Galectin-1 Drives Pancreatic Carcinogenesis through Stroma Remodeling and Hedgehog Signaling Activation

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Abstract

Despite some advances, pancreatic ductal adenocarcinoma (PDAC) remains generally refractory to current treatments. Desmoplastic stroma, a consistent hallmark of PDAC, has emerged as a major source of therapeutic resistance and thus potentially promising targets for improved treatment. The glycan-binding protein galectin-1 (Gal1) is highly expressed in PDAC stroma, but its roles have not been studied. Here we report functions and molecular pathways of Gal1 that mediate its oncogenic properties in this setting. Genetic ablation of Gal1 in a mouse model of PDAC (Ela-myc mice) dampened tumor progression by inhibiting proliferation, angiogenesis, desmoplastic reaction and by stimulating a tumor-associated immune response, yielding a 20% increase in relative lifespan. Cellular analyses in vitro and in vivo suggested these effects were mediated through the tumor microenvironment. Importantly, acinar-to-ductal metaplasia, a crucial step for initiation of PDAC, was found to be regulated by Gal1. Mechanistic investigations revealed that Gal1 promoted Hedgehog pathway signaling in PDAC cells and stromal fibroblasts as well as in Ela-myc tumors. Taken together, our findings establish a function for Gal1 in tumor-stroma crosstalk in PDAC and provide a preclinical rationale for Gal1 targeting as a microenvironment-based therapeutic strategy. Cancer Res; 74(13); 3512–24. ©2014 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer (95%), is one of the most aggressive tumors and the fourth leading cause of cancer-related mortality worldwide (1). Despite notable efforts to develop novel therapeutic targets, PDAC is still highly resistant to therapy, with a median survival of 4 to 6 months and a 5-year survival rate lower than 5% (2). Genome-wide analysis has identified a complex pattern of molecular alterations in PDAC including the previously well-known KRAS, CDKN2A, TRPS1, and SMAD4, and also the activation of other pathways as c-Myc or Hedgehog (Hh; ref. 3). At the histopathologic level, PDAC is characterized by an abundant stromal desmoplasia, a fibroin-inflammatory reaction composed of a dense extracellular matrix (ECM), fibroblasts/pancreatic stellate cells, immune cells, and endothelial cells. Recent data have demonstrated that PDAC-associated desmoplasis plays a crucial role in promoting tumor growth and progression, and also contributes to chemotherapy resistance, emerging as a promising target for pancreatic cancer treatment (4).

Galactins are a family of lectins defined by a highly conserved carbohydrate recognition domain that can localize intracellularly or extracellularly. Galectin-1 (Gal1), one of the best characterized members of this family, is a homodimer of 14 KDa subunits that can interact with carbohydrates from glycoconjugates located at the cell surface or in the ECM, regulating cell–cell and cell–ECM adhesion. By these interactions, it participates in different biologic functions like cell-cycle control, migration, invasion, angiogenesis, and immune system response, both in physiologic and pathologic situations (5, 6). Gal1 is overexpressed in many tumors (7), including PDAC (8–14), where a positive correlation with tumor stage has been found (15). Of note, Gal1 overexpression in PDAC was identified mostly in stromal cells. However, the biologic relevance of these findings remains elusive.

In this study, using Ela-myc mice, a well-characterized model of pancreatic cancer (16, 17) and Gal1 knockout mice, we define a novel Gal1-driven mechanism controlling desmoplasia in these tumors. Our data show that partial or complete depletion of Gal1 reduces in vivo tumorigenicity, leading to a significant increase in Ela-myc mice survival. Abolishment of Gal1 expression not only prevents tumor growth but also...
modulates the tumor microenvironment, hampering stromal activation and angiogenesis and favoring immune surveillance. Moreover, acinar-to-ductal metaplasia (ADM), a transdifferentiation process that likely triggers PDAC initiation, was severely impaired after Gal1 loss in Ela-myc mice, and further analysis suggest that EGFR and Pdx1 are the molecular pathways underlying Gal1-mediated ADM. In addition, in vivo and in vitro strategies indicate that stromal Gal1 is majorly responsible for its tumoral properties. Finally, high-throughput expression analysis and in vitro molecular assays identify Hh as a key signaling pathway involved in Gal1-regulated functions in pancreatic tumor epithelial and stromal cells. Together, these data shed light on the role and molecular mechanisms of Gal1 during pancreatic cancer progression through tumor microenvironment remodeling, suggesting that targeting Gal1 represents a promising therapeutic strategy for this deadly disease.

