# ENVIRONMENTAL FACTORS INFLUENCING TUMOR ASSOCIATED PERICYTE PHENOTYPE IN PANCREATIC CANCER

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**Title**

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**MASTER OF SCIENCE**

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## ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer death in the U.S. with a 5-year survival rate of 11%. PDAC patients suffer from the lack of effective treatment options largely due to the limited delivery efficacy of the drugs. Tumor vessels are abnormal, leaky, and lack proper pericyte coverage, contributing to elevated hypoxia and interstitial fluid pressure, promoting cancer progression and metastasis, and inhibiting drug delivery efficacy. In the case of PDAC, a large portion of blood vessels were covered by α-smooth muscle actin (α SMA) expressing pericyte, which is normally absent in capillary pericytes. We also showed that PDAC cancer cell-derived exosomes could induce αSMA expression in the pericyte suggesting the pericyte phenotype is influenced by the Tumor Microenvironment (TME). Induced αSMA expression in pericyte disrupted the 3D tube formation efficiency when cultured with endothelial cells suggesting $\alpha SMA^{high}$ pericyte confers a pathological feature of tumor blood vessels.

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## DEDICATION

In memory of Teresa E. Delgado-Perez, Julian Perez, and Yolanda Perez-Diaz.

Dedicated to Primitivo Delgado, Gerardo Delgado, Sergio Delgado, Lauren LeDeaux-Delgado, Teresa “Nena” Delgado, Araceli Delgado, Gina Delgado, Michele Delgado-Koszycki Samuel “Sam” LeDeaux, Patrick Mason, Veronica Pollock, Christian Bedford, Gabriel Delgado, Kyran LeDeaux, Kaycen LeDeaux, Andrea Delgado, Camden LeDeaux, Viviana Munson, Naya Mason, Elliot Black, Michael Scott, Stephen Scott, Audrone Scott-Brakauskas, Andrew Scott, Brian Sutton, Joseph “Joe” DiCosola, Robert “Robbie” DiCosola, Megan DiCosola-Korn, Adriana Torres-Castro, Audrey Karpf, and Robert “Bob” Trzaskus

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| | |
|---|---|
| hPC | Human Placenta Pericyte |
| HUVEC | Human Umbilical Vein Endothelial Cell |
| MMC | Mitomycin C |
| TME | Tumor Microenvironment |
| TGF-β | Transforming Growth Factor Beta |
| HSE | Normal Human Serum Exosomes |
| FGF | Fibroblast Growth Factor |
| VEGF | Vascular Endothelial Growth Factor |
| α-SMA | Alpha-Smooth Muscle Actin |
| Ang-1 | Angiopoietin 1 |
| Ang-2 | Angiopoietin 2 |
| PFA | Paraformaldehyde |
| BSA | Bovine Serum Albumin |
| PBS | Phosphate Buffered Saline |
| NG2 | Neural/Glial Antigen 2 |
| PDGFRβ | Platelet-Derived Growth Factor Receptor Beta |
| Col4α1 | Collagen, Type IV, Alpha 1 |
| PECAM | Platelet Endothelial Cell Adhesion Molecule |
| Tie-2 | Tyrosine Kinase with Ig and EGF Homology Domains-2 |
| Tie-1 | Tyrosine Kinase with Immunoglobulin Like and EGF Like Domains-1 |

# INTRODUCTION

## Pancreatic Cancer

The pancreatic cancer field is suffering from the lack of effective treatment options, in effective drug delivery to the tumor, and early detection methods[3]. It is estimated that in 2021 there will be 60,430 new cases and 48,220 pancreatic cancer associated deaths in the U.S. [10]. According to an NIH-SEER fact sheet (2021), while pancreatic cancer is ranked 11$^{th}$ for the most common types of cancers in occurrence of cases, cancers such as breast and melanoma are estimated to have 2-5x the number of cases of pancreatic cancer. Despite this comparatively lower rate of occurrence, pancreatic cancer is estimated to have the third highest death rate of all the listed cancers at approximately 7.8% of all cancer related deaths. Although there are environmental factors in play, along with lifestyle and genetic factors, one of the most damaging aspects of pancreatic cancer is the late-stage detection of the cancer [3]. While the direct causes for the onset of pancreatic cancer are unknown, what is known is that environmental factors such as smoking, excessive consumption of alcohol, family history and diabetes mellitus are associated with significantly increased risk for pancreatic cancer and hold synergistic relationships [11].

PDAC metastasizes to distant organs early, making most patients unsuitable for surgical resection. Also, many patients experience only nonspecific symptoms, or sometimes, no symptoms at all at early stages, which under either circumstance prevents them from seeking medical intervention before the cancer has already metastasized. In addition, a lack of sensitive tumor markers makes it harder to detect the PDAC at early stages [2]. The extremely hypoxic, heterogenous, and dense TME [12, 13] complicates the treatment strategies as well.

## Tumor Microenvironment

Tumor microenvironment (TME) is the site in which cancer cells grow and communicate with other cells. It is a complex system, which includes various components such as blood vessels, immune cells, fibroblasts, extracellular matrix (ECM), cytokines, growth factors, and hormones [14], that contribute to the tumor growth and progression. TME can be manipulated by cancer cells themselves or by cross communications between differet types of cells, in such a way that the cancer cells are able to survive and proliferate while regular healthy cells and tissue will fail and die. Mutational status of the cancer cells can have significant effects on their behavior and communication with cells within TME. Examples of these changes include situations in which negative feedback loops that ensure homeostatic security are disrupted, such as Ras proteins and p53 [15]. The mutations of these proteins allow for unregulated growth, as is the case with Ras, or allow for the cancer to evade growth suppressors, such as the case with p53. With both proteins being manipulated by the cancer cells, they can proliferate with few issues; and with their unregulated growth, there will come a time when the cells are unable to support themselves or leach from surrounding cells and become necrotic instead of undergoing apoptosis [15]. Necrotic cells burst and release their contents into the intracellular fluid, many of which are proinflammatory proteins that contribute further by recruiting immune cells to the microenvironment and tumor, allowing the cycle to continue and exacerbate [15]. Those changes caused by the cancer cells in the TME is what gives rise to pancreatic cancer's most distinguished features: drug resistance, hypoxia, aggressiveness, and abundance of stroma. The TME consists of many components that are sensitive to growth factors, feedback loops, and other components that produce certain stimuli and other cues that determine the health and function of other cells within the microenvironment [15, 20-21]

When this balance is altered, such as an overabundance of Transforming Growth Factor Beta (TGF-β) induced by cancer cells, processes that govern the health and condition of the microenvironment can be greatly altered or in some situations fail all together. Moreover, the abundance of TGF-β causes the cancer cells to proliferate at an increased rate, and consequently, causes a signaling cascade that leads to the creation of faulty endothelial tubes, which results in poor perfusion within the TME [15]. Due to the complexity and the varying individual differences of the microenvironment from person-to-person, there is still so little known about the many different components and interactions at play [16-21].

The TME consists of proliferating tumor cells, blood vessels, which are made from endothelial cells and supported by pericytes, fibroblasts, and immune cells with various ratio [22]. Since every person is different and the TME recruits various cells both from local and distant to the tumor, the progression and makeup of each TME is unique, along with the interactions that occur within it [22]. This is one of the many other factors contributing the complexity of TME and lack of comprehensive study of it. An example of such is how TME generate signals to recruit and transform immune cells through the use of TGF-β to manipulate the immune response, so the natural response of inflammation can result in the development, growth, and metastasis of the cancer [22, 23].

**Tumor Vasculature**

Tumor growth largely depends on the formation of the vascular system to meet the nutrient and oxygen demands posed by exponential growth [20, 24-27]. Despite the abundance of pro-angiogenic factors such fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) within the TME, PDAC is considered hypovascular in nature and contributes to the hypoxic TME. In addition, cancer cells quickly adapt to the hypoxic environment to survive.

Hypoxia is a condition in which cells and or tissues are starved of oxygen. This condition has been documented to exacerbate the aggressive nature of pancreatic cancer, which includes an increase in apoptosis, exponential increase in tumor growth, chemoresistance, and metastasis [17, 28-30]. In addition to a lack of adequate number of vessels, significantly defective vascular structure further contribute to inefficient oxygen and nutrient diffusion to surrounding tissues, which exacerbates the hypoxic and aggressive nature of PDAC. During tumor angiogenesis, vessels fail to acquire appropriate pericyte coverage leading to structural instability, leakiness, dialated and convoluted vessels[5-8], which in turn significantly effect TME and cancer cell behavior.

Vascular irregularities in the TME are not unique to pancreatic cancer. Other types of cancer such as breast cancer have similar conditions [31, 32]. Irregularities of the vasculature such as dilated, permeable, and convoluted vessels, exacerbate the issues and makes treatment more difficult. This is due to the cancer's domination over the TME in addition to the poor vascular perfusion causing signaling cascades that cause for inflammation to allow for immune cell recruitment and metastasis [8, 15]. Attempts to fix these vascular irregularities aimed to treat prancreatic cancer by inhibiting angiogenesis in the TME. However, these means of treatment for pancreatic cancer have mostly gone unsuccessful. For example, randomized phase III trials with a combinatorial treatment of bevacizumab, an anti-VEGF monoclonal antibody, and gemcitabine, a commonly used therapeutic agent for pancreatic cancer, was unsucesseful to surpass 6 months or provided little change from typical late-stage pancreatic cancer survival with the combination of both drugs [33-35]. Therefore, eliminating blood vessels, even the defective ones, may not be the best approach to target the TME. Instead, a different approach would be to strengthen the already present vessels by reinvesting in pericyte coverage and providing stronger

and more efficient vessels, which has been recorded to improve immune cell infiltration and function[6]. Pericyte reinvestment was attempted in breast tumor models, in which it was found that higher mature pericyte coverage achived by anti-Ang2 antibody treatment reduced hypoxia, vessel leakiness, and distance metastasis [43]. Thus, the presence of correct pericytes may be an important factor allowing the tumor vessels to be refunctionalized, alleviating hypoxia.

Pericytes in the TME can originate from bone marrow mesenchymal stem cells [36]. Depending on the type of blood vessels that the pericytes attach to, there are different phenotypic traits that are expressed. In high pressure locations such as the arteries, pericytes expressing a cytoskeletal protein responsible for contractile force, such as alpha-smooth muscle actin (α-SMA), are more common due to the elastic nature of arteries [37]. As blood surges through the arteries and causes the vessel to dilate, α-SMA ensures pericyte attachment through its contractile force [37]. In low pressure vessels such as veins and capillaries, α-SMA is not required since a low-pressure environment would not demand contractile strength that a pericyte would require in a high-pressure environment such as an artery [37].

Angiogenesis is the process that allows for the formation of new blood vessels and can be stimulated or manipulated by the TME. The angiogenic process is influenced by many circulating factors such as VEGF, FGF, angiopoietin 2 (Ang-2), angiopoietin 1 (Ang-1) and transforming growth factor beta (TGF-β). Due to the increased demand of blood vessels for tumor growth, an abundant presence of circulating angiogenic factors stimulates the proliferation of endothelial cells but does not allow the maintenance or maturation process in a timely manner. This results in suboptimal, convoluted, and nonuniform vessels that do not contribute to better oxygen and nutrient transport [38-40]. Reciprocal signal communication between tumor vessels

and cancer cells and other tumor stromal cells further affects vascular function leading to hypoxic conditions and chemoresistance [38].

What makes the relationship between endothelial cells and pericytes so pivotal is the role and signaling cascade of pericytes. The role of pericytes is to attach to the endothelial cells and provide physical security to the vasculature by reinforcing the tube structure and permeability of the vessels. This ability of the pericyte is essential for specialized protective structures such as the blood brain barrier or limiting the hypoxic environment within the TME [6, 37]. Another important role of the pericyte is the signaling cascade between itself and endothelial cells. There are two important ligands that are integral to the function and stability of the vasculature of the microenvironment and those are Ang-1 and Ang-2.

