Chapter 5

Galectin-4 expression is associated with reduced lymph node metastasis and modulation of Wnt/β catenin signalling in pancreatic adenocarcinoma

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Abstract

Galectin-4 (Gal-4) has been recently identified as a pivotal factor in the migratory capabilities of a set of defined pancreatic ductal adenocarcinoma (PDAC) cell lines using zebrafish as a model system. Here we evaluated the expression of Gal-4 in PDAC tissues selected according to their lymph node metastatic status (N0 vs. N1), and investigated the therapeutic potential of targeting the cross-link with the Wnt signaling pathway in primary PDAC cells.

Analysis of Gal-4 expression in PDACs showed high expression of Gal-4 in 80% of patients without metastasis, while 70% of patients with lymph node metastases had low Gal-4 expression. Primary PDAC cells with high expression of Gal-4 had less migratory/invasive ability in vitro and in vivo. This was counteracted by knockdown of Gal-4, resulting in significant increase of invasion (40%) and migration (50%, P<0.05). Accordingly, enforced expression by lentiviral transduction of Gal-4 in cells with low Gal-4 expression reduced the migratory and invasive behaviors compared to the control cells. Moreover Gal-4 markedly reduced the expression of β-catenin, counteracting the function of Wnt signaling, as also assessed by down-regulation of survivin and cyclin D1. Further, Gal-4 sensitized PDAC cells to the Wnt inhibitor ICG-001, which successfully disrupted the interaction between CBP and β-catenin.

Collectively, these findings provide novel insights into the role of Gal-4 in PDAC migration and invasion, and support the analysis of Gal-4 for rational targeting of Wnt/β-catenin signaling for the treatment of PDAC.

Key words: Pancreatic ductal adenocarcinoma, Galectin-4, migration, lymph node metastatic status, primary PDAC cells, Wnt/β-catenin pathway
**Introduction**

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-death, and has the worst prognosis of any major malignancy, with less than 5% of patients alive 5-years after diagnosis [Vincent et al., 2011]. One of the major hallmarks of PDAC is its early systemic dissemination, which is frequently coupled to extensive local tumor spread [Hidalgo, 2012]. These features explain why more than 80% of patients diagnosed with this disease cannot be offered surgical treatment and, therefore, are the main causes of the high mortality rate among PDAC patients.

Invasion and metastasis are complex processes, and further studies on their genetic and biochemical determinants are urgently needed. Loss of junctional contact between adjacent epithelial cells and cell-extracellular matrix association constitute fundamental prerequisites for cancer cell detachment from the primary tumor. However, not only adhesion molecules on the cell surface but also regulators of transmembrane signaling play a determinant role in tumor cell migration and regulate the potential for epithelial cells to metastasize [Valastyan and Weinberg, 2011].

Galec-tins consist of a large family of galactoside-binding soluble lectins that have been classified, based on their structure and carbohydrate-recognition domains, in prototype gaelectins (galectins-1, -2, -5, -7, -10, -11, -13, -14), chimera type (galectin-3), and tandem repeat type (galectins-4, -6, -8, -9, -12) [Cummings and Liu, 2009]. Galectins display a broad variety of functions, including mediation of cell–cell interactions, cell–matrix adhesion and transmembrane signaling [Baronds et al., 1994; Yang et al., 2008]. The expression of galectins is strictly regulated during the development of individual cells, but can be altered under different pathological conditions [Danguy et al., 2002]. In particular, galectins contribute to different key events in tumor cells, such as differentiation, angiogenesis, malignant transformation, apoptosis, tumor immune escape and resistance to anticancer drugs [Liu and Rabinovich, 2005]. Galectins have also been described to modulate homo- and heterotypic adhesion of tumor cells thereby mediating invasion and metastasis in several tumor types, including PDAC [Danguy et al., 2002; Barrow et al., 2011; Tang et al., 2014].

In this study we focus on the role of Galectin 4 (Gal-4) in PDAC. In healthy individuals Gal-4 is predominantly expressed in the luminal epithelia of the gastrointestinal tract, and not detected in healthy pancreas. Remarkably, in PDAC tissue a high expression of Gal-4 was observed both at the transcriptional and protein level mRNA [Lowe et al., 2007; Bauer et al., 2009]. High expression levels of Gal-4 were shown in PDAC and in intraductal papillary mucinous carcinoma, while the almost invariably benign cystic neoplasms and mucinous cystadenoma of the pancreas showed no Gal-4 expression [Bauer et al., 2009]. These results lead the authors to consider Gal-4 to be a typical example of a gradual increase in transcriptional activity and to be a factor that could account for the invasive ability of PDAC.

Our recent studies, however, demonstrated an opposite role for Gal-4 showing that it was overexpressed in less migrating PDAC cell lines, compared to those having metastatic abilities [Belo et al., 2013]. Moreover, Gal-4 delayed migration of PDAC cells both in vivo, using the zebrafish experimental model [Belo et al., 2013].

In the current research we have extended these studies by exploring the role of Gal-4 in
human PDAC tissues and in several primary PDAC cultures. Our data establish a role of Gal-4 as tumor suppressor in PDAC, since we showed that elevated Gal-4 levels correlated significantly with a reduced in vitro migratory and invasive behavior in primary PDAC cultures, as well as with a reduced lymph node metastasis in PDAC patients.

