HYPOTHESIS:

I **hypothesize** that PDAC cells intravasate by TMEM doorways which can be inhibited by Tie2 blockade. Testing this hypothesis will uncover PDAC vulnerabilities that may be leveraged with novel therapeutics.

SPECIFIC AIMS:

Aim 1: Identify patterns of tumor cell intravasation in mouse models of PDAC using large volume high-resolution intravital imaging (LVHR-IVI) and multiplex immunofluorescence (IF).
Aim 2: Determine whether Tie2 inhibition disrupts dissemination in PDAC.

a. Quantify vascular opening, tumor cell intravasation using LVHR-IVI, disseminated cancer cells (DTCs), metastasis and survival in mice treated with or without Rebastinib.
b. Quantify vascular opening, tumor cell intravasation using LVHR-IVI, disseminated cancer cells (DTCs), metastasis and survival in transgenic mice with a conditional macrophage Tie2 knockout.

BACKGROUND, SIGNIFICANCE and PRELIMINARY STUDIES:

Even though metastasis is a hallmark of PDAC, a detailed understanding of the mechanisms by which metastasis occurs is lacking. A critical step in the metastatic process involves the intricate mechanism(s) by which invasive cancer cells enter the bloodstream, or intravasate. The goal of this proposal is to determine the mechanism(s) by which PDAC cells intravasate, thus understanding the events that promote pancreatic cancer metastasis.

EMT in PDAC - Cancer cell intravasation depends on tumor cell-intrinsic *and* -extrinsic factors including the epithelial-mesenchymal transition (EMT) program which facilitates invasion by relieving tumor cells of their adhesive properties(5,6). Previous reports show PDAC cells can undergo EMT through one of two distinct mechanisms – a classical EMT ("C-EMT") that is associated with single cell invasion and a partial EMT ("P-EMT") that is associated with collective invasion(7). In order to study this, the KPCY model of PDAC was developed, which uses lineage tracing with a YFP lineage tracer to mark cancer cells regardless of changes in appearance due to EMT at single cell resolution(7). From this tumor model, cell lines were developed. With these models, one is able identify and isolate cells that have undergone EMT *in vivo and in vitro* in the context of stochastic tumor progression/metastasis. *However, the mechanism by which these single or collective metastatic cells intravasate is unknown.*

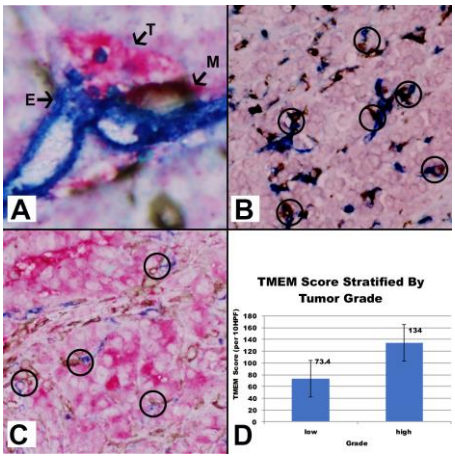

Fig. 1. (A) high power, high resolution TMEM doorway; T = tumor cell (pan-MENA), E = endothelial cell (CD31), M = macrophage (CD68). (B) TMEM doorway scoring by IHC in PDAC resected from patients. (C) TMEM doorway scoring by IHC in PDAC from KPC mouse pancreata. (D) TMEM doorway scores from PDAC tumors from patients who underwent curative intent resection; stratified by tumor grade.

The Tumor Microenvironment of Metastasis - While the mechanism of intravasation in PDAC is unknown, in breast cancer the mechanism is well established. In breast cancer, tumor cells enter the bloodstream only through specialized portals known as **T**umor **M**icro**E**nvironment of **M**etastasis (TMEM) doorways(8–10). TMEM doorways are stable tri-cellular interactions between a tumor cell (expressing Mena), macrophage (expressing Tie2), and endothelial cell, resulting in the creation of a trans-endothelial doorway through which tumor cells enter the bloodstream(9,11). These doorways and their functional properties can be readily imaged by LVHR-IVI(12–15). To initiate TMEM doorway activity, Tie2 -expressing macrophages secrete vascular endothelial growth factor A (VEGFA), which locally reduces cohesion of capillary endothelial adherens and tight junctions, thereby allowing a transient opening of the endothelial wall (~30 min "opening"). This opening is not related to vascular "leaking". It is a highly localized, deliberate, large opening in the tumor endothelial layer of sufficient size to allow passage of an intact tumor cell(16). Breast cancer cells with an invasive gene signature and a C-EMT phenotype then enter the bloodstream through these doorways(17). Disruption of Tie2 signaling significantly impairs TMEM doorway function(8,9,18,19). Importantly, the concentration of TMEM doorways in breast cancer is a validated, prognostic marker of metastasis and survival(20,21). Although, while these

discoveries have led to great progress in the understanding of metastasis and treatment of breast cancer, it is unknown if this biology is present in more lethal tumors such as PDAC.

