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Extracellular Matrix Composition Significantly Influences Pancreatic Stellate Cell (PSC) Gene Expression Pattern: Role of Transgelin in PSC Function

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Short running head: Pancreatic stellate cell and transgelin

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Abstract:

Activated pancreatic stellate cells (PSCs) are responsible for the fibrotic matrix of chronic pancreatitis and pancreatic cancer. *In vitro* protocols examining PSC biology have usually involved PSCs cultured on plastic, a non-physiological surface. However, PSCs cultured on physiological matrices e.g. Matrigel^{TM} (normal basement membrane) and collagen (fibrotic pancreas), may have distinctly different behaviours compared to cells cultured on plastic. Therefore, we aimed to i) compare PSC gene expression after culture on plastic, Matrigel^{TM} and collagen I; ii) validate the gene array data for transgelin, the most highly dysregulated gene in PSCs grown on activating versus non-activating matrices, at mRNA and protein levels; iii) examine the role of transgelin in PSC function; and iv) assess transgelin expression in human chronic pancreatitis sections. Culture of PSCs on different matrices significantly affected their gene expression pattern. 146, 619 and 432 genes respectively were differentially expressed ($p<0.001$) in PSCs cultured on collagen I *vs* Matrigel^{TM}, Matrigel^{TM} *vs* plastic and collagen I *vs* plastic. The highest fold change (12.5 fold upregulation) in gene expression in cells on collagen I vs Matrigel^{TM}, was observed for transgelin (an actin stress fibre associated protein). Transgelin was significantly increased in activated PSCs versus quiescent PSCs. Silencing transgelin expression decreased PSC proliferation and also reduced PDGF-induced PSC migration. Notably, transgelin was highly expressed in chronic pancreatitis in stromal areas and peri-acinar spaces but was absent in acinar cells. These findings suggest that transgelin is a potentially useful target protein to modulate PSC function so as to ameliorate pancreatic fibrosis.

Key words: Pancreatic fibrosis, pancreatic stellate cells, extracellular matrix, transgelin, chronic pancreatitis.

Introduction:

The architecture of the normal pancreas is maintained by a fine balance between extracellular matrix (ECM) synthesis and degradation. Alteration of this balance leads to the deposition of excessive amounts of ECM proteins and the development of fibrosis, a characteristic pathological feature of chronic pancreatitis and pancreatic cancer. It is now established that ECM in the pancreas is mainly modulated by pancreatic stellate cells (PSCs) (1). In health, PSCs maintain a balance between the synthesis and degradation of normal ECM (usually comprising collagen IV, laminin, fibronectin and glucosaminoglycans), since these cells synthesize ECM proteins as well as matrix degrading enzymes (matrix metalloproteinases, MMPs), including MMP2, MMP3, MMP9, and MMP13 and also their inhibitors (tissue inhibitors of metalloproteinases) TIMP1, TIMP2 (24) and RECK (19). In diseased states such as chronic pancreatitis and pancreatic cancer, PSCs are activated and secrete excessive amounts of ECM proteins, a predominant component of which is the fibrillar collagen, collagen I.

In order to investigate the influence of specific diseases on PSC function, researchers have usually utilized PSCs isolated from heterogeneous pancreatic tissues and cultured *in vitro* on plastic surfaces. However, it is now known that plastic itself induces PSC activation and does not replicate the microenvironment surrounding PSCs in normal or diseased conditions. Since the ECM is known to play a role in organising and maintaining normal cell function (5), it is possible that a lack of native tissue microenvironment (as in the case of culture on plastic) dramatically changes the pattern of gene expression and function of PSCs compared to that of cells *in situ* within the pancreas.

Therefore, the **aims** of this study were:

i) To compare PSC gene expression after culture on different matrices – a non-physiological matrix, plastic (traditional culture surface) and physiological matrices, Matrigel^{TM} (a basement membrane-like compound which mimics ECM in health) and collagen I (which mimics the ECM in diseased states);

ii) To validate the gene array data for transgelin, which we found to be the most highly dysregulated gene in PSCs grown on activating versus non-activating matrices, at both mRNA and protein levels. Transgelin (also known as SM22alpha) is a 22KDa actin stress fibre-associated protein of the calponin family (17). The calponin family of proteins is expressed both in the cytoskeleton and in the contractile apparatus of smooth muscle cells (10). Transgelin is the product of a single gene (designated TAGLN) that is conserved in yeast, drosophila, molluscs and humans (25). It is ubiquitously expressed in vascular and visceral smooth muscle (3) with high levels reported in the aorta, lung, uterus and intestine. In vitro studies have demonstrated high expression in freshly isolated smooth muscle cells (29) and presence in mesenchymal cells, fibroblasts (17) and tumour cells of epithelial origin (3). It has long been hypothesized that the function of transgelin is to regulate the development and contractile function of smooth muscle cells (17). Transgelin has been reported to be involved in cell migration and along with calponin forms podosomes (10) which are specialized structures that facilitate cell migration and invasion in smooth muscle cells. Transgelin is also a direct target of the transforming growth factor β (TGF-β)/smad3-dependent pathway for epithelial cell migration (34) and has been shown to promote cancer stem cell migration and invasion (18).

iii) To examine the role of transgelin in PSC function; and

iv) To assess the expression of transgelin in human chronic pancreatitis tissue sections.

Materials and methods:

Isolation and culture of rat PSCs

Rat PSCs were isolated from 5 week old male Sprague-Dawley rats as described previously by using density gradient centrifugation as described by us previously (2). Purity of PSC preparations was confirmed by morphology and immunocytochemical staining for the PSC selective marker glial fibrillary acidic protein (GFAP). Experiments were performed with freshly isolated stellate cells, 1 million PSCs were seeded on either plastic, Matrigel™ or collagen I, in 10cm Petri-dishes. PSCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal bovine serum (FBS) (Invitrogen) for 72 hours. Culture medium was changed every 24 hours and at the end of the culture period, cells were removed from plastic dishes by trypsinization, from Matrigel™ coated dishes using BD cell recovery solution as per manufacturer's instructions and from collagen I coated dishes using collagenase digestion (20mins at 37°C) Cells were centrifuged at 200g for 5 minutes at 4°C and cell pellets were lysed for RNA or protein extraction.

Total RNA isolation

Total RNA from stellate cells in 10 cm Petri dishes was isolated by Qiagen RNeasy Mini Plus kit as per manufacturer's instructions. The Agilent 2100 Bioanalyzer (Agilent Technologies Inc. Santa Clara, CA) was used for quality control of the isolated total RNA.

Microarray analysis

The gene expression profiles of rat PSCs cultured on Matrigel™, collagen I and plastic were analyzed by whole rat genome microarray purchased from Affymetrix (Rat Gene 1.0 ST Array). This array was able to detect 27,342 rat genes, with approximately 26 probes on average per gene (referred to as a probe set). The hybridization process was performed in the

Ramaciotti Centre, UNSW, according to an established protocol. Briefly, RNA samples (100ng) were first reverse transcribed into cDNA, and products were then transcribed and amplified *in vitro* to synthesize cRNA. These cRNA products were used for a second cycle of reverse transcription and *in vitro* transcription (IVT). The cRNA was also biotin-labeled during IVT. It was then fragmented by metal-induced hydrolysis and RNA fragments were loaded on the array chips for target hybridization. After hybridization, the chips were washed and stained. Signal was then detected and analyzed. The microarray data files have been submitted to the Gene Expression Omnibus; the accession number is GSE43496 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43496).

Statistical analysis of microarray data

Normalization and probe set summarization was performed using the robust multi-chip average (14) implemented in the Affy library (8) from R/ Bioconductor (9, 13). Differential gene expression was calculated between PSCs cultured on collagen I *vs* Matrigel^{TM}, Matrigel^{TM} *vs* plastic and collagen I *vs* plastic groups using an empirical Bayes, moderated t-statistic implemented in Limma (28). Genes were identified as differentially expressed if they had a fold change greater than two and a p-value <0.05. Highly dysregulated genes were identified by setting the fold change to greater than two, the p-value to less than 0.001 and the false discovery rate (FDR) to less than 0.25. Functions of differentially expressed genes were investigated by searching the Gene Ontology database (www.geneontology.org) and the biological significance of dysregulated genes was investigated by performing Gene Set Enrichment Analysis with the use of GSEA v2.0 software (downloaded from www.broad.mit.edu/gsea/).

Real time quantitative PCR

RNA extractions were performed using the Qiagen RNeasy Mini Plus Kit (Qiagen). cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed with the QuantiFast SYBR Green PCR Kit (Qiagen). Quantitect Primers were obtained from Qiagen. The calculated number of specific transcripts was normalized to the housekeeping gene 18s RNA. Statistical comparisons were made by t-test and a $p<0.05$ was regarded as significant.

Western blotting and densitometry

Five micrograms of total protein lysates were resolved on SDS-PAGE gels, transferred onto nitrocellular membranes, and then immunoblotted with mouse anti-transgelin (Abcam). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody. GAPDH was used as the loading control.

Immunocytochemistry for transgelin expression in rat PSCs

Rat PSCs cultured on coverslips were incubated with goat anti-mouse transgelin antibody (1:100) overnight at 4ºC. Mouse anti-goat IgG (1:500) was used as a secondary antibody. For negative controls, mouse IgG2a was used at the same concentration as the primary antibody.

Assessment of transgelin expression in quiescent *vs* activated rat PSCs

Since collagen I and plastic are both activating matrices for PSCs, it was of interest to determine whether PSCs activated by these matrices showed a similar trend in terms of upregulated transgelin expression compared to cells in their quiescent phase. In addition, whether this upregulation of transgelin is associated with PSC activation is still unknown.