Materials and Methods

Animals

Animal procedures were approved by the PRBB Ethical Committee for Animal Experimentation. Ela-myc mice were kindly provided by E. Sandgren (University of Wisconsin-Madison, Madison, WI). Animals were housed and fed as described previously (16, 18). For details on breedings, genotyping, and tumor sample collection, see Supplementary Materials and Methods.

Histopathology and immunohistochemistry

For histopathologic analysis, tumor sections from Ela-myc:Gal1+/+/ (n = 44), Ela-myc:Gal1−/− (n = 55), and Ela-myc:Gal1+/− (n = 43) mice were contrasted with hematoxylin and eosin staining and evaluated by two expert pathologists.

Immunohistochemistry (IHC) was performed as described previously (19). An Olympus BX61 microscope and CellSens software were used to acquire images. For antibody and IHC quantification details, see Supplementary Materials and Methods.

Cell lines

PANC-1, SK-PC-1, RWP-1, and HEK-293 T cells were obtained from the Cancer Cell Line Repository at IMIM-Hospital del Mar (Barcelona, Spain). F88.2 cells were a kind gift from F.X. Real (CN10, Spain) and human pancreatic stellate cells (HPSC) were generated as previously described (20). For cell line description see Supplementary Materials and Methods.

Gal1 knockdown by shRNA or siRNA

F88.2 and HPSC were transfected with 50 nmol/L of Gal1 siRNA or an irrelevant siRNA (SMARTpool, Dharmacon). For siRNA lentiviral infections, pLKO-1 vectors targeting Gal1 or a nontargeting shRNA were used (MissionRNAi, Sigma).

In vitro functional experiments

For proliferation, cells were incubated with bromodeoxyuridine (BrdUrd; 40 μmol/L) for 10 minutes. After fixation, BrdUrd immunofluorescence was performed with α-BrdUrd (Santa Cruz Biotechnology) as described previously (19). The percentage of positive cells was evaluated in 10 fields. For migration studies, 2D Gap Closure experiments (Radius Cell Migration Assay, Bionova) were performed. Wound closure was quantified using ImageJ software analysis after 24 hours. For time-lapse video microscopy, 15-minute frames were recorded for 12 hours with a Zeiss Cell Observer HS microscope. Twenty-five cell trajectories per group were analyzed using Manual Tracking (ImageJ). Anchorage-independent growth experiments were performed as described previously (19).

In vitro luciferase measure

RWP-1 cells transfected with an empty pcDNA3 or with pcDNA3-Gal1, were transfected with the vector p551LucII containing eight Gli-binding sites (21). After 48 hours, cells were lysed and luciferase and Renilla activity measured (Promega).

Microarray analysis

Microarray expression profiles were obtained using the Affymetrix Human Exon ST 1.0 arrays (Affymetrix) in Microarray facility of IMIM. Detailed description and validation by qRT-PCR is provided in Supplementary Materials and Methods.

Statistical analysis

Statistical analyses were performed with SPSS version 12.0. Statistical significance cut-off has been always considered when P < 0.05. Kaplan–Meier analyses were used for establishing survival curves and comparisons were performed using the log-rank test. Student t, Mann–Whitney, or χ2 tests were applied, as indicated.