Ang-1 is responsible for mediating maturation of well constructed and optimal vascular cells and pruning of cells that produce inefficient vasculature [37]. Ang-1 is released by pericytes and binds to the Tyrosine Kinase with Ig and EGF Homology Domains-2 (Tie-2) receptor that is on the surface of endothelial cells. Tie-2 is responsible for allowing the communication pathways to initiate that allow for the stabilization of blood vessels once Ang-1 binds [37]. Conversely, Ang-2 is responsible for the creation of new blood vessels during angiogenesis, which counteracts the function of Ang-1 [37]. Ang-2 is released by the endothelial cells and for the function of Ang-2 to initiate it must also binds to Tie-2 [37]. Lastly, there is the Tyrosine Kinase with Immunoglobulin Like and EGF Like Domains-1 (Tie-1) receptor that is considered an orphan receptor. This classification is due to the receptor not being able to bind directly with Ang-1 or Ang-2 but it has been documented to be able to both negatively and positively regulage Tie-2 during angiogenic processes or perpetuate Ang-1 and Tie-2 signaling [41, 42]. Although the contributions of Tie-1, since both ligands Ang-1 and Ang-2 require the receptor Tie-2 to

initiate the cascade, it experiences competitive binding. Due to the manipulation of the microenvironment by the tumor, more Tie-2 receptors are created and more Ang-2 is released since it is released by endothelial cells [37, 40, 43].

Another confounding factor is that there are no known marker genes that are solely expressed by pericytes. A reason for this could be the wide variation in pericyte function, location, and characteristics [37]. Pericytes do possess several genes that are commonly expressed, but they are not exclusive to pericytes. These genes include Desmin, Collagen, type IV, alpha 1 (Col4α1), Neural/glial antigen 2 (NG2), Platelet-derived growth factor receptor beta (PDGFRβ), and α-SMA [37, 44]. Pericytes that express Desmin are typically denoted to have reached maturity, which signifies a better and more secure attachment to endothelial cells [9, 37]. Desmin is also a cytoskeleton contractile filament protein [9, 37]. Conversely, Col4α1 is responsible for cellular elasticity. NG2 and PDGFRβ are more pericyte-specific genes and are considered responsible for pericyte recruitment. PDGFRβ is a receptor located on the surface of the plasma membrane of pericytes. The ligand, PDGF-BB, is released by the endothelial cell to initiate pericyte recruitment then binds to the receptor PDGFRβ. Lastly, α-SMA is responsible for the contractile force exerted by pericytes. In the focus of our research, α-SMA is an abnormal gene to be expressed in a low-pressure environment such as capillaries [9, 37]. Previous studies by Keskin et al. [45] and Kim et al. [9] showed that proper pericyte coverage of endothelial cells decreased the hypoxic nature of the breast tumor site. Furthermore, the secure vasculature then allowed for efficient administration of therapeutic agents. In this project, we hypothesized that with proper mature pericyte coverage, increased Desmin expression compared to α-SMA expression, there will be a greater probability to reduce the hypoxic nature of the pancreatic tumor microenvironment.

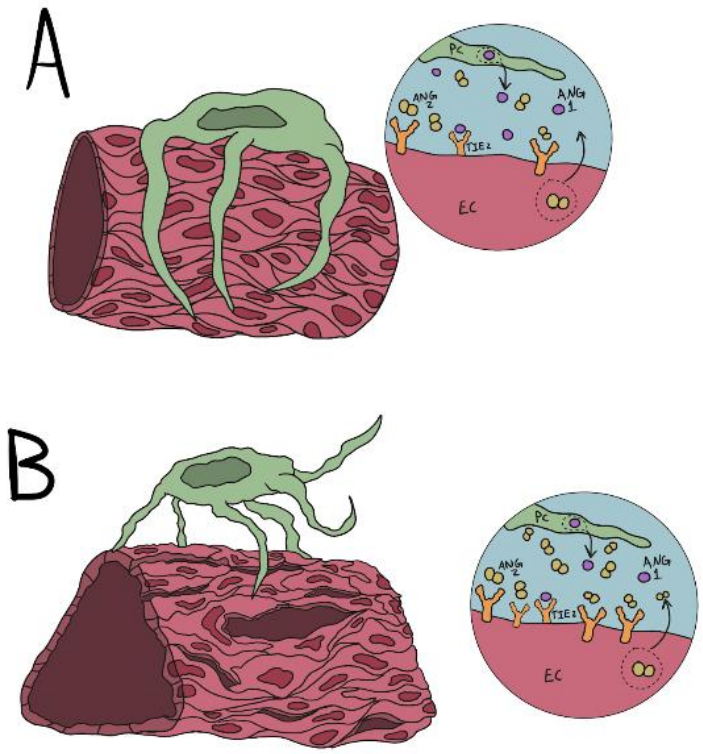


**Figure 1.** Function and Role of a Pericyte.
The pericyte (shown in green) rests on top of the endothelial cells (shown in red) that make up the vessels. Part A shows a healthy vessels in which the pericyte is able to attach properly to the vessel. To the right, in the circle showing a more zoomed in view of the communication site of the pericyte (abbreviated as PC) and endothelial cell (abbreviated as EC). Angiopoietin 1 (ANG 1; shown in purple) is being released by the pericyte and is able to bind to the receptor TIE2 (shown in orange) which is located on the membrane of the endothelial cell. Meanwhile Angiopoietin 2 (ANG 2) is being released by the endothelial cell. In the healthy scenario, as shown in part A, there is balance that exists between the two ligands to maintain the structural and functional integrity of the vessels. Part B shows an unhealthy pericyte that is unable to attach. As a result, the vessel has become nonuniform in shape, leaky and overall inefficient. The more zoomed in view shows an increase in ANG 2 released by the pancreatic tumor activated endothelial cell along with the increased numbers of TIE 2 receptors. This increased release of ANG 2 allows for destabilization of the vessels and conteracts the contribution of vascular integrity done by ANG 1. Additionally due to the greater abundance of ANG 2 and competive binding of ANG 1 and ANG 2 to TIE2, there is greater possibility for ANG 2 to bind than ANG 1.

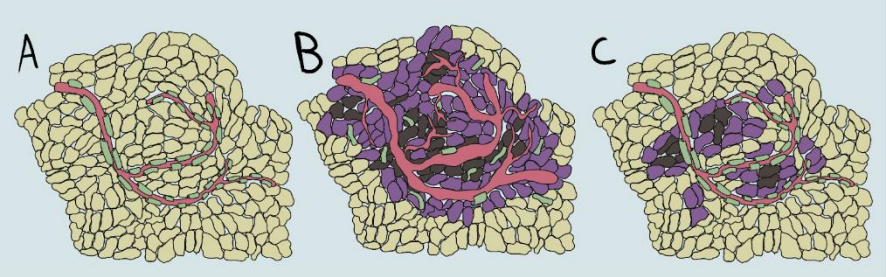


**Figure 2.** Vascular Renormalization.
Part A shows an example of the normal tissue vasculature, the red tubes represent the blood vessels (endothelium), the green portions represent pericytes, and the yellow shapes represent healthy cells of the tissue. The shape of the vasculature is uniform and expresses the proper phenotype that would allow for efficient delivery of oxygen and nutrients. Part B resembles the tumor microenvironment with the cancer cells (represented with the purple shapes) and necrotic cells (represented with dark brown color). While there is a greater number of branching blood vessels, the flow and delivery of oxygen and nutrients is not enough to maintain the tumor microenvironment as the vessels are damaged, inefficient, and leaky.This is evident with the presence of necrotic cell patches buried in the cancer cells. The increased number of necrotic cells would cause for greater potential of aggression of the tumor and greater chance of metastasis. Few pericytes are able to attach and as a result the microenvironment becomes susceptible to unregulated and unoptimized angiogenesis. Part C represents a scenario if the pericytes were reengineered to have a mature and healthy phenotype (proper green patches) that would allow the role of pericytes to function in the structural stability and pruning process for more optimized vessels. We hypothesize this would alleviate hypoxia, interstitial pressure allow for reduced cancer cell proliferation and formation of necrotic patches as the aggressiveness of the tumor is reduced and with the strengthened blood vessels, it would allow for a more secure means to administer a therapeutic agent such as a drug.

## Exosomes

Exosomes, also known as extracellular vesicles, are membrane encased pouches that are released by the cells of an organism, whether that organism is a plant, eukaryote, or prokaryote in origin [46]. Exosomes, as noted by Yáñez-Mó, et al. [46] are separated into three different main classes. Class one of exosomes are produced by the budding of the plasma membrane, making a bubble-like structure, then the fission and fusion of the plasma membrane to create the vesicle [44]. Class two of exosomes are vesicles that are formed in the endosomal network then these vesicles are released when multi-vesicular cells are fused together at the plasma membrane [44]. Class three is formed from apoptotic cells when they are in the process of apoptosis.

Exosomes will form from the dying cell as blebs then they are released into the surrounding extracellular fluid. One aspect that makes exosomes so notable is their ability to store important chemical communicators such as proteins and their specific markers that then allow for the location or origin of the exosomes to be determined [46]. The proteins that are commonly found in exosomes are: cytoskeletal, cytostolic, heat shock, and those originating from the plasma membrane [46]. But these proteins are not definitive to the cell that secretes the exosomes as there are other factors that contribute to the proteins present in exosomes. These factors include variables such as environmental stimuli (for example the oxygen concentration present), cell topography, or process activating stimuli such as something that could initiate apoptosis [46]. Usually, the process of shedding or the creation of exosomes is regulated in normally healthy cells, but in cancerous cells, the release of exosomes is unregulated mostly [47]. Most of these cancer derived exosomes are shed from the cell plasma membrane, and these exosomes in turn carry communication factors such as DNA, mRNA, microRNA, cytosolic proteins, and lipids [48]. These chemical communicators can promote tumor metastasis to other sites, promote cancer angiogenesis, and affect patient immune response that benefit tumor progression if these communicators are able to reach their target and bind with the necessary receptors [47, 48]. Taking these factors into consideration, we devised a plan to explore the effects of exosomes in a pericyte culture and determine if the effects brought by exosomes are enough to cause a noticeable effect in the phenotype and function of pericytes.

## MATERIALS AND METHODS

### Cell Culture

A note about sterility precautions: All cell work was conducted in a Thermo Fisher 1300 Series A2 hood to ensure sterility. All containers (excluding cell flasks) were washed with 70% ethanol/30% di-$H_2O$ mixture prior to entering the hood. Gloves were scrubbed with 70% ethanol prior to entering the hood. All media used were pre-warmed to 37°C before contact with the cells.

**Cancer Cell Lines (KPC 689, KPC 689 G/L, PANC-1, and PANC-1 G/L)**

Cells were stored frozen in liquid nitrogen in their respective mediumcontaining 5% DMSO. Cells were removed from liquid nitrogen and thawed in a 37°C water bath for approximately 100 seconds. Cells were then added to a Corning Falcon® 75$cm^2$ flask with 9mL media. Cells were incubated at 37°C at 5% $CO_2$ in a HERACELL VIOS 160i $CO_2$ incubator for 16 hours to ensure attachment. After the 16-hour period, media were removed via aspiration and 10mL fresh media were added. Media were changed every other day until desired confluency was achieved. Cells were cultured until 70%-80% confluent, then split into the container of choice.

To split cells, media were removed from cells via aspiration. Cells were washed in 5mL 37°C pre-warmed HyClone™ Phosphate buffered Saline (PBS). Next, 2mL pre-warmed 37°C 1x (0.025%) Gibco Trypsin-EDTA was added to the cells which were then incubated at 37°C with 5% $CO_2$ for 5 minutes. The flask was removed from the incubator and was then tapped laterally on the palm to free any remaining attached cells, which were then checked under a microscope to ensure complete removal. Cells were brought back into the hood and 5mL complete media were added to neutralize trypsin. Cells were then collected into a 15mL tube. The empty flask was

washed with 5mL PBS which was placed into the cell-containing 15mL tube. The cells were then centrifuged for 5 minutes at 400rcf. The supernatant was then removed via decanting. 5mL media were then added to the cells and resuspended via serological pipet. Cells were then placed in appropriate flasks or wells.

