECs. Colorimetric and scratch-wound assays revealed that secretion of Gal1 by HCC cells induced SEC proliferation and migration. Moreover, by fluorescence microscopy we demonstrated that Gal1 promoted glycan-dependent heterotypic adhesion of HepG2 cells to SK-HEP-1 SECs. Furthermore, TGF-β_1 induced Gal1 expression and secretion by HCC cells, and promoted HepG2 cell adhesion to SK-HEP-1 SECs through a Gal1-dependent mechanism. Finally, Gal1 modulated HepG2 cell proliferation and sensitivity to TGF-β_1-induced growth inhibition. Our results suggest that Gal1 and TGF-β_1 might function coordinately within the HCC microenvironment to regulate tumor growth, invasion, metastasis, and angiogenesis.

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Compelling evidence indicates that hepatocellular carcinoma (HCC) develops not only as a consequence of the molecular changes that occur in transformed hepatocytes, but also due to the cross-talk between diverse cellular and molecular pathways present in the liver tumor microenvironment (Hernandez-Gea et al., 2013). A chronic insult, such as infection with Hepatitis B or C virus or alcohol abuse, induces liver injury (El-Serag, 2012). This damage causes the activation of hepatic stellate cells and macrophages which produce matricellular components and growth factors that promote migration of liver sinusoidal endothelial cells (SECs), angiogenesis and fibrosis. These microenvironmental sources of chronic inflammation increase hepatocyte proliferation and can also lead to evasion of antitumor immune responses (Hernandez-Gea et al., 2013). Thus, the HCC microenvironment is a highly interconnected network that supports tumor growth, migration, invasion, and angiogenesis.

During carcinogenesis, transforming growth factor β (TGF-β), an ubiquitous cytokine, has a dual role. Although it initially suppresses tumorigenesis by inducing growth arrest and apoptosis, in advanced cancers it induces epithelial-mesenchymal transition (EMT), a crucial event that favors the metastatic spread of tumor cells (Thiery, 2002). Interestingly, the "TGF-β paradox" has been observed during HCC progression (Dooley and ten Dijke, 2012). TGF-β_1, a ligand of this family, strongly inhibits proliferation in rat epithelial

Abbreviations: Gal1, galectin-1; Gal3, galectin-3; HCC, hepatocellular carcinoma; SECs, sinusoidal endothelial cells; TGF-β_1, transforming growth factor-β_1; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase.

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hepatocytes (Carr et al., 1986; Oberhammer et al., 1992); however HCC cells may lose their cytostatic response to TGF-β_1 and undergo EMT (Xu et al., 2003). Moreover, an imbalance in the regulation of TGF-β_1-induced apoptosis has been observed in many HCC cells (Fabregat, 2009). Remarkably, TGF-β_1 is highly expressed in biological fluids of HCC patients and correlates with worse prognosis and shorter survival (Bedossa et al., 1995; Tsai et al., 1997; Dituri et al., 2014).

Galectin-1 (Gal1), a member of a conserved family of β-galactoside-binding lectins, is often a hallmark of malignant tumor progression as it is abundantly overexpressed in advanced stages of the disease (Demydenko and Berest, 2009). Within the tumor microenvironment, Gal1 plays critical roles in cell adhesion, tumor growth, migration, metastasis, and tumor-immune escape (Elola et al., 2007; Ito et al., 2012). A high expression of Gal1 in the peritumoral stroma and in tumor-associated vascular endothelial cells has been described for various neoplastic tissues (Clausse et al., 1999; Thijssen et al., 2006; Hsieh et al., 2008). Importantly, Gal1 plays a fundamental role in vascular biology and tumor angiogenesis through glycosylation-dependent mechanisms (Thijssen et al., 2006; Hsieh et al., 2008; Thijssen et al., 2010; Croci et al., 2012; Mathieu et al., 2012; D'Haene et al., 2013; Croci et al., 2014; Wu et al., 2014).

In human HCC tissues and cell lines, Gal1 is up-regulated (Chung et al., 2002; Kondoh et al., 2003). This overexpression correlates with HCC cell migration and invasion and, it is often associated with tumor invasiveness, metastasis, tumor recurrence, and shortened patient survival, suggesting a potential value for Gal1 as a biomarker for predicting poor prognosis of HCC (Spano et al., 2010; Wu et al., 2012). Previously, we demonstrated that Gal1 acts as a glycan- and integrin-dependent modulator of HepG2 HCC cell adhesion and polarization. Moreover, Gal1-overexpressing HepG2 cells inoculated into nude mice promoted tumor growth and metastasis (Espelt et al., 2011). Recently, we provided the first evidence of a role of Gal1 as an inducer of EMT in HepG2 cells (Bacigalupo et al., 2015). However, the specific role of Gal1 within the HCC microenvironment remains uncertain.

In this report, we found that secretion of Gal1 by HepG2 and HuH-7 HCC cells induced SK-HEP-1 SEC proliferation and migration. Moreover, Gal1 promoted glycan-dependent HCC cell adhesion to human SECs. In addition, we showed that TGF-β_1 induced Gal1 expression and secretion by HCC cells, an effect which may allow neoplastic hepatocytes to adhere to SECs, and to escape from TGF-β_1-induced tumor growth inhibition.

Materials and Methods

Reagents

Actinomycin D, bovine serum albumin (BSA), aprotinin, phenylmethylsulfonyl fluoride (PMSF), lactose, PD98059, wortmannin, anti-β-actin, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Sigma–Aldrich Co. (St. Louis, MO). Fetal bovine serum was from Natocor (Córdoba, Argentina). Dulbecco's modified Eagle medium (DMEM), from GIBCO®, and red and green calcein AM, from Molecular Probes®, were purchased from Life Technologies-Invitrogen Corporation (Carlsbad, CA). 1,4-diazabicyclo[2.2.2] octane (DABCO) was from Fluka, Germany. Human recombinant Gal1 (rGal1) was prepared as previously described (Bacigalupo et al., 2015). Human recombinant TGF-β_1 was obtained from R&D System (MN). Anti-Gal1 and anti-Gal3 antibodies and siRNAs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture

The human HCC cell lines HepG2/C3A (ATCC CRL-10741, a clonal derivative of HepG2 cell line ATCC HB-8065) and HuH-7 (JCRB 0403) and SK-HEP-1 liver SECs (ATCC HTB-52) were cultured in DMEM, 10% v/v serum, 2 mM L-glutamine and antibiotics in a humidified atmosphere of 5% CO_2 at 37°C. BAEC cells were maintained in 20% v/v serum-containing medium. For Gal1 secretion, subconfluent HCC cell monolayers were cultured with serum-free medium for 24 h and then, conditioned media (CM) were collected, centrifuged, and added to SK-HEP-1 cells.

Transfections

Transfections to overexpress or knockdown Gal1 were performed as previously described (Espelt et al., 2011; Bacigalupo et al., 2015). Briefly, cells were transfected with pcDNA3.1-*Lgals1* (Rabinovich et al., 1999) or pcDNA3.1 expression vector (Invitrogen Corporation, CA) alone as control, using Lipofectamine 2000 (Invitrogen Corporation). Stable Gal1-overexpressing cells were selected by G418 resistance. siRNA experiments were performed with a pool of 3 target-specific Gal1 siRNAs or nontargeting scrambled siRNA.

Western blot analysis

Cells were homogenized in lysis buffer (100 mM Tris, pH 7.4, 1% v/v Triton X-100, 10 mM EDTA, 100 mM sodium pyrophosphate, 2 mM PMSF and 0.012–0.034 TIU/ml aprotinin), centrifuged and supernatants were collected. To evaluate protein secretion, CM were collected as described above and sodium dodecyl sulfate (SDS) was added to 0.5% (w/v) final concentration, then heated at 100°C for 10 min, and diluted with methanol 1/10, followed by a −20°C overnight incubation. After centrifugation, pellets were recovered. To evaluate TGF-β_1 effects, HCC cells were synchronized by 24 h-serum starving and then incubated with 5 ng/ml TGF-β_1, in serum-free media. Proteins were separated by SDS-PAGE, transferred to membranes, and immunoblotted. Bands were detected by chemoluminiscence (Amersham ECL prime Western Blotting Detection Reagents, GE Healthcare, Uppsala, Sweden). Densitometric analysis of protein levels was performed using ImageJ software (U.S. NIH, MD; http://rbsweb.nih.gov/ij/).

Immunofluorescence

Cells cultured on coverslips were fixed with 4% w/v formaldehyde. Then, cells were incubated in PBS-0.1% v/v Triton X-100 containing 1% w/v BSA and incubated with anti-Gal1 and the corresponding FITC-conjugated anti-IgG antibodies (BD, Pharmigen Biosciences). Coverslips were mounted with DABCO on glass slides and observed on a FluoView 1000 confocal microscope (Olympus, Japan).

Cell proliferation and viability assay

To evaluate SK-HEP-1 cell proliferation, cells were cultured in 96-well plates, synchronized by 24 h-serum starving, then cultured with CM from HCC cells or with soluble rGal1 for 72 h. To analyze saccharide-dependent Gal1 interactions, soluble rGal1 and CM were pre-incubated for 30 min with lactose (100 mM). To assess the effect of Gal1 expression on HepG2 cell proliferation, cells were synchronized by 24 h-serum starving and then cultured with serum for 72 h. To determine the effect of Gal1 expression on HepG2 cell resistance to TGF-β_1-induced cell death, cells were incubated with TGF-β_1 (5 ng/ml) in the absence of serum for 48 h. We used the CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS assay) (Promega Corp., Madison, WI) and expressed the results as percentage of proliferating viable cells or cell viability as indicated.