TMEM Doorways in PDAC - Given the clinical problem of metastasis in PDAC and the gap in knowledge regarding mechanisms of intravasation and dissemination, we evaluated human and mouse tissue samples by immunohistochemistry (IHC) for the presence of TMEM doorways(22). In human samples, TMEM doorways were detected in primary tumors (**Fig.1**). Murine tumors from the genetically engineered KPC model also harbored TMEM doorways (**Fig. 1**). The concentration of TMEM doorways was associated with tumor grade and much higher than previously observed in breast cancer cohorts (**Fig. 1**)(21). This indicates TMEM doorways are more prevalent in PDAC than in breast cancer.

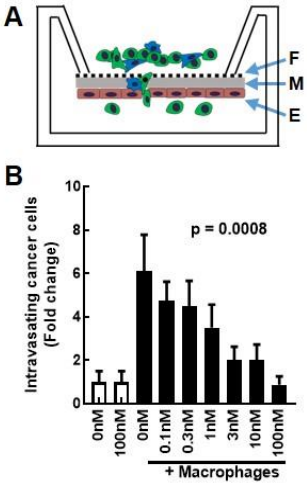

Fig. 2. PDAC transendothelial migration can be enhanced by macrophages and blocked by Rebastinib. (A) Schematic of the *in vitro* intravasation-oriented transendothelial migration assay. Green = tumor cells, Blue = macrophages, F = filter (10 µm pores), M = matrigel (10 µm), E = human umbilical vein endothelium. (B) Fold change of PDAC cells intravasating (open bars) compared to PDAC cells co cultured with macrophages (black bars) with increasing doses of Rebastinib. Tumor cells and BAC 1.2F5 macrophages (where indicated) were plated on top of the filter and co-cultured for 4 hours with and without increasing doses of Rebastinib. Comparison was done by one-way ANOVA. Error bars represent standard deviations.

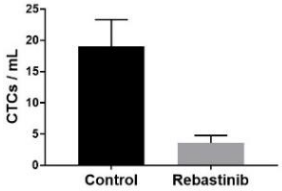

Fig. 3. CTC counts. EpCAM+ /CD45- CTCs in KPC mice (Rebastinb, N=3; control, N=2) treated with 3 doses of oral Rebastinib (10mg/kg).

To further test the hypothesis that PDAC cells disseminate via TMEM doorways, we determined whether their transendothelial migration requires the TMEM doorway associated Tie2+ macrophages. Using a previously established *in vitro* transendothelial migration assay (iTEM) to measure TMEM function(16,23) (**Fig. 2)**, we observed significantly more PDAC cells crossing the endothelial layer when macrophages (TMEM) were present (**Fig. 2**). To determine whether Tie2 signaling mediates this TMEM macrophage-assisted transendothelial migration, we used Rebastinib, a highly selective, potent inhibitor of Tie2 and TMEM doorway function *in vivo*(9). Rebastinib induced a dose-dependent inhibition of transendothelial migration of PDAC cells only in the presence of macrophages, indicating that Tie2 signaling is essential for TMEM macrophage-mediated transendothelial migration *in vitro* (**Fig. 2**). Since we observed the presence of TMEM doorways in PDAC and showed that their function depends on Tie2 signaling in breast cancer, we asked whether Rebastinib could inhibit PDAC tumor cell entry into the bloodstream. We treated tumor bearing KPC mice(24) with or without Rebastinib then collected circulating tumor cells (CTCs). Rebastinib markedly reduced CTC number (**Fig. 3**), suggesting that intravasation in PDAC requires Tie2 signaling and TMEM doorway function. *While these discoveries support the notion that intravasation in PDAC is TMEM doorway mediated via Tie2 signaling, we have not directly tested this. We propose to address this critical gap in knowledge in this current research.*

A. RESEARCH DESIGN AND METHODS

Aim 1: Identify patterns of tumor cell intravasation in mouse models of PDAC using LVHR-IVI and multiplex IF. How metastatic cells with differing EMT programs enter the blood stream is unknown. The goal of this aim is to *directly assess* TMEM doorway activity in PDAC intravasation in the context of collective (P-EMT) *and* single-cell (C-EMT) migration. By taking an agnostic experimental approach, we will determine whether TMEM doorways are the sole portal by which PDAC cells access the circulation, or whether PDAC cells intravasate by TMEM-independent mechanisms. With the help of [redacted] (see Letter of Support), I will use permanently implantable abdominal imaging windows to study tumor cell intravasation and IF of resected mouse tumors from implantable models using P-EMT or C-EMT PDAC cell lines.