Therefore, transgelin expression was assessed in quiescent rat PSCs compared to activated rat PSCs. Rat PSCs were isolated and an aliquot seeded onto two Petri dishes (1.2 million cells per dish). The remaining cells were cultured in a T75 flask. After 24 hours, protein and RNA were collected from each Petri dish (these were considered as quiescent cells since activation of PSCs as assessed by αSMA expression occurs after 48 hours of culture on plastic (2). When the cells in the T75 flask attained confluence (about 72 hours), they were passaged and seeded at a density of 1.2 million/dish onto another two Petri dishes. RNA and protein were collected when the cells attained confluence (considered as activated cells). Transgelin expression at mRNA level and protein level were assessed as described earlier.

Inhibition of transgelin expression in rat PSCs using small interfering RNA (siRNA)

Rat PSCs were transfected with On-TARGET Plus (Thermo Fisher Scientific) siRNA for transgelin or control (non-silencing) siRNA (100nM) using Lipofectamine (Invitrogen). At the end of a 5h incubation period, medium was replaced with fresh IMDM containing 10% FBS. At 48 and 72h after transfections with siRNA, mRNA and protein levels for transgelin, were assessed.

Effect of silencing transgelin on PSC functions

i) αSMA and collagen I expression: To examine the effects of silencing transgelin on PSC function, α smooth muscle actin and collagen I expression was assessed by western blotting of cell lysates using relevant primary antibodies, at 48 and 72 hours post-transfection with transgelin siRNA.

ii) Cell proliferation: At 48 hours after transfections, PSC proliferation was assessed using the cell counting kit-8 (CCK-8) according to the manufacturer's instructions. To determine whether any effect of transgelin silencing on PSC proliferation was influenced

by the culture matrix, PSCs transfected with siRNA for transgelin, were cultured on Matrigel™ or collagen I for 48 hours and cell proliferation assessed using the CCK-8 kit.

iii) Cell migration: PSC migration was assessed using a modified Boyden chamber method to follow the movement of fluorescently labeled PSCs across a porous insert (8 μm), in the presence and absence of the potent chemotactic agent platelet derived growth factor (PDGF). At 48 hours after transfections, PSCs were labeled with CytoTracker™ (fluorescence) according to the manufacturer's instructions and 100,000 labeled hPSCs were placed over the porous membrane in each chamber. The bottom well of the Boyden chamber contained culture medium without or with 10 ng/ml PDGF. After 18 hours, non-migratory cells were removed by cotton-tipped swabs from the top of the inserts, and the inserts were transferred into a well containing 200 μl lysis buffer. After incubation for 5 minutes, 150 μl aliquots were transferred into an opaque 96 plate and the fluorescent signal was measured using the SpectraMax M2 microplate reader (480 nm/520 nm, Molecular Devices, Sunnyvale, CA).

Immunohistochemistry for transgelin expression in human pancreatic tissue sections

Chronic pancreatitis tissue sections were collected by surgical removal. In each tissue, sequential paraffin sections were prepared and stained with hematoxylin and eosin (H&E), Sirius Red for collagen. Pancreatic sections were incubated in 60ºC oven for 30 minutes followed by immersion three times in fresh xylene. Sections were then dipped in absolute ethanol followed by two immersions in 95% ethanol. After another two immersions in 70% ethanol, sections were rinsed in tap water. Antigen retrieval was performed by soaking the sections in heated 10mM citrate buffer for 15 minutes in an oven set to 104ºC. Sections were then incubated in methanol containing 1% H_2O_2 for 10 minutes to inhibit endogenous peroxidase reactivity followed by incubation in a blocking solution containing PBS and 10%

goat serum for 30 minutes. Sections were then incubated with rabbit anti-transgelin antibody (1:200) overnight at 4ºC. After 3 washes in phosphate buffered saline, sections were incubated with biotin-labelled goat anti-rabbit IgG (1:200) for 1h at room temperature, followed by 5 minutes incubation with ABC kit (Vector Laboratories) to amplify the signal, Colour was developed using the 3, 3-diaminobenzidine tetrahydrochloride / DAB) substrate chromogen system (DAKO). Negative controls were sections incubated with rabbit isotype IgG. Coverslips were mounted using histomount media. Whole slides were then scanned using the Aperio ScanScope XT system and images obtained using Aperio software.

Statistical Analysis

Data for transgelin experiments are expressed as the mean ± SEM and analyzed as appropriate by the Kruskal–Wallis test followed by the Dunn post hoc test, 1-way analysis of variance followed by the Tukey post hoc test, or the Student *t* test (for paired data). The analyses were performed using Graphpad Prism software.

Ethics approval: The use of male Sprague-Dawley rats for PSC isolation for this study was approved by the University of New South Wales Animal Care and Ethics Committee. The use of human chronic pancreatitis tissue sections was approved by the University of New South Wales Human Research Ethics Committee.

Results

Quality of PSCs and RNA Extracts: Consistently pure PSC preparations were obtained as assessed by morphology and GFAP positivity. The average RNA yield from 1-2 million

PSCs was 4µg. The average A260/A280 ratio of all RNA preparations was 2.08 ± 0.12 and the RNA Integrity Number (RIN) score were >9.

Gene Expression Profiles via Microarray Analysis

Gene expression profiles were visualized as heat maps and a hierarchical cluster analysis was performed to demonstrate the pattern of gene cluster (average linkage) (Figure 1). As expected, genes from cells cultured on each of the matrices clustered together. The widest separation of clusters was seen with genes of cells grown on plastic versus cells grown on Matrigel™ or collagen. When comparing rat PSCs cultured on collagen I *vs* Matrigel™, Matrigel™ *vs* plastic and collagen I *vs* plastic, 293, 839 and 533 genes respectively were differentially expressed. These genes exhibited a fold change greater than two (FC > 2), p value < 0.05, and a false discovery rate (FDR) < 0.25. To identify significantly dysregulated genes, the above genes were further filtered by adjusting the p-value to less than 0.001. This yielded 146 (collagen I *vs* Matrigel™), 619 (Matrigel™ *vs* plastic) and 432 (collagen I vs plastic) differentially expressed genes respectively (Supplementary Table 1-3). The top 15 dysregulated genes between cells grown on the physiologically relevant matrices i.e. Matrigel™ and collagen I are shown in Table 1. The highest fold change in gene expression in cells cultured on collagen I vs Matrigel™, was observed for transgelin (12.5 fold upregulation).

Biological Functions of Differentially Expressed Genes

In order to investigate the functions of differentially expressed genes (which could provide important clues about their roles in cell-ECM interactions and PSC activation), a search was conducted using the Gene Ontology (GO) database. The biological contexts as well as the inter-relationships of the dysregulated genes were assessed by Gene Set Enrichment Analysis

(GSEA). The significantly ($p<0.05$, FDR<0.25) altered functional pathways are listed in Table 2 (collagen I *vs* Matrigel™), Table 3 (Matrigel™ *vs* plastic) and Table 4 (collagen I *vs* plastic). Interestingly, when using Matrigel™ as normal PSC gene expression profile, and comparing it to activated PSCs (cultured on activating matrices- collagen I or plastic), certain altered pathways are common to both collagen and plastic (Table 5).

Validation of Transgelin Expression in Rat PSCs

In order to confirm the significant upregulation of transgelin expression revealed by microarray analysis, validation studies were conducted to assess mRNA and protein levels of transgelin in PSCs. Freshly isolated rat PSCs were cultured on either Matrigel™ or collagen I for 72 hours. Both mRNA and protein levels of transgelin were significantly upregulated in PSCs cultured on collagen I compared to PSCs grown on Matrigel™ (Figure 2).

Assessment of Transgelin Expression in Quiescent vs Activated Rat PSCs

To determine whether the upregulation of transgelin expression was associated with PSC activation, the expression of transgelin in activated PSCs was compared to that in quiescent (freshly isolated) PSCs which were cultured on plastic. The results showed a significant upregulation of transgelin mRNA assessed by real-time PCR (20.16 ± 5.94 fold, $p<0.05$, $n=4$ separate rat PSC preparations) and significantly increased protein levels assessed by immunoblotting (2.957 ± 0.29 fold, $p<0.05$, $n=5$ separate rat PSC preparations), in activated PSCs compared to quiescent PSCs (Figure 3).

Expression of Transgelin by Immunocytochemistry

Immunostaining of cultured PSCs revealed prominent cytoplasmic staining for transgelin in the rat PSCs. Both cytoplasmic as well as nuclear staining was evident (Figure 4).

Inhibition of Transgelin Expression by Transgelin siRNA

In order to study the role of transgelin in PSC activation, PSCs were transfected with transgelin siRNA to inhibit the expression of the protein. 48 hours post-transfection, PSCs exhibited a 97.814±0.41% ($p<0.001$, tagln siRNA *vs* ns siRNA; n=3 separate rat PSC preparations) knockdown of transgelin gene expression, and this effect was sustained over 72 hours (94.638±0.89% knockdown; $p<0.001$, tagln siRNA *vs* ns siRNA; n=3 separate rat PSC preparations) (Figure 5A). To confirm whether this knockdown also influenced protein expression, western blotting was performed. The results demonstrated a 78.52±11.07% and 84.29±5.77% ($p<0.001$, tagln siRNA *vs* ns siRNA; n=3 separate rat PSC preparations) knockdown at protein level at 48 hours and 72 hours post-transfection (Figure 5B).

