UNIVERSIDADE D
COIMBRA

Patrícia Fernanda Carneiro Vieira

EXOMET
UNDERSTANDING THE ROLE OF PANCREATIC CANCER METASTASIS EXOSOMES IN ITS ESTABLISHMENT, SURVIVAL AND GROWTH AT SECONDARY ORGANS

Dissertação no âmbito do Mestrado em Investigação Biomédica com especialização em Oncobiologia orientada pela Professora Doutora Sónia Melo e pelo Doutor Henrique Girão apresentada à Faculdade de Medicina da Universidade de Coimbra.

Julho de 2019

EXOMET

UNDERSTANDING THE ROLE OF PANCREATIC CANCER METASTASIS EXOSOMES IN ITS ESTABLISHMENT, SURVIVAL AND GROWTH AT SECONDARY ORGANS

Patrícia Fernanda Carneiro Vieira

Thesis submitted to Faculty of Medicine of University of Coimbra for the Master's degree in Biomedical Research

Supervisor: Sónia Melo, PhD

Internal Supervisor: Henrique Girão, PhD

Institute of Pathology and Molecular Immunology of the University of Porto (IPATIMUP), Porto, Portugal

Instituto de Investigação e Inovação em Saúde (i3S), Porto, Portugal

July 2019

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive"

Ricardo Reis

Acknowledgments

Neste agitado ano, foram vários aqueles que desempenharam um papel para que fosse possível chegar aqui e aos quais estarei eternamente grata.

Em primeiro lugar, quero agradecer à minha orientadora, Sónia Melo. Obrigada por me ter recebido no grupo, pela sua orientação e pelo saber que transmitiu. Acima de tudo, obrigada por sempre se mostrar disponível, por me ter ensinado que não existem limites para aquilo que somos capazes de fazer e que existe sempre algo que podemos melhorar.

Agradeço ao professor Henrique Girão, pela oportunidade de integrar este mestrado e por, mesmo longe, estar sempre pronto para ajudar em qualquer situação.

Aos Melos, obrigada por me terem integrado desde o primeiro dia no grupo e por, além de todo o ensinamento profissional, me terem ensinado que as frustrações e conquistas são melhores quando partilhadas. Bárbara, a minha mentora, a pessoa que tem dentro de si um mundo que não cabe no seu tamanho. Obrigada por me guiares neste ano de cores que nem sempre foram brilhantes. Nuno, o melhor amigo destes últimos meses. Obrigada pela confiança, companhia e pelas longas conversas sobre o sentido da vida. Carolina, obrigada por seres tão bondosa e estares sempre disponível para me ajudar em todas as situações. Inês, obrigada por estares sempre disposta a ajudar-me quando parecia mais perdida. Ana Rita, obrigada por mostrares que nunca é tarde para novos desafios. Continua a tomar conta de todos, como fizeste desde o primeiro dia.

Ao grupo Gedy, muito obrigada por serem um grupo unido e por me integrarem nesta grande família, foi um prazer.

A todos aqueles que já me acompanham há mais tempo. Raquel, Inês e Maria, obrigada pelo companheirismo durante os últimos cinco anos.

A todos os meus amigos, peço desculpa por todo o desaparecimento ao longo deste ano. À Mila, Rafa e Jorge, obrigada pelo apoio, por ouvirem todos os meus desabafos, partilharem as minhas frustrações e me incentivaram todos os dias. À Tati pela amizade, por toda a preocupação e por compreender sempre todos os cafés cancelados. À Ana por partilhar esta experiência comigo e por ouvir todos os meus devaneios. À restantes pessoas que de alguma forma me acompanharam, obrigada.

Por último, um agradecimento especial à minha família, por serem presentes e se orgulharem das minhas conquistas como se de todos fossem.

Ao meu irmão e aos meus pais. Por serem modelos de coragem, pelo apoio incondicional, por acreditarem em mim e me guiarem nesta caminhada, obrigada. Obrigada por me ensinarem que todos os dias são um novo dia e que com persistência e resiliência somos capazes de mover montanhas.

A todos, um obrigado do fundo do meu coração.

Abstract

Exosomes are extracellular vesicles produced in the endosomal compartment of all cell types, and are involved in cell to cell communication. Exosomes carry information between close or distant cells, contributing for the reprograming of recipient cells. These vesicles have been described to regulate the immune system, particularly in tumorigenesis, as well as modulate the tumor microenvironment and cancer cells metastasis to other organs. Tumor exosomes interact with various cells in the microenvironment that contribute to cancer invasion, induction of angiogenesis, survival in the bloodstream and formation of a metastatic favoured environment at secondary organs. Cancer exosomes prepare the organs of metastasis for the incoming cancer cells. The role of tumor exosomes has been extensively investigated. However, how exosomes secreted by the metastasis influence its establishment and growth is still largely unexplored. To address this question, we used orthotopic and genetically engineered mouse models (GEMMs) of pancreatic cancer. The KPC-ExoBow GEMM is a CD63 multireporter transgenic mouse model that develops pancreatic ductal adenocarcinoma and secretes color-coded pancreatic cancer exosomes. Cancer cells express one of the fusion proteins CD63-mCherry, -phiYFP, -eGFP or -mTFP wherein their exosomes express respectively the same color. Using this animal model, as well as the Mia PaCa-2 with tagged CD63-eGFP human orthotopic model, we attempted to map the communication established *in vivo* by exosomes derived from pancreatic cancer metastasis, the ExoMet, at the metastatic sites and to other organs. To access this, we collected the metastasis from the lung and liver of KPC-ExoBow mice and orthotopically injected the cells in the lung and liver, respectively, of syngeneic wild-type animals. The same is true for human PDAC Mia PaCa-2 CD63-eGFP cells orthotopically injected in immunodeficient animals (nude CBA mice). At euthanasia, we collected the metastatic organs. Most importantly, we have also analysed *in vitro* the influence of CD63 overexpression and the CD63-tagged proteins resulting from distinct recombination events in the secretion of exosomes and communication in the KPC-ExoBow model.

Key-words: tumor exosomes; color-coded exosomes; metastasis; pancreatic cancer; CD63 tetraspanin

Sumário

Exossomas são vesículas extracelulares produzidas no compartimento endossomal de todas as células, envolvidos na comunicação intercelular. Os exossomas transportam informação entre células próximas ou distantes, o que permite a troca de informação que contribui para a reprogramação das células recipientes. Estas vesículas foram descritas como reguladoras do sistema imunitário, particularmente durante a tumorigénese, assim como moduladoras do microambiente tumoral e do processo de metastização. Exossomas produzidor por células tumorais interagem com várias células no seu microambiente que contribuem para os processos de invasão, indução da angiogénese, sobrevivência na corrente sanguínea e formação de um ambiente metastático favorável em órgãos secundários. Exossomas do tumor preparam os órgãos das metástases para a chegada das células cancerígenas. O papel dos exossomas tem sido extensivamente estudado. Contudo, como os exossomas libertados pelas metástases influenciam o estabelecimento e crescimento da metástase continua por explorar. Para abordar esta questão, foram utilizados modelos de murganho ortotópicos e geneticamente modificados do cancro do pâncreas. O KPC-ExoBow é um modelo animal transgénico *multireporter* que desenvolve adenocarcinoma ductal em que as células cancerígenas libertam exossomas marcados fluorescentemente, isto é, com cor. As células do tumor expressam uma das seguintes proteínas de fusão, CD63-mCherry, -phiYFP, -eGFP ou -mTFP em que os seus exossomas expressam a mesma cor. Através deste modelo animal, assim como do modelo ortotópico das células humanas Mia PaCa-2 que expressam CD63-eGFP, é pretendido estabelecer as vias de comunicação mediadas pelos exossomas das metástases do cancro pancreático *in vivo*, a ExoMet, no microambiente da metástase como para outros órgãos. Com este intuito, foram recolhidas as metástases do pulmão e do fígado de animais KPC-ExoBow e de seguida foram injetadas ortotópicamente no pulmão e no fígado, respetivamente, em animais singénicos *wild-type*. O mesmo procedimento foi seguido para as células humanas Mia PaCa-2 CD63-eGFP injetadas ortotópicamente em animais imunodeficientes (nude CBA). Na eutanásia, os órgãos metastáticos foram recolhidos. Ainda, também foi analisada *in vitro* a influência da sobre expressão de CD63 e das proteínas CD63 marcadas com a proteína de fusão resultante de diferentes eventos de recombinação, na secreção dos exossomas e na comunicação.

Palavras-chave: exossomas tumorais; exossomas com cor; metástases; cancro do pâncreas; tetraspanina CD63

Table of contents

Table of Figures

Glossary of abbreviations and acronyms

A

α-SMAD – α-smooth muscle actin

ABs – Apoptotic bodies

ApoE - Apolipoprotein

ATCC - American Type Culture Collection

B

BMDC – Bone marrow derived cells

BSA - Albumine Bovine Fraction

C

CAFs – Cancer-associated fibroblastos

CCL2 – Chemokine C-C motif ligand 2

CTLA-4 – Cytotoxic T-Lymphocyte antigen 4

D

DGAV - Direção-Geral de Alimentação e Veterinária

DMEM - Dulbecco's modified Eagle's medium

DMEM/F-12 - Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

E

ECL - Enhanced chemiluminescence

ECM – Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

EMT – Epithelial to mesenchymal transition

ESCRT – Endosomal sorting complex required for transport

EVs – Extracellular vesicles

F

FACS - Fluorescence-activated cell sorting

FBS - Fetal Bovine Serum

FITC - Fluorescein Isothiocyanate

Flp – Flippase

G

GEMM – Genetically engineered mouse model

GPC1 – Glypican 1

GrB – Granzyme B

H

HBSS - Hank's Balanced Salt Solution

HRP - Horseradish Peroxidase

HSP – Heat shock proteins

Hu – human

I

IL-10 – Interleukin-10

ILVs – Intraluminal vesicles

K

KPC – $Pdx1^{cre/+}$; $LSL\text{-}Kras^{G12D/+}$; $Trp53^{R172H/+}$ mouse model

KPC-ExoBow - *R26$^{CD63\text{-}XFP/+}$; Pdx-1$^{Flp/Cre}$; LSL-Kras$^{G12D/+}$; Trp53$^{R172H/+}$* mouse model

KRAS – Kirsten rat sarcoma viral oncogene homolog

M

MET – Tyrosine-protein kinase Met

MIF – Macrophage migration inhibitor

MMP – Metalloproteinases

mRNAs – Messenger RNAs

Ms - mouse

MVBs – Multivesicular bodies

MVs – Microvesicles

N

NF-kB – Nuclear factor-kB

NK – Natural killer

NTA - Nanoparticle Tracking Analysis

P

PanIN – Pancreatic intraepithelial neoplasia

PBS - Phosphate-Buffered Saline

PC – Pancreatic cancer

PDAC – Pancreatic ductal adenocarcinoma

Pdx – Pancreatic duodenal homeobox promoter

PFA – Paraformaldehyde

PMSF - Phenylmethanesulfonyl Fluoride

R

RPMI - Roswell Park Memorial Institute

S

SDF-1 – Stromal cell-derived factor 1

SDS - Sodium Dodecylsulphate

SMAD4 – Mothers against decapentaplegic homolog 4

T

TDEs – Tumor-derived exosomes

TGF-β – Tumor growth factor β

TLR-2 – Toll-like receptor 2

TP53 – Tumor promoting p53 gene

Treg – Regulatory T cells

Tspan8 – tetraspanin 8

V

VEGFs – Vascular endothelial growth factors

WNT5A – Wnt family member 5A

1. Introduction

1. Introduction

1.1. Exosomes

Communication is an integral part of the life of a cell. It is an essential process for cells to maintain their normal growth and function. Cells can communicate through different mechanism which may involve gradients of soluble factors or transport of molecular and genetic information encapsulated in vesicles (Camussi et al. 2010). Over the past decade, a growing number of studies suggest the transfer of extracellular vesicles (EVs) as a fundamental mechanism for intercellular communication (Graça Raposo and Stoorvogel 2013; El Andaloussi et al. 2013). EVs are composed by a lipid bilayer containing transmembrane and cytosolic proteins and nucleic acids (Colombo, Raposo, and Théry 2014). Although first classified as a way for cells to eliminate unnecessary compounds such as membrane debris or other unneeded structures (Johnstone et al. 1987), EVs are now recognized as key players of numerous physiological processes in pathological and nonpathological conditions (De Toro et al. 2015). EVs have molecules at their surface which support their interaction and/or recognition by recipient cells. When EVs bind to the target cell they can be internalized by endocytosis, phagocytosis or fuse with the membrane where release their content to the cytosol, resulting in changes in the cellular physiology (Meldolesi 2018). The term EVs refer to a heterogenous group of secreted membrane vesicles divided in microvesicles (MVs) and exosomes (van Niel, D'Angelo, and Raposo 2018). Microvesicles are 150 nm to 1000 nm vesicles originated from the direct budding from the plasma membrane whereas exosomes are small vesicles ranging in size from 50 nm to 150 nm in diameter formed by inward budding of the endosomal membrane (Tricarico, Clancy, and D'Souza-Schorey 2017). The term exosomes should not be confused with the exosome complex involved in RNA degradation (Wasmuth, Januszyk, and Lima 2014; Decker 2004).

This thesis focuses on the role cancer exosomes have as important mediators of information exchange to support metastasis survival. In the last years, interesting findings demonstrate the involvement of exosomes in various cellular activities from the immune system modulation (Robbins and Morelli 2014) to the pathogenesis of several diseases such as cardiovascular diseases (Zhu and Fan 2011), neurodegenerative diseases (Ghidoni, Benussi, and Binetti 2008) and infectious diseases (Mahmoodzadeh Hosseini et al. 2013). Nevertheless, is in cancer that exosomes have been thoroughly studied. Exosomes are able to mediate the communication between cancer cells and

their microenvironment, contributing for tumor progression and metastasis (Brücher and Jamall 2014).

1.1.1. Exosomes biogenesis

Exosomes were first described in the 1980s, but only in the last decades their biological significance started to be addressed. The first breakthrough in the field happened when exosomes secreted by Epstein-Barr virus (EBV)-transformed B-lymphocytes were shown to transport essential molecules for the adaptive immune response (G Raposo et al. 1996). Right after, it was demonstrated that dendritic cells secrete exosomes with MHC-peptide complexes, capable of inducing antitumor immune responses (Zitvogel et al. 1998). These findings were the first evidence that related exosomes with a possible role in intercellular communication. The second major advance happened when messenger RNAs (mRNAs) and microRNAs were found in exosomes, and transfer of genetic information between cells was demonstrated (Valadi et al. 2007).

Exosomes biogenesis is associated with the endocytic pathway (Johnstone et al. 1987) (**Figure 1**). When endocytosis starts, endocytic vesicles bud with the early endosome. Early endosomes differentiate into multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) in their lumen. ILVs are formed by the inward budding of clathrin-coated domains of the endosomal membrane during the maturation of the early endosome (Stoorvogel et al. 1991; Colombo, Raposo, and Théry 2014). When MVBs fuse with the plasma membrane, the internal vesicles containing lipids, proteins, DNA, mRNAs and microRNAs are released in the form of exosomes. Alternatively, these vesicles can fuse with lysosomes leading to their degradation. An important player during the formation of ILVs and endosomes maturation is the endosomal sorting complex required for transport (ESCRT) (Colombo et al. 2013). The ESCRT machinery is composed by four complexes of ESCRT (0 through III) that together with accessory proteins (ALIX, TSG101, VPS4, and VTA1) localize in the cytoplasm of the endosome, participate in the MVB formation, vesicle budding, protein cargo sorting and even in the release of exosomes into the endosomal lumen (Hurley and Hanson 2010). Another family of proteins that participate in exosomes biogenesis is the Rab family of small GTPase proteins (Blanc and Vidal 2018). Rab proteins are involved in different steps of the endocytic pathway, such as budding, endosomes mobility, docking and fusion with the plasma membrane and consequent exosomes secretion. Rab4 and Rab5 are proteins located in the early endosome, which control fast recycling and endosomal fusion, respectively (McCaffrey et al. 2001; Gorvel et al. 1991). Rab11 and Rab35 are

also associated with the early endosome stage but are involved in slow recycling and sorting, respectively (Stenmark 2009). On the other hand, Rab7 is involved in the late endocytic pathway regulating the secretion of ALIX- and syntenin-positive exosomes in cancer (Baietti *et al.*, 2012). However, this protein was also shown to be involved in the movement of cargo from the MVBs to the lysosome (Vanlandingham and Ceresa 2009). Rab27a and Rab27b are involved in late endosomal and secretory pathway, and mediate the fusion and docking of MVBs to the plasma membrane and exosomes release (Ostrowski et al. 2010). Besides the set of proteins already mentioned, exosomes are also enriched in other proteins involved in membrane transport and fusion process, such as integrins, tetraspanins (CD9, CD63, CD81, CD82) and heat shock proteins (HSP70, HSP90) (Simons and Raposo 2009; Colombo, Raposo, and Théry 2014) (**Figure 2**). Tetraspanins are widely used as exosomal marker proteins (Kowal et al. 2016).

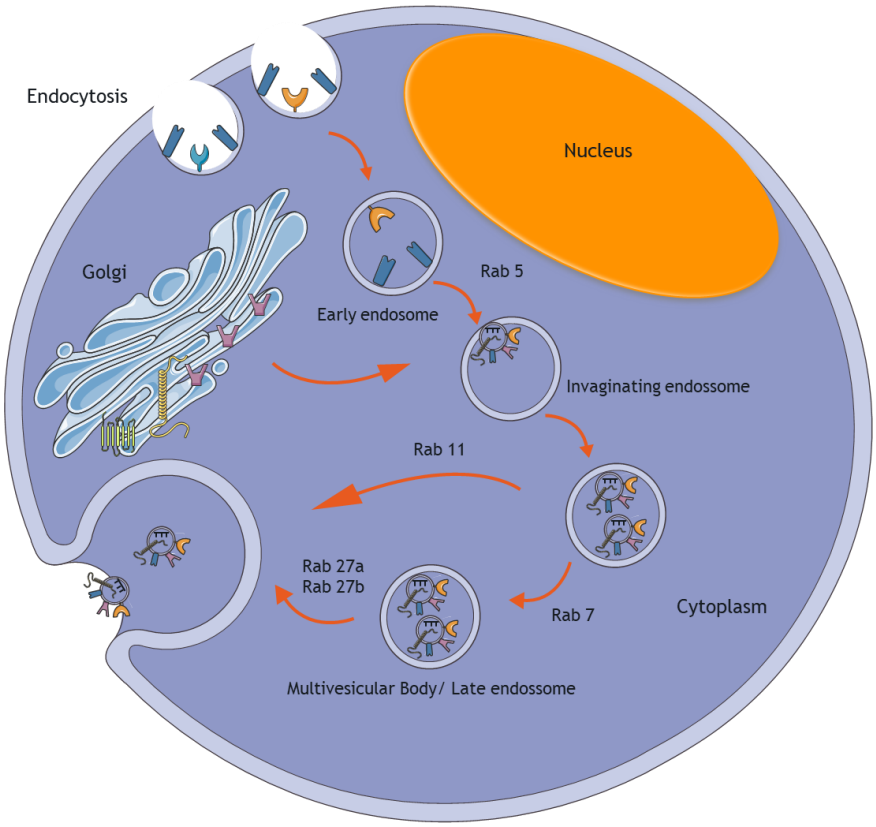


Figure 1. **Exosomes biogenesis.** After invagination, the endocytic vesicles fuse with the early endosome. During maturation steps, early endosomes differentiate into multivesicular bodies/late endosomes. When the multivesicular body fuse with the plasma membrane, the exosomes containing functional proteins, RNA and DNA are released to the extracellular space.

Over the past years, exosomes have been isolated from almost all body fluids, including urine (Pisitkun, Shen, and Knepper 2004), saliva (Ogawa et al. 2011), cerebrospinal fluid (Vella et al. 2007), breast milk (Admyre et al. 2014), ascites (Andre et al. 2002), blood (Caby et al. 2005) and semen (Madison, Jones, and Okeoma 2015). Despite carrying microRNAs, exosomes have the capacity to process precursor microRNAs (pre-microRNAs) to mature microRNAs, allowing to rapidly reprogram the transcriptome of recipient cells by cancer cells (Melo et al. 2014). Ultimately, once released into the extracellular space exosomes are able to interact with neighbour cells or enter in circulation to reprogram distant cells.