Results

Gal1 deficiency increases Ela-myc mice survival and impairs tumor proliferation

c-myc oncogene plays a key role in the initiation and progression of PDAC (22, 23) and it is frequently overexpressed in human tumors (3, 24). In mouse, expression of c-myc using pancreas-specific elastase promoter (Ela-myc model) leads to the generation of acinar tumors and ductal tumors (16) as well as ADM (Fig. 1A, a–c; ref. 17). We analyzed Gal1 expression by IHC in acinar and ductal tumors, as well as in metaplastic lesions (Fig. 1A, d–f). We found that Gal1 was expressed in the stromal compartment of all samples and, in particular, it was highly expressed in ductal tumors due to its abundant stroma composition. Importantly, this expression pattern in Ela-myc ductal tumors was similar to the one found in human PDACs (Supplementary Fig. S1A; refs. 8, 11, 13) and in tumors from a PDAC K-Ras–driven mouse model (K-Ras<sup>A146C;G12D</sup>; Elas-tTA/tetO-Cre)^<sup>3</sup>; Supplementary Fig. S1A; ref. 18).

To define the role of Gal1 in pancreatic cancer development and progression, Ela-myc transgenic mice were crossed with Gal1 knockouts to obtain Ela-myc:Gal1+/− (n = 80), Ela-myc: Gal1+/− (n = 64), and Ela-myc:Gal1−/− (n = 54). Remarkably, a significant increase in animal survival was observed after loss of either one or both Gal1 alleles (Fig. 1B, left; P < 0.001). These differences were even more evident when considering longtime survivors; Ela-myc:Gal1−/− mice rarely survived more...
Figure 1. Gal1 deficiency increases pancreatic cancer survival and decreases cell proliferation in Ela-myc model. A, hematoxylin and eosin (H&E; a–c) and Gal1 staining (d–f) of acinar, metaplastic, or ductal areas of Ela-myc transgenic mice. Scale bars, 50 μm. B, Kaplan–Meier survival curves (left) from Ela-myc:Gal1+/+, Ela-myc:Gal1+/−, and Ela-myc:Gal1−/−. P values (log-rank test) are relative to Ela-myc:Gal1+/+. Pie charts (right) specify the percentage of animals of each genotype that died before four months (<4 months), between the 4th and 5th month (4–5 months), between the 5th and 6th month (5–6 months) or those animals that survived more than 6 months (>6 months). C, immunostaining for P-Histone H3 in acinar or Ki67 in ductal tumors of each genotype. Scale bars, 50 μm. Right, quantification of the proliferation rates in acinar and ductal areas shown by the percentage of positive cells per field. Bar plots represent mean ± SEM. P values (Mann–Whitney test) are relative to Ela-myc:Gal1+/+. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
than 6 months (4%), whereas long-term survival raised to 20% in Ela-myc:Gal1+/− (or Ela-myc:Gal1−/−) (Fig. 1B, right). The increased survival obtained in Ela-myc:Gal1−/− compared with wild type (wt) mice, indicates that Gal1 haploinsufficiency impairs pancreatic tumor development. Gal1 expression in Ela-myc:Gal1+/− tumors was confirmed by IHC to rule out the inactivation of the wt allele (Supplementary Fig. S1B). Taken together, these data demonstrate that total or partial abolishment of Gal1 alleles (Fig. 1C, a), using the M-phase specific marker P-Histone H3, we observed a significant decrease on the number of cells upon loss of one or two Gal1 alleles (Fig. 1C, a-c and right). Furthermore, ductal tumors from Ela-myc:Gal1+/− or Ela-myc:Gal1−/− displayed a significant reduced Ki67 immunostaining compared with Ela-myc:Gal1+/+ counterparts (Fig. 1C, d-f and right).

We also analyzed whether Gal1 expression could affect tumor infiltration and metastasis and no significant differences were observed (Supplementary Text and Supplementary Table S1).

Altogether, these data indicate that reduced expression of Gal1 affects proliferation of PDAC tumors in Ela-myc mice, resulting in a slower tumor growth and increased survival.