To determine if EMT subtype affects the mode of PDAC intravasation, we will generate orthotopic tumors from C-EMT and P-EMT PDAC cell lines. Cancer cells (all YFP+) will be implanted into sygeneic mice. At the time of injection of tumor cells into mouse pancreata, an abdominal imaging window will be placed. Using LVHR-IVI approaches, we will quantify the following endpoints: (**i**) the number of acute vascular "opening" events; (**ii**) the duration of "opening" (20-30 min is characteristic of TMEM doorway opening in breast cancer models); and (**iii**)

the number of cancer cells intravasating at and away from "opening" sites. Intravasation events will be scored as either TMEM-associated or non-TMEM-associated based on these characteristics for both C-EMT and P-EMT cell lines. We will confirm the site of vascular opening and intravasation is at TMEM doorways or other sites by IVITA methodology as discussed below. These experiments will allow a direct evaluation of the interplay between EMT phenotype, patterns of invasion (single cell or collective), and path of vascular entry (TMEM doorway or non-TMEM doorway).

Expected outcomes, potential pitfalls, and alternative approaches: We expect to observe PDAC cells entering the bloodstream only at TMEM doorways regardless of EMT program. However, although TMEM-independent mechanisms of cancer cell dissemination have not been observed in breast cancer, most of our prior studies have been limited to C-EMT (single cell invasion) models. Thus, TMEM-independent mechanisms of vascular invasion may exist in PDAC, particularly in our P-EMT models. Moreover, single tumor cell intravasation at TMEM doorways can lead to the formation of CTC clusters as a result of cell-cell adhesion between cancer stem cells in the CTC population. Therefore, it is possible that both C- and P-EMT associated intravasation may involve the crossing of single tumor cells through the endothelium at TMEM doorways one at a time in PDAC, even in models where clustered tumor cells are observed in the CTC population. We are able to monitor for this due to the stable, high resolution imaging of our LVHR-IVI methodology. Given our prior experience with these models and LVHR-IVI, reflected in prior publications and those in revision, we do not expect any issues with feasibility.

Statistical analyses: We will compare the vascular opening and intravasation endpoints at and away from TMEM doorways between P- and C-EMT tumors. We will use two-tail t-test to compare each of the outcome variables. With 8 mice per group, our study has 80% power to detect a minimum effect size of 1.5 SD between the two groups.

Large-Volume High-Resolution Intravital Imaging (LVHR-IVI). [redacted] (collaborator) and [redacted] (Senior Scientist Mentor, see letter of support) have developed several surgical protocols for stabilizing tissues that enable extended time-lapse intravital multiphoton imaging in soft tissues such as lymphatic tissue, lymph nodes, lungs, livers, and mammary fat pads. The complete immobilization of these live tissues allows the use of LVHR-IVI to generate a large-scale view of the tissue at high-resolution (0.24 µm/pixel), allowing a much more complete investigation of the tumor than possible with traditional intravital imaging showing the capture of time-lapsed movies of large volumes of tissue at subcellular resolution(25). Combining LVHR-IVI with our previously published imaging windows, extends these studies to multiple imaging sessions spanning days to weeks. [redacted] has trained me in these techniques. Utilizing my surgical expertise, we collaborated on a new technique for a stabilized window for IVI of the murine pancreas (SWIP) which is in revision at a peer reviewed journal currently. With [redacted] guidance, I will use SWIP for the proposed LVHR-IVI experiments to ensure stable single cell dynamic imaging of PDAC tumors *in vivo*.

Intravital Imaging Tissue Alignment (IVITA): While histochemistry and immunostaining show extreme versatility for labeling, they cannot capture cellular or tissue dynamics. A novel method was developed for mechanically sectioning the exact tissue slice probed with LVHR-IVI. This process involves marking the live tissue with a high-power laser ("branding") at the end of the LVHR-IVI session and then re-locating this mark in the fixed and mechanically sectioned tissue. IVITA aligns the fixed tissue block to the microtome cutting plane during mechanical sectioning. This approach increases by orders of magnitude the number of cells that can be correlated between LVHR-IVI and multiplex IF (manuscript submitted).

Aim 2: Determine whether Tie2 inhibition disrupts dissemination in PDAC.