**Primary Cell Culture: Human Pericyte from Placenta (hPC) and Human Umbilical Vein Endothelial Cell (HUVEC)**

Cells frozen in GC Lymphotec Inc. BAMBANKER® cell freezing medium. Cells were removed from liquid nitrogen and thawed in 37°C water bath for approximately 100 seconds. Cells were then added to a Corning Falcon® 25cm$^2$ flask with 4mL media. Cells were incubated at 37°C at 5% $CO_2$ in a HERACELL VIOS 160i $CO_2$ incubator for 16 hours to ensure attachment. After the 16-hour period, media were removed via aspiration and 5 mL fresh media were added. Media were changed every other day until desired confluency was achieved. Cells were cultured until 70%-80% confluent, then transferred into Corning Falcon® 75cm$^2$ flask.

To transfer cells, media were removed from cells via aspiration. Cells were washed in 3mL 37°C pre-warmed HyClone™ Phosphate buffered Saline (PBS) 1x 0.067M ($PO_4$), which was vacuum aspirated after washing cells. Next, 2mL Innovative Cell Technologies Inc. Accutase® was added to the cells, which were then incubated at 37°C with 5% $CO_2$ for 5 minutes. After 5 minutes passed, the flask was tapped laterally on the palm to free any remaining attached cells, which were then checked under microscope to ensure complete removal. Cells were then collected into a 15mL tube. The empty flask was washed with 3mL PBS which was placed into the cell-containing 15mL tube. The cells were then centrifuged for 5min at 400rcf. The supernatant was then removed via decanting. 5mL media were then added to the cells and resuspended via serological pipet. Cells were then added to a Corning Falcon® 75cm$^2$ flask with

5mL media. Cells were then cultured with media changes every other day until 70-80% confluency. Cells were then transferred into appropriate flasks or wells.

**Culture Medium for Each Cell Line**

***hPC***

Cells were cultured in Pericyte Growth Medium 2 containing PromoCell SupplementMix C-39841.

***HUVEC***

Cells were cultured in PromoCell Endothelial Cell Basal Medium 2 containing PromoCell SupplementPack Endothelial Cell GM 2 (1x FCS-10, 1x hEGF-2.5, 1x HC-100, 1x VGEF-0.25, 1xhbFGF-5, 1x R3 IGF-1, 1x AA-500, 1x Hep-11.25).

***PANC-1***

Cells were cultured in HyClone™ DMEM/High Glucose Media Supplemented with 10% Atlanta® Biologicals FBS and 100x Corning Pennicillin/Streptomycin mixture diluted to 1x in media.

***PANC-1 G/L***

Cells were cultured in HyClone™ DMEM/High Glucose Media Supplemented with 10% Atlanta® Biologicals FBS and 100x Corning Pennicillin/Streptomycin mixture diluted to 1x in media and 1ug/mL InvivoGen Puromycin.

***KPC***

Cells were cultured in HyClone™ RPMI 1640 Media (1x) Supplemented with 10% Atlanta® Biologicals FBS and 100x Corning Pennicillin/Streptomycin mixture diluted to 1x in media.

***KPC G/L***

Cells were cultured in HyClone™ RPMI 1640 Media (1x) Supplemented with 10% Atlanta® Biologicals FBS, 100x Corning Pennicillin/Streptomycin mixture diluted to 1x in media, and 1ug/mL InvivoGen Puromycin.

***HPNE***

Cells were cultured in HyClone™ DMEM/High Glucose Media Supplemented with 10% Atlanta® Biologicals FBS and 100x Corning Pennicillin/Streptomycin mixture diluted to 1x in media and 0.75ug/mL InvivoGen Puromycin.

## General Techniques

### RNA Extraction

Medium was aspirated from each well in 6-well plate that RNA was to be extracted from. Next, 0.7mL Life technologies™ ambion® TRIzol® Reagent was added to each well. The TRIzol was then washed over the cells via pipet to ensure complete removal of all cells from the 6-well plate. The cells containing TRIzol were then stored in 1.5mL microcentrifuge tubes. The RNA was either extracted immediately, or the TRIzol with RNA was stored in -80$^{o}$C from 1-7 days before RNA extraction. RNA extraction was performed using Zymo Research Direct-zol RNA MiniPrep Kit and according to manufacturer instructions. RNA was eluted using 20µL RNAse free water.

### cDNA Synthesis

If frozen, RNA and cDNA synthesis reagents were thawed on ice. RNA concentrations were measured on Thermo Scientific Nano Drop 1000 Spectrophotometer. Equal amounts of RNA were added to each reaction tube and di-$H_2O$ was added for a total volume of 10µL. In a separate master mix was made using reagents from the High-Capacity cDNA Reverse

Transcription Kit with RNase Inhibitor (10x Buffer, 25x dNTP (100mM), 10x RT Random Primer, and Reverse Transcriptase) and reagents were added to a total volume of 20µL. cDNA synthesis thermal profile was 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, followed by an indefinite temperature hold at 4°C. Samples were then stored in -20°C for long term or 4°C for short term.

**Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)**

cDNA and primers were removed from -20°C and thawed on ice. Primers were diluted to 10µM using di-$H_2O$ (refer to table below for primer sequences), and cDNA was diluted 1:25 using di-$H_2O$. 4µL 1:25 cDNA from each sample were added in a technical triplicate to three respective wells, followed by the addition of a master mix containing 1µL of 10µM forward primer, 1µL 10µM reverse primer, 10µL PowerUp™ SYBR™ Green Master Mix, and 4µL di-$H_2O$. Each qRT-PCR reaction had a total well volume of 20µL. qRT-PCR thermal profile was 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds to 60°C for 1 minute, 95°C for 30 seconds, 60°C for 1 minute, and finally 95°C for 30 seconds. Threshold for all CT values was set to 0.5. The average CT value of each technical triplicate was calculated, then compared to the values of the biological triplicate.

**Statistical Analysis**

All statistics were calculated using GraphPad Prism 8. Tests that were used were ordinary one-way ANOVA, t-test with Welch correction, and multiple t-test. For data collected comparing the mean of three or more groups or treatments, an ordinary one-way ANOVA was used. For data consisting of means of two groups or treatments, a t-test was used.

## Exosome Extraction

### *Cell Culture Exosomes*

PANC-1 and KPC cells were cultured in 225cm$^2$ flasks until 50-70% confluency. Media were aspirated, and cells were washed with PBS, and then aspirated. Cells were placed in their respective media containing Gibco Exosome-Depleted FBS at 10% in place of the standard FBS. Cells then remained in the incubator for 48 hours. Media were removed and transferred to 50mL tubes.

Medium was centrifuged for 5 minutes at 800rcf, then 10 minutes at 2000rcf to pellet out dead cells and debris. Media were then vacuum filtered through a 0.2μm filter, then either stored at 4°C, or immediately prepared for ultracentrifugation. Media were ultracentrifuged at 28,000rpm using a Beckman SW28 rotor at 4°C for 16-20 hours. After ultracentrifugation, supernatant was decanted, and pellet was resuspended in 150-200μL PBS. Exosomes from the same cell line were combined and mixed and were stored at 80°C. Protein content in exosomes was measured using Pierce™ BCA Protein Assay Kit, according to the manufacturer's instructions.

### *Human Serum Exosomes*

Sigma-Aldrich Human Serum Cat# H4522 was centrifuged at 2,000rpm at 4°C for 30 minutes. Serum was then mixed with PBS in a 1:20 ratio; and then, filtered through a 0.2μm filter. Filtrate was then centrifuged at 40,000 RRM using Beckman SW 41 Ti. After ultracentrifugation, supernatant was decanted, and pellets were resuspended in 500μL PBS. Exosomes were stored at -80°C. Protein content in exosomes was measured using Pierce™ BCA Protein Assay, according to the manufacturer's instructions.

**Exosome Treatment Experiment**

Gibco™ Attachment Factor Protein (1x) was added at 0.5mL to each well in a standard 6-well plate and was incubated at 37°C for 30 minutes. Excess liquid was then aspirated. 180,000 cells were seeded in each well of various attachment factors treated 6-well plates (either 180,000 hPC, 180,000 HUVEC, or 90,000 hPC + 90,000 HUVEC) in 2mL PromoCell Endothelial Cell Basal Medium containing PromoCell SupplementPack Endothelial Cell GM 2. Three wells were seeded for each experimental group. Cells were allowed to reach 70% confluency, then media were aspirated and replaced with PromoCell Endothelial Cell Basal Medium without PromoCell SupplementPack Endothelial Cell GM 2. Cells were left undisturbed for 6 hours and were then treated with 200μg protein's worth of exosomes in 30-70μL PBS. Cells were then placed back into the incubator at 37°C and 5% $CO_2$ and were undisturbed for 24 hours. Cells were photographed using ZOE™ Fluorescent Cell Imager, then prepared for RNA extraction.

**Culture Insert Experiment**

hPC and or HUVEC were removed from the flask using the method above for primary cells. If a single culture of hPC, then 180,000 cells were seeded into each of a Falcon® 6-well TC-treated Polystyrene Permeable Support Companion Plate. If a co-culture was being seeded, then 90,000 hPC and HUVEC each were then seeded into each well then incubated for approximately 16 hours in 2ml per well of HyClone™ DMEM/High Glucose Media supplemented with 10% Atlanta® Biologicals FBS and 100x Corning Pennicillin/Streptomycin mixture diluted to 0.8x in media. The cells were checked under a microscope to ensure all cells were attached and healthy. If the cells were not fully attached, the cells would continue to incubate until a total of 24 hours. The medium in each well was aspirated out then replenished

with new medium, approximately 1.4 mL. PANC-1, KPC, were then seeded into 6.5 mm Transwell® with 0.4 µm Pore Polyester Membrane Insert with 0.6 mL of media in each culture insert. 285,000 PANC-1 and 512,960 KPC cells were seeded into respective treatment wells then incubated for an additional 48 hours. RNA extraction, cDNA synthesis, and qRT-PCR were completed from the cells in the wells.

**Endothelial Tube Formation Assay**

At least a day before seeding HUVEC and or hPC, the cells were labeled with a fluorescent marker. Invitrogen CellTracker™ CM-Dil and DiO'; $DiOC_{18}(3)$ were used, and the cell tracking dyes were diluted in DMSO to the designated concentration as called for in the product manual. These dyes (red and green respectively) were used to label hPC or HUVEC without the need of immunocytochemistry, ensuring greater tube structural stability as the washes could weaken or break vessels. 1.5 µL of stock dye was added for every 1mL of media that would be used to cover the growing surface for the cells (125µM concentration). Before the addition of the dye solution, old media in the culture flasks would be aspirated then washed with PBS. If a T25 flask was being used, 3 µL of dye was added in 2 mL of the respective cell line's media (PromoCell Endothelial Cell Basal Medium 2 for HUVEC and Pericyte Growth Medium 2 containing PromoCell SupplementMix C-39841). Cell dye media solution was properly mixed then added to each flask. The process was done in minimal light to protect the light sensitive dyes. The flasks were then incubated at 37°C for 5 minutes then transferred to a 4°C refrigerator for 15 minutes to limit endocytosis of the dye. Once done, the flasks were returned to the hood and the dye/media solution in each flask was aspirated out and an additional PBS wash was conducted. The cells/wells were replenished with fresh media and stored in the 37°C incubator until needed. Additionally, extracellular matrix solution, a 96 well plate, and a box of 200µL

pipettes were placed into a container with ice overnight and chilled at 4°C. This was done in order to allow the extracellular matrix solution to thaw without the risk of the solution solidifying and any materials that come in contact with the solution will not allow for premature solidification of the extracellular matrix solution. The day of seeding, a standard cell culturing protocol for HUVEC was followed as stated previously. Before the cells were seeded, a centrifuge needed to have been pre chilled to 4°C and the plate prepared. 50µL of the extracellular matrix solution was added to each well using a chilled pipette tip. After the matrix was added, the plate was carefully inspected for any bubbles. If there were bubbles present in the extracellular matrix solution, then the plate was placed into the pre chilled 4°C centrifuge and spun at 300rcf for 10 minutes. After centrifugation, the plate was placed in the hood to incubate for 10 minutes at room temperature then transferred to the incubator for another 30 minutes at 37°C. Once the extracellular matrix solution-treated 96 well plate was placed into the incubator, HUVEC and hPC were accutased via the method stated above. 10,000 cells per well were added. For co-culture wells, 2,000 hPC and 10,000 HUVEC were seeded per well. Each well had a total of 100µL of cell suspension in PromoCell Endothelial Cell Basal Medium 2 containing PromoCell SupplementPack Endothelial Cell GM 2. The seeded plate was then returned to the 37°C incubator until pictures were taken at 7, 10, and 14 hours after initial seeding. Before the last set of pictures were taken, NucBlue was added to each well. To do this, for every 1 mL of PromoCell Endothelial Cell Basal Medium 2, two drops of NucBlu were added. The solution was then mixed well and 100µL of the dye solution were added to each well, and the plates were incubated at room temperature for 15 minutes in the dark. Once the incubation period was completed, the final set of pictures were taken.

