The Role of the Stroma and CYR61 in Chemoresistance in Pancreatic Cancer

By

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2016
ABSTRACT

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal cancer in part due to inherent resistance to chemotherapy, including the first-line drug gemcitabine. Gemcitabine is a nucleoside pyrimidine analog that has long been the backbone of chemotherapy for PDAC, both as a single agent, and more recently, in combination with nab-paclitaxel. Since gemcitabine is hydrophilic, it must be transported through the hydrophobic cell membrane by transmembrane nucleoside transporters. Human equilibrative nucleoside transporter-1 (hENT1) and human concentrative nucleoside transporter-3 (hCNT3) both have important roles in the cellular uptake of the nucleoside analog gemcitabine. While low expression of hENT1 and hCNT3 has been linked to gemcitabine resistance clinically, mechanisms regulating their expression in the PDAC tumor microenvironment are largely unknown. We identified that the matricellular protein cysteine-rich angiogenic inducer 61 (CYR61) negatively regulates expression of hENT1 and hCNT3. CRISPR/Cas9-mediated knockout of CYR61 significantly increased expression of hENT1 and hCNT3 and cellular uptake of gemcitabine. CRISPR-mediated knockout of CYR61 also sensitized PDAC cells to gemcitabine-induced apoptosis. Conversely, adenovirus-mediated overexpression of CYR61 in PDAC cells decreased hENT1 expression and reduced gemcitabine-induced apoptosis. We demonstrate that CYR61 is expressed primarily by stromal pancreatic stellate cells (PSCs) within the
PDAC tumor microenvironment, with transforming growth factor-β (TGF-β) inducing the expression of CYR61 in PSCs through canonical TGF-β-ALK5-Smad signaling. Activation of TGF-β signaling or expression of CYR61 in PSCs promotes resistance to gemcitabine in an in vitro co-culture assay with PDAC cells. Our results identify CYR61 as a TGF-β induced stromal-derived factor that regulates gemcitabine sensitivity in PDAC and suggest that targeting CYR61 may improve chemotherapy response in PDAC patients.
Dedication

I dedicate this dissertation to Katherine Hesler, Stephen Hesler, Mary Hesler, Greg Hesler, and Joseph Johnson. I thank all of you for your support and encouragement throughout my thesis research. I could not have done this without you.
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List of Abbreviations

PDAC  pancreatic ductal adenocarcinoma
PanIN  pancreatic intraepithelial neoplasia
FOLFIRINOX  fluorouracil, irinotecan, oxaliplatin, and leucovorin
hNT  human nucleoside transporter
hENT  human equilibrative nucleoside transporter
hCNT  human concentrative nucleoside transporter
dCK  deoxycytidine kinase
RRM1, RRM2  ribonucleotide reductase subunits
EMT  epithelial-to-mesenchymal transition
CYR61  cysteine-rich angiogenic inducer 61
CTGF  connective tissue growth factor
NOV  nephroblastoma overexpressed
WISP  WNT1 inducible signaling pathway protein
HSPG  heparin sulfate proteoglycan
SP  N-terminal secretory signal peptide
IGFBP  insulin-like growth factor-binding domain
vWC  von Willebrand factor type C repeat
TSR  thrombospondin type I repeat
<table>
<thead>
<tr>
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<th>Description</th>
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<tr>
<td>CT</td>
<td>cysteine knot-containing carboxyl domain</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia inducible factor 1</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element-binding protein</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>PSC</td>
<td>pancreatic stellate cell</td>
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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>Cal</td>
<td>calcipotrial</td>
</tr>
<tr>
<td>Smo</td>
<td>smoothened</td>
</tr>
<tr>
<td>Hh</td>
<td>hedgehog</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>TβRII</td>
<td>type II TGF-β receptor</td>
</tr>
<tr>
<td>ALK5</td>
<td>type I TGF-β receptor (TβRI)</td>
</tr>
<tr>
<td>CAFs</td>
<td>carcinoma-associated fibroblasts</td>
</tr>
<tr>
<td>NBMPR</td>
<td>S-(4-Nitrobenzyl)-6-thioinosine (hENT1 inhibitor)</td>
</tr>
<tr>
<td>GEO</td>
<td>Gene Expression Omnibus</td>
</tr>
<tr>
<td>ICGC</td>
<td>International Cancer Genome Consortium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PDX</td>
<td>patient-derived xenograft</td>
</tr>
<tr>
<td>CA-ALK5</td>
<td>constitutively active version of the TGF-β receptor ALK5</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescent</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β Activated Kinase 1</td>
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1. Introduction

1.1 Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth leading cause of cancer death in the United States, with 41,780 patient deaths estimated in 2016 (Siegel et al., 2014), and it is projected to become the second leading cause of cancer death by 2030 due to increased incidence and lack of improvement in survival compared to other cancers (Rahib et al., 2014). PDAC has a five-year survival rate of only 7%, and this has remained largely unchanged over the last decades (Siegel et al., 2014), illustrating the need for continued research on what makes PDAC so resistant to therapy. PDAC originates in the pancreatic ductal epithelium of the exocrine pancreas and most commonly develops from precursor lesions called pancreatic intraepithelial lesions (PanIN) (Hidalgo, 2010). At lower frequencies, PDAC can also develop from intraductal papillary mucinous neoplasms or mucinous cystic neoplasms (Gharibi et al., 2016).

The transition from early PanIN lesions to invasive carcinoma occurs through a stepwise progression involving the accumulation of mutations. In early PanIN-1 lesions, mutation of the KRAS oncogene occurs in 90-95% of PDAC patients and results in activation of signaling pathways regulating proliferation and survival. During the transition to PanIN-2 and PanIN-3 lesions, mutations frequently occur to delete or inactivate the tumor suppressors CDKN2A/INK4, TP53, and SMAD4. CDKN2A/INK4
normally inhibits cell cycle progression through the G1-S checkpoint, and mutations causing loss of function occur in over 90% of PDAC, usually during the PanIN-2 stage (Gharibi et al., 2016). TP53 is a key regulator of DNA damage and can promote cell cycle arrest and apoptosis, and it is inactivated in 50%-75% of patients (Gharibi et al., 2016). SMAD4 is a transcription factor that mediates the growth inhibition induced by the transforming growth factor-β (TGF-β) signaling pathway, and it is lost in 55% of patients, most commonly in the PanIN-3 stage (Gharibi et al., 2016). Additional mutations occurring at a lower frequency have also been identified in PDAC patients (Gharibi et al., 2016), and the collective accumulation of these mutations promotes the transition from PanIN lesions to invasive carcinoma.

Only 9% of PDAC is diagnosed at an early stage while still confined to the pancreas, and the 5-year survival rate of these patients is still quite low at 26% (Gharibi et al., 2016). PDAC is most often diagnosed after it is already locally advanced or metastatic because it is so difficult to detect during the pre-invasive stage due to lack of physical symptoms and lack of reliable biomarkers or imaging techniques. Since the majority of patients are diagnosed after the cancer has spread from the pancreas, systemic chemotherapy is frequently the first-line treatment (Garrido-Laguna et al., 2015).
1.2 Gemcitabine Chemotherapy

1.2.1 Clinical Overview

Gemcitabine (2’,2’-difluoro-2’-deoxycytidine) is a nucleoside pyrimidine analog of deoxycytidine created by replacing the hydrogen atoms on the 2’ carbon of deoxycytidine with fluorine atoms (de Sousa Cavalcante et al., 2014). For the last two decades, gemcitabine has been the backbone of chemotherapy for PDAC, and it is indicated for first and second line treatment, as well as adjuvant treatment, for patients with locally advanced or metastatic PDAC (Silvestris et al., 2016). Gemcitabine was approved for PDAC in 1995 after a randomized phase III trial demonstrated an improvement in survival over fluorouracil treatment (Burris et al., 1997). It works by interfering with DNA synthesis and inducing apoptosis of dividing cells. While gemcitabine is one of the most effective and commonly used treatments for PDAC, as a single agent it prolongs median survival by just over a month on average (Burris et al., 1997) and is not effective for all patients. Attempts to enhance gemcitabine efficacy with targeted agents or other cytotoxic agents, with the exception of nab-paclitaxel (Garrido-Laguna et al., 2015), have had limited success. Recently, a chemotherapy regimen without gemcitabine, consisting of fluorouracil, irinotecan, oxaliplatin, and leucovorin (FOLFIRINOX), was approved for PDAC patients, but this regimen is very toxic and still not effective for all patients (Conroy et al., 2011). The high rate of therapy resistance is a major clinical problem in PDAC, and understanding the molecular mechanisms that
drive resistance to gemcitabine will help to identify drug targets that will improve the response rate.

1.2.2 Molecular Mechanism of Action

1.2.2.1 Cellular uptake

Since gemcitabine is hydrophilic (de Sousa Cavalcante et al., 2014), it must be transported into the cell through the hydrophobic plasma membrane by transmembrane nucleoside transporters (hNT) (Figure 1). The equilibrative nucleoside transport (hENT) family mediates bidirectional transport of nucleosides across the plasma membrane along the concentration gradient (Baldwin et al., 2004), while the concentrative nucleoside transport (hCNT) family concentrates nucleosides in the cell by coupling transport of nucleosides and cations down the electrochemical gradient (Gray et al., 2004). The hENT family has four members (hENT1, hENT2, hENT3, hENT4), but hENT3 and hENT4 are localized to intracellular organelles and do not mediate cellular uptake of gemcitabine (Endo et al., 2007). hENT1 has a much higher affinity for gemcitabine than hENT2 and mediates the cellular uptake of the majority of gemcitabine in PDAC cells (de Sousa Cavalcante et al., 2014). The hCNT family has three members (hCNT1, hCNT2, hCNT3), but hCNT2 only binds to purine nucleosides and is not able to transport gemcitabine since it is an analog of the pyrimidine nucleoside deoxycytidine (Gray et al., 2004). Transport assays examining gemcitabine import using radiolabeled
$^3$H-gemcitabine indicate that the hENT1 and hCNT3 nucleoside transporters both have important roles in the cellular uptake of gemcitabine (Paproski et al., 2013).

1.2.2.2 Intracellular mechanisms

Once inside the cell, gemcitabine is phosphorylated by the enzyme deoxycytidine kinase (dCK), which is the rate-limiting step in the production of gemcitabine triphosphate (de Sousa Cavalcante et al., 2014) (Figure 1). Once it is in the triphosphate form, gemcitabine is incorporated into DNA during DNA synthesis. After addition of gemcitabine triphosphate to the DNA, DNA polymerase is only able to add one more deoxynucleotide, resulting in masked-chain termination and induction of apoptosis. Due to the addition of this single additional deoxynucleotide during masked chain termination, DNA repair enzymes are not able to remove gemcitabine triphosphate, and DNA synthesis is stopped. Gemcitabine can also covalently bind to the active site of the enzyme ribonucleotide reductase, which catalyzes the conversion of ribonucleotides to deoxyribonucleotides. The binding of gemcitabine to ribonucleotide reductase blocks activity and results in a decrease in the intracellular deoxynucleotide pool, which further inhibits DNA synthesis (de Sousa Cavalcante et al., 2014).
Gemcitabine enters the cell through the nucleoside transporters hENT1 and hCNT3. Phosphorylation of gemcitabine by deoxycytidine kinase (dCK) is the rate-limiting step in the production of active gemcitabine triphosphate. Gemcitabine induces apoptosis of cells by blocking DNA synthesis and inhibiting the enzyme ribonucleotide reductase. Figure adapted from (de Sousa Cavalcante et al., 2014)

**Figure 1: Gemcitabine Metabolism**

![Diagram of Gemcitabine Metabolism](image)
1.2.3 Mechanisms of Resistance

PDAC patients with low hENT1 and hCNT3 protein expression have significantly worse survival after gemcitabine treatment compared to patients with high hENT1 and hCNT3 protein expression (Fujita et al., 2010; Z. Q. Liu et al., 2014; Marechal et al., 2012; Marechal et al., 2009), suggesting low expression of these nucleoside transporters mediates resistance clinically. Patients with high expression of both hENT1 and hCNT3 protein do significantly better than patients with high expression of only one of these nucleoside transporters (Marechal et al., 2009) (Figure 2). Therefore, it will be important to understand what regulates their expression in order to develop strategies to increase their expression in PDAC.
A cohort of PDAC patients receiving adjuvant gemcitabine after surgery. hENT1 and hCNT3 expression evaluated by immunostaining (Marechal et al., 2009) (A) Graph shows overall survival hazard ratio for multivariate analysis of hENT1 high (n=19) and low (n=26) patients and hCNT3 high (n=22) and low (n=26) patients. Hazard ratio (95% CI) for low hENT1 is 3.42 (1.44-8.81) p=0.005. The hazard ratio (95% CI) for low hCNT3 is 2.65 (1.19-5.87) p=0.017. (B) % Survival at 3 years after surgery for patients with low expression of both hENT1 and hCNT3 (n=11), high expression of either hENT1 or hCNT3 (n=19), and high expression of both hENT1 and hCNT3 (n=15). The 3-year survival was 23.1% (95% CI, 11.5-34.7; P < 0.0001), 24.9% (95% CI, 14.1-36.7), and 81.1% (95% CI, 71.4-90.8).
A recent meta-analysis of 12 studies shows that low hENT1 expression is significantly associated with worse survival in patients receiving gemcitabine (Z. Q. Liu et al., 2014). In fact, hENT1 expression is being considered as a promising biomarker to predict patient response to gemcitabine (Elnaggar et al., 2012). Overexpression of hENT1 in xenograft model of PDAC improves gemcitabine efficacy (Perez-Torras et al., 2008), suggesting that identifying methods to enhance hENT1 levels clinically might improve efficacy of treatment. In addition to nucleoside transporter expression, low expression of the dCK, the kinase that phosphorylates gemcitabine, is associated with poor survival in patients receiving gemcitabine (Fujita et al., 2010; Marechal et al., 2012). High expression of the subunits of ribonucleotide reductase (RRM1, RRM2) is also associated with resistance to gemcitabine (Duxbury et al., 2004; X. Zhang et al., 2013b). Studies also indicate that increased expression of drug-efflux pumps that are capable of exporting gemcitabine from the cell is associated with resistance. The drug efflux pumps ABCB1 (MDR1/P-glycoprotein) and ABCC1 (MRP1) are important in the resistance of PDAC cells to gemcitabine (Nath et al., 2013; W. Zhang et al., 2013a).

### 1.2.4 Regulation of hENT1 and hCNT3 Nucleoside Transporters

The molecular mechanisms regulating the expression of the hENT1 and hCNT3 nucleoside transporters in the PDAC microenvironment are largely unknown. A recent study suggests that the epithelial-to-mesenchymal transition (EMT) negatively regulates hENT1 and hCNT3 expression. The authors blocked EMT by deleting the EMT master
transcription factors Twist and Snail in the KPC mouse model of PDAC (Ptf1a<sup>cre</sup>;LSL-Kras<sup>G12D/+;Trp53<sup>R172H/+</sup></sup>) and found this increased hENT1 and hCNT3 protein expression in vivo by two fold (Zheng et al., 2015). However, further studies are needed to identify mechanisms that regulate their expression in the PDAC microenvironment and identify therapeutic targets to up-regulate their expression and improve gemcitabine efficacy. In particular, high expression of both hENT1 and hCNT3, not just one, is associated with better gemcitabine response (Figure 2), so targeting negative regulators common to both might achieve the best result.

Several studies have examined the molecular mechanisms regulating hENT1 expression in cell contexts other than PDAC. In endothelial cells, TGF-β (Vega et al., 2009), high D-glucose (Puebla et al., 2008) and hypoxia-inducible factor-1 (HIF-1) (Eltzschig et al., 2005) negatively regulate adenosine uptake through hENT1 by decreasing hENT1 transcription, but their contribution to regulation of hENT1 expression and gemcitabine transport in PDAC remains to be studied. In ovarian cancer cells, the nuclear transcription factor PPARα (Peroxisome Proliferator Activated Receptor α) induces hENT1 expression by binding to its promoter (Montero et al., 2012), but this has not been examined in PDAC. In addition, protein kinase C is emerging as an inducer of hENT1 activity through phosphorylation of serine and threonine residues (Coe et al., 2002; Hughes et al., 2015; Reyes et al., 2011). In mouse myeloid leukemia cells, hENT1 is negatively regulated at the transcriptional level by c-Jun N-terminal
kinase (JNK) signaling (Leisewitz et al., 2011). Less is known about the molecular mechanisms regulating hCNT3 expression and function. It has recently been demonstrated that Erb-b2 receptor tyrosine kinase 2 (ErbB2, HER2), a member of the epidermal growth factor receptor family, negatively regulates hCNT3 expression in PDAC cells (Skrypek et al., 2015). In addition, increased expression of the membrane-bound glycoprotein Mucin 4 (MUC4) negatively regulates hCNT3 expression in PDAC (Skrypek et al., 2013). The sorting of hCNT3 to the cell surface and regulation of its turnover at the plasma membrane are regulated by an interaction with Galectin-4 (Fernandez-Calotti et al., 2015).

Since gemcitabine will continue to be the backbone of treatment and will be used in many clinical studies in PDAC, it is urgent that we identify the mechanisms regulating the expression of nucleoside transporters whose down-regulation mediates clinical resistance. Currently, little is known about the molecular mechanisms regulating their expression in the PDAC microenvironment.