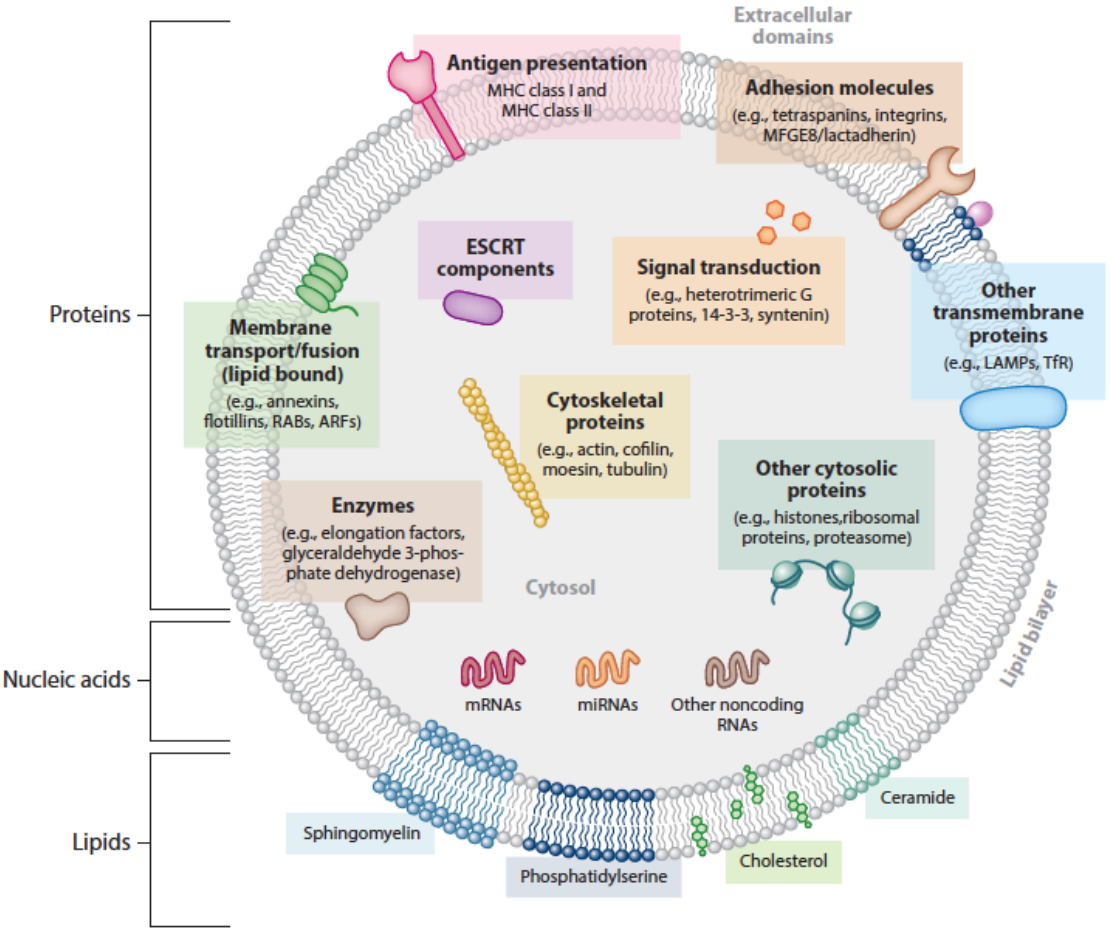


Figure 2. Overall composition of exosomes. Exosomes have a lipid bilayer membrane and are enriched in a subset of proteins from the endocytic system, plasma membrane and cytosol as well as cell-specific proteins. Exosomes also carry mRNAs, microRNAs, DNA and other noncoding RNAs. Taken from Colombo et al. (2014).

1.2. Cancer exosomes and the tumor microenvironment

Originally described as a mechanism for cellular release of waste, exosomes are now perceived as important mediators of intercellular communication (Bastos et al. 2018). In cancer, intercellular communication is an essential event for tumor formation, progression and metastasis. Cancer exosomes behave as reprogramming messengers, enabling the communication between cancer cells with local or distant cells/tissues/organs, transporting cancer-related DNA, microRNAs, mRNAs and proteins. It has been demonstrated that cancer exosomes are essential in the different steps of cancer progression. From tumor microenvironment remodelling, angiogenesis, invasion, intravasation and extravasation, to colonization at distant organs and immunosurveillance escape, cancer exosomes seem to be functionally relevant in tumorigenesis (Silva and Melo 2017). Moreover, exosomes are present in various body fluids and may serve as liquid biopsy for cancer diagnosis (Corcoran et al. 2011; Skog et al. 2008; Nilsson et al. 2009). In fact, the concentration of exosomes is elevated in the serum of cancer patients, mostly because the exosomes are from the tumor, enhancing its potential utility to detect and monitor the disease (Melo et al. 2015; Taylor and Gercel-Taylor 2008).

Tumor-derived exosomes can transfer oncogenic proteins to their surrounding cells and alter the microenvironment. Indeed, exosomes from prostate cancer cells containing tumor growth factor β1 (TGF-β1) protein where shown to trigger the differentiation of fibroblasts to activated-fibroblasts or myofibroblasts (J. Webber et al. 2010). Activated fibroblasts secrete tumor growth factors and chemokines which contribute for tumor progression. Breast cancer exosomes can induce the alteration of adipose tissue-derived mesenchymal stem cells into myofibroblasts via a SMAD2 activation (Cho et al. 2012). SMAD pathway leads to the production of vascular endothelial growth factor (VEGF) and TGF-β supporting tumorigenesis. The tumor growth depends on the access to nutrients and oxygen, enabled by a highly vascularized microenvironment. Myofibroblasts can release angiogenic growth factors and remodel the extracellular matrix (ECM), supporting tumor angiogenesis (Vong and Kalluri 2011). Furthermore, cancer cells release exosomes containing epidermal growth factor receptor (EGFR) which interact with endothelial cells, activating the MAPK and Akt signalling pathways (Al-Nedawi et al. 2009). The transfer of EGFR is accompanied with VEGF expression in endothelial cells which support tumor angiogenesis. More recently, microRNA-9 exosomes from melanoma cells were shown to activate JAK-STAT cascade, promoting endothelial cell migration and tumor angiogenesis (Zhuang et al. 2012). The role of exosomes in cell-to-cell communication in the tumor microenvironment has been highly

studied. Cancer cells produce exosomes with different molecular content prepared to interact with the local tumor environment and with distant organs targeted as future metastasis site.

1.3. Exosomes throughout the metastatic cascade

Metastasis is the leading cause of cancer-related death. The metastatic cascade is a multistep process where cancer cells first set free from the primary tumor, enter the bloodstream, survive and travel in circulation until they extravasate to the distant organ. In the host-organ, cancer cells can die, remain dormant or proliferate to form metastasis (**Figure 3**). Tumor-derived exosomes have been extensively described as key players in the metastatic process.

1.3.1. Invasion

One of the first steps in metastasis is tumor invasion. Cancer cells must overcome the ECM barrier to reach a vessel and intravasate. Tumor exosomes mediate the ECM remodelling through invadopodia formation (D. Hoshino et al. 2013). Invadopodia are subcellular structures mainly composed of actin which degrade ECM. Being rich in proteases, including matrix metalloproteinases (MMP) and cathepsins, tumor exosomes modulate the ECM by degradation of collagen, laminin and fibronectin, increasing cell proliferation and migration (Mu, Rana, and Zöller 2013). Moreover, cancer-associated fibroblasts (CAFs) and immune cells, also contribute for ECM remodelling and invasion (Orimo et al. 2005). CAFs influence the epithelial-mesenchymal-transition (EMT) of cancer cells. Under EMT, epithelial cells undergo various biochemical changes to acquire mesenchymal characteristics, such as the expression of vimentin, α-smooth muscle actin (α-SMA), N-cadherin and the activation of the Snail, Zeb and Twist pathways (Lee et al. 2006; Micalizzi, Farabaugh, and Ford 2010). The cellular phenotypic alteration enhanced cell motility, invasiveness and resistance to apoptosis (Kalluri and Neilson 2003). In fact, CAFs from breast cancer cells secrete exosomes which stimulate the activation of the Wnt pathway, increasing the migration capacity of breast cancer cells (Luga and Wrana 2013; Luga et al. 2012).

Recent studies suggest a main role for tumor exosomes in the EMT pathway. Urothelial cells treated with exosomes isolated from invasive bladder cancer cells presented an increased expression of mesenchymal markers (e.g. Vimentin, Snail and

Twist) while the expression of epithelial markers decreased (e.g. E-cadherin and β-catenin) (Franzen et al. 2015). Similarly observations were made with exosomes from lung cancer serum, which induced an EMT effect in human epithelial cells, enhancing cancer cell migration, invasion and proliferation (Rahman et al. 2016).

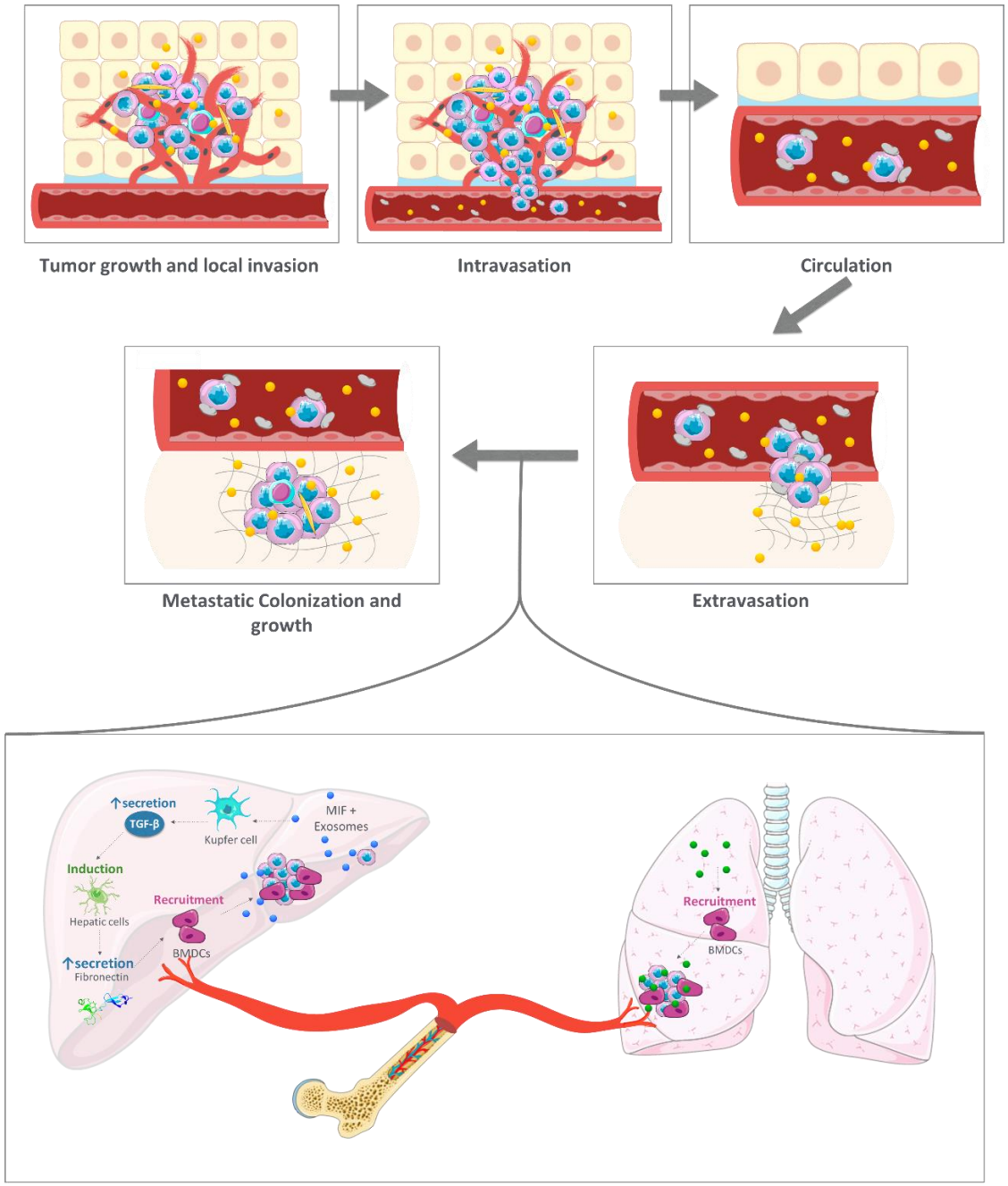


Figure 3. Tumor exosomes mediate the metastatic process. Exosomes can play several roles in the formation of a metastatic niche at distant sites of future metastasis. Exosomes can transport different cargo that promotes tumor growth, invasion, intravasation into blood vessels, survival in circulation, extravasation and modulation of the metastatic site. Exosomes reprogram the host organ making it more prone to receive the metastatic cells.

Besides being able to modulate cancer cells phenotype, tumor exosomes influence stromal fibroblasts. Tumor exosomes carrying TGF-β at the surface triggered the expression of α-SMA in fibroblasts, leading to the differentiation into CAFs (J. Webber et al. 2010; Borges et al. 2013). Interestingly, soluble TGF-β1 alone was not able to drive fibroblasts into a cancer-associated phenotype and the elimination of cancer exosomes even in the presence of TGF-β1 abolished differentiation. Therefore, these results confirm that the differentiation of stromal fibroblasts is mediated through TGF-β1 positive exosomes (J. P. Webber et al. 2015).

1.3.2. Angiogenesis

Cancer cells require oxygen and nutrients to survive. Angiogenesis is the process by which the formation of new blood vessels establishes a constant supply of oxygen and nutrients necessary during tumor progression. Accumulating evidence shows the involvement of tumor exosomes in the tumor microenvironment vascularity, especially in hypoxic conditions. For example, the uptake of pancreatic cancer (PC) exosomes expressing Tetraspanin 8 (Tspan8) induced the regulation of several angiogenesis-related genes in endothelial cells (Nazarenko et al. 2010). The cargo of exosomes can activate VEGFs which can cause endothelial cell proliferation, migration and maturation (Nazarenko et al. 2010). In melanoma cells, WNT5A (Wnt family member 5A) signalling induces the secretion of exosomes containing immunomodulatory and pro-angiogenic proteins including IL-6, VEGF and MMP2 through a CA^{2+}-dependent pathway (Ekström et al. 2014). In many cancers, hypoxia itself also plays a major role in tumor vascularization and metastasis (Finger and Giaccia 2010). For instance, exosomes released by a leukemia cell line under hypoxic conditions enhanced tube formation in endothelial cells (Tadokoro et al. 2013). Glioblastoma multiforme exosomes mediated hypoxia-dependent intercellular signalling. Exosomes derived from glioblastoma cells grown in hypoxic conditions can modulate the endothelial cells to a pro-angiogenic and migratory phenotype (Kucharzewska et al. 2013).

1.3.3. Intravasation and Extravasation

Towards a metastatic phenotype, cancer cells acquire additional characteristics which allow their proliferation from the primary tumor, intravasation to the bloodstream, circulation and extravasation at the metastatic site. Exosomes from breast cancer containing microRNA-105 decreased ZO-1 expression in endothelial cells therefore,

disrupting endothelial tight junctions and amplifying vascular permeability (Zhou et al. 2014). Tumor exosomes from metastatic hepatocellular carcinoma contain tumorigenic mRNAs and proteins, such as MET protooncogene. Exosomes carrying MET can mobilize normal hepatocytes, which may facilitate cancer cell motility through liver parenchyma during metastasis (M. He et al. 2015). Recently, cell-intrinsic change of *Met* expression was associated with the metastatic phenotypes (Adachi et al. 2016). In melanoma, low expression of *Met* was associated with angiogenesis, rapid tumor growth and chemotherapeutic resistance while with high expression of *Met*, cancer cells had a differentiated phenotype and were resistant to B-RAF inhibitors, increasing metastasis in the lungs. This suggests that Met exosomes can promote endothelial cells transformation and movement through the vascular structures which facilitate the metastatic process.

1.3.4. Organ colonization

Once cancer cells leave the primary tumor their utmost aim is to survive. For that, they should be able to establish at a secondary organ site. To prepare the metastatic sites, the primary tumor secretes signals such as exosomes. These secreted signals travel to specific organs where they induce changes to prepare the environment to receive the cancer cells. Recruitment of different cells to the metastatic niche, such as endothelial progenitor cells, fibroblasts, macrophages and bone marrow-derived cells (BMDC) is promoted by tumor exosomes (Costa-Silva et al. 2015; Peinado et al. 2012). Melanoma exosomes prepare sentinel lymph nodes for tumor metastasis (Hood, San Roman, and Wickline 2011). Homing of melanoma exosomes promotes melanoma cells recruitment, ECM deposition and increase vascular proliferation factors in the lymph nodes (Hood, San Roman, and Wickline 2011). Moreover, melanoma exosomes educate and mobilize BMDCs towards a pro-vasculogenic phenotype that induces vascular leakiness at the metastatic site (Peinado et al. 2012). Another study showed that pancreatic ductal adenocarcinoma (PDAC) exosomes initiate the formation of metastasis in the liver (Costa-Silva et al. 2015). In prostate cancer, exosomes secreted by cancer cells decreased the expression of markers for osteoclast fusion and differentiation but promoted osteoblast activity and regulation of bone metastases microenvironment (Karlsson et al. 2016; Ye et al. 2017). Tumor exosomes can also reprogram glucose metabolism at the metastatic site. Breast cancer exosomes carrying microRNA-122 suppress glucose uptake by non-cancer cells increasing nutrient availability to the recipient cell at the metastatic site (Fong et al. 2015). Interestingly, exosomes from

stromal cells can regulate metastasis survival, dormancy or growth. Exosomes released by brain astrocytes containing microRNA-19 silence the PTEN expression in metastatic cells. PTEN downregulation increased secretion of the chemokine CCL2 which recruits myeloid cells that promote the growth of brain metastasis (Zhang et al. 2015). These evidences demonstrate the remarkable plasticity of the tumor microenvironment, where exosomes from non-neoplastic cells may affect the cancer cells (Zhang et al. 2015).

The distribution of cancer cells to specific organs is not a random process, and it is known as metastatic organotropism. The role of tumor exosomes in metastatic organotropism has been explored. Tumor exosomes express integrins that associated with molecules, such as laminin and fibronectin, could predict the metastatic site (A. Hoshino et al. 2015). Exosomes' integrins α6β1 and α6β4 were correlated with lung metastasis, while integrins αvβ5 and αvβ3 were associated with liver metastasis (A. Hoshino et al. 2015). Once the secondary organ is colonized by the metastatic cells, they frequently present epithelial characteristics, suggesting a mesenchymal to epithelial transition. Metastatic cells return to a less migratory phenotype but higher proliferation capacity, enabling metastasis development (Wells, Yates, and Shepard 2008). In prostate cancer, patient adipose-derived stem cells developed cytogenic aberrations and mesenchymal to epithelial transition, expressing epithelial, neoplastic and vasculogenic markers (Elmageed et al. 2014). The trafficking of prostate cancer exosomes was associated with these phenotypic alterations (Elmageed et al. 2014). After the metastasis is well established, neoangiogenesis is necessary to provide the nutrients and oxygen required to avoid cancer necrosis and allow metastasis growth (Vong and Kalluri 2011). Breast cancer-associated fibroblasts stimulate neoangiogenesis through secretion of exosomal stromal cell derived factor-1 (SDF-1) (Cho et al. 2012). Stromal myofibroblasts are sources of angiogenic growth factors, ECM remodelling enzymes, inflammatory cytokines and chemokines which recruit infiltrating immune cells. In addition, stromal myofibroblasts recruit endothelial cells to migrate and form new blood vessels (Vong and Kalluri 2011). Neoangiogenesis results not only from myofibroblasts exosomes stimulating factors but also from microRNA transport directly to the surrounding stroma, influencing direct gene expression (Skog et al. 2008).

1.3.5. Exosomes in tumor immunity

Exosomes and their role in the immune system was first described in the late 1990s, when a study showed that exosomes from human and murine B lymphocytes contained antigen-specific MHC class I and II (G Raposo et al. 1996). For instance,

treatment of dendritic cells derived from the bone marrow with tumor exosomes triggered T-cell mediated antitumor immune response (Wolfers et al. 2001). Since then, the involvement of exosomes in immune regulation from tumor proliferation to metastasis establishment, has been extensively studied.

To effectively reach in the host organ, cancer cells need to escape immunosurveillance and survive in the blood circulation. In this regard, exosomes released by platelets seem to be key players in this event. Platelet-derived exosomes express P-selectin and glycoprotein IIb-IIIa, which interact with endothelial cells, cancer cells and leucocytes (Dean et al. 2009; Dovizio et al. 2015). In the blood stream, platelets escort cancer cells and assist cell adhesion at the vessel endothelium, via P-selectin, allowing extravasation into the metastatic organ (Erpenbeck and Schön 2010; Gay and Felding-Habermann 2011). Additionally, platelet interaction with metastatic cells, protect them from natural killer (NK) cells enabling the survival in the blood circulation (Borsig et al. 2002). After extravasation to the metastatic site, a process not yet elucidated, tumor exosomes activate immunosuppressive pathways to enable metastatic cells proliferation. Exosomes secreted from acute myeloid leukemia cells were capable of inducing apoptosis of activated T cells. These cancer exosomes inhibited signalling and proliferation of $CD8^+$ but not $CD4^+$ T cells, inducing antitumor $CD8^+$ effector T cells apoptosis (Wieckowski et al. 2009). In fact, exosomes from a human prostate cancer cell line decrease T-cell proliferation in a dose-dependent manner through Fas/FasL-mediated apoptosis (Abusamra et al. 2005). Also, cancer exosomes promoted $CD4^+$ T cells differentiation into regulatory T-cells (Treg) which express interleukin-10 (IL-10), TGF- β1, cytotoxic T-Lymphocyte antigen 4 (CTLA-4) and granzyme B (GrB)/perforin. Altogether, decreased cytotoxic activity of NK cells and, consequently improved cancer cells survival (Whiteside 2013). In addition to these immune cells, macrophages are also important in tumor progression and metastasis. Macrophages are activated by cancer cells, releasing growth factors, cytokines, proteolytic enzymes and pro-inflammatory mediators. Cancer macrophages can modify the ECM, stimulate angiogenesis, promote cancer cell invasion and suppress the immune system (Noy and Pollard 2014; Pollard 2004). The presence of high infiltration of macrophages has been correlated with metastasis and poor prognosis in several human cancers (Qian and Pollard 2010; Ojalvo et al. 2009). Moreover, breast cancer exosomes stimulated NF-kB (nuclear factor-kB) activation in macrophages resulting in pro-inflammatory cytokines release. *In vivo* experiments showed that Toll-like receptor-2 (TLR-2), a critical signalling adaptor in the NF-kB pathway, is responsible for the interaction of exosomes with macrophages (Chow et al. 2014). Also, cancer-associated macrophages were described to transfer

apolipoprotein E (ApoE) to gastric cancer cells through exosomes. M2 macrophages express high levels of ApoE and the release of exosomal ApoE activates the PI3K/Akt signalling pathway in the recipient cancer cell, which facilitates cytoskeleton remodelling and increase gastric cancer cells migration (Zheng et al. 2018). Overall, exosomes from cancer macrophages contribute to a pro-inflammatory response and support cancer survival and aggressiveness.