Cell migration analysis

SK-HEP-1 cell migration was evaluated by the scratch-wound assay. Cells were cultured in 96-well plates in the presence of

serum and were allowed to grow to confluence. A scratch was performed in the monolayers, which were then incubated with CM from HCC cells or rGal1 at varying concentrations, in the absence of serum for 9 h. To assess saccharide-dependent modulation of Gal1 function, CM and soluble rGal1 were pre-incubated for 30 min with lactose (100 mM). Monolayers were photographed at 0 and 9 h. ImageJ software was used to determine the percentage of reduction of the wound area at 9 h versus 0 h, [% of closure: $100\times$ (area t_{0h}–area t_{9h})/area t_{0h}].

HCC cell adhesion to SK-HEP-1 cells

Subconfluent HCC cell monolayers were harvested with PBS-EDTA (1 mM) which releases cells from the culture plates while keeping the cell membrane proteins intact. Then, cells were labeled with red calcein AM (5 μM) for 1 h at room temperature. After washing, 1.5×10^4 viable HCC cells were added to SK-HEP-1 cell monolayers cultured on coverslips, pre-labeled with green calcein AM (5 μM). In some experiments, HCC cells were pre-incubated with rGal1 (30 min), lactose (100 mM, 30 min), wortmannin (0.1 μM, 15 min), PD98059 (2.5 μM, 15 min), or cultured with TGF-β_1 (5 ng/ml, 48 h). After the indicated times, cells were washed and observed with a Nikon TE-200 epifluorescence inverted microscope. Sixteen fields per coverslip were photographed and three coverslips per treatment of, at least, three independent experiments were examined. HCC cell adhesion to SK-HEP-1 cells was determined by counting the number of red-fluorescent HCC cells adhered to SK-HEP-1 green-fluorescent monolayers.

Statistical analysis

Data were analyzed using GraphPad Prism Software (GraphPad Software Inc., CA). Results are expressed as the mean ± SEM from, at least, three independent experiments. Statistical analysis was performed using *t* test or one-way analysis variance, with the pertinent post-test. *P* values <0.05 were considered as significant.

Results

Modulation of Gal1 in HepG2 and HuH-7 HCC cells

To unravel the function of Gal1 within HCC microenvironment, we first assessed its expression in HepG2 and HuH-7 HCC cells. Gal1 protein expression in HuH-7 cell lysates was significantly lower than in HepG2 cell lysates (Fig. 1A). Gal1 is a typical cytosolic protein, although its presence has also been described in the extracellular compartment (Hughes, 1999). Interestingly, Gal1 secretion was substantially higher in HuH-7 cultures with respect to that observed in HepG2 cultures (Fig. 1B). Then, we stably transfected HepG2 cells with Gal1 sense cDNA. HepG2-Gal1 cells showed higher expression and secretion of Gal1 to serum-free CM compared to non-transfected and transfected cells with empty vector (HepG2-M) (Fig. 1C and D). Gal1 immunostaining of HepG2, HepG2-M and HuH-7 cells showed positive cytoplasmic localization and a faint nuclear staining (Fig. 1E). Interestingly, in HepG2-Gal1 cells, Gal1 appeared mainly localized at the proximity of the cell surface (Fig. 1E).

Galectin-3 (Gal3), another member of the galectin family, is also overexpressed in HCC tumors (Hsu et al., 1999; Matsuda et al., 2008; Bacigalupo et al., 2013; Jiang et al., 2014). We observed higher levels of intracellular Gal3 expression in HuH-7 cells (124 ± 11%) than in HepG2 cells (100%) and interestingly, HepG2 cells overexpressing Gal1 showed a significant decrease in Gal3 expression (53 ± 8%) (Fig. 1F). However, Gal3 could not be detected in the CM from HepG2, HepG2-Gal1 (Fig. 1G), or HepG2-M cells (data not shown), suggesting that this galectin may not be secreted or might be secreted at undetectable levels by these cells. On the contrary, HuH-7 cells secreted considerable amounts of Gal3 to the extracellular milieu (Fig. 1G). Thus, we used CM from HuH-7 cells to evaluate the effects of both Gal1 and Gal3 in the HCC microenvironment. Conversely, as HepG2-Gal1 cells release high quantities of Gal1 and negligible amounts of Gal3, they represent a reliable model to study the effects of Gal1 in the HCC microenvironment.

Gal1 expression, secretion, and localization in SK-HEP-1 SECs

The immortal human cell line SK-HEP-1 derives from the ascitic fluid of a patient with liver adenocarcinoma. These cells show fenestrations, rudimentary sieve plates, micropinocytotic vesicles, and high endocytic activity, which are typical structural and functional features of liver SECs (Heffelfinger et al., 1992; Cogger et al., 2008). Besides, they express endothelial antigens including von Willebrand factor, vascular cell adhesion molecule, intercellular adhesion molecule-1 and vascular endothelial growth factor receptor-2 (Heffelfinger et al., 1992; Cogger et al., 2008). The lack of expression of the typical vascular endothelial marker CD31 is another characteristic of liver SECs (Lalor et al., 2006). Thus, to evaluate the influence of HCC-derived Gal1 on SEC biology within the HCC microenvironment, we used SK-HEP-1 cells.

SK-HEP-1 cells expressed high amounts of intracellular Gal1 which was secreted at greater levels as compared with normal bovine aortic endothelial cells (BAEC) (Fig. 2A and B). Gal1 immunostaining showed both cytoplasmic and nuclear localization (Fig. 2C). Additionally, culturing SK-HEP-1 cells under serum-free conditions promoted a significant increase in Gal1 expression (133 ± 5%) respect to cultures with 10% (100%) and 20% serum-containing media (91 ± 7%) (Fig. 2D), suggesting that deprivation of nutrients might induce Gal1 expression in SECs. Besides, while CM from HepG2 cells did not alter Gal1 expression in SK-HEP-1 cells (94 ± 11%) with respect to serum-free control medium (100%), we observed that CM from HepG2-Gal1 cells showed a tendency to increase Gal1 levels in SK-HEP-1 cells (123 ± 14%), and this effect reached statistical significance using HuH-7 cell CM (143 ± 16%) (Fig. 2E). Next, to evaluate whether increased Gal1 expression levels observed in SK-HEP-1 cells cultured with CM from HuH-7 cells were due to transcriptional regulation, we used actinomycin D. We observed that treatment of SK-HEP-1 cells with the transcription inhibitor actinomycin D (0.01 μg/ml) for 24 h significantly inhibited the effect of CM from HuH-7 cells (110 ± 6%), reaching similar levels of Gal1 expression to those observed with control medium (100%). Therefore, we conclude that Gal1 secreted from HCC cells is capable of inducing its own expression in SK-HEP-1 cells acting at the transcriptional level.

HCC-derived Gal1 induces proliferation and migration of SK-HEP-1 SECs

Gal1 plays critical roles in the control of vascular endothelial cell biology (Thijssen et al., 2006; Thijssen et al., 2010; Croci et al., 2014); nevertheless its function within the liver microenvironment remains uncertain. To determine whether HCC-derived Gal1 modulates SEC physiology, we further evaluated its effect on SK-HEP-1 cell proliferation and migration. SK-HEP-1 cells were synchronized by 24 h-serum starving, then cultured with CM from HCC cells for 72 h and the percentage of proliferating viable cells was assessed by MTS assay. To evaluate cell migration, scratch-wound assays were carried out on SK-HEP-1 monolayers incubated with CM from HCC cells for 9 h. We observed that CM from HepG2 cells promoted an increase in proliferating viable (126±4%) and migrating (141 ± 15%) SK-HEP-1 cells, respect to cells cultured with serum-free DMEM (100%) (Fig. 3A and C). Similar effects were

