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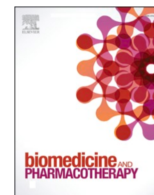

Interplay in galectin expression predicts patient outcomes in a spatially restricted manner in PDAC

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ABSTRACT

Background: Galectins (Gal's) are a family of carbohydrate-binding proteins that are known to support the tumour microenvironment through their immunosuppressive activity and ability to promote metastasis. As such they are attractive therapeutic targets, but little is known about the cellular expression pattern of galectins within the tumour and its neighbouring stromal microenvironment. Here we investigated the cellular expression pattern of Gals within pancreatic ductal adenocarcinoma (PDAC).

Methods: Galectin gene and protein expression were analysed by scRNAseq (n=4) and immunofluorescence imaging (n=19) in fibroblasts and epithelial cells of pancreatic biopsies from PDAC patients. Galectin surface expression was also assessed on tumour adjacent normal fibroblasts and cancer associated primary fibroblasts from PDAC biopsies using flow cytometry.

Results: scRNAseq revealed higher Gal-1 expression in fibroblasts and higher Gal-3 and −4 expression in epithelial cells. Both podoplanin ($PDPN^+$, stromal/fibroblast) cells and $EpCAM^+$ epithelial cells expressed Gal-1 protein, with highest expression seen in the stromal compartment. By contrast, significantly more Gal-3 and −4 protein was expressed in ductal cells expressing either EpCAM or PDPN, when compared to the stroma. Ductal Gal-4 cellular expression negatively correlated with ductal Gal-1, but not Gal-3 expression. Higher ductal cellular expression of Gal-1 correlated with smaller tumour size and better patient survival.

Conclusions: In summary, the intricate interplay and cell-specific expression patterns of galectins within the PDAC tissue, particularly the inverse correlation between Gal-1 and Gal-4 in ducts and its significant association with patient survival, highlights the complex molecular landscape underlying PDAC and provides valuable insights for future therapeutic interventions.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a tendency to metastasise early in pathology [1], resulting in high mortality and low 5-year survival [2]. The PDAC tumour microenvironment itself is highly complex, exhibiting elements of tumour protective and tumour suppressive responses interacting to create a highly treatment refractory, aggressive, immune evasive cancer [3]. The oncogenic mechanisms underpinning PDAC results in extensive remodelling of the tumour microenvironment leading to fibrosis [4], as well as immune evasion. Current therapeutic options for PDAC remain severely limited, and targeting of the stroma has been

Abbreviation: CAF, Cancer associated fibroblast; EpCAM, epithelial cell adhesion molecule; EMT, epithelial-mesenchymal transition; Gal, galectin; PDAC, pancreatic ductal adenocarcinoma; PDPN, podoplanin; ANF, tumour adjacent normal fibroblast.

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ineffective/inadequate in mouse models [5,6] or in human clinical trials (reviewed in [7]). Numerous studies have attributed roles for individual galectin (Gal) family members in PDAC pathology in isolation, however, redundancy within the family is a concern given that many members are expressed in the intracellular compartments of multiple cell types. For example, we have previously shown in global Gal-1 deficient mice, induction of paw oedema resulted in compensatory over expression of Gal-9 promoting T cell apoptosis in the absence of Gal-1 [8]. Thus, there is an urgent need to analyse multiple galectin family members within individual patients to ascertain their combined role in PDAC.

Galectins are a family of β-galactoside binding proteins that play diverse roles in acute inflammatory responses [9], immune-mediated inflammatory diseases [10,11], and within cancer. In the context of PDAC, Gal-1 and −3 have emerged as pivotal pro-tumorigenic players, with substantive evidence demonstrating their involvement in immune evasion, remodelling of the tumour microenvironment resulting in tissue fibrosis, and their ability to promote the migratory and invasive properties of tumour cells contributing to metastasis [12–15]. Comparative analysis has shown increased expression of Gal-9 and programmed death ligand 1 (PD-L1) levels in PDAC tissue in contrast to normal pancreatic tissue [16]. Notably, the authors also demonstrated a significant reduction in tumour growth in a murine model of PDAC when combining therapeutic targeting of Gal-9 and PD-L1 with antibodies, as opposed to treatment with either therapy alone [16]. Much less is known about the impact other galectin family members have in onset or progression of PDAC – despite increased expression of Gal-4 [17,18], −7 [17,18], −8 [19], and −9 [17] being reported. In contrast to the above, Gal-4 displays anti-tumorigenic properties, limiting tumour cell proliferation, migration and metastasis [20]. A more coordinated approach is required to ascertain the significance of each family member in concert in PDAC disease progression.

Many of the studies looking at galectins in PDAC focus on a single family member rather than looking at the interplay across the family. Furthermore, many studies fail to appropriately define whether they are analysing cell surface or soluble galectins, and some even interchange between both throughout the study applying similar roles for both forms of the galectin. However, the binding interactions and functional response elicited by intracellular/extracellular galectins is very different, for instance intracellular Gal-3 inhibits T cell apoptosis [21], whilst extracellular Gal-3 promotes it [22]. Here we investigated the cellular and anatomical expression pattern of multiple galectins in serial sections within the same individual with PDAC, and *ex vivo* in tumour adjacent normal fibroblasts (ANF) and cancer associated fibroblasts (CAF). We observed that multiple galectins display distinct anatomical expression patterns. Moreover, cellular expression of Gal-1 and −4 in the ducts were negatively correlated, where high Gal-1 and low Gal-4 cellular expression within a patient was associated with good survival outcome.

2. Methods

2.1. Patient samples

Pancreatic tissue samples or fibroblasts were obtained from patients undergoing pylorus-preserving pancreatico-duodenectomy (PPPD) for treatment of pancreatic ductal adenocarcinoma (Supplementary Table 1). Samples were obtained with written, informed consent and approval from Human Biomaterial Resource Centre (Birmingham, UK - REC 16/WM/0214 ethically approved Northwest - Haydock Research Ethics Committee 20/NW/0001) or by University of Birmingham Local Ethics Board (18−304) in compliance with the Declaration of Helsinki.

2.2. Isolation of cells

Pancreatic tissues were stored overnight at 4°C in MACS Tissue storage solution (Miltenyi) supplemented with primocin (50 mg/ml; InvivoGen), 1% penicillin-streptomycin (Sigma-Aldrich). Subsequently, tissue was cut into 2–3 mm pieces and digested by gentle agitation using a cocktail of 1x collagenase/hydaluronidase (StemCell), 1x liberaseTL and benzonase (50 U/ml; both from Merck) diluted in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Gibco) and 1% penicillin-streptomycin at 37°C for 30 min. Samples were filtered and washed by centrifugation at 250 g for 5 min prior to counting using a haematocytometer. Cells were used for scRNAseq or fibroblast culture either immediately or stored in −80°C until use.