1.4. In vivo tools to study exosomes

Despite the increasing interest in research of cancer exosomes, the tools and methodologies available are based in the administration of labeled exosomes purified from cell lines into animal's circulation, or the injection of genetically engineered cells that produce labeled exosomes (Adem and Melo 2017). Recently, the Cre-loxP system together with a color based methodology was used (Zomer et al. 2016). In this model, EVs from donor cells are Cre-recombinase positive labelled with CFP and when taken up by non-recombined Cre-reporter cells, the DsRed-stop codon is removed, and the recombinant cells express eGFP (Zomer et al. 2016). Moreover, using this approach, it was shown that EVs released by malignant tumor cells are taken up by less malignant tumor cells at the same site or at a distance, which enhanced the migratory behavior and metastatic capacity of those cells (Zomer et al. 2015). This study showed for the first time evidences of *in vivo* cargo transfer through EVs *in situ* (Zomer et al. 2015). The use of a cancer cell line engineered with an exosomal marker fuse with a fluorescent protein has already been performed (Suetsugu et al. 2013). Nonetheless, results from *in vitro* experiments need to be carefully interpreted when trying to extrapolate to the *in vivo* context.

1.5. Pancreatic cancer

Pancreatic cancer is one of the most lethal cancers in the world with a 5-year survival around 8% (J. He et al. 2014). Pancreatic cancer is an extremely aggressive tumor and difficult to treat, and it is predicted to become the second leading cause of cancer death in the next decade (Rahib et al. 2014). The diagnosis is usually associated with the appearance of vague symptoms, which include fatigue, indigestion, abdominal and back pain, and jaundice that are often attributed to other factors (Siegel and Miller 2018). Therefore, diagnosis often occurs at late stages, when the disease is already metastatic and treatment indications are mostly palliative. Around 20% of the patients are diagnosed

with resectable disease, a point at which surgery can be a curative treatment, although many relapse within five years (J. He et al. 2014). Pancreatic cancer is characterized by genetic and epigenetic alterations and a complex and dense stroma. All these factors confer resistance to most standard treatments, including chemotherapy, radiotherapy and molecularly targeted therapy. At least 10% of pancreatic cancer patients have a family history of pancreatic cancer (Turati et al. 2013). Patients that smoke cigarettes (Bosetti et al. 2012), with advanced age, male sex or with diabetes mellitus (Bosetti et al. 2014) and chronic pancreatitis (Raimondi et al. 2010) have higher risk to develop pancreatic cancer. Despite all the efforts, the incidence and overall prognosis of pancreatic cancer remains extremely poor.

The most common subtype of PC is pancreatic ductal adenocarcinoma (PDAC). The majority of PC cases evolve through pre-neoplastic lesions, most frequently pancreatic intraepithelial neoplasia (PanIN), but can also arise from larger precursor lesions, such as intraductal papillary mucinous neoplasms or mucinous cystic neoplasms (Hezel et al. 2006; Tanaka 2014). The driving oncogene in PC is dominated by activating mutations in *KRAS*, present in >90% of the tumors while 50-80% of PC cases present inactivating mutations of *TP53*, *CDKN2A* and *SMAD4*, while other genes, such as *ARID1A*, *MLL3* and *TGFBR2* are mutated in 10% of the tumors (Graham et al. 2015). KRAS mutation activates multiple signalling pathways to affect cell proliferation, growth, survival and motility, among others (Suda, Tomizawa, and Mitsudomi 2010). Pancreatic tumors are characterized by an abundant and dense desmoplastic reaction (stroma) that result in a hypoxic and nutrient-deprived microenvironment. This stroma is composed by ECM proteins, pancreatic stellate cells, fibroblasts, infiltrating immune cells and endothelial cells (Seymour et al. 1994; Feig et al. 2012). Pancreatic stellate cells play a crucial role in disease progression and chemotherapy resistance, they can increase cancer cells survival by inhibiting cell apoptosis, thereby facilitating the formation of a cancer cell niche (Hamada et al. 2012). Moreover, stellate cells can travel from the primary tumor to distant sites where they promote the growth of metastatic cancer cells (Xu et al. 2010).

1.5.1. Exosomes in Pancreatic cancer

Exosomes have been acknowledged as crucial players in the intercellular communication between pancreatic cancer cells, its microenvironment and the metastatic site. Furthermore, exosomes carrying specific cargo were identified as potential biomarkers for disease prognostic. A study using pancreatic cancer cell lines and serum from PDAC patients detected exosomes containing genomic DNA with *KRAS*

and *TP53* mutations (Kahlert et al. 2014). In fact, serum from PDAC patients contained genomic DNA spanning all chromosomes. These results suggest that PC exosomes from serum can be used to detect genomic DNA mutations for cancer prediction. Furthermore, a cell surface proteoglycan, glypican-1 (GPC1), was identified in the exosomes from serum of pancreatic cancer patients. $GPC1^+$ circulating exosomes distinguished healthy individuals from pancreatic cancer patients, and most importantly enabled disease detection before imaging techniques could detect any lesion in the pancreas (Melo et al. 2015). The levels of GPC1 were correlated with tumor burden and the survival of surgical patients. This study propose $GPC1^+$ circulating exosomes as a potential non-invasive diagnostic and screening tool to detect early stages of PC (Melo et al. 2015).

The primary tumors release a subset of molecules that prepare the metastatic site microenvironment to receive the metastatic cells. Tumor exosomes have been characterized as drivers of the metastatic process. A recent study demonstrated that PDAC exosomes initiate the formation of the metastatic niche in the liver and consequently increased liver metastatic burden (Costa-Silva et al. 2015). PDAC exosomes express macrophage migration factor (MIF) that is taken up by Kupffer cells in the liver, causing the secretion of TGF-β. In turn, TGF-β increases fibronectin production by hepatic stellate cells which bind to BMDCs and migrate to the liver. These sequential events establish the formation of a niche suitable for liver metastasis. Moreover, MIF was also found to be highly expressed in exosomes from PDAC patients with progressive disease suggesting that exosomal MIF may be a possible prognostic marker for PDAC liver metastasis (Costa-Silva et al. 2015).

1.5.2. In vivo study of PDAC: the KPC mouse model

Our understanding of pancreatic cancer pathogenesis has improved over the years. However, the overall survival of PC patients remains very modest (Rawla, Sunkara, and Gaduputi 2019). Model systems of pancreatic cancer have been developed, from the establishment of the traditional cell lines and xenograft models, to genetic engineered mouse models (GEMMs), organoid cultures and patient-derived xenografts (PDXs) (Boj et al. 2015; Westphalen and Olive 2012). Mouse models of pancreatic cancer that target somatically mutant alleles to the mouse pancreas have revealed that $Kras^{G12D}$ mutation in enough to initiate PanIN, which can progress to fully invasive and metastatic disease (Hingorani et al. 2003; Guerra et al. 2007). Unfortunately, mice expressing $Kras^{G12D}$ developed an aggressive, locally invasive and undifferentiated disease, occasionally with micrometastases (Aguirre et al. 2003).

Thereby, additional mutations may be required to develop a fully penetrant disease. Hingorani et al., developed a mouse model with the *Kras*^{G12D} and the *Trp53*$^{R172H/+}$ mutation that are targeted to the mouse pancreas using the Cre-Lox technology. The *Kras*^{G12D} mutation drives neoplastic transformation and the *Trp53*$^{R172H/+}$, an ortholog of one of the most common *TP53* mutations in human PDAC, leads to tumor progression. The KPC (*LSL-Kras*$^{G12D/+}$*; Trp53*$^{R172H/+}$*; Pdx1*$^{Cre/+}$) model is a GEMM that develop spontaneous PDAC with full penetrance that recapitulates the clinical and histological features of the human disease (Hingorani et al. 2005). These animals express Cre recombinase under the control of the mouse pancreatic-duodenal homeobox promoter (Pdx). The activation of the *Kras*$^{G12D/+}$ and *Trp53*$^{R172H/+}$ alleles occurs in the progenitor cells of the mouse pancreas in development, due to the Pdx promoter that is only activated at embryonic stages. The KPC mice have significant advantages over other models of PDAC. The pancreas of newly born KPC mice is histologically normal, only by 8 to 10 weeks of age, the mice develop precursor lesions such as PanINs, in the pancreas. Around 16 weeks of age, most KPC mice have developed invasive PDAC with a dense desmoplastic reaction. At this time, the KPC model can recapitulate many clinical features such as ascites development, cachexia, bowel and biliary obstruction, jaundice and weight loss. In addition, PDAC tumors can metastasize to multiple sites including liver, lung, lymph nodes and peritoneum. The tumors from KPC mice present differentiated ductal morphology with extensive stromal desmoplasia, similar to what is observed in the human cancer. The tumors in mice express many of the immunohistochemical markers observed in the human disease, harbour complex genomic rearrangements indicative of genomic instability and are mostly resistant to chemotherapy (Westphalen and Olive 2012; Hingorani et al. 2005). All these characteristics seen in the KPC model reflect tumor progression in human PDAC making this a good model to investigate PDAC metastasis.

1.6. The ExoBow mouse model

The current state of the art relative to the available techniques to study exosomes biological significance *in vivo* do not reflect the correct location, concentration and nature of exosomes released by tumor cells. Therefore, it is of great interest the development of a mouse model that enables exosomes study closer to the normal biological system. Dr. Melo´s team has developed a unique GEMM which allows the study of exosomes distribution *in vivo*, in both pathological and non-pathological conditions, the ExoBow (**Figure 4**). The ExoBow is a multireporter mouse that produces color-coded pancreas-

derived exosomes. The ExoBow transgene contains the tetraspanin CD63 open reading frame (ORF), an exosomal marker, cloned into intron 1 of ROSA26 locus under the action of the CAG promoter. A neomycin resistance cassette with a stop-codon flanked by FRT sites is upstream of the CD63 ORF. The CD63 tetraspanin is followed by four different fluorescent reporter proteins, mCherry, phiYFP, eGFP and mTFP flanked with distinct and incompatible lox recombination sites (LoxN, Lox2272 and Lox5171). Through the action of the recombinase flippase (Flp) the stop cassette is deleted allowing the expression of CD63-mCherry fusion protein (Cai et al. 2013; Livet et al. 2007). Thus, the expressing cell will be CD63-mCherry positive and the respective exosomes will have the same label of the cell of origin. Cre recombinase can mediate the deletion of one of the three pairs of the mutually exclusive lox sites, allowing the expression of one of the three fusion proteins CD63-phiYFP, -eGFP or -mTFP. Hence, the resulting cell will have one of the three colors and produce exosomes labeled with the same color. The ExoBow (*R26*$^{CD63\text{-}XFP/+}$; *Pdx-1*$^{Flp/+;Cre/+}$) can be tissue specific and also inducible.

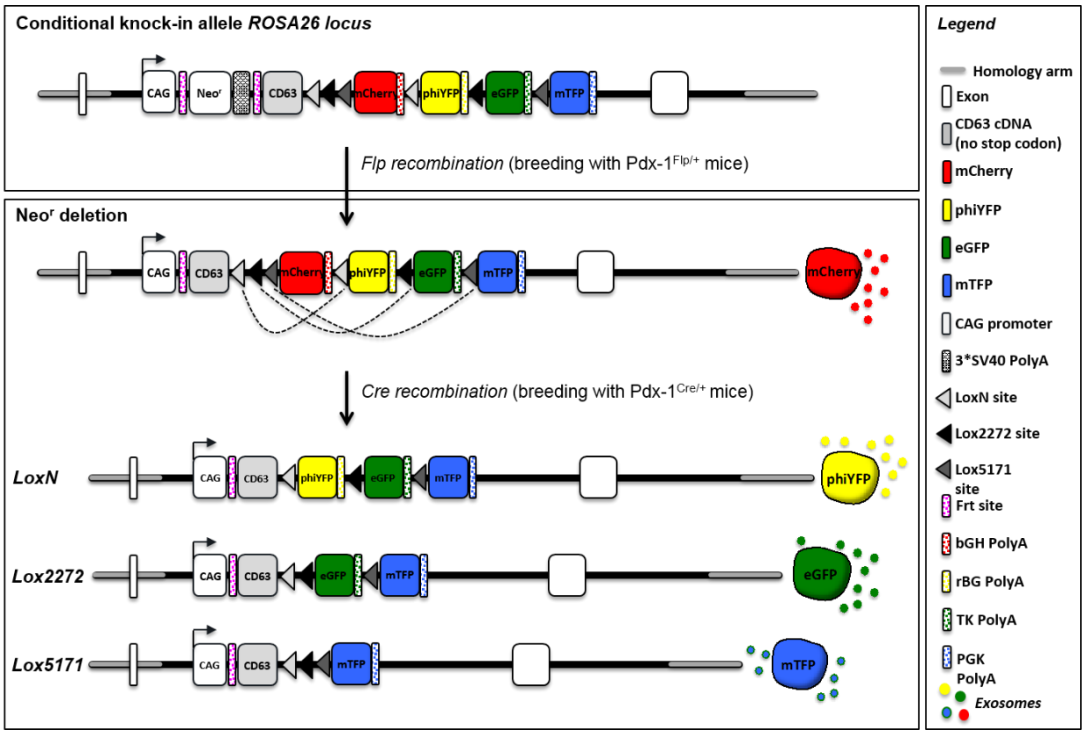


Figure 4. ExoBow knock-in allele at ROSA26 locus. The ExoBow construct is under the action of a stronger promoter, CAG. Upstream of the CD63 gene is a neomycin resistance cassette with a stop-codon flanked by FRT sites. Each fluorescent protein its between different Lox recombination sites (LoxN, Lox2272 and Lox5171). In the presence of Flp recombinase the FRT sites are removed allowing the expression of CD63-mCherry. Cre recombinase mediate one of the three deletions to allow CD63-phiYFP, CD63-eGFP or CD63-mTFP expression. The expressing cell will be fluorescently label and produce exosomes with the same color.

1.6.1. KPC-ExoBow mouse model

In order to identify the biodistribution and network of communication of PDAC metastasis exosomes the ExoBow model was crossed with the KPC model to generate the KPC-ExoBow (*R26*$^{CD63\text{-}XFP/+}$*;LSL-Kras*$^{G12D/+}$*;Trp53*$^{R172H/+}$*;Pdx-1*$^{Flp/Cre}$) mice, in which cancer cells secrete color-coded exosomes. Cre and Flp recombinases are under the control of the Pdx-1 promoter which is characteristic of the KPC model. Therefore, in the KPC-ExoBow mice in which both Flp and Cre recombination occur, the pancreatic cancer cells will produce CD63-eGFP, -phiYFP or -mTFP positive exosomes. This model will allow the identification of cancer cell communication by unravelling the routes established by cancer exosomes during tumor progression and metastasis.

1.7. Hypothesis

The role of cancer exosomes during the progression of the disease is well-documented. Nevertheless, with respect to metastasis most studies are focused on the role of tumor exosomes in the metastatic niche formation and metastasis establishment, while the role of exosomes from the metastatic site remains mostly unexplored. We hypothesize that metastasis-derived exosomes may be important in the survival and growth of the metastasis. To test our hypothesis, we will identify the communication established *in vivo* by exosomes derived from pancreatic cancer metastasis, the ExoMet, at both local and distant sites.

Our work has the possibility to unravel new and important fragilities in the establishment of the metastasis and its growth, opening new perspectives for the treatment of the deadliest process in cancer, the metastasis.

2. Material and Methods

2. Materials and Methods

2.1. Cell Culture

In this study three different PDAC cell lines were used, BxPC-3, Mia PaCa-2 and KPC. BxPC-3 and Mia PaCa-2 cell lines were purchased from ATCC (American Type Culture Collection) and have been authenticated by STR Profiling, as well as routinely mycoplasma tested. KPC cell line was purchased from Ximbio. BxPC-3 and Mia PaCa-2 cell lines were cultured in RPMI (Roswell Park Memorial Institute) 1640 (1x) medium (Gibco®) and KPC cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) (1x) medium (GE Healthcare Life Sciences). The medium was supplemented with 10% FBS (fetal bovine serum) (Gibco®) and 1% penicillin-streptomycin (Gibco®). The cells were maintained at 37 ºC in a humidified chamber with 5% CO_2.

2.2. Cell line transfection

BxPC-3 cells were transfected with the mouse (ms) vectors CD63-XFP: msCD63-mCherry, msCD63-phiYFP, msCD63-eGFP and msCD63-mTFP in the pRP[Exp]-Puro-CAG plasmid backbone. Each plasmid (2.5 µg DNA/1.5 x 10^5cells) was separately transfected using cells in suspension using Invitrogen Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions. 72h after transfection, cells were maintained in selection medium containing 10 mg/mL of puromycin (Sigma-Aldrich®). The transfected cells were also selected by cell sorting using the FACS ARIA sorter (Advanced Flow Cytometry Unit of i3S, Porto). Clones were considered stable when >90% cells were positive for the respective fluorescent marker for up to three cell passages with no selection.

2.2.1. Flow cytometry

For each clone, transfected cells expressing the respective fluorescent marker were selected via cell sorting performed as follows. When at 70-90% confluency, each transfected cell line was trypsinized, using 0.05% (w/v) Trypsin-EDTA (0.5%) (1×) (Gibco®), then washed 2 times with NaCl 0.9% (B.Braun®) and filtered through blue cap FACS tube. The parental cell line (no color) was used as control for the selection of positive cells (with color). The sorted cells were plated and maintained under selection

conditions until the end of the process of stable transfection. Data from flow cytometry acquisition was analysed using the FlowJo software (version 10, BD).

2.3. Protein extraction and quantification

Cells were washed with NaCl 0.9%, trypsinized and pellet down by centrifugation at 1200 rpm for 5 minutes. Using RIPA buffer (AMRESCO®) supplemented with phosphatase inhibitor phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich®) and protease inhibitor cOmplete™ (Roche), cells were lysed. The cells lysates were incubated on ice for 30 minutes, followed by a 30 minutes centrifugation at 17 000 g, 4 ºC. The supernatant was transferred to a new tube and the pellet discarded. The total protein concentration in the supernatant was measured using an adaptation of the Lowry's method (DC™ Protein Assay Reagent, BIO-RAD®) according to the manufacturer's instructions.

2.4. Exosomes isolation from cell culture medium

BxPC-3 msCD63-XFP cells were cultured in exosomes-free medium (RPMI medium supplemented with 10% FBS depleted of exosomes and 1% penicillin-streptomycin). After 72 hours the medium was collected and centrifuged at 2500g for 10 minutes followed by a 5 minutes centrifugation at 4000g. Subsequently, the medium was filtered through a 0.2 µm filter (GE Healthcare Whatman™) directly to an ultra-clear centrifuge tube (Beckman Coulter®). The samples were centrifuged overnight at 100 000g, 4 ºC using the Optima™ L-80 XP ultracentrifuge, Beckman Coulter. The supernatant was carefully removed, and the pellet kept for downstream analysis.

2.4.1. Sucrose gradient

Exosomes isolated from BxPC-3 msCD63-XFP cells by ultracentrifugation were subjected to a continuous sucrose gradient (0.25 – 2 M) (Théry et al. 2006). The pellet was first resuspended in 2 mL of HEPES/Sucrose stock solution (HEPES 20 mM/protease-free sucrose 2.5M, pH 7.4) and transferred to an ultra-clear centrifuge tube. Using a gradient maker, 2 M sucrose solution was loaded in the proximal cavity while 0.25 M sucrose solution was loaded in the distal compartment with a magnet stirrer. The ultra-clear centrifuge tube was placed under the outer tubing of the gradient maker.

The shutter between the proximal and distal compartments was opened followed by the opening of the outer shutter. A continuous sucrose solution (2 M to 0.25 M) was dispensed into the ultracentrifuge tube with the exosomes suspension. The tubes containing the exosomes sucrose gradient were centrifuged overnight (> 14 hours) at 210 000g, 4 ºC. After ultracentrifugation, 1 mL of gradient fractions, from top to bottom, were collected. 50 µL of each fraction was used to measure the refractive index in a refractometer. Each fraction was individually placed in an ultra-clear centrifuge tube, diluted in NaCl 0.9% and centrifuged at 100 000g for 2 hours, at 4 ºC. The resultant pellet was resuspended in 30 µL 2.5% SDS/8 M Urea and incubated for 30 minutes on ice, followed by a 30 minutes centrifugation at 17 000g, 4 ºC. The supernatant was stored at -20 ºC.

2.4.2. Exosomes quantification by Nanosight

The pellet of exosomes that resulted from the ultracentrifugation of the cell culture medium, was resuspended in 200 µL of NaCl 0.9%. Samples were diluted in NaCl 0.9% in a ratio 1:20 and their size and concentration were measured using the Nanoparticle Tracking Analysis (NTA) technology (NanoSight NS300 particles counter). Measurements are done in quadruplicate, three independent movies and analyses of constant flowing sample measured, and data on size is presented as mode and concentration as particles/mL upon calculation having into account the respective dilution factor.