1.3 Cysteine-Rich Angiogenic Inducer 61

1.3.1 Cysteine-Rich Angiogenic Inducer 61

Cysteine-rich angiogenic inducer 61 (CYR61) is a member of the CCN family of secreted matricellular proteins, which includes CTGF (Connective tissue growth factor), NOV (Nephroblastoma overexpressed), WISP-1 (WNT1 inducible signaling pathway protein 1), WISP-2, and WISP-3 (Lau, 2011). The CCN family regulates many diverse
cell behaviors, primarily through interacting with integrins and heparin sulfate proteoglycans (HSPGs) on the cell surface. The CYR61 protein contains 5 structural domains: an N-terminal secretory signal peptide (SP), an insulin-like growth factor-binding domain (IGFBP), a von Willebrand factor type C repeat (vWC), a thrombospondin type I repeat (TSR), and a cysteine knot-containing carboxyl domain (CT) (Figure 3) (Leask et al., 2006). Deletion of CYR61 in mice is embryonic lethal, with embryos displaying vascular defects, including failing to form chorioallantoic fusion or developing placental vascular insufficiency, loss of vascular integrity, hemorrhage, and severe cardiac atroventricular septal defect (Mo et al., 2002). CYR61 heterozygous mice are viable, but 20% of the mice exhibit persistent ostium primum atrial septal defects (Mo et al., 2006).
The matricellular protein CYR61 contains 5 domains: N-terminal secretory signal peptide (SP), insulin-like growth factor-binding protein (IGFBP), von Willebrand factor type C repeat (vWC), thrombospondin type I repeat (TSR), and cysteine knot containing carboxy terminal domain (CT). CYR61 binds to integrins and heparin sulfate proteoglycans (HSPGs) on the cell surface, which leads to activation of downstream signaling, including the FAK, Akt, and Erk signaling pathways. Figure adapted from (Leask et al., 2006)

CYR61 does not bind integrins through a canonical RGD sequence, but non-canonical binding sites for integrins αvβ3, αvβ5, α6β1, αvβ5, and αMβ2 have been identified in CYR61 (N. Chen et al., 2000; Jedsadayanmata et al., 1999; Kireeva et al., 1998; Leask et al., 2006; Schober et al., 2002). In addition, CYR61 can interact with the heparin sulfate proteoglycans (HSPGs) syndecan 4 and perlecan which act as co-receptors, through a heparin-binding domain (N. Chen et al., 2000). CYR61 has diverse and sometimes conflicting functions in biology. These conflicting roles of CYR61 in biology can be partially explained by the interaction with different integrins and HSPGs in different cell
contexts (Lau, 2011) (Table 1). The biological effects of CYR61 can also depend on the presence of other growth factors and cytokines as CYR61 can regulate biology by modifying the function of ECM components and growth factors, for example interacting with fibronectin or TGF-β (Figure 3).
Table 1: CYR61 Responses and Integrins Involved. Adapted from Lau, 2011.

<table>
<thead>
<tr>
<th>CYR61 Cellular responses</th>
<th>Cells types and cell-surface receptors involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell adhesion</td>
<td>Fibroblasts – α6β1 and HSPGs</td>
</tr>
<tr>
<td></td>
<td>Activated endothelial cells – αvβ3; unactivated endothelial cells – α6β1</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle cells – α6β1 and HSPGs</td>
</tr>
<tr>
<td></td>
<td>Platelets – αIIbβ3</td>
</tr>
<tr>
<td></td>
<td>Monocytes – αMβ2</td>
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<tr>
<td></td>
<td>Macrophages – αMβ2</td>
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<tr>
<td></td>
<td>Macrophage foam cells – αDβ2</td>
</tr>
<tr>
<td>Migration</td>
<td>Fibroblasts – αvβ5</td>
</tr>
<tr>
<td></td>
<td>Microvascular endothelial cell chemotaxis – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle cell chemotaxis – α6β1 and HSPGs</td>
</tr>
<tr>
<td>DNA synthesis/proliferation</td>
<td>Fibroblasts – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Astrocytoma cells – α5, α6, and β1</td>
</tr>
<tr>
<td>Survival</td>
<td>Endothelial cells – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Breast cancer cells – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Cardiac myocytes – β1</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Fibroblasts – α6β1 and syndecan 4</td>
</tr>
<tr>
<td></td>
<td>Synergism with TNF-α in fibroblasts – α6β1, αvβ5, and syndecan-4</td>
</tr>
<tr>
<td></td>
<td>Synergism with FasL in fibroblasts – α6β1 and HSPGs</td>
</tr>
<tr>
<td></td>
<td>Synergism with TRAIL in PC3 cells – αvβ3, α6β4 and syndecan-4</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Osteoblastic differentiation – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Endothelial tubule formation – αvβ3</td>
</tr>
<tr>
<td>Gene expression</td>
<td>Chondrosarcoma cells, MMP13 expression – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Macrophages, M1 genetic program – αMβ2</td>
</tr>
<tr>
<td></td>
<td>MCF7 breast cancer cells – αvβ3</td>
</tr>
<tr>
<td>Invasiveness</td>
<td>Gastric adenocarcinoma cells – αvβ3</td>
</tr>
<tr>
<td></td>
<td>Oral squamous cell carcinoma line – αvβ5</td>
</tr>
<tr>
<td>Senescence</td>
<td>Fibroblasts – α6β1 and HSPGs</td>
</tr>
</tbody>
</table>
Major biological roles of CYR61 include promoting adhesion, ECM interaction, and migration across many cell types (Table 1). CYR61 can also stimulate angiogenesis by promoting endothelial cell migration, adhesion, tube formation, and expression of vascular endothelial growth factor (VEGF) (Leu et al., 2002). Mutation of the integrin αβ3-binding domain of CYR61 abrogates the angiogenic activity of CYR61 (N. Chen et al., 2004). CYR61 is also an important regulator of inflammation and wound repair. During wound healing, myofibroblast cells in the wound proliferate and synthesize ECM components to promote repair of the tissue. This ECM synthesis must be suppressed during completion of the wound healing to prevent fibrosis, scarring, and loss of tissue function (Gurtner et al., 2008). Studies suggest that CYR61 can promote senescence of myofibroblasts in later stages of wound healing (Jun et al., 2010). In knockin mice where CYR61 is replaced with an allele of CYR61 that is not capable of integrin αβ1- HSPG interaction, CYR61 cannot induce senescence of myofibroblasts (Jun et al., 2010).

CYR61 has also been demonstrated to induce or suppress apoptosis in a context- and cell-specific manner. For example, binding of CYR61 to integrin αβ3 induces cell survival in endothelial cells (Leu et al., 2002) and breast cancer cells (M. T. Lin et al., 2004), while binding of CYR61 to integrin αβ1 can promote apoptosis in fibroblasts through p53-dependent activation of Bax and release of cytochrome c (Todorovic et al., 2005). The apoptotic function of CYR61 is relatively modest without combination of
other apoptotic factors, like TNF-α, FasL, and TRAIL (C. C. Chen et al., 2010). Knockin mice with CYR61 replaced with a an allele of CYR61 that is not capable of binding integrin α6β1 or HSPGs are resistant to concanavalin A-induced TNF-α-dependent hepatocyte apoptosis (Sherman et al., 2014).

In summary, CYR61 influences a wide range of biological processes in a context-specific manner. Given the important biological functions of CYR61, further studies are needed to clarify the impact of CYR61 in diseases like PDAC.

1.3.2 Role of CYR61 in Pancreatic Ductal Adenocarcinoma

Only a few studies have examined the role of CYR61 in PDAC, and little is known about its function in tumor biology. The first observation of a role for CYR61 in PDAC was a study demonstrating a six fold increase in expression of CYR61 in peritoneal metastases in an orthotopic xenograft mouse model using the Capan-1 cell line (Holloway et al., 2005), suggesting a potential role in metastasis. Inhibition of CYR61 through shRNA knockdown or neutralizing antibody made PANC1 cancer cells more epithelial and inhibited their migration (Haque et al., 2011). In this model, CYR61 expression was higher in a side population of PANC1 cells enriched for cancer stem cell traits and tumor initiating capability, and CYR61 knockdown inhibited tumor formation in a subcutaneous xenograft assay (Haque et al., 2011). Knockdown of CYR61 also decreased Sonic Hedgehog (Shh) signaling by decreasing expression of the Shh ligand, the Smoothened receptor, and the nuclear transcription factor GLI family zinc finger
(Gli) in PANC1 cells (Haque et al., 2012). Recombinant CYR61 induced expression of Shh and active Notch-1, which was inhibited by an integrin β3 blocking antibody (Haque et al., 2012). CYR61 derived from PANC1 cells has also been implicated in promoting endothelial cell migration and angiogenesis in an in vitro endothelial tube formation assay using human umbilical vein endothelial cells (HUVEC) (Maity et al., 2014). One recent report suggests that CYR61 promotes cell proliferation in PDAC cells by activating PI3K-Akt signaling to promote nuclear exclusion of the cell cycle inhibitor p27, although this study was performed in PANC1 cells which have very high basal CYR61 expression (Shi et al., 2014). In summary, CYR61 promotes EMT and angiogenesis in PDAC and mediates the Shh, Notch-1, and Akt signaling pathways. These studies have only begun to examine the role of CYR61 in PDAC, and additional studies are needed to determine whether CYR61 has the potential to be a therapeutic target in PDAC.

1.3.3 Role of CYR61 in Other Cancers

While the role of CYR61 in PDAC is still largely unknown, many studies have examined the role of CYR61 in other cancer contexts. Regulation of cancer cell migration is one major role that has emerged for CYR61 in cancer. Knockout and overexpression studies have demonstrated that CYR61 promotes cell migration in oral squamous cell carcinoma (Chuang et al., 2012; Tanaka et al., 2013), thyroid cancer (Chin et al., 2015), chondrosarcoma (Tan et al., 2009), prostate cancer (Sun et al., 2008),
osteosarcoma (J. Chen et al., 2013), and breast cancer (Jim Leu et al., 2013). Mechanistically, this enhanced cell migration frequently occurs via induction of EMT and activation of signaling downstream of integrins, including focal adhesion kinase, Rac, RhoA, and Erk. Further, CYR61 promotes tumorigenesis in xenograft models of breast cancer (J. Lin et al., 2012; D. Xie et al., 2001), gastric cancer (Babic et al., 1998), prostate cancer (Sun et al., 2008), and glioma (D. Xie et al., 2004a). In vitro studies indicate that CYR61 promotes anchorage-independent growth in breast cancer (D. Xie et al., 2001), prostate cancer (Sun et al., 2008), esophageal squamous cell carcinoma (J. J. Xie et al., 2011b), and glioma (D. Xie et al., 2004a) cell lines. Consistent with its established role in angiogenesis, several studies have demonstrated that CYR61 promotes vascularization and angiogenesis in cancer, including breast cancer (D. Xie et al., 2001) and glioma (D. Xie et al., 2004a). Clinical data indicates that high expression of CYR61 is associated with poor prognosis in colorectal cancer (Jeong et al., 2014), ovarian cancer (Shen et al., 2014), esophageal squamous cell carcinoma (J. J. Xie et al., 2011a), endometrioid adenocarcinoma (Watari et al., 2009), and glioma (D. Xie et al., 2004b). A functional polymorphism in the promoter region of CYR61 results in lower expression, and men carrying this allele have a significantly lower risk of developing prostate cancer (Tao et al., 2013).

Taken together, these studies suggest a tumor-promoting role for CYR61 across many cancer types. However, other studies have found a tumor suppressor role for
CYR61. In non-small cell lung cancer, ectopic expression of CYR61 inhibits colony formation and proliferation *in vitro* and inhibits tumor growth in a xenograft assay, and CYR61 expression is decreased at the mRNA level in tumor samples compared to matched normal lung tissue (Tong et al., 2001). Supporting this work, another study demonstrated that lung cancer patients with high CYR61 expression have longer survival (P. P. Chen et al., 2007). CYR61 expression is decreased in hepatocellular carcinoma (HCC) tumors, and overexpression of CYR61 in HCC cell lines inhibited proliferation, anchorage-independent growth, migration, and invasion (Feng et al., 2008). In addition, mice with hepatocyte-specific deletion of CYR61 have increased tumor formation following treatment with the carcinogen diethylnitrosamine, and this effect was phenocopied in mice with a knock-in mutant CYR61 that is not able to bind integrin α6β1 (C. C. Chen et al., 2015). The authors found that CYR61 functions to inhibit epidermal growth factor receptor (EGFR)-dependent hepatocyte proliferation, and the EGFR inhibitor erlotinib blocked the increased diethylnitrosamine-induced tumor formation in mice with hepatocyte-specific deletion of CYR61. Another study contradicted this finding by demonstrating that CYR61 expression in increased in HCC, and overexpression of CYR61 in HepG2 HCC cells promoted xenograft formation (Li et al., 2012). The role of CYR61 in HCC needs to be further examined to understand these conflicting studies. In multiple myeloma, overexpression of CYR61 was associated with longer overall survival, and overexpression of CYR61 in multiple myeloma cells
inhibited tumor growth in a mouse xenograft model (Johnson et al., 2014). Similarly, in melanoma, CYR61 overexpression reduced tumor growth, metastases, cell motility, invasion, and angiogenesis while increasing apoptosis (Dobroff et al., 2009). These studies suggest that CYR61 can act a tumor suppressor in lung cancer, HCC, multiple myeloma, and melanoma.

CYR61 has a complex role in cancer, where it regulates a wide range of biological processes and can act as either a tumor suppressor or a tumor promoter in different contexts. In prostate cancer, CYR61 can both promote proliferation and adhesion while also promoting apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Franzen et al., 2009), illustrating that CYR61 can have multiple effects even within one type of cancer. It will be essential to gain an understanding of its function and contribution to cancer cell biology to determine whether CYR61 is a promising target in various cancers.

### 1.3.4 Role of CYR61 in Chemoresistance

While it is not known whether CYR61 regulates gemcitabine resistance in PDAC, CYR61 has recently been implicated in therapy resistance in other cancers, as summarized in Figure 4. In renal cell carcinoma, CYR61 confers resistance to vinblastine chemotherapy via up-regulation of the drug-efflux pump MDR1 (P-glycoprotein/ABCB1) downstream of integrin αvβ3–PI3K-Akt signaling (Q. Z. Long et al., 2013). Several reports have also implicated CYR61 in therapy resistance in breast
cancer. CYR61 overexpression in breast cancer cells promotes resistance to paclitaxel, adriamycin, and β-lapachone through induction of the anti-apoptotic protein XIAP downstream of integrin-NFκB signaling (M. T. Lin et al., 2004). Another report shows that CYR61 promotes resistance to taxol-induced cytotoxicity in breast cancer through the integrin αvβ3–Erk signaling pathway (Menendez et al., 2005). Recently, CYR61 has been demonstrated to promote mitoxantrone resistance in acute myeloid leukemia via activation of Spleen Tyrosine Kinase (X. Long et al., 2015). In ovarian cancer, CYR61 protects against carboplatin-induced apoptosis by decreasing expression of the pro-apoptotic factor Bax and increasing expression of the anti-apoptotic factors BxL-xL, Mcl-1, and Bxl-2 (K. B. Lee et al., 2012). These studies suggest a potential role for CYR61 in promoting therapy resistance across several cancer types, so it is important to identify what role CYR61 plays in PDAC, particularly in resistance to gemcitabine since it is the most common chemotherapy drug in PDAC.
CYR61 promotes resistance to therapy in several cancer contexts, including breast cancer, ovarian cancer, and renal cell carcinoma. Many of these effects are mediated through integrin αvβ3 signaling.

1.3.5 Regulation of CYR61 Expression

The molecular mechanisms regulating CYR61 in the PDAC microenvironment remain to be studied. In other cellular contexts, the TGF-β and the Hippo-YAP/TAZ pathways are major regulators of CYR61 transcription (Figure 5). TGF-β induces CYR61 transcription through binding of Smad proteins to consensus sequences in the promoter (Bartholin et al., 2007). In osteosarcoma cells, TGF-β induces CYR61 expression in a p38 MAPK-dependent manner (J. Chen et al., 2013). The Hippo pathway was found to activate CYR61 transcription in breast cancer cells through binding of a YAP/TAZ-
TEAD4 complex to two TEAD response elements in the CYR61 promoter (Lai et al., 2011)

While TGF-β and Hippo-YAP/TAZ signaling are established transcriptional regulators of CYR61, other factors regulate CYR61 expression in various cell contexts. Inflammatory cytokines like interleukin-1, tumor necrosis factor α as well as growth factors like platelet-derived growth factor, fibroblast growth factor, and VEGF all been reported to regulate CYR61 expression in various cell contexts (Lau, 2011). Hypoxia can induce CYR61 through binding of a complex containing HIF-1α, c-Jun/AP-1 to the promoter of CYR61 in monkey choroidal retinal vascular endothelial cells (You et al., 2010). Further, CYR61 is induced by estrogen in breast and ovarian cancer cell lines (Gery et al., 2005; D. Xie et al., 2001). In hepatocellular carcinoma, CYR61 expression is induced by β-catenin binding to TCF4 binding sites in the CYR61 promoter (Li et al., 2012), although reports of increased CYR61 expression in HCC are conflicting. In thyroid cancer, CYR61 is induced by EGF via Erk signaling and cAMP-response element-binding protein (CREB) (Chin et al., 2015). However, in melanoma, CREB directly binds to the CYR61 promoter and negatively regulates its transcription (Dobroff et al., 2009). Hypoxia has also been demonstrated to induce CYR61 through CREB binding to the promoter (Meyuhas et al., 2008), which suggests that CREB can both induce and inhibit CYR61 expression in a context-specific manner.
In summary, CYR61 transcription is regulated in a context-specific manner by numerous growth factors and transcription factors. Alternative splicing and post-translational glycosylation have been reported for CYR61 (Lau, 2011), but there is very little information on the significance of these modifications or the post-transcriptional regulation of CYR61 expression. Studies to date suggest that CYR61 is largely regulated transcriptionally, but additional work needs to be done to identify how CYR61 translation and protein stability are regulated.