2.3. Single cell RNA sequencing and data processing

Cells were stained with the following antibodies for 20 min at 4 °C prior to washing in MACS buffer and incubation with propidium iodide: anti-CD45 BV785 (clone: 2D1), anti-EpCAM APC (clone: 9C4), and anti-podoplanin AF488 (clone:NC-08, all from BioLegend). $CD45^+$ and $CD45^-$ populations were sorted using the BD FACSMelody and adjusted to 1×10^3 cells/μL. Samples with a viability of >85% were processed at the Genomics Birmingham Sequencing Facility (University of Birmingham, United Kingdom) for gene expression profiling using the 10X Genomics platform. Samples (1.7×10^4 cells) were processed using the Chromium Controller for a recovery of 1×10^4 cells per sample, and library preparation was performed using the Chromium Single Cell 3′ Library & Gel Bead Kit v2 (CG00052, 10X Genomics) according to the manufacturer's instructions. Library quantification was performed using TapeStation (Agilent). Samples were sequenced on an Illumina NextSeq 500 (150 bps, paired-end) at a sequencing depth of >50,000 raw reads/cell.

Raw reads were processed to identify high-level cell type populations as outlined in [23] using CellRanger (v3, 10X Genomics) for alignments to human genome GRCh38 and feature count matrix generation followed by downstream analysis with the R (v3.6) package Seurat [24,25]. *SCTransform IntegrateData* workflow for normalisation, scaling and integration procedure was applied to the unique molecular identifier (UMI) raw count data by constructing a generalised linear model (GLM) for each gene relating sequencing depth to molecule counts using regularized negative binomial distributions to model UMI counts [26]. Pearsons residuals of this model represent a variance-stabilised transformation and are used for downstream dimensionality reduction by principal component analysis (PCA) and Uniform Manifold Approximation and Projection (UMAP) embedding determined using PC's 1:20. A shared nearest neighbour graph based on euclidean distance in PCA space was constructed using the function *FindNeighbours* on PCs 1:20 and modules within this graph representing clusters were identified using the function *FindClusters*. Clusters were annotated with their high-level cell type using canonical marker gene expression profiles for T lymphocytes (*CD3D*), natural killer (NK) cells (*GNLY, XCL1*), T/NK mixed (*CD3D, GNLY, XCL1, FGFBP2*), B lymphocytes (*MS4A1, IGKC*), myeloid (*LYZ*), endothelial cells (*PECAM1*), mast cells (*TPSAB1*), epithelial cells (*EpCAM*), cycling cells (*MKI67*) and fibroblasts (*DCN, PDPN*) (Supplementary Fig 1).

2.4. Immunofluorescence imaging

FFPE pancreas tissue Section 5 mm in depth were subjected to deparaffinisation using histoclear (Sigma) followed by incubation in decreasing concentrations of ethanol prior to antigen retrieval using a 10 mM citrate buffer at pH6 for 15 min. Slides were blocked using 10% normal horse serum in 2% BSA/PBS for 10 min, prior to incubation at 4°C overnight with goat anti-Gal-1 (1:75, AF1152, R&D systems), goat anti-Gal-3 (1:75, AF1154, R&D systems) or goat anti-Gal-4 (1:20, AF1227, R&D systems) antibodies diluted in PBA with 1% BSA. Following 3 ×5-min washes in PBS, slides were incubated for 45 min in the dark at room temperature with donkey-anti-goat IgG AF647 (1:400, A-21447, Invitrogen). Slides were subsequently washed as described, incubated with sheep IgG isotype (31243, ThermoFisher Scientific,

Loughborough UK), Rabbit anti-EpCAM (1:250, ab71916, ABCAM), Rat anti-PDPN (1:100, 14–9381–82, Invitrogen), and Mouse anti-CD45 (1:400, 14–0459–82, Invitrogen) antibodies for 1 h as above. Following 3 ×5-min washes in PBS, slides were incubated for 45 min in the dark at room temperature with goat anti-rabbit IgG AF488 (1:200, A11034, Invitrogen), goat anti-mouse IgG AF546 (1:400, A-11003, Invitrogen) goat anti-rat IgG AF594 (1:400, #A-11081, Invitrogen) antibodies. Slides were washed and mounted using ProLong™ Diamond Antifade Mountant with DAPI and stored at −20°C.

Slides were imaged using Zeiss LSM 780 at 25x magnification using the same acquisition parameters for each slide. Images were initially processed with ZEN Software (Black Edition v2.2) into digital arrays, which were subsequently analysed with the machine learning software QuPath (v0.3.2). Cell detection was defined based on a nuclear area of 8 µm using DAPI and a diameter of 13 µm (Supplementary Fig 2). Background fluorescence was set using isotype control-stained sections for each protein of interest. Regions of interest (ROI) denoting ductal or stromal areas where manually annotated prior to training Qupath Object classification AI (Supplementary Fig 2). Average number of cells positive for each marker or a combination of markers across the whole tissue or within ROI were determined and expressed as either percentage of ROI or percentage of parent population.

2.5. Flow cytometric analysis of fibroblasts

Fibroblasts were isolated as described above and cultured in DMEM (D6429–500 ml, Sigma-Aldrich) supplemented with 10% FBS (F7524–500 ml, Sigma-Aldrich), 1% Glutmax (35050061), and 1% penicillin-streptomycin (15070063, both from ThermoFisher Scientific). Fibroblasts were used between passage 4–6. Fibroblasts were incubated with 2x Trypsin/EDTA (Gibco) for 5 min, retrieved, washed in PBS, and incubated with LIVE/DEAD™ Fixable Near-IR (L34975, ThermoFisher Scientific) for 30 min. Following washing, cells were resuspended in MACs buffer, then blocked with FcR blocker (Miltenyi Biotec) for 2 min prior to staining with unconjugated rabbit anti-human Gal-4 (1:100, PA5–119685, Invitrogen) for 30 min. The samples were stained with one of the following of antibodies for 30 min at 4°C prior to washing and fixation with 4% PFA diluted in PBS: anti-Gal-1 PE (1:50, IC1245P, R&D systems), anti-Gal-3 AF488 (1:50, M3/38, Biolegend), goat anti-rabbit secondary AF594 (1:200, A11012, Invitrogen), anti-Gal-9 BV421 (1:50, 9M1–3), anti-podoplanin PerCpCy5.5 (1:50, NC-08), anti-EpCAM-1 PE-Cy7 (1:20, 9C4, all from Biolegend) and anti-CD90 APC (1:50, 5E10, BD Biosciences). In some cases, samples were permeabilised with 1x permeabilization buffer (00–8333–56, eBioscience) and stained with anti-Gal-4 as described above. Cells were washed and then subsequently stained with one of the following antibodies, anti-Gal-1 or anti-Gal-3 as described above. Due to autofluorescence within the fibroblast populations, cells were used as compensation controls. Samples were filtered and acquired using Fortessa-X20 and data analysed offline using FlowJo (V-10.2.6) (Supplementary Fig 3) Median Fluorescence intensity (MFI) was calculated by subtracting the isotype control for the target antibody MFI.