Moreover, we hypothesized that Gal-4 might inhibit metastasis by down-regulation of Wnt signaling target genes, as already shown for colon rectal cancer [Satelli et al, 2011]. Recently, it was demonstrated that the activation of Wnt/β-catenin pathway, which plays an essential role in proliferation and differentiation of many organs [Clevers, and Nusse, 2012], is required for progression of PDAC [Zhang et al, 2013]. In the absence of Wnt stimuli, GSK3-β phosphorylates β-catenin in order to degrade it. However, activation of this pathway results in dephosphorylation of β-catenin, followed by accumulation and translocation into the nucleus. Interaction of accumulated β-catenin with CREB binding protein (CBP) leads to an active transcriptional complex for downstream target genes [Hecht et al, 2000], and appears a key step to activate transcription of target genes involved in PDAC development [Zhang et al, 2013]. Enhanced Wnt/β-catenin signaling has been observed in human PDAC tissues and preclinical models, while inhibition of Wnt signaling through transfection with the Wnt inhibitors Icat and dn-Lef-1, or knockdown of β-catenin, increased apoptosis and decreased PDAC cells proliferation [Pasca di Magliano et al, 2007].

Thus, inhibition of Wnt/β-catenin signaling by novel anticancer agents might have a therapeutic impact on suppression of PDACs driven by this pathway, and key factors to identify these tumors are warranted. To this end, in the current study we explored the interaction of Gal-4 with the Wnt/β-catenin signaling and we demonstrated that Gal-4 sensitized PDAC cells to the Wnt inhibitor ICG-001, which successfully disrupted the interaction between CBP and β-catenin.

Materials and Methods

Studies in clinical samples

Immunohistochemistry (IHC)

In order to evaluate the Gal-4 expression in human PDAC tissues, immunohistochemistry (IHC) was executed according to standard procedures in formalin-fixed paraffin embedded (FFPE) sections of 20 primary PDACs radically resected patients, carefully selected according to their clinicopathological characteristics. All these patients underwent radical surgical resection with curative intent (pancreatice-duodenectomy, total pancreatectomy and distal pancreatectomy) at the Department of General Surgery and Transplant, University Hospital of Pisa (Pisa, Italy), between 2000 and 2010 and were retrospectively reviewed using electronic medical records. Among them, we selected 10 patients with T3N0Mx stage, whereas the other 10 patients had N1 lymph node metastasis (i.e. T3N1Mx stage, according to the American Joint Committee on Cancer (AJCC) TNM staging system).

Staining with the goat anti-human Gal-4 (1:100) was visualized with the BenchMark Special Stain Automation, and evaluated using a four-tier system, including positive cells number and intensity, as described previously [Giovannetti et al, 2014]. Negative controls
were obtained by replacement of primary antibody with buffer. All slides were reviewed by two researchers (N.F and E.G) who also evaluated the amount of tissue loss, background staining and overall interpretability before the formal Gal-4 reactivity evaluation. Additional digital analyses were performed with a computerized high-resolution acquisition system (D-Sight, Menarini, Florence, Italy), equipped with the automated quantitative image analysis software algorithm DSight software 2.1.0. The two groups of analyses were compared by t-test (P<0.05 for significant results).

**Preclinical studies**

**Chemicals and reagents**

RPMI-1640 and DMEM medium, foetal bovine serum (FBS), penicillin (50 IU/mL) and streptomycin (50 µg/mL) were purchased from Gibco (Gaithersburg, MD). The Wnt inhibitor ICG001 was obtained from Selleckchem (Bio-Connect Diagnostics BV, Huissen, The Netherlands). Gal-4 siRNA and Silencer®Negative control were from Ambion (Life Technology, Bleiswijk, The Netherlands). Lipofectamine® LTX and Opti-MEM® were purchased from Invitrogen (Life Technology).