**Immunocytochemistry**

An alcohol lamp was filled with 100% ethanol just up to the wick then lit under the laminar flow hood. 12mm diameter Micro Coverglass by Electron Microscopy Sciences slips were saturated with 100% ethanol then grasped using forceps and exposed to the flame to sanitize via immolation. The sanitized coverglass was then inspected to ensure there were no cracks formed after the exposure to the flame and that no ash accumulates on any of the surfaces. Once the inspection was over, each coverglass was then placed into an empty well of a 24-well, flat bottom Tissue Culture Plate by Olympus Plastics. Once the coverglasses were placed into the desired wells, 50µL of Gibco$^{TM}$ Attachment Factor Protein (1x) were added to each coverglass. Next, a pipette tip was flattened using an empty well and used to spread the gelatin over the coverglass like a spatula. The attachment factor was then incubated for at least 1 hour and up to 24 hours. Overnight was the most optimal condition. Each well was washed with 1mL PBS then removed via aspiration all while being mindful to not touch the coverglass. This PBS wash was repeated one additional timebefore cells were seeded directly on the glass, with a cell density of 40,000 cells/mL. A total of 1mL of cell suspension was added to each well in which cells were seeded. The cells were then incubated between 36-48 hours at 37°C to fully attach.

After the allotted time for the cells to attach, 4% paraformaldehyde (PFA) was thawed in a water bath at 37°C, occasionally vortexed, then once there is no precipitate left the PFA is stored at 4°C . Media was removed from each well via aspiration, whilebeing careful to not touch the coverglass to ensure that the cells were not disturbed or removed. 1mL of PBS was thenadded to each well for a 5-minutesbefore being aspirated. To fix the cells, 500µL of 4% PFA was pipetted overthe cells on each coverclass for 15 minutes . After PFA exposure 1mL of PBS was added to each well to wash for 5 minutes then aspirated out. This wash was repeated an

additional 2 times. Remaining PBS was aspirated from each well and then the wells were treated with 500µL of 0.1% Triton for 10 minutes to destroy the plasma membrane of the cells. The triton was removed and three additional PBS washes were completed using 1mL of PBS per well for 5 minutes before being aspirated out.

After PBS washing, 500µL of 3% Bovine serum albumin (BSA) in PBS was added to each well then allowed to sit at a minimum of 2 hours or up to 16 hours to block any nonspecific receptors. If the wells were treated for over 2 hours in BSA, the plate was covered and parafilm was wrapped around the perimeter of the plate and stored at 4°C. Half of the coverglasses were then randomly selected to be treated with 100µL of a primary antibody solution diluted in 3% BSA. Before the primary antibodies can be added, the BSA was aspirated out. Table 1 lists the primary antibodies used and the working dilutions. After the antibody solution was added to each treatment coverglass, the plate was once again covered and the perimeter wrapped in parafilm and stored 4°C overnight.

**Table 1.** Antibodies and Dilutions.

| Antibody | Dilution |
|---|---|
| Monoclonal Anti-Desmin Antibody [Mouse] | 1:100 (3µL/300µL) |
| PDGFRβ Antibody (G.290.3) [Rabbit] | 1:100 (3µL/300µL) |

The next day, three PBS washes were completed using 1mL of PBS per well for 5 minutes followed by PBS aspiration. Calculating for the proper silution, secondary antibodies were mixed in 3% BSAfor fluorescence. The antibodies used and dilutions made were listed in Table 2. All liquid was carefully aspirated out from each well then 100µL of secondary antibody solution was added to each coverglass. The plates were then covered and obscured from light for at least an hour at room temperature. After allowing the secondary antibodies to attach, three

1mL PBS washes were done; but while the PBS rested for 5 minutes, it was important to ensure that the cells were shaded from the light similar to when the secondary antibodies were added.

**Table 2.** Secondary Antibodies.

| **Antibody** | **Dilution** | **Color** |
|---|---|---|
| Alexa Fluor 546 goat anti-mouse IgG (H+L) | 1:250 | Red |
| Cy5 goat anti-rabbit IgG (H+L) Anti-rabbit 694 | 1:250 | Far Red |
| Monoclonal Anti-Actin, α-Smooth Muscle - FITC antibody produced in mouse | 1:500 (0.6µL/300µL) | Green |

After the last wash the coverglass was mounted onto glass slides. Two drop of 5µL Vectashield with DAPI was placed onto a labeled coverslip. To remove the coverglass from each well, a pair of forceps were used to gently move the coverglass against the wall of the well, then coaxed from the well to be lifted up then removed from the well. Coverglasses were then placed cells side down on the glass slides and sat at room temperaturein the dark for 1 hour. Kim Wipes were used to dry off any excess Vectashield DAPI that squeezed out from under the coverglass, and nailpolish was applied to the perimeter of the coverglass to fix and protect the coverglass to the slide. The slides were stored and shielded from the light at 4°C.

# RESULTS

## Establishing the Gene Expression Profile of Pericytes and Endothelial Cells

We utilized pericytes (hPC) and endothelial cells (HUVEC) to establish a vascular co-culture system. First, to confirm the identity of the cells and set the baseline of a unique gene expression profile of each cell line, we performed qRT-PCR using total RNA exptracted from hPC and HUVEC without any treatment. The genes expected to be expressed by the pericytes used (hPC) were: NG2, PDGFRβ, Desmin, and α-SMA. In case of the endothelial cells (HUVEC), genes such as Platelet endothelial cell adhesion molecule (PECAM) and Col4α1 are expected to be expressed. PECAM makes up a large portion of endothelial cell intercellular junctions, thus widely ustilized endothelial cell marker. The results are shown in Figure 3. As expected, PECAM was exclusively expressed by HUVEC; however, there was no significant difference in Col4α1 expression between pericytes and endothelial cells (red bars). Meanwhile, NG2, PDGFRβ, Desmin, and α-SMA were predominantly expressed by pericytes (blue bars) and negligible amount was detected by endothelial cells. This result confirms the cellular identity and provides the normal expression level of these genes of interest in each cell lines when the cells are in basal culture condition without specific stimulation.

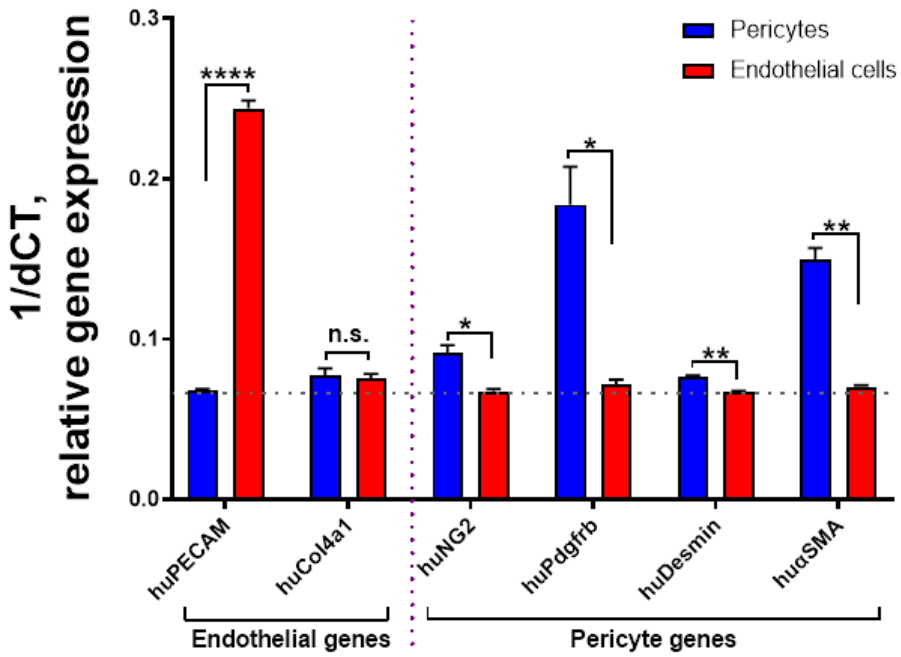


**Figure 3.** Gene Expression Profile of Pericytes and Endothelial Cells.
Baseline gene expression for endothelial cells (HUVEC) in red and pericyte (hPC) cells in blue. Both cells were cultured in their designated culture medium without further stimulation. Total RNA were extracted and used to perform qRT-PCR for PECAM, Col4α1, NG2, PDGFRβ, Desmin, and α-SMA. The data are represented as the mean ± standard deviation of each gene tested (n=3) determined by multiple t tests. The genes are separated between what was significantly, or exclusively, by which cell line.

## Co-Cultures

After establishing the baseline expression differences between hPC and HUVEC , an experiment was performed to test the expression level of these gens in hPC by itself compared to a co-culture of hPC and HUVEC. Additionally, hPC were treated with the antineoplastic drug mitomycin C, which acted as a proxy for HUVEC. Intimate communication with endothelial cells prmotes pericyte maturation and quiescent. Thus, the hypothesis was that mitomycin C would force pericyte maturation by inhibiting the cell cycle, similar as to HUVEC would. The expression of α-SMA and NG2 in pericytes were significantly affected by MMC treatment and the presence of HUVEC (Figure 4). hPC by itself had greater expression of α-SMA than hPC+MMC and the co-coculture of hPC+HUVEC. Similarly, hPC by itself had greater

expression of NG2 than hPC+MMC and the co-coculture of hPC+HUVEC. The significance of this experiment was calculated using an ordinary one-way ANOVA between the three treatments. It is also important to note that the influence of MMC treatment and co-culture with HUVEC was not identical even though the MMC treatment was to serve as a proxy or alternative to the co-culture environment to force the pericyte maturation/quiescence. This indicated pericyte maturation or differentiation requires more than cell cycle inhibition. It involves further factors or means of cell to cell communication and more approaches needed to be considered and tested.