2.6. Statistical analysis

Data were analysed using GraphPad Prism and presented as mean ± SEM for n independent experiments. Normality was assessed using D'Agostino and Pearson test. Univariate analysis was performed using unpaired, paired t-test, Wilcoxon matched pairs test (also known as signed rank test), Pearson's or Spearman's correlation. $p<0.05$ was deemed statistically significant.

3. Results

3.1. Galectin gene expression across immune and stromal compartments in PDAC

Initially we investigated the expression pattern of the galectins across the immune and stromal cell subpopulations found within PDAC tissues using scRNA-seq. Expression of *LGALS1, 3, 4,* and *9* was observed (Fig. 1) with each gene displaying a distinct expression pattern. *LGALS1* and *3* showed the widest expression and were detectable in both immune and stromal cells (Fig. 1A-B). Expression of *LGALS1* was most marked in fibroblast and myeloid cells with limited expression within the epithelial compartment (Fig. 1A). By contrast, *LGALS3* was mostly highly expressed by epithelial cells, fibroblasts, and myeloid cells (Fig. 1B). Expression of *LGALS4* was restricted to the epithelial compartment (Fig. 1C), whilst *LGALS9* was expressed in myeloid and epithelial cells, albeit at much lower levels than seen with the other 3 galectins (Fig. 1D). Visualisation of the relative average *LGALS* expression across all subpopulations can be seen in Fig. 1E. We also detected very low levels of *LGALS2, 8, 9B, 9 C* and *12* in immune and stromal cells, whereas low *LGALS7* expression was restricted to the epithelial compartment [data not shown]. Of note, the fibroblast, but not the epithelial cells, expressed several genes associated with epithelial-to-mesenchymal transition (EMT), namely *ZEB1, ZEB2, TWIST1, SNAI1* (Supplementary Fig 4). This agrees with previous data [27] suggesting that the fibroblasts were main contributor to the EMT signature within the PDAC microenvironment.

3.2. Differential expression of galectins in the stromal compartments in PDAC

To further investigate the contribution of multiple galectins to PDAC pathogenesis in distinct anatomical regions, we subsequently examined the expression pattern of galectins 1, 3, and 4 in stromal and ductal sections from patients with PDAC. Overall tissue architecture revealed that ∼ 40% of PDAC tissue in our sample images was ductal, whilst the remaining 60% was stromal (Supplementary Fig 5A). We observed distinct histological separation in the expression of galectins 1, 3, 4 (Fig. 2). Gal-1 expression was significantly higher in the stromal region when compared to the ductal region (Fig. 2B). In contrast, significantly more Gal-3 and −4 staining was seen in the ductal regions than the stromal (Fig. 2C-D), with Gal-4 barely detectable in the stroma (Fig. 2D). Notably there was an inverse correlation between Gal-1 and Gal-4 expression in the ducts (Fig. 2E) but not the stroma (Fig. 2F), indicating these galectin family members might act in concert to support PDAC pathogenesis. By contrast, there was no correlation between Gal-3 expression with either Gal-1 or Gal-4 in either anatomical region (data not shown).

Next, we examined the cellular expression pattern of galectins. Within the ductal regions most of the cells were positive for the epithelial marker EpCAM (∼80%), with expression of the fibroblast marker podoplanin (PDPN) varying across samples from 30%−90% (Supplementary Fig 5B). Of note, EpCAM^{+} cells also tended to express PDPN (data not shown). By contrast, only 20% of the cells in the stromal region were EpCAM^{+}, with the majority staining positive for PDPN (Supplementary Fig 5C). Once again, EpCAM^{+} cells tended to co-express PDPN (data not shown). In line with results from the scRNAseq analysis, significantly more PDPN^{+} fibroblast/stromal cells expressed Gal-1 compared to EpCAM^{+} epithelial cells across the tissue (Supplementary Fig 6A), whilst the reverse was true for Gal-3 protein expression (Supplementary Fig 6B). We saw no difference in Gal-4 expression between these two cell markers across the entire tissue section (Supplementary Fig 6C). Analysis of galectin expression on EpCAM^{+} and PDPN^{+} cells revealed that galectin expression mirrored that seen in the regional analysis: where higher expression of Gal-1 was detected in stroma on both EpCAM^{+} or PDPN^{+} cells compared to these cells within ductal

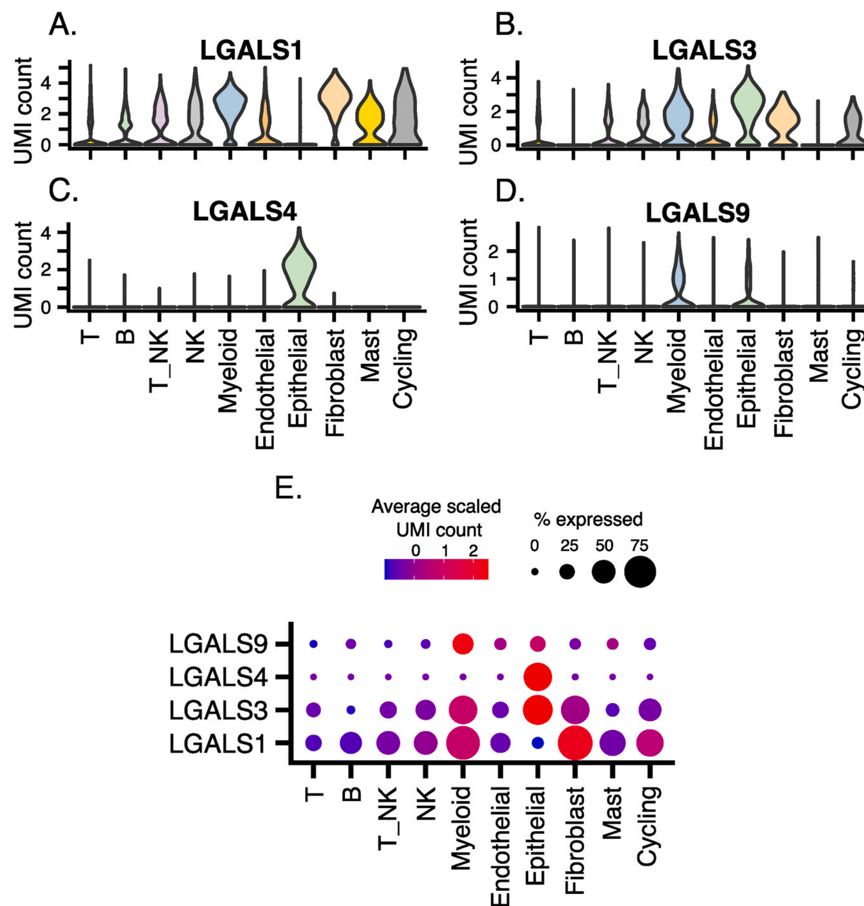

Fig. 1. Galectin gene expression is restricted to distinct cell lineages in PDAC. PDAC tissue was analysed by scRNAseq, data annotated by cell type and expressed in violin plots as a log relative expression vs cell type for (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS4*, and (D) *LGALS9* for n=4 donors. (E) Relative *LGALS* expression profile of the average UMI count. Dot size indicates the percentage of the cell type expressing the given gene.

regions (Fig. 3A-B), and conversely highest Gal-3 and -4 expression was seen in EpCAM$^+$ (Fig. 3D-E) or PDPN$^+$ (Fig. 3G-H) cells in ductal regions compared to stroma. The same pattern was seen for PDPN$^+$EpCAM$^+$ double positive cells in these regions (Fig. 3C, F, I). Confirming these data, we observed a negative correlation in the expression of Gal-1 and Gal-4 on both PDPN$^+$ and EpCAM$^+$ cells within the ducts (Fig. 4A-B). Whilst a positive association was seen for Gal-1 and Gal-3 on both PDPN$^+$ and EpCAM$^+$ cells, this was not statistically significant (Fig. 4C-D). These data suggest that anatomical location of cells is the driving factor influencing galectin expression, rather than being restricted to a given cell type.