**Cell culture**

Eight primary PDAC cell cultures (PDAC1, PDAC-2, PDAC-3, PDAC-4, PDAC-5, PDAC-6, PDAC-7 and PDAC-8) were isolated from patients at the University Hospital of Pisa (Pisa, Italy), as described previously [Avan et al, 2013A], while the human pancreatic duct epithelial-like cells hTERT-HPNE, and the PDAC cell lines Pa-Tu-8988T (PaTu-T) and Pa-Tu-8988S (PaTu-S) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), respectively. The primary cells were cultured in RPMI-1640, supplemented with 10% heat-inactivated (HI) FBS and 1% streptomycin/penicillin at 37°C, in a 5% CO₂ humidified atmosphere and harvested with trypsin-EDTA in their exponentially growing phase. The cell lines hTERT-HPNE, Pa-Tu-8988S (PaTu-S) and Pa-Tu-8988T (PaTu-T), transduced PaTu-T/Gal-4 and PaTu-T/Mock were cultured in DMEM high glucose (GIBCO, Invitrogen), with 10% HI-FBS and 1:100 streptomycin/penicillin.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted using TRIzol® (Life Technology), and its yields and purity were checked at 260-280 nm with NanoDrop®-1000-Detector (NanoDrop-Technologies, Wilmington, NC). One µg of RNA was reverse transcribed using the DyNAme cDNA Synthesis Kit (Thermo Scientific, Vantaa, Finland), according to the manufacturers’ instruction. In order to evaluate whether the expression of Gal-4 was similar in the primary cells and the tumors from which they were derived, we also extracted RNA from these 8 tumors, after laser-microdissection with a Leica LMD7000 instrument (Leica, Wetzlar, Germany), using the QiaAmp RNA micro-Kit (Qiagen, San Diego, CA). Because of the long experience of our laboratory with this method, there was no difficulty in selecting areas with morphological defined cancer cells, and the precision of the laser beam resulted in the capture of individual cells with high degree of accuracy [Funel et al, 2008]. qRT-PCR of Gal-4 was performed with the SYBR Green method in an ABI-7900HT sequence detection system (Applied Biosystems, Life Technology, Forster City, CA), as described previously [Belo et al, 2013].
Additional qRT-PCR studies evaluated the mRNA expression of cyclin-D1 (CCDN1, NCBI Reference Sequence: NM_053056.2) and survivin (BIRC5, NCBI Reference Sequence: NM_001012271.1) using primers and probes from Applied Biosystems Assay-on-Demand Gene expression products (Hs00765553_m1 and Hs00153353_m1, respectively). The PCR was performed in a 25 μl reaction volume containing TaqMan Universal master mix (Applied Biosystems), in triplicate using the ABI PRISM-7500 sequence detection system instrument (Applied Biosystems), as described previously [Avan et al, 2012].

Array Comparative-Genomic-Hybridization (aCGH)

In order to evaluate whether different expression levels of Gal-4 were determined by copy number variation of this gene, genomic DNAs were extracted from PDAC-1 and PDAC-2 cells using Ambion®-RecoverAll kit (Life Technologies). The quantity and purity of extracted DNAs were determined as described above. DNAs were subjected to array Comparative Genomic Hybridization, using Agilent 4x180K platform (Agilent, Santa Clara, CA), as described previously [Avan et al, 2013A]. The slides were scanned on Agilent Microarray Scanner, followed by data extraction and normalization by Feature Extraction v10.5 software (Agilent). Nexus Copy Number™ software was used to analyze the DNA copy number variations (BioDiscovery, Hawthorne, CA).

Western blot

Further analyses on Gal-4 and β-catenin protein expression were performed in PDAC-1, PDAC-2, Patu-S and Patu-T cells, which were selected for their differential Gal-4 mRNA expression and migration capabilities as described earlier [Belo et al, 2013, Avan et al, 2013A].

Briefly, 30 μg of proteins was separated on a 10% SDS-polyacrylamide gel and transferred onto PVDF membrane (Immobilion®-FL, Millipore, Billerica, MA). The membrane was blocked with Rockland (Rockland inc., Pennsylvania, USA), and incubated with goat anti-human Galectin-4 (1:1000; BD Biosciences, Erembodegem, Belgium), mouse anti-β-catenin (1:1000; Cell Signaling, Danvers, MA) and mouse anti-β-actin (1:50000; Sigma–Aldrich Chemicals). The membrane was probed with the anti-mouse-InfraRedDye (1:10000, Westburg, The Netherlands) or anti-goat InfraRedDye (1:10000) secondary antibodies. Fluorescent proteins were determined by an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE), at 84-μm resolution, 0-mm offset, using high quality settings. The intensities of protein bands were quantified using the Odyssey v.3.0 software (LI-COR Bioscience), as described earlier [Avan et al, 2013B].

Immunocytochemistry and IHC in cells and xenografts

For immunocytochemical studies (ICC) of Gal-4 expression, the cells were seeded in 8-well chamber slides (Lab-Tek-II Chamber Slide System, Nalge-Nunc, Naperville, IL). After 24 hours, the cells were fixed with 70% ethanol for 10 minutes. ICC was performed using goat anti-human Gal-4 (1:100), as described above for IHC. The cells were then stained with avidin-biotin-peroxidase complex (Ultramarque™HRP-Detection, Greenwood, AR), as described previously [Avan et al, 2013B]. Negative controls were obtained replacing the primary antibody with PBS. The sections were reviewed and scored blindly by comparing the staining of PDAC-1 cells versus PDAC-2 and HPNE cells.

Additional IHC analyses of Gal-4 expression were performed in FFPE sections of PDAC
specimens obtained from orthotopic mouse models of PDAC-1 and PDAC-2 cells, as described previously [Avan et al, 2013B].

**Overexpression and knock down of Gal-4 in PDAC cells**

In order to investigate the effects of the modulation of Gal-4 expression on cellular processes, a specific human Galectin-4 construct was generated and transduced into PDAC-2 and PaTu-T cells, as described previously [Belo et al, 2013]. Briefly the human Galectin-4 (hGal-4) gene was cloned by inserting entire hGal-4 open reading frame in the vector pRRL-cPPT-CMV-X2-PRE-SIN-IRES-eGFP under a constitutive active CMV promoter. Lenti-viral productions containing the Gal-4 insert and the empty vector as well as subsequent infection of the primary PDAC-2 cells with the viral construct resulted in the cell line PDAC-2/Gal-4 and the control cell line (PDAC-2/mock) respectively. Moreover, in order to reduce the expression of Gal-4 we used RNA-mediated interference, as described previously [Belo et al, 2013]. Transfections were performed according to Invitrogen guidelines for reverse transfection in a 24-wells plate using 1 µl Lipofectamine RNAiMax and 100 µl Opti-MEM medium. A negative control (scramble-A together with negative control siRNA#1) was included in the experiments. Gal-4 mRNA and protein levels were measured using qRT-PCR and Western blotting at several time points during these experiments.