**Figure 5: Regulation of CYR61 Transcription. Adapted from (Jun et al., 2011)**

CYR61 transcription is regulated by the Transforming Growth Factor-β (TGF-β) signaling pathway and the Hippo-YAP/TAZ signaling pathway. Additionally CYR61 expression can be regulated hypoxia as well as signaling downstream of receptor tyrosine kinases, interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα).
1.4 *Pancreatic Stellate Cells in the Fibrotic Tumor Stroma*

1.4.1 The Role of Pancreatic Stellate Cells in PDAC

PDAC is characterized by an abundant fibrotic stroma that can comprise up to 80% of the tumor volume (Erkan et al., 2012) making this stroma perhaps the most prominent of all epithelial cancers. The stroma is largely composed of extracellular matrix (ECM) proteins like collagen and fibronectin. There is great interest in understanding how the fibrotic tumor stroma influences cancer progression and resistance to therapy.

The pancreatic stellate cell (PSC) is the predominant cell type responsible for secretion of the ECM components that comprise the fibrotic stroma (Apte et al., 2004; Bachem et al., 2005; Yen et al., 2002). Quiescent PSCs normally are located in the periacinar space in the exocrine pancreas, where they make up around 4% of pancreatic cells and function as a storage site for vitamin A lipid droplets (Apte et al., 2004; Bachem et al., 1998). In PDAC and fibrotic conditions, including acute and chronic pancreatitis, PSCs are activated to a myofibroblast state that is characterized by increased proliferation and excessive secretion of ECM components, matrix-remodeling enzymes, growth factors, and cytokines (Omary et al., 2007). Myofibroblast activation of PSCs is a normal aspect of injury repair, but persistent, pathological activation results in fibrosis. Activation from the quiescent to the myofibroblast state is marked by expression of the
cytoskeletal protein α-smooth muscle actin (α-SMA) and disappearance of the cytoplasmic vitamin A lipid droplets (Omary et al., 2007).

Several in vitro co-culture studies have demonstrated that activated PSCs can promote cancer progression and resistance to chemotherapy (McCarroll et al., 2014; Vonlaufen et al., 2008; H. Zhang et al., 2015). Treatment with conditioned media from PSCs increased proliferation, migration, and invasion of PDAC cells (Vonlaufen et al., 2008). PSCs secrete stromal cell-derived factor 1 α (SDF-1α), which signals to C-X-C chemokine receptor type 4 (CXCR4) on PDAC cells to promote chemoresistance to gemcitabine (H. Zhang et al., 2015). In addition, co-injection of PSCs in xenograft assays with PDAC cells increased tumor frequency, tumor size, and metastasis (Bachem et al., 2005; Hwang et al., 2008; Schneiderhan et al., 2007; Vonlaufen et al., 2008), suggesting that PSCs promote tumor progression. Chronic pancreatitis is a major risk factor for the development of PDAC, which supports that fibrosis and PSC activation promote cancer initiation and progression (Lowenfels et al., 2005). However, a recent study indicated that PSCs might play a more complicated role in cancer progression than previously appreciated. The authors depleted α-SMA positive PSCs in two genetic mouse models of PDAC, the PKT model (Ptf1a<sup>cre/+</sup>; LSL-Kras<sup>G12D+/+</sup>; Tgfbr2<sup>flx/flx</sup>) and the KPC model (Ptf1a<sup>cre/+</sup>; LSL-Kras<sup>G12D+/+</sup>; Trp53<sup>R172H+/+</sup>). Surprisingly, depletion of myofibroblast PSCs led to more undifferentiated tumors, reduced survival, and failed to improve the efficacy of gemcitabine (Ozdemir et al., 2014). This study contradicted the results of the xenograft
assays and *in vitro* studies that found PSCs promoted cancer growth and progression. A more recent study converted PSCs from the activated myofibroblast state to the normal quiescent state by treating with the vitamin D analog calcipotriol (Cal) in the KPC genetic mouse model of PDAC (Sherman et al., 2014). While treatment with Cal alone demonstrated no effect, treatment with Cal in combination with gemcitabine prolonged survival of the mice and reduced tumor volume. This study suggests that activated myofibroblast PSCs normally promote resistance to gemcitabine, and that converting myofibroblast PSCs to their quiescent state might have a clinical benefit in PDAC. The authors argue that stromal reprogramming, not stromal depletion, will enhance chemotherapy efficacy. Taken together, these *in vitro* and *in vivo* studies suggest that there are aspects of the interaction of PSCs with cancer cells that contribute to cancer progression and therapy resistance and aspects that regulate normal tissue homeostasis. Identifying which aspects of the stroma to target clinically will be important for development of effective therapies in PDAC. Further studies are needed to understand the complex signaling interaction of the fibrotic stroma with cancer cells, particularly in PDAC where the stroma comprises up to 80% of the tumor volume.

### 1.4.2 Targeting the PDAC Fibrotic Stroma in Clinical Trials

#### 1.4.2.1 Sonic Hedgehog

Hedgehog (Hh) signaling is activated when the Hh ligand binds to the Patched 1 protein on the cell surface, leading to activation of the Smoothened (Smo) receptor and
downstream activation of the GLI transcription factors (Hwang et al., 2012). Pancreatic cancer cells express high levels of the Hedgehog (Hh) ligand while PSCs express high levels of the Smo receptor (Hwang et al., 2012). While overexpression of Hh ligand in PDAC promoted tumorigenesis, deletion of Smo from the tumor epithelium had no effect on PDAC progression (Nolan-Stevaux et al., 2009), which indicates that Hh signaling affects cancer progression primarily through signaling in stromal PSCs. In 2009, a group found that the efficacy of gemcitabine in the KPC mouse model of PDAC could be improved by co-treatment with the Hh inhibitor IPI-926 through depletion of the stroma and transiently increasing intratumoral vascular density to enhance delivery of gemcitabine to the tumor cells (Olive et al., 2009). However, a phase II clinical trial of IPI-926 in combination with gemcitabine for metastatic PDAC patients demonstrated that patients receiving IPI-926 surprisingly had shorter median overall survival than the gemcitabine control arm. Similarly, another clinical trial using the small molecule Hh inhibitor GDC-0449 combined with gemcitabine in metastatic PDAC demonstrated no improvement over gemcitabine alone (E. J. Kim et al., 2014). These results demonstrate that targeting Hh signaling in the stroma may actually promote cancer progression. Two recent reports further studied the role of inhibiting stromal Hh signaling using genetic mouse models of PDAC. Inhibition of the Hh pathway genetically or through pharmacological inhibition resulted in accelerated tumor progression (J. J. Lee et al., 2014; Rhim et al., 2014). Interestingly, treatment of mice with the Hh inhibitor
suppressed stromal desmoplasia while promoting increased tumor progression, and conversely a small molecule agonist enhanced stromal desmoplasia while inhibiting cancer progression (J. J. Lee et al., 2014). These studies suggest that Hh signaling in the stroma can restrain tumor growth and could explain the surprising results of the recent clinical trials. These studies also caution that incorrectly targeting signaling pathways in the stroma might risk accelerating tumor growth, which means that preclinical studies must carefully identify the signaling mechanisms that mediate crosstalk between PSCs and cancer cells.

1.4.2.2 Hyaluronic Acid

The fibrotic stroma secreted by PSCs creates high interstitial fluid pressures in PDAC, which limits ability of small molecule drugs to diffuse into the tumor. Hyaluronic acid (HA) is a component of the ECM that contributes to interstitial fluid pressure, and HA is elevated in PDAC compared to normal pancreas (Theocharis et al., 2000). Enzymatically degrading stromal HA in a genetic mouse model of PDAC using PEGPH20, a PEGylated form of recombinant hyaluronidase, normalized the interstitial fluid pressure and nearly doubled overall survival (Provenzano et al., 2012). Currently, PEGPH20 is being evaluated in clinical trials in combination with gemcitabine/nab-paclitaxel and FOLFIRINOX in stage IV metastatic PDAC patients (NCT01839487). For patients with high HA, there was an increase in median progression-free survival from 4.3 months to 9.2 months when treated with PEGPH20 in combination with
gemcitabine/nab-paclitaxel vs. gemcitabine/nab-paclitaxel alone (Hingorani et al., 2016; Sato et al., 2016). There was an increased rate of thromboembolic events, but this risk is being successfully managed by treatment with low molecular weight heparin (Sato et al., 2016). These studies indicate that stromal hyaluronic acid within the tumor stroma has potential as a therapeutic target.

1.4.2.3 Connective Tissue Growth Factor

Studies demonstrate that connective tissue growth factor (CTGF) is elevated in PDAC, and PSCs have been demonstrated to be the source of CTGF within the tumor (Hartel et al., 2004; Neesse et al., 2013). Results from a preclinical mouse model of pancreatic cancer demonstrated that inhibiting CTGF with the neutralizing antibody FG-3019 enhanced response to gemcitabine (Neesse et al., 2013). FG-3019 can inhibit tumor growth, metastasis, and angiogenesis in subcutaneous and orthotopic xenograft models of PDAC (Aikawa et al., 2006; Dornhofer et al., 2006). Results from a phase I clinical trial indicated that FG-3019 did not demonstrate toxicity in PDAC patients treated with gemcitabine and erlotinib (Dimou et al., 2013). A phase 2 study of FG-3019 in combination with erlotinib and gemcitabine in PDAC patients with locally advanced or metastatic PDAC found a dose-dependent increase in survival (NCT01181245). Therefore, this strategy of inhibiting stromal-derived CTGF remains promising.
1.4.2.4 Summary and Perspective on Targeting Stroma

The recent preclinical and clinical results of stromal-targeting agents reveal the complexities of the stroma and suggest that further studies are required to identify aspects of the stroma that promote cancer progression and limit drug delivery in pancreatic cancer. However, caution is required in targeting stromal components that may promote normal tissue homeostasis or even restrain tumor growth. Therefore, it is increasingly important to investigate the mechanisms of crosstalk between stromal cells and cancer cells to effectively target aspects of this interaction that enable chemoresistance.

1.5 Transforming Growth Factor-β Signaling in PDAC

1.5.1 Transforming Growth Factor-β Signaling

The transforming growth factor-β (TGF-β) signaling pathway regulates numerous biological processes in a cell- and context-dependent manner and plays an important role in development and many disease settings, including fibrosis and cancer. TGF-β signaling is initiated when the extracellular TGF-β ligand binds to the type II TGF-β receptor (TβRII) on the cell surface (Figure 6). Ligand binding results in complex formation between TβRII and the type I TGF-β receptor (ALK5, TβRI). TβRII is a constitutively active serine-threonine kinase, and complex formation allows TβRII to phosphorylate and activate ALK5. Activated ALK5 can then phosphorylate intracellular transcription factors called Smad2 and Smad3. Once phosphorylated,
Smad2 and Smad3 complex with Smad4 and translocate to the nucleus to regulate transcription of a wide variety of target genes (Figure 6). TGF-β can also activate non-canonical, Smad-independent signaling pathways, such as p38 MAPK and Akt, in a cell- and context-dependent manner (Y. E. Zhang, 2009). Activation of downstream signaling by TGF-β can regulate diverse cell behaviors including proliferation, apoptosis, migration, ECM production, and differentiation (Blobe et al., 2000).
Figure 6: Transforming Growth Factor-β Signaling Adapted from (Blobe et al., 2000)

Transforming growth factor-β (TGF-β) signaling occurs when the extracellular TGF-β ligand binds to the type II TGF-β receptor (TβRII) on the cell surface. Ligand binding allows a complex to form between TβRII and the type I TGF-β receptor (ALK5), and ALK5 is phosphorylated and activated by TβRII. Once activated, ALK5 phosphorylates intracellular transcription factors Smad2 and Smad3. When phosphorylated, Smad2 and Smad3 can complex with Smad4 and translocate to the nucleus where the complex regulates transcription of a variety of target genes.
1.5.2 The Dichotomous Role of TGF-β in Pancreatic Ductal Adenocarcinoma

Early in cancer, TGF-β acts as a tumor suppressor by strongly inhibiting proliferation of epithelial cells, largely through inducing G1 cell cycle arrest by regulating the activity of cyclin-dependent kinases and cyclins (Blobe et al., 2000). However, in later stages of tumorigenesis, TGF-β can promote cancer progression by increasing cell motility through induction of EMT, leading to enhanced migration and invasion of cancer cells. Several mechanisms through which cancer cells escape the growth inhibitory signaling of TGF-β have been identified. In some cancers, TGF-β signaling is inactivated through genetic mutations in pathway components, while other cancer cells are able to suppress the growth inhibitory effects of TGF-β signaling while still retaining a functional TGF-β signaling pathway, which allows TGF-β signaling to promote metastasis later in cancer progression (Blobe et al., 2000).

In the early stages of PDAC tumor initiation, TGF-β inhibits growth, but in the transition from precursor PanIN lesions to carcinoma in situ, cancer cells lose TGF-β growth inhibition and TGF-β acts to promote invasion, angiogenesis, and immunosuppression (Figure 7). TGF-β ligand expression is elevated in PDAC, and patients with high levels of TGF-β1 ligand in their serum have a significantly worse prognosis (Javle et al., 2014), suggesting TGF-β1 promotes tumor progression. This concept is being explored in a phase Ib/II clinical trial to compare overall survival of patients with stage II to IV unresectable PDAC when treated with the ALK5 inhibitor
LY2157299 in combination with gemcitabine versus gemcitabine alone (NCT01373164) (Michl et al., 2013).

![Diagram showing the progression from normal ductal epithelium to invasive metastatic adenocarcinoma](image)

**Figure 7**: TGF-β in PDAC. Adapted from (Truty et al., 2007)

### 1.5.3 Inactivating Mutations in TGF-β in PDAC

#### 1.5.3.1 Smad4 Mutations

Around 55% of PDAC patients have inactivating mutations in mothers against decapentaplegic homolog 4 (SMAD4), also called Deleted in Pancreatic Carcinoma locus 4 (DPC4) (Hahn et al., 1996). Smad4 is inactivated in PDAC through homozygous deletion of both alleles or through point mutations that disrupt its function or stability. For example, a missense mutation in an arginine residue in the MH1 domain of SMAD4 was identified in PDAC patients, and it resulted in rapid degradation of Smad4 through ubiquitination by the UbcH5 family of ligases (J. Xu et al., 2000). Similarly, a nonsense mutation that results in a C-terminal truncation of Smad4 has been identified in PDAC patients, and has been demonstrated to both prevent homomeric and heteromeric Smad
complex formation and lead to instability and rapid degradation of the Smad4 protein through the ubiquitin-proteasome pathway (Hahn et al., 1996; Maurice et al., 2001). Re-expressing Smad4 in a panel of PDAC cells with homozygous deletion of SMAD4 inhibited tumor growth and reduced invasion and angiogenesis in xenograft assays (Duda et al., 2003). Smad4 re-expression in PDAC cell lines also resulted in reduced migration and a TGF-β-dependent G1-arrest via up-regulation of CDKN1A/p21 (Fullerton et al., 2015). These studies suggest a tumor suppressor role for Smad4 and indicate that Smad4 expression and function might be lost during cancer progression to escape TGF-β-mediated growth inhibition. There is a higher frequency of loss of SMAD4 expression in poorly differentiated and metastatic PDAC patients, suggesting loss of SMAD4 occurs in later stages of PDAC carcinogenesis (Hua et al., 2003). One study demonstrated that all PanIN-1 and PanIN-2 lesions expressed Smad4, but Smad4 expression was lost in 30% of PanIN-3 lesions (Wilentz et al., 2000).

1.5.3.2 Type II TGF-β Receptor Mutations

The type II TGF-β receptor (TβRII) is inactivated in around 4% of PDAC patients through homozygous deletion or homozygous frameshift mutations (Goggins et al., 1998). One study demonstrated that 4% of PDAC tumors have mutations in the polyA region of TGFBR2, which is targeted by microsatellite instability (Venkatasubbarao et al., 1998). While pancreas-specific expression of mutant Kras (G12D) under the Ptf1a promoter in mice resulted in only intraepithelial neoplasia within one year, combining
the \( Kras^{G12D} \) mutation with pancreas-specific homozygous deletion of \( TGFB\)R2 resulted in the development of PDAC with 100% penetrance and median survival of only 59 days (Ijichi et al., 2006). These studies suggest that T\( \beta \)RII functions as a tumor suppressor and that inactivation of T\( \beta \)RII occurs in some PDAC patients.