Effect of Transgelin Inhibition on α Smooth Muscle Actin and Collagen I Expression by Rat PSCs Cultured on Plastic

The effect of decreased transgelin expression on PSC αSMA and Collagen I levels was assessed by western blotting at 48 hours and 72 hours after transfection. Inhibition of transgelin had no effect on PSC αSMA or collagen I expression at both time points (Figure 6).

Effect of Transgelin Inhibition on Proliferation of Rat PSCs Cultured on Different Matrices

Inhibition of transgelin expression resulted in a significant reduction (26.41 ± 3.72%, $p<0.001$, n=4 separate rat PSC preparations) of PSC proliferation compared to control cells (mock transfected cells), as assessed at 72 hours post-transfection. To determine whether the

effect of transgelin on PSC proliferation was influenced by the culture surface on which the cells were grown, PSCs were transfected with siRNA for transgelin and then cultured on Matrigel^{TM} or collagen I-coated plates for 48 hours. Similar to the results observed for PSCs cultured on plastic, significant reductions of PSC proliferation were observed in cells cultured on Matrigel^{TM} and collagen I (by 19.38 ± 2.6% and 28.47 ± 5.1% respectively, $p<0.05$ *vs* ns siRNA, n=4 separate rat PSC preparations). The type of matrix (fibrotic or basement membrane like) did not appear to influence this effect (Figure 7).

Effect of Transgelin Inhibition on Basal and PDGF-Stimulated Migration of Rat PSCs

Inhibition of transgelin expression did not influence basal migration of PSCs but significantly inhibited PDGF-induced migration of the cells (Figure 8).

Transgelin Expression in Human Chronic Pancreatitis Tissue Sections

In chronic pancreatitis tissue sections (n=5 patients), transgelin was expressed in fibrotic areas and peri-acinar spaces but *not in acinar cells*. To confirm that transgelin was mainly expressed in fibrotic areas in the pancreas, serial sections were stained for total collagen using Sirius red (n=5 patients); these showed that the areas that stained positive for transgelin were also positive for Sirius red (Figure 9).

Discussion

The biology of PSCs, the key players in pancreatic fibrogenesis, has usually been studied using cells cultured on plastic. This surface is known to transform quiescent PSCs into an activated myofibroblast-like phenotype that secretes excessive ECM proteins (2). However, culture on plastic does not replicate the complex microenvironment in which PSCs normally reside – namely, basement membrane in health and a fibrotic matrix in diseased states.

356

357 This study compared, for the first time, gene expression in PSCs cultured for 72 hours on
358 different matrices (on plastic, collagen I and Matrigel^{TM}). We have found that 432 genes
359 (FC>2, P<0.001, FDR<0.25) were dysregulated between collagen I *vs* plastic, suggesting that
360 activation of PSCs on plastic may not fully represent the *in vivo* activation process occurring
361 in a fibrotic matrix. However, there were several altered pathways common to cells grown on
362 collagen and plastic as listed in Table 5 (such as actin binding, cell division and cell
363 migration), indicating that there are similarities between PSCs grown on the two 'activating'
364 surfaces. Therefore, while PSCs cultured on plastic may not be an ideal experimental set up
365 to replicate the in vivo microenvironment, it remains a valuable in vitro tool to study PSC
366 biology.

367

368 As expected, a large number of genes (619 genes) were found to be dysregulated (FC>2,
369 P<0.001, FDR<0.25) between cells grown on the quiescence inducing, basement membrane-
370 like surface Matrigel^{TM} *vs* the activating surface plastic. Interestingly, 146 genes (FC>2,
371 p<0.001, FDR<0.25), were found to be dysregulated in cells cultured on basement membrane
372 (Matrigel^{TM}) *versus* a fibrotic matrix (collagen I), supporting the concept that the composition
373 of ECM per se can influence cell behaviour.

374

375 In order to understand the cell biological implications of Matrigel^{TM} induced quiescence and
376 collagen I induced activation, differentially expressed genes were classified into several
377 functional categories. As the biological processes of PSC activation have not been clarified in
378 previous microarray experiments performed with primary PSCs, GO classification of the
379 genes was used for analyzing microarray results. Not surprisingly, pathways related to cell -
380 ECM interactions were dysregulated, such as Extracellular_Region, Extracellular_Matrix and

Extracellular_Region_Part, confirming that the ECM plays a crucial role in regulating PSC phenotype.

A similar study comparing gene expression patterns in human hepatic stellate cell (HSC) lines cultured on plastic *vs* Matrigel™ for 3 days has identified several dysregulated pathways, such as Muscle_Development, Cellular_Morphogenesis, Organ_Development and Regulation_of_Cell_Growth (27). These pathways were also found altered in the current study, which confirmed that stellate cells from different organs behave in a similar way when cultured on physiological or non-physiological activating matrices.

Gene expression of MMPs (2, 3, 9, and 13) and their inhibitors TIMP1, 2 as well as RECK showed no differences between the cells cultured on Matrigel™ *vs* collagen I. One possible reason for this is that these enzymes are tightly controlled at three levels: mRNA, protein and activity. For example, MMP9 is secreted as a zymogen and maintained in the latent form presumably by the interaction of a conserved cysteine in its N-terminal prodomain with the zinc atom in the catalytic sites. Activation by cleavage of the prodomain is essential for enzymatic activity (21). Therefore, even though microarray results showed no change at gene level, it does not rule out possible changes in enzyme activity.

One of the most consistent features of PSC activation is the progressive loss of vitamin A (retinyl ester) stores (2). Interestingly, GO pathways related to retinol metabolism did not show significant dysregulation between the 3 matrices, which was consistent with a previous study on gene expression profile of PSCs activated by culture on plastic (11). One possible reason for this could be that loss of vitamin A lipid droplets is a consequence rather than the driving force for PSC activation.

406

407 The top 15 dysregulated genes identified in this study are likely the key regulators in the
408 process of PSC transformation from a quiescent to an activated state. Of these 15 genes, we
409 chose to validate the most highly dysregulated gene in PSCs cultured on collagen I or plastic
410 versus Matrigel^{TM}, i.e. transgelin. This gene was upregulated by 12.5 fold in PSCs cultured
411 on collagen I and by 11. 8 fold in PSCs cultured on plastic versus cells cultured on
412 Matrigel^{TM}. As noted earlier, transgelin is thought to regulate contractile function, migration
413 and invasion of smooth muscle cells, but there is no information in the literature about the
414 role of this protein in PSCs.

415

416 The upregulation of transgelin gene expression observed by microarray was confirmed by
417 real-time PCR whereby mRNA levels for transgelin in PSCs cultured on collagen I were
418 significantly increased above levels in cells cultured on Matrigel^{TM} Furthermore,
419 immunoblotting results confirmed that mRNA changes were translated into increases in
420 transgelin protein levels in the cells grown on the activating surfaces. However, the
421 immunoblots yielded an interesting finding - a double band for transgelin was observed in
422 activated PSCs while only one band was observed in quiescent PSCs, indicating that there are
423 two isoforms of transgelin in activated PSCs. More interestingly, transgelin siRNA only
424 depleted the band that was not seen in the quiescent PSCs. It has previously been reported
425 that rat tissue extracts have two transgelin homologs, sized 22KDa and 20KDa. The 20KDa
426 band presumably results from proteolytic removal of the *C*-terminal end of the 22KDa
427 polypeptide (12). A more recent paper has also reported the same result, i.e. a shorter protein
428 formed through cleavage of the COOH terminus (7). The properties of these two polypeptides
429 may be different; the smaller isoform of bovine transgelin protein homolog showed stronger

F-actin gelling activity than the larger form (16), suggesting that different isoforms of transgelin may have different key functions *in vivo*.

Immunocytochemical analysis showed that transgelin was expressed in the cytoplasm as well as in the nuclear compartment. Transgelin is known to localize in cytoplasm, where it binds to actin (26). Expression of this protein in the nucleus has also been described in other cell types, such as H9c2 cells (derived from embryonic rat ventricle) (4), bone marrow-derived mesenchymal stem cells (30) and colorectal cancer cells (20). Indeed, many of the actin-binding proteins (more than 60 classes) localize to the nucleus (31) and these nuclear actin-binding proteins are associated with transcriptional regulation and DNA repair (33).

To examine the function of transgelin in PSCs, siRNA techniques were used which demonstrated that inhibition of transgelin expression significantly reduced PSC proliferation and PDGF-stimulated PSC migration, but did not influence αSMA or collagen expression in the cells. Our observation that transgelin mediates PDGF-induced migration of PSCs, is consistent with the previously reported role of transgelin in the migration of other cell types. However, the observed increase in cell proliferation and growth by transgelin are contrary to studies reporting an inhibitory effect of transgelin on cell growth, of HepG2 cells (15), LNCaP cells (human prostate adenocarcinoma cell line) (32) and vascular smooth muscle cells (6). The reason for the discrepant findings is not known, but may be related to variable effects of transgelin on the structure of the actin filament in different cells. The effect of transgelin inhibition on growth of PSCs on different matrices was also assessed in this study. Transgelin knockdown not only decreased the proliferation of PSCs cultured on plastic, but also of PSCs cultured on the physiological matrices Matrigel^{TM} and collagen I.

A consequence of the observed inductive effects of transgelin on PSC proliferation and migration would be significantly increased numbers of PSCs within areas of pancreatic damage, which would then play an important role in pancreatic fibrosis. Therefore, the results of our studies suggest that transgelin may represent a valuable target to inhibit/retard pancreatic fibrosis.