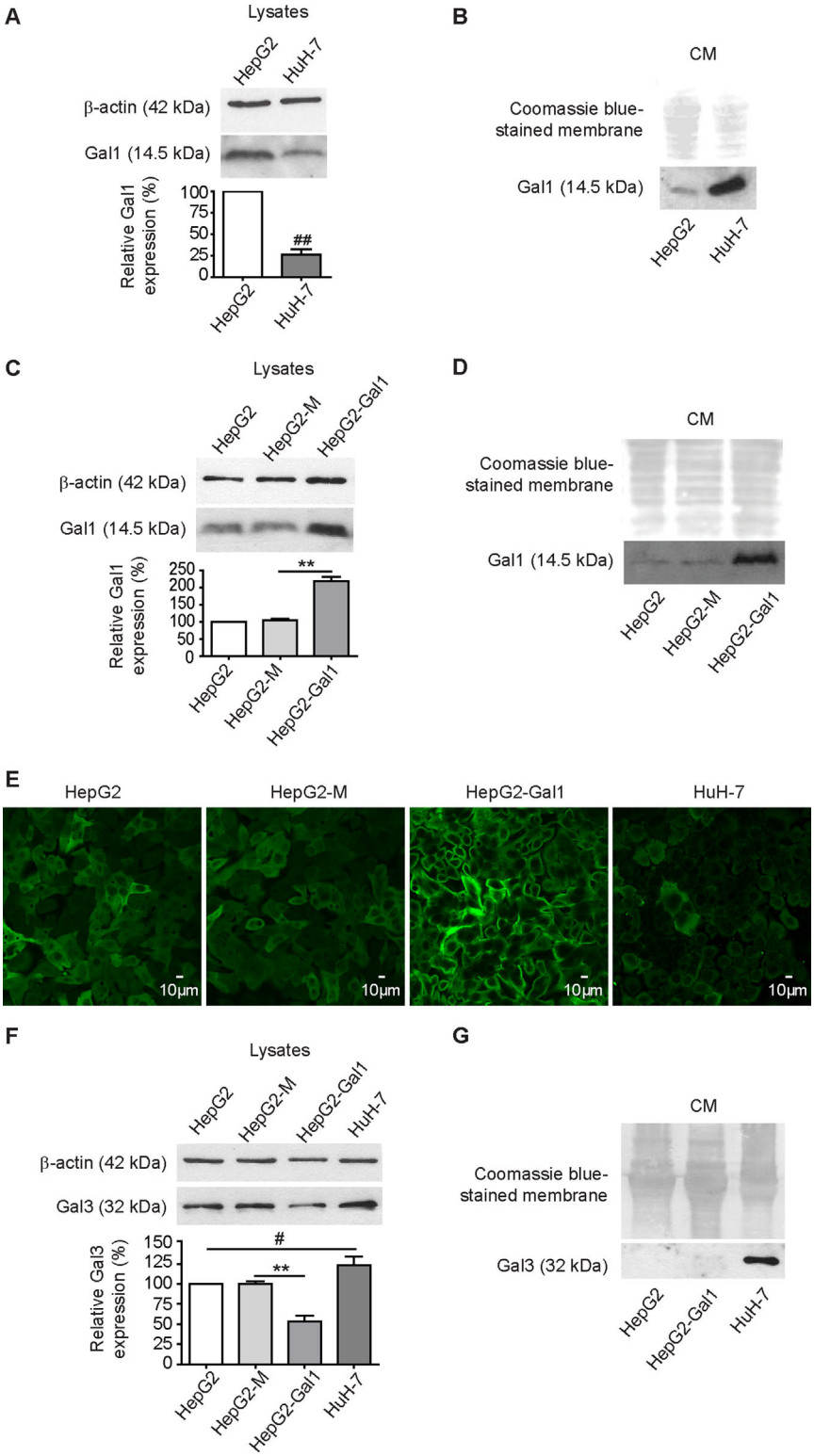

Fig. 1. Gal1 expression, secretion, and localization in HCC cells. (A, C) Western blot and densitometric analysis showing Gal1 expression in HCC cell lysates (n = 5). (B, D) Secretion of Gal1 evaluated by Western blot in serum-free conditioned media (CM) from HCC cell cultures (n = 5). HepG2 cells were transfected with pcDNA3.1-*Lgals1* (HepG2-Gal1) or empty vector (HepG2-M). β-actin and Coomassie blue-stained membranes were used as loading controls. (E) Gal1 subcellular distribution was visualized by confocal microscopy in HCC cells cultured with 10% serum (400×). Photographs are representative of three independent experiments. Western blot showing (F) Gal3 expression in HCC cell lysates (n = 5) and (G) secretion to CM (n = 3). $^{\#}P < 0.05$; $^{**,\#\#}P < 0.01$.

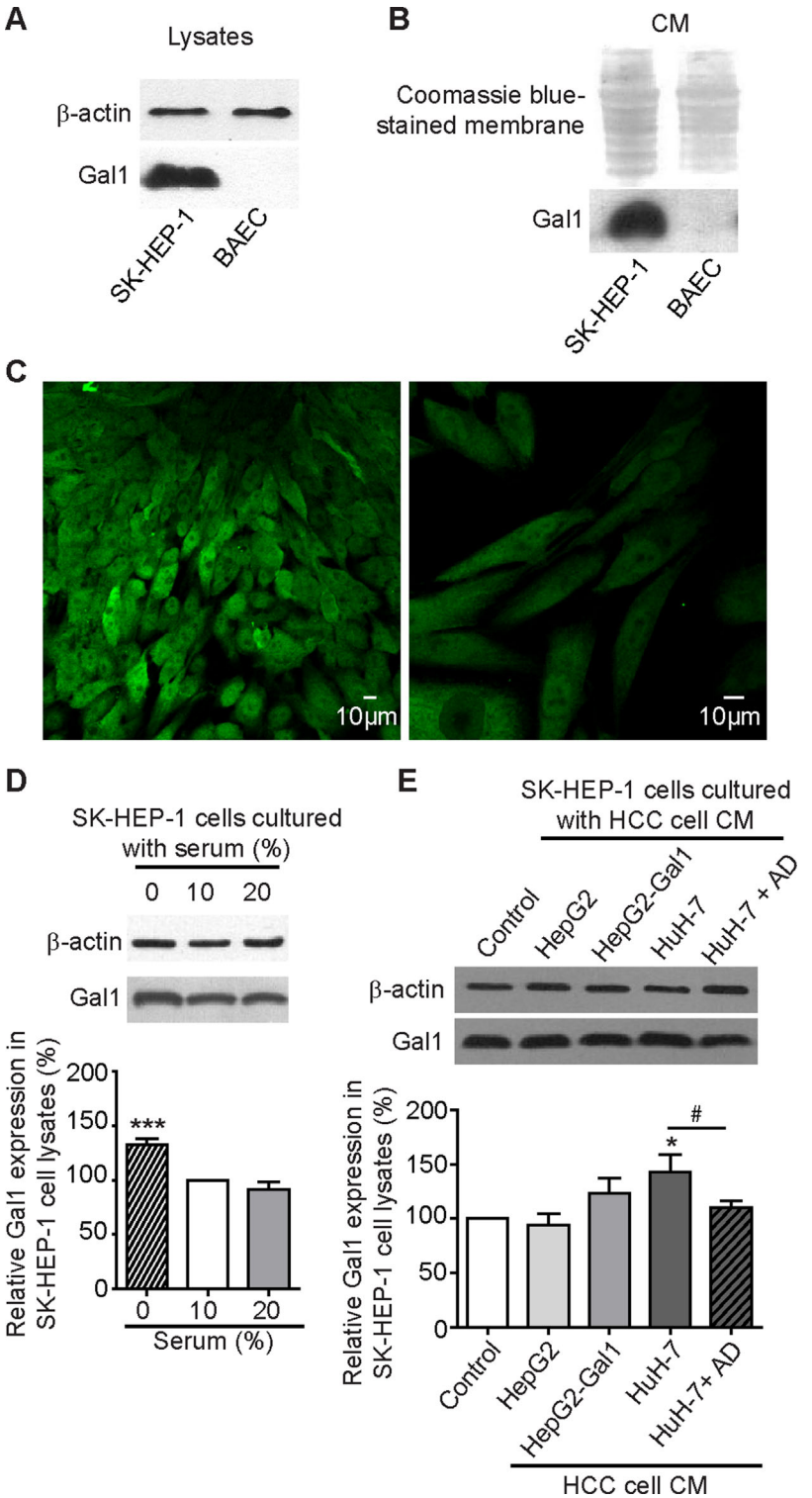

Fig. 2. Expression, secretion, and localization of Gal1 in SK-HEP-1 cells. Western blot showing (A) Gal1 expression in SK-HEP-1 and BAEC lysates (n = 3) and (B) secretion to CM (n = 3). β-actin and Coomassie blue-stained membranes were used as loading controls. (C) Gal1 localization in SK-HEP-1 cells cultured with 10% serum-containing media, visualized by confocal microscopy (400×, left; 680× right). Photographs are representative of three independent experiments. Western blot and densitometric analysis showing Gal1 expression relative to β-actin in SK-HEP-1 cells cultured for 24 h, (D) with varying serum concentration-containing media (n = 6) or (E) with serum-free control media or CM from HCC cells, in the presence or the absence of 0.01 μg/ml actinomycin D (AD) (n = 6). $^{*,\#}P < 0.05$, $^{***}P < 0.001$.

obtained with CM from HepG2-M cells respect to HepG2 CM (Fig. 3A and C). Interestingly, SK-HEP-1 cells exposed to HepG2-Gal1 or HuH-7 cell CM (which secrete higher levels of Gal1 than HepG2 cells, Fig. 1B and D), led to increased endothelial cell proliferation (142 ± 4% and 153 ± 6%, respectively) and migration (175 ± 28% and 215±22%, respectively) (Fig. 3A, C, and E). These effects involved carbohydrate-dependent interactions as they were completely prevented by pre-treatment with lactose, a galectin-specific inhibitor (Fig. 3A and C). On the other hand, culturing SK-HEP-1 cells in the presence of CM from Gal1 siRNA-treated HepG2 cells (50 ± 6% diminished Gal1 expression, Fig. 3A inset) led to a significant decrease in endothelial cell proliferation (118 ± 2%) and migration (93 ± 19%) respect to scrambled siRNA-transfected HepG2 cells (132 ± 6% and 133 ± 18%, respectively) (Fig. 3A and C). Moreover, exogenous recombinant Gal1 (rGal1) also augmented the percentage of proliferating viable SK-HEP-1 cells and stimulated cell migration in a carbohydrate-dependent manner (Fig. 3B and D). These results suggest that Gal1 derived from tumoral hepatocytes can modulate SEC proliferation and migration.