**Gal1 deficiency impairs ADM in Ela-myc mice**

PDAC is likely to originate from transdifferentiation of acinar cells into ductal cells, a process known as ADM (25). This mechanism has been also described in the Ela-myc model, where formation of ductal tumors is preceded by transdifferentiation of acinar cells (17). To assess whether Gal1 is involved in ADM in pancreatic cancer, we performed a detailed histopathologic analysis and classification of acinar and ductal tumors from Ela-myc:Gal1+/−, Ela-myc:Gal1−/−, and Ela-myc:Gal1+/+ mice (Fig. 2A, a–f). Remarkably, we found that Gal1 deficiency, either one or two alleles, resulted in a dramatic reduction of Ela-myc ductal tumors (Fig. 2B). Of note, the percentage of tumors with high ductal component was reduced in Ela-myc:Gal1−/− mice compared with Ela-myc: Gal1+/− and Ela-myc:Gal1+/+ mice (Fig. 2C). These results strongly suggest a role for Gal1 in ADM during c-myc-driven pancreatic carcinogenesis.

To get insights into the molecular signaling pathways impairing ADM after Gal1 downregulation in Ela-myc tumors, we selected several genes previously reported to be involved in this process (26) and compared their expression by qRT-PCR in tumors from Ela-myc:Gal1+/−, Ela-myc:Gal1−/−, and Ela-myc: Gal1+/+ mice. Interestingly, EGFR and Pdx1 RNA levels were downregulated in tumors with low levels of Gal1, and MMP7 was upregulated (Fig. 2D), whereas the other genes analyzed were not affected (Supplementary Fig. S2A). Finally, considering the paramount importance of EGFR in pancreatic ADM (27) and PDAC progression (28), we further validated its downregulation at the protein level by IHC in Ela-myc: Gal1−/− tumors (Supplementary Fig. S2B).

**Loss of Gal1 modulates pancreatic tumor microenvironment**

The stroma is the major component of the tumor mass in PDAC and it represents a promising target for therapy (4, 29). Considering high Gal1 levels of expression in pancreatic stroma (Fig. 1A, d–f) and our previous in vitro data supporting a role for this lectin in the tumor–stroma crosstalk (12), we evaluated the overall contribution of Gal1 to tumor microenvironment during in vivo pancreatic carcinogenesis. First, tumor vascularization was analyzed using von Willebrand factor (vWF) staining. A significant reduction of angiogenesis was observed both in acinar and ductal tumors from Ela-myc: Gal1−/− and Ela-myc:Gal1+/+ mice compared with Ela-myc: Gal1+/+ (Fig. 3A, a–f and right). Accordingly, mice with reduced Gal1 levels displayed significantly less intraperitoneal hemorrhages (Supplementary Text and Supplementary Table S1) and increased tumoral necrosis (Supplementary Fig. S3A) likely due to reduced tumor vascular network. Second, we analyzed activated fibroblast and stellate cells in these tumors using α-SMA immunostaining (Fig. 3B, a–f). Activated stromal cells were reduced after Gal1 depletion in acinar and ductal lesions, although significance was only reached in the latter (Fig. 3B, right). Moreover, we found that vimentin-positive cells (which label all fibroblasts independently of their activation status) were also reduced upon Gal1 loss (Supplementary Fig. S3B), showing a very similar distribution compared with α-SMA (Fig. 3B). Indeed α-SMA and vimentin stainings on serial sections revealed that all fibroblasts in these tumors were activated (Supplementary Fig. S3C). Third, we analyzed the effects of Gal1 depletion in infiltrating tumor immune cells. Quantification of intratumoral T-lymphocytes in ductal lesions, detected by IHC against CD3, showed a significant and dose-dependent increase in this population in the absence of Gal1 (Fig. 3C a–c, right). Acinar lesions showed reduced immune cell infiltration, although the same pattern was observed (data not shown). Similarly, neutrophil quantification by MPO staining revealed a significant increase of this cell population in ductal tumors after Gal1 loss (Fig. 3C, d–f and right). No differences were observed in intratumoral macrophages or B cells between different genotypes (data not shown). These results demonstrate the involvement of Gal1 in maintaining pancreatic tumor immune privilege by hampering T-cell and neutrophil-mediated immune response during in vivo cancer progression.

Altogether, these data indicate that modulation of Gal1 expression in pancreatic cancer has a crucial impact on remodeling in vivo the tumor microenvironment, through regulation of angiogenesis, fibroblasts activation, and immune response.