Although transgelin protein expression has been reported to be upregulated in pancreatic cancer (particularly in stromal areas (22)), there have been no reports to date about the expression of transgelin in chronic pancreatitis. This study is the first to report that transgelin is highly expressed in human chronic pancreatitis tissues. The expression is strongest in stromal areas and peri-acinar spaces but absent in acinar cells. Transgelin expression has been shown to be upregulated in fibrotic areas of hepatic sections from liver cirrhosis patients showing intense staining in broad cirrhotic septa. In contrast, in normal liver, transgelin is expressed in the wall of blood vessels, in portal areas and in some fibroblasts in portal areas but not in epithelial cells and hepatocytes (23). Transgelin expression was also found to be upregulated in the lungs of patients with idiopathic pulmonary fibrosis and its expression was localized to fibroblastic foci, and smooth muscle (34). The findings of upregulated transgelin expression in pancreatic fibrosis concur well with the studies noted above and suggest that transgelin may play a role in fibrogenesis in the pancreas. More importantly, the absence of transgelin expression in normal acinar cells may ensure specific targeting of stellate cells *in vivo*, with minimal off target effects.

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587
588
589

590 **Figure Legends:**

591 **Figure 1**: Hierarchical clustering of all probe sets. The line distance between each member
592 indicates the similarity/dissimilarity between each cluster. n=4, 3 or 2 separate preparations
593 of rat PSCs cultured on plastic, collagen I and Matrigel^{TM} respectively. As expected, genes
594 from cells cultured on same matrices (e.g. plastic, collagen or Matrigel^{TM}) clustered together.
595 However, there is a large difference in cells cultured on different matrices.

596

597 **Figure 2**: **A)** Expression of transgelin in rat PSCs cultured on Matrigel^{TM} vs collagen I for 72
598 hours. Transgelin mRNA expression was assessed by real-time quantitative PCR and showed
599 a significant upregulation in rat PSCs cultured on collagen I vs PSCs cultured on Matrigel^{TM}
600 (*$p<0.05$, collagen I vs Matrigel^{TM}; n=4 separate rat PSC preparations). Transgelin mRNA
601 levels were normalized against house keeping gene 18S levels.

602 **B)** Expression of transgelin in rat PSCs cultured on Matrigel^{TM} vs collagen I for 72 hours.
603 The figure shows a representative western blot and densitometry analysis of transgelin
604 expression. Transgelin protein levels were significantly upregulated in rat PSCs cultured on
605 collagen I vs PSCs cultured on Matrigel^{TM} (*$p<0.05$, collagen I vs Matrigel^{TM}; n=3 separate
606 rat PSC preparations). GAPDH was used as a loading control.

607

608 **Figure 3**: A) Normalized transgelin mRNA expression in quiescent vs activated rat PSCs.
609 Transgelin mRNA expression was significantly increased in activated PSCs compared to
610 quiescent PSCs (*$p<0.05$; n = 4 separate rat PSC preparations). Transgelin mRNA levels
611 were normalized against housekeeping gene 18S levels.

B) Expression of transgelin protein in quiescent vs activated rat PSCs. Transgelin expression was significantly increased in activated rat PSCs compared to quiescent PSCs (*$p<0.05$; n=5 separate rat PSC preparations). A double band for transgelin was observed in activated PSCs, while only one band was observed in quiescent PSCs (details in discussion section).

Figure 4: Expression of transgelin by rat PSCs. Strong positive staining for transgelin was observed in the cytoplasm and nucleus of PSCs using immunocytochemistry. The panel on the left shows no staining in the negative control. Magnification×100.

Figure 5: A) Effect of transfection of rat PSCs with siRNA for transgelin (tagln) on transgelin mRNA expression. Rat PSCs were transfected with lipofectamine only (mock), non-silencing siRNA (ns siRNA) and transgelin siRNA (tagln siRNA). Transfection of PSCs with siRNA for transgelin significantly decreased transgelin mRNA expression compared to PSCs transfected with ns siRNA at both 48 and 72 hours (**$p<0.001$, tagln siRNA vs ns siRNA; n=3 separate rat PSC preparations).

B) Effect of transfection of rat PSCs with siRNA for transgelin (tagln) on transgelin protein expression. The figure shows a representative western blot and densitometry analysis of transgelin expression. Transfection of rat PSCs with siRNA for transgelin significantly decreased transgelin protein expression in PSCs at both 48 and 72 hours (**$p<0.001$, tagln siRNA vs ns siRNA; n=3 separate rat PSC preparations).

Figure 6: Effect of transfection of rat PSCs with siRNA for transgelin on αSMA and collagen I expression, assessed 48 hours and 72 hours post transfection by western blotting..

635 Inhibition of transgelin expression had no effect on **(A)** αSMA expression or **(B)** collagen I
636 expression by rat PSCs (n=4 separate rat PSC preparations).
637
638 **Figure 7:** Effect of silencing transgelin (tagln) on proliferation of rat PSCs cultured on
639 different matrices (plastic, Matrigel^{TM} and collagen I). Inhibition of transgelin expression
640 significantly decreased PSC proliferation compared to non-silencing siRNA (ns siRNA)
641 control of PSCs cultured on different matrices (**p<0.001, tagln siRNA vs ns siRNA; n=4
642 separate rat PSC preparations).
643
644 **Figure 8:** Effect of silencing transgelin (tagln) on basal and PDGF-stimulated PSC
645 migration. Inhibition of transgelin expression did not influence basal PSC migration but
646 abolished the PDGF-induced migration of PSCs (n=4 separate rat PSC preparations).
647
648 **Figure 9: A)** Expression of transgelin in human chronic pancreatitis tissue sections. The
649 figure shows representative paraffin-embedded chronic pancreatitis tissue sections
650 immunostained for transgelin. Strong positive staining for transgelin was observed in stromal
651 areas, duct and periacinar space, but not in acinar cells. The panel on the left shows no
652 staining in the negative control (Scale bar =100μm).
653 **B)** Co-localization of transgelin and Sirius red (stain for collagen) on human chronic
654 pancreatitis serial tissue sections. The figure shows representative immunostaining for
655 negative isotype control (on the top), transgelin (middle) and collagen (Sirius red on the
656 bottom). The stromal areas which are positive for transgelin also showed positive staining for
657 collagen (Scale bar =100μm).

Hierarchical Clustering of Gene Array Probe Sets

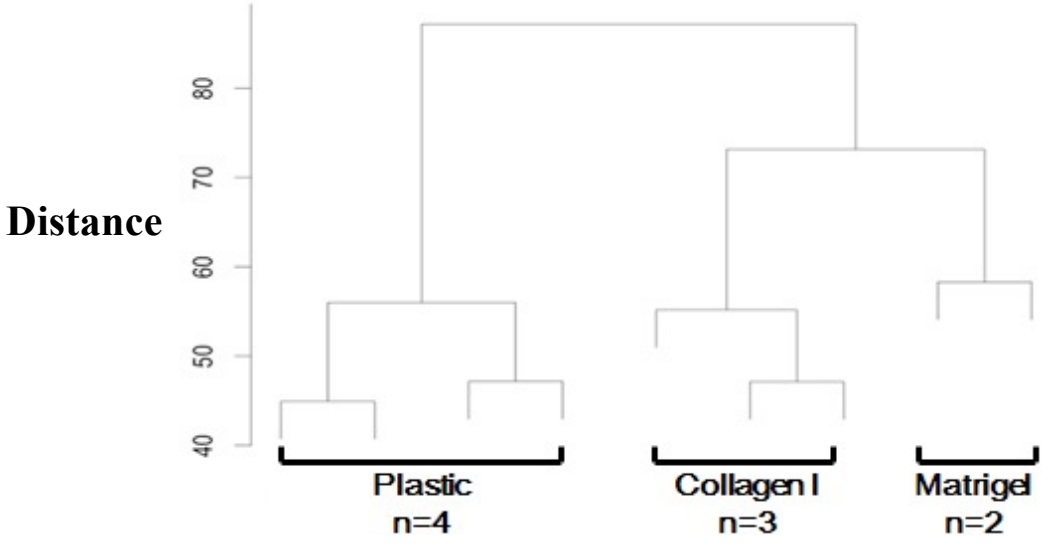


Table 1: **Top 15 Dysregulated Genes in Rat PSCs Cultured on Collagen I vs Matrigel™**

Direction	Gene (FC>2, p<0.001, FDR<0.25)
Up	Transgelin
	FBJ osteosarcoma oncogene B
	Similar to cysteine-rich protein 1 (cysteine-rich intestinal protein) (CRIP)
	FBJ osteosarcoma oncogene
	Lumican
	Interleukin-1 alpha
Down	Chymotrypsinogen B1
	Fatty acid binding protein 4, adipocyte
	Carboxypeptidase A1
	Ischemia related factor vof-16
	Solute carrier family 7, member 11
	Glycerol-3-phosphate dehydrogenase 1 (soluble)
	Phospholipase A2, group VII (plasma)
	Islet amyloid polypeptide
	Proprotein convertase subtilisin/kexin type 2

Table 2: Altered Functional Pathways in Comparison of Collagen I vs Matrigel™ (p<0.05, FDR<0.25)

Upregulated Pathways	
NAME	SIZE*
REGULATION_OF_SECRETION	17
CYTOKINESIS	15
TRANSLATION	129
CARBOHYDRATE_BIOSYNTHETIC_PROCESS	28
MACROMOLECULE_BIOSYNTHETIC_PROCESS	216
STRUCTURAL_MOLECULE_ACTIVITY	136
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	75
MOTOR_ACTIVITY	18
ACTIN_BINDING	51
CHEMOKINE_RECEPTOR_BINDING	19
ACTIN_FILAMENT_BINDING	17
CHEMOKINE_ACTIVITY	19
G_PROTEIN_COUPLED_RECEPTOR_BINDING	22
CYTOKINE_ACTIVITY	47
HYDROLASE_ACTIVITY__HYDROLYZING_O_GLYCOSYL_ COMPOUNDS	20