Gal1 promotes HepG2 cell adhesion to SK-HEP-1 SECs

Recently, we demonstrated that HepG2 cells overexpressing Gal1 can trigger EMT, a key event for tumor cell dissemination (Bacigalupo et al., 2015). Since heterotypic cell adhesion of cancer cells to the endothelium represents another important step for metastasis, we evaluated the role of Gal1 on HCC adhesion to SECs. Whereas no differences were observed between HepG2 and HepG2-M cell adhesion to confluent monolayers of SK-HEP-1 cells, reinforced expression of Gal1 in HepG2 cells significantly increased adhesion after 15 min culture in serum-free conditions (146 ± 5% respect to HepG2-M cell adhesion; 100%) (Fig. 4A–C). This effect was preserved even after 60 min of culture (127 ± 7%) and was also evident in the presence of serum (123 ± 5%, 15 min) (Fig. 4B). Moreover, the percentage of HuH-7 cells adhered to SK-HEP-1 cells was significantly greater than HepG2 cells (167 ± 7%) (Fig. 4C). Remarkably, these pro-adhesive effects were completely abrogated by lactose (Fig. 4C), suggesting that specific protein-glycan interactions control heterotypic cell adhesion of HCC to liver endothelial cells. Moreover, knocking down Gal1 expression significantly decreased HepG2 cell adhesion to SECs (59 ± 10%) respect to control cells (scrambled siRNA, 95 ± 11%) (Fig. 4C). Furthermore, HepG2 cell adhesion to SK-HEP-1 cells substantially increased in the presence of rGal1 (3.5×10^{-4}–3.5×10^{-3} μM). However, higher concentrations of rGal1 showed the opposite effect (Fig. 4D).

To investigate the signaling pathways mediating HCC cell adhesion to SECs, assays were performed in the presence of pharmacological inhibitors. Notably, pre-treatment with the PI3K inhibitor, wortmannin, or with the ERK1/2-specific mitogen-activated protein kinase (MAPK) kinase inhibitor, PD98059, significantly decreased HepG2-M cell adhesion to SK-HEP-1 cells (63 ± 5 and 52 ± 8%), respectively; versus vehicle control (87 ± 6%) (Fig. 4E), indicating that PI3K and MAPK are involved in the process of HepG2 cell adhesion to SECs. When we analyzed the effect of Gal1 overexpression in HepG2-Gal1 cell adhesion to SK-HEP-1 cells we observed that wortmannin partially inhibited the increased cell adhesion of HepG2-Gal1 cells (85 ± 6% vs. HepG2-M cells + Wort: 63 ± 5%). On the contrary, the pro-adhesive effect of Gal1 was completely abolished by PD98059 pre-treatment (46 ± 7% vs. HepG2-M cells + PD98059: 52 ± 8%) (Fig. 4E). In conclusion, Gal1 modulates HepG2 cell adhesion to SK-HEP-1 SECs, one of the first processes during HCC dissemination, through mechanisms involving specific protein-carbohydrate interactions and signaling through the MAPK pathway.

TGF-β_1 increases Gal1 expression and secretion by HCC cells

As TGF-β_1 is elevated in the HCC microenvironment (Bedossa et al., 1995; Tsai et al., 1997; Dituri et al., 2014), we investigated whether it could modulate Gal1 expression in HCC cells. When HepG2 cells were cultured with TGF-β_1 (5 ng/ml) for 24 h, the expression of Gal1 significantly increased (151 ± 17%), being this effect more pronounced after 48 h-treatment (230 ± 21%) (Fig. 5A). The continuous presence of TGF-β_1 was essential for supporting increased Gal1 expression increased, as tumor cells cultured with TGF-β_1 for 24 h and then deprived of this cytokine showed considerably diminished levels of Gal1 (134 ± 18%) with respect to 48 h-treatment (Fig. 5A). Worth mentioning is that TGF-β_1 had no effect on Gal1 levels in HepG2-Gal1 siRNA cells, which maintained low levels of Gal1 even when they were cultured in the presence of this cytokine for 48 h (Fig. 5A). TGF-β_1 also augmented Gal1 expression in HuH-7 cells (180 ± 14%) and interestingly, Gal1 secretion to CM of HepG2 and HuH-7 cultures was significantly higher (141 ± 4% and 262 ± 39%, respectively) in TGF-β_1-treated versus untreated cells (Fig. 5A). Of note, TGF-β_1 had no effect on the expression of Gal3, at least under our experimental conditions (data not shown). Whereas no apparent changes were observed in the localization of Gal1 in TGF-β_1-treated HuH-7 cells under serum-free conditions, Gal1 localized underneath the cell membrane in some HepG2 cells treated with this cytokine (Fig. 5B). This distribution pattern in cell membranes resembled that observed for HepG2-Gal1 cells (Fig. 1E), which was more pronounced following serum deprivation (Fig. 5B). These results suggest that TGF-β_1 induces Gal1 expression and secretion by HCC cells.

TGF-β_1 induces HepG2 cell adhesion to SK-HEP-1 SECs through a Gal1-dependent mechanism

We hypothesized that TGF-β_1 effects on HCC progression could take place via indirect mechanisms involving, at least in part, Gal1 expression and secretion by tumoral hepatocytes. Therefore, we investigated whether TGF-β_1 might induce HCC dissemination by promoting tumor hepatocyte adhesion to SECs in a Gal1-dependent fashion. Our results show that TGF-β_1 significantly increased HepG2 cell adhesion to SK-HEP-1 monolayers (118 ± 4%) respect to untreated HepG2 cells. On the contrary, TGF-β1 was unable to increase adhesion of HepG2-Gal1 siRNA cells to SK-HEP-1 cells and even reduced heterotypic adhesion of these cells (59 ± 5%) when compared to HepG2-Gal1 siRNA cells (73 ± 3%) (Fig. 5C). Thus, since HepG2-Gal1 siRNA cells maintained low levels of Gal-1 after TGF-β_1 treatment (Fig. 5A), our results show that HepG2 cell adhesion to SK-HEP-1 SECs induced by TGF-β1 involves, at least in part, expression of Gal1. Hence, Gal1 up-regulation and secretion by HCC cells and consequently, promotion of tumor hepatocyte adhesion to SECs may represent a novel mechanism triggered by TGF-β_1 to induce HCC dissemination.

Gal1 modulates HepG2 cell proliferation and sensitivity to TGF-β_1-induced growth inhibition

TGF-β_1 induces apoptosis in some human liver tumor cells; however HepG2 cells are refractory to the suppressive and pro-apoptotic effects of this cytokine (Zhang et al., 2004; Caja et al., 2011; Dzieran et al., 2013). As TGF-β_1 increases Gal1 expression and secretion by HCC cells, we next investigated