**Migration and invasion assays**

The effects of Gal-4 on migration and invasion were evaluated as described previously [Maftouh et al, 2014]. Migration was investigated using the LeicaDMI300B (Leica) migration station integrated with the Scratch-Assay 6.1 software (Digital-Cell Imaging Labs, Keerbergen, Belgium). Cells were plated at a density of 6x10^4 cells/well onto 96-well plates, and after 24 hours, artificial wound tracks were created by scraping with a specific scratcher within the confluent monolayers. After removal of the detached cells by gently washing with PBS, the cells were treated with fresh medium. The ability of the cells to migrate into the wound area was determined by comparing the pixels in the images taken at the beginning of the exposure (time 0), with those taken after 4, 6, 8, 24 and 48 hours.

For invasion assay, transwell chambers with polycarbonate membranes and 8 µm pores were used. The assays were performed through coated transwell filters, with 100 µL of 0.1 mg/mL collagen I solution. Hundred thousand cells were plated on the upper side of the filter. After 24 hours, cells migrated into the lower side were fixed with paraformaldehyde and stained with Giemsa in 20% methanol. The filters were photographed and cells were counted.

**Inhibition of Wnt signaling studies**

The effect of the Wnt inhibitor ICG-001 on cell growth was evaluated in PDAC-1, PDAC-1 transfected with anti-Gal-4 siRNA, PDAC-2 and PDAC-2 transduced with the Gal-4 gene (PDAC-2/Gal-4), using sulforhodamine-B (SRB) assay. The cells were plated in triplicate at a density of 5x10^3 cells in a 96-well plate. After 24 hours, the cells were treated for 72 hours with ICG-001 (0.05-100 µM). Then plates were processed for the SRB assay, as described previously [Giovannetti et al, 2011]. Optical density was measured at 540 nm using the Tecan SpectraFluor (Tecan, San Diego, CA). The 50% inhibitory concentration of cell growth (IC50) values of the drug was expressed as the concentration needed for a 50% reduction of cell growth after treatment relative to untreated controls, and calculated by
non-linear least squares curve fitting (GraphPad PRISM, Intuitive Software for Science, San Diego, CA, USA).

**Immunofluorescence of β-catenin**

Immunofluorescence analysis of β-catenin was performed in cells seeded in 8-well chamber slides (Lab-Tek-II), fixed in formaldehyde/PBS and incubated in a methanol/acetone solution for 15 minutes. Cells were incubated with anti-β-catenin (1:500 dilution), washed in PBS and then probed with AlexaFluor 594 antibodies (Life Technologies). Nuclei were counterstained with 0.1 mg/ml 4',6-diamidino-2-phenylindole (DAPI, Life Technologies), as described previously [Giovannetti et al, 2014].

**Immunoprecipitation**

In order to reveal the inhibitory effect of ICG-001 on β-catenin/CBP complex, immunoprecipitation (IP) was performed in PDAC-2 nuclear extract, as described previously [Emami et al, 2004]. The immunoprecipitated antibody-antigen complex was also subjected to a reversal of the cross-link, followed by Western blot analysis as described above using either the polyclonal anti-β-catenin (dilution 1:1000), or polyclonal anti-CBP (1:1000) as primary antibodies (Santa Cruz Biotechnology).

**Statistical analysis**

All experiments were performed in triplicate and repeated at least twice. Data were expressed as mean values ± SEM and analysed by Student’s t-test or ANOVA followed by the Tukey’s multiple comparison test. The level of significance was P<0.05.

**Ethics**

All the patients gave their informed consent to the sample collection and analysis, and the study has received approval from the Ethics committee of Pisa University Hospital as a follow-up study of the research protocol entitled “Pharmacogenetics of gemcitabine-related genes in pancreas cancer: correlation with clinical outcome and tolerability” [Giovannetti et al., 2012]. Animal experiments were carried out according to a protocol approved by the by the local animal welfare committee (Animal Experimental licensing Committee, DEC) of the VU University medical center, Amsterdam, The Netherlands [Avan et al, 2013A].

**Results**

**Gal-4 expression in PDAC patients is associated with lack of tumor invasion in the lymph nodes**

To explore the role of Gal-4 in PDAC invasive behavior we evaluated its expression in 20 PDAC patients selected according to their differential lymph node metastatic status. A wide range of Gal-4 expression levels was observed in our collection of human PDACs. As exemplified by the IHC pictures in the Fig. 1A, some PDACs showed a negative or very weak staining, with a few positive cells, while other tumors had a higher number of positive cells, characterized by much stronger staining intensity. Patients were categorized into two subgroups with respect to the median protein expression (low vs. high Gal-4 expression).