**Gal1 cell-autonomous and non–cell autonomous effects during pancreatic tumorigenesis**

Gal1 is a secreted protein; therefore, Gal1 found in tumoral stroma can originate from epithelial, stromal cells, or both. In fact, we have previously reported that Gal1 is highly expressed
in human pancreatic cell lines (12) and primary cultures from
Ela-myc tumors (unpublished data). To analyze the cell-auton-
omous and non–cell autonomous contribution of Gal1 in
epithelial pancreatic cancer cell oncogenesis, we knocked
down its expression in the human cell line PANC-1 and tested
its tumorigenic properties in vitro and in vivo. Cells were
transfected with a shRNA control (shCtl) or with two different
Gal1 shRNA sequences (shGal1#1 and shGal1#2), which ef-

ciently reduced Gal1 protein expression (>90%; Fig. 4A).

In vitro characterization of these cells showed that while downregu-
lation of Gal1 expression did not affect cell proliferation (Fig. 4B),
it significantly reduced soft agar colony formation (Fig. 4C) and
cell migration and motility (Fig. 4D and E and Supplementary
Videos S1–S3). These functional effects were also confirmed in
another PDAC cell line (SK-PC-1; Supplementary Fig. S4 and
Supplementary Videos S4–S6). To further analyze the specific
contribution of Gal1 expressed by epithelial pancreatic cancer
cells to in vivo tumorigenesis, we injected PANC-1 cells with
normal or downregulated Gal1 levels into nude mice. Unex-
pectedly, no differences in tumor formation, animal survival
(Supplementary Text and Supplementary Fig. S5), or histo-
pathologic hallmarks (Supplementary Fig. S6) were observed
between wt and shGal1 cells, suggesting that the effects of Gal1
depletion in vivo are mainly non–cell autonomous.

Characterization of the molecular pathways triggered by
Gal1 in pancreatic carcinogenesis

To characterize the molecular pathways affected by Gal1 in
PDAC cells, we compared the global gene expression profile by
microarray analysis of PANC-1 cells with endogenous Gal1
levels (shCtl) and after Gal1 knockdown (shGal1; Fig. 5). We
found 547 (175 upregulated and 372 downregulated) genes
differentially expressed (P < 0.0005) in Gal1 downregulated

cells (Fig. 5A and Supplementary Table S2). Gene ontology
analysis of Gal1 target genes identified a significant (P < 0.0001)
enrichment in genes involved in regulation of cell adhesion,
migration, and cell signaling pathways (Fig. 5B) (Supplemen-
tary Table S3). Interestingly, we found many significantly

Figure 2. Gal1 loss impairs ADM.
A, hematoxylin and eosin staining
of acinar (a–c) and ductal regions
(d–f) of Ela-myc:Gal1+/+,
Ela-myc:
Gal1+/–, and Ela-myc:Gal1–/–
transgenic mice. Scale bars,
50 μm. B, pie charts show the
percentage of ductal (white) versus
acinar (gray) areas in tumors of
each genotype. C, quantification of
the percentage of entirely ductal
tumors (100%), tumors with a
ductal component over the 70%
(>70%) or over the 50% (>50%) in
the different genotypes. D, analysis
of EGFR, Pdx1, and MMP7 by qRT-
PCR in RNA extracts from tumors
of Ela-myc:Gal1+/–, Ela-myc:
Gal1–/–, and Ela-myc:Gal1–/–
mice. Bar plots represent
mean ± SEM of three independent
experiments. P values (B, Mann–
Whitney; C, χ2; D, Student t test)
are relative to Ela-myc:Gal1+/+.
*, P < 0.05; **, P < 0.01;
***, P < 0.001.
Figure 3. Gal1 modulates the tumor microenvironment. A, immunostaining for vWF in acinar and ductal parts of tumors of Ela-myc:Gal1+/+, Ela-myc:Gal1+/−, and Ela-myc:Gal1−/− mice. Scale bars, 200 μm. B, α-SMA IHC in Ela-myc:Gal1 acinar and ductal tumors. Scale bars, 50 μm (a–c), 100 μm (d–f). C, IHC to detect T lymphocytes with CD3 (a–c) or neutrophils with MPO (d–f) antibodies in Ela-myc:Gal1 ductal tumors. Scale bars, 20 μm. Bar plots on the right (A–C), show quantifications, which are represented as the mean positively stained area ± SEM. P values (Mann–Whitney test) are relative to Ela-myc:Gal1+/+. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
altered pathways involved in cellular response to cytokines and inflammation/immune processes, one of the best characterized functions of Gal1 (30). Validation by qRT-PCR of several of these genes demonstrated that Gal1 levels correlates with genes involved in cell migration, adhesion, and malignant transformation (Supplementary Text and Supplementary Fig. S7), and, remarkably, with genes of the Hh-Gli axis, a key pathway in the initiation and progression of PDAC (31). In particular, Disp1, which is involved in Hh ligand secretion and signaling (32), and cyclin-D2, a downstream Gli target gene (33), showed reduced levels in cells knocked down for Gal1 (Fig. 5C), suggesting Gal1 may regulate Hh pathway in pancreatic tumoral cells.