1.5.3.3 Summary and Perspective

While high TGF-\( \beta \) confers a worse prognosis in PDAC, inactivation of canonical TGF-\( \beta \) signaling occurs in cancer cells in more than half of PDAC patients, which suggests that TGF-\( \beta \) may influence cancer progression in part through stromal cells with intact TGF-\( \beta \) signaling, including PSCs. Further studies are needed to understand the role of stromal TGF-\( \beta \) signaling in PDAC progression and chemoresistance.

1.5.4 Role of TGF-\( \beta \) in Stellate Cells and Cancer-Associated Fibroblasts

TGF-\( \beta \) signaling plays a central role in promoting activation of stellate cells from the quiescent state to the myofibroblast state both \textit{in vitro} and \textit{in vivo} (Bachem et al., 1993; Menke et al., 2002). However, little is known about how TGF-\( \beta \) signaling induces activation in PSCs and how TGF-\( \beta \) signaling in PSCs contributes to PDAC. Overexpression of TGF-\( \beta 1 \) ligand was sufficient to promote formation of a fibrotic stroma in an orthotopic xenograft assay using PANC1 cells, which suggests that TGF-\( \beta \) signaling is sufficient to initiate myofibroblast activation of resident PSCs (Lohr et al., 2001), but further studies are needed to better understand the role of stromal TGF-\( \beta \) signaling in PDAC. While stellate cells are only present in the pancreas and liver,
carcinoma-associated fibroblasts (CAFs) function similarly in the stroma of many other tumors, including breast, colorectal, prostate, and ovarian cancer. Several studies have examined the role of TGF-β signaling in CAFs.

Deletion of TGFBR2 specifically in fibroblasts of Tgfbr2^floxE2/floxE2 mice was achieved by expressing Cre recombinase under the FSP1 (fibroblast specific protein 1) promoter (Bhowmick et al., 2004). Deletion of TGFBR2 in fibroblasts lead to spontaneous cancer initiation in the forestomach and prostate without addition of any other genetic mutations in epithelial cells (Bhowmick et al., 2004), suggesting that TGF-β signaling in fibroblasts normally functions to inhibit tumor formation in neighboring epithelial cells. Follow-up studies demonstrated that deletion of TGFBR2 in fibroblasts in this mouse model led to inflammation and DNA damage in the neighboring epithelia as well as induction of the cytokine CXCL10, which stimulates proliferation and migration (Achyut et al., 2013; B. J. Xu et al., 2010).

Conversely, many studies have demonstrated that TGF-β signaling within cancer-associated fibroblasts promotes cancer progression by playing an important role in metastasis. In colorectal cancer, TGF-β induced CAFs to secrete Interleukin 11, which triggers GP130/STAT3 signaling in tumors cells and led to a survival advantage during metastasis (Calon et al., 2012). In fact, the authors found that metastasis depended on a gene expression program that was expressed in CAFs after TGF-β stimulation, demonstrating the importance of stromal TGF-β signaling in metastasis. Similarly, in
ovarian cancer, TGF-β induced CAFs to express the ECM protein versican, which promoted migration and invasion of ovarian cancer cells through NF-κB signaling and induction of CD44 and MMP-9 (Yeung et al., 2013).

These studies indicate that TGF-β has a dichotomous role in stromal CAFs within the tumor microenvironment that is similar to its role in the tumor epithelia. The role of TGF-β signaling in the stromal PSCs in PDAC is not well understood. Given the controversial role of PSCs in PDAC, further studies are needed to identify the role of TGF-β signaling in PSCs on cancer progression and therapy resistance.
2. Materials and Methods

2.1 Cell Culture and Reagents

PANC1, MiaPaCa-2, BxPC3, CFPAC-1, and 293T cells were obtained from ATCC and were short tandem repeat (STR) verified. L3.6p cells were provided by Dr. Isaiah Fidler (MD Anderson) (Bruns et al., 1999). RLT-PSC cells were provided by Dr. Ralf Jesenofsky (University of Heidelberg) (Jesnowski et al., 2005); HPSC-T cells were provided by Dr. Rosa Hwang (MD Anderson) (Hwang et al., 2008); LTC-14 cells were provided by Dr. Gisele Sparman (University Hospital of Rostock) (Sparmann et al., 2004); imPSC cells were provided by Dr. Raul Urrutia (Mayo Clinic) (Mathison et al., 2010). All cells were grown at 37°C at 5% CO2. PANC1, L3.6p, LTC-14, HPSC-T, RLT-PSC, and imPSC cells were grown in DMEM with 1 mM sodium pyruvate and 10% fetal bovine serum (FBS). MiaPaCa-2 cells were grown in DMEM with 1 mM sodium pyruvate, 10% FBS, and 2.5% horse serum. CFPAC-1 cells were grown in IMDM with 10% FBS. BxPC3 cells were grown in RPMI-1640 media containing 1 mM sodium pyruvate, 10 mM HEPES, and 10% FBS. Conditioned media from LTC-14 cells was concentrated by centrifugation using a Millipore Amicon Ultra-15 cellulose filter with a molecular weight cut-off of 3kDa. Chemical inhibitors against ALK5 (SB431542, #14775), p38 MAPK (SB203580, #5633) and PI3K (LY294002, #9901) were purchased from Cell Signaling Technology. TGF-β1 ligand was purchased from R&D Systems (#240-B-010). Gemcitabine was purchased from the Duke Hospital Pharmacy Store Room. The
hENT1 inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) was purchased from Sigma (#N2255) and dissolved in DMSO. Recombinant CYR61-Fc protein and IgG1-Fc control protein were purchased from R&D Systems (#4055-CR-050, #110-HG-100).

2.2 Adenovirus

HA-tagged constitutively-active ALK5 adenovirus (HA-ALK5T204D) was provided by Dr. Carlos Arteaga (Vanderbilt University) (Ueda et al., 2004). The luciferase control and mouse CYR61 adenoviruses were provided by Dr. Brahim Chaqour (SUNY Downstate) (Hasan et al., 2011; H. Liu et al., 2008). Adenoviruses were generated and purified using the Adeno-X Maxi Purification Kit from Clontech (#631542). Adenovirus titer was determined using the Adeno-X Rapid Titer Kit from Clontech (#632250), and cells were infected at the indicated multiplicity of infection (MOI).

2.3 Lentivirus

YAP5SA lentiviral construct and luciferase lentiviral control were a gift of Kris Wood (Duke University). Lentivirus CRISPR constructs targeting CYR61 were made using the LentiCRISPRv2 vector (Addgene Plasmid #52961) following the GeCKO protocol (Sanjana et al., 2014; Shalem et al., 2014). Briefly, the lentiCRISPRv2 vector was digested by BsmB1 and treated with Calf-intestinal alkaline phosphatase (CIP) to remove phosphate groups. Single guide RNA (sgRNA) target sequences against CYR61 were designed using the Human GeCKO library (Sanjana et al., 2014; Shalem et al., 2014) (sequences listed in Table 1), and the synthesized oligos were annealed and
phosphorylated using T4 Polynucleotide Kinase. The annealed sgRNA target sequence oligos were ligated into the digested lentiCRISPRv2 backbone using T4 DNA ligase. The ligated DNA was transformed into One Shot Stabl3 competent cells (Invitrogen #C7373-03) and selected on lysogeny broth-ampicillin (LB-Amp) plates. Each construct was sequenced to verify correct incorporation of the sgRNA target sequence into the lentiCRISPRv2 vector. To generate lentivirus for each lentiCRISPRv2 construct, a 10 cm dish of 293T cells was transfected with 4.5 μg of the respective lentiCRISPRv2 construct along with 2.25 μg PAX2, 0.75 μg pMD2.G, and 18 μL Xtremegene (Roche, #11580600). Media was changed on the 293T cells the morning after transfection. At 48 hr and 72 hrs later, the media from these cells containing lentivirus was harvested and filtered through a 0.45 μm cellulose filter. The media was then applied to MiaPaCa-2 or PANC1 cells with 6 μg/ml polybrene. Stably infected PDAC cells were selected using 2 μg/ml puromycin, and single cell clones were isolated to generate cultures with complete knockout of CYR61.

**Table 2: CYR61 CRISPR Sequences**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>hCYR61-1-crisprF</td>
<td>CACCGGTGTGCATTGGTAACTCGTG</td>
</tr>
<tr>
<td>hCYR61-1-crisprR</td>
<td>AAACCACGAGTTACCAATGACAAACC</td>
</tr>
<tr>
<td>hCYR61-2-crisprF</td>
<td>CACCGATGCCTCCGCTGGAAGAA</td>
</tr>
<tr>
<td>hCYR61-2-crisprR</td>
<td>AAACTCTTCGCAGCGGAACCGCAGCATC</td>
</tr>
<tr>
<td>hCYR61-3-crisprF</td>
<td>CACCGAAGTACTGCCTTCTGCG</td>
</tr>
<tr>
<td>hCYR61-3-crisprR</td>
<td>AAACCGCAGGAACCGCAGTACTTG</td>
</tr>
</tbody>
</table>
2.4 Western Blotting

Total cell lysates were harvested, boiled in sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, blocked in 5% milk in Tris-buffered saline, and incubated overnight with the primary antibody of interest in 5% bovine serum albumin (BSA) in Tris-buffered saline/0.1% TWEEN. Quantification was performed using the LICOR Odyssey software by taking the integrated intensity of each band and normalizing to the integrated intensity of the β-actin band. Antibodies against cleaved caspase 3 (1:1000, #9664), P-Smad2 (1:1000, #3101), Total Smad2 (1:2000, #3103), P-p38 MAPK (1:2000, #4511), Total p38 MAPK (1:2000, #9212), P-Akt (1:2000, #4058), Total Akt (1:2000, #4691), MDR1 (1:1000, #13978), P-Stat3 (1:2000, #9145), Total Stat3 (1:2000, #9139), P-Erk (1:2000, #9101), T-Erk (1:2000, #9102), and YAP (1:1000, #4912) were all purchased from Cell Signaling Technology. Antibodies against human CYR61 (1:2000, #sc-13100), dCK (1:1000, #sc99008), and hENT1 (1:1000, #sc-134501) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against hCNT3 (1:500, #HPA024729), β-actin (1:10,000, #A5441), and α-SMA (1:5000, #5228) were purchased from Sigma-Aldrich. Antibodies against rat/mouse CYR61 (1:2000, #ab24448) and fibronectin (1:2000, #ab2413) were purchased from Abcam. The antibody for E-cadherin (1:2000, #610182) was purchased from BD Transduction. Anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from Cell Signaling (#5470, #5151) and LICOR (#926-32212, #926-32213).
2.5 Microarray and RNAseq Dataset Analysis

Patient mRNA microarray expression data was obtained from publically available datasets on NCBI Gene Expression Omnibus (GEO) for GDS4103 and GSE43288 (Badea et al., 2008; Crnogorac-Jurcevic et al., 2013). The GDS4103 platform was Affymetrix Human Genome U133 Plus 2.0 Array. The GSE43288 platform was Affymetrix Human Genome U133A Array (GPL96). All microarray data were log2 transformed. We queried the GDS4103 dataset using the gene probe IDs listed in Table 3. In dataset GSE43288, we queried the dataset for CYR61 using gene probe ID 210764_s_at. Survival analysis of PDAC patients based on CYR61 expression was obtained using publically available RNAseq data in the ICGC PACA-AU Data Portal (Scarlett et al., 2011). Patients were divided in half into high and low CYR61 expressing groups based on normalized read count of CYR61 expression using Gene ID ENSG00000142871. For analysis of the cellular source of CYR61 within the tumor, CYR61 mRNA expression in isolated pancreatic stellate cells, patient-derived xenografts, PDAC samples, and tumor-derived PDAC cell lines was obtained from previously published RNAseq expression data (Moffitt et al., 2015).
We used the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) to analyze a publically-available microarray dataset of PDAC tumor samples (Mixed pancreatic ductal - Badea - 78 - MAS5.0 - u133p2, NCBI GEO GDS4103) (Badea et al., 2008) to identify genes whose expression significantly correlated with expression of either \textit{hENT1} (\textit{SLC29A1}) or \textit{hCNT3} (\textit{SLC28A3}). We ranked the significantly correlated genes based on their correlation coefficient R and took the top 10\% of the positively-correlated genes and the top 10\% of the negatively-correlated genes. We narrowed this
list to only genes whose expression is significantly altered in PDAC tumor compared to
normal adjacent tissue using the Kruskal test (p<0.01) and corrected for multiple testing
using the False Discovery Rate. We confirmed that the genes were significantly
changed in cancer by performing a Wilcoxon Matched-Pairs Signed Rank Test. We
identified 25 genes whose expression significantly correlates with both hENT1
(SLC29A1) and hCNT3 (SLC28A3) and whose expression is significantly altered in cancer

2.6 Gemcitabine Transport Assay

[3H]gemcitabine (16.32 µg/mL, 16.2 Ci/mmol) was purchased from Moravek
Biochemicals Inc. Transport buffer (20 mM Tris/HCl, 3mM K2HPO4, 5 mM glucose, 130
mM NaCl, 1 mM MgCl2·6H2O, 2 mM CaCl2) was made as previously described [31].
Cells were plated in 12 well plates at 150,000 cells/well (MiaPaCa-2) or 90,000 cells/well
(PANC1). The following day, cells were rinsed in transport buffer then incubated with
100 nM [3H]gemcitabine in transport buffer for 2 mins (MiaPaCa-2) or 30s (PANC-1).
When indicated cells were pre-treated for 10mins with doses of the hENT1 inhibitor
S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) dissolved in diethyl sulfoxide (DMSO) or
DMSO control in transport buffer, and NBMPR or DMSO was included in the 100 nM
[3H]gemcitabine incubation. After incubation with [3H]gemcitabine, cells were
rinsed 3 times with transport buffer containing 5µM NBMPR to inhibit efflux of
[3H]gemcitabine. Cells were lysed in 1% (v/v) Triton-X-100, and protein concentration
was determined using a BCA assay (Thermo Scientific #23227). Cell lysates were added
to Ultima Gold (Perkin #6013326) and cell-associated radioactivity in counts per minute (CPM) was determined using a liquid scintillation counter. 

\[^3\text{H}\]gemcitabine transport was calculated by normalizing CPM to protein concentration for each well. Each condition was performed in triplicate, and the experiment was repeated 3 times.

2.7 Titer Glow Cell Viability Assay

Cells were plated in 96 well opaque plates (#6005680 Perkin Elmer) and treated in triplicate for 48 hrs with indicated doses of gemcitabine. The viability of cells was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (#G7571 Promega) and normalized to untreated condition.

2.8 CYR61 ELISA

The CYR61 ELISA kit was purchased from R&D Systems (DCYR10), and the ELISA was performed according to kit instructions. Serum samples were obtained from Dr. Becky White at Duke University. All patient samples were de-identified, and the study was conducted with approval of the Duke IRB. For ELISA performed on conditioned media, cells were incubated in serum-free media for 24hrs. Conditioned media was filtered through a 0.45 μM cellulose filter and frozen in liquid nitrogen.

2.9 In Vitro Co-Culture Assay

LTC-14 or imPSC pancreatic stellate cells were infected with adenovirus at indicated MOIs. After 24 hrs, adenovirus was washed off cells with PBS and media was replaced. After 24hrs, conditioned media was harvested, filtered through a 0.45 μM
cellulose filter, and applied to PDAC cells for 24hrs. After 24 hrs, media was replaced with a second round of PSC conditioned media following same collection protocol. PDAC cells were then treated with gemcitabine for 48hrs at indicated doses. Adherent and floating PDAC cells were collected for Western analysis of cleaved caspase 3 levels.

2.10 RT-PCR

RNA was extracted using the Quick-RNA™ MiniPrep kit (Zymo Research, #R1055) according to kit instructions. 500 ng of RNA was reversed transcribed using the iScript cDNA Synthesis Kit (BioRad #170-8891) following kit instructions. Each PCR reaction contained 1 µl of cDNA, 8 µl H2O, 10 µl SYBRGreen Mix (BioRad #170-8882), and 0.5 µl each of respective forward and reverse primers. Primer sequences are listed in Table 4. PCR was performed as follows: 2 mins at 94°C then 50 rounds of 94°C for 45 sec, 56.8°C for 45 sec, 72°C for 45 sec, then 7 mins at 72°C. The fold change in expression was determined by calculating $2^{\Delta\Delta CT}$, with GAPDH used as a reference gene. RT-PCR was performed for each gene in triplicate in three or more independent experiments.
Table 4: Primer Sequences

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAPDH Forward</td>
<td>GAGTCAACGGATTTGTCGT</td>
</tr>
<tr>
<td>hGAPDH Reverse</td>
<td>TTGATTTTGGAGGGATCTCG</td>
</tr>
<tr>
<td>hRRM2 Forward</td>
<td>GACACAAGGCATCGTTTCAA</td>
</tr>
<tr>
<td>hRRM2 Reverse</td>
<td>TCTATGGCTTCCAAATTGCC</td>
</tr>
<tr>
<td>hABCC1 Forward</td>
<td>TTCTGGCTGAGGCTAGCGAGGATTA</td>
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<tr>
<td>hABCC1 Reverse</td>
<td>TGGACTCTGGGCGAGGATTA</td>
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<tr>
<td>hRRM1 Forward</td>
<td>TACCAACCGCCCACAACCTTT</td>
</tr>
<tr>
<td>hRRM1 Reverse</td>
<td>TCTCAGCATCGGTACAAAGGC</td>
</tr>
</tbody>
</table>

2.11 Immunofluorescent Staining

LTC-14 and imPSC cells were plated on glass coverslips. The following day, the cells were serum starved in 1% FBS for 4 hrs then treated with 100 pM TGF-β for 24 hrs. After 24 hrs, media was removed and cells were fixed in 4% paraformaldehyde in PBS for 15 mins, washed 3 times in PBS, then permeabilized in 0.1% Triton-X PBS for 5 mins. After being washed 3 times in PBS, cells were blocked overnight at 4°C in 1% BSA-PBS. The following day, cells were incubated for 1 hr at room temperature in the α-SMA primary antibody (Sigma #5228) at a 1:500 dilution in 1% BSA-PBS. Cells were washed 3 times in PBS then incubated for 30 min with Alexa-Fluor conjugated secondary donkey anti-mouse 594 antibody (Invitrogen #A21203) in the dark at room temperature. Cells were again washed 3 times in PBS, then counterstained for nuclei with DAPI at a 1:100 dilution in PBS for 5 mins then washed 3 times with PBS. The glass coverslips
containing cells were mounted on slides using Prolong Gold Antifade Reagent (Life Technologies #P36930). Immunofluorescence images were obtained for α-SMA using a 200 ms exposure on a Nikon inverted microscope at 60x magnification.