Downregulated Pathways	
NAME	SIZE
SECONDARY_ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	22
ATPASE_ACTIVITY__COUPLED_TO_MOVEMENT_OF_SUBSTANCES	28
ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	97
HYDROLASE_ACTIVITY__ACTING_ON_ACID_ANHYDRIDES__CATALYZI NG_TRANSMEMBRANE_MOVEMENT_OF_SUBSTANCES	27
ATPASE_ACTIVITY__COUPLED_TO_TRANSMEMBRANE_MOVEMENT_OF _IONS	15
CHROMATIN_BINDING	24
ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	66
INOSITOL_OR_PHOSPHATIDYLINOSITOL_KINASE_ACTIVITY	16
SH3_SH2_ADAPTOR_ACTIVITY	23
SUBSTRATE_SPECIFIC_TRANSPORTER_ACTIVITY	167

* Size: Number of genes in the functional pathway

METAL_ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	43
SYMPORTER_ACTIVITY	15
TRANSFERASE_ACTIVITY__TRANSFERRING_GROUPS_ OTHER_THAN_AMINO_ACYL_GROUPS	37
TRANSMEMBRANE_TRANSPORTER_ACTIVITY	154
SUBSTRATE_SPECIFIC_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	141
MOLECULAR_ADAPTOR_ACTIVITY	26
CATION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	77
ACETYLTRANSFERASE_ACTIVITY	19
SEQUENCE_SPECIFIC_DNA_BINDING	30
SMALL_GTPASE_BINDING	23
ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	18
TRANSCRIPTION_COFACTOR_ACTIVITY	168
MAGNESIUM_ION_BINDING	38
TRANSCRIPTION_REPRESSOR_ACTIVITY	111

Table 3: Altered Functional Pathways in Comparison of Matrigel™ vs Plastic (p<0.05, FDR<0.25)

Upregulated Pathways	
NAME	SIZE
CELL_DIVISION	17
CELL_MIGRATION	53
ACTIN_BINDING	51

Downregulated Pathways	
NAME	SIZE
SECONDARY_ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	22
ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	66
SYMPORTER_ACTIVITY	15
ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	97
ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	18
SUBSTRATE_SPECIFIC_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	141
TRANSCRIPTION_ACTIVATOR_ACTIVITY	116
CATION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	77
GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY	36
SUBSTRATE_SPECIFIC_TRANSPORTER_ACTIVITY	167
TRANSCRIPTION_REPRESSOR_ACTIVITY	111
TRANSCRIPTION_FACTOR_ACTIVITY	179
N_ACETYLTRANSFERASE_ACTIVITY	16
TRANSMEMBRANE_TRANSPORTER_ACTIVITY	154
SEQUENCE_SPECIFIC_DNA_BINDING	30
HYDROLASE_ACTIVITY__ACTING_ON_ACID_ANHYDRIDES__ CATALYZING_TRANSMEMBRANE_MOVEMENT_OF_SUBSTANCES	27
INOSITOL_OR_PHOSPHATIDYLINOSITOL_KINASE_ACTIVITY	16
ATPASE_ACTIVITY__COUPLED_TO_MOVEMENT_OF_SUBSTANCES	28
DNA_BINDING	357
METAL_ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	43
ATPASE_ACTIVITY__COUPLED_TO_TRANSMEMBRANE_MOVEMENT	15
METALLOENDOPEPTIDASE_ACTIVITY	19
PRIMARY_ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	28
METALLOPEPTIDASE_ACTIVITY	35

TRANSCRIPTION_FACTOR_BINDING	228
TRANSCRIPTION_COFACTOR_ACTIVITY	168
RNA_POLYMERASE_II_TRANSCRIPTION_FACTOR_ACTIVITY	119
TRANSCRIPTION_COACTIVATOR_ACTIVITY	87
TRANSFERASE_ACTIVITY__TRANSFERRING_GROUPS_OTHER_THAN AMINO_ACYL_GROUPS	37
GTPASE_REGULATOR_ACTIVITY	89

Table 4: Altered Functional Pathways in Comparison of Collagen I vs Plastic ($p<0.05$, FDR<0.25)

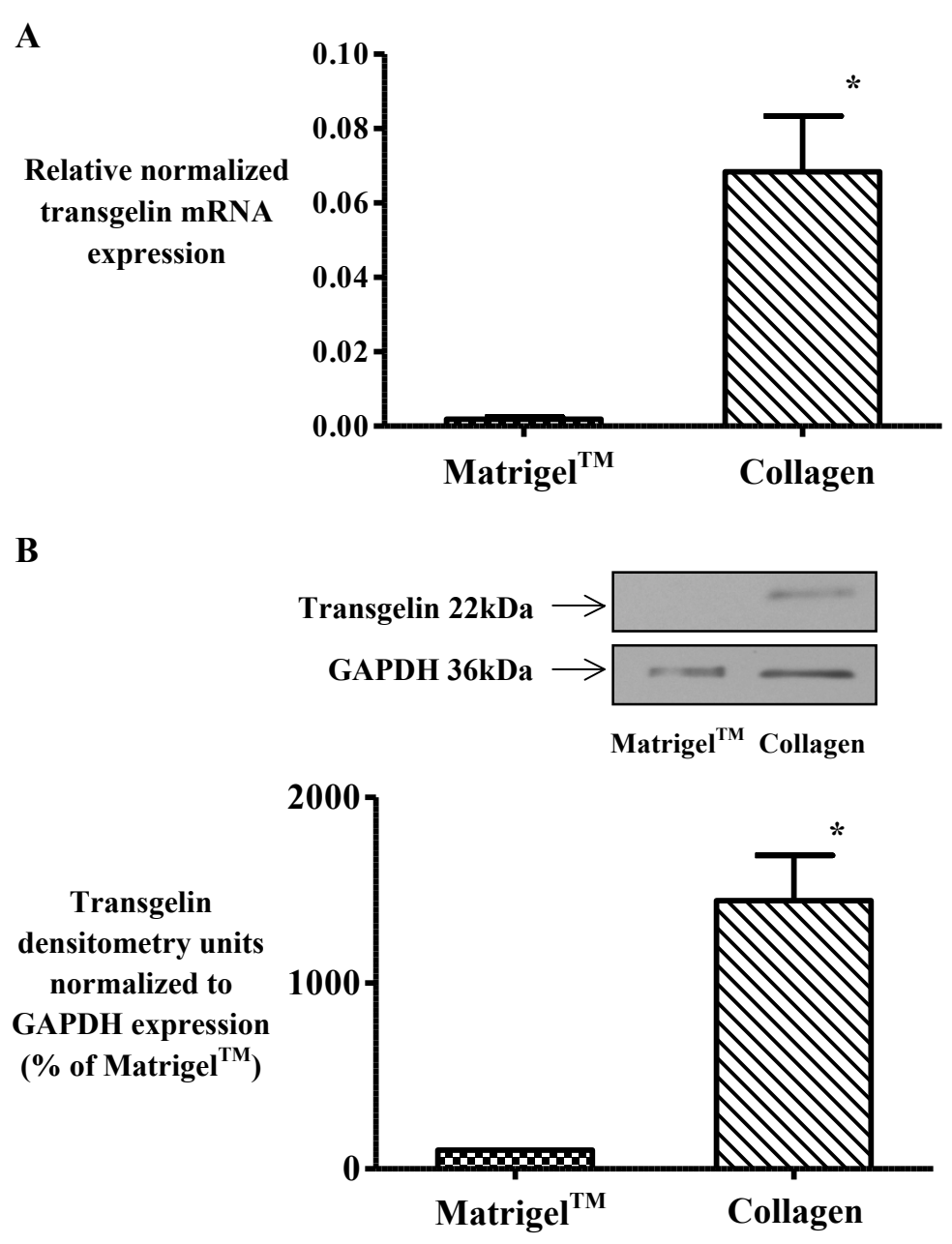
Upregulated Pathways	
NAME	SIZE
SKELETAL_DEVELOPMENT	57
NEGATIVE_REGULATION_OF_CELL_DIFFERENTIATION	15
REGULATION_OF_SECRETION	17
REGULATION_OF_CELL_DIFFERENTIATION	34
POSITIVE_REGULATION_OF_PROTEIN_MODIFICATION_PROCESS	15
REGULATION_OF_ANGIOGENESIS	16
MULTICELLULAR_ORGANISMAL_DEVELOPMENT	480
METALLOPEPTIDASE_ACTIVITY	35
METALLOENDOPEPTIDASE_ACTIVITY	19
AMINE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	20
CYTOKINE_ACTIVITY	47
CHEMOKINE_RECEPTOR_BINDING	19
CHEMOKINE_ACTIVITY	19
G_PROTEIN_COUPLED_RECEPTOR_BINDING	22
GROWTH_FACTOR_ACTIVITY	17