2.5. Western Blot

For western blot analysis, 30 µg of each protein sample from cells was used. For analysis of the exosomes fraction obtained by sucrose gradient, the total volume of 30 µL of each sample was used. All samples were incubated with laemmli buffer without β-mercaptoethanol (ratio 4:1) for 10 minutes at 95 ºC. Proteins were separated by 7.5% (w/v) sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis at constant 120 V until the protein of interest was conveniently separated. The molecular weight estimation of the obtained bands was made using Precision Plus Protein™ Dual Color Standards (BIO-RAD®). After separation by electrophoresis, proteins were transferred onto nitrocellulose membranes 0.2 µm (GE Healthcare®) using a wet electrophoretic transfer for 90 minutes at 100 V. Ponceau S staining was used to confirm an effective protein transfer. Subsequently, the nitrocellulose membranes were blocked with 5% non-

fat dry milk in phosphate-buffered saline (PBS) 1x/0.1% Tween 20 (Sigma-Aldrich) for 1 hour at room temperature. After blocking, membranes were incubated overnight at 4 ºC on a shaker with the following primary antibodies: mouse anti-CD63 (dilution 1:500, BD Pharmingen™ 551458), anti-mCherry (dilution 1:500, Biorbyt orb116118), anti-eGFP (dilution 1:500, Abcam ab13970), anti-phiYFP (dilution 1:1000, Evrogen AB603) and anti-mTFP (dilution 1:500, kindly provided by Cai Laboratory, University of Michigan Medical School, Michigan, USA). After 4 washes with PBS 1X/0.1% Tween 20 for 10 minutes intervals on an orbital shaker, membranes were incubated 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies: anti-rat (dilution 1:2000, GenScript a00167), anti-goat (dilution 1:5000, Abcam ab6741), anti-chicken (dilution 1:5000, Sigma-Aldrich A906), anti-rabbit (dilution 1:5000, Cell Signalling 7074) and anti-rat (dilution 1:5000, GenScript a00167), respectively. Membranes were washed 4 times at 10 minutes intervals with PBS 1X/0.1% Tween 20 on an orbital shaker. In the end, all membranes were incubated with Clarity™ Western Enhanced chemiluminescence (ECL) Substrate (BIO-RAD), according to the manufacturer's recommendations, to detect the bands using GE Healthcare Amersham™ Hyperfilm™ ECL.

2.6. Immunofluorescence

A cover slip was placed into a 24-well plate in sterile conditions. A total of 40 000 BxPC-3 msCD63-XFP cells were plated separately in each well. The cells were maintained in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin in a 5% CO_2 humidified incubator at 37°C for 24 hours. Cells were washed with cold PBS 1x for 5 minutes and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 15 min at room temperature. After fixation, the cells were washed three times with cold PBS 1x for 5 minutes and then, incubated with a quenching solution of 0.1M glycine for 5 minutes at room temperature. The cells were permeabilized with a solution of Triton-X (VWR) 0.1% followed by a 45 minutes incubation at room temperature with 10% Albumine Bovine Fraction V (BSA) (NZYTech). After blocking, cells were incubated overnight at 4 ºC with the following primary antibodies: human anti-CD63 (Novus Biologicals® H5C6), anti-mCherry, anti-eGFP (Bio-Rad 81/4745-1051), anti-phiYFP and anti-mTFP, in a dilution of 1:500 in 2% BSA. Anti-mCherry, anti-phiYFP and anti-mTFP antibodies were developed and kindly provided by Cai Laboratory, University of Michigan Medical School, Michigan, USA. Next day, after washed, cells were incubated 45 minutes at room temperature with the respective secondary antibodies: anti-mouse

Alexa-Fluor® 594 (Abcam ab150108), anti-chicken Alexa-Fluor® 633 (Sigma, SAB4600127), anti-sheep Alexa-Fluor® 488 (Jackson Immunoresearch, 713-545-003), anti-rabbit Alexa-Fluor® 488 (Jackson Immunoresearch, 711-545-152) and anti-rat Alexa-Fluor® 488 (Invitrogen, A21208), at a 1:500 dilution in 2% BSA. Counterstain was achieved using Hoechst solution (dilution 1:10 000, Thermo Scientific) for 10 minutes. Between each incubation, the cells were washed 3 times at 5 minutes intervals with PBS 1x. The cover slips were mounted using a drop of VECTASHIELD mounting medium (VECTOR Laboratories®) and sealed with nail polish to prevent drying. Samples were stored protected from light at 4 ºC until observation at the spectral confocal microscope Leica TCS-SP5 AOBS (Bioimaging Center, i3S, Porto).

2.7. In vitro co-culture

To analyse the communication between BxPC-3 msCD63-XFP cells, co-cultures of two distinct msCD63-XFP stable clones were performed. A total of 100 000 BxPC-3 msCD63-XFP cells were mixed and plated in a 35 mm cell imaging dish with a glass bottom (Ibidi®) in a proportion of 1:1. When analysing the communication between BxPC-3 msCD63-XFP with BxPC-3 human (hu) CD63-XFP, a proportion of 4:1 was used. Each co-culture was incubated during 96 hours in a 5% CO_2 humidified incubator at 37°C. Afterwards, the cell cultures were observed in the spectral confocal microscope Leica TCS-SP5 AOBS (Bioimaging Center, i3S, Porto) and a post image processing was performed using NIH ImageJ software.

2.8. KPC-ExoBow mice

2.8.1. Monitoring

KPC-ExoBow mice were monitored. The animal behaviour, appearance, body weight, food and water consumption were assessed to follow the disease progression and to assure that the welfare of the animal was maintained. The presence of a mass in the pancreas was analysed also by palpation of the abdomen.

All procedures using mice models were approved by Direção-Geral de Alimentação e Veterinária (DGAV ref. 015225/2017-06-30) and the animal facility at i3S, Porto, and I have accredited authorization for mice handling (FELASA B).

2.8.2. Euthanasia

When the animal health and welfare were compromised, and the humane endpoints were reached the animals were euthanized. At time of euthanasia, animals were anaesthetised in 5% isoflurane and blood was collected retro-orbitally followed by cervical dislocation. The abdominal cavity was exposed, and necropsy was performed. The pancreas, liver and lung were collected. Liver and lung metastasis and ¼ of the pancreas were collected and maintained on ice for downstream processing for FACS analysis. The remaining organ collected was placed in formalin 20% v/v for tissue fixation.

2.9. Metastasis digestion and flow cytometry

The liver, lung and tumor samples collected were diced with sterile scalps into 3x3 mm pieces. The tissues were digested using 0.012% w/v dispase-collagenase XI buffer (Sigma-Aldrich), incubated at 37 ºC for 15 minutes with vortex agitation intervals of 5 minutes each. The digestion reaction was stopped with DMEM F12 Glutamax medium (Gibco®) and then centrifuged at 600g for 5 minutes. The digested tissues were smash and filtered through a 70 µm cell strainer (Falcon®) with the help of a syringe embolus followed by centrifugation. Afterwards, cells were incubated 3 minutes with Red Blood Lysis buffer (pH 7.2) and the reaction was stopped with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco®). Cells were spun down and washed twice with Hank's Balanced Salt Solution (HBSS) 1x. The final samples were resuspended in HBSS 1x and filtered through a 70 µm cell strainer to a FACS tube. The sample of each organ was then analysed and sorted using the FACS Aria sorter (Advanced Flow Cytometry Unit of i3S, Porto). Cell viability was assessed using the fixable viability dye eFluor™ 780 (ThermoFisher 65-0865-14). Cells from the liver and lung positive for the fluorescein isothiocyanate (FITC) channel were collected. Tumor, liver and lung from a KPC ExoBow negative for flippase mouse was used as control for the fluorescence. Liver metastatic cells were resuspended in 20 µL of Matrigel™ Basement Membrane Matrix (BD) while lung metastatic cells were resuspended in 100 µL of NaCl 0.9%. Cells were maintained on ice until orthotopic injection into mice.

2.10. Orthotopic injection of metastatic cells

2.10.1. Intrahepatic injection

Wild-type mice were anaesthetized by intraperitoneal administration of a ketamine/xylazine (125mg/kg/12.5mg/kg) solution followed by the subcutaneous administration of an analgesic, buprenorphine (0.08mg/kg). The mice fur was shaved in the ventral abdomen area and sterilized with betadine solution and 70% ethanol. Using a sterile scalpel, a transverse bilateral incision was made in the skin, a similar incision was performed in the muscle layers using scissors to enter the peritoneal cavity, exposing the liver. The liver metastatic cells were resuspended and 20 µL of cells suspension in Matrigel was loaded into a Hamilton syringe. The needle of the syringe was inserted into the liver and the cells were gently injected. The needle was carefully removed from the liver and gentle pressure with a cotton-swab was applied followed by the placement of a hemostatic gauze (Celox™ Rapid Hemostatic Gauze) in the injection site to stop bleeding. When the hemostasis was achieved, the liver was carefully placed inside the abdominal cavity and the incision was closed using 6-0 PGA sutures (Surgicryl PGA 6-0). After surgery, the anaesthetic effect was reversed through subcutaneous administration of atipamezole (2.5 mg/kg). During 3 days post-surgery, buprenorphine was administered to the animal every 12 hours.

2.10.2. Intravenous injection

The animals were placed under an infrared lamp used to stimulate the dilatation of the tail vein. After a few minutes the veins were vasodilated, and it was possible to identify them. The animals were placed in a restrainer device, the cells were resuspended and injected with a 0.5mL Insulin Syringe & Needle 30G (BD). The tail was disinfected with ethanol 70%, the needle was inserted in the vein and the 100 µL of lung metastatic cells were injected. After injection the needle was withdrawn, and a gauze was placed immediately at the site of injection with gentle pressure to stop the bleeding.

2.11. H&E staining

The paraffin-embedded organs were sectioned in 4 µm slices using a microtome Microm HM335E (HEMS, i3S, Porto) and transferred to KP frost slides (Klinipath). The slides were incubated at 37 ºC overnight and stained using the haematoxylin-eosin

staining as briefly described next. Sections were deparaffinized with xylene, followed by dehydration by submersion in solutions of decreasing alcohol concentrations (100%, 100%, 70%) and rinsed in running water. The sections were then stained with Modified Gill II Hematoxylin (Merck Millipore, Burlington, MA, EUA), washed in running water and dehydrated using increasing concentrations of alcohol (70%, 100%, 100%). The counterstain of the tissue was performed in alcoholic eosin solution (Thermo Scientific), quickly rinsed in 100% ethanol and then twice with xylene. The sections were mounted using DPX mounting medium (Sigma-Aldrich) with a glass coverslip (Normax). The samples were analysed using the Light microscope Olympus DP 25 Camera Software Cell B (Histology and Electron Microscopy facility, i3S, Porto).

2.12. Immunohistochemistry (IHC)

Sections 4 µm thick were performed using a paraffin microtome Microm HM335E (HEMS, i3S, Porto) and transferred to coated slides (Thermofisher). The slides were incubated at 37 ºC overnight. Sections were deparaffinized with xylene, followed by dehydration in solutions of decreasing alcohol concentrations (100%, 100%, 70%) and then rinsed with running water to hydrate. Sections were subjected to antigen retrieval by placing the slides in an antigen unmasking solution of sodium citrate buffer pH 6.0 (Sigma-Aldrich) at a ratio of 1:100, inside a water vaporizer machine at approximately 99 ºC for 40 minutes. The slides were removed from the vaporizer machine and allowed to cool down at room temperature for 20 minutes, followed by two washes of 5 minutes with PBS 1X/0.1% Tween 20. To permeabilize the tissue and inhibit endogenous peroxidases, the slides were placed in a humid chamber and incubated with a 3% hydrogen peroxide in methanol solution (H_2O_2 [Sigma-Aldrich]; CH_3OH [VWR]) for 15 minutes. The slides were washed twice in PBS1X/0.1% Tween 20 as previously mentioned. Tissue sections were delimited with a hydrophobic pen (VECTOR Laboratories®), placed in the humid chamber and incubated with Ultravision Protein-block solution (Thermofisher) for 1 hour at room temperature. Sections were incubated in a humidified chamber, at 4 ºC for 1 day and half with a mix of primary antibodies: anti-mCherry (dilution 1:500), anti-eGFP (dilution 1:300), anti-phiYFP (dilution 1:300) and anti-mTFP (dilution 1:500) diluted in PBS1X/0.1% Tween 20. All antibodies were antibodies were developed and kindly provided by Cai Laboratory, University of Michigan Medical School, Michigan, USA. After primary antibody incubation, slides were washed three times with PBS1X/0.1% Tween 20 for 10 minutes. In the humid chamber, a mix of secondary antibodies was added to the tissue sections: anti-chicken Alexa-Fluor® 633

(Sigma, SAB4600127), anti-sheep Alexa-Fluor® 488 (Jackson Immunoresearch, 713-545-003), anti-rabbit Alexa-Fluor® 546 (ThermoFisher Scientific, A10040) and anti-rat Alexa-Fluor® 594 (ThermoFisher Scientific, A21209), in a dilution 1:500. After overnight incubation, the slides were washed with PBS1X/0.1% Tween 20 as described before. The nucleus was counterstained with Hoechst solution (dilution 1:10 000, Thermo Scientific) for 15 minutes. The slides were mounted with VECTASHIELD mounting medium (VECTOR Laboratories®) and sealed with nail polish to prevent drying. Samples were stored protected from light at 4 ºC until observation at the spectral confocal microscope Leica TCS-SP5 AOBS (Bioimaging Center, i3S, Porto).

2.13. Orthotopic injection of Mia PaCa-2 huCD63-GFP cells

Mia PaCa-2 huCD63-GFP cells were grown under normal conditions (as described in 1.) up to 70-80% confluence upon which they were trypsinized. The number of cells and their viability was assessed using Trypan Blue Assay and a haemocytometer. A total of 2 x 10^6 cells for each animal were resuspended in NaCl 0.9% and maintained on ice until injection. For this procedure, three nude CBA immunodeficient mice were orthotopically injected in the pancreas. Before surgery, mice were weighted and anaesthetized by intraperitoneal administration of a ketamine/xylazine (125mg/kg/12.5mg/kg) solution followed by the subcutaneous administration of buprenorphine (0.08mg/kg). The left side of mice abdomen was sterilized with betadine solution and 70% ethanol. A small incision in the left abdominal flank was made and the pancreas tissue was exposed. The cell suspension on ice was resuspended with a Hamilton syringe and the needle was carefully inserted into the pancreas. The final volume of 12 µL containing 2 x 10^6 cells was slowly injected in the mouse pancreas. The pancreas was returned to the peritoneal cavity and the peritoneal and the skin layer were sequentially closed with 6-0 PGA sutures (Surgicryl PGA 6-0). After surgery, the anaesthetic effect was reversed through subcutaneous administration of atipamezole (2.5 mg/kg). During three days all mice were administered with analgesic every 12 hours. All mice were monitored once a week until tumor detection by palpation, after which all mice were monitored every other day. The tumor growth was accessed once a week through Micro Ultrasound Vevo 2100 (Animal Facility, i3S, Porto).

2.14. Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 6 Software (version 6.01). One Way A-NOVA non-parametric test was used for comparison of each clone with the parental cell line.

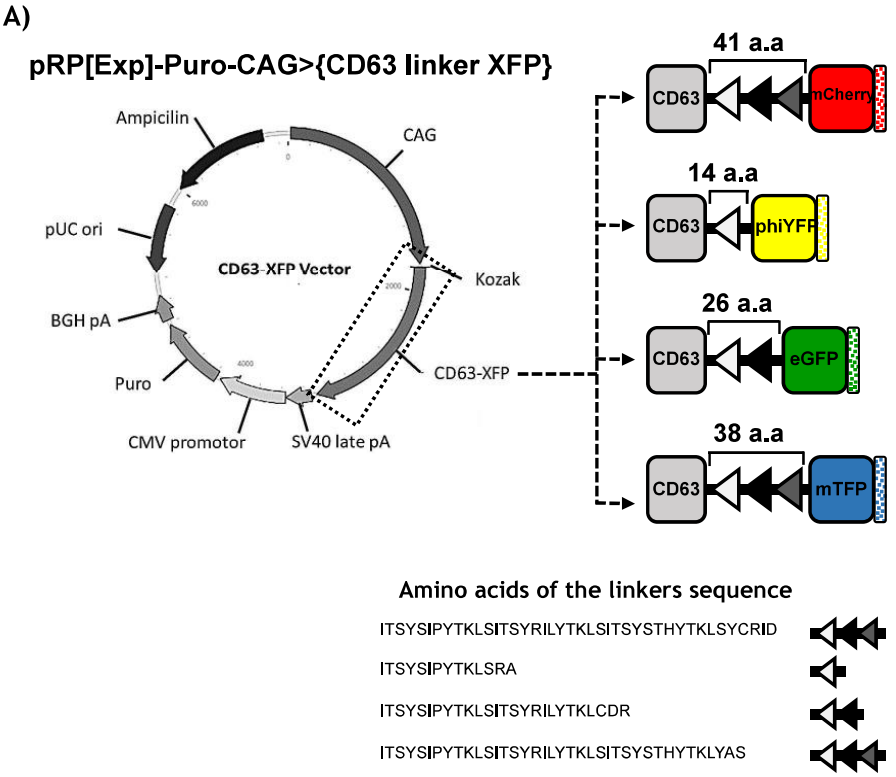
3. Results

3. Results

3.1. Establishment of stable clones of the BxPC-3 msCD63-XFP pancreatic cancer cell lines.

The ExoBow transgene is composed of the CD63 tetraspanin, a well described exosomal marker, followed by four different fluorescent reporters, mCherry, phiYFP, eGFP and mTFP. These reporters are flanked by different lox recombination sites that are excised when the Cre recombinase is present. The deletion of the lox sites in addition to the Flp-mediated recombination, allows the expression of one of the four fusion proteins CD63-mCherry, -phiYFP, -eGFP or -mTFP. Since each one of the fluorescent reporters is flanked by distinct lox sites, the resulting fusion protein will present a different linker between the CD63 and the reporter. To control for the expression and location of each fusion protein western blot and optical microscopy was used. With this purpose, each sequence of msCD63-XFP was cloned into the pRP[Exp]-Puro-CAG backbone vector and used to transfect the BxPC-3 cell line, a pancreatic cancer cell line (**Figure 5A**).

The BxPC-3 cells were separately transfected with the msCD63-mCherry, -phiYFP, -eGFP and -mTFP plasmids. Positive msCD63-XFP cells were selected using the antibiotic puromycin and through enrichment by flow cytometry sorting until stable clones were obtained. CD63-XFP expression of each clone was analysed by immunofluorescence and it was observed that the CD63-XFP fusion proteins localize in the endosomal compartment, with a speckle-like pattern inside the cell, as expected for endogenous CD63 protein expression (**Figure 5B**). This pattern of expression is identical to the one observed when the endogenous huCD63 of the parental cell line was analysed (**Figure 5B**).



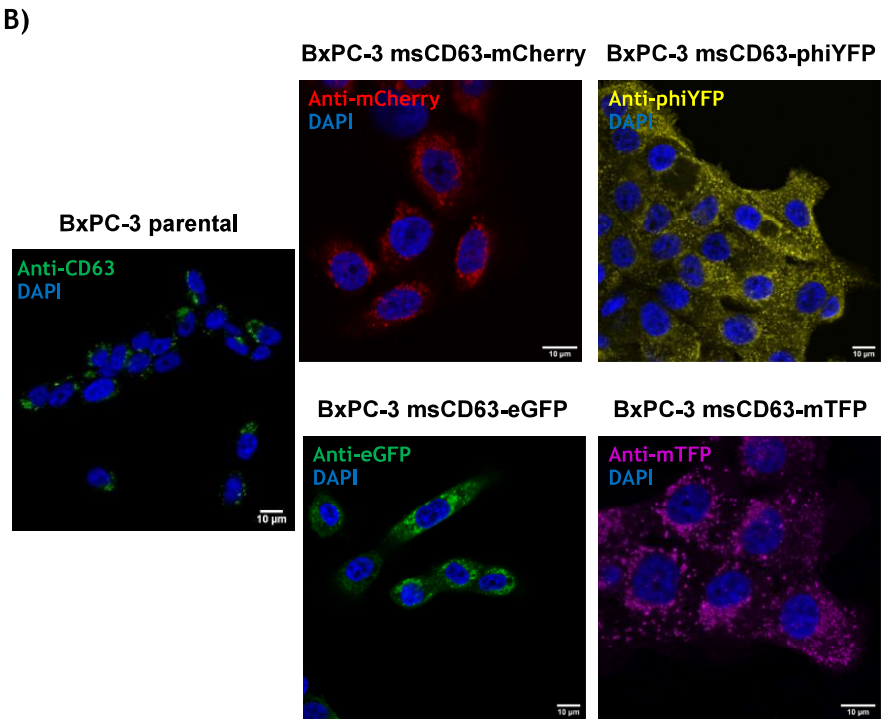


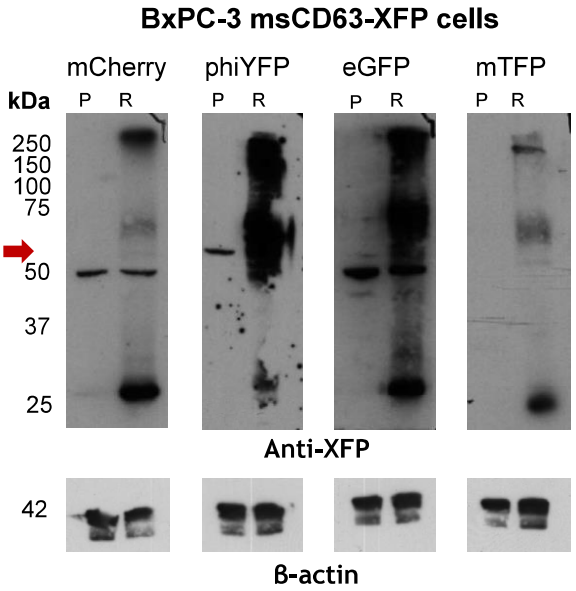
Figure 5. Establishment of CD63-XFP PDAC cell lines. (A) Each fluorescent protein (mCherry, phiYFP, eGFP and mTFP) is fused to the C-terminal of the mouse CD63. This plasmid confers resistance to the antibiotic puromycin. (B) Confocal microscopy images of BxPC-3 parental cells immunostained against anti-human CD63 (left), and fluorescent endogenous levels of each BxPC-3 msCD63-XFP clone: msCD63-mCherry (red), msCD63-phiYFP (yellow), CD63-eGFP (green) and msCD63-mTFP (magenta). Scale bar, 10µm.