2.12 Invasion Assay

50,000 PANC1 cells were plated on matrigel invasion chambers in media containing 0.5% FBS and allowed to invade for 24 hrs. PANC1 cells invaded towards bottom chambers containing control media (0.5% or 10% FBS) or conditioned media (CM) from HPSC-T cells. Conditioned media contained 0.5% FBS and was collected after 48 hrs on HPSC-T cells. Prior to start of 48 hrs collection of CM, HPSC-T were treated with or without 50pM TGF-β for 24 hrs following 30 min pretreatment with DMSO or 10µM SB203580 dissolved in DMSO. The number of invaded PANC1 cells was quantified for 3 independent experiments.

2.12 Statistics

All statistical analyses were conducted with GraphPad Prism software. For all experiments, significance was set at p<0.05. All in vitro experiments were analyzed using parametric statistics (t test or ANOVA with indicated post hoc test,) and expressed as the mean ± SEM. ELISA and microarray expression data were analyzed using nonparametric statistics (Mann-Whitney U, Wilcoxon matched pairs signed rank test, or Kruskal-Wallis global test). Linear regression was performed on microarray data with
the $R^2$ value, p value, and slope for the line of best fit reported for each comparison.

Survival curves were analyzed with log-rank statistics.
3. The Role of CYR61 in Gemcitabine Resistance

3.1 CYR61 negatively regulates expression of hENT1 and hCNT3

3.1.1 Bioinformatic Analysis of hENT1 and hCNT3 Regulators

Low expression of hENT1 and hCNT3 has been linked to clinical resistance to gemcitabine in PDAC, but little is known about what regulates their expression in the PDAC microenvironment. To identify potential regulators of hENT1 and hCNT3 in the PDAC microenvironment, we analyzed a publically-available microarray dataset of PDAC tumor samples (Badea et al., 2008) to identify genes whose expression significantly correlated with expression of $hENT1$ (SLC29A1) and $hCNT3$ (SLC28A3) and whose expression is significantly altered in PDAC tumor samples compared to normal adjacent tissue (Figure 8). We identified 25 genes whose expression significantly correlated with both hENT1 and hCNT3 and whose expression is significantly altered in cancer (Table 5).
A microarray dataset of PDAC tumor samples was analyzed to identify genes whose expression significantly correlated with expression of either hENT1 (SLC29A1) or hCNT3 (SLC28A3). The top 10% of the correlated genes were selected based on their correlation coefficient R, and this list was narrowed to only genes whose expression is significantly altered in PDAC tumor compared to normal adjacent tissue. The analysis identified 25 genes whose expression significantly correlates with both hENT1 (SLC29A1) and hCNT3 (SLC28A3) and whose expression is significantly altered in cancer.

Figure 8: Bioinformatic Analysis of hENT1 and hCNT3 Regulators
Table 5: Genes Correlated with hENT1 and hCNT3 Whose Expression Changes in Cancer

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
<th>PDAC versus Normal Adjacent</th>
<th>Correlation with hENT1 and hCNT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYR61</td>
<td>cysteine-rich, angiogenic inducer, 61</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>KCNMB4</td>
<td>potassium channel subfamily M regulatory beta subunit 4</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>NIN</td>
<td>ninein</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>STK10</td>
<td>serine/threonine kinase 10</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>RAB8B</td>
<td>RAB8B, member RAS oncogene family</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>LY96</td>
<td>lymphocyte antigen 96</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>ETS1</td>
<td>v-ets avian erythroblastosis virus E26 oncogene homolog 1</td>
<td>Increased</td>
<td>Negative</td>
</tr>
<tr>
<td>RAC2</td>
<td>ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)</td>
<td>Increased</td>
<td>Negative</td>
</tr>
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<td>HBEGF</td>
<td>heparin-binding EGF-like growth factor</td>
<td>Increased</td>
<td>Negative</td>
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<td>CD163 molecule</td>
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<td>S100 calcium binding protein A9</td>
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<td>Negative</td>
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We were particularly interested in investigating CYR61 because its expression was increased in cancer and negatively correlated with hENT1 and hCNT3 expression.
Further, it is a secreted protein that can be targeted using a neutralizing antibody, which indicates it has the potential to be targeted clinically. The mRNA expression of \( SLC29A1 \) (hENT1) and \( SLC28A3 \) (hCNT3) negatively correlated with \( CYR61 \) mRNA expression in PDAC patient samples (Figure 9), indicating that \( CYR61 \) may play a role in suppressing expression of the nucleoside transporters that mediate cellular uptake of gemcitabine in the PDAC tumor microenvironment. \( CYR61 \) expression did not significantly correlate with the expression of other nucleoside transporters tested in PDAC patient samples (\( hCNT1, hCNT2, hENT2, hENT3, hENT4 \)) (Figure 10), suggesting specific regulation of hENT1 and hCNT3.

![Graphs showing correlation between CYR61 and nucleoside transporter mRNA expression in PDAC samples](image.png)

**Figure 9: hENT1 and hCNT3 Expression Negatively Correlate with CYR61 Expression in PDAC Samples**

(A) Linear regression using the microarray dataset GDS4103 for hENT1 (\( SLC29A1 \)). (B) Linear regression using the microarray dataset GDS4103 for hCNT3 (\( SLC28A3 \)). \( n=39 \) patient samples
3.1.2 CYR61 regulates hENT1 and hCNT3 expression

Based on this correlation, to examine whether CYR61 negatively regulated hENT1 and hCNT3 expression, we used CRISPR/Cas9 technology (Shalem et al., 2014) to knock out CYR61 expression in PDAC cell lines with high CYR61 expression. CRISPR-mediated knockout of CYR61 significantly increased hENT1 and hCNT3 expression in PANC1 (Figure 11A). In PANC1 cells, 48 hrs treatment with gemcitabine induced down-regulation of hENT1, but CRISPR knockout of CYR61 kept hENT1 levels relatively high (Figure 11B). Knockout of CYR61 also significantly increased hENT1
expression in MiaPaCa-2 cells, and increased hCNT3 expression, albeit with larger increases in CRISPR 2 cells (Figure 11C). In a reciprocal manner, adenovirus-mediated overexpression of CYR61 in BxPC3 and CFPAC cells, which have low basal CYR61 expression, decreased hENT1 expression (Figure 12). All cell lines had low basal expression of hCNT3 as previously reported for in vitro cell culture conditions (Zheng et al., 2015), so overexpression of CYR61 in BxPC3 and CFPAC cells was not able to further decrease these low basal levels of hCNT3 expression.
Figure 11: CRISPR Knockout of CYR61 Increases hENT1 and hCNT3 Expression

(A) Western blots for CYR61 (Santa Cruz antibody), hENT1, hCNT3 in PANC1 NTC and CYR61 CRISPR 1 knockout cells. t test hENT1 *p<0.0001, t test hCNT3 *p=0.006 (B) Western for hENT1 and CYR61 (Santa Cruz) in PANC1 NTC and CYR61 CRISPR cells treated with 10µg/ml gemcitabine for 48 hrs. n= 5 independent replicates (C) Western blots for CYR61 (Santa Cruz antibody), hENT1, hCNT3 in MiaPaCa-2 cells for NTC, CYR61 CRISPR 1, and CRISPR 2 knockout cells. ANOVA, Fishers LSD for hENT1 NTC versus CR1 *p=0.0147, NTC versus CR2, *p=0.0108, ANOVA, Fishers LSD for hCNT3 NTC versus CR1 p= 0.7371, NTC versus CR2. n= 4 independent replicates p=0.0581
Figure 12: Overexpression of CYR61 Down-Regulates hENT1

(A) Western blots for CYR61 (Abcam antibody), hENT1, and hCNT3 in BxPC3 cells infected with CYR61 adenovirus or control luciferase adenovirus at an MOI of 100 for 48hrs. hENT1 t test *p=0.004. n= 3 independent replicates (B) Western blots for CYR61 (Abcam antibody), hENT1, and hCNT3 in CFPAC cells infected with CYR61 adenovirus or control luciferase adenovirus at an MOI of 100 for 48hrs. hENT1 t test *p=0.0476. n= 3 independent replicates
3.2 **CYR61 inhibits cellular uptake of gemcitabine**

To determine if this increase in hENT1 and hCNT3 in cells with CYR61 knockout resulted in higher cellular uptake of gemcitabine, we performed gemcitabine transport assays using radiolabeled $^{3}$H-gemcitabine as previously described (Mackey et al., 1998). Increasing doses of the hENT1 specific inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) decreased the levels of $^{3}$H-gemcitabine transported into the cell, demonstrating specificity of the assay and is consistent with hENT1 being a major gemcitabine transporter in MiaPaCa-2 and PANC1 cells (Figure 13A). CRISPR-mediated knockout of CYR1 significantly increased the amount of $^{3}$H-gemcitabine transported into the cell in PANC1 cells (Figure 13B) and MiaPaCa-2 cells (Figure 13C). These data indicate that the up-regulation of hENT1 and hCNT3 following CRISPR knockout of CYR61 result in enhanced cellular uptake of gemcitabine.
Figure 13: CYR61 Knockdown Increases Cellular Uptake of Gemcitabine

(A) Gemcitabine transport assay in MiaPaCa-2 and PANC1 cells using hENT1 inhibitor NBMPR at indicated doses. (B) Gemcitabine transport assay in PANC1 cells. T test *p=0.0039. (C) Gemcitabine transport assay in MiaPaCa-2 cells. ANOVA/Fisher’s LSD, NTC vs. CR1 *p=0.0023, NTC vs. CR2 *p<0.0001. For all assays, n= 3 independent replicates, each performed in triplicate.
3.3 **CYR61 promotes resistance to gemcitabine-induced apoptosis**

As CYR61 regulated the activity of the nucleoside transporters that import gemcitabine into PDAC cells, we examined whether CYR61 regulates gemcitabine-induced apoptosis in PDAC cells. CRISPR-mediated knockout of CYR61 in PANC1 and MiaPaCa-2 cells resulted in decreased cell viability in response to gemcitabine (Figure 14).

![Figure 14: Knockout of CYR61 Decreases Cell Viability in Response to Gemcitabine](image)

(A) Cell titer glo assay measuring cell viability of PANC1 cells after 48 hr treatment with a dose course of gemcitabine. 2 way ANOVA, effect of CRISPR *p*<0.0001, interaction of CRISPR and gemcitabine treatment *p*=0.0320. (B) Cell titer glo assay measuring cell viability of MiaPaCa-2 cells after 48hr treatment with a dose course of gemcitabine. 2-way ANOVA, effect of CRISPR *p*<0.0001, interaction of CRISPR and gemcitabine treatment *p*=0.0127. *n* = 3 independent replicates, each performed in triplicate

Knockout of CYR61 also increased levels of gemcitabine-induced apoptosis as demonstrated by an increase in the levels of cleaved caspase 3 (Figure 15A,B). In a
reciprocal manner, adenovirus-mediated expression of CYR61 in CFPAC cells decreased gemcitabine-induced apoptosis (Figure 15C). In addition, treatment of BxPC3 cells with a recombinant CYR61 protein reduced gemcitabine-induced apoptosis (Figure 15D).
Figure 15: CYR61 Promotes Resistance to Gemcitabine-Induced Apoptosis

(A) Western blot analysis of cleaved caspase 3 and CYR61 (Santa Cruz) for PANC1 NTC and CYR61 CRISPR cells treated with a dose course of gemcitabine for 48 hrs. 2 way ANOVA, effect of CRISPR* p=0.0108. n= 4 independent replicates (B) Western blot analysis of cleaved caspase 3 and CYR61 (Santa Cruz) for MiaPaCa-2 NTC and CYR61 CRISPR cells treated with a dose course of gemcitabine for 48 hrs. 2 way ANOVA, effect of CRISPR *p<0.0001, Fisher’s LSD NTC versus CR1 *p<0.0001, NTC versus CR2 p=0.0965. n=3 independent replicates (C) Western blot analysis of cleaved caspase 3 for CFPAC cells treated with 1µg/ml gemcitabine for 48 hrs. CFPAC cells were infected with CYR61 adenovirus or control luciferase adenovirus at MOI of 100 48 hrs prior to gemcitabine treatment  (D) BxPC3 cells were treated with 5 µg/ml recombinant CYR61-Fc or IgG1-Fc control for 48 hrs then treated with 10 µg/ml gemcitabine for 48 hrs.
3.4 CYR61 expression is increased in PDAC

Bioinformatic analysis of the microarray dataset demonstrated that CYR61 expression was increased in PDAC samples compared to matched, normal adjacent tissue (Figure 16A,B), supporting increased CYR61 expression in PDAC, consistent with a prior report (Haque et al., 2011). Further analysis demonstrated that patients with familial PanIN precursor lesions (Crnogorac-Jurcevic et al., 2013) have an intermediate level of CYR61 (Figure 16C). While these assessments at the mRNA level were suggestive, serum protein levels of CYR61 in PDAC patients have not been investigated. Here we demonstrate that CYR61 protein expression is significantly elevated in the serum of PDAC patients, with a mean expression of 857.5 ng/ml compared to a mean expression of 508.5 ng/ml for healthy volunteers (Figure 16D). Further, survival data from the ICGC PACA-AU dataset (Scarlett et al., 2011) suggested that PDAC patients with high levels of CYR61 had a lower media survival time relative to patients with low CYR61 expression (Figure 16E).
Figure 16: CYR61 is Increased in PDAC

(A) Microarray dataset analysis (GDS4103) for CYR61 expression in PDAC tumors. Mann Whitney test was performed, *p<0.0001. n= 39 patient samples (B) Microarray dataset analysis (GDS4103) for CYR61 expression in PDAC tumors, comparing matched normal adjacent tissue to tumor tissue. Wilcoxon matched pairs signed rank test was performed, *p<0.0001. 29/39 samples had increased CYR61 expression. (C) Microarray dataset analysis (GSE43288) for CYR61 expression in normal pancreas, familial PanIN lesions, and PDAC tumors. Kruskal-Wallis test and Dunn’s Multiple Comparison test were performed. *p=0.0051 for Normal versus PDAC. n=3 for normal pancreas, n=13 for PanIN, n=4 for PDAC (D) ELISA was performed on serum samples from healthy volunteers and PDAC patients. Mann Whitney test was performed, *p=0.0142. n=8 healthy volunteer samples, n=9 PDAC samples (E) Survival analysis of PDAC patients split by CYR61 expression from RNAseq analysis of the ICGC PACA-AU dataset. p=0.2389.
3.5 CYR61 does not regulate expression of other gemcitabine resistance factors

While nucleotide transporters are an important mechanism for regulating entry of gemcitabine into pancreatic cancer cells, there are several additional mechanisms that regulate clinical resistance to gemcitabine. dCK is the kinase that phosphorylates gemcitabine to its active form once it enters the cytoplasm, and low expression of dCK is associated with worse survival after gemcitabine treatment (Fujita et al., 2010; Marechal et al., 2012). High expression of the ribonucleotide reductase subunits RRM1 and RRM2 are also associated with gemcitabine resistance in patients (Duxbury et al., 2004; Nakahira et al., 2007; H. Xie et al., 2013). In addition, the expression of ATP-binding cassette (ABC) transporters, which act as drug-efflux pumps, are associated with drug resistance in PDAC (Konig et al., 2005). The drug efflux pumps ABCB1 (MDR1/P-glycoprotein) and ABCC1 (MRP1) are important in the resistance of PDAC cells to gemcitabine (Nath et al., 2013; W. Zhang et al., 2013a). CYR61 has been previously reported to regulate expression of the drug efflux pump MDR1/P-glycoprotein in renal cell carcinoma (Q. Z. Long et al., 2013), but it has not been studied in PDAC. We examined the effect of CYR61 on the expression of these factors associated with gemcitabine resistance. Either overexpression of CYR61 or CRISPR-mediated knockdown of CYR61 did not alter the expression levels of MDR1 or dCK at the protein level (Figure 17). In addition, there was no effect of CYR61 expression on the mRNA level of RRM1, RRM2, or ABCC1 (Figure 18). Moreover, in PDAC patient samples, the
level of CYR61 did not significantly correlate with expression of gemcitabine resistance factors, including RRM1, RRM2, DCK, ABCB1 (MDR1), and ABCC1 (Figure 19). These studies suggest that CYR61 functions to mediate resistance to gemcitabine largely through its effects on the nucleotide transporters hENT1 and hCNT3.