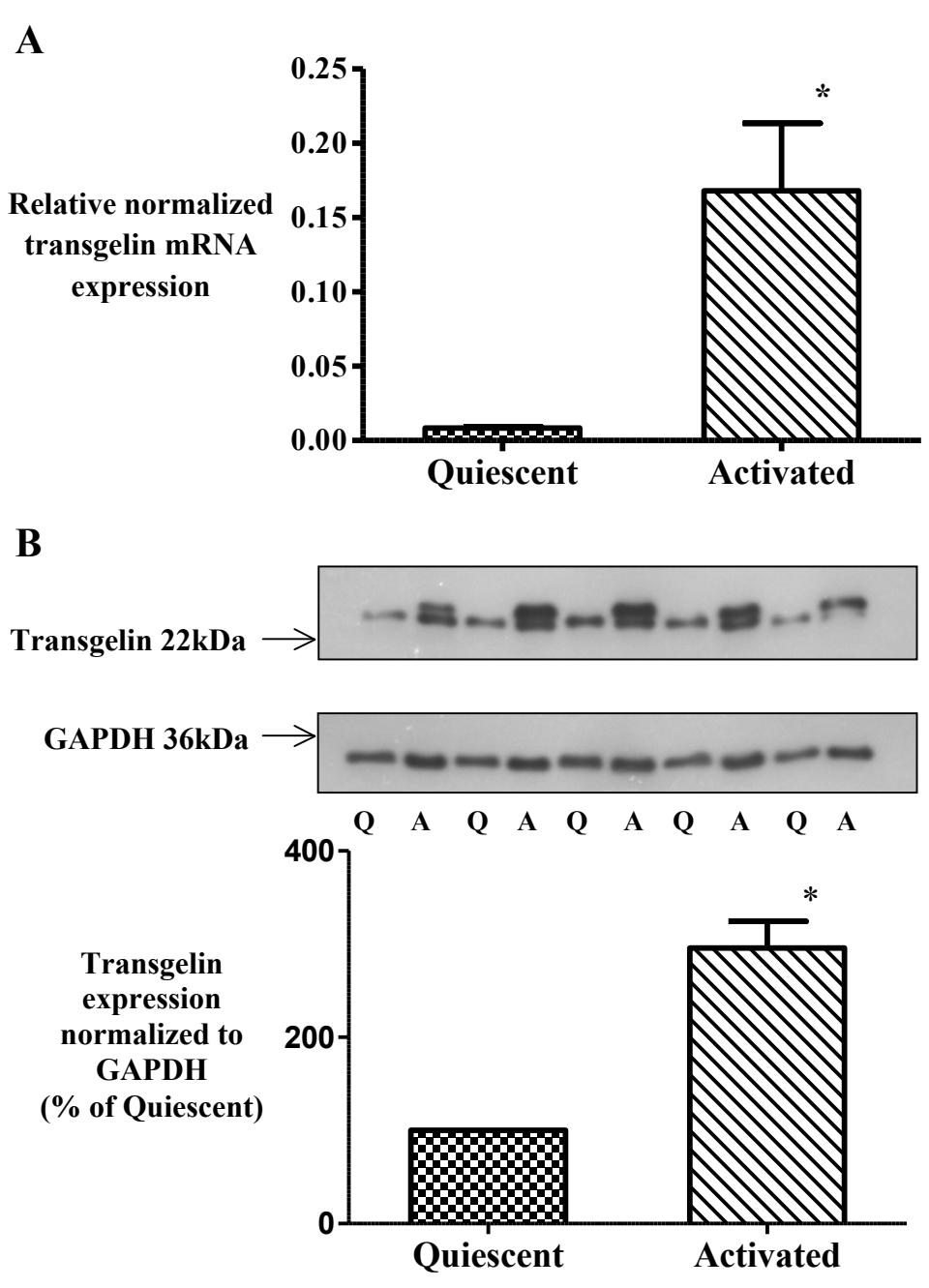
Downregulated Pathways	
NAME	SIZE
ELECTRON_CARRIER_ACTIVITY	58
OXIDOREDUCTASE_ACTIVITY__ACTING_ON_CH_OH_GROUP_OF _DONORS	41

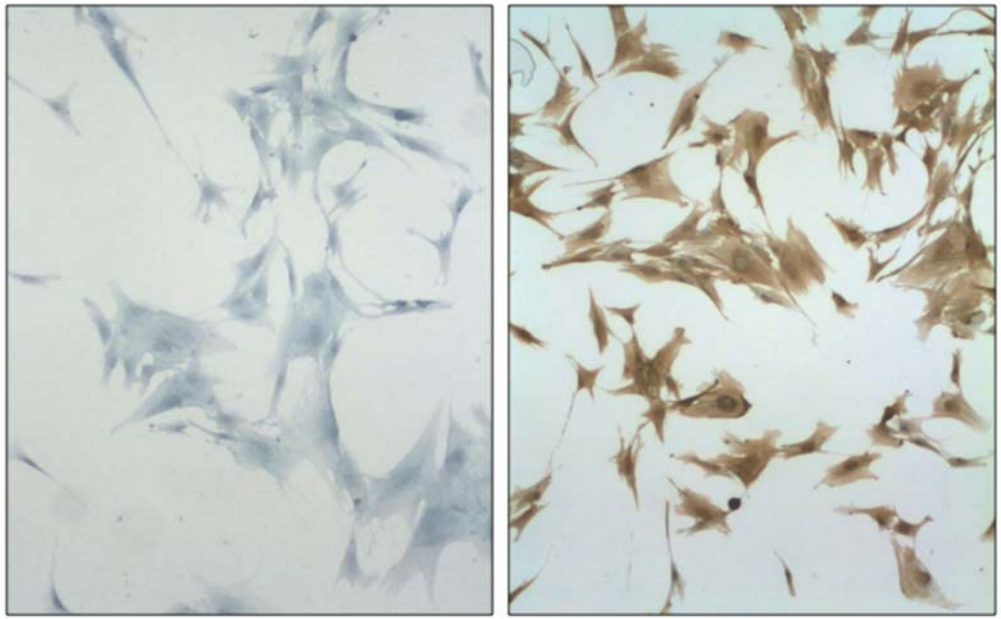
Table 5: Common Altered Pathways in Activated Rat PSCs

Collagen I *vs* Matrigel™	Plastic *vs* Matrigel™
Actin binding ↑	Actin binding ↑
Cytokinesis ↑	Cell division ↑
Regulation of cell migration ↑	Cell migration ↑
Extracellular Matrix ↑	
Chemokine activity ↑	
Regulation of secretion ↑	
	Regulation of cell differentiation ↓
Drug resistance and metabolism ↓	Drug resistance and metabolism ↓
ABC transporters general ↓	ABC transporters general ↓

Table 5 Legend: Common altered pathways in the comparison of PSCs cultured on Collagen I vs Matrigel™ and Plastic vs Matrigel™. Upregulated pathways are in grey boxes while downregulated pathways are in white boxes. When comparing activated PSCs (cultured on collagen I and plastic) to quiescent PSCs (cultured on Matrigel™), certain altered pathways are common, such as actin binding, cell migration, drug resistance and metabolism and ABC transporters general.

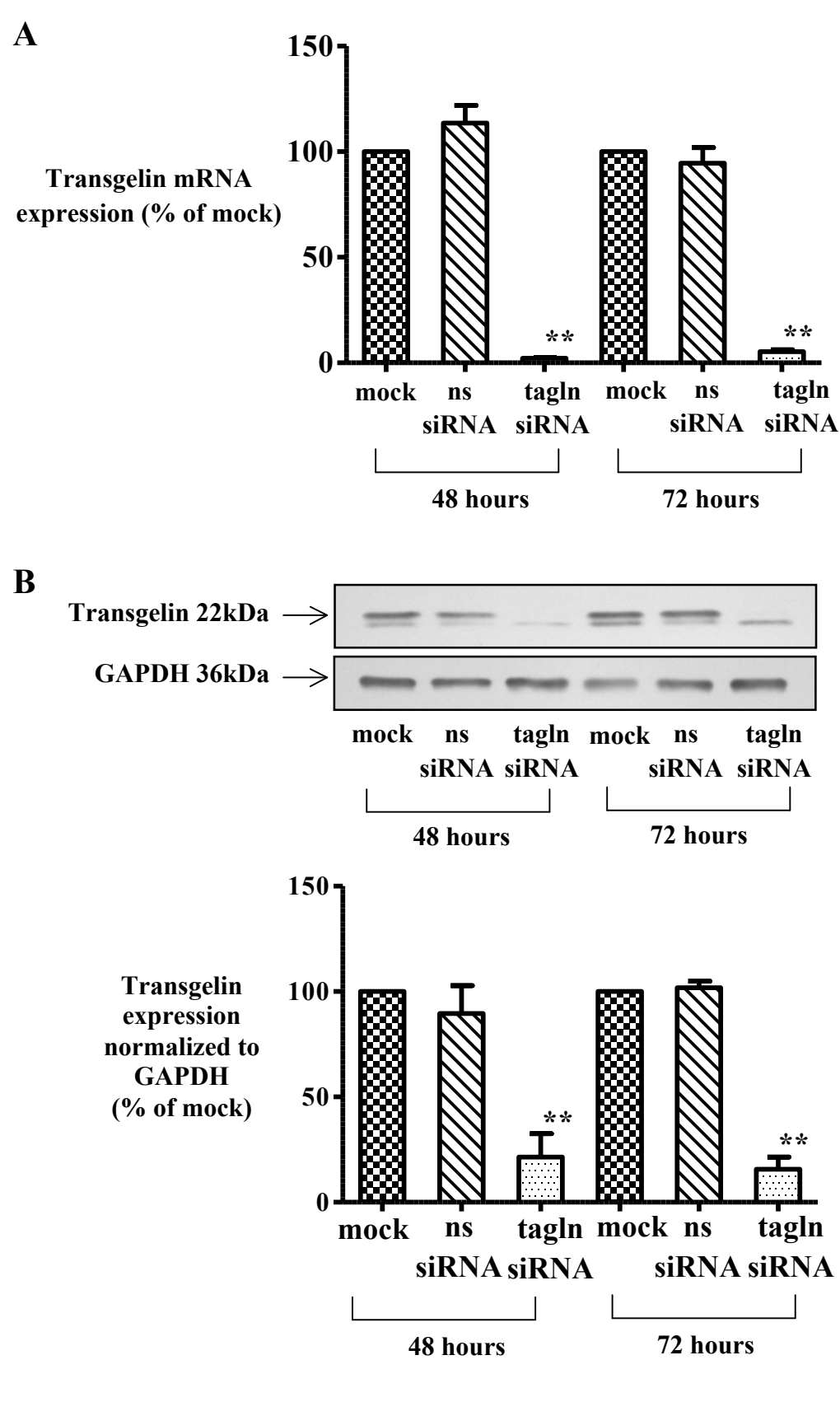

A
Relative normalized transgelin mRNA expression
0.10
0.08
0.06
0.04
0.02
0.00
*
Matrigel™
Collagen
B
Transgelin 22kDa
GAPDH 36kDa
Matrigel™
Collagen
Transgelin densitometry units normalized to GAPDH expression (% of Matrigel™)
2000
1000
0
*
Matrigel™
Collagen