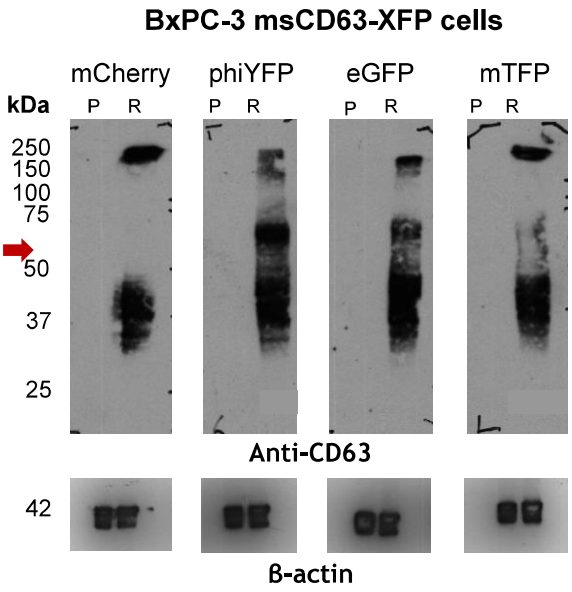
3.2. BxPC-3 msCD63-XFP cell lines secrete CD63-XFP positive exosomes.

In order to confirm and validate the expression of color-coded CD63 in our clones, western blots of each BxPC-3 msCD63-XFP cell line were performed (**Figure 6A**). The predicted molecular weight for each fusion protein is around 56 kDa, which may at times appear as a smear due to the glycosylated nature of the CD63 protein. The results were confirmed using anti-CD63 antibody, as well as against the fluorescent protein, anti-mCherry, -phiYFP, -eGFP or -mTFP, according to the respective clone. A specific band was detected at the expected molecular weight in all cell lines (**Figure 6A**).

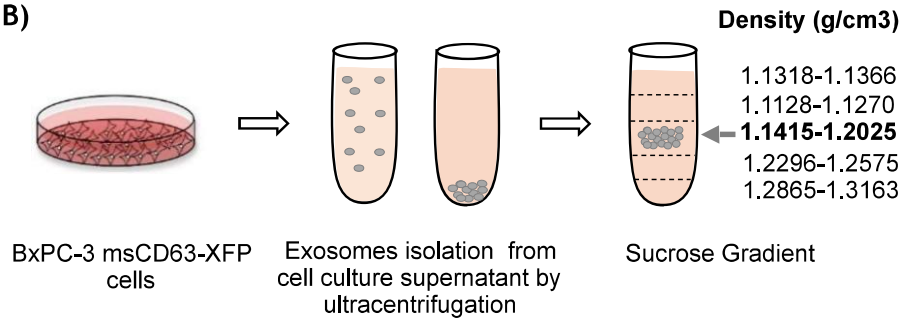
One aim of our work was to assess exosomes biodistribution, therefore the presence of CD63-XFP color-coded exosomes derived from BxPC-3 msCD63-XFP cell lines was analysed by western blot (**Figure 6B-C**). For this purpose, exosomes were isolated by ultracentrifugation followed by continuous sucrose gradient. This methodology allows the separation of exosomes from other vesicles to obtain a pure fraction of exosomes (**Figure 6B**). Exosomes float at a buoyant density between 1.1415 and 1.2025 g/cm^3. Western blot against fluorescent reporter proteins demonstrated that all BxPC-3 msCD63-XFP cell lines secreted color-coded exosomes (**Figure 6C**). It was observed that bands appear as smear, which represents the distinct glycosylated patterns of CD63 protein that have been previously mentioned (**Figure 6C**).

A)


BxPC-3 msCD63-XFP cells
mCherry
phiYFP
eGFP
mTFP
kDa
P R
250
150
100
75
50
37
25
Anti-XFP
42
ß-actin


BxPC-3 msCD63-XFP cells
mCherry
phiYFP
eGFP
mTFP
kDa
P R
250
150
100
75
50
37
25
Anti-CD63
42
ß-actin

B)


Density (g/cm3)
1.1318-1.1366
1.1128-1.1270
1.1415-1.2025
1.2296-1.2575
1.2865-1.3163
BxPC-3 msCD63-XFP cells
Exosomes isolation from cell culture supernatant by ultracentrifugation
Sucrose Gradient

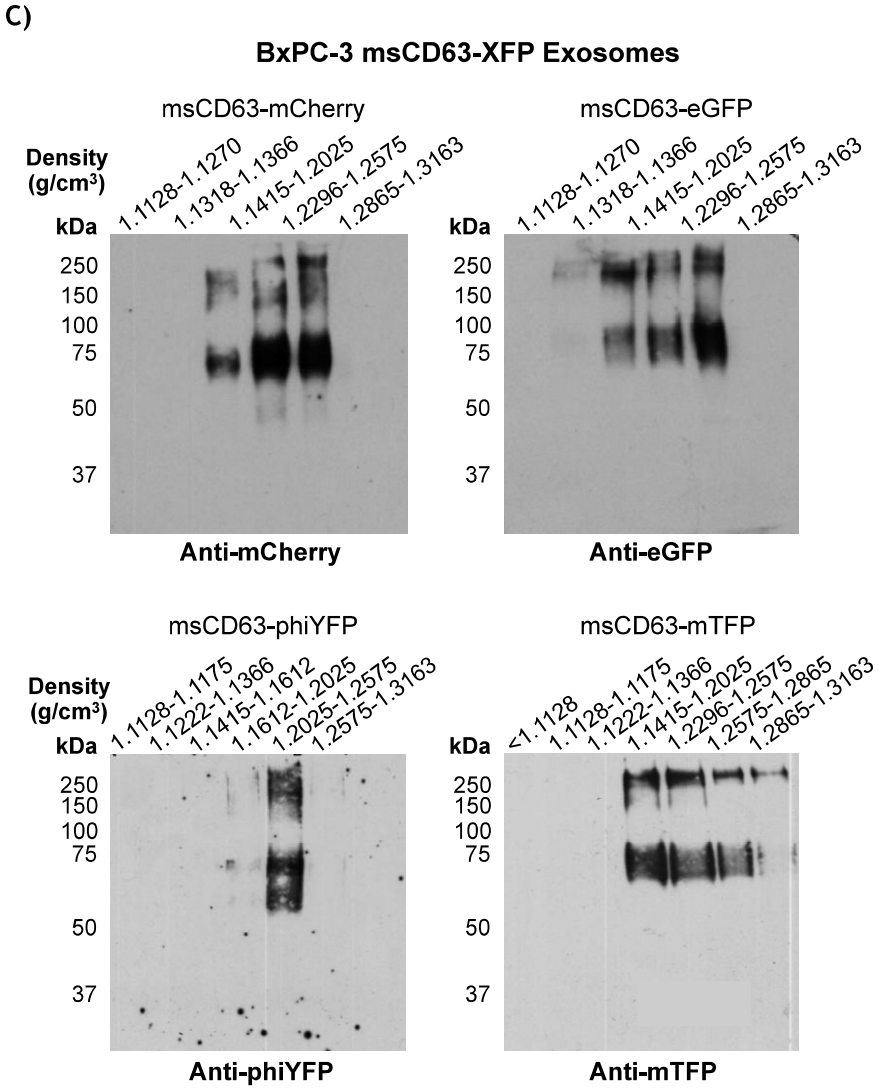


Figure 6. **BxPC-3 msCD63-XFP pancreatic cancer cell lines secrete CD63-XFP positive exosomes. (A)** Western blot of CD63-XFP expression in BxPC-3 parental cell line (P) and BxPC-3 msCD63-XFP cell lines (R). The predicted molecular weight for the CD63-XFP fusion protein is 56 kDa (red arrow). Anti-mCherry, anti-eGFP, anti-phiYFP and anti-mTFP were used to identify CD63-XFP protein expression. β-actin was used as loading control. **(B)** Schematic representation of exosomes isolation through continuous sucrose gradient ultracentrifugation. **(C)** Representative western blot of CD63-XFP expression in exosomes from BxPC-3 msCD63-XFP cell lines. Individual 1 mL fractions were collected and after ultracentrifugation were loaded on gels for electrophoresis. Exosomes are located in fractions between 1.1415 and 1.2025 g/cm3 density. Anti-mCherry, anti-eGFP, anti-phiYFP and anti-mTFP antibodies were used in the respective clone.

Moreover, it was evaluated if the overexpression of CD63 and the different lox sites between each fusion protein and CD63 would affect the size of secreted exosomes or even their amount, for each clone. Using NTA the number of exosomes was quantified from the culture medium of BxPC-3 msCD63-XFP cell lines (**Figure 7A**). It was observed that exosomes secretion in not altered by the msCD63-XFP fusion proteins overexpression and the distinct linkers between each BxPC-3 msCD63-XFP cell line do not affect exosomes secretion or size (**Figure 7B**).

Therefore, it was demonstrated that pancreatic cancer cell lines with msCD63-XFP expression secrete color-coded exosomes labelled with the same fusion protein.

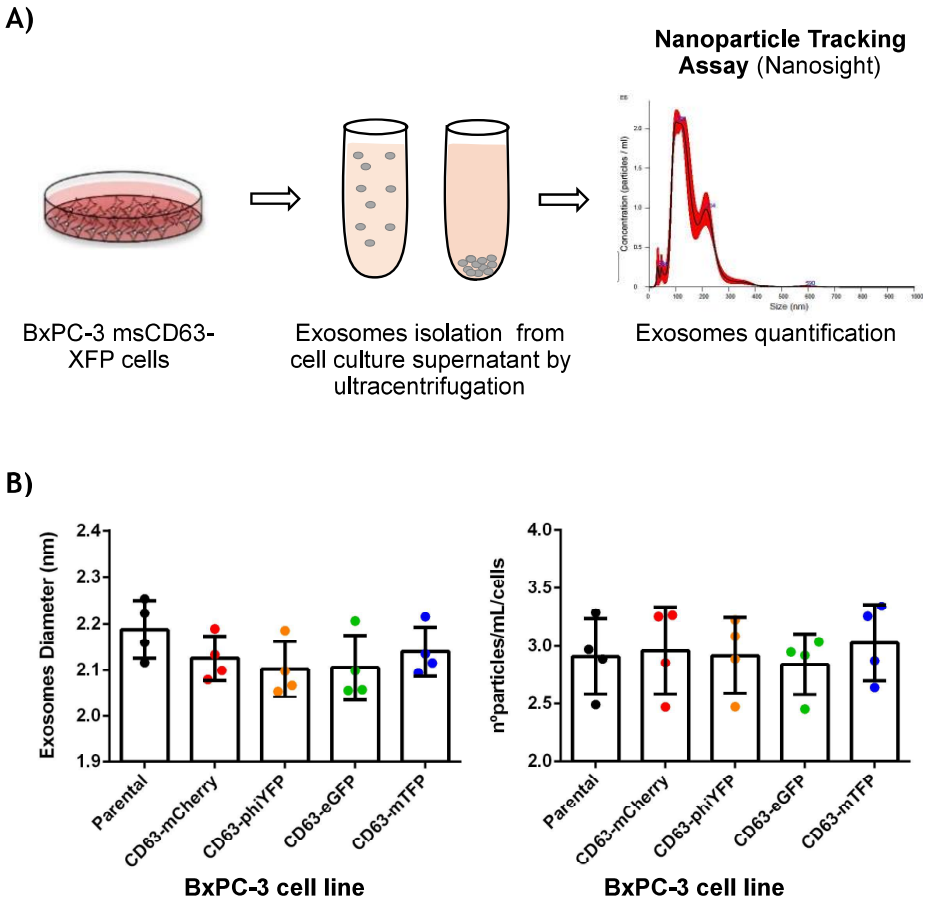


Figure 7. Exosomes secretion is not affected by CD63 overexpression in BxPC-3 msCD63-XFP cell lines. (A) Exosomes quantification of each clone and parental cell line was assessed by Nanopaticle Tracking Assay (Nanosight) after ultracentrifugation of the culture medium. **(B)** Size and number of exosomes quantification using NTA. The number of particles/mL was normalized to the number of cells**.** Results are presented in logarithmic scale. Each bar represents mean ratio and error bars indicates standard deviations (n=4±SD, the analysis was assessed using quadruples for each cell line). One Way ANOVA non-parametric test was performed among each clone with the parental cell line and showed non-statistical significant.

3.3. CD63-XFP fusion proteins co-localize with endogenous CD63.

Once the stable clones were established, the intracellular location of the CD63-XFP proteins were analysed. For each BxPC-3 msCD63-XFP cell line immunofluorescence using anti-XFP and the human anti-CD63 antibodies was performed. It was observed that msCD63-XFP proteins co-localize with the endogenous huCD63 of the cell (**Figure 8**).

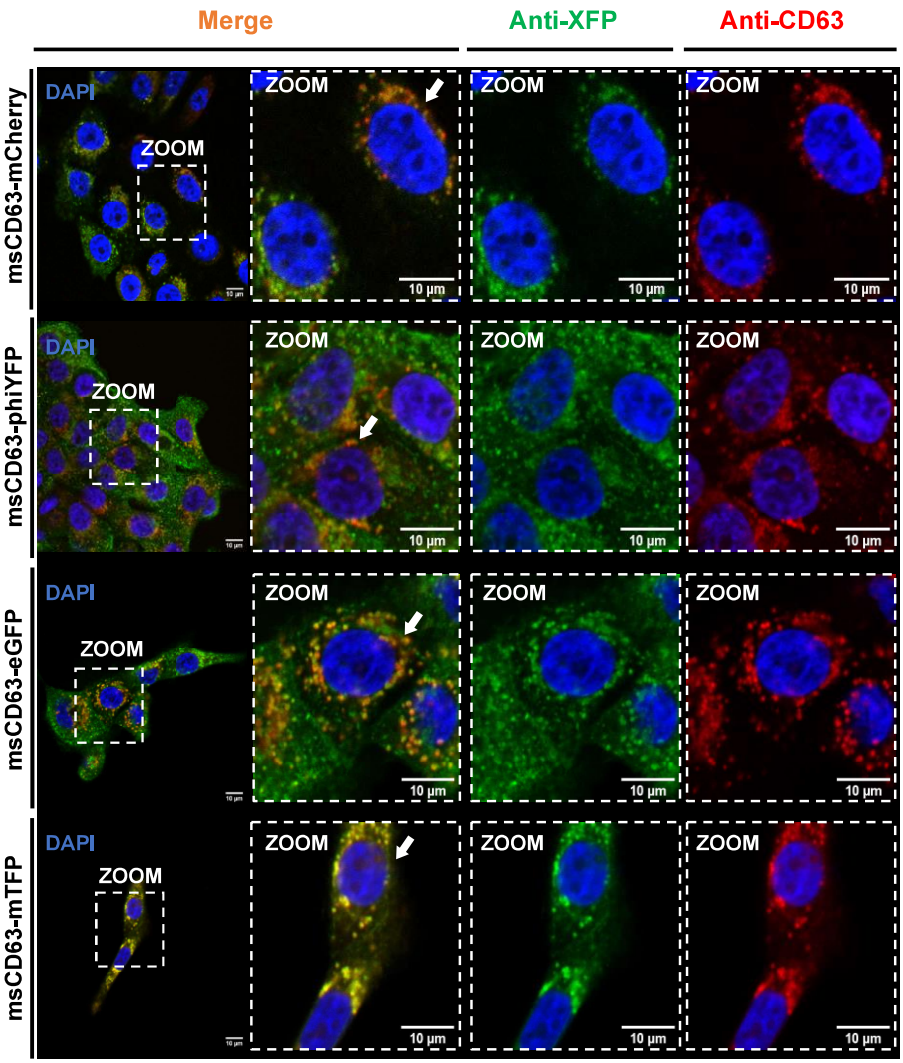


Figure 8. CD63-XFP fusion proteins co-localize with endogenous CD63. Confocal microscopy images of BxPC-3 msCD63-XFP cells. Immunostaining using each anti-XFP antibody and the anti-human CD63 antibody. The nucleus was stained with DAPI. Arrows indicate the examples of co-localization sites. Scale bar, 10µm.

3.4. BxPC-3 msCD63-XFP cells exchange color-coded exosomes.

After confirmation that BxPC-3 msCD63-XFP cells secreted CD63-XFP labelled exosomes to the extracellular space, and that CD63-XFP was being expressed in the expected intracellular region of the cell, it was further analysed if the different clones communicated between them through CD63-XFP positive vesicles. Therefore, a 96 hours co-culture of BxPC-3 msCD63-mCherry with each remaining clone: msCD63-eGFP, -phiYFP or -mTFP was performed. Live cell imaging acquisition was performed by confocal microscopy (**Figure 9**). All cells secreted fluorescently labelled exosomes that travel to recipient cells. In case of the BxPC-3 co-culture of msCD63-mCherry with msCD63-eGFP cells it was observed that mCherry positive exosomes travel to the BxPC-3 msCD63-eGFP cells (**Figure 9A**). While in the BxPC-3 co-culture of msCD63-mCherry with msCD63-phiYFP or with msCD63-mTFP, the phiYFP and mTFP positive exosomes communicate with the BxPC-3 msCD63-mCherry recipient cells (**Figure 9B-C**). These results show that BxPC-3 msCD63-XFP cells exchange information between them through exosomes. Despite this observation, the amount of communication identified between the clones was limited. Not only that, as it appears not to occur randomly, since the same clone might communicate or not depending on which clone it is in contact with. Since mouse and human CD63 protein sequence similarity is only 79.41%, at this point we wondered if this could be interfering with the amount of communication detect. Therefore, the communication between the BxPC-3 msCD63-XFP cells with human pancreatic cancer cells stably expressing the human plasmids huCD63-eGFP or huCD81-Tomato was analysed. BxPC-3 huCD63-eGFP and BxPC-3 huCD81-Tomato cell lines were previously developed and validated in the lab to produce and exchange color-coded exosomes. Therefore, distinct co-cultures were performed of the different BxPC-3 clones: msCD63-mCherry with huCD63-eGFP (**Figure 10A**), msCD63-phiYFP with huCD81-Tomato (**Figure 10B**), msCD63-eGFP with huCD81-Tomato (**Figure 10C**) and msCD63-mTFP with huCD81-Tomato (**Figure 10D**). Using confocal microscopy, it was observed in the majority of the co-cultures the exchange of color-coded exosomes between cancer cells. Nevertheless, the pattern of communication was different between the different cells. The most striking observation is that cells expressing the human version of the tagged CD63 communicate with other cells more frequently while the exosomes with the mouse CD63 proteins communicate less. For instance, BxPC-3 huCD63-eGFP cells secreted more exosomes that communicate with the msCD63-mCherry cells (**Figure 10A**). Similar observations were made on the BxPC-3 msCD63-eGFP with huCD81-Tomato co-culture (**Figure 10C**). However, in the case of the BxPC-3 co-culture huCD81-Tomato with msCD63-phiYFP

(**Figure 10B**) or with msCD63-mTFP cells (**Figure 10D**) the flow of exosomes between the cancer cells was lower. Particularly, BxPC-3 huCD81-Tomato and BxPC-3 msCD63-phiYFP demonstrated mutual communication, with different recipient cells receiving huCD81-Tomato and msCD63-phiYFP positive exosomes (**Figure 10B**).

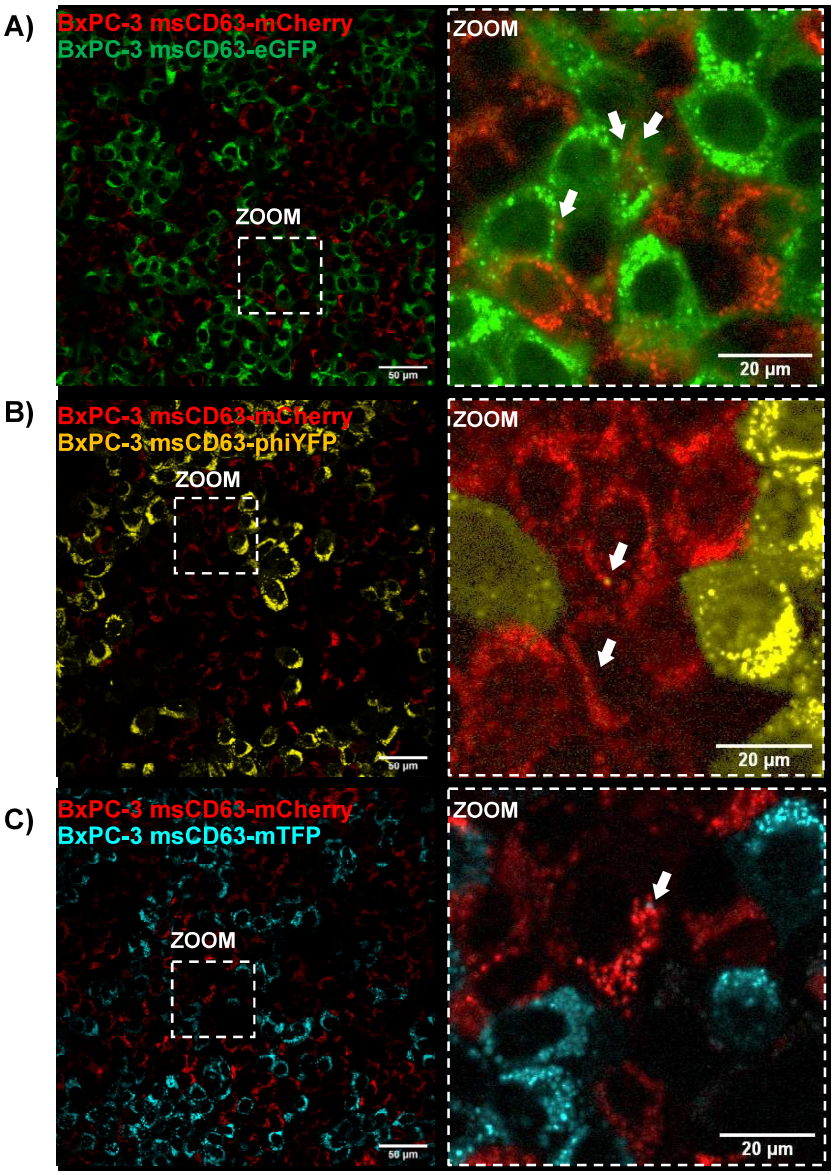


Figure 9. BxPC-3 msCD63-XFP cells communicate via exosomes. Confocal microscopy images of 96 hours live co-culture of BxPC-3 msCD63-mCherry cell line with one of the BxPC-3 msCD63-XFP clones: **(A)** msCD63-eGFP, **(B)** msCD63-phiYFP or **(C)** msCD63-mTFP (ratio 1:1). Arrows indicate examples of exosomes that flew from the donor cell to the recipient cell. Scale bar, 50µm (left) and 20µm (right).