Gal1 activates Hh signaling pathway in epithelial and fibroblastic cells

Taking into account the reported autocrine and paracrine effects reported for Hh signaling pathway, we aimed to evaluate more in detail whether Gal1 expression regulates Hh pathway in PDAC epithelial cells and in fibroblastic stromal cells. First, RWP-1 control cells (RWP-1 pcDNA3) or overexpressing Gal1 (RWP-1_Gal1) cells were transfected with a Gli-luciferase reporter cassette to analyze the effects of Gal1 expression in Gli1 transcriptional activity (Fig. 6A, left). Cells overexpressing Gal1 showed a significant increase in Gli-driven luciferase activity (Fig. 6A, right). These data, together with those of microarray validation, indicate that Gal1 triggers Gli...
expression and activity in pancreatic epithelial tumoral cells. Second, we analyzed whether Gal1 can modulate Hh-Gli pathway in tumor-associated fibroblasts. For this purpose, Gal1 expression was knocked down in F88.2 (tumor-derived fibroblasts) and HPSC (Fig. 6B), and effects on Hh signaling were analyzed. Importantly, we found that levels of Gli1 and the Hh receptor Patched1 (Ptc1) were dramatically reduced after Gal1 downregulation (Fig. 6C), indicating that Gal1 also activates Hh pathway in tumor-associated fibroblasts. Gal1 regulation of Gli1 and Ptc1 levels were confirmed in vivo in the Ela-myc model. Interestingly, Gli1 and Ptc1 protein expression was significantly reduced in Ela-myc;Gal1+/− and Ela-myc:Gal1−/− in comparison with Ela-myc;Gal1+/* (Fig. 6D). Altogether these results strongly suggest a role for Gal1-mediated activation of the Hh signaling pathway in epithelium and stroma in vitro as well as during in vivo pancreatic carcinogenesis in the Ela-myc model.

Discussion
Despite major efforts to unveil the molecular mechanisms underlying initiation and progression of pancreatic cancer, very little progress has been made in treatment, and it still remains an incurable disease. Given the persistent desmoplastic response that characterizes PDAC and its role as a physical barrier for drug delivery (4, 34), tumoral stroma has emerged as a novel promising target (29, 35). Here, we...
Figure 6. Gal1 activates Hedgehog signaling pathway in pancreatic tumoral cells and fibroblasts. A, reporter plasmid pΔ51/LucII showing eight Gli-binding sites, the α-crystallin basal promoter, and the luciferase gene (left). Luciferase activity driven by the Gli reporter cassette after transfection of pΔ51/LucII in RWP-1 cells transfected with empty pcDNA3 or pcDNA3-Gal1 (Gal1; right). B, Western blot analysis of Gal1 downregulation after siRNA Gal1 transfection in F88.2 and HPSC cells. Tubulin levels are shown as the loading control. Bottom, quantification is shown. C, Gli1 or Ptch1 RNA levels assessed by qRT-PCR in control cells (shCtl) or fibroblasts with downregulated Gal1 levels (shGal1). D, Gli1 and Ptch1 IHC in tumors from Ela-myc:Gal1+/+, Ela-myc:Gal1+/−, and Ela-myc:Gal1−/− mice. Scale bars, 25 μm (a–c, g–i), 50 μm (d–f, j–l). Quantifications of nuclear staining (Gli1) or cytoplasmic (Ptch1; H-score) are shown on the right. Bar plots (A, C, and D) represent mean ± SEM. P values (determined by Student t test) are relative to shCtl. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
identify Gal1 as a novel player in tumor–stroma crosstalk in pancreatic cancer, supporting tumor progression (Fig. 7). Moreover, our data hold the in vivo therapeutic benefits of Gal1 downregulation, as its deficiency results in stroma remodeling, tumor size reduction, and increased survival.