3.3. Galectin expression on PDAC CAF

To further assess the relationship between fibroblasts and galectin expression, we opted to further analyse galectin surface and total (surface and intracellular) expression on cultured tumour adjacent normal fibroblasts (ANF) and CAFs isolated from PDAC tissues using flow cytometry (Fig. 5). Pancreatic fibroblasts were defined based on positive expression for CD90 and negative expression for EpCAM (Supplementary Fig 3). Gal-1 was highly expressed on the cell surface of both ANF and CAF, with little if any found intracellularly (Fig. 5A). In contrast, significantly more Gal-3 and Gal-4 were found intracellularly in both ANF and CAF (Fig. 5B-C). Whilst the majority of ANF and CAF expressed Gal-1 (Fig. 5D) and intracellular Gal-4 (Fig. 5F), low numbers of fibroblasts expressed either surface (<5%) or total (~<30%) Gal-3 (Fig. 5E). Of note, protein expression of Gal-3 and −4 contrasts with that seen at a transcriptional level, where we detected substantive amounts of *LGALS3* and minimal *LGALS4* within the fibroblast population (Fig. 1B-C). These data indicate that fibroblasts contribute to Gal-1, Gal-3 and -4 expression within PDAC tissues, although Gal-3 and Gal-4 are likely to be released as soluble, rather than surface bound, galectins.

3.4. Galectin expression correlates with patient survival

Studies have indicated that Gal-1 and −4 expression correlates with patient survival. Across whole pancreatic tissue sections high total Gal-1 expression (i.e. both extracellular and cell associated/intracellular) in PDAC has been shown to correlate with early patient death [28]. By contrast, the prognostic importance of Gal-4 expression is uncertain with conflicting reports correlating high intracellular Gal-4 protein expression in PDAC with better patient outcomes [29], conversely high Gal-4 gene expression [18] in PDAC were linked to reduced survival. Here we assessed whether cellular expression of the different galectins within the ducts correlated with patient survival (Fig. 6). Cellular expression of Gal-1 on both PDPN$^+$ and EpCAM$^+$ cells within the ducts positively correlated with patient survival (Fig. 6A-B), whereby high expression on PDPN$^+$ cells was associated with a smaller tumour size (Fig. 6C). Conversely, we observed a tendency for patients with poorer survival to display higher frequency of Gal-4$^+$PDPN$^+$ or Gal-4$^+$EpCAM$^+$ cells within the ducts, albeit this was not statistically significant (Fig. 6D-E). Cellular expression of Gal-3 in the ducts did not correlate with patient survival (data not shown). Of note, we observed no correlation between galectin expression and sex or lymph node invasion (data not shown). Due to the majority of our patients being diagnosed with a TMN of stage 2b or 3, we were unable to correlate TMN stage with