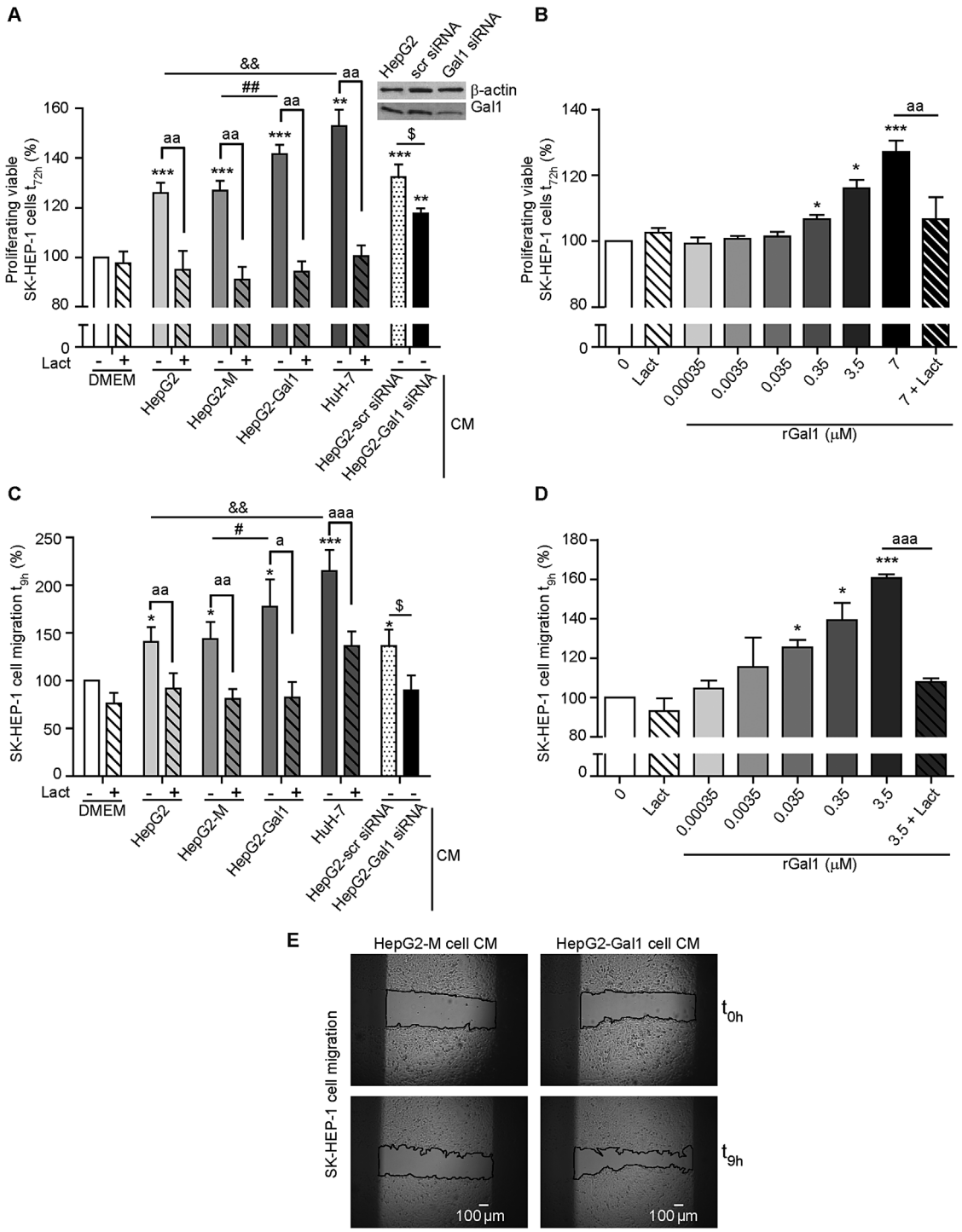

Fig. 3. HCC cell-derived Gal1 induces SK-HEP-1 cell proliferation and migration. Proliferating viable SK-HEP-1 cells by the MTS assay, evaluated after incubation for 72 h with (A) CM from HCC cultures or (B) recombinant Gal1 (rGal1), with or without pre-treatment with lactose (Lact). Results are expressed as percentage of proliferating viable cells respect to DMEM or absence of rGal1 (100%) (n = 4). Inset panel shows Gal1 expression in Gal1 knocked down HepG2 cells (HepG2-Gal1 siRNA). Cells transfected with nontargeting scrambled siRNA (HepG2-scr siRNA) were used as control. Monolayers of SK-HEP-1 cells were incubated for 9 h with (C) CM from HCC cell cultures or (D) rGal1, with or without lactose. Cell migration was evaluated by scratch-wound assay. Results are expressed as the percentage of cell migration (n = 5), calculated as % of wound area closure respect to DMEM or absence of rGal1 (100%). (E) Representative photographs of five independent experiments showing the wound area of SK-HEP-1 cells cultured with CM from HepG2-M and HepG2-Gal1 cell cultures (40×). $^{*,a,\#,\$}P < 0.05$; $^{**,aa,\#\#,\&\&}P < 0.01$, $^{***,aaa}P < 0.001$.

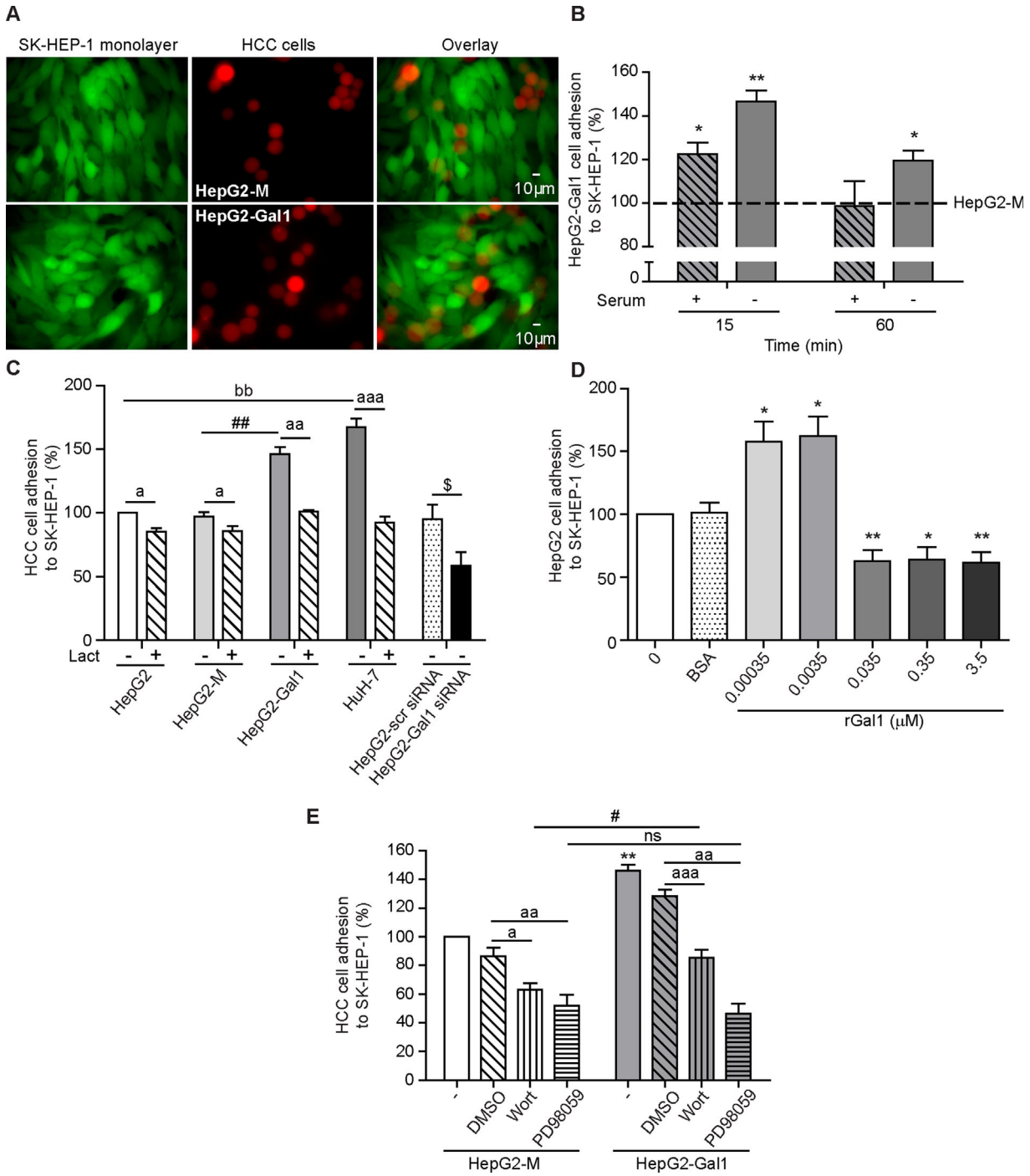

Fig. 4. Gal1 promotes HCC cell adhesion to SK-HEP-1 SECs. HCC cells adhered to SK-HEP-1 cell monolayers were (A) visualized by fluorescence microscopy (400×) after 15 min co-culture, in the absence of serum, and (B) quantified, after 15 or 60 min co-culture, with or without serum, and expressed as percentage (n = 3) respect to HepG2-M cell adhesion to SECs (100%). (C) HCC cell adhesion to SK-HEP-1 cells after 15 min of serum-free co-culture, with or without 30 min-pre-treatment with lactose. D: HepG2 cells were pre-incubated with rGal-1 for 30 min, with or without lactose, and cell adhesion to SK-HEP-1 cells, in the absence of serum, was determined after 15 min. BSA was used as negative control. (E) HepG2-M or HepG2-Gal1 cells were pre-treated with wortmannin (Wort) or PD98059 for 15 min. Cell adhesion to SK-HEP-1 cells was determined after 15 min of serum-free co-culture, and expressed as percentage (n = 3) respect to HepG2-M cell adhesion without treatment (100%). DMSO was used as vehicle control. $^{*}P < 0.05$, $^{**}P < 0.01$ respect to HepG2-M (B and E) and BSA (D); $^{a,\#,\$}P < 0.05$; $^{aa,bb,\#\#}P < 0.01$; $^{aaa}P < 0.001$.