There was no difference in Gal-4 expression levels according to grade (P=0.436, Chi-Square two-sided test), as well as for age, sex, neural infiltration, resection margins and
occurrence of Pancreatic Intraepithelial Neoplasia (PanIN) lesions. However, a trend toward a significantly higher percentage of tumors with vascular infiltration (P=0.064) was observed in patients’ tumors with lower expression levels of Gal-4 (Table 1). Furthermore, 80% of the patients without lymph node metastasis (N0) had high Gal-4 expression, while only two patients of this group had low Gal-4 expression. Conversely, considering the group of patients with lymph node metastasis (N1), most patients (70%) had high level of Gal-4 expression (P=0.025; Fig. 1B). Since recent studies showed that not the lymph node involvement per se but especially the lymph nodes ratio is an independent prognostic factor after resection of pancreatic cancers. In this group we performed also the analysis of the ratio between metastatic and examined lymph nodes (LNR ratio), which has been recently identified as one of the strongest prognostic factor after resection of pancreatic cancer [Riediger et al, 2009]. Patients with low Gal-4 expression had a significantly lower LNR ratio than patients with high Gal-4 expression (Fig. 1C).

**Expression of Gal-4 in PDAC cells and xenografts**

Gal-4 was expressed in all the primary PDAC cell cultures, as well as in their originator tissues. However, this expression differed among cells, ranging from 0.006 a.u. in PDAC-2 cells, to 0.190 a.u. in PDAC-1 cells (Fig. 2A). The mean (0.059±0.10 a.u.) and median (0.058 a.u.) expression levels in the tumor cells were significantly higher than the expression measured in the immortalized normal ductal cells hTERT-HPNE (0.002 a.u., P<0.01). Remarkably, Gal-4 gene expression in the 8 primary tumor cells and their originator tumors showed a similar pattern and resulted highly correlated with Spearman analysis (R2>0.96, P<0.01), suggesting that these cells represent optimal preclinical models for studies on PDAC.

<table>
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PanIN, Pancreatic Intraepithelial Neoplasia
WHO, World Health Organization
*P calculated with the two-sided Pearson Chi-Square text
Figure 1. Patients with PDACs that highly express Gal-4 have a significantly decreased number of malignant cells in the lymph nodes, compared to patients with low Gal-4-PDACs. (A) Representative pictures of immunohistochemical analysis (IHC) for Gal-4 expression in PDAC patients, showing differential Gal-4 expression (negative, weak, intermediate, strong). (B) Patients were classified in two groups, i.e. with (N1) or without (N0) lymph metastasis, and the IHC showed that eight patients without lymph node metastasis had high Gal-4 expression, while two patients had low Gal-4 expression. (C) Analysis of the LNR ratio showed that in the group of patients with lymph node metastasis three patients had high Gal-4 expression while seven patients had low Gal-expression.

PDAC-1 and PDAC-2 cells were selected for further studies, since they had the highest and lowest Gal-4 expression, respectively. In these cells we explored copy number variations in the Gal-4 gene, which is included in the cytoband 19q13.2, and we observed a copy number gain (4N) in PDAC-1 cells. Conversely, no changes were identified in PDAC-2 (Fig. 2B). This data might at least in part explain the overexpression of Gal-4 in PDAC-1 compared to PDAC-2 cells.

To investigate whether mRNA expression differences of Gal-4 observed in PDAC cells were reflected in differences in protein levels, we analyzed the protein expression of Gal-4 by Western blotting, ICC and IHC. These studies were performed in the PDAC cells and in their originator tumors, and demonstrated that PDAC-1 had markedly higher expression of Gal-4 with respect to PDAC-2 cells and tumors (Fig. 2C).
Figure 2. Gal-4 was expressed in all the primary PDAC cell cultures, as well as in their originator tissues, and PDAC-1 and PDAC-2 cells were selected since they had the highest and lowest Gal-4 expression, respectively. (A) Gal-4 mRNA expression in primary tumor cultures (white bars), and their originator tissues (black bars). Bars represent the arithmetic means ± SEM of two independent experiments performed in triplicate. (B) Copy number variations in the Gal-4 gene, included in the cytoband 19q13.2, as detected by array-CGH analysis, showing a copy number gain (4N) in PDAC-1 cells. Left shifts and red color indicate the deleted segments, while right shifts and blue color indicate the gains/amplifications. The complete array-CGH database is available at Gene Expression Omnibus (GEO) with accession number GSE44587. (C) Representative blots of Gal-4 protein expression in the PDAC-1 and PDAC-2 cells. As a loading control β-actin levels are indicated. (D) Representative pictures of Gal-4 protein expression in the PDAC-1 and PDAC-2 originator tissues and primary cells. Original magnification, 40X.

**Gal-4 expression correlates with invasive/migratory capabilities of PDAC cells**

Our previous studies showed that low and high expression of Gal-4 in the PDAC cell lines PaTu-T and PaTu-S were correlated respectively with high and low migration and metastatic ability [Belo et al. 2013], here we further evaluated the invasive properties of these PDAC cells as well as the invasion and migration of our primary PDAC cells, PDAC-1 and PDAC-2. Our data showed that PDAC-1 and PaTu-T cells, characterized by high Gal-4 expression, were less invasive compared to PDAC-2 and PaTu-S cells, harboring low Gal-4 expression (Fig. 3A). Similarly, the ability of PDAC-1 cells to migrate to the wound
area after 24 hours was significantly (P<0.05) lower compared to PDAC-2 cells (Fig. 3B).