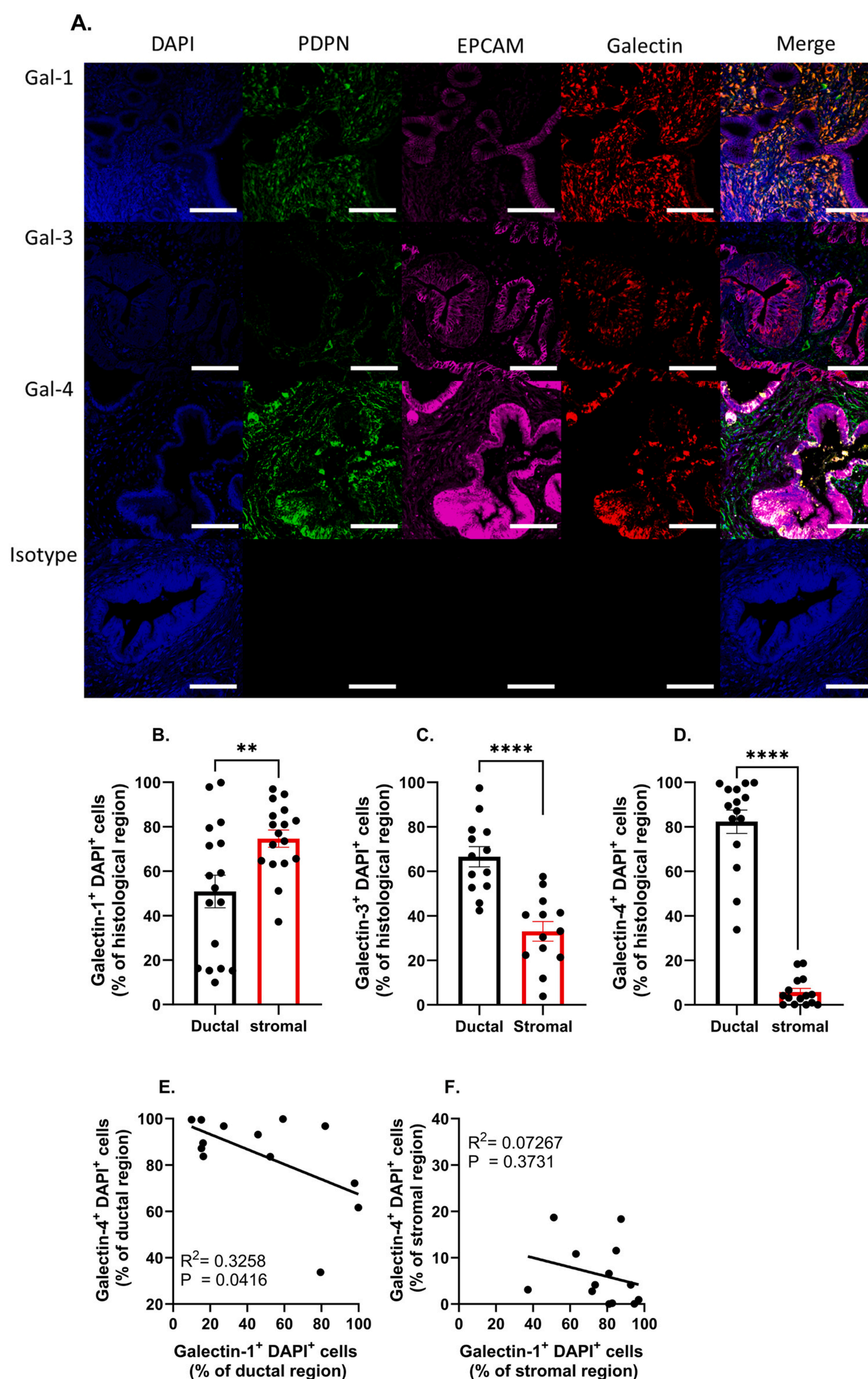

Fig. 2. Galectin expression is influenced by histological region. Paraffin embedded tissue sections from PDAC patients were stained for (A) DAPI (blue), podoplanin (PDPN, green), epithelial cell adhesion molecule (EpCAM, purple) and galectin (Gal, Red). Expression of each marker was assessed within ductal and stromal regions of PDAC tissue sections by confocal microscopy. Scale bars = 100 μm. The percentage of $DAPI^{+}$ cells expressing Gal (B) −1 (n=17), (C) −3 (n=13) and (D) −4 (n=15) within the ductal and stromal regions. Pearson r correlation of Gal-1 with Gal-4 expression on cells in (E) ducts and (F) stroma was performed, where p and R^2 values are denoted on the graphs. Data are mean ± SEM. **= p<0.01 and **** p<0.0001 by unpaired t-test or (D) by Wilcoxon matched pairs.

galectin expression.

4. Discussion

There is an urgent unmet clinical need for better diagnosis and treatment options for PDAC. Many groups have postulated that specific targeting of the stroma and tumour will be therapeutically valuable. Here we investigated for the first time the cellular and histological expression pattern of multiple galectins in serial sections of PDAC patients. We demonstrate that Gal-1 expression is highest within the

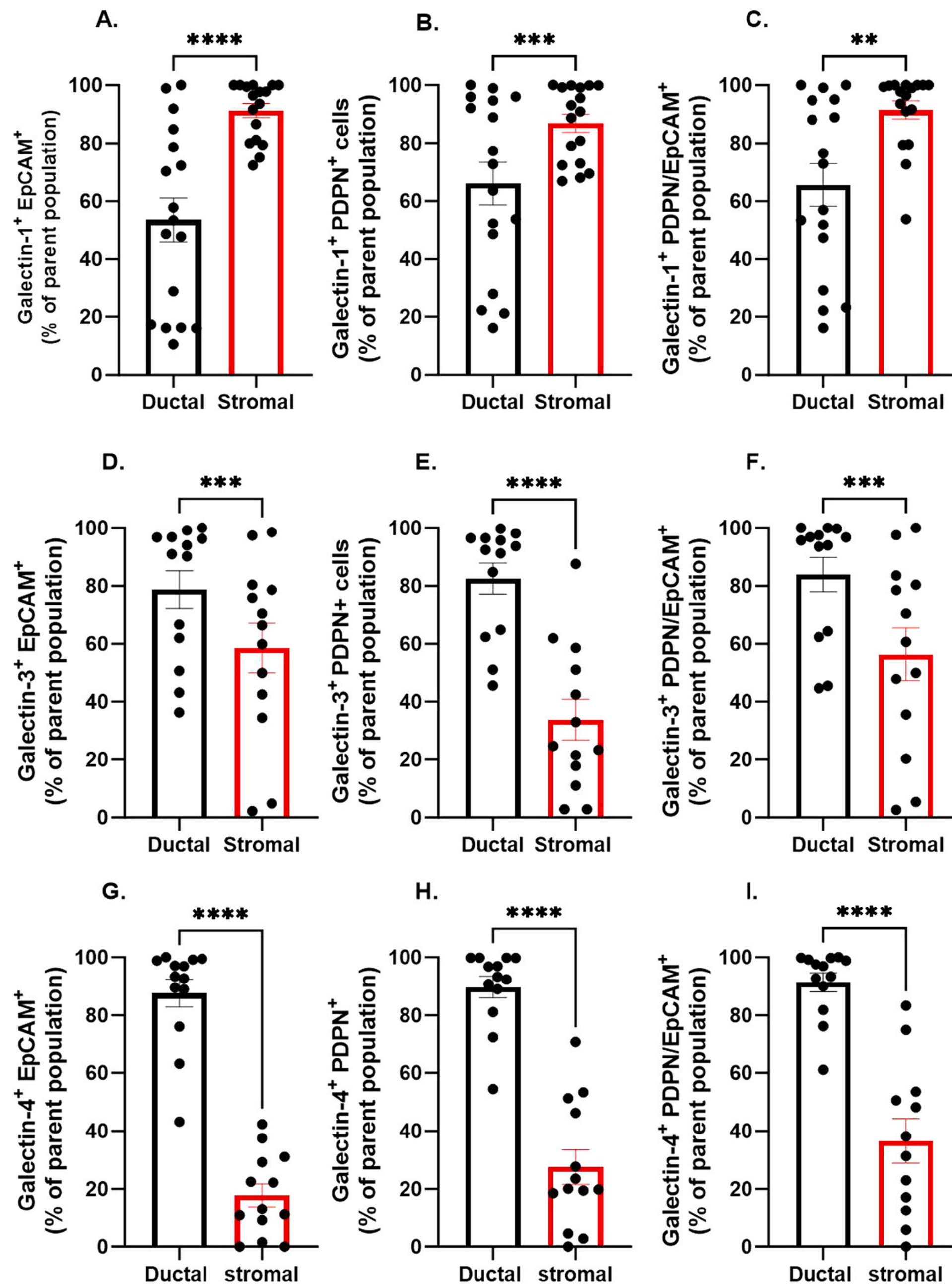

Fig. 3. Cellular expression of galectins differs based on histological region. Paraffin embedded tissue sections from PDAC patients were stained for Gal (A-C) −1 (n=17), (D-F) −3 (n=13) and (G-I) −4 (n=13) alongside cell specific markers (A, D, G) $EpCAM^+$, (B, E, H) $PDPN^+$ or (C, F, I) $PDPN^+EpCAM^+$ cells. Co-expression of galectin with epithelial ($EpCAM^+$), stromal ($PDPN^+$) or transitional ($PDPN^+EpCAM^+$) cells within ductal and stromal regions of PDAC tissue was analysed by confocal microscopy and expressed as a percentage of the parent. Data are mean ± SEM. **= $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ by paired t-test or (G-I) by Wilcoxon matched pairs.

stromal compartment on both $PDPN^+$ and $EpCAM^+$ cells, and crucially that higher expression within ductal cells correlated with smaller tumour size and better patient survival. By contrast, Gal-3 and −4 expression were highest in the ductal regions, with high Gal-4 expression negatively correlating with Gal-1 expression and tending to be highest in those patients with the shortest survival. Further work, with larger patient cohorts, is now required to truly dissect the dynamic interplay between the galectin family members in PDAC pathology and ascertain whether their combined cellular expression patterns are viable prognostic biomarkers.