The Ela-myc model overexpresses c-myc oncogene in acinar cells and it is the only single transgene model that develops PDAC with short latency and high penetrance. Moreover, this model displays ADM (17) and it has been one of the first models to demonstrate the acinar origin of PDAC. Importantly, c-myc overexpression has been frequently found in PDAC and pancreatic cell lines (3, 24) and recent data have reported that it is essential for initiation, maintenance, and recurrence of PDAC (22) as well as for K-Ras–induced carcinogenesis (23, 36, 37). In this study, we show that loss of one or two Gal1 alleles ameliorates c-myc–driven pancreatic carcinogenesis by decreasing tumor cell growth, angiogenesis, and stroma activation, and increasing immune surveillance (Fig. 7). Importantly, most of Gal1-mediated protumoral functions are non-cell autonomous, as in vitro downregulation of Gal1 levels in human PANC-1 cells has no effect on cell proliferation and these cells develop similar tumors than those from wt cells when injected into nude mice. These results highlight the crucial contribution of the tumor microenvironment to Gal1 effects in pancreatic carcinogenesis. The lack of effect of Gal1 knockdown in xenograft models could be due to Gal1 expressed by host stromal fibroblastic cells (Supplementary Fig. S6B and S6C) or to the absence of T-lymphocytes in nude mice, which could hide Gal1-mediated regulation of the immune system during cancer progression (6). In this regard, we have found that in Ela-myc mice, which are immunocompetent, pancreatic tumors with reduced Gal1 show a significant increased number of intratumoral T-lymphocytes and neutrophils in comparison with wt tumors. A role for Gal1 in tumor growth through its immunomodulatory function has been also recently proposed in other tumors (38–40).

An important issue that comes out from our data is what type of cells produces Gal1 found in the stroma of pancreatic cancer in patients and mice (Fig. 1 and Supplementary Fig. S1A; refs. 8, 11, 13). Gal1 can be secreted and can bind to the ECM through protein and keratan sulfate recognition. Activated tumor fibroblasts or stellate cells have been proposed as a source of stromal Gal1 in pancreatic cancer (41). Notwithstanding, tumoral cells may also express high Gal1 levels and secrete it to the extracellular milieu. In accordance, we have previously reported high secretion of Gal1 in conditioned medium of human tumoral pancreatic cell lines (12). Similarly, a paracrine mechanism involving uptake of Gal1 secreted by tumoral cells has been described in endothelial cell activation (42). Remarkably, reported data showing that Gal1 induces chemokine production, pancreatic stellate cell proliferation (43), and stimulation of collagen and fibronectin synthesis (44), indicates that Gal1 generates a positive feedback loop exacerbating tumor desmoplastic reaction. In agreement with this scenario, we report here that abolishment of Gal1 expression in Ela-myc pancreatic tumors results in decreased desmoplasia.