**Figure 17: CYR61 Does Not Regulate dCK or MDR1 in PDAC**

(A) Western blot of CYR61 (Abcam antibody), MDR1, and dCK in CFPAC after 48hrs infection with CYR61 adenovirus or luciferase control adenovirus at MOI 100. (B,C) Western blot of CYR61 (Santa Cruz antibody), MDR1, and dCK in PANC1 and MiaPaCa-2 cells with NTC or CRISPR knockout of CYR61. Results are representative of 3 independent replicates.
Figure 18: CYR61 Does Not Regulate RRM1, RRM2, or ABCC1 mRNA in PDAC Cells

RT-PCR on RRM1, RRM2, and ABCC1 normalized for GAPDH. For each experiment, n=2 independent replicates, each gene performed in triplicate. (A) PANC1 NTC and CRISPR 1 bulk cells (74.8% knockdown of CYR61). t test RRM1 p=0.567. t test RRM2 p=0.5213. t test ABCC1 p=0.6135. (E) MiaPaCa-2 NTC and CRISPR 1 bulk cells (69.2% knockdown of CYR61). t test RRM1 p=0.5635. t test RRM2 p=0.556. t test ABCC1 p=0.3317. n=2 independent replicates, each gene performed in triplicate (F) CFPAC cells after 48hrs infection with CYR61 adenovirus or luciferase control adenovirus at MOI 100. T test RRM1 p=0.47. T test RRM2 p=0.6987. T test ABCC1 p=0.0042. n=2 independent replicates, each gene performed in triplicate.
Pancreatic stellate cells are the primary source of CYR61 in the PDAC tumor microenvironment

PDAC is characterized by an abundant fibrotic stroma that can comprise up to 80% of the tumor volume (Erkan et al., 2012) making this stroma perhaps the most prominent of all epithelial cancers. This stroma contains PSCs, which are the predominant cells responsible for secretion of the ECM components that comprise the fibrotic stroma (Apte et al., 2004; Bachem et al., 2005; Yen et al., 2002). The microarray
dataset used to examine CYR61 expression analyzed whole-tissue tumor samples that include both cancer and stromal cells (Badea et al., 2008), suggesting that PSCs might be a source of CYR61. To determine which cells within the tumor microenvironment secrete CYR61, we examined recent RNAseq data that analyzed gene expression in PDAC tumors as well as three cell populations isolated from the tumor: PSCs, tumor epithelial cells grown in patient-derived xenografts (PDX), and tumor epithelial cells grown in in vitro cell culture (Moffitt et al., 2015). Isolated PSCs, identified as α-SMA positive, vimentin positive, and EpCam negative (Moffitt et al., 2015), expressed significantly higher levels of CYR61 compared to the human tumor epithelium from patient-derived xenografts (Figure 20A). PDAC samples also expressed a significantly higher level of CYR61 than PDX samples, suggesting the CYR61 from these samples is derived from stromal cells present in the samples. To investigate expression of CYR61 at the protein level, we compared expression of CYR61 in two human PSC cell lines, HPSC-T (Hwang et al., 2008) and RLT-PSC (Jesnowski et al., 2005), to expression of CYR61 in five common PDAC cell lines. The mesenchymal PSCs expressed high levels of fibronectin, while most PDAC cells express higher levels of the epithelial marker E-cadherin (Figure 20B). The PSC cell lines had much higher CYR61 expression than the majority of PDAC cell lines (Figure 20B). Interestingly, the PANC1 cell line also had high expression of CYR61 (Figure 20B). Isolated tumor epithelial cells grown in vitro (Cancer Cell) also had higher CYR61 expression than tumor cells grown in PDX (Figure
20A), suggesting that some PDAC epithelial cell lines express CYR61 in \textit{in vitro} cell culture conditions as compensation for the lack of stromal-derived factors present \textit{in vivo}. Consistent with a role for CYR61 in gemcitabine resistance, pancreatic cancer cell lines that express higher levels of CYR61 \textit{in vitro} were more resistant to gemcitabine-induced apoptosis in cell culture (Figure 20C). MiaPaCa-2 demonstrate high induction of CYR61 in response to gemcitabine (Figure 15B), while PANC1 cells have high basal CYR61 levels (Figure 15A).
Figure 20: CYR61 is Expressed by Pancreatic Stellate Cells

(A) CYR61 expression in fragments per kilobase of transcript per million mapped reads (FPKM) in PDAC samples, patient-derived xenografts (PDX), pancreatic stellate cells (PSCs), and isolated cancer cells in vitro. PDX samples were processed with Xenome to sort human- and mouse-specific expression. Kruskal-Wallis and Multiple Comparison Test were performed, *PDX vs. PSC p<0.0001, PDX vs. PDAC p<0.0001. (RNA seq data from (Moffitt et al., 2015). n=15 for PDAC, n=37 for PDX, n= 6 for PSCs, n= 3 for Cancer Cells (B) Western blot of CYR61 (Santa Cruz antibody) in immortalized human PSC cell lines (HPSC-T, RLT-PSC) and PDAC cell lines (CFPAC, BxPC3, L3.6p, MiaPaCa-2, and PANC1). Lysates were also probed for the mesenchymal marker fibronectin and the epithelial marker E-cadherin. n=3 independent replicates (C) Western blot analysis of cleaved caspase 3 for PDAC cell lines treated with or without 20µg/ml gemcitabine for 48 hrs. Results are representative of three independent experiments.
3.7 TGF-β signaling induces CYR61 expression in PSCs in the PDAC tumor microenvironment

CYR61 has been demonstrated to be regulated by both the TGF-β and Hippo-YAP/TAZ signaling pathways (Bartholin et al., 2007; Jun et al., 2011). TGF-β ligand expression is elevated in PDAC, and patients with high levels of TGF-β1 ligand in their serum have a significantly worse prognosis (Javle et al., 2014). However, mutations that inactive the canonical TGF-β-Smad signaling pathway are common in PDAC, with around 55% of PDAC patients having inactivating mutations in SMAD4 (Hahn et al., 1996). Therefore, elevated TGF-β may negatively affect PDAC progression or therapy response in part through stromal cells with intact Smad4, including PSCs. Consistent with this hypothesis, in the microarray dataset that analyzed gene expression in whole-tissue PDAC samples, CYR61 expression significantly correlated with expression of the TGFβ1 ligand and also with the well-established TGF-β target genes SERPINE1 (PAI-1) and SMAD7 (Figure 21). Moreover, in the rat PSC cell line LTC-14 (Sparmann et al., 2004) and the mouse PSC cell line imPSC (Mathison et al., 2010), both with low basal CYR61 expression, TGF-β induced CYR61 expression in a dose dependent fashion (Figure 22A). Activation of Hippo-YAP/TAZ signaling by expression of a constitutively active YAP (YAP5SA) only weakly induced CYR61 in comparison to TGF-β treatment (Figure 22B). Human PSC cell lines HPSC-T and RLT-PSC, which were isolated from PDAC and chronic pancreatitis, had high basal CYR61 expression (Figure 20B, Figure 23A). However, ELISA shows that activation of TGF-β signaling by expression of a constitutively active
version of the TGF-β receptor ALK5 (CA-ALK5) induced even higher secretion of CYR61 (Figure 23A). TGF-β did not induce CYR61 in Smad4-null cells (CFPAC and BxPC3) or TGF-β non-responsive MiaPaCa-2 cells (Simeone et al., 2000) (Figure 23).

Figure 21: CYR61 Correlates with TGF-β Activation in PDAC Samples

Linear regression was performed using the microarray dataset GDS4103 for (A) TGFB1, (B) SERPINE1, and (C) SMAD7. n=39 patient samples
Figure 22: TGF-β Induces CYR61 Expression in PSCs

(A) Western blot analysis of CYR61 (Abcam antibody) in LTC-14 and imPSC cells that were serum starved in 1% FBS then treated with indicated doses of TGF-β1 for 16hrs. (B) Western blot of CYR61 (Abcam antibody) and YAP in LTC-14 cells after stable expression of constitutively active YAP (YAP5SA) or luciferase control lentivirus. Cells were serum starved in 1% FBS and treated with or without 100 pM TGF-β1 for 16 hrs. Results representative of 3 independent experiments.
Figure 23: TGF-β Does Not Induce CYR61 in SMAD4-Null Cells

(A) ELISA for CYR61 on conditioned media from indicated cell lines with or without 48 hrs of CA-ALK5 adenovirus expression. (B) Western blot and quantification for CYR61 (Santa Cruz antibody) and Smad4 in PDAC cells. Cells were serum starved in 1% FBS then treated with 100 pM TGF-β1 for 24 hrs. n= 3 independent replicates
TGF-β induces activation of canonical Smad signaling in PSCs, but TGF-β also induces activation of several non-canonical signaling pathways, including p38 MAPK and PI3K-Akt signaling, in the PSCs (Figure 24A,B). To determine which downstream signaling pathways were important for TGF-β-induced CYR61 expression in PSCs, we pre-treated the PSCs with kinase inhibitors against ALK5 (SB431542), p38 MAPK (SB203580), and PI3K-Akt (LY294002) and examined the effect on TGF-β-induced CYR61 expression. Treatment with the ALK5 inhibitor, but not the p38 MAPK or PI3K-Akt inhibitor, blocked TGF-β-induced CYR61 expression (Figure 24C,D), while expression of CA-ALK5 induced CYR61 expression in LTC-14 and imPSC cells (Figure 24E,F). These results suggest that TGF-β induces CYR61 expression in PSCs through canonical TGF-β-ALK5-Smad signaling.
Figure 24: TGF-β Induces CYR61 Through Canonical ALK5-Smad Signaling

(A,B) Western blot analysis of downstream TGF-β signaling. LTC-14 and imPSC cells were serum starved in 1% FBS then treated with 100 pM TGF-β1 ligand for indicated times. (C,D) Western blot analysis of CYR61 (Abcam antibody) in LTC-14 and imPSC cells pretreated with DMSO vehicle control or inhibitors against ALK5 (20 μM SB431542), p38 MAPK (10 μM SB203580), or PI3K (10 μM LY294002) for 30 mins then treated with 100 pM TGF-β1 for 16 hrs. (E,F) Western blots analysis for CYR61 (Abcam antibody) and HA. LTC-14 and imPSC cells were infected with HA-tagged CA-ALK5 adenovirus for 48 hrs or treated with 100 pM TGF-β for 30 mins or 24 hrs. Cells were serum starved in 1% FBS. All Western results are representative of three independent experiments.
3.8 TGF-β-induced CYR61 promotes gemcitabine resistance in an in vitro co-culture assay

Our data indicate that CYR61 is expressed predominately by stromal PSCs in the tumor microenvironment, and that TGF-β signaling can drive high expression of CYR61 in PSCs. To examine the role of stromal TGF-β-induced CYR61, we established an in vitro co-culture assay by treating PDAC cells with conditioned media collected from PSCs and examined the effect of the conditioned media on gemcitabine-induced apoptosis (Figure 25). LTC-14 PSCs were infected with adenoviruses to express luciferase control, CYR61, or CA-ALK5 (Figure 26A). We verified that activation of TGF-β signaling in LTC-14 PSCs thorough TGF-β1 ligand treatment or expression of CA-ALK5 releases soluble CYR61 into the conditioned media (Figure 26B). Conditioned media from PSCs with CYR61 expression or CA-ALK5 protected CFPAC and BxPC3 cells from gemcitabine-induced apoptosis as demonstrated by reduced levels of cleaved caspase 3 (Figure 26C,D). We confirmed the effect of TGF-β activation in PSCs on chemoresistance of cancer cells by performing the co-culture assay with L3.6p PDAC cells and using the imPSC stellate cell line (Figure 27A,B). We also activated TGF-β signaling in LTC-14 PSCs with TGF-β ligand treatment and found the same effect as expression of CA-ALK5 (Figure 27C). Finally, in silico analysis of whole tissue-PDAC samples demonstrated that TGFB1 ligand expression negatively correlates with expression of SLC29A1 (hENT1) and SLC28A3 (hCNT3) (Figure 28), suggesting that TGF-β plays a role in regulating these nucleoside transporters in vivo.
Figure 25: In Vitro Co-Culture Model

An *in vitro* co-culture assay was established by treating PDAC cells with conditioned media from pancreatic stellate cell lines for 24 hrs before treating with gemcitabine for 48 hrs and measuring gemcitabine-induced apoptosis.
Figure 26: TGFβ-Induced CYR61 Promotes Gemcitabine Resistance

(A) Western blot analysis of CYR61 (Abcam antibody) in LTC-14 cells. LTC-14 cells were infected with CA-ALK5 or luciferase control adenovirus or CYR61 or luciferase control adenovirus at MOI 100 for 48 hrs. (B) Western blot of CYR61 (Abcam antibody) in LTC-14 condensed conditioned media following treatment with 100 pM TGF-β1 or infected with CA-ALK5 adenovirus at MOI 25 for 48 hrs. (C) Western blot analysis of cleaved caspase 3 in CFPAC cells treated with 10 µg/ml gemcitabine for 48 hrs. CFPAC cells were pretreated for 24 hrs with conditioned media (CM) collected from LTC-14 or non-conditioned media (non-CM) as a control. CM from LTC-14 cells infected with adenoviruses as indicated in panel B. (D) Western blot analysis of cleaved caspase 3 in BxPC3 cells after 48 hrs treatment with 20 µg/ml gemcitabine. PDAC cells were pretreated for 24 hrs with conditioned media (CM) collected from LTC-14 PSCs or non-conditioned media (non-CM) as a control. CM was collected from LTC-14 PSCs infected with CA-ALK5 adenovirus (MOI 25), CYR61 (MOI 100), or control luciferase adenovirus (MOI 100). All Western results are representative of 3 independent experiments.
Figure 27: TGF-β Signaling in PSCs Inhibits Gemcitabine-Induced Apoptosis in Cancer Cells

Western blots analysis of cleaved caspase 3 in cells from in vitro co-culture assay after 48 hrs treatment with indicated doses of gemcitabine. (A) L3.6p cells were treated with conditioned media collected from LTC-14 cells or non-conditioned media (non-CM) as a control. LTC-14 cells were infected with CA-ALK5 adenovirus or control luciferase adenovirus (MOI 25). (B) CFPAC cells were treated with conditioned media collected from imPSC cells or non-conditioned media (non-CM) as a control. CM was collected from imPSC cells infected with CA-ALK5 adenovirus or control luciferase adenovirus (MOI 25). (C) BxPC3 cells were treated with conditioned media from LTC-14 cells that were treated with or without 100 pM TGF-β1 for 24 hrs before start of conditioned media collection. All Western results are representative of three independent experiments.
Interestingly, activation of TGF-β signaling in PSCs resulted in a decrease in dCK levels in PDAC cells in our co-culture model (Figure 29). We saw no effect on dCK when altering CYR61 levels (Figure 17), suggesting that TGF-β might induce other factors in PSCs in addition to CYR61 that regulate gemcitabine response in PDAC cells through distinct mechanisms. Additional studies are needed to identify what other factors downstream of TGF-β might regulate dCK expression and gemcitabine resistance.