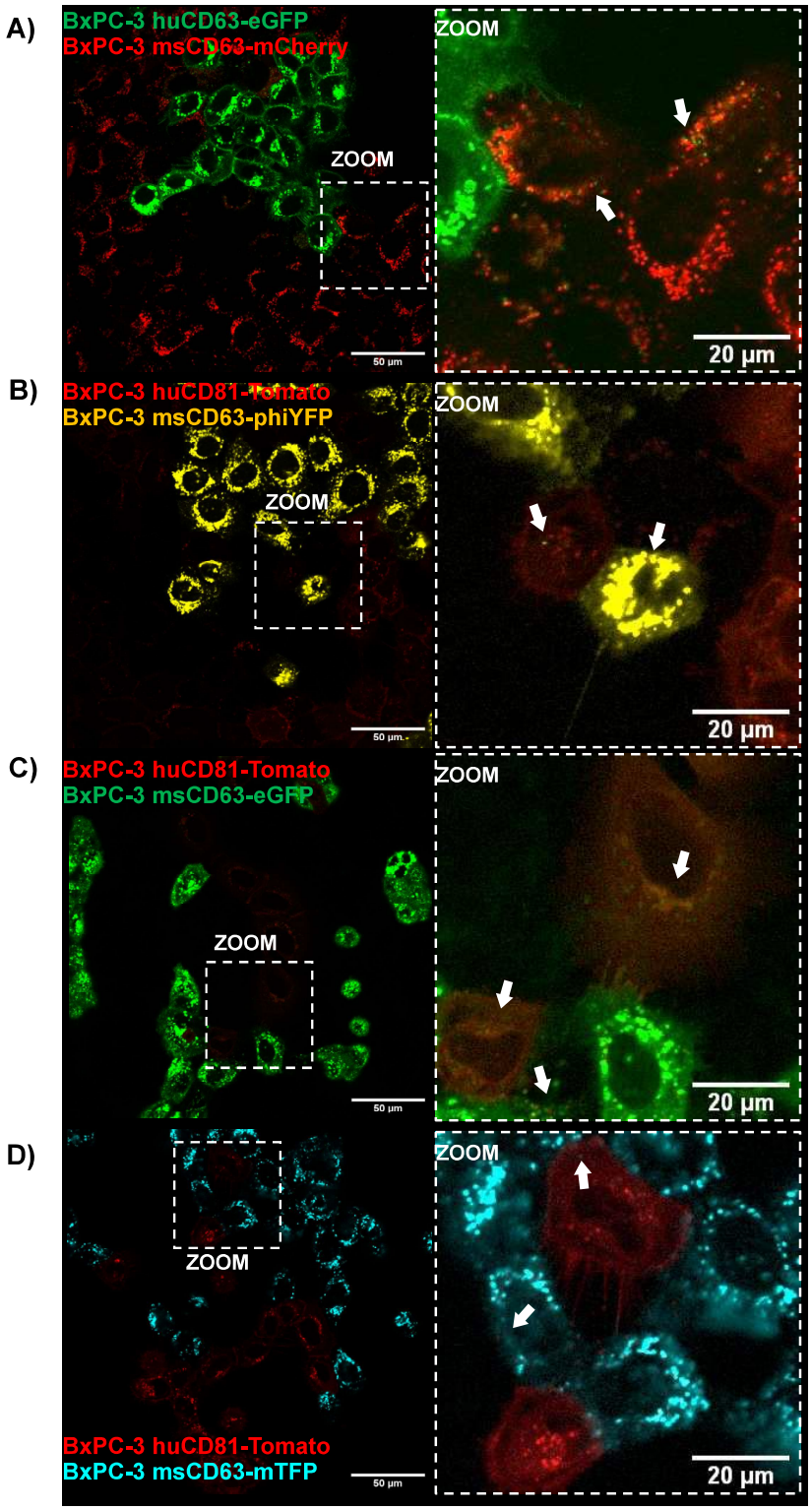


Figure 10. BxPC-3 msCD63-XFP cells communicate with BxPC-3 huCD63-eGFP and BxPC-3 huCD81-Tomato cell lines. Confocal microscopy images of 96 hours live BxPC-3 co-culture of **(A)** msCD63-mCherry cell line with huCD63-eGFP cell line; **(B)** msCD63-phiYFP cell line with huCD81-Tomato cell line **(C)** msCD63-eGFP cell line with huCD81-Tomato cell line; or **(D)** msCD63-mTFP cell line with huCD81-Tomato cell line (ratio 4:1). Arrows indicate examples of exosomes that flew from the donor cell to the recipient cell. Scale bar, 50µm (left) and 20µm (right).

Having into consideration these results we have started to transduce the KPC cell line with the msCD63-XFP plasmids. This cell line derives from a PDAC tumor of a KPC mouse and therefore should be the best model for the *in vitro* experiments. The KPC msCD63-mCherry and -eGFP clones are already established (**Figure 11**). The establishment of the remaining msCD63-XFP clones is ongoing. Of note, at the time we started this work the lab did not have the cell line available yet.

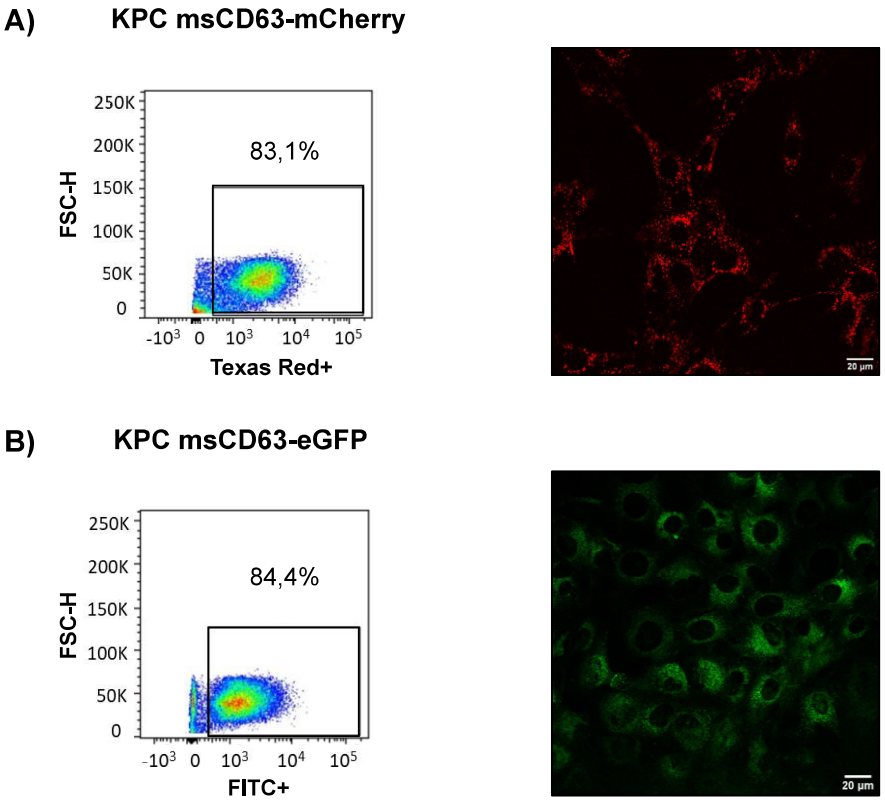


Figure 11. Establishment of CD63-XFP mouse PDAC cell lines. Flow cytometry (left panel) analysis and representative images by confocal microscopy (right panel) of the **(A)** KPC msCD63-mCherry and **(B)** KPC msCD63-eGFP clones. Scale bar, 20µm.

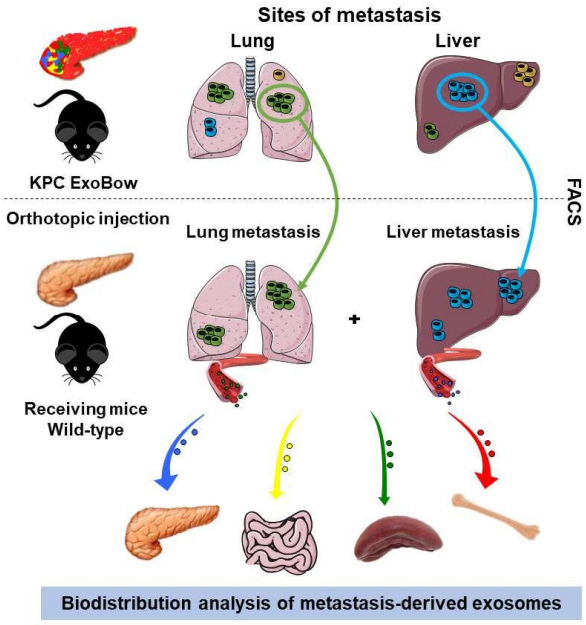
3.5. Intrahepatic implantation of liver and lung metastasis from KPC-ExoBow mice to determine the ExoMet.

The KPC-ExoBow mice is a GEMM developed by the lab. The multireporter mouse spontaneously develops pancreatic cancer and produces color-coded pancreas derived exosomes. The labelled cells and its derived exosomes allow the study of exosomes communication and biodistribution during disease progression. The main objective of this project is the identification of the biodistribution of exosomes derived from the

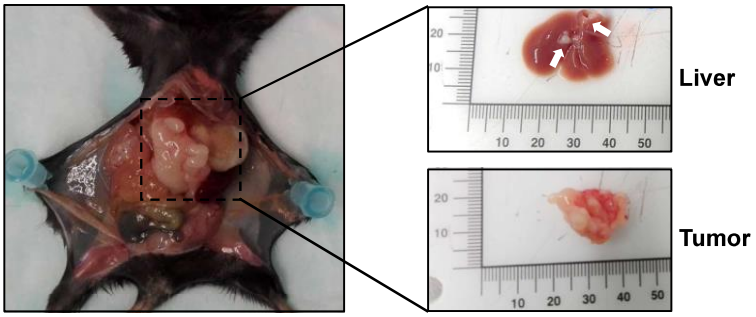
metastasis. For this purpose, we used the KPC-ExoBow and waited until the animal showed signs of discomfort and the humane endpoint was reached to euthanize it, which is on average 25 weeks of age (**Figure 12A**). At time of euthanasia the lung and liver metastasis were collected as well as the primary tumor (**Figure 12A-B**). The cancer cells were sorted using FACS to detect fluorescent cells in the liver and lungs. The positive cells were collected and injected orthotopically in the liver or lungs in syngeneic wild-type mice. Overall, 5 KPC-ExoBow mice were euthanized and in those in which we observed metastasis macroscopically, it was injected orthotopically in the liver of 7 wild-type mice, by intrahepatic injection, and in the lung of 2 wild-type mice, by tail vein injection (**Figure 12C**). The number of cells injected were dependent of the number and size of the original metastasis as well as the number of viable cells upon sorting. All of the euthanized animals had a tumor in the pancreas (**Figure 12B and 13**). We could detect liver or lung macrometastasis in only 4 KPC-ExoBow mice. The PDAC tumors are characterized by a strong desmoplastic reaction with fibroblasts infiltration and deposition of extracellular matrix (Feig et al. 2012). Histology of the primary tumor, liver and lung from the KPC-ExoBow demonstrated the PDAC histopathologic phenotype (**Figure 13**). The tumors predominantly have stroma, and it was also noted some necrotic tissue due to their size, while the amount of cancer cells was low (**Figure 13**). Using FACS the CD63-phiYFP, -eGFP and -mTFP positive cancer cells from the metastasis were collected. From the same animal it was obtained liver metastatic cells or lung metastatic cells or both (**Figure 10**). A very small part of the liver or lung was collected for histology and it was possible to identify micrometastasis in some animals (**Figure 13**). The primary tumor from each KPC-ExoBow animal also demonstrated a small percentage of color-coded cancer cells when analysed in FACS (**Figure 14**). These results were confirmed by immunofluorescence of the tumor tissue using antibodies against the four fluorescence proteins (**Figure 15**). Despite the low amounts of positive cells, it was possible to identify CD63-eGFP and -mTFP cancer cells in the tumor. The KPC-ExoBow negative for the flippase allele (Pdx-$1^{flp/+}$) was used as control.

In general, we observed that all KPC-ExoBow mice hac color-coded cells in the primary tumor and in the metastatic sites but in very small percentages. Consequently, the number of metastatic cancer cells injected intrahepatic or tail vein were very limited, therefore, in order to obtain a considerable growth of the injected cells several animals need to be performed and the timeline of the experiments extended. This work is still ongoing in the lab.

A)



B)



C)

Animal	Tumor volume (mm^3)	Number of macrometastasis		Local of Injection	Number of animals	Number of cells
		Liver	Lung			
3326 TEDFD	2584	40	0	Liver	4	3000
2554 FDTD	3163	1	1	Liver	1	3000
2966 FEDTD	5689	6	0	Liver	1	4100
2592 FETE	8525	2	3	Liver	1	8000
				Lung	1	8400
3602 TE	1599	0	0	Lung	1	4000

Figure 12. KPC-ExoBow mouse model in the ExoMet. (A) Schematic representation of the procedure for the collection and orthotopic injection of metastatic cells from liver and lung metastasis of the KPC-ExoBow mouse model **(B)** Representative image of KPC-ExoBow pancreatic tumor and liver. Arrows indicate liver macrometastasis. **(C)** Summary table of the total KPC-ExoBow mice euthanized, respective number of lung and liver metastasis and the number of cells injected in wild-type mice.

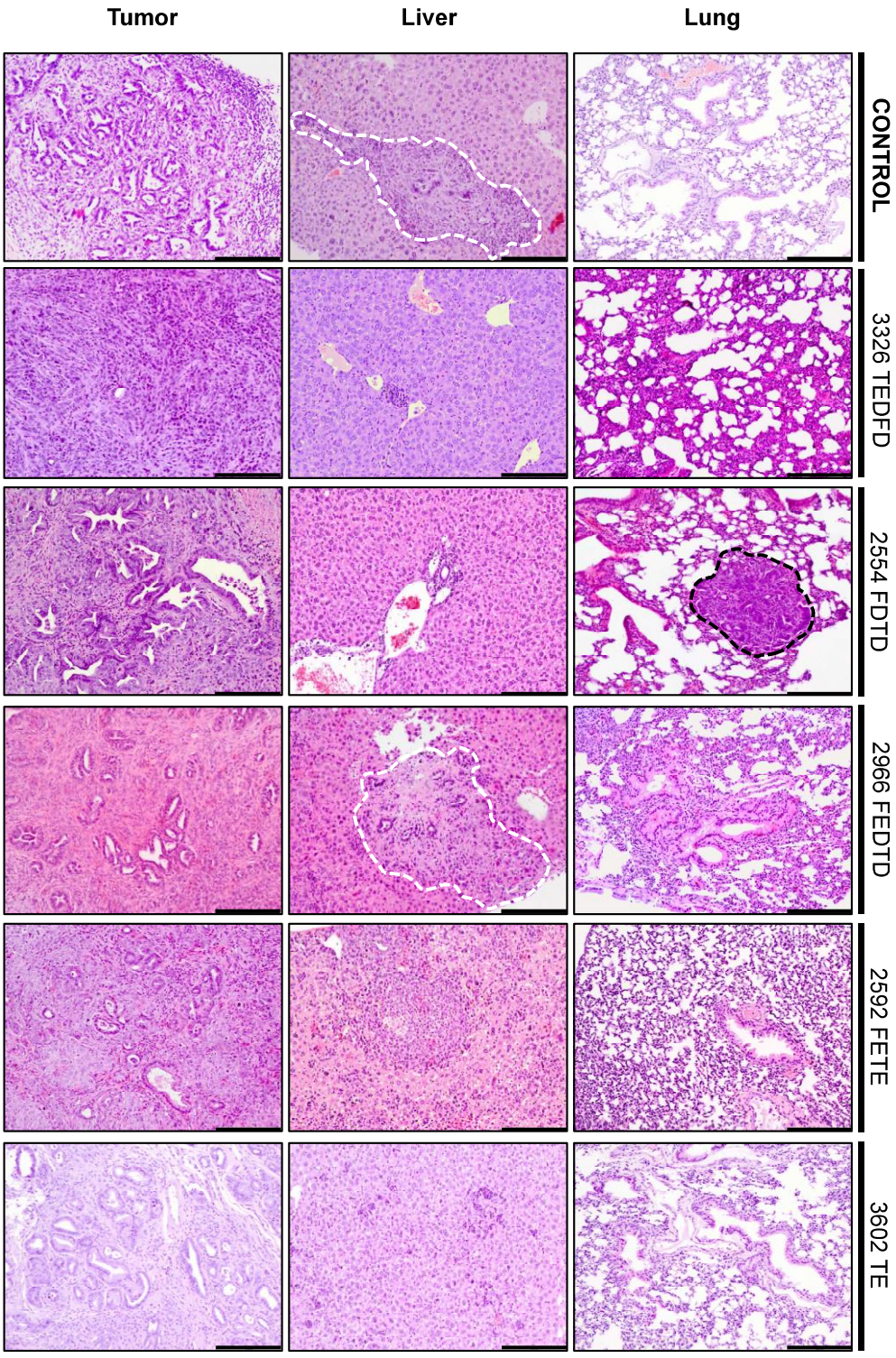


Figure 13. KPC-ExoBow organs histology. Representative H&E images from the 5 KPC-ExoBow and the control, the KPC-ExoBow flippase negative. Primary tumor (left panel), liver (middle panel) and lung (right panel). Liver metastasis (dashed white line) and lung metastasis (dashed black line) are delineated. Scale bar, 200µm.

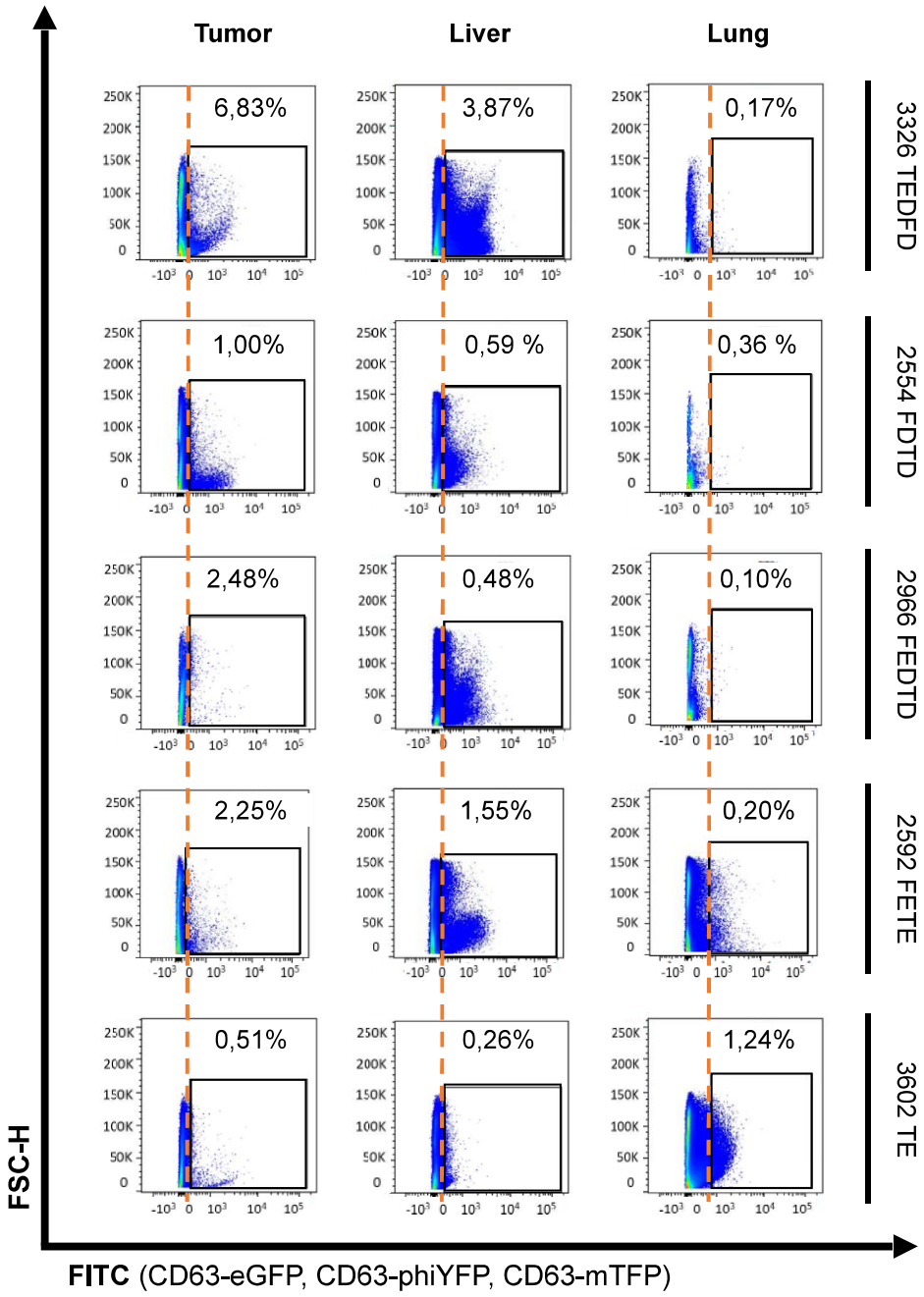


Figure 14. Flow Cytometry analysis of KPC-ExoBow primary tumor, liver and lung. Tumor, liver metastasis and lung metastasis were collected from KPC-ExoBow mice and sorted using FACS. The CD63-eGFP, CD63-phiYFP and CD63-mTFP positive cells were collected and orthotopically injected in wild-type animals. A total of 5 KPC-ExoBow mice euthanized. KPC-ExoBow flippase negative was used as control.