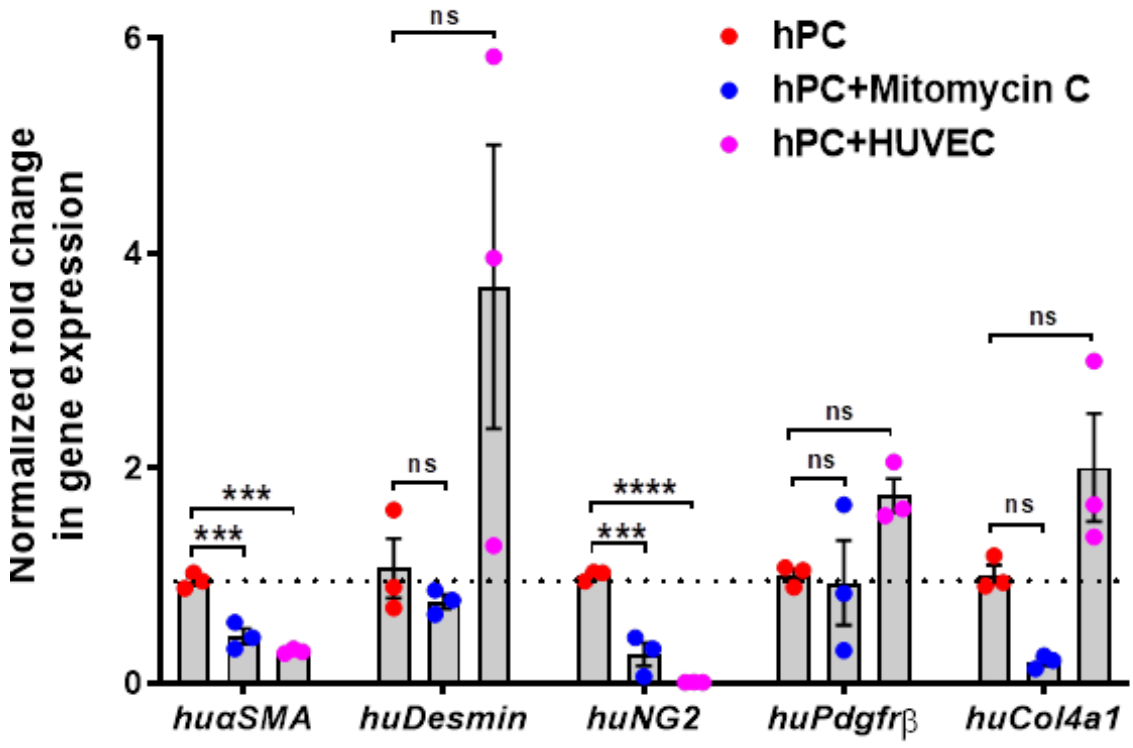


**Figure 4.** Differntial Gene Expression by Pericytes When Treated with MMC or Co-Cultured with HUVEC.
A bar graph with whiskers visualizing the normalized gene expression (α-SMA, Desmin, NG2, PDGFRβ, and Col4α1) for hPC cultured by itself (red), hPC treated with Mitomycin C (blue), and hPC co-cultured with HUVEC(pink). Each data point (n=3) is the mean of three technical triplicates for one of the biological triplicates. The whiskers denote the range of error for each data point when using an ordinary one-way ANOVA.

Additionally, a ratio of Desmin to α-SMA was used to represent the maturity of pericytes. Desmin is predominantly expressed by mature and differentiated pericytes, where as α-SMA is known to be up-regulated in activated pericytes, such as the ones in the pathological sites. The higher the value of the ratio would determine that there is greater desmin expression within that treatment, thus, greater pericyte maturity. The results of which are shown in Figure 5. There was a statistically significant difference between all three treatments. Significance determination was conducted with an ordinary one-wayANOVA. Desmin/ α-SMA ratio was the greatest when hPC was cultured with HUVEC followed by hPC treated with MMC, and hPC alone. This data strongly support the idea that the presence of endothelial cells contribute significantly to pericyte maturation. While the inhibition of the cell cycle by MMC shift the ratio of Desmin/ α-SMA toward maturation, more complex cell-cell communication is provided by endothelial cells.

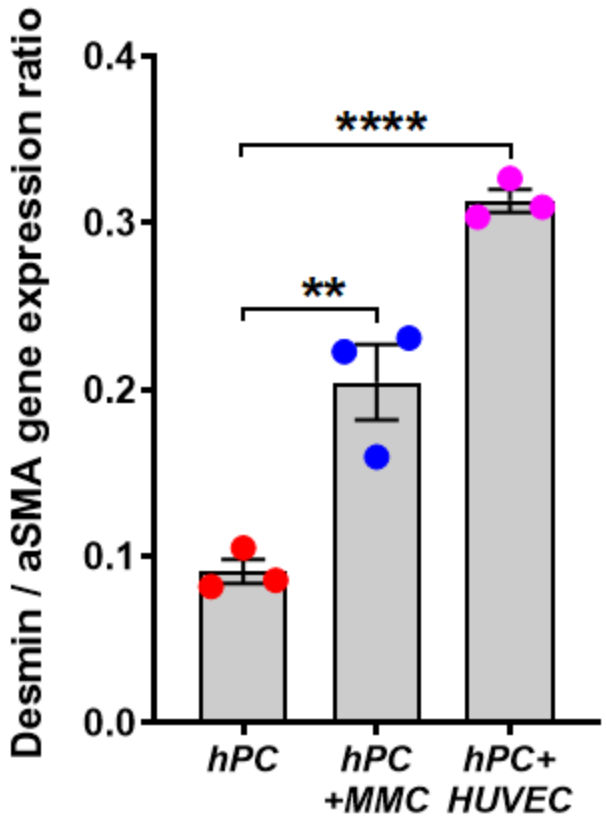


**Figure 5.** Desmin/α-SMA Ratio of hPC+/- MMC by Itself or Co-Cultured with HUVEC. A bar graph with whiskers showing three different treatments, hPC, hPC treated with MMC, and hPC co-cultured with HUVEC. Each data point shows the mean of three technical triplicates for one of the biological triplicates (n=3) measuring the Desmin:α-SMA ratio. The hypothesis was that the greater the ratio of Desmin: α-SMA the more mature the pericytes are in a given treatment, such as in hPC co-cultured with HUVEC. The whiskers for each bar represent the range of error for each treatment in regards to using an ordinary one-way ANOVA.

Next, we determined the effects of pathological conditions on the pericyte phenotype by co-culturing pericyte with cancer cells. hPC was cultured by itself then compared to a co-culture of hPC with KPC 698, a murine pancreatic cancer cell line. Considering atypical expression of α-SMA by pericytes, we expected, due to the addition of pancreatic cancer cells, hPC would express atypical gene expression such as elevated levels of α-SMA expression. What was noticed, shown in Figure 6, NG2 and PDGFRβ expression were significantly higher in the hPC and KPC 689 co-culture (near 10 fold increase and over 10 fold increase, respectively). A visual

patter was noticed with the amount of α-SMA expressed in hPC co-cultured with KPC as two data points were grouped at a near 20-fold difference.But due to an outlier, the relationship was not statistically significant. Nevertheless, this visual difference, along with the statistically significant difference in NG2 and PDGFRβ, warranted enough evidence to continue testing and explore further how these cells behave and interact with each other.

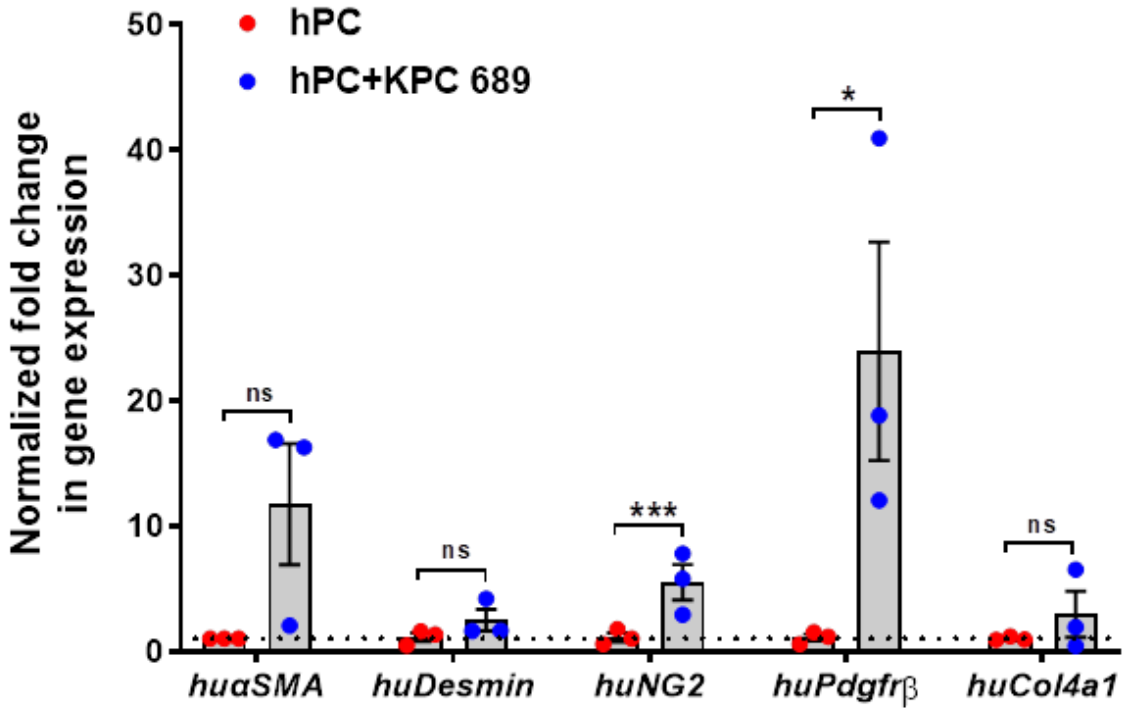


**Figure 6.** Gene Expression of hPC Cultured by Itself or Co-Cultured with KPC 689. Difference of gene expression of α-SMA, Desmin, NG2, PDGFRβ, and Col4α1 of hPC gene expression cultured by itself, simulating a "healthy" scenario, or hPC co-cultured with KPC 689, simulating an "unhealthy" cancer environment, represented with a bar graph and whiskers. Each data point represents the mean of three technical triplicates of one of the biological triplicates (n=3). The whiskers represent the range of error as determined by Welch's t test.

It is worth noting that the changes of gene expression pattern for NG2 and α-SMA were the opposite between hPC + HUVEC and hPC + KPC 689. A higher level of NG2 and α-SMA expression might represet the pathological phenotype of the tumor associated pericytes. Considering the significant effects of cancer cells on pericyte phenotype changes, we attempted

to visualize the physical contact and behavior of the cells. KPC 689 was transfected with GFP to facilitate the visualization of the cancer cells. Figure 7A shows hPC cultured by itself whereas Figure 7B shows a co-culture of hPC and HUVEC. hPC and HUVEC were intermingled with each other as we could not delineate individual cells. However, in the triple co-culture of hPC, HUVEC, and KPC 689, islands of KPC 689 were noticed, as indicated with the red dotted shapes in Figure C and these same dotted shapes around the green fluorescence islands in part D. Cell organization occurred that looked similar to tumor cross sections as hown in parts E and F. These islands or clusters of KPC 689 were spontaneously organized.

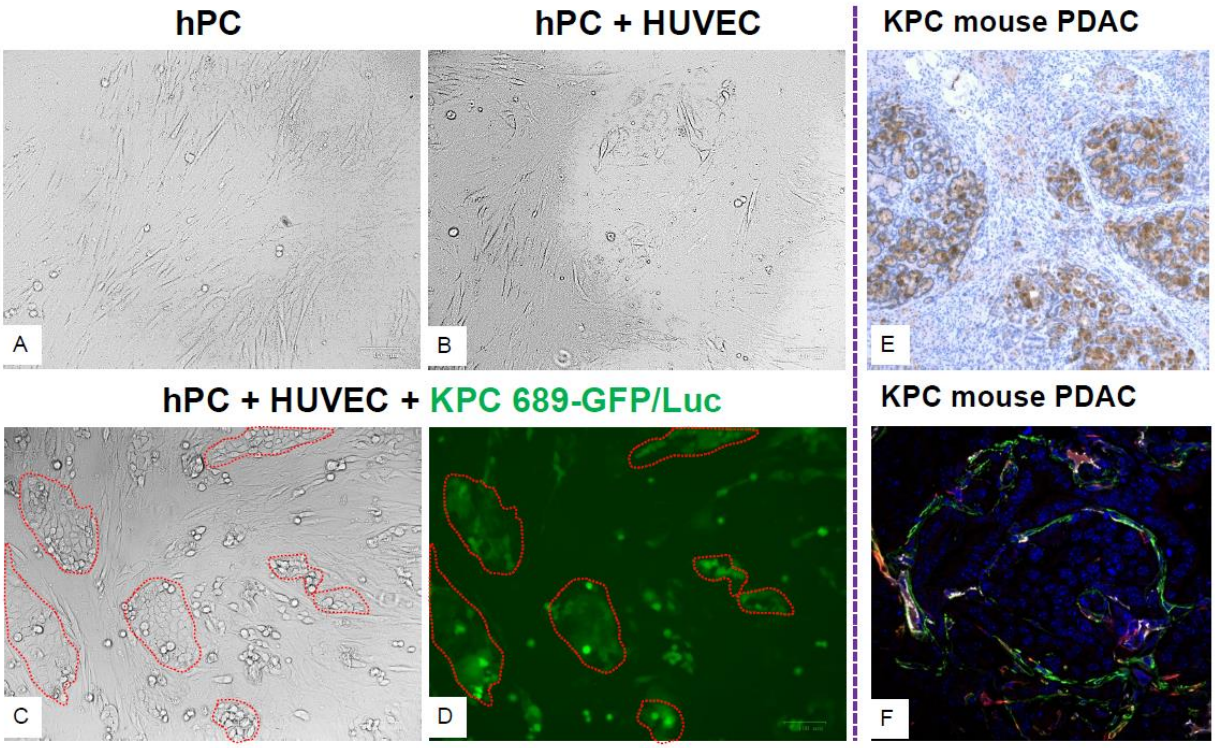


**Figure 7.** Triple Co-Culture of hPC, HUVEC, and KPC 689.
hPC was cultured by itself (A), with HUVEC (B), or with HUVEC + KPC 689 all together (C-D). A red line denote the clusteres of KPC 689 under a light microscope (C) then the same image under florescence (D). The similarity in cell island clusters from part C and D can then be compared to tumor sections shown in parts E and F, with part F showing the fluorescence of the tumor tissue.