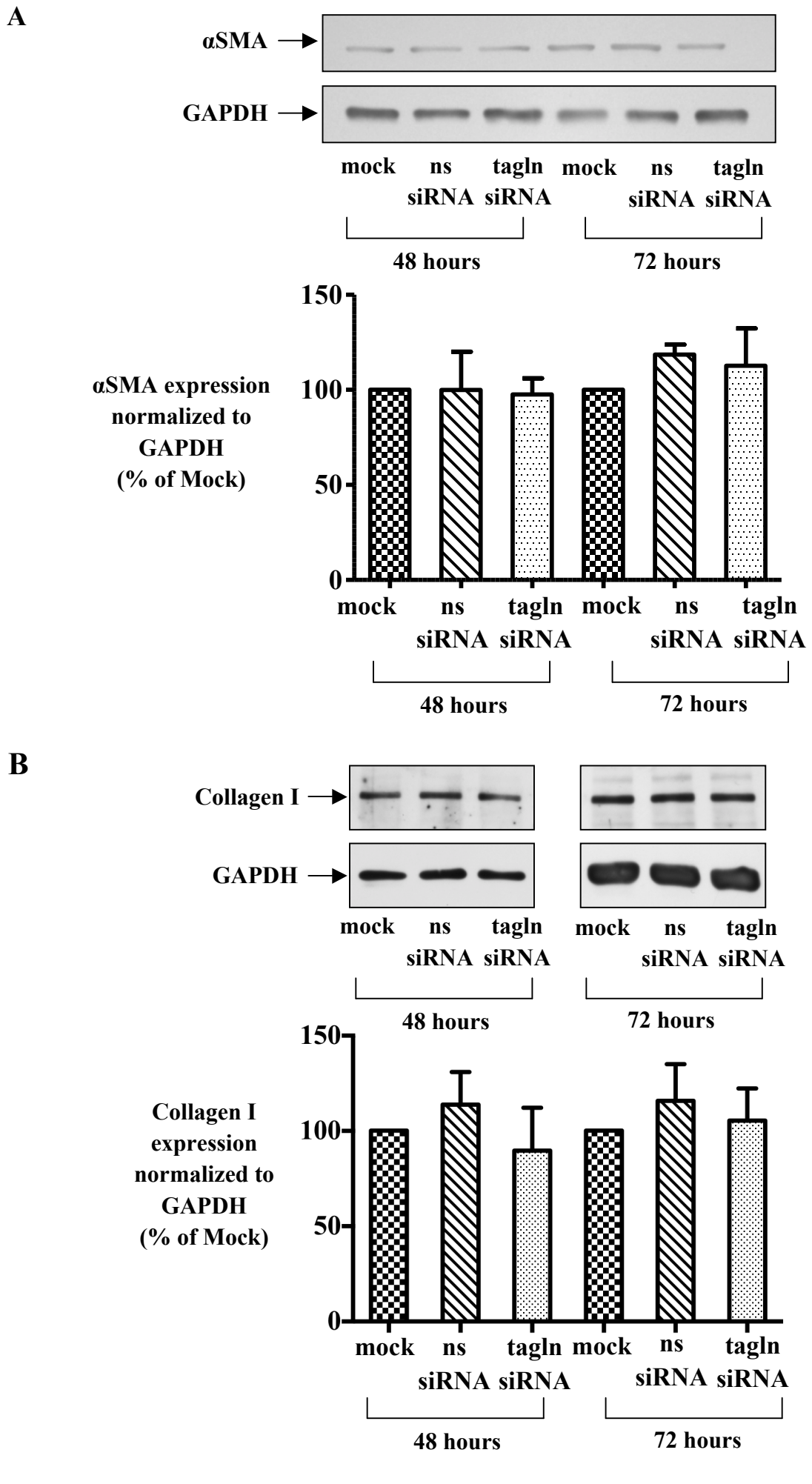

A
Relative normalized transgelin mRNA expression
0.25
0.20
0.15
0.10
0.05
0.00
*
Quiescent
Activated
B
Transgelin 22kDa
GAPDH 36kDa
Q A Q A Q A Q A Q A
Transgelin expression normalized to GAPDH (% of Quiescent)
400
200
0
*
Quiescent
Activated