Rationale: Our preliminary data shows that trans-endothelial migration of PDAC cells is macrophage dependent and blocked by the specific inhibitor of Tie2, Rebastinib. Also, our preliminary data shows that CTCs decrease in response to Rebastinib in PDAC harboring mice. Rebastinib (DCC-2036), is a "switch-pocket" inhibitor originally designed to bind to Arg386/Glu282 of ABL1 and improve treatment for acquired resistance to imatinib (Gleevec) in chronic myeloid leukemia. However, recent *in vivo* analysis of Rebastinib has shown a potent and highly selective, picomolar inhibition of the Tie2 receptor tyrosine kinase; specifically, Rebastinib has approximately a 62-fold more selective binding to Tie2 compared to BCR-ABL (9). In fact, Rebastinib is currently being tested in clinical trials for breast and gynecologic cancers (NCI NCT03717415, NCT03601897) at Montefiore-Einstein and other sites. However, Tie2 inhibition in PDAC may effect vascular normalization by other non-TMEM doorway mediated mechanisms. We will evaluate these possibilities in this aim. The impact of Tie2 inhibition will give insight into the mechanism of TMEM doorway activity in PDAC and establish a [redacted]

PDAC.

***2a*. Quantify vascular opening, tumor cell intravasation using LVHR-IVI, disseminated cancer cells (DTCs), metastasis and survival in mice treated with or without Rebastinib.**

We will inject metastatic YFP-labeled PDAC cells into pancreata of C57Bl/6 mice along with placement of abdominal imaging windows. Once recovered from surgery and tumors develop, mice will be treated with or without Rebastinib (chow for 21 days. Rebastanib will be provided as a chow formulation for murine use by Deciphera™). After treatment, mice will undergo LVHR-IVI and IVITA as described in Aim 1. Vascular opening and intravasation events at and away from TMEM doorway sites will be quantified and compared between treated and untreated mice as described in Aim 1. In parallel, we will inject a metastatic YFP-labeled PDAC cell line into pancreata of C57Bl/6. Primary tumors in this model tend to grow quickly and mouse morbidity is usually due to local effects of the tumor rather than metastatic progression. To address this limitation in the mouse model, once recovered from surgery, mice will be treated with or without Rebastinib. Once primary tumors are established (about 10 days after injection) the control and treated mice will undergo a second surgical procedure to remove their primary tumor (distal pancreatectomy). The resected primary tumors will be evaluated for TMEM score (Mena, CD68, endomucin IHC) and mean vessel density (endomucin).

Once recovered from the second surgery, control and treated mice will be divided randomly and stratified based on gender into 2 cohorts. **(i):** mice will be treated with or without Rebastinib for 40 days then sacrificed with collection the liver and lungs. Liver and lung tissue will be evaluated for the number of gross metastases. Liver and lung will also be evaluated for the number of DTCs by multiplex IF of YFP, endomucin and Dapi. **(ii):** mice will be treated until moribund with or without Rebastinib to evaluate overall survival from the time of distal pancreatectomy.

Expected outcomes, potential pitfalls, and alternative approaches: We expect to observe a decrease in vascular opening and intravasating PDAC cells in response to Rebastinib therapy. This will show directly *in vivo* the mechanism of action of Rebastinib. We expect to observe a decrease in DTCs and metastasis in mice treated with Rebastinib. Additionally, we expect an increased median overall survival for those mice treated with Rebastinib. This will also provide the rationale to complement standard of care chemotherapy with Rebastinib in clinical trial for metastatic, locally advanced, and resectable PDAC with the goal of decreasing overwhelming metastatic burden in all patients with PDAC. Since we have robust experience with the methods proposed and use of Rebastinib, we do not anticipate pitfalls. However, we note that Rebastinib may have off target effects which will be assessed (mean vessel density, macrophage count, tumor volume). Previous work has showed that Rebastinib does not affect TMEM doorway assembly and number(26). Therefore, we will still be able to evaluate TMEM doorway function substaniating the need to clinically develop of Rebastinib in PDAC. We anticipate morbidity related to manipulating the pancreas which may increase morbidity and mortality of the mice. This pitfall will require more mice to mitigate variability in these aspects.

Statistical analyses: We will compare vascular opening and intravasation at *and* away from TMEM doorways between Rebastinib-treated *and* untreated control mice. We will use ANOVA and two-tail t-test to compare each of the outcome variables and treatment groups. With 8 mice per group, our study has 80% power to detect a minimum effect size of 1.5 SD between the two groups. We will compare DTCs, and the number of gross metastases between mice treated with or without Rebastinib. We will use two-tail t-test to compare each of the outcome variables. With 8 mice per group in cohort **i**, our study has 80% power to detect a minimum effect size of 1.5 SD between the two groups. We will compare overall survival from the time of distal pancreatectomy between treated and untreated mice using Kaplan-Meier estimates. We expect an improvement in median overall survival of one month between groups. The sample size of cohort **ii** is determined to ensure estimating the posterior distribution of the risk of mortality in treated mice. Therefore, we expect a sample size of 10 mice per treatment group to provide a 75% chance that mice treated with Rebastinib have a greater median overall survival than control mice taking into account variability in tumor implantation, perioperative survival, and oncologic outcomes.