## In-Direct Co-Cultures

### Using Transwell Membrane Culture Inserts

Noticing that there is a difference in gene expression when pericytes are co-cultured with pancreatic cancer cells and HUVEC, all under physical touch, what would happen if hPC was co-cultured with HUVEC and pancreatic cancer cell chemical communicators were only allowed to interact with the co-culture? To answer this question, hPC was co-cultured with HUVEC in a 6 well plate with a permeable trans-well membrane. The function of the trans-well membrane is to prohibit physical touch between the vascular cells and cancer cells but still allow for chemical communicators to pass through the membrane and interact between the two groups of cells. hPC and HUVEC co-cultured together were compared between the presence or absence of cancer cells seeded in the transwell membrane using an ordinary one-way ANOVA. The cancer cells used were the murine cell line KPC 689 and the human cell line PANC-1. hPC + HUVEC coculture with KPC 689 seeded on the transwell membrane showed an approximate 4-fold increase in Desmin expression, and a near 2-fold increase in α-SMA expression compared to hPC + HUVEC in the absence of the cancer cells (control). Meanwhile, PANC-1 seeded on the transwell membranes resulted in an approximate 2-fold difference in Desmin expression and an approximate 2-fold difference in α-SMA than control situation (hPC + HUVEC only). The expression of Desmin and α-SMA in these experiments are shown in Figure 8.

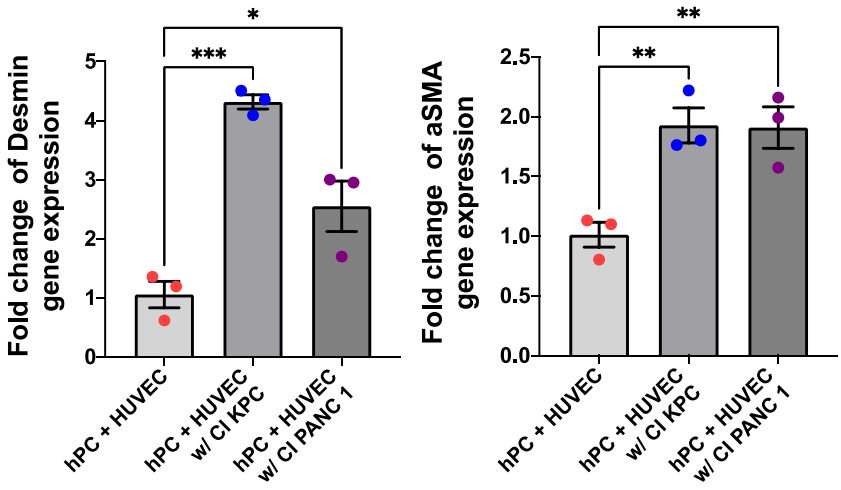


**Figure 8.** Fold change of Desmin and α-SMA Gene Expression Between hPC Co-Culture with HUVEC with or without the Addition of Pancreatic Cancer Cells.
The graph on the left shows fold change in Desmin gene expression while the graph on the right shows fold change in α-SMA expression. Each graph depicts the mean of three technical triplicates of one of the biological triplicates (n=3) and shows the difference between three treatments of hPC co-cultures. The hPC co-cultured with only HUVEC served as the control, healthy environment. The hPC co-cultured with HUVEC with a culture insert containing either KPC 689 or PANC-1 were the treatment groups that represented the unhealthy cancer environment. The whiskers shows the range of error as calculated using an ordinary one-way ANOVA.

**Using Exosomes for In-Direct Co-Cultures**

Knowing that physical touch and chemical communicators were able to influence pericyte phenotype, what specific chemical communicators were present that allowed for these changes? We attempted to isolate a possible indirect communicator that was responsible for the change in gene expression in pericytes. As indicated previously, cancer cell-derived exosomes are known to elicit a parenchymal signaling response in the TME of primary and metastatic sites. In addition, tumor-derived exosomes significantly influence distant organs to create a pre-metastatic niche, suggesting exosomes are a potent TME influencer. To test our hypothesis that PDAC-derived exosomes stimulate the tumor-promoting pericyte phenotype, an experiment was conducted using exosomes isolated from PANC-1 pancreatic cancer cells with negative controls such as PBS and normal Human Serum Exosomes (HSE). There was no change in α-SMA,

Desmin, and NG2 expression for hPC treated with PBS or HSE (controls). However, hPC treated with PANC-1 exosomes showed a statistically significant increase in α-SMA, Desmin, and NG2 expression. α-SMA expression in hPC treated with PANC-1 Exosomes had a near 10-fold increase in gene expression. As for Desmin, PANC-1 Exosome treated hPC experienced an approximate 2.5-fold increase in expression and lastly there was an approximate 1.5 fold-change in NG2 expression.

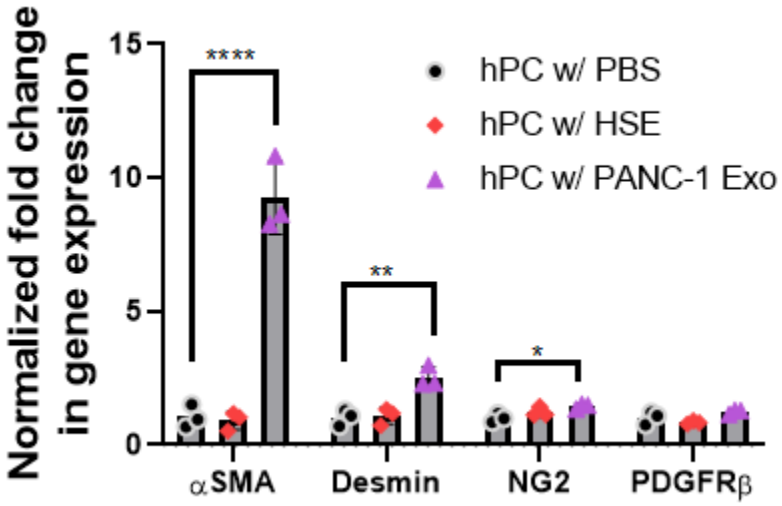


**Figure 9.** Effects of Cancer Cell-Derived Exosomes (PANC-1 Exo) on the Gene Expression Profile of hPC.
The difference in α-SMA, Desmin, NG2, and PDGFRβ gene expression of hPC treated with PBS, HSE, or PANC-1 exosomes were measured by qRT-PCR. Each point is the mean of three technical triplicates for one of the biological triplicates (n=3), and the whiskers present for each bar denotes the range of error as calculated using an ordinary one-way ANOVA.

The significant increase of α-SMA expression brought into question if this effect is specific due to the addition of PANC-1 exosomes. To then test the specificity of the PANC-1 exosomes, hPC was cultured by itself and varying concentrations of exosomes were added. The concentration of exosomes ranged from 0.5X, 1X (same conditions as figure 9), and 2X, with 1X being 100μg/μL of exosome protein. Figure 10 shows the results of α-SMA expression in hPC among the three different condition. As the concentration of PANC-1 Exosomes increased, so

did the expression of α-SMA, going as high as an approximate 20-fold change when looking to the 2X treatment. The statistical significance of these data were calculated using an ordinary one-way ANOVA.

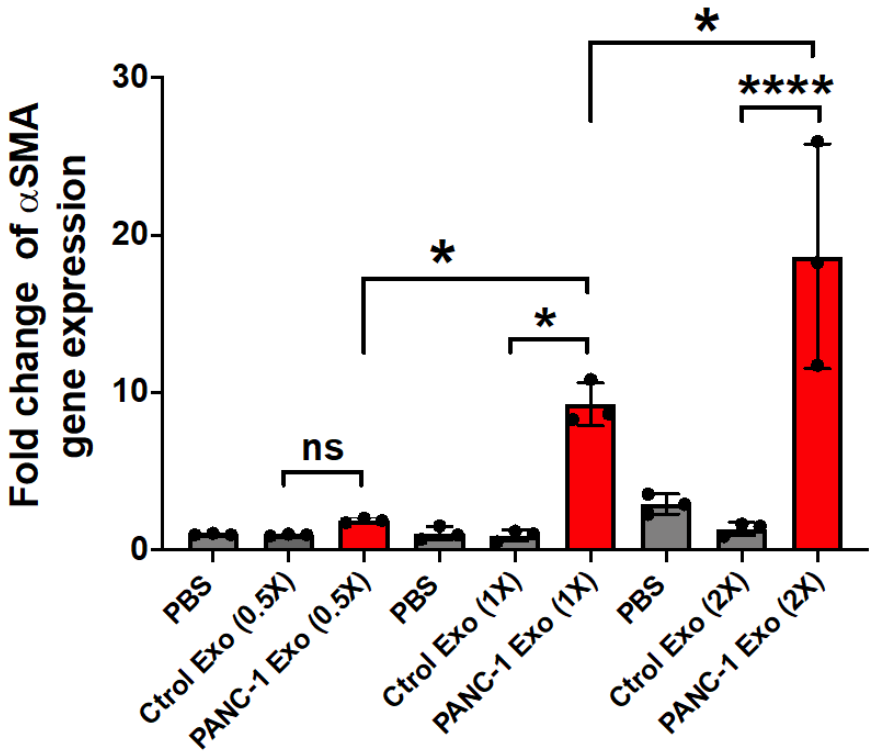


**Figure 10.** Specific Atypical α-SMA Expression of hPC in the Presence of PANC-1 Exosomes. A bar graph with whiskers showing changes in α-SMA gene expression when hPC was cultured with varying concentrations of PBS, HSE, and PANC-1 exosomes. Each data point represents the mean of three technical triplicates of one of the biological triplicates. The whiskers for each bar is the range of error as determined using an ordinary one-way ANOVA.

To determine the influence of PANC-1 exosomes on a vascular like environment, hPC was co-cultured with HUVEC and treated with PANC-1 exosomes (Figure 11). There was a statistically significant increase in atypical α-SMA expression when hPC + HUVEC co-culture was treated with PANC-1 exosomes, compared to the same co-culture treated with PBS or HSE along with other genes. α-SMA expression increased a near 2-fold, while Desmin and NG2 both had an approximate 1.5 fold increase in their respective expression. There was no significant change in expression of any of the geness tested in co-cultures treated with either PBS and HSE

treatments. There was also no significant difference for PBS vs PANC-1 exosome treatment for PDGFRβ. It is worth noting that the presence of HUVEC seems to suppress the induction of α-SMA expression as PANC-1 exosomes only increased α-SMA expression by 2-fold, whereas the increase was nearly 10-fold when pericytes were exposed to the PANC-1 exosomes alone.