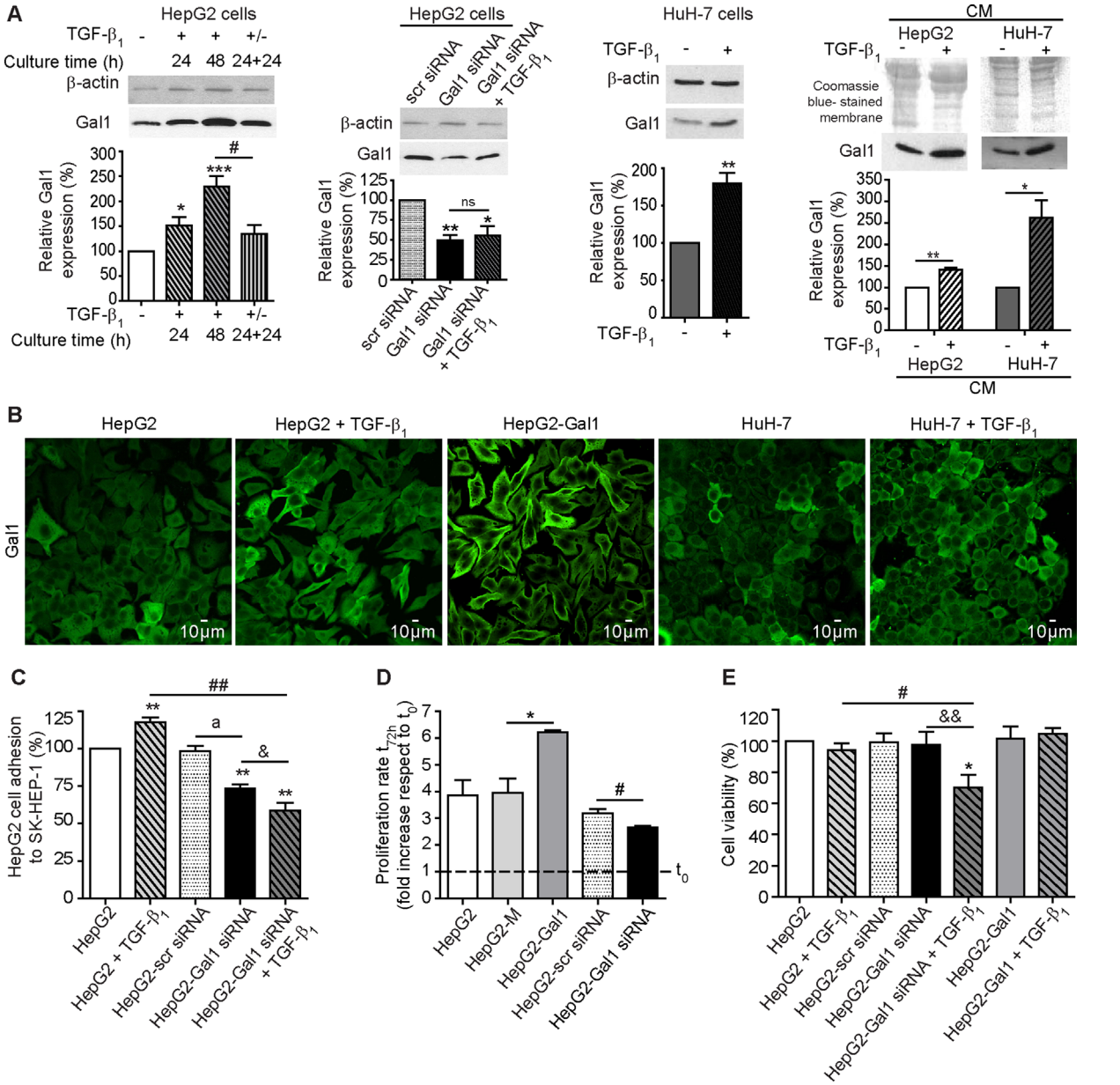

Fig. 5. HepG2 cell adhesion to SK-HEP-1 cells and resistance to growth inhibition induced by TGF-β_1 depend on Gal1 expression and secretion by HCC cells. (A) Western blot and densitometric analysis showing Gal1 expression relative to β-actin in HepG2 cells cultured without serum for 24 or 48 h in the presence of TGF-β_1, or cultured for 48 h, with TGF-β_1 only during the first 24 h (n = 4) (left panel); Gal1 expression in HepG2-siRNA (middle left panel) and HuH-7 cells (middle right panel) treated or not with TGF-β_1 for 48 h (n = 3), and Gal1 secretion to CM from HCC cells treated or not with TGF-β_1 for 48 h (n = 4) (right panel). Coomassie blue-stained membranes were used as loading controls for CM. (B) Gal1 localization in HCC cells cultured with or without TGF-β_1 in serum-free media for 48 h visualized by confocal microscopy (400×). Photographs are representative of four independent experiments. C: HepG2, HepG2-scr siRNA cells or HepG2-Gal1 siRNA cells, with or without pre-treatment with TGF-β_1 for 48 h, were co-cultured with SK-HEP-1 cells in serum-free media for 15 min. Cell adhesion determined by fluorescence microscopy is expressed as percentage (n = 3) respect to HepG2 cell adhesion to SECs (100%). D: Non-transfected and transfected HepG2 cells were cultured in the presence of serum for 72 h and cell proliferation was evaluated by the MTS assay. Results are expressed as proliferation rate (n = 3), determined as fold increase proliferation at t_{72h} respect to t_0 (onefold) for each cell type. (E) Gal1-silenced, Gal1-overexpressing and control HepG2 cells were cultured with TGF-β_1 for 48 h in serum-free media and cell viability was evaluated (MTS assay). Results are expressed as percentage of cell viability (n = 4) respect to HepG2 cells (100%). $^{*,\#,\&,a,}P < 0.05$; $^{**,\#\#,\&\&}P < 0.01$; $^{***}P < 0.001$.

whether this galectin could mediate tumor cell resistance to TGF-β_1-induced growth inhibition. First, we analyzed if endogenous Gal1 levels could modulate the number of proliferating viable HepG2 cells. While overexpression of Gal1 in HepG2 cells significantly increased their proliferation rate at 72 h (6.02 ± 0.10-fold increase respect to t_0), compared to non-transfected and HepG2-M cells (3.86 ± 0.55 and 3.96 ± 0.53, respectively) (Fig. 5D), Gal1 knockdown cells showed slower proliferation rate (2.66 ± 0.06) compared to HepG2-scrambled siRNA cells (3.19 ± 0.15) (Fig. 5D). Next, we studied the

relevance of Gal1 expression in HepG2 cell viability under non-proliferative (serum-free) conditions. We found that knocking down or overexpressing Gal1 did not significantly alter tumor cell viability under serum-free conditions (Fig. 5E). However, addition of TGF-β_1 to Gal1-silenced HepG2 cells significantly reduced cell viability ($70 \pm 8\%$), whereas this cytokine showed no effect on Gal1-overexpressing HepG2 cells (Fig. 5E). These results show that Gal1 silencing in HepG2 cells turns these cells sensitive to the growth inhibitory effects of TGF-β_1, indicating that the pro-tumorigenic effects of this cytokine during HCC progression may be modulated, at least in part, by Gal1 expression in tumor hepatocytes.

Discussion

In this study we reported the effects of Gal-1 in liver endothelial cell biology and the interplay between this lectin and TGF-β_1 in the regulation of tumor-endothelial cell interactions in the HCC microenvironment. Seeking to study the influence of HCC-derived Gal1 on liver SEC biology within the HCC microenvironment, we used SK-HEP-1 cells. These cells were derived from a patient with hepatic adenocarcinoma and were demonstrated to be of endothelial origin. Although SK-HEP-1 cells do not contain mRNA for hepatocyte-specific proteins such as albumin, α- or γ-fibrinogen, and show features consistent with an endothelial origin, like pinocytotic vesicles and Weibel-Palade bodies (Heffelfinger et al., 1992), some reports considered them as hepatic cells. Nevertheless, a proteomic analysis revealed that SK-HEP-1 cells are markedly different from normal liver tissue and other hepatoma cells (Seow et al., 2001). Moreover, based on a cDNA microarray analysis, it was concluded that these cells are α-fetoprotein-negative and that their gene expression profile is different from that observed for hepatoma cell lines (Kawai et al., 2001). Furthermore, these cells were stained for several endothelial antigens including von Willebrand factor, vimentin, cytokeratin, ELAM-1, vascular cell adhesion molecule, intercellular adhesion molecule-1 and vascular endothelial growth factor receptor-2 (Heffelfinger et al., 1992; Watchorn et al., 2002; Cogger et al., 2008). Moreover, it was also demonstrated that SK-HEP-1 cells show fenestrations, rudimentary sieve plates, micropinocytotic vesicles, and high endocytic activity, which are typical structural and functional features of liver SECs (Cogger et al., 2008). Thus, taking into account that tumor-derived endothelial cells are morphologically and functionally unique; and based on the fact that SK-HEP-1 cell line, derived from a patient with hepatic adenocarcinoma, has many typical structural and functional features of liver SECs, these cells represent a suitable model to study tumor-endothelial cell interactions.

We showed that these endothelial cells derived from the ascitic fluid of a patient with a liver tumor, expressed high intracellular levels of Gal1 with respect to non-tumoral endothelial cells (BAEC). Accordingly, Thijssen et al. (2006) described that Gal1 was abundantly expressed in vascular endothelial cells in response to growth factor activation. However, we observed that Gal1 levels increased when SK-HEP-1 cells were cultured under serum-free conditions. These results suggest that growth factors can differentially modulate Gal1 expression in normal or tumor-associated, vascular or sinusoidal endothelial cells. It might be speculated that inside the liver tumor, a nutrient-poor microenvironment may contribute to increase Gal1 levels in SECs. Besides, we showed that exposure of SK-HEP-1 cells to CM from HepG2-Gal1 and HuH-7 HCC cells augmented Gal1 expression by regulating its own transcription. Clausse et al. (1999) observed a similar effect when human umbilical vascular endothelial cells (HUVEC) were incubated with CM from prostate carcinoma cells. Thus, Gal1 secreted by tumor hepatocytes may induce its own expression in SECs.