Figure 28: Correlation of hENT1 and hCNT3 with TGFB1

Linear regression using the microarray dataset GDS4103 for (A) hENT1 (SLC29A1) and (B) hCNT3 (SLC28A3). n=39 patient samples
Figure 29: Activation of TGF-β Signaling in PSCs Decreases dCK in PDAC Cells in Co-culture Assay

Western blot of dCK in BxPC3 cells after 48 hr treatment with conditioned media (CM) from LTC-14 PSCs expressing CA-ALK5 or luciferase control. dCK expression normalized to β-actin. ANOVA, Tukey’s multiple comparison test, *p=0.0142 for Non-CM versus CA-ALK5 CM. n= 3 independent replicates

In addition to TGF-β signaling, CYR61 expression is also known to be regulated by Hippo-YAP/TAZ signaling (Jun et al., 2011). Indeed CYR61 expression significantly correlated with expression of the Hippo target gene AXL in PDAC samples (Figure 30A). However, activation of Hippo signaling in LTC-14 PSCs through expression of constitutively-active YAP5SA only moderately induced CYR61 expression relative to TGF-β treatment (Figure 22B) and expression of YAP5SA in LTC-14 PSCs did not affect the gemcitabine-induced apoptosis of PDAC cells in our in vitro co-culture model (Figure 30B), suggesting that TGF-β signaling was the primary pathway inducing CYR61 expression in our PSC model.
Figure 30: Hippo Signaling in PSCs Did Not Promote Gemcitabine Resistance

(A) Linear regression using the microarray dataset GDS4103 for AXL. (B) Western blot analysis of cleaved caspase 3 in BxPC3 cells after 48 hrs treatment with 10 μg/ml gemcitabine. BxPC3 cells were pretreated with non-conditioned media (non-CM) or conditioned media from LTC-14 cells expressing luciferase control or YAP5SA with or without adenovirus expression of CA-ALK5.
3.9 CYR61 regulates gemcitabine-induced Mcl-1 expression in PDAC cells

The mitochondrial or intrinsic pathway of apoptosis involves the release of cytochrome c from the mitochondrial intermembrane into the cytoplasm, where it facilitates activation of the caspase cascade (Figure 31). The release of cytochrome c is regulated by the Bcl-2 family, which is comprised of pro-apoptotic members Bax and Bak as well as anti-apoptotic members Bcl-2, Bcl-xL, and Mcl-1. The balance of these pro- and anit-apoptotic family members regulates the cell decision of survival or apoptosis (Figure 31).
Figure 31: The Apoptotic Pathway. Adapted from (Correia et al., 2015)
We examined whether the ability of CYR61 to inhibit gemcitabine-induced apoptosis involves regulation of these Bcl-2 family members. We found that gemcitabine induced down-regulation of Mcl-1 in PDAC cell lines that are sensitive to gemcitabine-induced apoptosis, like BxPC3 and CFPAC, while it failed to down-regulate Mcl-1 in gemcitabine resistant cell lines, like PANC1 and MiaPaCa-2 (Figure 32A). Gemcitabine did not induce down-regulation of the other anti-apoptotic family members Bcl-2 and Bcl-xL in sensitive cell lines (Figure 32B). Adenovirus-mediated overexpression of CYR61 in CFPAC cells inhibited the gemcitabine-induced down-regulation of Mcl-1 (Figure 32C). Similarly, CRISPR-mediated knockdown of CYR61 in MiaPaCa-2 cells sensitized cells to gemcitabine-induced down-regulation of Mcl-1 (Figure 32D). CRISPR knockout of CYR61 in PANC1 cells modestly sensitized cells to gemcitabine-induced down-regulation of Mcl-1 (Figure 32E). Further, MCL1 expression strongly correlated with CYR61 expression in the microarray dataset of PDAC tumors (Figure 32F). These data suggest that CYR61 may play a role in regulating the expression of Mcl-1 in PDAC.
Figure 32: CYR61 Regulates Gemcitabine-Induced Mcl-1 Down-Regulation

(A) Western blot analysis for Mcl-1 in cell lines after treatment with 10 µg/ml gemcitabine for 48 hrs. (B) Western blots analysis for Bcl-2 and Bcl-xL in BxPC3 and CFPAC cells after 48 hrs treatment with 10 µg/ml gemcitabine (C) Western blot analysis for Mcl-1 and CYR61 (Abcam) in CFPAC cells infected with CYR61 adenovirus or luciferase control adenovirus at MOI 100 and treated with 10 µg/ml gemcitabine for 48 hrs. (D) Western blot analysis for Mcl-1 and CYR61 (Santa Cruz) in MiaPaCa-2 NTC and CYR61 CRISPR 2 cells treated with 10 µg/ml gemcitabine for 48 hrs. (E) Western blot anlaysis for Mcl-1 and CYR61 (Santa Cruz) in MiaPaCa-2 NTC and CYR61 CRISPR 2 cells treated with 10 µg/ml gemcitabine for 48 hrs. (F) Linear regression using the microarray dataset GDS4103 for MCL1 and CYR61
3.10 Regulation of signaling by CYR61 in PDAC cells

The signaling mechanisms downstream of CYR61 involved in regulating the expression of Mcl-1, hENT1, and hCNT3 remain to be determined. Using our adenovirus-mediated overexpression and CRISPR-mediated knockout models, we examined the role of CYR61 in several signaling pathways. Future studies should examine the contribution of these signaling pathways, and other pathways, to regulation of hENT1, hCNT3, and Mcl-1 expression and gemcitabine resistance. We first looked at Stat3 signaling because this has been reported to be an important inducer of Mcl-1 expression (Yu et al., 2004). CRISPR-mediated knockout of CYR61 in MiaPaCa-2 cells decreased levels of P-Stat3 (Figure 33A), and conversely adenovirus-mediated overexpression of CYR61 in CFPAC cells induced P-Stat3 (Figure 33B). However, CRISPR knockout of CYR61 in PANC1 cells did not affect P-Stat3 levels (Figure 33C), suggesting the potential regulation of Stat3 signaling by CYR61 is not consistent in all cell types.
Figure 33: CYR61 May Regulate Stat3 Signaling in PDAC

(A) Western blot analysis for P-Stat3, total Stat3, and CYR61 (Santa Cruz) in MiaPaCa-2 NTC, CRISPR 3, and CRISPR 1 cells. (B) Western blot analysis for P-Stat3, total Stat3, and CYR61 (Abcam) for CFPAC cells infected with CYR61 adenovirus or luciferase control at MOI 100 for 48 hrs. (C) Western blot analysis for P-Stat3, total Stat3, and CYR61 (Santa Cruz) in PANC1 NTC and CRISPR 1 cells.

Figure 34: CYR61 Regulation of Akt and Erk Signaling in PDAC

(A) Western blot analysis for phospho/total Akt and Erk and CYR61 (Abcam) for CFPAC cells infected with CYR61 adenovirus or luciferase control at MOI 100 for 48 hrs. (B) Western for phospho/total Akt and Erk and CYR61 (Santa Cruz) for PANC1 NTC and CRISPR 1 cells.
We also examined the phosphorylation of the Akt and Erk pathway because they are commonly activated by CYR61-integrin signaling in other cell types (Lau, 2011). Over-expression of CYR61 in CFPAC cells increased levels of P-Akt (Figure 34A), and conversely CRISPR-mediated knockout of CYR61 decreased levels of P-Akt in PANC1 cells (Figure 34B), suggesting that CYR61 may regulate Akt signaling in PDAC cells. Overexpression of CYR61 increased P-Erk levels in CFPAC cells, but CRISPR knockout had the same effect in PANC1 cells, suggesting that CYR61’s effect on Erk signaling might be different in these cell lines (Figure 34). These data indicate that CYR61 may regulate gene expression via activation of Stat3, Akt, or Erk signaling, but the mechanisms might be cell-dependent and depend on context. Future studies should manipulate activation of these pathways using inhibitors and dominant-negative or constitutively-active constructs to examine their contribution to CYR61’s regulation of hENT1, hCNT3, and Mcl-1 expression as well as gemcitabine resistance.

3.11 Summary

We have identified that the secreted matricellular protein CYR61 negatively regulates the expression of the nucleoside transporters hENT1 and hCNT3 in PDAC cells. CRISPR-mediated knockout of CYR61 in PANC1 and MiaPaCa-2 cells resulted in increased influx of gemcitabine into the cell as measured by radiolabeled ³H-gemcitabine transport assays. Cells with knockout of CYR61 had a greater loss of cell viability and induction of cleaved caspase 3 in response to gemcitabine treatment, suggesting that
CYR61 promotes resistance to gemcitabine-induced apoptosis. In PDAC patient samples, expression of hENT1 (SLC28A1) and hCNT3 (SLC28A3) negatively correlated with expression of CYR61. CYR61 expression was also increased in PDAC tumor tissue compared to normal adjacent tissue, and precursor PanIN lesions had an intermediate level of CYR61. We have identified that CYR61 is significantly elevated in the serum of PDAC patients compared to healthy volunteers. In the in vivo tumor microenvironment, CYR61 was expressed primarily by PSCs. We demonstrate that TGF-β signaling induced CYR61 expression in PSCs, and expression of CYR61 positively correlated with the expression of TGFB1 ligand and the TGF-β-responsive genes SERPINE1 and SMAD7 in PDAC patient samples. In PSCs, TGF-β induced both canonical Smad signaling as well as activation of non-canonical PI3K-Akt and p38 MAPK signaling. As an ALK5 kinase inhibitor blocked TGF-β induction of CYR61 expression in PSCs, but PI3K-Akt and p38 MAPK inhibitors failed to decrease induction, TGF-β likely regulates CYR61 expression via canonical ALK5-Smad signaling. We established an in vitro co-culture assay to examine the effect of TGF-β signaling in PSCs on gemcitabine resistance in by PDAC cells. Activation of TGF-β signaling and overexpression of CYR61 in PSCs protected PDAC cells from gemcitabine-induced apoptosis. In PDAC patient samples, expression of hENT1 (SLC28A1) and hCNT3 (SLC28A3) negatively correlated with expression of TGFB1, suggesting this regulation might be relevant in vivo. CYR61 also regulated the gemcitabine-induced down-regulation of the anti-apoptotic Bcl-2 family
member Mcl-1 in PDAC cells. Gemcitabine treatment induced down-regulation of Mcl-1 in gemcitabine-sensitive CFPAC and BxPC3 cells, but gemcitabine did not down-regulate Mcl-1 in the resistant MiaPaCa-2 and PANC1 cells. Overexpression of CYR61 prevented gemcitabine-induced down-regulation of Mcl-1 in CFPAC cells, and conversely CRISPR-knockdown of CYR61 sensitized MiaPaCa-2 and PANC1 cells to gemcitabine-induced down-regulation of Mcl-1. While we have identified a role for CYR61 in regulating hENT1, hCNT3, and Mcl-1 expression in PDAC, we have not identified the signaling mechanisms through which CYR61 mediates these effects. Our preliminary studies demonstrated that CYR61 regulated Stat3, Akt, and Erk signaling in PDAC cells in a context-specific manner. CYR61 induced Stat 3 signaling in MiaPaCa-2 and CFPAC cells but did not regulate Stat3 signaling in PANC1 cells. CYR61 promoted Akt signaling in both PANC1 and CFPAC cells, but both overexpression in CFPAC cells and knockout of CYR61 in PANC1 cells activated Erk signaling. Future studies should use dominant-negative and constitutively-active constructs to identify the signaling mechanisms through which CYR61 regulates hENT1, hCNT3, and Mcl-1 expression in PDAC.

3.12 Future Directions

Prior studies have demonstrated that CYR61 mediates many of its cellular effects by signaling through integrins, particularly integrin $\alpha_v\beta_3$ and $\alpha_v\beta_1$. A mutation that converts the aspartate 125 residue of CYR61 to alanine (D125A) prevents soluble CYR61
from binding to integrin αvβ3 (N. Chen et al., 2004), while mutating three sites within the TSP1 and CT domains creates a mutant CYR61 that is defective in binding to integrin α6β1-HSPG but is still capable of interacting and signaling through integrin αvβ3 (Leu et al., 2004). These mutated CYR61 constructs could be used to examine whether each specific integrin binding is required for CYR61’s effects on nucleoside transporter expression and gemcitabine resistance. In additional, studies using existing blocking antibodies against integrin αvβ3 or integrin β1 could be used to determine if inhibition of either integrin phenocopies the effect of CYR61 knockout and whether they are sufficient to abrogate the effect of CYR61 overexpression. In a xenograft mouse model of PDAC, expression of integrin αvβ3 promoted primary tumor growth and metastasis via activation of Src (Desgrosellier et al., 2009), suggesting that activation of integrin αvβ3 by CYR61 might have important biological effects in PDAC. If CYR61 does not regulate hENT1 and hCNT3 through binding to integrin αvβ3 or integrin α6β1, the contribution of other integrins or growth factor interactions could be examined.

It would also be of interest to examine the role of CYR61 in PDAC using a genetic mouse model of PDAC where CYR61 signaling is abrogated by conditionally knocking out CYR61 or by treating the mice with a CYR61 neutralizing antibody to inhibit downstream integrin signaling. A global knockout of CYR61 is embryonic lethal (Lau, 2011), so a conditional knockout model would be necessary for this study. CYR61 could be knocked out in myofibroblast PSCs specifically using the α-SMA promoter, but since
it is a soluble factor that is present in the serum (Figure 16D), a global conditional knockout model might be required. In these genetic mouse studies, it would be important to identify the effects on primary tumor growth, metastasis, angiogenesis, and the effectiveness of gemcitabine.

Data from the ICGC database suggested that patients with high expression of CYR61 have lower overall survival (Figure 16E), but the available data did not include what treatment these patients received. While many patients likely received gemcitabine, it would be interesting to examine whether CYR61 expression significantly relates to survival specifically in a patient population receiving gemcitabine treatment. For example, the expression of hENT1 is significantly associated with survival only in patients receiving gemcitabine but has no association with survival in patients receiving other treatment (Fujita et al., 2010; Marechal et al., 2012). Our data suggest that we might see a larger association between CYR61 expression and survival if we focus on the group of patients receiving gemcitabine. To identify potential drug targets, it would also be important to identify whether other factors in addition to CYR61 are responsible for regulating hENT1 and hCNT3 expression in the PDAC microenvironment.

CYR61-integrin α6β1 signaling promotes senescence in myofibroblasts during late stages of wound healing and recently has been implicated in regression of liver fibrosis by inducing senescence or apoptosis of hepatic stellate cells (Borkham-Kamphorst et al., 2016; K. H. Kim et al., 2013). We have identified that CYR61 is expressed in response to
TGF-β in PSCs and is elevated in PDAC, and it would be interesting to examine whether CYR61 similarly induces senescence of PSCs via integrin α6β1 signaling during pancreatic fibrosis and cancer. The recent identification that ablation of myofibroblasts (using the α-SMA promoter) in a PDAC genetic mouse model accelerated tumor progression and lowered survival suggests that senescence or apoptosis of myofibroblasts may promote tumor progression. Therefore, it will be important to examine whether targeting CYR61 might be beneficial in regulating the interaction between stromal and cancer cells in PDAC.

To perform a rescue experiment, hENT1 and hCNT3 expression could be knocked down in CYR61 CRISPR cells to return to levels in the NTC control cells. Experiments could evaluate whether this knockdown of hENT1 and hCNT3 rescues the effects of CYR61 knockout on gemcitabine uptake and levels of gemcitabine-induced apoptosis. Similarly, cells with CYR61 knockout could be treated with the hENT1-specific inhibitor NBMPR to determine whether inhibiting hENT1 activity rescues the effect of CYR61 on gemcitabine-induced apoptosis. PDAC cells could also be treated with recombinant CYR61 to determine whether that treatment decreases hENT1 and hCNT3 expression. Our data indicate that CYR61 is predominantly expressed by stromal pancreatic stellate cells within the tumor microenvironment. Immunohistochemical staining of PDAC tumor tissue could further examine the cellular source of CYR61 within the tumor.
4. The Role of TGF-β Signaling in PSCs

PSCs exist in a quiescent state in the normal pancreas, but they are activated to a myofibroblast state in disease settings, such as acute pancreatitis, chronic pancreatitis, and PDAC (Omary et al., 2007). This activation is characterized by excessive secretion of ECM components and expansion of the numbers of PSCs in the pancreas. Activation of PSCs to the myofibroblast state is a normal aspect of promoting injury repair in the pancreas, but the persistent, pathological activation that occurs during fibrosis and cancer leads to excessive deposition of the ECM components. The role of myofibroblast PSCs and the fibrotic stroma in PDAC progression has been of high interest recently, with studies having conflicting results on whether myofibroblast PSCs promote cancer progression or act to retrain growth and maintain normal tissue homeostasis. TGF-β induces myofibroblast activation of PSCs as well as hepatic stellate cells in the liver and fibroblasts in many other organs (Omary et al., 2007). TGF-β signaling is a major driver of the expression of ECM proteins like collagen and fibronectin. We have identified that TGF-β induces PSCs to secrete CYR61, which promotes chemoresistance in cancer cells. However, it is important to gain a broader understanding of how TGF-β signaling in PSCs contributes to cancer progression, including therapy resistance and metastasis. Patients with elevated TGF-β ligand in their serum have significantly worse prognosis (Javle et al., 2014). However, mutations inactivate the TGF-β signaling pathway in cancer cells in over half of PDAC patients. Therefore, it is important that we understand
how TGF-β signaling in stromal cells affects cancer progression and therapy response.

In a broader sense, it is important that we understand what role quiescent vs. myofibroblastic PSCs have in PDAC. TGF-β is a well-known driver of myofibroblast activation of both stellate cells and fibroblasts, but the signaling mechanisms downstream of TGF-β that regulate myofibroblast activation are not well studied in PSCs.

4.1 TGF-β induces myofibroblast activation of PSCs through ALK5 and p38 MAPK signaling

To study the role of downstream signaling pathways in TGF-β-induced myofibroblast activation in PSCs, we established an in vitro model of TGF-β-induced myofibroblast activation using the LTC-14 cell line. Activation from the quiescent to the myofibroblast state is marked by expression of the cytoskeletal protein α-SMA (Omary et al., 2007). In our model, treatment with TGF-β induced abundant α-SMA positive stress fibers, as seen through immunofluorescent (IF) staining for α-SMA (Figure 35A). Further, robust increases in expression of α-SMA and the ECM protein fibronectin were detectable by Western blot (Figure 35B).
Our data indicate that TGF-β induces activation of both canonical ALK5-Smad signaling and non-canonical p38 MAPK signaling in PSCs (Figure 24A,B). While canonical ALK5-Smad signaling is known to contribute to TGF-β-induced myofibroblast activation of PSCs (Ohnishi et al., 2004), the role of TGF-β-p38 MAPK signaling has not been examined in PSCs. In fibroblasts, TGF-β-p38 MAPK signaling has been demonstrated to be important in TGF-β-induced myofibroblast activation (Meyer-Ter-Vehn et al., 2006; Meyer-ter-Vehn et al., 2011). Therefore, we were interested in examining whether TGF-β-p38 MAPK signaling is involved in TGF-β-induced myofibroblast activation of PSCs. Treatment with either the p38 MAPK inhibitor (SB203580) or the ALK5 inhibitor (SB431542) blocked TGF-β-induced myofibroblast activation of PSCs.
activation of LTC-14 PSCs, as demonstrated by decreased levels of α-SMA positive stress fibers in IF staining (Figure 36). Western blot confirmed that the p38 MAPK inhibitor blocked TGF-β-induced α-SMA expression (Figure 38A).