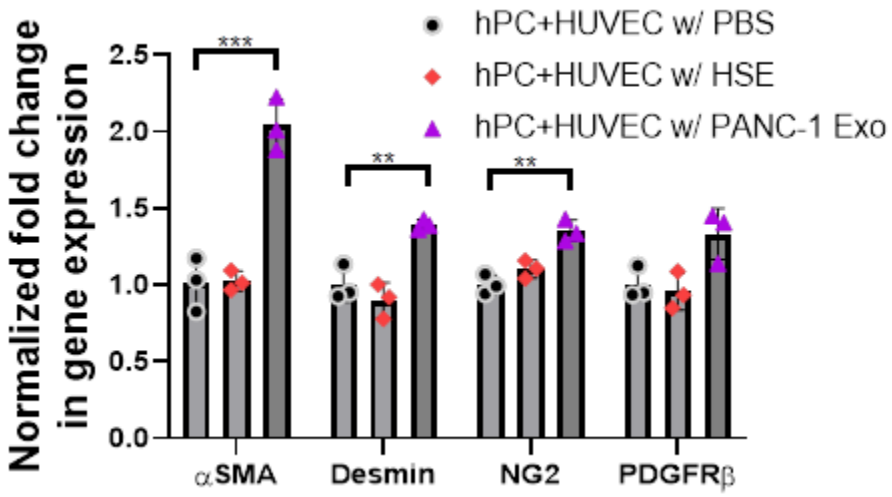


**Figure 11.** Changes in Pericyte Gene Expression Profile When hPC + HUVEC Were Treated with PANC-1 Exosomes.
hPC co-culture with HUVEC with treatments of PBS, HSE, and PANC-1 exosomes measuring and comparing the difference in fold change of gene expression in α-SMA, Desmin, NG2 and PDGFRβ gene expression. Each data point is the average of three technical triplicates of one of the biological triplicates (n=3). Each bar possesses a set of whiskers which show the range of error as determined using an ordinary one-way ANOVA.

Considering a significant changes in pericytes phenotype in the presence of cancer cell-derived exosomes, we hypothesized that the cancer derived exosomes will result in the functional defect in pericytes. To test this, an endothelial tube formation assay was performed. This is a quanitiative method to determine what aspects that effect angiogenesiss. Aspects such as genes and pathways are examples of some of the components that control signaling for the production of tube like structures [49].

HUVEC were seeded by themesleves to see what the effects of exosomes are on tube formation, serving as a control. The tubes formed over a time range of 16 hours and were

observed from 6, 8, 14 and 16 hours after exosomes were added. The same experimental procedure was applied when HUVEC and hPC were seeded together to form the tubes. In the HUVEC only wells, there were minor phenotypic changes cultured alone or treated with either PBS, HSE, or PANC-1 exosomes. These phenotypic changes were present in both light and fluorescent microscope pictures in Figure 12. The DiO'; $DiOC_{18}(3)$ (green dye) wase used to label HUVEC. This allowed for isolated visualizations of HUVEC when viewed under florescence conditions. The slight phenotypic change was observed when tubes were treated with PANC-1 exosome. Over time, there was a darkening of the tube structures throughout the 8-16 hour range indicating degredation of the tube structures.

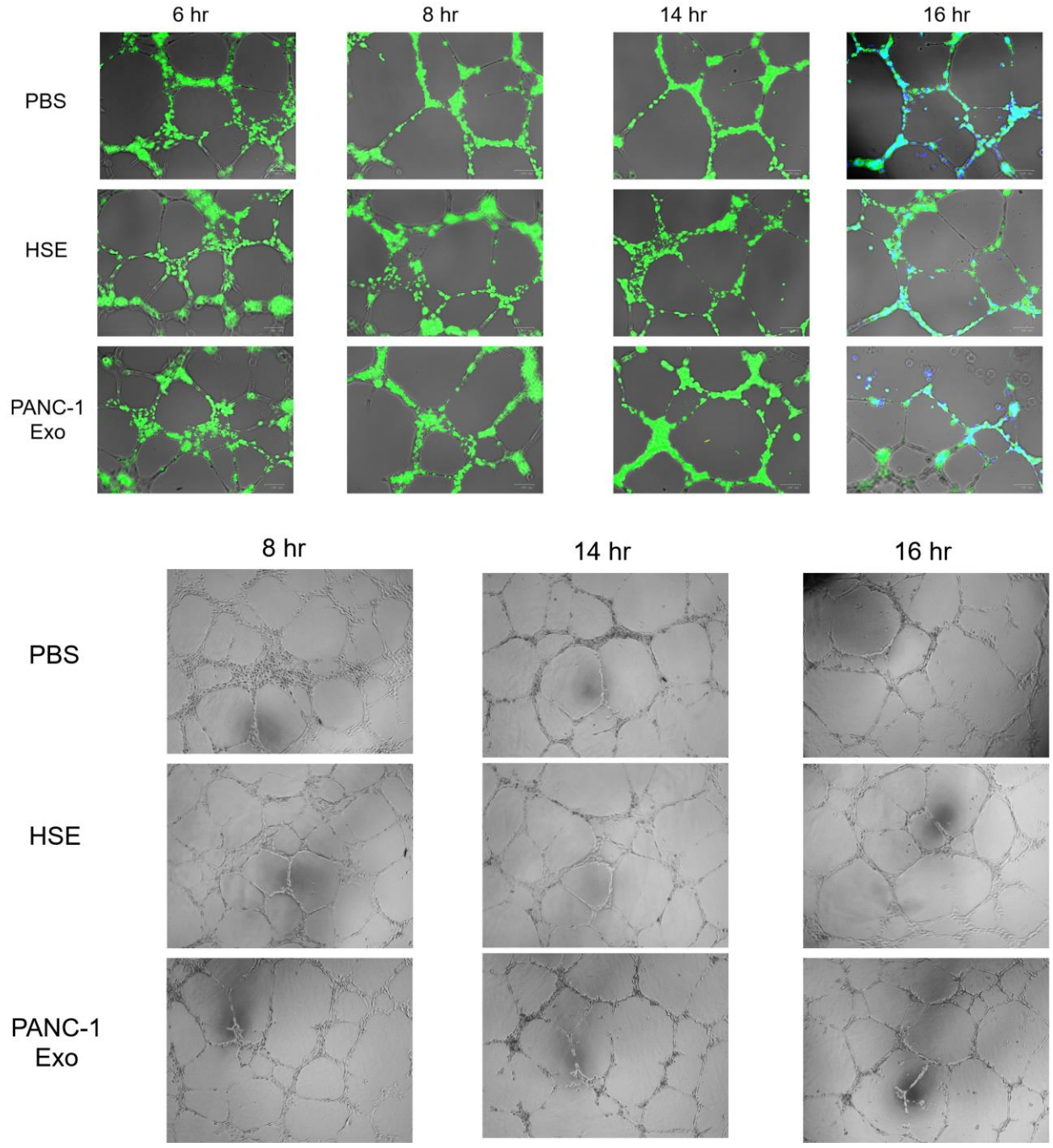


**Figure 12.** Effect of PANC-1 Exosomes on Tube Formation Capacity of HUVEC.
HUVEC only tube formation with green fluorescence staining of HUVEC with DiO'; $DiOC_{18}(3)$ and blue fluorescence staining of nuclei with NucBlue. Pictures taken range from 6 hour to 16 hour after initial seeding. Light microscopy show the approximate same area from 8 hours to 16 hours after initial seeding between three different treatments of HUVEC treated with PBS, HSE, or PANC-1 exosomes. As time passes this is minimal change in tube structure as control and PANC-1 exosomes are added. Degradation of tubes happen naturally over time.

The phenotypic change was much more obvious when hPC and HUVEC were co-cultured together in a 1:5 ratio. DiO'; $DiOC_{18}(3)$ was used to label HUVEC green and CM-Dil was used to label hPC red. The usage of these dyes allowed for isolated visualizations of HUVEC and hPC when viewed under floresenct conditions. When the cultures were treated with PANC-1 exosomes, there was greater tube deterioration and clustering, as can be seen in Figure 13. From hour 8, after cells were seeded, to hour 14, the tubes that formed had collapsed and pulled together. By hour 16, most of the tubes had collapsed and clustered together. Conversely, this phenotypic change was not as noticeable or present in PBS or HSE treatments.

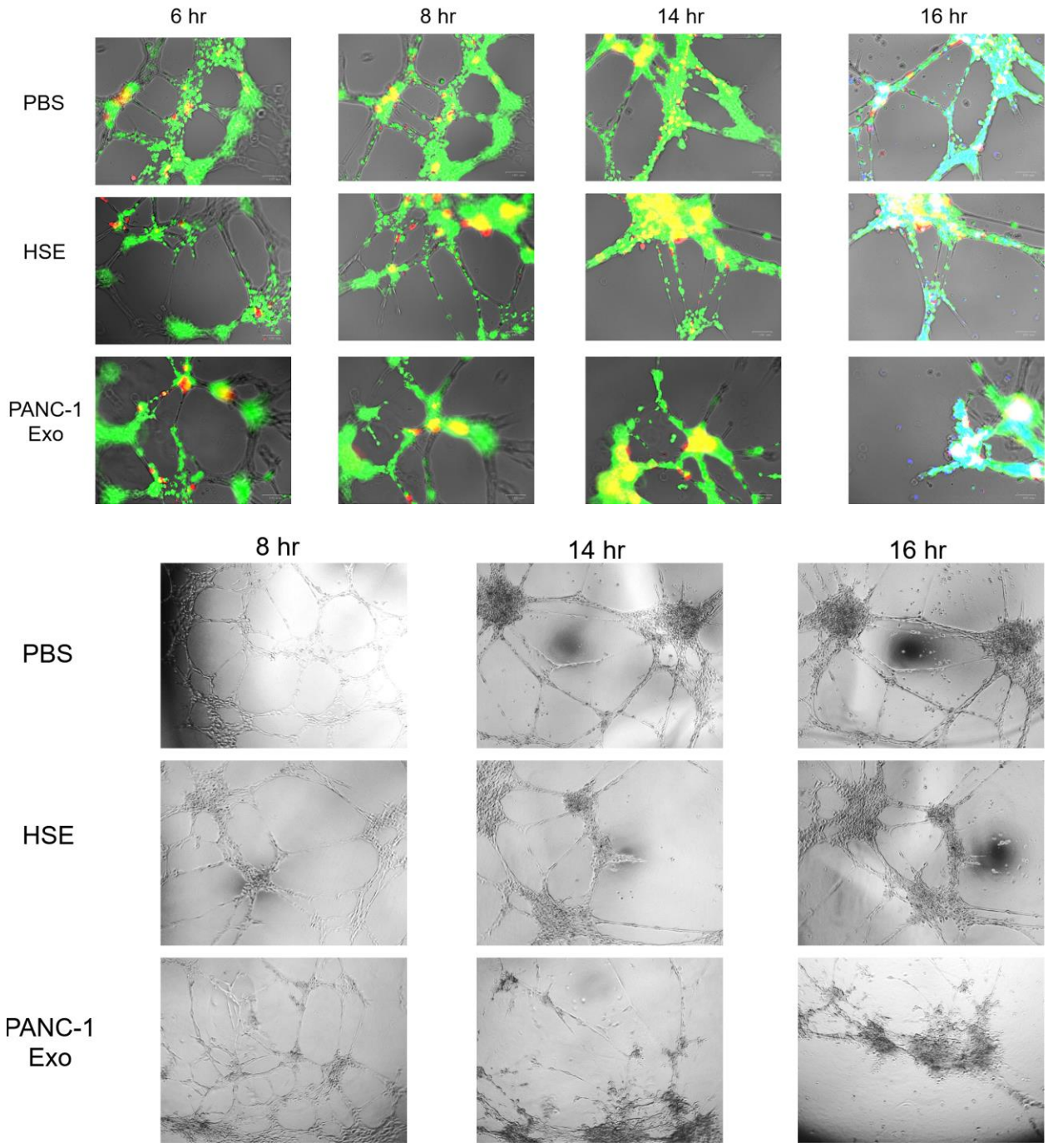


**Figure 13.** Effect of PANC-1 Exosomes on Tube Formation Capacity of Endothelial Cells and Pericytes.
HUVEC and hPC co-culture tube formation with green fluorescence staining of HUVEC with DiO'; $DiOC_{18}(3)$, red fluorescence staining of hPC with CM-Dil, and blue fluorescence staining of nuclei with NucBlue. Imaging for floresecent images were takenfrom 6 hour to 16 hours after initial seeding. Images obtained using light microscopy were done so at the approximate the same area from 8 hours to 16 hours after initial seeding between three different treatments of HUVEC co-cultured with hPC treated with PBS, HSE, or PANC-1 exosomes.