Broadly speaking Gal-1 and −3 are considered pro-tumorigenic, thought to suppress the activation and function of effector T-cell and support the expansion of Treg, thus limiting the immune response to the tumour [30–32]. Moreover, these galectins have also been associated with enhanced migration and/or metastasis of tumour cells [33–35]. In the context of PDAC, studies have undertaken bulk RNA sequencing demonstrating elevated gene expression of several galectins 1, 3, 4, 7, and 9 when compared to healthy pancreatic tissue [17,18]. Despite evidence indicating a role for galectins in the pathogenesis of PDAC, very few studies have assessed their anatomical or cellular distribution patterns. Here we observed discrete anatomical expression patterns for galectins: Gal-3 and −4 were typically expressed highest within the ductal regions, whilst Gal-1 highest within the stromal compartment. Of the other two published studies looking at anatomical expression of galectins in patient tissues – one shows Gal-8 expression within the ducts of PDAC tissues [19], whilst the other reports that Gal-1 is restricted to

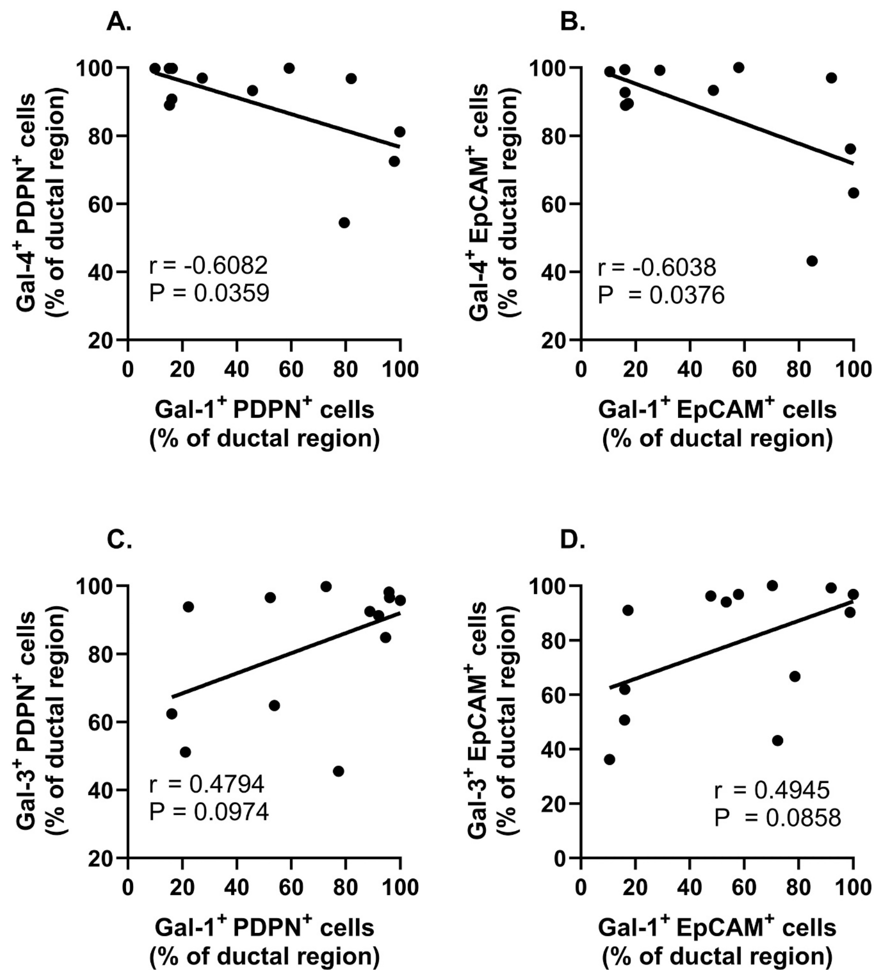

Fig. 4. Gal-1 regulates the expression of Gal-3 and −4 in the ducts. Expression of Gal-1, −3 and −4 (n=13) alongside cell specific markers PDPN$^+$ or EpCAM$^+$ with the ducts of PDAC tissue was analysed by confocal microscopy. Pearson r correlation of Gal-1 expression with (A-B) Gal-4 or (C-D) Gal-3 on (A,C) PDPN$^+$ and (B,D) EpCAM$^+$ cells in the ducts was performed, where p and R^2 values are denoted on the graphs.

the stroma using immunohistochemistry [28]. The latter study disagrees with our findings, whereby we see highest cellular expression of Gal-1 in the stroma, but also see expression within the ducts, similar to what has been observed using murine models of PDAC [36]. Differences in methodology (IHC vs confocal) and quantification (cellular frequency vs total expression based on visualised numerical scoring model) might account for the discrepancies reported between our study and Chen et al., 2012. The specific pattern of galectin tissue distribution in PDAC may provide a critical diagnostic tool for tumour progression and survival. Clearly significant future work is required to determine the significance of the anatomical expression pattern of galectins in PDAC pathology, response to treatment and thus patient prognosis.

Using a scRNAseq approach, we report for the first time the cellular distribution of galectin gene expression within PDAC. Existing literature predominantly associates Gal-1 expression with pancreatic stellate cells (PSC – often located in the perivascular ductal region of the pancreas), it is known to inhibit effector T cell function and pro-inflammatory cytokine production [32]. Our findings suggest a broader cellular distribution in both hematopoietic and stromal compartments, indicating a much broader functional role for Gal-1 within PDAC pathology. Moreover, studies have also reported a crucial role for Gal-1 mediated tumour-stromal cross talk, where Gal-1 expressing PSC increased both tumour size and metastasise when co-injected with tumour cells in xenograft and transgenic murine models of PDAC [12,32,35]. In line with this, histologically Gal-1 expression was significantly elevated within the stromal compartment on all cell types analysed (e.g., EpCAM$^+$ epithelial cells, PDPN$^+$ stromal cells and PDPN$^+$EpCAM$^+$ cells) and also on *ex vivo* cultured CAF [28,37]. Of relevance here, Gal-1 has been reported to drive stromal cell/fibroblast activation resulting in remodelling of the extracellular matrix leading to fibrosis [38]. Moreover, tumour-derived Gal-1 induces PD-L1 expression on neighbouring blood vessels, actively inhibiting T-cell influx into the tumour and further contributing to immune evasion [39]. Further supporting its role in pathogenesis, higher total tissue expression of Gal-1 correlated with poorer patient survival/outcomes [40], where the tissues from majority of 10 year + survivors expressed low or minimal Gal-1 within the stroma [28]. However, others report no correlation between patient survival and Gal-1 tissue expression [37] or serum levels [38]. Conversely, our data suggests that high cellular expression of Gal-1 within the ducts is associated with smaller tumour size and better patient survival, albeit in a small patient cohort. To truly understand the importance of Gal-1 in PDAC more detailed studies using larger patient cohorts are urgently needed to ascertain the isoform of Gal-1 (soluble vs membrane bound) expressed in each anatomical region and their relationship to patient survival.