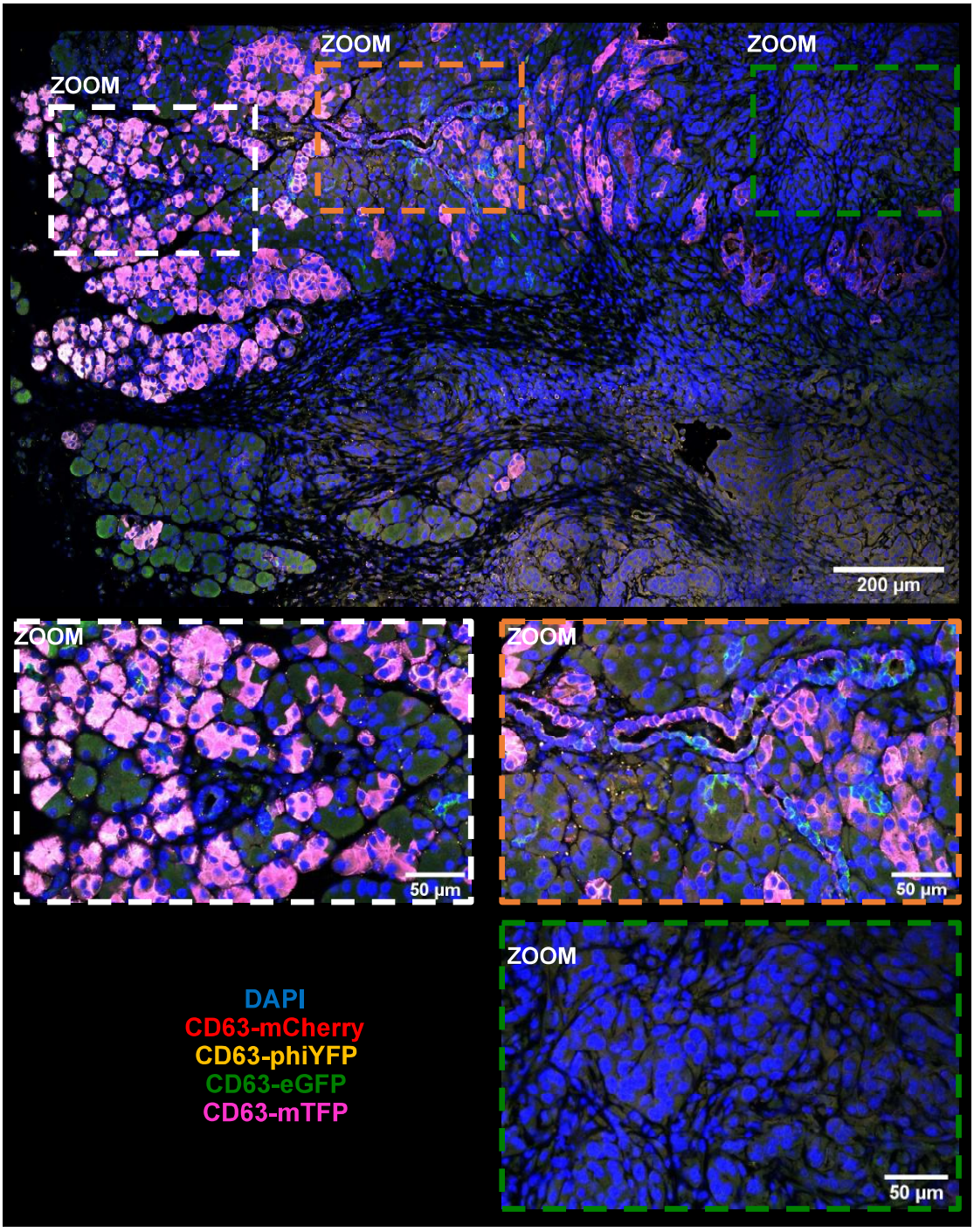
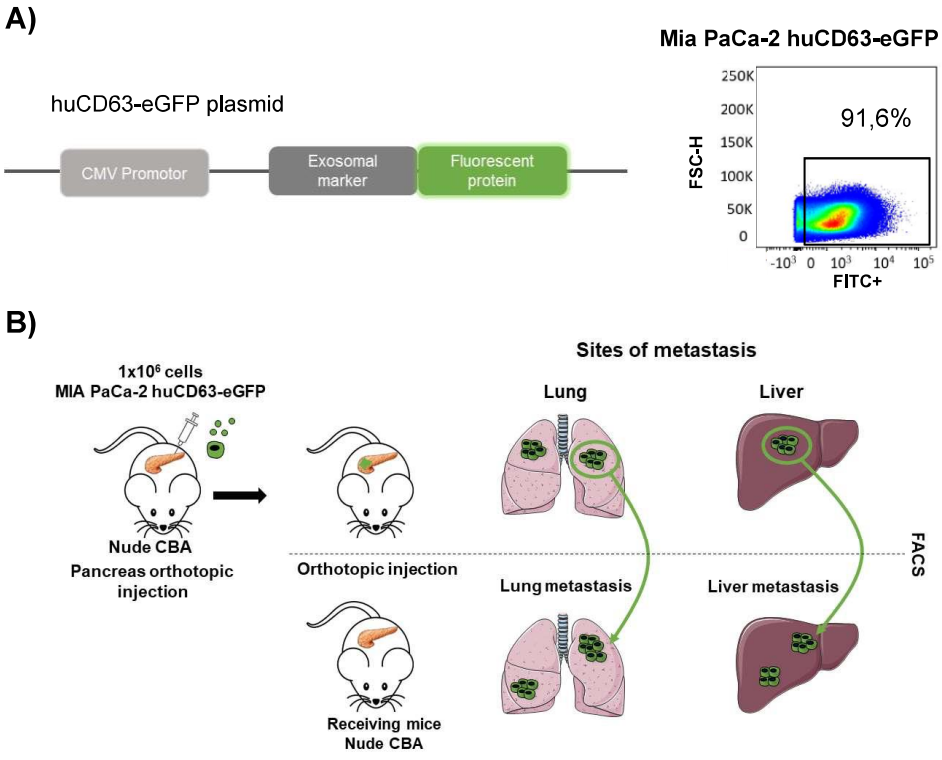


Figure 15. KPC-ExoBow primary tumor expresses CD63-XFP fluorescent proteins. Confocal microscopy images of a KPC-ExoBow primary tumor. Immunofluorescence against the four fluorescent proteins mCherry, phiYFP, eGFP and mTFP. Cell nucleus counterstained with DAPI. Representative image from “normal tissue” (no-histological disease, white), pancreatic precursor lesions (orange) and adenocarcinoma (green). Scale bar, 100µm and 50µm.

3.6. Orthotopic injection of Mia PaCa-2 huCD63-eGFP pancreatic cancer cells.

Since the GEMMs take several months for the disease to progress, we decided to use also a human pancreatic cancer cell line that stably expresses huCD63-eGFP, the Mia PaCa-2 cell line (**Figure 16A**). This cell line was established and previously validated in the lab. FACS analysis demonstrated 91.6% positive huCD63-eGFP cells. We orthotopically injected 1x10^6 Mia PaCa-2 huCD63-eGFP cells in the pancreas of nude CBA immunodeficient mice (**Figure 16B**). The aim was to euthanize the animals when the tumor reached a volume between 1000 and 1500 mm^3 to collect the macrometastasis from the liver and lung, sort the eGFP positive cells by FACS, and inject them in another nude CBA animal (**Figure 16B**). The approach is the same used for the KPC-ExoBow mice. The tumor growth and volume were monitored using the ultrasound technique from the moment a palpable mass was detected (**Figure 16C**). Tumor volume was measured weekly and increased at an exponential rate as expected (**Figure 16D**). At the moment we have euthanised one animal, which presented macrometastasis in the liver. Interestingly, despite the fact that the primary tumor is in fact FITC positive by flow cytometry analysis (cancer cells were >90% huCD63-eGFP positive) the metastasis had no color whatsoever, and therefore these cells were not re-injected in another animal as initially planned (**Figure 16E**). We have injected a total of 3 animals and euthanised only one until now.



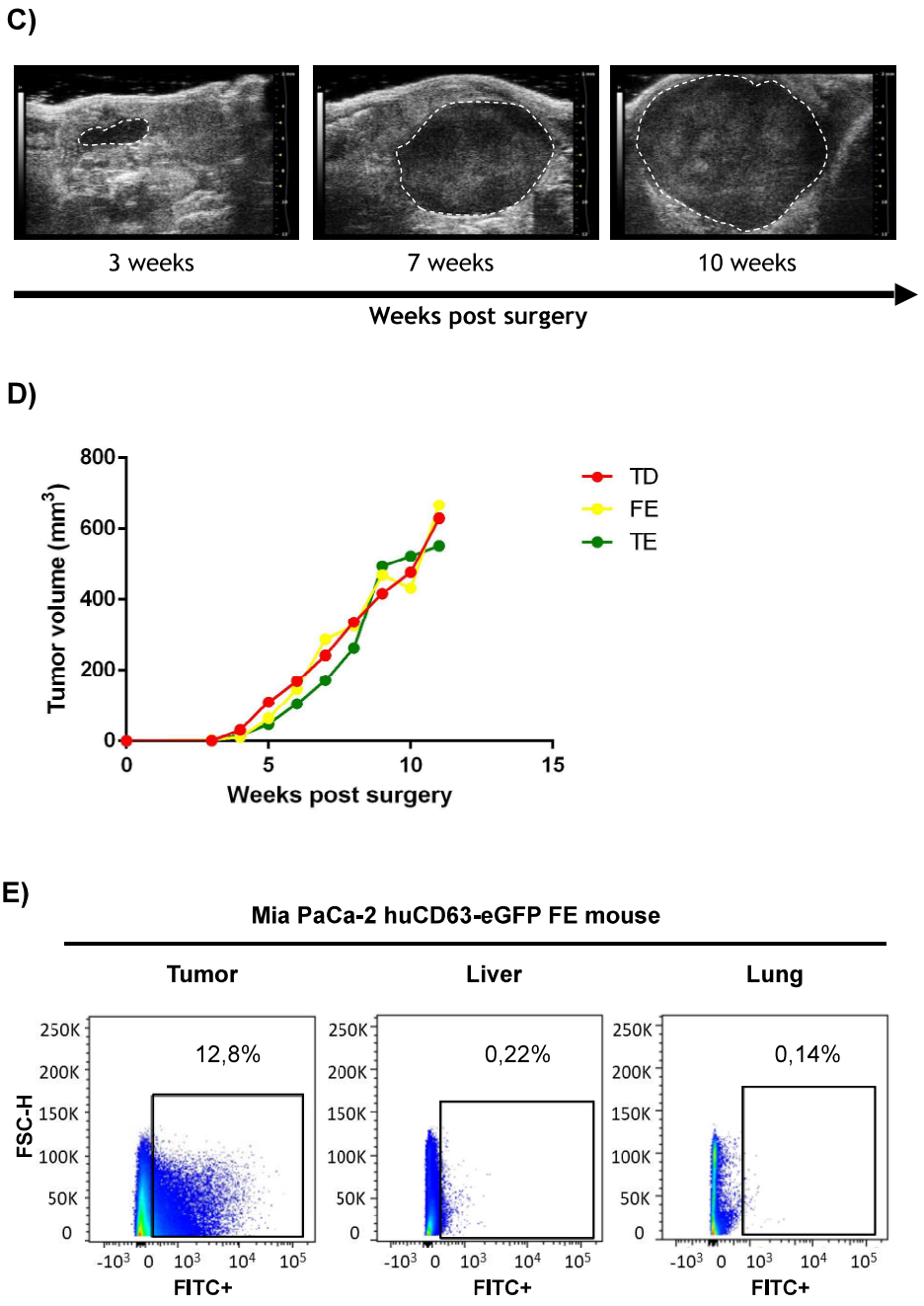


Figure 16. Orthotopic injection of Mia PaCa-2 huCD63-eGFP cells in the pancreas. (A) The eGFP fluorescent protein is fused to the C terminal of the human CD63. In the Mia PaCa-2 huCD63-eGFP clone, 91,6% of the cells were eGFP positive **(B)** A total of $1x10^6$ Mia PaCa-2 huCD63-eGFP cells were orthotopically injected in the pancreas of 3 nude CBA mice. After tumor implantation and growth animals are euthanized and metastasis from the lung and liver are collected, sorted and injected in receiving mice. **(C)** Representative timeline of ultrasound tumor growth monitoring of a pancreas orthotopically injected animal with Mia PaCa-2 huCD63-eGFP cells. **(D)** Tumor volume (mm^3) was measured through ultrasound since 3 weeks post surgery. Each line represents a different animal. **(E)** Flow cytometry analysis of the primary tumor, liver and lung from the FE mouse orthotopically injected with Mia PaCa-2 huCD63-eGFP cells, for the presence of eGFP positive cells.

4. Discussion

4. Discussion

In the last decade, exosomes were described has being important mediators of communication between different cells in a vast subset of biological processes from healthy to pathological contexts (De Toro et al. 2015). Moreover, cancer exosomes appear to mediate the communication between the tumor and its microenvironment (Silva and Melo 2017). It has also been shown that cancer cells release exosomes capable of exchanging information with the surrounding area of the tumor which influences tumor growth and proliferation, as well as invasion to other organs, where the metastasis is established. Exosomes seem to pave the road that cancer cells follow during the metastatic process until the secondary host-organ, with the sole function to prepare the new environment to receive the incoming cancer cells (Peinado, Lavotshkin, and Lyden 2011). In pancreatic cancer, it was already shown that exosomes are responsible for the formation of a metastatic niche in the liver and for the increased liver metastatic burden (Costa-Silva et al. 2015). Indeed, the role of tumor exosomes during disease progression and metastasis has been extensively addressed. However, if and how exosomes derived from metastasis influence the metastasis survival and growth is still an unexplored field. To address this question, we have delineated a series of experiments to determine if metastasis exosomes communicate with other cells of the host-organ and also other organs. This information can then be further dissected to determine what is the role of this communication in metastasis.

CD63 is a tetraspanin characterized by four transmembrane (TM) domains with a short N and C terminal tail, a small extracellular loop between TM1 and TM2 region, a small intracellular loop between TM2 and TM3 region, and a large extracellular loop between TM3 and TM4 (Hemler 2005). Tetraspanins are one of the most commonly proteins found in exosomes which also follow the endosomal system (Pols and Klumperman 2009). In general, endocytic vesicles fuse with early endosomes, which contain ILVs that bud into MVBs. CD63 protein is internalized in ILVs. After maturation, MVBs can be sorted to the lysosomal complex or can fuse with the plasma membrane and release exosomes into the extracellular environment. CD63, among other tetraspanins including CD81, CD82, CD9 are considered exosomal markers (Théry, Zitvogel, and Amigorena 2002). Preliminary data acquired in the lab demonstrated that CD63 overexpression in a human pancreatic cancer cell line orthotopically implanted in the pancreas does not alter primary tumor growth kinetics when compared to CD81 or CD82. Nonetheless, the data collected from the KPC-ExoBow model raises some doubts about this phenomenon in a GEMM model of PDAC. The low percentages of color in the

tumors that were identified in the KPC-ExoBow mice can indicate one of two scenarios: one is that the recombination levels driven by flippase are not being efficient in our animal model (the allele driving flippase is the Pdx1-Flp); or that the overexpression of the CD63 protein is impairing growth of cancer cells in which recombination has occurred. In fact, overexpression of CD63 *in vivo* was shown to suppress tumorigenesis in athymic nude mice injected with human melanoma cells overexpressing CD63 (Hotta et al. 1991). The same was not seen in the preliminary results of the lab, in which a pancreatic cancer cell line overexpressing CD63 (Mia PaCa-2) did not show any alteration in the kinetics of growth of the primary tumor when compare to the parental cell line. We understand that this data, since it was performed in one cell line only, might not reflect the real role of CD63 in pancreatic cancer, which in fact we believe is what we are observing in the KPC-ExoBow. To address this question, and to overcome the limited number of cancer cells with color that the model has, the lab is developing the same KPC-ExoBow animal but inducible, in which the Pdx1-Flp allele is being replaced by the Rosa-LSL-FlpoERT2 allele, the KPC-iExoBow. In this scenario the mice will spontaneously develop PDAC as the KPC mouse model, with no CD63 overexpression, and some days before euthanasia mice will be treated with tamoxifen. Upon tamoxifen treatment, the ERT2 receptor is activated and the FlpoERT2 travel to the nucleus where it drives the expression of the ExoBow transgene, and therefore the overexpression of the color-coded CD63 proteins. The inducible system has shown to be efficient with other models in the lab, showing good recombination levels. In this way the overexpression of a protein that might affect disease progression is avoided since the aim is to merely tag exosomes with color. In this way, it is expected that the KPC-iExoBow mice have a significantly higher percentage of cancer cells with color as well as the metastasis.

Up until now, our results show that the distinct mouse CD63 fusion proteins (CD63-XFP) present an intracellular location in the endosomal area of the cell with a speckle-like pattern. This pattern of expression is identical to the one observed for the endogenous CD63 in the BxPC-3 parental cell line. This pattern and location indicate that the fusion protein is following the endocytic pathway, and therefore, could be included into exosomes. These results were supported by western blot analysis of BxPC-3 msCD63-XFP-derived exosomes isolated by sucrose gradient. Also, in the cells, the western blots for the four cell lines using an anti-XFP antibody that detects the mCherry, phiYFP, eGFP or mTFP, or an CD63 anti-mouse antibody detected a band with the molecular weight expected for the fusion protein, regardless of the antibody used. Bands are not detected in the parental cell line confirming the expression of CD63-XFP in all clones. When using CD63 anti-mouse antibody, we do not detect a band in the parental

cell line because the antibody used is specific for mouse species and the cell lines are of human origin. In all western blots, either for cells or exosomes, we always observed bands at higher molecular weights as it was expected. These bands can correspond to post-translation modifications that occur in the CD63 fusion proteins. In fact, CD63 has two glycosylation sites in the large extracellular membrane portion, which makes them a target for glycosylation (Pols and Klumperman 2009). Additionally, using NTA it was also shown that levels of exosomes secreted from each BxPC-3 msCD63-XFP clone are similar between them. Using One Way ANOVA test, there is no statistical significance between each clone and the parental cell line in terms of numbers or size of the secreted exosomes. In sum, we have clearly demonstrated that independently of the identity and length of the linker amino acids generated by recombination of the lox sites between CD63 and the fluorescent reporter, the cells secrete CD63-XFP color-coded exosomes.

Having in mind these results, we next evaluated the communication between BxPC-3 msCD63-XFP cells. It is known by the literature that these vesicles can travel to local or distant sites where they deliver their content to recipient cells. Co-cultures of BxPC-3 msCD63-XFP clones demonstrated that exosomes flow between clones because we could identify double positive cells. Despite the positive results, the frequency of this communication is significantly less than it was initially expected. Because this result could be due to the fact that CD63 in the ExoBow transgene is from mouse background, and somehow this, despite not affecting their secretion from cells, would affect their uptake by recipient cells, co-cultures with the mouse clones (msCD63-XFP) and two human clones (huCD63-eGFP and huCD81-Tomato) were performed. Indeed, it was possible to determine that, despite the ratio of cells used being 4:1, msCD63-XFP and huCD63-eGFP or huCD81-Tomato, respectively, exosomes from the human clones could easily be detected in the mouse CD63-XFP clones, hinting for the possibility that in fact the low communication frequency previously observed is due to the protein being of mouse origin. One other possible explanation could be that the mechanisms of uptake by cells do not recognize this protein and exosomes are not internalized, or they might be fast degraded once inside the cells. Nonetheless, the exact same clone when in contact with two different clones (mouse or human) behaves in distinct ways supporting the idea that the communication through exosomes is not a random process. All these possibilities are currently being tested in the lab, and a KPC mouse cell line is currently being used to test the mouse CD63-XFP proteins.

Finally, as a complementary method to the KPC-ExoBow mouse, an orthotopic model with the human Mia PaCa-2 huCD63-eGFP pancreatic cancer cell clone was used. This clone had been previously established in the lab and presented with 91.6%

of CD63-eGFP positive cells. Despite very preliminary, the data collected on the first euthanised animal is very interesting and complements well with the KPC-ExoBow data. When the animal was euthanised, some small macrometastasis in the liver were observed. Nonetheless, upon analysis of the metastasis we were able to conclude that metastatic cancer cells do not have color. If this data is confirmed on the rest of the animals to be euthanised, it might indicate that overexpression of CD63, despite not altering primary tumor growth rate in orthotopic models using cell lines, it impairs metastasis. Indeed, a correlation between higher levels of CD63 and lower cell motility and metastatic capacity of cancer cells was already observed in *in vitro* studies (Radford, Thome, and Hersey 1997; Jang and Lee 2003). The second hypothesis would be that for cancer cells to metastasize they silence CD63 by epigenetic mechanisms. All of these hypotheses are currently being tested.

5. Conclusion and Future Perspectives

5. Conclusion and Future Perspectives

The data gathered in this thesis has made an important contribution to determine that CD63-XFP proteins produced by the ExoBow transgene are indeed secreted in exosomes. Most importantly, that the assessment of the biodistribution of exosomes, by the primary tumor or by the metastasis as initially planned, will need to be determined using an inducible model in which the overexpression of the CD63-XFP proteins only occurs few days before euthanasia. Based on these results, that model is currently being developed. Most interestingly, the data generated by this thesis has also unravelled a possible role of the CD63 protein in PDAC pathogenesis, which could be further dissected.