Furthermore, we evaluated whether the differential invasive potential and expression levels of Gal-4 were retained in bioluminescent orthotopic mouse models which we recently developed using PDAC-1 and PDAC-2 cells [Avan et al, 2013A]. Most of our orthotopic tumors metastasized to other organs such as lymph nodes, and liver, as detected via bioluminescence (Fig. 3C), and then confirmed via light microscopy of three non-sequential serial sections stained with hematoxilin and eosin. In particular, macroscopic metastases were observed in all the livers of the PDAC-2 mice, while no liver metastases were detected in 33% of the mice of the PDAC-1 group. Moreover the LNR ratio in the PDAC-2 models was 2.5-fold higher than in the PDAC-1 models (Fig. 3D). These mouse models (3 mice for each group) showed key histopathological features of human PDAC in terms of tumor infiltration, PDAC-associated desmoplastic reaction, ductal characteristics and adenocarcinoma differentiation. All these tumors react positively to human specific antibodies directed against CK8/18, CK7, CK19, Ca19.9, EGFR, and CEA (data not shown). However, the PDAC-1 tumors showed a strong staining for Gal-4, while the PDAC-2 tumors had only a weak staining (Fig. 3E), and thus recapitulated the immunophenotypes of the PDAC cells and originator tumors.

**Figure 3. Gal-4 expression correlates with invasive/migratory capabilities of PDAC cells.** (A) Results of invasion studies in primary PDAC-1 and PDAC-2 cells, characterized by high and low Gal-4 expression, respectively. (B) Results of wound-healing assay in PDAC-1 and PDAC-2 cells (C) Representative Firefly-luciferase bioluminescence images of orthotopic mouse models, derived from PDAC-1 and PDAC-2, characterized by low and high metastatic properties, respectively. (D) LNR ratio in PDAC-1 and PDAC-2 orthotopic mouse models. Columns, mean values obtained from three independent experiments; bars, SEM. *Significantly different from PDAC-2. (E) Representative IHC pictures of Gal-4 protein expression in PDAC-1 and PDAC-2 orthotopic mouse models. Original magnification, 40X.
**Modulation of Gal-4 alters the migratory/invasive behavior of PDAC cells**

In order to gain further insights on the role of Gal-4 on the migratory and invasive behaviors of PDAC, we used gain- and loss-of-function models using recombinant human Gal-4 gene transduction and siRNA.

As shown in the Fig. 4A, we successfully established Gal-4-overexpressing subclones and empty vectors, with more than 70% efficiency, in PDAC-2 cells. These subclones were tested for their differential migratory and invasion abilities using the wound healing and Boyden-chamber assays, as described above. The migration of PDAC-2-Gal-4 cells was significantly compared to the PDAC-2-mock cells (i.e., -15% after 20 hours, Fig. 4B). These Gal-4 overexpressing cells also had a 20% reduction (P<0.05) in the invasive behavior compared to the control cells (Fig. 4C). Similar results were obtained in PaTu-T cells transduced with Gal-4 (Fig. 4C).

To generate loss-of-function phenotypes we transduced PDAC-1 and PaTu-S cells with a specific siRNA against Gal-4. As shown in Figure 4D, Gal-4 expression was significantly (P<0.05) decreased in both cells, compared to the control, as determined by qRT-PCR. The Gal-4-siRNA transfection increased the invasive behavior of both PDAC-1 (+43%) and PaTu-S (+24%) cells (P<0.05, Fig. 4E). Moreover, the knockdown of Gal-4 resulted in increased migratory ability (about 20% after 8 and 20 hours, in PDAC-1 and PaTu-S cells compared to their control cells, P<0.05, Fig. 4F).

**Gal-4 modulates β-catenin expression and sensitivity of PDAC cells through modulation of the Wnt pathway**

Since previous studies suggested that Gal-4 modulated the β-catenin expression [Satelli et al, 2011], we evaluated the expression of β-catenin in PDAC-1, PDAC-2 and PDAC-2-Gal-4 by Western blotting. As illustrated in Fig. 5A, PDAC-2 cells presented a band with high density, while a fade band was observed in PDAC-1 cells. However, the transduction of the PDAC-2 cells with human Gal-4 significantly (P<0.05) reduced the expression of β-catenin (Fig. 5A). PDAC-2 cells were characterized also by higher accumulation of β-catenin into the nucleus, whereas Gal-4 transduction reduced this accumulation, as demonstrated by immunofluorescence analysis (Fig. 5B).

Importantly, Gal-4 over-expression in PDAC-2 cells transduced with Gal-4 vector leaded to down-regulation of key target genes in the Wnt pathways, such as survivin and cyclin D1 (Fig. 5C). Keeping with these findings, we observed that cells overexpressing Gal-4 were arrested at G1/S (data not shown).