Several reports have involved transdifferentiation of acinar cells into ductal cells in the onset of pancreatic cancer (17, 25, 45, 46). Our data show that loss of expression of Gal1 in Ela-myc mice leads to ADM blockade, adding Gal1 as a novel gene in the increasing list of molecules triggering this process (26). Furthermore, our molecular analysis from Ela-myc tumors suggests that Gal1-mediated ADM involves positive regulation of EGFR and Pdx1. In contrast, the opposite

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**Figure 7.** Role of Gal1 in ADM and PDAC–involvement of molecular pathways. Gal1 favors ADM, a key step for PDAC initiation, likely through activation of EGFR, Pdx1, and Hh pathways. In PDAC, Gal1 expression favors tumor progression by promoting tumoral cell proliferation, angiogenesis and Hh-mediated stroma activation, and blocking immune surveillance.
correlation found between Gal1 and MMP7 levels suggest that MMP7 pathway should be upstream of Gal1 and may be upregulated in the absence of the lectin as a compensatory mechanism. Altogether, these data identify for the first time Gal1 and EGFR-Pdx1 axis in ADM, suggesting a putative role of Gal1 in PDAC initiation.

We further evaluated the molecular pathways underlying Gal1 promalignant functions during pancreatic carcinogenesis. We found that Gal1 downregulation in Panc-1 cells results in increased expression of fibronectin-1, integrin-co3 thrombospondin-1, and E-cadherin (Supplementary Fig. 7), which could be responsible for the inhibition of cell motility. Interestingly, Gal1 downregulation in glioblastoma cells also increases ECM and cell adhesion molecules (47), suggesting a common Gal1-related gene signature between different tumors.

One of our major findings is the activation of Hh-Gli pathway by Gal1. In PDAC, deregulated Hh signaling has been found in precursor lesions and primary tumors (31). Interestingly, Hh signaling in PDAC can be activated in autocrine and paracrine ways supporting tumor epithelium–stroma crosstalk and tumor progression (48, 49). Our findings demonstrate that Gal1 has a direct role in activating Hh-Gli pathway in pancreatic tumors and fibroblasts, as shRNA downregulation of Gal1 decreases the expression of upstream and down-stream effectors of Hh-Gli axis and overexpression of Gal1 in PDAC cells leads to increased Gli activity. Surprisingly, despite the well-reported data of Hh inhibition negatively regulating cell proliferation (31), we did not observe any effect in the in vitro cell growth in Panc-1 (Fig. 4B) or HPSC cells (data not shown) after Gal1 downregulation and subsequent Hh inhibition. These results can be explained either by insufficient reduction of Hh pathway-Gli levels after Gal1 knockdown, or by the modulation of other cellular pathways that could compensate for Hh-mediated inhibition of cell growth. Importantly, we have found that in vivo Gal1 downregulation in Ela-myc mice results in decreased tumor cell proliferation (Fig. 1C) and in an impairment of the Hh signaling pathway (Fig. 6D). These different effects on cell proliferation that we found after in vitro and in vivo Gal1 downregulation strongly suggest that the tumor–stroma crosstalk might be essential for the Hh-mediated increase in cell proliferation in PDAC. Finally, as Hh activation has been previously related to stroma activation and ADM (50), it is tempting to speculate that in vivo Gal1 effects on stroma remodeling and ADM reported here are also triggered by Hh signaling.

In conclusion, our data identify a crucial role for Gal1 in promoting pancreatic carcinogenesis through the activation of tumor and microenvironment crosstalk, favoring key steps of cancer progression as proliferation, angiogenesis, desmplasia, immune evasion, and ADM (Fig. 7). These pleiotropic functions of Gal1 in PDAC, together with increasing evidences supporting the tumoral stroma, where Gal1 is highly expressed, as a potential target to surmount PDAC resistance to therapy, lead us to propose Gal1 as a promising molecular target for pancreatic cancer treatment. For instance, our results showing that heterozygous deletion of Gal1 also inhibits pancreatic tumor progression indicate that partial abolishment of this lectin may be therapeutically effective. Moreover, lessons from Gal1 knockout mice indicate that this protein is not essential for animal survival, suggesting that Gal1 inhibitors should be a safe therapy lacking associated undesirable side effects. Last but not least, given Gal1 overexpression in many different tumors, our data have broader implications in the use of this lectin as a novel molecular target for general cancer diagnosis and therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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