Substantial evidence has shown Gal-3 is expressed within tumour epithelial cells in PDAC [41,42]. Collectively, our transcript and protein data support this view, with 80% ductal cells expressing Gal-3 for all cell types analysed (e.g., EpCAM$^+$ epithelial cells, PDPN$^+$ stromal cells and PDPN$^+$EpCAM$^+$ cells). Interestingly whilst our scRNAseq suggests that fibroblasts express *Gal-3* transcript, we detected relatively little surface protein expression on *ex vivo* cultured CAFs using flow cytometry. This

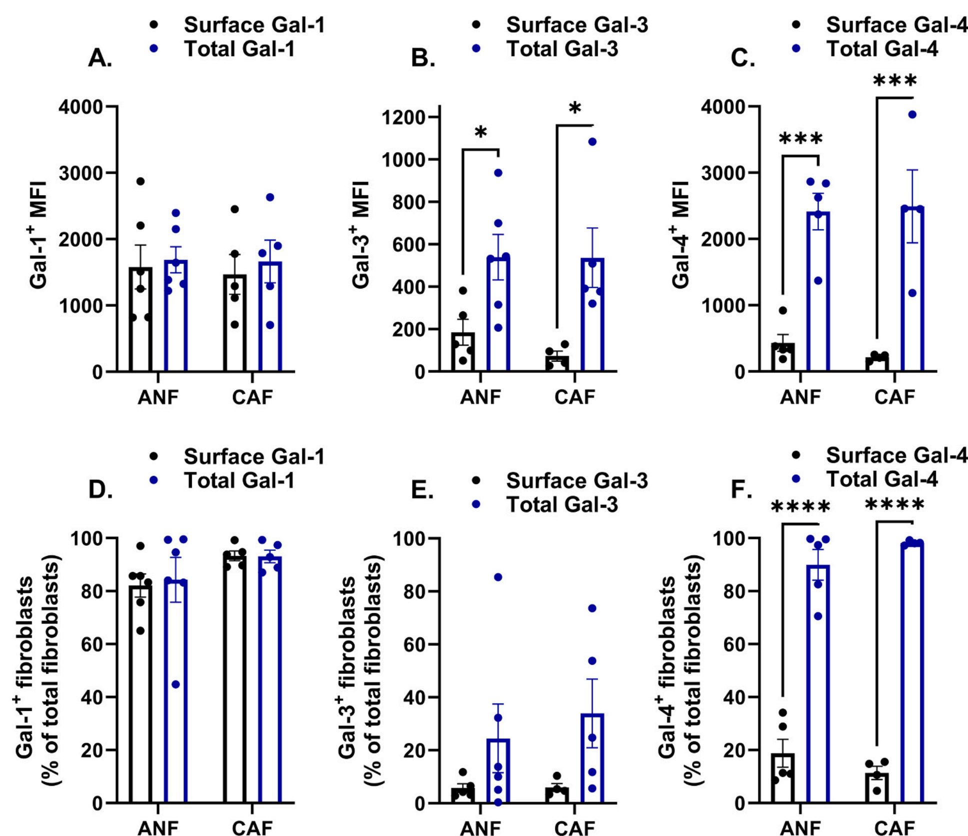

Fig. 5. Gal-1 is surfaced expressed, whilst Gal-3 and Gal-4 are largely intracellular in tumour adjacent and cancer associated fibroblasts. Isolated tumour adjacent normal fibroblasts (ANF) and CAF from PDAC patients were analysed for the expression of surface (black) and total (surface and intracellular, blue) Gal (A,D) −1, (B, E) −3 and (C,F) −4 protein by flow cytometry. Data were expressed median fluorescence intensity (A-C) or as (D-F) percentage of the total fibroblast population. In B-C, F, ANOVA shows a significant effect of expression location, but not fibroblast type, $p<0.01$. Data are mean ± SEM, where n=4–6. *= $p<0.05$, ***= $p<0.001$ and **** $p<0.0001$ by Bonferroni post-test.

lack of cell surface expression may be the result of constitutive release of soluble Gal-3 into the microenvironment, which has been widely reported to drive the pro-inflammatory/pro-fibrotic functions of Gal-3 [43,44]. Soluble Gal-3 plays a critical role in remodelling the tumour microenvironment – promoting PSC activation and extracellular matrix deposition resulting in aberrant tissue fibrosis, as well as driving PSC migration and invasion *in vitro* using in a wound repair assay and Matrigel invasion chamber, respectively [42]. Indeed, blocking Gal-3 with antibodies [45], interfering RNA [14,46], or chemical inhibitors [47,48] inhibits/limits the proliferation, migration and invasive potential of various pancreatic cancerous cell lines *in vitro* and also in patient-derived xenograft models [47,48]. Of interest here is our data showing the tendency for high cellular Gal-3 expression to be correlated with high cellular Gal-1 expression within the ducts and stroma of the same patient, further supporting the widely accepted view that both galectins are pro-tumorigenic [30–32], but importantly highlighting that both galectins may operate in concert with one another to drive pathology. Furthermore, Gal-3 expression has been reported to increase with disease progression [49], thus collectively indicating that targeting Gal-3 might have therapeutic value. Despite these data, expression of Gal-3 in either the ducts or stromal compartment appears to have no impact on patient survival/outcome. Our findings agree with the literature, where multiple studies and a meta-analysis revealed that Gal-3 expression had limited prognostic value, no correlation with either patient survival or clinical characteristics [37,40,41]. Several possible explanations could be posed for the discrepancies between the oncogenic mechanism of Gal-3 and the clinical relevance [e.g., dynamic role in pathology, anatomical location of expression, and/or heterogeneity in patient population (e.g., ethnicity)], which need to be carefully considered and consistently reported in future studies prior to any further development of Gal-3 targeting therapies.

Although less well studied, Gal-4 shares a similar expression profile to that of Gal-3 - predominantly seen on tumour cells [29,50] in the ducts and based on our data appears to be restricted to epithelial cells at least at the transcript level. Elegant studies revealed the importance of intracellular Gal-4 in driving metastasis – where patient derived Gal-4 low expressing PDAC cells had higher migratory and invasive capabilities *in vitro* and enhanced metastasis *in vivo* when compared to patient derived Gal-4 high expressing PDAC cells [20,50]. By contrast it has been recently reported that higher extracellular Gal-4 is deposited within the extracellular matrix proximal to the tumour/ducts compared to distal to the ducts , and contributes to immune evasion through excluding T-cells and M1-like macrophages from the tumour microenvironment [18]. Moreover, the presence of Gal-4 influences the subtype of CAF within the tumour, whereby Gal-4 knockout in orthotopic transplants had reduced inflammatory CAF and increased myofibroblastic CAF compared to the scrambled control [18], thereby creating a less inflammatory and more fibrotic tumour microenvironment. Indeed, patients lacking Gal-4 expression in tissue sections died sooner than those with Gal-4 [29]. However, this study used a binary measurement of Gal-4 (positive or negative) and failed to quantify the degree or the anatomical/cellular location of expression in relationship to patient outcome measurements. Maftouh et al. revealed that high ductal Gal-4 was associated with less lymph node involvement resulting in patients tending to survive better, although there was no statistically significant correlation between Gal-4 expression levels and survival [50] agreeing with our findings. Interestingly in our cohort, Gal-4 cellular expression negatively correlated with Gal-1 within the same patient highlighting a