Finally, we evaluated the role of Gal-4 expression on cell proliferation as well as on sensitivity to the specific Wnt inhibitor ICG-001. To this end, PDAC-1, PDAC-1-siRNA-Gal-4, PDAC-2 and PDAC-2-transduced with Gal-4 were used in growth inhibition studies. As shown in Fig. 5D-E, ICG-001 inhibited PDAC-1 cell growth with IC50 values of 2.3 μM and 11.3 μM in PDAC-1 and PDAC-1-siRNA cells, respectively. This compound was able to inhibit the cell proliferation with IC50 values of 22.8 μM and 0.3 μM in PDAC-2 and PDAC-2-Gal-4 cells, respectively. Therefore, knockdown of Gal-4 in PDAC-1 cells increased IC50 values more than 5-fold, while enforced expression of Gal-4 in PDAC-2 cells decreased the IC50 of about 76-fold. Our results showed also for the first time in PDAC-2 cells that ICG-001 disrupted β-catenin/CBP complex, as assessed by immunoprecipitation (Fig 5F).
Figure 4. Modulation of Gal-4 expression alters the migratory/invasive behavior of PDAC cells. (A) Representative pictures of fluorescence microscopy in PDAC-2 cells transduced with Gal-4-GFP vector. (B) Results of migration assay in PDAC-2 cells transduced with Gal-4 vector. The insert shows a representative picture at 24 hours. *Significantly different from PDAC-2 mock. (C) Results of invasion assay in PDAC-2 and PaTu-T cells transduced with Gal-4 vector. (D) Gene expression of Gal-4 in PDAC-1 and PaTu-S transfected with a specific siRNA anti-Gal4, as determined by qRT-PCR. *Significantly different from PDAC-2 and PaTu-S mock, respectively. (E) Results of invasion and migration studies in PDAC-2 and PaTu-T cells transfected with siRNA anti-Gal4. The insert shows a representative picture at 24 hours. Significantly different from PDAC-2 mock. Points and columns, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls.
Figure 5. Gal-4 modulates β-catenin and sensitivity of PDAC cells to the Wnt inhibitor ICG-001.
(A) Representative pictures of β-catenin protein expression as detected by Western blot in PDAC-1, PDAC-2 and PDAC-2 cells transduced with Gal-4 vector. As a loading control β-actin levels are indicated. (B) Immunofluorescence analysis of the accumulation of β-catenin (red) into the nucleus (blue), in PDAC-1, PDAC-2 and PDAC-2 cells transduced with Gal-4 vector. (C) Modulation of survivin and cyclin-D1 gene expression in PDAC-2 cells transduced with Gal-4 vector, as detected by quantitative RT-PCR (D) Inhibition of cell proliferation in PDAC-1 and PDAC-1 transfected with Gal4-siRNA, after 72 hours exposure to the Wnt inhibitor ICG-001. (E) Inhibition of cell proliferation in PDAC-2 and PDAC-2 transduced with Gal-4 vector cells, after 72 hours exposure to the Wnt inhibitor ICG-001. (F) Modulation of β-catenin in PDAC-2 cells treated with ICG-001, as assessed by immunoprecipitation.

Discussion

This is the first study demonstrating the role of galectin 4 (Gal-4) in the inhibition of the invasive/metastatic behavior of primary PDAC cell cultures, in vitro and in vivo, as well as in human samples. Remarkably, enforced expression of Gal-4 reduced cell migration and invasion. Furthermore, Gal-4 sensitized PDAC cells to the Wnt inhibitor ICG-001. As Gal-4 markedly reduced the expression of β-catenin, our data suggest that Gal-4 interferes with the interaction between CBP and β-catenin.

Recent gene array analyses of pancreatic cancer specimens, searching for genes that are linked to tumor progression, have raised interest in Gal-4. Gal-4 is a member of a large family of soluble carbohydrate-binding proteins that are well known to control tumor progression by promoting transformation, angiogenesis and immune escape (Belo et al., 2013; Danguy et al., 2002; Delacour et al., 2005). In healthy individuals Gal-4 is predominantly expressed in the luminal epithelia of the gastrointestinal tract, where it acts as an innate immune mediator with bactericidal activity (Stowell et al., 2010). Remarkably, whereas Gal-4 is not found in healthy pancreas, it shows a significantly higher expression in...
cystic tumors of the human pancreas and in PDAC, compared to normal pancreas and benign neoplasms (Bauer et al., 2009). Similarly, Gal-4 expression is aberrantly induced in several tumors outside the pancreas, including breast and colorectal cancers (Barrow et al., 2011; Satelli et al., 2011). The latter data suggest that tumors may benefit from high expression of Gal-4. However, Gal-4 expression is strongly reduced in colon adenomas, and essentially undetectable in invasive colon carcinomas (Satelli et al., 2011), indicating a function of Gal-4 as tumor repressor. Similarly, a genomic study showed a down-regulation of the expression of the Gal-4 gene (LGALS4) in never-smoker lung tumors, suggesting the role of DNA methylation as a potential regulator of the expression of this gene (Selamat et al., 2012).

We recently showed that Gal-4 expression inhibits the invasive behavior in two established PDAC cell lines, PaTu-S and PaTu-T, using in vitro migration assays and a zebrafish metastasis model (Belo et al., 2013). Collectively, these data raise questions about the putative biological role of Gal-4 in PDAC, compared to its role in colon cancer and other tumor types.