# DISCUSSION

## Preliminary Data

Establishing basal gene expression profile of pericytes and endothelial cells is critical to understanding the normal phenotype of these cells before they are altered by external conditions and treatments. qRT-PCR using total RNA extracted from each cell line cultured in their normal condition confirmed their identity. PECAM was only significantly expressed by HUVEC, whereas NG2, PDGFRβ, Desmin, and α-SMA expression was predominantly detected by pericytes (Figure 3). Col4A1 is the subunit of the collagen IV, which is the major component of the basement membrane between endothelial cells and pericytes and is known to be expressed by both endothelial cells and pericytes [37, 50, 51]. Thus, no significant differences in expression level between two cell lines agree with the previous finding.

## Co-Cultures

Endothelial cells and pericytes are the two major components of blood vessels and their physical contact and communication with one another is critical for the vascular structure and stability. To simulate the in vivo blood vessels more closely, we established a endothelial cell-pericyte co-culture model. The presence of endothelial cells facilitates pericyte maturation, differentiation process and contributes to the vascular stabilization. To test if the cell cycle arrest is sufficient to force pericyte maturation/differentiation in the absence of endothelial cells, pericytes were mitotically arrested using MMC, an antineoplastic drug that limits cell proliferation. If this is the case, we would expect to see the similar phenotype changes between pericytes co-cultured with endothelial cells and pericytes treated with MMC.

Both groups, pericytes co-cultured with endothelial cells and pericytes treated with MMC, yield significant reduction of α-SMA and NG2 expression in the pericytes compared to

pericytes cultured alone. This result suggests that the cross-communication between healthy endothelial cells and pericytes is important to suppress α-SMA expression, which is abnormal for the capiliary pericytes. In addition, endothelial cells seem to exert more potent effect on α-SMA expression than MMC treatment, indicating the presence of endothelial cells provide more factors than just stopping the pericyte proliferation. While the difference was not statistically significant for PDGFRβ, Desmin, and Col4α1 expression, both endothelial cells and MMC treatment influenced the level of these gene expression, encouraging further studies on various ways for two cells to communicate with each other. As these marker proteins are involved in different cellular functions, the ratio or the combination of certains proteins can dictate the functional and structural status of the pericytes. Since Desmin is known to be expressed by mature and functional pericytes, and α-SMA is often observed in pathological pericytes, we measured the ratio between Desmin and α-SMA in different conditions. Between all three samples, there were significant differences in the Desmin:α-SMA ratio. The pericyte and endothelial cell co-culture showed the highest Desmin:α-SMA ratio, thus the greatest pericyte maturity and least pericyte stress and fewer atypical phenotypes (Figure 5).

The fact that a unique pericyte phenotype ($Desmin^{low}$/ $\alpha\text{-}SMA^{high}$) is observed in TME suggest that the influence might be coming from the cancer cells. In fact, many studies have shown that cancer cells contribute to creating a pathological TME in which they can thrive. Thus, we explored the effect of pancreatic cancer cells on pericyte phenotype. To limit gene expression from other human cell interference, a murine (mouse) cell line was used since human gene expression could not be detected from murine cell lines. KPC 689 was the murine cancer cell line used, and in this experiment these cells were cultured directly with pericytes. Due to outliers present in some of the gene comparisons in Figure 6, some results had no significant

differencesThere was enough of a difference to show a potential pattern. This is most notably the case with α-SMA where some of the samples had a nearly 17-fold change in gene expression – a considerable increase in overall α-SMA, which we assume is due to the influence of KPC 689. Additionally, there is statistically significant differences between the treatments when looking at NG2 and PDGFRβ. This difference in gene expression between the two groups denotes that with the introduction of KPC 689 to pericyte caused for an increase in expression of both genes which means that additional cell to cell communication is present.

Keeping these changes in mind, we designed our next experiment to mimic the tumor microenvironment by culturing KPC 689 with pericytes and endothelial cells and see how they behave and interact with each other.

A triple co-culture was done using pericytes, endothelial cells, and KPC 689. While there were technical difficulties when quantifying gene expression, pictures of the cultures were taken and observations were record. Figure 7 shows hPC cultured in part A appeared normal as did the co-culture of hPC and HUVEC in part B. Though it is part C and D, with the triple co-culture of hPC, HUVEC and KPC 689, there is cell organizations that resemble the tumor sections in parts E and F. The red outlines in part C show the KPC 689 cell clusters. Part D shows those same cell clusters with fluorescence due to the KPC 689 having been modified with GFP and luciferase. The clustering of the cancer cells (Figure 7C and D) resembled the pattern in human tissues (Figure 7E and F) which indicate that in the presence of pancreatic cancer cells (KPC 689), there is manipulation and forced organization infuced by the cancer cells that could cause for a potential phenotypic change.

## In-Direct Co-Cultures

### Using Transwell Membrane Culture Inserts

To confirm the possible similarity of KPC 689 to PANC-1 being able to induce atypical expression of α-SMA in pericytes, both pancreatic cancer cell lines were used as treatments for the co-culture of pericytes and endothelial cells when seeded on a transwell membrane culture insert. The transwell membrane would prevent direct physical touch of the co-culture cells (hPC and HUVEC) from the pancreatic cancer cells (PANC-1 and KPC 689). Results from the transwell membrane experiments show an increase in both Desmin and α-SMA expression in pericytes(Figure 8). Since both of these genes encode cytoskeletal proteins, we hypothesized that if expression of one of these genes was enhanced, the other may also increase through similar signaling mechanisms. Thereafter, the Desmin:α-SMA ratio was not further repeated, but instead separate genes are showcased. Desmin expression increased for the pericytes when in the presence both cancer cell lines. Furthermore, Desmin expression was greater in KPC 689 cultured inserts than PACN-1 cultured inserts. Though the atypical α-SMA expression fold change of hPC was similar between KPC 689 and PANC-1 cell inserts. Therefore, we speculated that atypical α-SMA expression in hPC would take place if hPC cultured directly with PANC-1 as was done with KPC 689 prior.

### Using Exosomes for In-Direct Co-Cultures

After confirming that there were chemical communicators in play affecting gene expression, an additional factor was used to determine what specific communicator could be causing this increased atypical pericyte phenotype. Exosomes were extracted and isolated from PANC-1, then used as a treatment condition for pericytes cultured by themselves. The results showed that the exosomes by themselves were able to influence atypical pericyte phenotype,

with an approximate 8 fold change in α-SMA expression compared to pericytes treated with PBS as a negative control and HSE as treatment control (Figure 9). There was no significant change in gene expression in any of the genes tested between HSE and PBS treated pericytes. This indicates that the cancer derived exosomes alone were able to simulate culture conditions similar to pericytes cultured directly with cancer cells (KPC 689, previous experiments) or indirectly (PANC-1 seeded in a culture well).

These same conditions were repeated using varying concentrations (0.5, 1 and 2 times) of exosomes per well, with 1X concentration consisting of 100μg/μL of exosome protein (Figure 10). As the concentration of exosomes increased, the fold change for α-SMA also increased. This trend indicates that the effect of exosomes on pericytes is specific to the presence of these exosomes and that the amount of exosomes are able to alter the phenotype of pericytes. Relating back to an *in* vivo model, if more cells are present that expel exosomes capable of inducing atypical vessel phenotype. Likewise, if a greater amount of exosomes is present within the microenvironment, then the effect on pericyte by inducing atypical up regulations of pericyte genes, such as α-SMA, will be stronger.

To reconfirm these findings and to determine if the suspected stabilizing effect between pericytes and endothelial cells can counteract the effect of the exosomes, a co-culture of pericytes and endothelial cells were treated with PBS, HSE, KPC 689 or PANC-1 exosomes. Greater expression of α-SMA was present in the co-cultures treated with KPC 689 or PANC-1 exosomes (Figure 11).

To determine what the function and physical effect of pancreatic cancer exosomes on vasculature, an endothelial tube formation assay was conducted with an endothelial cell only culture (Figure 12), or in a co-culture condition with pericytes and endothelial cells (Figure 13).

Focusing on the HUVEC only endothelial tube formation assay in Figure 12, the integrity of the tubes are expected to diminish as time passes and endothelial cells undergo apoptosis. There seems to be no noticeable difference between PBS and HSE treated tubes, while there was only a slight, though not significant, change between the control treatments (PBS and HSE) and PANC-1 exosome treated wells.These differences are more noticeable in the co-culture samples in Figure 13. There is a clear morphological difference between PANC-1 treated tubes between the treatments at hours 14-16, when compared to either PBS or HSE exosome treated well. While all wells have some clumping, the PANC-1 exosome treated wells showed greater tube instability, as any of the small branching tubes seen at hour 8 have collapsed and clustered at hour 14 or completely failed by hour 16. In PBS and HSE treated wells, the tubes got darker and clustered with more cells but never collapsed. This effect could be due to the cancer derived exosomes caused for atypical pericyte phenotypes that when in the presence of endothelial cells caused greater instability of endothelial tubes, potentially affecting the cell to cell communication like Ang-1, Ang-2 and Tie-2 signalling as is commonly noticed in cancer angiogenesis.

In conclusion, from these approaches, it was established that pericytes are sensitive to its environment. This is supported by the presence of atypical expression of α-SMA that are observed in a direct co-culture where physical touch is present (Figure 6), in an in-direct co-culture is present and chemical communication is allowed to intereact with the pericytes (Figure 8), and lastly, the presence of pancreatic cancer cell exosomes alone are able to cause a specific change in atypical α-SMA in pericytes (Figures 9-11). The trend that caused for this atypical increase of α-SMA in pericytes were cancerious conditions. These conditions, especially noticed with pancreatic cancer derived exosomes, were specific and potent enough to induce pericyte

phenotypic changes and affect its functioning potential, which is also noted in the observations in Figures 9 and 13.

The use of these observations can aid in better understanding the processes that affect pericyte function, especially in considerationto delivering drugs to the TME. Bettering the functioning potential of the vasculature will not just be beneficial to pancreatic cancer, but other forms of cancer as well. While the TME studied may be unique for pancreatic cancer, tumor angiogenesis is not specific to pancreatic cancer. The potential for vascular renormalization to be considered as a treatment option for other cancers, in which the vasculature is inefficient and impacts drug deliver, could be a useful source of treatment options. This approach especially holds potential since the work conducted by Kim, et al. showed overall greater suvivalibility of patients when administered cancer treatment when they posses a high Desmin to PDGFRβ ratio [9]. The use of vascular renormalization could also be used for other vascular diseases and conditions where inefficient pericyte attachment is the root of the problem or contributes to its worsening condition.

To reach that goal, more effort and research will be required since there are not any known pericyte specific gene markers. An approach that may be useful to determine potential specific pericyte markers would be to attempt single cell sequencing. It is a technique that sequences the genome for one cell and can do this up to several thousand cells and compare the differences between the sequenced cells and possibly compare them to a data bank of other genomes. That data could allow for a breakdown of a more narrow range of genes to then test what other genes are atypically expressed in a cancerous environment. One other future route to consider is determining what exosomal components cause for these atypical pericyte phenotype. Communicating molecules such as DNA, RNA, and proteins could be isolated to see what their

effects are on pericytes and then compare the severity or potency of the effect of these communicatin molecules. Additionally, specific proteins or sequences of DNA and RNA could be  then isolated to determine their effect on pericyte function.

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