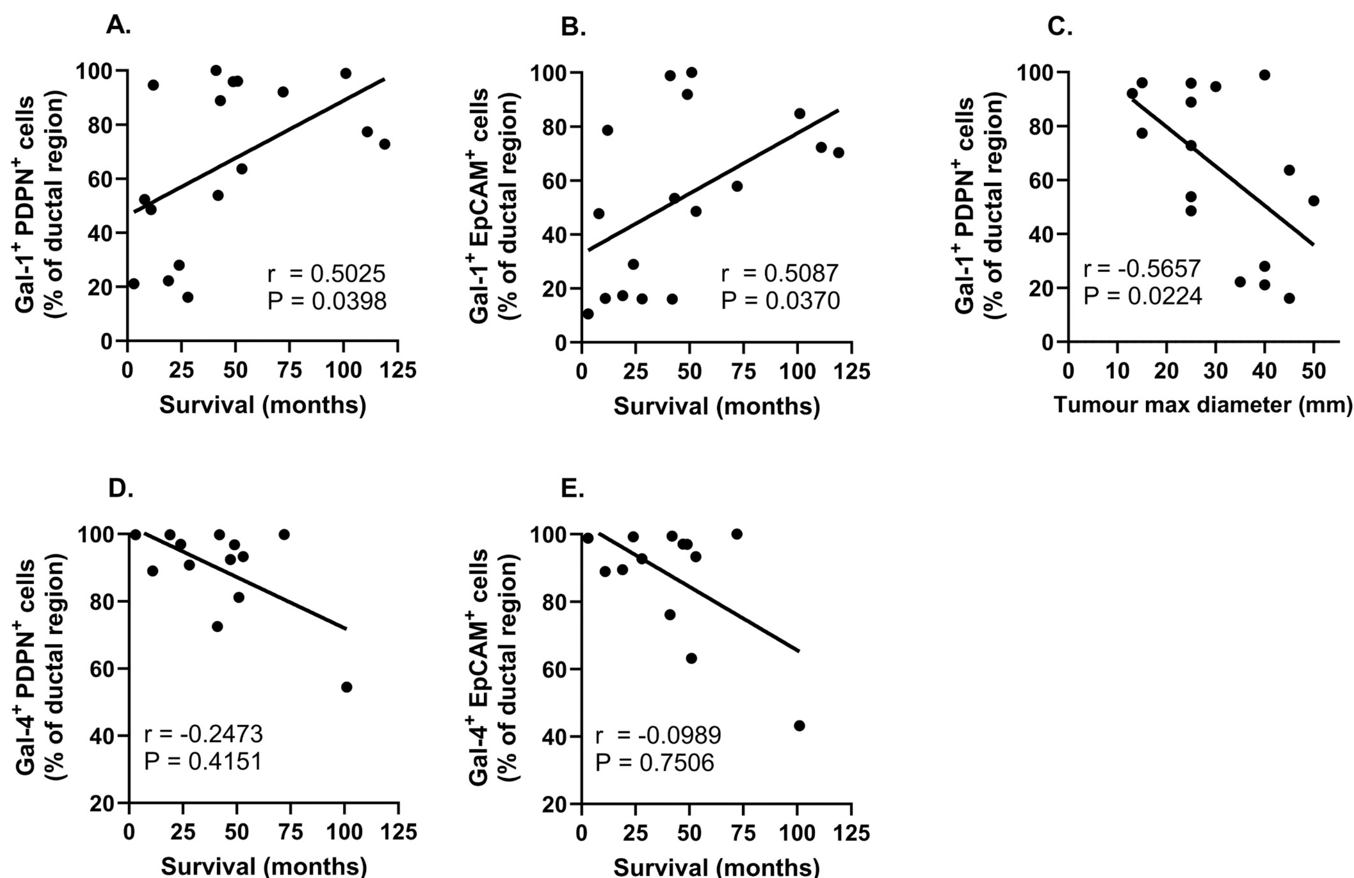

Fig. 6. Low cellular galectin 1 expression is correlated with poor survival and large tumour diameter. Expression of Gal-1 (n=17) and −4 (n=13) alongside cell specific markers PDPN$^+$ or EpCAM$^+$ with the ducts of PDAC tissue was analysed by confocal microscopy. Pearson r (A-C) or Spearman r (D-E) correlation of cellular expression of (A-C) Gal-1 or (D-E) Gal-4 on (A,D) PDPN$^+$ and (B,E) EpCAM$^+$ cells in the ducts (A-B, D-E) patient survival in months or (C) maximum tumour size was performed, where p and r values are denoted on the graphs.

potential important interaction between these two galectins in PDAC pathology. Further work is now required to fully elucidate the oncogenic role of Gal-4 in PDAC, its interplay with the other galectin family members and its potential prognostic and/or therapeutic value. Such experiments could include knockdown and overexpression studies in relevant PDAC cell culture models to investigate how differences in the pattern of galectin expression impacts cancer cell behaviour, such as proliferation, migration or invasion. Alternatively, utilising animal models, such as xenografts or genetically engineered mice, to mimic the PDAC tumour microenvironment *in vivo* could further elucidate the role of galectins in tumour onset and progression.

5. Conclusions

As mentioned earlier galectins exhibit redundancy, meaning that multiple galectin family members may share similar functions or compensate for each other. It is possible that a combination the high cellular Gal-1 and Gal-3, with low cellular Gal-4 act in concert to reduce oncogenic state in PDAC to create a state from which patients are better able to survive following resection and/or chemotherapy. In the context of therapeutic targeting, understanding this redundancy is crucial. It implies that targeting one specific galectin or its isoform might have limited efficacy, as other galectins could compensate for the targeted one. It is important to note that galectins have specific and multiple binding partners [51], and their glycosylation state within the wider tumour microenvironment plays a crucial role in PDAC pathology. Understanding the intricate network of interactions between galectins and their diverse binding partners is essential for unravelling the complexities of PDAC progression. Therefore, effective therapeutic strategies may need to consider the broader galectin and binding partner landscape, including whether the glycoprotein is expressed extracellular or cellularly, and develop approaches that address the redundancy to achieve optimal outcomes.

Ethics approval statement

Samples were obtained with written, informed consent and approval from Human Biomaterial Resource Centre (Birmingham, UK) in compliance with the Declaration of Helsinki.

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Authors did not used any AI tool/service and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AJI and HMM have received funding from Roche. All authors have no conflict of interests to declare.

Data Availability

Data are available within this publication or previous publications (e.g. scRNAseq raw data) cited within the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116283.

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