Consistent with our findings in SK-HEP-1 cells, several studies demonstrated that exogenously added rGal1 induces HUVEC proliferation and migration (Hsieh et al., 2008; Thijssen et al., 2010; Croci et al., 2012; D'Haene et al., 2013). Although this galectin promoted comparable effects on HUVEC and SK-HEP-1 cell proliferation, it had a higher impact on SK-HEP-1 cell migration, since lower concentrations promoted substantial changes in the migratory phenotype. Moreover, we found that HCC-derived Gal1 induced SK-HEP-1 cell proliferation and migration. These effects were dependent on the levels of Gal1 expression by HCC cells and mediated by galectin-saccharide interactions. In this regard, a previous study, using lung carcinoma and melanoma models demonstrated that tumor cells secrete Gal1, and that this lectin can be taken up by endothelial cells, stimulating proliferation and migration (Thijssen et al., 2010). Thus, elevated levels of Gal1 within HCC microenvironment may have critical implications in tumor SEC angiogenesis.

Since cancer cell adhesion to the endothelium is an important step for metastasis, we analyzed the role of Gal1 on HepG2 cell adhesion to SK-HEP-1 cells. We found that Gal1 expression modulates HepG2 cell adhesion to SK-HEP-1 monolayers through mechanisms involving the MAPK signaling pathway. Moreover, rGal1 showed a biphasic effect on HepG2 cell adhesion to SECs. Lower concentrations than those required to promote SEC proliferation and migration, favored HCC cell adhesion to SK-HEP-1 monolayers. Conversely, concentrations required for SK-HEP-1 cell proliferation and migration, inhibited HepG2 cell adhesion to SECs. Consistent with these findings, rGal1 has previously been demonstrated to have biphasic effects on proliferation, migration and adhesion of different cell types, including HUVEC (Biron et al., 2006; Thijssen et al., 2006; Elola et al., 2007). Thus, Gal1 may differentially control SEC biology within HCC microenvironment.

Interestingly, SK-HEP-1 cell proliferation and migration were increased when these cells were cultured with CM from HuH-7 cells, compared to HepG2 cell CM. Also, HuH-7 cell adhesion to SK-HEP-1 monolayers significantly increased with respect to HepG2 cells. Since HuH-7 cells export Gal1 and Gal3, both galectins may have combined effects on SK-HEP-1 cell proliferation, migration and adhesion. Moreover, since Gal3 was undetectable in the CM from HepG2 cells, it is likely that the effects on SK-HEP-1 cell proliferation, migration and adhesion are due to increased Gal1 secretion from HepG2-Gal1 cells. Thus, our findings suggest that Gal1 expression and secretion by tumoral hepatocytes may represent key steps associated with HCC dissemination. These data are in line with the results obtained by Spano et al. (2010), who observed that Gal1 was accumulated within the stroma of neoplastic hepatocytes of HCC tumors and its expression correlated with the presence of metastasis in HCC patients.

TGF-β_1 orchestrates a favorable microenvironment for HCC cell growth and EMT. We recently demonstrated that Gal1 up-regulation in HepG2 cells triggers EMT (Bacigalupo et al., 2015). Here, we found that TGF-β_1 substantially increased Gal1 expression and secretion by HCC cells. Similar effects were observed in mammary adenocarcinoma (Daroqui et al., 2007) and pancreatic cells (Tang et al., 2014), and also, in embryonic fibroblasts (Lim et al., 2014). In the latter, Lim et al. (2014) found that TGF-β_1 treatment also triggered nuclear translocation of Gal1. Moreover, this lectin was strongly increased and translocated to nucleus in renal epithelial cells treated with high glucose and TGF-β_1 (Okano et al., 2010). In contrast, TGF-β_1-treated HepG2 cells evidenced a major localization of Gal1 close to the cell membrane, whereas no apparent changes in localization were observed in HuH-7 cells. These results indicate that, although the effects of TGF-β_1 on Gal1 localization are cell-, tissue-, and context-specific, this

cytokine governs expression of this endogenous lectin in several different tissues.

Interestingly, we demonstrated that TGF-β_1 induced HepG2 cell adhesion to SK-HEP-1 SECs through a Gal1-dependent mechanism. Similarly, by inducing collagen III and fibronectin expression, TGF-β_1 enhanced gastric cancer cell adhesion to mesothelial cells (Lv et al., 2011). In addition, TGF-β_1 was found to increase non-small-cell lung cancer cell adhesion to lymphatic endothelial cell monolayers through a β_3 integrin-dependent mechanism (Salvo et al., 2014). Thus, in addition to the well-established role of TGF-β_1 in HCC dissemination through induction of EMT in tumoral hepatocytes, this cytokine may also contribute to HCC metastasis by up-regulating Gal1 expression and favoring HCC cell adhesion to liver endothelium.

Escape from the anti-proliferative and pro-apoptotic actions of TGF-β_1 is a prerequisite for HCC progression (Xu et al., 2003; Dooley and ten Dijke, 2012). Here, we demonstrated that Gal1 levels modulate HepG2 cell proliferation when cultured in serum-rich media. These data confirm our previous findings showing that Gal1-overexpressing HepG2 cells injected in nude mice promote tumor growth in vivo (Espelt et al., 2011). On the other hand, neither Gal1 silencing nor TGF-β_1 treatment influenced the viability of HepG2 cells in non-proliferative conditions (absence of serum). Our results are consistent with previous findings demonstrating that HepG2 cells are refractory to 48 h-culture with TGF-β_1 probably because of the activation of the Ras/ERK1/2 pathway (Zhang et al., 2004; Caja et al., 2011; Dzieran et al., 2013). Interestingly when Gal1 expression declined, HepG2 cells became sensitive to TGF-β_1-induced growth inhibition.

In conclusion, up-regulation of Gal1 within HCC microenvironment controls proliferation and migration of SECs, and favors HCC cell proliferation and adhesion to sinusoidal endothelium. Therefore, Gal1 and TGF-β_1 may work in concert within the HCC microenvironment to promote tumor growth, metastasis and angiogenesis.

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Literature Cited

Bacigalupo ML, Manzi M, Rabinovich GA, Troncoso MF. 2013. Hierarchical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation of hepatocellular carcinoma. World J Gastroenterol 19:8831–8849.

Bacigalupo ML, Manzi M, Espelt MV, Gentilini LD, Compagno D, Laderach DJ, Wolfenstein-Todel C, Rabinovich GA, Troncoso MF. 2015. Galectin-1 triggers epithelial-mesenchymal transition in human hepatocellular carcinoma cells. J Cell Physiol 230:1298–1309.

Bedossa P, Peltier E, Terris B, Franco D, Poynard T. 1995. Transforming growth factor-beta 1 (TGF-beta 1) and TGF-beta 1 receptors in normal, cirrhotic, and neoplastic human livers. Hepatology 21:760–766.

Biron VA, Iglesias MM, Troncoso MF, Besio-Moreno M, Patrignani ZJ, Pignataro OP, Wolfenstein-Todel C. 2006. Galectin-1: Biphasic growth regulation of Leydig tumor cells. Glycobiology 16:810–821.

Caja L, Sancho P, Bertran E, Fabregat I. 2011. Dissecting the effect of targeting the epidermal growth factor receptor on TGF-beta-induced-apoptosis in human hepatocellular carcinoma cells. J Hepatol 55:351–358.

Carr BI, Hayashi I, Branum EL, Moses HL. 1986. Inhibition of DNA synthesis in rat hepatocytes by platelet-derived type beta transforming growth factor. Cancer Res 46:2330–2334.

Clausse N, van den Brule F, Waltregny D, Garnier F, Castronovo V. 1999. Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell–cell adhesion. Angiogenesis 3:317–325.

Cogger VC, Arias IM, Warren A, McMahon AC, Kiss DL, Avery VM, Le Couteur DG. 2008. The response of fenestrations, actin, and caveolin-1 to vascular endothelial growth factor in SK Hep1 cells. Am J Physiol Gastrointest Liver Physiol 295:G137–G145.

Croci DO, Salatino M, Rubinstein N, Cerliani JP, Cavallin LE, Leung HJ, Ouyang J, Ilarregui JM, Toscano MA, Domaica CI, Croci MC, Shipp MA, Mesri EA, Albini A, Rabinovich GA. 2012. Disrupting galectin-1 interactions with N-glycans suppresses hypoxia-driven angiogenesis and tumorigenesis in Kaposi's sarcoma. J Exp Med 209:1985–2000.

Croci DO, Cerliani JP, Dalotto-Moreno T, Mendez-Huergo SP, Mascanfroni ID, Dergan-Dylon S, Toscano MA, Caramelo JJ, Garcia-Vallejo JJ, Ouyang J, Mesri EA, Junttila MR, Bais C, Shipp MA, Salatino M, Rabinovich GA. 2014. Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. Cell 156:744–758.

Chung EJ, Sung YK, Farooq M, Kim Y, Im S, Tak WY, Hwang YJ, Kim YI, Han HS, Kim JC, Kim MK. 2002. Gene expression profile analysis in human hepatocellular carcinoma by cDNA microarray. Mol Cells 14:382–387.

D'Haene N, Sauvage S, Maris C, Adanja I, Le Mercier M, Decaestecker C, Baum L, Salmon I. 2013. VEGFR1 and VEGFR2 involvement in extracellular galectin-1- and galectin-3-induced angiogenesis. PLoS ONE 8:e67029.