**Figure 36: TGF-β Induces Myofibroblast Activation of PSCs Through ALK5 and p38 MAPK Signaling**

Immunofluorescent staining for α-SMA (red) and DAPI (blue, nuclei) (A) LTC-14 cells were serum starved in 1% FBS then pre-treated with DMSO or p38 MAPK inhibitor (10 μM SB203580) for 30 mins then treated with 50 pM TGF-β1 for 24hrs. (B) LTC-14 cells were serum starved in 1% FBS then pre-treated with DMSO or ALK5 inhibitor (20 μM SB431542) for 30 mins then treated with 50 pM TGF-β1 for 24 hrs

**4.2 TGF-β induces p38 MAPK activation through TRAF6-TAK1 signaling**

The mechanisms through which TGF-β activates p38 MAPK in PSCs have not been examined previously. TGF-β can activate p38 MAPK in other cell types through interaction of the E3 ubiquitin ligase TRAF6 (TNF receptor-associated factor 6) with the TGF-β receptors (Figure 37). TRAF6 associates with the TGF-β receptor complex that
forms following TGF-β ligand binding, and this association induces K63-linked polyubiquitination of TRAF6, which in turn promotes ubiquitination and activation of downstream kinase TAK1 (TGF-β Activated Kinase 1) (Y. E. Zhang, 2009). TAK1 phosphorylates the kinases MKK3/6, which then phosphorylate and activate p38 MAPK.

Figure 37: Mechanism of TGF-β-Induced p38 MAPK Activation. Adapted from (Y. E. Zhang, 2009)

We examined whether this pathway was active in promoting p38 MAPK activation downstream of TGF-β in PSCs. To examine the role of TRAF6, we expressed either WT TRAF6 or a dominant-negative version of TRAF6 (C70A) that lacks E3 ligase activity. TRAF6 C70A inhibited TGF-β-induced phosphorylation of p38 MAPK (Figure 38B). Similarly, treatment of PSCs with the TAK1 inhibitor 5z-7-oxozeaenol reduced
TGF-β induced phosphorylation of p38 MAPK (Figure 38C). These data indicate that TGF-β-induced activation of p38 MAPK signaling in PSCs occurs via activation of the E3 ubiquitin ligase TRAF6 and the downstream kinase TAK1.

Figure 38: TGF-β Activates p38 MAPK Through TRAF6-TAK1 Signaling in PSCs

(A) LTC-14 cells were serum starved for 6 hrs, pretreated with DMSO or p38 MAPK inhibitor (10 µM SB203580) for 30 mins, then treated with 50 pM TGF-β1 for 24hrs. (B) LTC-14 cells were transfected with pcDNA, pcDNA-WT TRAF6, or pcDNA-C70A TRAF6. 48 hrs after transfection, cells were serum-starved and then treated with 50 pM TGF-β1 for 45 mins. (C) LTC-14 cells were serum starved for 6 hrs, pretreated with DMSO or 100nM 5z-7-oxozeaenol for 30 mins then treated with 50 pM TGF-β1 for the indicated times.
4.3 Activation of TGF-β-p38 MAPK signaling in PSCs promotes invasion of PDAC cancer cells

Our data point to an important role for TGF-β-induced p38 MAPK signaling in myofibroblast activation of PSCs. Next, we examined whether TGF-β-induced p38 MAPK signaling in PSCs regulates invasion of neighboring cancer cells, which is important for formation of metastases in PDAC. To examine invasion, we plated PANC1 cells in a Matrigel-coated transwell and measured the number of cells that invaded through the transwell in response toward conditioned media collected from PSCs. The PSC conditioned media contained 0.5% FBS, and we used 0.5% FBS and 10% FBS non-conditioned media as controls. Conditioned media was collected for 48hrs from PSCs following 24hrs treatment with TGF-β after pre-treatment with the p38 MAPK inhibitor (SB203580) or DMSO control. Conditioned media from PSCs treated with TGF-β for 24hrs induced higher invasion of PANC1 cells than conditioned media from untreated PSCs (Figure 39). However, after pretreatment with the p38 MAPK inhibitor, TGF-β treatment of PSCs induced significantly less invasion of PANC1 cancer cells in our model (Figure 39). These data suggest that TGF-β induces PSCs to secrete soluble factors that induce invasion of neighboring cancer cells, and this induction of these soluble factors requires downstream TGF-β-p38 MAPK signaling. Additional studies are necessary to identify the soluble factor(s) that mediate(s) the conditioned media-induced increase in invasion.
Figure 39: Inhibition of TGF-β-Induced p38 MAPK Signaling in PSCs Inhibits Invasion of PDAC Cells

Matrigel invasion assay using PANC1 cells invading towards bottom chambers containing control media (0.5% or 10% FBS) or conditioned media (CM) from HPSC-T cells (0.5% FBS) collected after the indicated treatments.

4.4 Summary and Future Directions

We established an in vitro model of TGF-β-induced myofibroblast activation using the LTC-14 and imPSC cell lines (Figure 35). This model will be particularly useful in future studies to examine both the mechanism of TGF-β-induced myofibroblast activation and the role of TGF-β-induced myofibroblast activation on the biology of neighboring cancer cells. Our data indicate that TGF-β induces myofibroblast
activation of PSCs through both the canonical ALK5-Smad pathway and the non-canonical p38 MAPK pathway. Future studies could examine the contribution of other signaling pathways downstream of TGF-β, including PI3K-Akt signaling, as well as use dominant-negative and constitutively-active kinases to examine the role of each pathway further. We have demonstrated that activation of p38 MAPK by TGF-β occurred via TRAF6-TAK1 pathway in PSCs. These data suggest that inhibiting this TGF-β-TRAF6-TAK1-p38 MAPK pathway might block TGF-β-induced myofibroblast activation, and further studies should examine the role of TRAF6 and TAK1 in myofibroblast activation and induction of invasion in cancer cells. Our results demonstrated that TGF-β signaling in PSCs induced invasion of cancer cells in an in vitro invasion assay, but inhibition of TGF-β-p38 MAPK signaling abrogated this induction of invasion in PDAC cells. Future studies could focus on identifying what soluble factors induced by TGF-β signaling in PSCs are responsible for promoting invasion of neighboring cancer cells.

Additional studies are required to clarify the role of TGF-induced myofibroblast activation in PDAC cancer progression and determine whether targeting TGF-β signaling in PSCs is a therapeutic option in PDAC patients. One interesting approach is to knock out TGF-β signaling in PSCs in a genetic mouse model of PDAC and examine the effect on tumor growth, metastasis, and chemotherapy response. This could be accomplished through conditional deletion of TGFBR2 in PSCs. However, the choice of
promoter to specifically alter gene expression in PSCs remains a challenge. In previous studies, α-SMA is commonly used to alter gene expression in PSCs. However, α-SMA is only expressed in myofibroblast PSCs not quiescent PSCs, so this would only alter gene expression in cells that have already undergone activation. Further, α-SMA is a well-established marker of smooth muscle cells and pericytes, so it is likely there will be non-specific knockout of TGF-β signaling in other cell types.
5. Conclusions and Significance

5.1 The Role of Stromal Pancreatic Stellate Cells in PDAC

The high rate of therapy resistance is a major clinical problem in PDAC. Even one of the commonly used and most effective first-line therapies available, gemcitabine, has a very low response rate and only modestly prolongs survival. Therefore, it is important to understand the cellular mechanisms that regulate resistance to gemcitabine. Our results demonstrated that stromal-derived CYR61 promoted resistance to gemcitabine predominantly by modulating the levels of the nucleoside transporters that mediate cellular uptake of gemcitabine. The role of the stroma in therapy resistance is an emerging area of interest in PDAC with recent genetic mouse models and clinical trials that target PSCs having conflicting results, with both positive and negative effects on cancer progression and response to gemcitabine (Ozdemir et al., 2014; Provenzano et al., 2012; Rhim et al., 2014; Sherman et al., 2014). While in vitro and xenograft studies have demonstrated that PSCs promote cell survival and metastasis, depletion of myofibroblast PSCs in a genetic mouse model of PDAC surprisingly led to lower survival. The current consensus in the field is that there are aspects of the fibrotic stroma that promote cancer progression and therapy resistance and other aspects that promote normal tissue homeostasis or restrain tumor growth. Understanding what aspects of PSCs promote therapy resistance and metastasis and identifying signaling mechanisms that regulate these will be important to effectively target the stroma. We
have demonstrated that the stromal-derived factor CYR61 has an important role in promoting gemcitabine resistance through down-regulation of the nucleoside transporters hENT1 and hCNT3. This contributes to our understanding of the complex interaction between PSCs and cancer cells in the tumor microenvironment.

We have also demonstrated that activation of TGF-β-p38 MAPK signaling in PSCs can promote invasion of cancer cells through Matrigel-coated transwells (Figure 39). TGF-β promoted myofibroblast activation of PSCs (Figure 35), and the p38 MAPK inhibitor blocked this TGF-β-induced myofibroblast activation (Figure 36, Figure 38A). Future in vitro and in vivo studies should focus on identifying how factors secreted from PSCs influence the migration and invasion of PDAC cells. Over the last few years, it has become clear that therapies that attempt to ablate the stroma in PDAC will have complex effects on PDAC patients, so efforts should focus on identifying mechanisms of signaling crosstalk between PSCs and cancer cells and evaluating how these affect cancer progression and therapy resistance in relevant model systems.

5.2 The Role of TGF-β in Gemcitabine Resistance

We have demonstrated that TGF-β strongly induced CYR61 expression in PSCs (Figure 22), and expression of TGFβ1 ligand negatively correlated with expression of the nucleoside transporters hENT1 and hCNT3 in PDAC patient samples (Figure 28). Inhibition of TGF-β signaling in combination with gemcitabine is currently being investigated in a clinical trial for PDAC patients (NCT0137314). Due to the pleotropic homeostatic functions of TGF-β, global inhibition of TGF-β signaling does have the
potential to have side effects. Therefore, understanding the specific downstream
effectors of TGF-β signaling in PDAC is important for development of future therapies.
Our results indicate that stromal TGF-β signaling down-regulates the expression of
hENT1 and hCNT3 in PDAC cells via induction of CYR61. TGF-β is a major driver of
EMT (Lamouille et al., 2014), and EMT has recently been demonstrated to regulate
gemcitabine resistance and expression of hENT1 and hCNT3 in in vivo models of PDAC
(Zheng et al., 2015). Whether additional TGF-β-induced genes promote gemcitabine
resistance through regulation of these nucleoside transporters remains to be determined.
Treatment with recombinant CYR61 has been demonstrated to induce EMT of BxPC3
cells (Haque et al., 2011). Whether CYR61 mediates the effects of TGF-β on EMT in
PDAC also remains to be established. Interestingly, hENT1 and hCNT3 expression are
much higher in a genetic mouse model of PDAC with abrogated TGF-β signaling (Ptf1a
(P48)-cre; LSL-KrasG12D;Tgfbr2L/L) compared to the KPC mouse model (Ptf1a<sup>cre+</sup>;LSL-
KrasG12D<sup>;</sup>Trp53R172H<sup>+</sup>) with intact TGF-β signaling (Zheng et al., 2015), which suggests
that TGF-β signaling in cancer cells may negatively regulate hENT1 and hCNT3
expression in PDAC, a hypothesis that has not yet been examined. One group
demonstrated that treating PDAC cells with an ALK5 inhibitor (SB525334) in
combination with gemcitabine sensitized the cells to gemcitabine-induced apoptosis and
decreased cell viability, but no mechanism was proposed (Y. J. Kim et al., 2012). It
would be interesting to examine whether TGF-β signaling directly regulates hENT1 and hCNT3 expression in vitro and/or in vivo.

### 5.3 The Role of the CCN Family in Therapy Resistance

CYR61 is a member of the CCN family of matricellular proteins, which includes CTGF. Targeting the CCN family member CTGF in combination with gemcitabine in PDAC has demonstrated promise in a preclinical mouse model (Neesse et al., 2013), and the CTGF neutralizing antibody FG-3019 is being tested in combination with gemcitabine in an ongoing clinical trial by FibroGen (Dimou et al., 2013). However, although CTGF and CYR61 are structurally similar, the FG-3019 neutralizing antibody does not interact with CYR61 (Wang et al., 2011). Our results regarding the role of CYR61 in gemcitabine resistance provide a rationale for inhibiting CYR61 or both CTGF and CYR61 in combination with gemcitabine (and nab-paclitaxel) in PDAC patients. In vitro cell culture studies and mouse models should examine whether inhibition of both CTGF and CYR61 could be a promising treatment option in PDAC.

A role for CYR61 in regulating therapy resistance in other cancers has been identified, including vinblastine in renal cell carcinoma; paclitaxel, adriamycin, and β-lapachone, and taxol in breast cancer; mitoxantrone in acute myeloid leukemia; and carboplatin in ovarian cancer (Figure 4). Our data demonstrated that CYR61 specifically regulated gemcitabine resistance via the nucleoside transporters but also suggested a broader role of CYR61 in cell survival via regulation of the pro-survival Bcl-
family member Mcl-1 (Figure 32). Our findings support a previous study demonstrating that CYR61 positively regulates Mcl-1 expression in ovarian cancer cells (K. B. Lee et al., 2012), so it will be interesting to determine if CYR61 regulates cancer cell survival across cancer types through regulation of Mcl-1 expression. The role of CYR61 in regulating cell survival and apoptosis is context-specific and depends on specific integrin interactions. Therefore, the mechanisms through which Cyr61 regulates Mcl-1 in cancer cells will be important to elucidate. Stat3 signaling is known to be an important regulator of Mcl-1 transcription (Yu et al., 2004), and we have identified that CYR61 may regulate Stat3 signaling in the CFPAC and MiaPaCa-2 cell lines (Figure 33). A recent study has identified that BxPC3 PDAC cells that develop resistance to gemcitabine are very sensitive to inhibition of Stat3 (Ioannou et al., 2016), and it would be interesting to determine whether Stat3 signaling plays a role in chemoresistance in PDAC through regulating Mcl-1 expression. Future studies should examine the mechanism through which CYR61 regulates Mcl-1 and identify the contribution of CYR61 to cell survival.

5.4 The Regulation of hENT1 and hCNT3 in PDAC

In our bioinformatics screen examining potential regulators of hENT1 and hCNT3 in PDAC, we identified several genes whose expression is both altered in cancer and correlated with expression of the hENT1 and hCNT3 nucleoside transporters (Figure 8, Table 5). We examined the role of one of the identified genes, CYR61, and found it is a negative regulator of hENT1 and hCNT1 expression in PDAC. Several
other factors on this list have potential interest to study further. In particular, Heparin-binding EGF-like growth factor (HBEGF) is interesting because it is elevated in PDAC and negatively correlates with both hENT1 and hCNT3 (Table 5). HBEGF is a secreted factor in the epidermal-growth factor pathway that can be targeted by inhibitors and neutralizing antibodies. The role of HBEGF in PDAC and chemo-resistant tumor is not well studied, but a recent report shows that pancreas-specific overexpression of HBEGF in a mouse model was sufficient to promote Kras-initiated tumorigenesis (Ray et al., 2014) suggesting a pro-tumorigenic role. HBEGF signals by binding to the EGFR and ErbB4 receptors on the cell surface, and signaling is enhanced when the ErbB2 receptor heterodimerizes with EGFR and ErbB4 (Miyamoto et al., 2006). Interesting, knockdown of ErbB2 in PDAC cells increases hCNT3 expression and sensitizes the cells to gemcitabine (Skrypek et al., 2015). Whether HBEGF signaling down-regulates hCNT3 and hENT1 through ErbB2 signaling remains to be examined, and investigating the broader role of epidermal growth factor signaling in the regulation of nucleoside transporters will be important. Treatment of PDAC patients with the epidermal growth factor inhibitor erlotinib in combination with gemcitabine significantly improves overall survival and progression-free survival compared to gemcitabine alone in a randomized phase III trial (Moore et al., 2007), but the mechanism of this effect is not fully understood. Future studies could examine whether patients treated with erlotinib have higher expression of the nucleoside transporters hENT1 and hCNT3. One study
conversely demonstrated that erlotinib treatment actually blocked cellular uptake of gemcitabine, although these studies focused on lung cancer cell lines (Damaraju et al., 2014). Further studies are needed to identify whether HBEGF signaling regulates nucleoside transporter expression and gemcitabine sensitivity in PDAC.
References


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Biography

Rachel Anne Hesler

Rachel was born to Stephen and Katherine Hesler on June 18th, 1988 in Rochester, NY. In 2010, she graduated Summa Cum Laude with honors from Clemson University with a Bachelor of Science in Biochemistry and a minor in Genetics. Rachel is scheduled to receive her Doctor of Philosophy in Molecular Cancer Biology from Duke University On April 4, 2016.

Publications:

• Cheryl Ingram-Smith, Jeffrey Wharton, Christian Reinholz †, Tara Doucet †, Rachel Hesler † and Kerry Smith. The Role of Active Site Residues in ATP Binding and Catalysis in the Methanosarcina thermophila Acetate Kinase. Life 2015, 5(1), 861-871. † These authors contributed equally to this work.

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- James B. Duke Fellowship
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