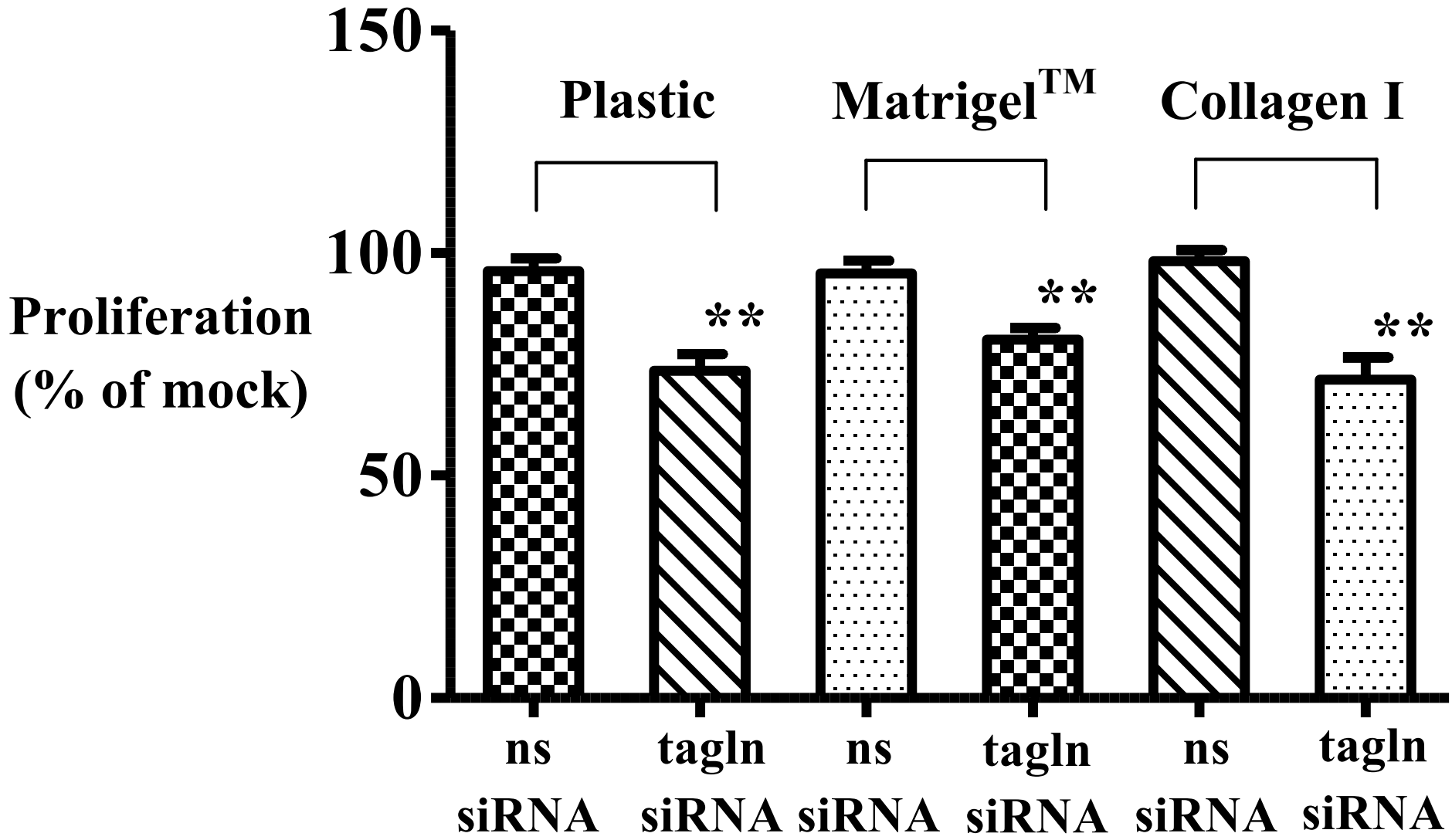


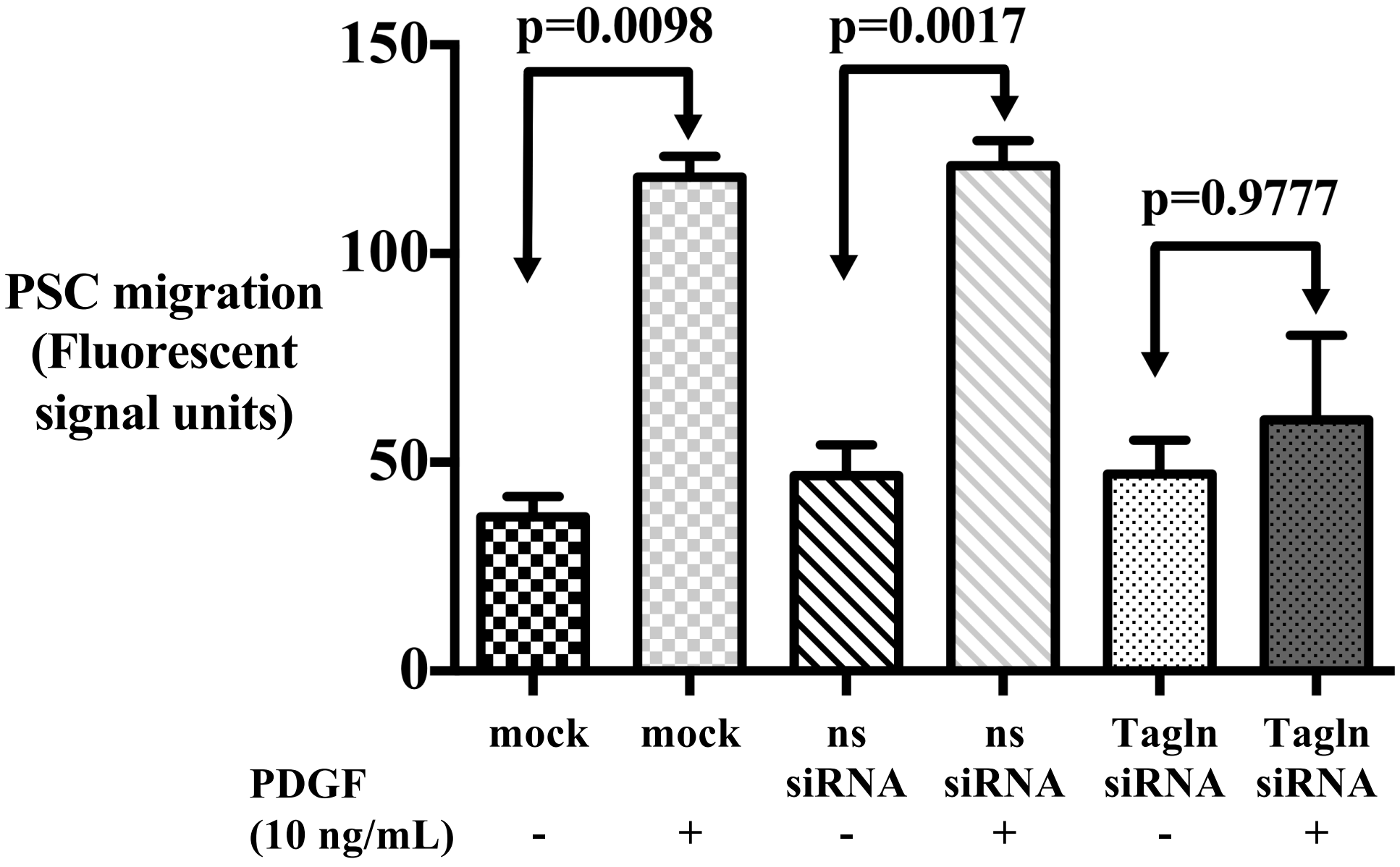
Negative control (rabbit IgG)

Transgelin

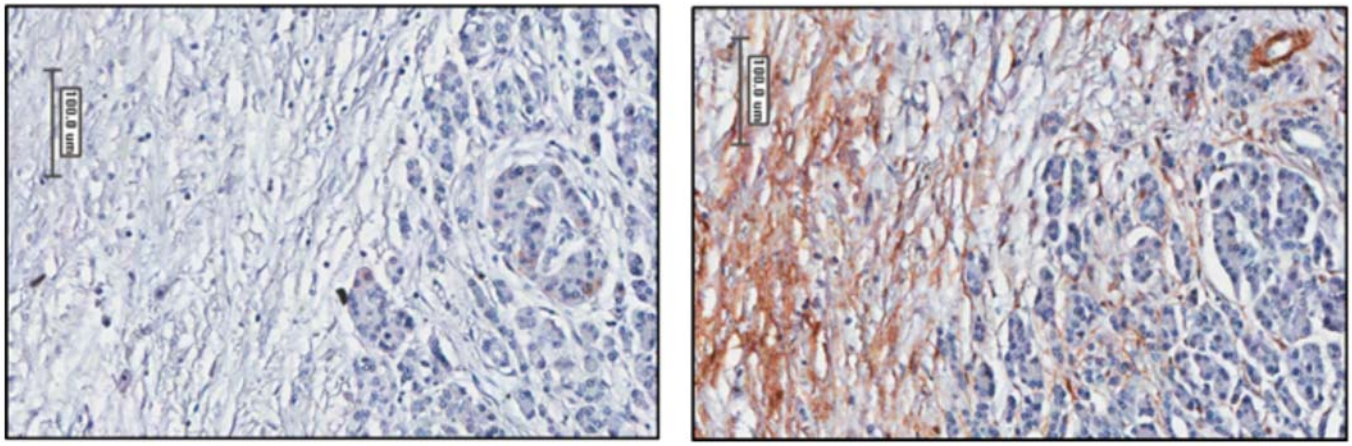

A
Transgelin mRNA expression (% of mock)
150
100
50
0
**
**
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours
B
Transgelin 22kDa
GAPDH 36kDa
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours
Transgelin expression normalized to GAPDH (% of mock)
150
100
50
0
**
**
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours


A
αSMA
GAPDH
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours
αSMA expression normalized to GAPDH (% of Mock)
150
100
50
0
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours
B
Collagen I
GAPDH
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours
Collagen I expression normalized to GAPDH (% of Mock)
150
100
50
0
mock
ns siRNA
tagln siRNA
mock
ns siRNA
tagln siRNA
48 hours
72 hours


150
100
50
0
Proliferation
(% of mock)
Plastic
Matrigel™
Collagen I
**
**
**
ns siRNA
tagln siRNA
ns siRNA
tagln siRNA
ns siRNA
tagln siRNA


p=0.0098
p=0.0017
p=0.9777
150
100
50
0
PSC migration (Fluorescent signal units)
mock
mock
ns siRNA
ns siRNA
Tagln siRNA
Tagln siRNA
PDGF (10 ng/mL)
-
+
-
+
-
+

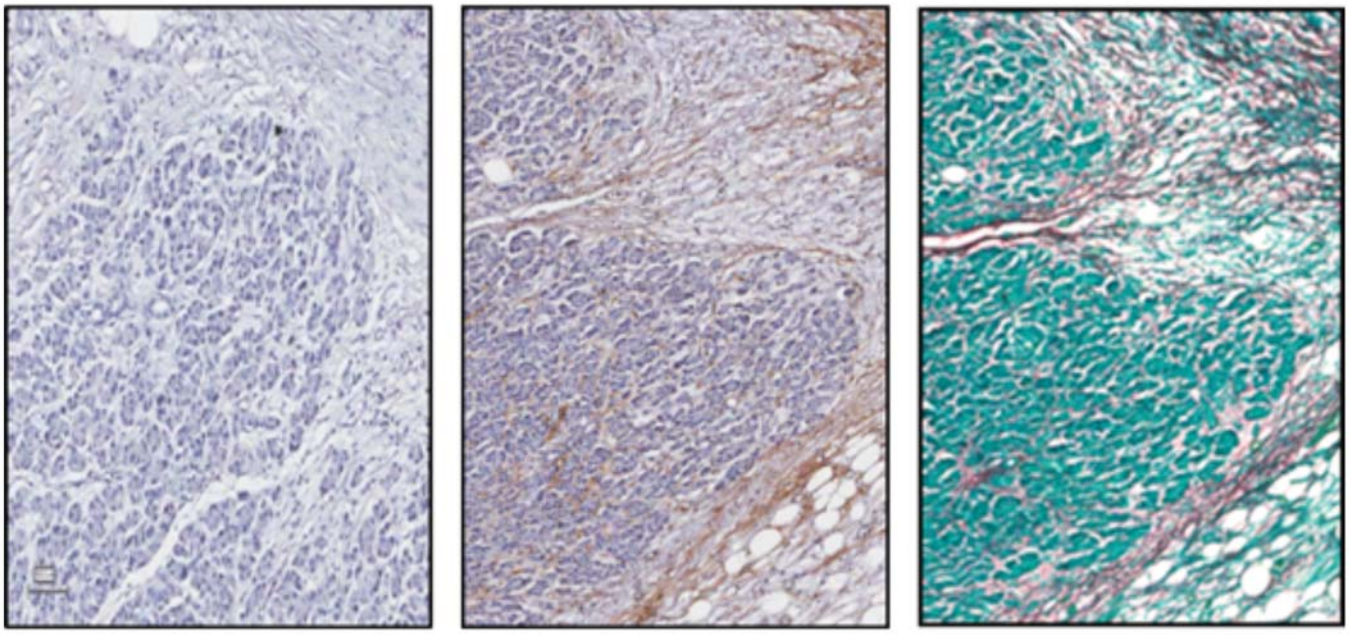
A



Isotype Negative Control

Transgelin

B



Isotype Negative Control

Transgelin

Sirius Red