Daroqui CM, Ilarregui JM, Rubinstein N, Salatino M, Toscano MA, Vazquez P, Bakin A, Puricelli L, Bal de Kier Joffe E, Rabinovich GA. 2007. Regulation of galectin-1 expression by transforming growth factor beta1 in metastatic mammary adenocarcinoma cells: Implications for tumor-immune escape. Cancer Immunol Immunother 56:491–499.

Demydenko D, Berest I. 2009. Expression of galectin-1 in malignant tumors. Exp Oncol 31:74–79.

Dituri F, Serio G, Filannino D, Mascolo A, Sacco R, Villa E, Giannelli G. 2014. Circulating TGF-beta1-related biomarkers in patients with hepatocellular carcinoma and their association with HCC staging scores. Cancer Lett 353:264–271.

Dooley S, ten Dijke P. 2012. TGF-beta in progression of liver disease. Cell Tissue Res 347:245–256.

Dzieran J, Fabian J, Feng T, Coulouarn C, Ilkavets I, Kyselova A, Breuhahn K, Dooley S, Meindl-Beinker NM. 2013. Comparative analysis of TGF-beta/Smad signaling dependent cytostasis in human hepatocellular carcinoma cell lines. PLoS ONE 8:e72252.

El-Serag HB. 2012. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology 142:1264–1273.e1261.

Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA. 2007. Galectins: Matricellular glycan-binding proteins linking cell adhesion, migration, and survival. Cell Mol Life Sci 64:1679–1700.

Espelt MV, Croci DO, Bacigalupo ML, Carabias P, Manzi M, Elola MT, Munoz MC, Dominici FP, Wolfenstein-Todel C, Rabinovich GA, Troncoso MF. 2011. Novel roles of galectin-1 in hepatocellular carcinoma cell adhesion, polarization, and in vivo tumor growth. Hepatology 53:2097–2106.

Fabregat I. 2009. Dysregulation of apoptosis in hepatocellular carcinoma cells. World J Gastroenterol 15:513–520.

Heffelfinger SC, Hawkins HH, Barrish J, Taylor L, Darlington GJ. 1992. SK HEP-1: A human cell line of endothelial origin. In Vitro Cell Dev Biol 28A:136–142.

Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. 2013. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. Gastroenterology 144:512–527.

Hsieh SH, Ying NW, Wu MH, Chiang WF, Hsu CL, Wong TY, Jin YT, Hong TM, Chen YL. 2008. Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. Oncogene 27:3746–3753.

Hsu DK, Dowling CA, Jeng KC, Chen JT, Yang RY, Liu FT. 1999. Galectin-3 expression is induced in cirrhotic liver and hepatocellular carcinoma. Int J Cancer 81:519–526.

Hughes RC. 1999. Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim Biophys Acta 1473:172–185.

Ito K, Stannard K, Gabutero E, Clark AM, Neo SY, Onturk S, Blanchard H, Ralph SJ. 2012. Galectin-1 as a potent target for cancer therapy: Role in the tumor microenvironment. Cancer Metastasis Rev 31:763–778.

Jiang SS, Weng DS, Wang QJ, Pan K, Zhang YJ, Li YQ, Li JJ, Zhao JJ, He J, Lv L, Pan QZ, Xia JC. 2014. Galectin-3 is associated with a poor prognosis in primary hepatocellular carcinoma. J Transl Med 12:273.

Kawai HF, Kaneko S, Honda M, Shirota Y, Kobayashi K. 2001. Alpha-fetoprotein-producing hepatoma cell lines share common expression profiles of genes in various categories demonstrated by cDNA microarray analysis. Hepatology 33:676–691.

Kondoh N, Hada A, Ryo A, Shuda M, Arai M, Matsubara O, Kimura F, Wakatsuki T, Yamamoto M. 2003. Activation of Galectin-1 gene in human hepatocellular carcinoma involves methylation-sensitive complex formations at the transcriptional upstream and downstream elements. Int J Oncol 23:1575–1583.

Lalor PF, Lai WK, Curbishley SM, Shetty S, Adams DH. 2006. Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo. World J Gastroenterol 12:5429–5439.

Lim MJ, Ahn J, Yi JY, Kim MH, Son AR, Lee SL, Lim DS, Kim SS, Kang MA, Han Y, Song JY. 2014. Induction of galectin-1 by TGF-beta1 accelerates fibrosis through enhancing nuclear retention of Smad2. Exp Cell Res 326:125–135.

Lv ZD, Na D, Ma XY, Zhao C, Zhao WJ, Xu HM. 2011. Human peritoneal mesothelial cell transformation into myofibroblasts in response to TGF-b1 in vitro. Int J Mol Med 27:187–193.

Mathieu V, de Lassalle EM, Toelen J, Mohr T, Bellahcene A, Van Goietsenoven G, Verschuere T, Bouzin C, Debyser Z, De Vleeschouwer S, Van Gool S, Poirier F, Castronovo V, Kiss R, Feron O. 2012. Galectin-1 in melanoma biology and related neo-angiogenesis processes. J Invest Dermatol 132:2245–2254.

Matsuda Y, Yamagiwa Y, Fukushima K, Ueno Y, Shimosegawa T. 2008. Expression of galectin-3 involved in prognosis of patients with hepatocellular carcinoma. Hepatol Res 38:1098–1111.

Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R. 1992. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. Proc Natl Acad Sci USA 89:5408–5412.

Okano K, Tsuruta Y, Yamashita T, Takano M, Echida Y, Nitta K. 2010. Suppression of renal fibrosis by galectin-1 in high glucose-treated renal epithelial cells. Exp Cell Res 316:3282–3291.

Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J, Chernajovsky Y. 1999. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. J Exp Med 190:385–398.

Salvo E, Garasa S, Dotor J, Morales X, Pelaez R, Altevogt P, Rouzaut A. 2014. Combined targeting of TGF-beta1 and integrin beta3 impairs lymph node metastasis in a mouse model of non-small-cell lung cancer. Mol Cancer 13:112.

Seow TK, Liang RC, Leow CK, Chung MC. 2001. Hepatocellular carcinoma: From bedside to proteomics. Proteomics 1:1249–1263.

Spano D, Russo R, Di Maso V, Rosso N, Terracciano LM, Roncalli M, Tornillo L, Capasso M, Tiribelli C, Iolascon A. 2010. Galectin-1 and its involvement in hepatocellular carcinoma aggressiveness. Mol Med 16:102–115.

Tang D, Zhang J, Yuan Z, Gao J, Wang S, Ye N, Li P, Gao S, Miao Y, Wang D, Jiang K. 2014. Pancreatic satellite cells derived galectin-1 increase the progression and less survival of pancreatic ductal adenocarcinoma. PLoS ONE 9:e90476.

Thiery JP. 2002. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454.

Thijssen VL, Barkan B, Shoji H, Aries IM, Mathieu V, Deltour L, Hackeng TM, Kiss R, Kloog Y, Poirier F, Griffioen AW. 2010. Tumor cells secrete galectin-1 to enhance endothelial cell activity. Cancer Res 70:6216–6224.

Thijssen VL, Postel R, Brandwijk RJ, Dings RP, Nesmelova I, Satijn S, Verhofstad N, Nakabeppu Y, Baum LG, Bakkers J, Mayo KH, Poirier F, Griffioen AW. 2006. Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. Proc Natl Acad Sci USA 103:15975–15980.

Tsai JF, Jeng JE, Chuang LY, Yang ML, Ho MS, Chang WY, Hsieh MY, Lin ZY, Tsai JH. 1997. Elevated urinary transforming growth factor-beta1 level as a tumour marker and predictor of poor survival in cirrhotic hepatocellular carcinoma. Br J Cancer 76:244–250.

Watchorn TM, Waddell I, Ross JA. 2002. Proteolysis-inducing factor differentially influences transcriptional regulation in endothelial subtypes. Am J Physiol Endocrinol Metab 282: E763–E769.

Wu H, Chen P, Liao R, Li YW, Yi Y, Wang JX, Sun TW, Zhou J, Shi YH, Yang XR, Jin JJ, Cheng YF, Fan J, Qiu SJ. 2012. Overexpression of galectin-1 is associated with poor prognosis in human hepatocellular carcinoma following resection. J Gastroenterol Hepatol 27:1312–1319.

Wu MH, Ying NW, Hong TM, Chiang WF, Lin YT, Chen YL. 2014. Galectin-1 induces vascular permeability through the neuropilin-1/vascular endothelial growth factor receptor-1 complex. Angiogenesis 17:839–849.

Xu Z, Shen MX, Ma DZ, Wang LY, Zha XL. 2003. TGF-beta1-promoted epithelial-to-mesenchymal transformation and cell adhesion contribute to TGF-beta1-enhanced cell migration in SMMC-7721 cells. Cell Res 13:343–350.

Zhang H, Ozaki I, Mizuta T, Yoshimura T, Matsuhashi S, Eguchi Y, Yasutake T, Hisatomi A, Sakai T, Yamamoto K. 2004. Transforming growth factor-beta 1-induced apoptosis is blocked by beta 1-integrin-mediated mitogen-activated protein kinase activation in human hepatoma cells. Cancer Sci 95:878–886.