Here we demonstrated for the first time that the LNR ratio (ratio between metastatic and examined lymph nodes) was significantly lower in PDAC patients with high expression of Gal-4, compared to patients with low Gal-4 expression, suggesting its role as a prognostic factors in resected PDAC patients. Interestingly, Gal-4 expression was not correlated with grading or other clinicopathological features, suggesting its potential role as a novel marker for disease characterization. According to the final results of the CONKO-001 and ESPAC-3 trials, adjuvant chemotherapy increased disease-free survival and overall survival duration (Oettle et al., 2013; Valle et al., 2014). Still, the identification of new prognostic factors for survival appears to be critical for the selection of patient subsets for better clinical management. The most biologically aggressive PDACs, such as those that recur soon after resection, should be treated initially with systemic therapy, as opposed to major surgery, which exposes the patient to substantial operative risk with little expected benefit. On the other hand, patients with indolent cancers may benefit from an aggressive surgical approach (Belli et al., 2013). Moreover, prognostic biomarkers provide mechanistic insights into cancer progression, and might unravel molecular targets for novel treatment strategies (Kelloff and Sigman, 2013).

High impact bench-to-bedside research on hundreds of patient samples improved prognostic capabilities in several tumor types, such as breast cancer (Volinia and Croce, 2013). Similar studies are difficult in PDAC, an organ with high endogenous nuclease activity, and a very small amount of tumor tissue available. A compendium of ~2500 published candidate biomarkers in PDAC was compiled (Harsha et al., 2009), but several studies used tissues that were not microdissected to separate cancer from stroma, and 74% of the biomarkers in this compendium are based solely on mRNA evidence. Molecules that have both mRNA and protein evidence, such as Gal-4, are high-priority candidates for further testing. In particular, prospective studies in independent cohorts of patients will be crucial to validate our clinical findings on Gal-4.

In order to increase our understanding on the molecular mechanisms underlying PDAC biological aggressiveness as well as for rational planning of future therapeutic strategies, we performed comprehensive preclinical studies on Gal-4 activity in PDAC. Exploration of the expression and function of Gal-4 in eight primary PDAC cultures and their originator PDAC tissues indicated that the level of Gal-4 expression showed a similar pattern in the primary PDAC cultures and their originator tumors, establishing the value of the primary
cell lines as a model to study PDAC. The tissue and resulting cell culture PDAC-1 was found to have the highest Gal-4 expression, which may be caused by amplification at 19q13.2. Conversely, the PDAC-2 tissue and cells showed the lowest expression levels of Gal-4. Importantly, we here show that Gal-4 expression inhibits the capacity for migration and invasion in primary PDAC cells. Furthermore, in the present study we demonstrated that tumors in a PDAC-1 mouse model showed a strong staining for Gal-4 and no liver metastases were detected in 33% of the mice, while the PDAC-2 tumors had only a weak staining and metastases were found in all livers. These results are in agreement with our previous data showing that cytosolic Gal-4 inhibits migration and metastasis in the PDAC cell lines PaTu-S and PaTu-T, using in vitro scratch assays, and an in vivo Danio rerio metastasis model, respectively (Belo et al., 2013).

Activation of Wnt/β-catenin pathway plays an important role in progression of PDAC (Zhang et al., 2013). Preclinical models throughout the last decade have established this pathway as an attractive drug target. However, therapies meant to attenuate the Wnt pathway have remained largely theoretical and preclinical (Anastas and Moon, 2013), and key factors to identify tumors driven by this pathway are warranted. Therefore, in the present study we hypothesized that Gal-4 might inhibit metastasis by down-regulation of Wnt signaling target genes, as shown for colorectal cancer (Satelli et al., 2011). In keeping with this hypothesis, we show evidence that Gal-4 interferes with Wnt/β-catenin signaling in primary PDAC cultures. The expression of β-catenin and its accumulation in the nucleus was significantly lowered in primary PDAC-1 cells that express higher protein levels of Gal-4. This is of significant relevance because the availability of β-catenin to translocate to the nucleus and activate downstream Wnt signaling target genes is required to initiate and critical for progression of PDAC (Zhang et al., 2013).

In addition, Gal-4 expression increased the sensitivity to the specific Wnt inhibitor small molecule ICG-001. This small molecule is known to bind specifically to CBP disrupting the interaction of CBP with β-catenin. Treatment with ICG-001 induces apoptosis in colon carcinoma cells but not in normal colonic epithelial cells (Emami et al., 2004). We showed an inverse correlation between Gal-4 expression and sensitivity of PDAC cells to the inhibitor ICG-001. In particular, IC50 values were 2 fold decreased when Gal-4 was forcedly expressed in PDAC-2, while suppressing Gal-4 expression in PDAC-1 cells increased the IC50 value more than 5 fold. This inverse correlation clearly shows that Gal-4 expression sensitizes tumor cells to this drug. Thus, inhibition of Wnt/β-catenin signaling by novel anticancer agents might have a therapeutic impact on suppression of PDACs driven by this pathway in Gal-4 high expressing pancreatic tumors.

In summary, our data establish a role of Gal-4 as tumor suppressor in PDAC, since we showed that elevated Gal-4 levels correlated significantly with reduced lymph node metastasis in PDAC patients, as well as with reduced in vitro migratory and invasive behavior in primary PDAC cultures, and reduced liver metastasis in mice. In addition our data support a role of Gal-4 in inhibition of the Wnt signaling pathway. Inhibition of Wnt/β-catenin signaling by novel anticancer agents might have therapeutic impact on suppression of PDACs driven by this pathway, and future translational and clinical studies on the role of Gal-4 in this process are warranted.
References


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