***2b*. Quantify vascular opening, tumor cell intravasation using LVHR-IVI, disseminated cancer cells (DTCs), metastasis and survival in transgenic mice with a conditional macrophage Tie2 knockout.**

To further test the hypothesis that the TMEM doorway macrophage Tie2 receptor is a critical regulator of TMEM doorway function and downstream intravastation events in PDAC, we will conditionally knock out Tie2 in tumor associated macrophages. This requires development of a transgenic mouse that currently is not available. Therefore, we will design a transgenic mouse with the extrcellular IgG binding domains of Tie2 (the binding site for Ang1 and Ang2) will be flanked with LoxP sites(27). This mouse will be generated in the Montefiore-

Einstein Transgenic Core which provides success rates of +95%. This mouse will be crossed with our tamoxifen regulated Cre-CSF1R mouse. Resultant mice will have macrophage Tie2 expression knocked out by tamoxifen treatment. We will inject a metastatic YFP-labeled PDAC cell line into pancreata of this Tie2-Cre CSF1R mouse. Mice will be handled and treated with tamoxifen similar to mice treated with or without Rebastinib in Aim 2a. The endpoints of this study will be the same as described in Aim 2a using LVHR-IVI, IVITA, gross metastatic burden, DTC, and survival to determine if macrophage Tie2 is mediating intravasation and metastasis of PDAC.

Expected outcomes, potential pitfalls, and alternative approaches: We expect to observe a decrease in vascular opening and intravasating PDAC cells in response to macrophage Tie2 knockout therapy. This will show directly *in vivo* macrophage Tie2 signaling is critical for metastasis of PDAC. We expect to observe a decrease in DTCs and metastasis in mice in knockout mice. Additionally, we expect an increased median overall survival for knockout mice. This will show that Tie2 inhibition is crucial for decreasing PDAC metastasis which will further support development of Rebastinib and other Tie2 inhibitors clinically. Since we have robust experience with the methods proposed, we do not anticipate pitfalls. However, we will also evaluate in vitro and in vivo Tie2 expression and activity in knockout mice to ensure efficacy of our transgenic construct.

Statistical analyses: We will compare vascular opening and intravasation at *and* away from TMEM doorways between control mice and knockout mice. We will use ANOVA and two-tail t-test to compare each of the outcome variables and treatment groups. With 8 mice per group, our study has 80% power to detect a minimum effect size of 1.5 SD between the two groups. We will compare DTCs, and the number of gross metastases. We will use two-tail t-test to compare each of the outcome variables. With 8 mice per group in cohort **i**, our study has 80% power to detect a minimum effect size of 1.5 SD between the two groups. We will compare overall survival from the time of distal pancreatectomy between treated and untreated mice using Kaplan-Meier estimates. We expect an improvement in median overall survival of one month between groups. The sample size of cohort **ii** is determined to ensure estimating the posterior distribution of the risk of mortality in treated mice. Therefore, we expect a sample size of 10 mice per treatment group to provide a 75% chance that knockout mice have a greater median overall survival than control mice taking into account variability in tumor implantation, perioperative survival, and oncologic outcomes.

B. RELEVANT WORK AND COLLABORATIONS

I have been collaborating and leading a new research team at [redacted] ([redacted]) on development and clinical utilization of Tie2 inhibitors for PDAC which is supplemented by sponsored research agreement with [redacted]. To complement and augment this initiative, I collaborate with [redacted] (*Maddipati Cancer Discovery 2021*) and [redacted] (NCI U01 supplement) to better understand the PDAC microenvironment, EMT, and dissemination. I was able to present our preliminary results at the annual the Pancreatic Cancer Microenvironment Network (PaCMEN) meeting twice (https://pacmen.org/home.html).

Collaborations at [redacted] have led to a co-authored manuscript accepted to Science Translational Medicine evaluating immune therapy delivery using a Listeria bacterial platform (*Gravekamp C., In Press*). I am also co-author of another manuscript in revision at Open Biology presenting our novel surgical and imaging protocol for a permanent abdominal window for intravital microscopy of PDAC.

C. REFERENCES

1. [redacted]