6. References

6. References

Abusamra, Ashraf J., Zhaohui Zhong, Xiufen Zheng, Mu Li, Thomas E. Ichim, Joseph L. Chin, and Wei Ping Min. 2005. “Tumor Exosomes Expressing Fas Ligand Mediate CD8+ T-Cell Apoptosis.” *Blood Cells, Molecules, and Diseases*. https://doi.org/10.1016/j.bcmd.2005.07.001.

Adachi, Eri, Katsuya Sakai, Takumi Nishiuchi, Ryu Imamura, Hiroki Sato, and Kunio Matsumoto. 2016. “Different Growth and Metastatic Phenotypes Associated with a Cell-Intrinsic Change of Met in Metastatic Melanoma.” *Oncotarget*. https://doi.org/10.18632/oncotarget.12221.

Adem, Bárbara, and Sónia A. Melo. 2017. “Animal Models in Exosomes Research: What the Future Holds.” In *Novel Implications of Exosomes in Diagnosis and Treatment of Cancer and Infectious Diseases*. https://doi.org/10.5772/intechopen.69449.

Admyre, C., S. M. Johansson, K. R. Qazi, J.-J. Filen, R. Lahesmaa, M. Norman, E. P. A. Neve, A. Scheynius, and S. Gabrielsson. 2014. “Exosomes with Immune Modulatory Features Are Present in Human Breast Milk.” *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.179.3.1969.

Aguirre, Andrew J., Nabeel Bardeesy, Manisha Sinha, Lyle Lopez, David A. Tuveson, James Horner, Mark S. Redston, and Ronald A. DePinho. 2003. “Activated Kras and Ink4a/Arf Deficiency Cooperate to Produce Metastatic Pancreatic Ductal Adenocarcinoma.” *Genes and Development*. https://doi.org/10.1101/gad.1158703.

Al-Nedawi, K., B. Meehan, R. S. Kerbel, A. C. Allison, and J. Rak. 2009. “Endothelial Expression of Autocrine VEGF upon the Uptake of Tumor-Derived Microvesicles Containing Oncogenic EGFR.” *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.0804543106.

Andaloussi, Samir El, Imre Mäger, Xandra O. Breakefield, and Matthew J.A. Wood. 2013. “Extracellular Vesicles: Biology and Emerging Therapeutic Opportunities.” *Nature Reviews Drug Discovery*. https://doi.org/10.1038/nrd3978.

Andre, Fabrice, Noel E.C. Schartz, Mojgan Movassagh, Caroline Flament, Patricia Pautier, Philippe Morice, Christophe Pomel, et al. 2002. “Malignant Effusions and Immunogenic Tumour-Derived Exosomes.” *Lancet*. https://doi.org/10.1016/S0140-6736(02)09552-1.

Baietti, Maria Francesca, Zhe Zhang, Eva Mortier, Aurélie Melchior, Gisèle Degeest, Annelies Geeraerts, Ylva Ivarsson, et al. 2012. “Syndecan-Syntenin-ALIX Regulates the Biogenesis of Exosomes.” *Nature Cell Biology*. https://doi.org/10.1038/ncb2502.

Bastos, Nuno, Carolina F. Ruivo, Soraia da Silva, and Sonia A. Melo. 2018. “Exosomes in Cancer: Use Them or Target Them?” *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2017.08.009.

Blanc, Lionel, and Michel Vidal. 2018. “New Insights into the Function of Rab GTPases in the Context of Exosomal Secretion.” *Small GTPases*. https://doi.org/10.1080/21541248.2016.1264352.

Boj, Sylvia F, Chang-Il Hwang, Lindsey A Baker, Iok In Christine Chio, Dannielle D Engle, Vincenzo Corbo, Myrthe Jager, et al. 2015. “Organoid Models of Human and Mouse Ductal Pancreatic Cancer.” *Cell*. https://doi.org/10.1016/j.cell.2014.12.021.

Borges, Fernanda T., Sonia A. Melo, Berna C. Özdemir, Noritoshi Kato, Ignacio Revuelta, Caroline A. Miller, Vincent H. Gattone, Valerie S. LeBleu, and Raghu Kalluri. 2013. “ TGF- β 1–Containing Exosomes from Injured Epithelial Cells Activate Fibroblasts to Initiate Tissue Regenerative Responses and Fibrosis .” *Journal of the American Society of Nephrology*. https://doi.org/10.1681/asn.2012101031.

Borsig, L., R. Wong, J. Feramisco, D. R. Nadeau, N. M. Varki, and A. Varki. 2002. “Heparin and Cancer Revisited: Mechanistic Connections Involving Platelets, P-Selectin, Carcinoma Mucins, and Tumor Metastasis.” *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.061615598.

Bosetti, C., E. Lucenteforte, D. T. Silverman, G. Petersen, P. M. Bracci, B. T. Ji, E. Negri, et al. 2012. “Cigarette Smoking and Pancreatic Cancer: An Analysis from the International Pancreatic Cancer Case-Control Consortium (PANC4).” *Annals of Oncology*. https://doi.org/10.1093/annonc/mdr541.

Bosetti, C., V. Rosato, D. Li, D. Silverman, G. M. Petersen, P. M. Bracci, R. E. Neale, et al. 2014. “Diabetes, Antidiabetic Medications, and Pancreatic Cancer Risk: An Analysis from the International Pancreatic Cancer Case-Control Consortium.” *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO*. https://doi.org/10.1093/annonc/mdu276.

Brücher, Björn L.D.M., and Ijaz S. Jamall. 2014. “Cell-Cell Communication in the Tumor Microenvironment, Carcinogenesis, and Anticancer Treatment.” *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000362978.

Caby, Marie Pierre, Danielle Lankar, Claude Vincendeau-Scherrer, Graça Raposo, and Christian Bonnerot. 2005. “Exosomal-like Vesicles Are Present in Human Blood Plasma.” *International Immunology*. https://doi.org/10.1093/intimm/dxh267.

Cai, Dawen, Kimberly B Cohen, Tuanlian Luo, Jeff W Lichtman, and Joshua R Sanes. 2013. “Improved Tools for the Brainbow Toolbox.” *Nature Methods*, no. May: 1–10. https://doi.org/10.1038/nmeth.2450.

Camussi, Giovanni, Maria C. Deregibus, Stefania Bruno, Vincenzo Cantaluppi, and Luigi Biancone. 2010. “Exosomes/Microvesicles as a Mechanism of Cell-to-Cell Communication.” *Kidney International*. https://doi.org/10.1038/ki.2010.278.

Cho, Jung Ah, Ho Park, Eun Hye Lim, and Kyo Won Lee. 2012. “Exosomes from Breast Cancer Cells Can Convert Adipose Tissue-Derived Mesenchymal Stem Cells into Myofibroblast-like Cells.” *International Journal of Oncology*. https://doi.org/10.3892/ijo.2011.1193.

Chow, Amy, Weiying Zhou, Liang Liu, Miranda Y. Fong, Jackson Champer, Desiree Van Haute, Andrew R. Chin, et al. 2014. “Macrophage Immunomodulation by Breast Cancer-Derived Exosomes Requires Toll-like Receptor 2-Mediated Activation of NF-κ B.” *Scientific Reports*. https://doi.org/10.1038/srep05750.

Colombo, Marina, Catarina Moita, Guillaume van Niel, Joanna Kowal, James Vigneron, Philippe Benaroch, Nicolas Manel, Luis F Moita, Clotilde Théry, and Graça Raposo. 2013. “Analysis of ESCRT Functions in Exosome Biogenesis, Composition and Secretion Highlights the Heterogeneity of Extracellular Vesicles.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.128868.

Colombo, Marina, Graça Raposo, and Clotilde Théry. 2014. “Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles.” *Annual Review of Cell and Developmental Biology*. https://doi.org/10.1146/annurev-cellbio-101512-122326.

Corcoran, Claire, Anne M. Friel, Michael J. Duffy, John Crown, and Lorraine O’Driscoll. 2011. “Intracellular and Extracellular MicroRNAs in Breast Cancer.” *Clinical Chemistry*. https://doi.org/10.1373/clinchem.2010.150730.

Costa-Silva, Bruno, Nicole M. Aiello, Allyson J. Ocean, Swarnima Singh, Haiying Zhang, Basant Kumar Thakur, Annette Becker, et al. 2015. “Pancreatic Cancer Exosomes Initiate Pre-Metastatic Niche Formation in the Liver.” *Nature Cell Biology*. https://doi.org/10.1038/ncb3169.

Dean, William L., Menq J. Lee, Timothy D. Cummins, David J. Schultz, David W. Powell, and Bill Dean. 2009. “Proteomic and Functional Characterisation of Platelet Microparticle Size Classes.” *Thrombosis and Haemostasis*. https://doi.org/10.1160/TH09-04-243.

Decker, Carolyn J. 2004. “The Exosome: A Versatile RNA Processing Machine.” *Current Biology*. https://doi.org/10.1016/s0960-9822(98)70149-6.

Dovizio, Melania, Sara Alberti, Angela Sacco, Paloma Guillem-Llobat, Simone Schiavone, Thorsten J. Maier, Dieter Steinhilber, and Paola Patrignani. 2015. “Novel Insights into the Regulation of Cyclooxygenase-2 Expression by Platelet-Cancer Cell Cross-Talk.” *Biochemical Society Transactions*. https://doi.org/10.1042/bst20140322.

Ekström, Elin J., Caroline Bergenfelz, Verena von Bülow, Filiz Serifler, Eric Carlemalm, Göran Jönsson, Tommy Andersson, and Karin Leandersson. 2014. “WNT5A Induces Release of Exosomes Containing Pro-Angiogenic and Immunosuppressive Factors from Malignant Melanoma Cells.” *Molecular Cancer*. https://doi.org/10.1186/1476-4598-13-88.

Elmageed, Zakaria Y.Abd, Yijun Yang, Raju Thomas, Manish Ranjan, Debasis Mondal, Krzysztof Moroz, Zhide Fang, et al. 2014. “Neoplastic Reprogramming of Patient-Derived Adipose Stem Cells by Prostate Cancer Cell-Associated Exosomes.” *Stem Cells*. https://doi.org/10.1002/stem.1619.

Erpenbeck, Luise, and Michael P. Schön. 2010. “Deadly Allies: The Fatal Interplay between Platelets and Metastasizing Cancer Cells.” *Blood*. https://doi.org/10.1182/blood-2009-10-247296.

Feig, Christine, Aarthi Gopinathan, Albrecht Neesse, Derek S Chan, Natalie Cook, and David A Tuveson. 2012. “The Pancreas Cancer Microenvironment Tumor Microenvironment.” *Clin Cancer Res*. https://doi.org/10.1158/1078-0432.CCR-11-3114.

Finger, Elizabeth C., and Amato J. Giaccia. 2010. “Hypoxia, Inflammation, and the Tumor

Microenvironment in Metastatic Disease." *Cancer and Metastasis Reviews*. https://doi.org/10.1007/s10555-010-9224-5.

Fong, Miranda Y, Weiying Zhou, Liang Liu, Aileen Y Alontaga, Manasa Chandra, Jonathan Ashby, Amy Chow, et al. 2015. "Breast-Cancer-Secreted MiR-122 Reprograms Glucose Metabolism in Premetastatic Niche to Promote Metastasis." *Nature Cell Biology*. https://doi.org/10.1038/ncb3094.

Franzen, C. A., R. H. Blackwell, V. Todorovic, K. A. Greco, K. E. Foreman, R. C. Flanigan, P. C. Kuo, and G. N. Gupta. 2015. "Urothelial Cells Undergo Epithelial-to-Mesenchymal Transition after Exposure to Muscle Invasive Bladder Cancer Exosomes." *Oncogenesis*. https://doi.org/10.1038/oncsis.2015.21.

Gay, Laurie J., and Brunhilde Felding-Habermann. 2011. "Contribution of Platelets to Tumour Metastasis." *Nature Reviews Cancer*. https://doi.org/10.1038/nrc3004.

Ghidoni, Roberta, Luisa Benussi, and Giuliano Binetti. 2008. "Exosomes: The Trojan Horses of Neurodegeneration." *Medical Hypotheses*. https://doi.org/10.1016/j.mehy.2007.12.003.

Gorvel, Jean Pierre, Philippe Chavrier, Marino Zerial, and Jean Gruenberg. 1991. "Rab5 Controls Early Endosome Fusion in Vitro." *Cell*. https://doi.org/10.1016/0092-8674(91)90316-Q.

Graham, J. S., N. B. Jamieson, R. Rulach, S. M. Grimmond, D. K. Chang, and Andrew V. Biankin. 2015. "Pancreatic Cancer Genomics: Where Can the Science Take Us?" *Clinical Genetics*. https://doi.org/10.1111/cge.12536.

Guerra, Carmen, Alberto J. Schuhmacher, Marta Cañamero, Paul J. Grippo, Lena Verdaguer, Lucía Pérez-Gallego, Pierre Dubus, Eric P. Sandgren, and Mariano Barbacid. 2007. "Chronic Pancreatitis Is Essential for Induction of Pancreatic Ductal Adenocarcinoma by K-Ras Oncogenes in Adult Mice." *Cancer Cell*. https://doi.org/10.1016/j.ccr.2007.01.012.

Hamada, Shin, Atsushi Masamune, Tetsuya Takikawa, Noriaki Suzuki, Kazuhiro Kikuta, Morihisa Hirota, Hirofumi Hamada, Masayoshi Kobune, Kennichi Satoh, and Tooru Shimosegawa. 2012. "Pancreatic Stellate Cells Enhance Stem Cell-like Phenotypes in Pancreatic Cancer Cells." *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2012.04.014.

He, Jin, Nita Ahuja, Martin A. Makary, John L. Cameron, Frederic E. Eckhauser, Michael A. Choti, Ralph H. Hruban, Timothy M. Pawlik, and Christopher L. Wolfgang. 2014. “2564 Resected Periampullary Adenocarcinomas at a Single Institution: Trends over Three Decades.” *HPB*. https://doi.org/10.1111/hpb.12078.

He, Mian, Hao Qin, Terence C.W. Poon, Siu Ching Sze, Xiaofan Ding, Ngai Na Co, Sai Ming Ngai, Ting Fung Chan, and Nathalie Wong. 2015. “Hepatocellular Carcinoma-Derived Exosomes Promote Motility of Immortalized Hepatocyte through Transfer of Oncogenic Proteins and RNAs.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgv081.

Hemler, Martin E. 2005. “Tetraspanin Functions and Associated Microdomains.” *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm1736.

Hezel, Aram F, Alec C Kimmelman, Ben Z Stanger, Nabeel Bardeesy, and Ronald a Depinho. 2006. “Genetics and Biology of Pancreatic Ductal Adenocarcinoma Genetics and Biology of Pancreatic Ductal Adenocarcinoma.” *Cold Spring Harbor Laboratory Press*. https://doi.org/10.1101/gad.1415606.

Hingorani, Sunil R, Emanuel F Petricoin, Anirban Maitra, Vinodh Rajapakse, Catrina King, Michael A Jacobetz, Sally Ross, et al. 2003. “Preinvasive and Invasive Ductal Pancreatic Cancer and Its Early Detection in the Mouse.” *Cancer Cell*.

Hingorani, Sunil R, Lifu Wang, Asha S Multani, Chelsea Combs, Therese B Deramaudt, Ralph H Hruban, Anil K Rustgi, Sandy Chang, and David A Tuveson. 2005. “Trp53 R172H and Kras G12D Cooperate to Promote Chromosomal Instability and Widely Metastatic Pancreatic Ductal Adenocarcinoma in Mice” 7 (May): 469–83. https://doi.org/10.1016/j.ccr.2005.04.023.

Hood, Joshua L., Susana San Roman, and Samuel A. Wickline. 2011. “Exosomes Released by Melanoma Cells Prepare Sentinel Lymph Nodes for Tumor Metastasis.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-10-4455.

Hoshino, Ayuko, Bruno Costa-Silva, Tang Long Shen, Goncalo Rodrigues, Ayako Hashimoto, Milica Tesic Mark, Henrik Molina, et al. 2015. “Tumour Exosome Integrins Determine Organotropic Metastasis.” *Nature* 527 (7578): 329–35. https://doi.org/10.1038/nature15756.

Hoshino, Daisuke, Kellye C. Kirkbride, Kaitlin Costello, Emily S. Clark, Seema Sinha, Nathan Grega-Larson, Matthew J. Tyska, and Alissa M. Weaver. 2013. “Exosome

Secretion Is Enhanced by Invadopodia and Drives Invasive Behavior." *Cell Reports*. https://doi.org/10.1016/j.celrep.2013.10.050.

Hotta, H., I. Hara, H. Miyamoto, and M. Homma. 1991. "Overexpression of the Human Melanoma-Associated Antigen ME491 Partially Suppresses in Vivo Malignant Phenotypes of H-Ras-Transformed NIH3T3 Cells in Athymic Nude Mice." *Melanoma Research*. https://doi.org/10.1097/00008390-199106000-00007.

Hurley, James H, and Phyllis I Hanson. 2010. "Membrane Budding and Scission by the ESCRT Machinery: It's All in the Neck." *Nature Reviews. Molecular Cell Biology*. https://doi.org/10.1038/nrm2937.

Jang, Hwa In, and Hansoo Lee. 2003. "A Decrease in the Expression of CD63 Tetraspanin Protein Elevates Invasive Potential of Human Melanoma Cells." *Experimental and Molecular Medicine*. https://doi.org/10.1038/emm.2003.43.

Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr, and C. Turbide. 1987. "Vesicle Formation during Reticulocyte Maturation. Association of Plasma Membrane Activities with Released Vesicles (Exosomes)." *Journal of Biological Chemistry*.

Kahlert, Christoph, Sonia A. Melo, Alexei Protopopov, Jiabin Tang, Sahil Seth, Oritz Koch, Jianhua Zhang, et al. 2014. "Identification of Doublestranded Genomic Dna Spanning All Chromosomes with Mutated KRAS and P53 DNA in the Serum Exosomes of Patients with Pancreatic Cancer." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.C113.532267.

Kalluri, Raghu, and Eric G. Neilson. 2003. "Epithelial-Mesenchymal Transition and Its Implications for Fibrosis." *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI200320530.

Karlsson, Terese, Marie Lundholm, Anders Widmark, and Emma Persson. 2016. "Tumor Cell-Derived Exosomes from the Prostate Cancer Cell Line TRAMP-C1 Impair Osteoclast Formation and Differentiation." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0166284.

Kowal, Joanna, Guillaume Arras, Marina Colombo, Mabel Jouve, Jakob Paul Morath, Bjarke Primdal-Bengtson, Florent Dingli, Damarys Loew, Mercedes Tkach, and Clotilde Théry. 2016. "Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes." *Proceedings of the National Academy of Sciences*.

https://doi.org/10.1073/pnas.1521230113.

Kucharzewska, P., H. C. Christianson, J. E. Welch, K. J. Svensson, E. Fredlund, M. Ringner, M. Morgelin, E. Bourseau-Guilmain, J. Bengzon, and M. Belting. 2013. “Exosomes Reflect the Hypoxic Status of Glioma Cells and Mediate Hypoxia-Dependent Activation of Vascular Cells during Tumor Development.” *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1220998110.

Lee, Jonathan M., Shoukat Dedhar, Raghu Kalluri, and Erik W. Thompson. 2006. “The Epithelial-Mesenchymal Transition: New Insights in Signaling, Development, and Disease.” *Journal of Cell Biology*. https://doi.org/10.1083/jcb.200601018.

Livet, Jean, Tamily A. Weissman, Hyuno Kang, Ryan W. Draft, Ju Lu, Robyn A. Bennis, Joshua R. Sanes, and Jeff W. Lichtman. 2007. “Transgenic Strategies for Combinatorial Expression of Fluorescent Proteins in the Nervous System.” *Nature*. https://doi.org/10.1038/nature06293.

Luga, Valbona, and Jeffrey L. Wrana. 2013. “Tumor-Stroma Interaction: Revealing Fibroblast-Secreted Exosomes as Potent Regulators of Wnt-Planar Cell Polarity Signaling in Cancer Metastasis.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-13-1791.

Luga, Valbona, Liang Zhang, Alicia M. Viloria-Petit, Abiodun A. Ogunjimi, Mohammad R. Inanlou, Elaine Chiu, Marguerite Buchanan, Abdel Nasser Hosein, Mark Basik, and Jeffrey L. Wrana. 2012. “Exosomes Mediate Stromal Mobilization of Autocrine Wnt-PCP Signaling in Breast Cancer Cell Migration.” *Cell*. https://doi.org/10.1016/j.cell.2012.11.024.

Madison, Marisa N., Philip H. Jones, and Chioma M. Okeoma. 2015. “Exosomes in Human Semen Restrict HIV-1 Transmission by Vaginal Cells and Block Intravaginal Replication of LP-BM5 Murine AIDS Virus Complex.” *Virology*. https://doi.org/10.1016/j.virol.2015.03.040.

Mahmoodzadeh Hosseini, Hamideh, Abbas Ali Imani Fooladi, Mohammad Reza Nourani, and Faezeh Ghanezadeh. 2013. “The Role of Exosomes in Infectious Diseases.” *Inflammation & Allergy-Drug Targets*. https://doi.org/10.2174/1871528111312010005.

McCaffrey, Mary W., Anna Bielli, Giuseppina Cantalupo, Silvia Mora, Vera Roberti, Mariarosaria Santillo, Frances Drummond, and Cecilia Bucci. 2001. “Rab4 Affects

Both Recycling and Degradative Endosomal Trafficking." *FEBS Letters*. https://doi.org/10.1016/S0014-5793(01)02359-6.

Meldolesi, Jacopo. 2018. "Exosomes and Ectosomes in Intercellular Communication." *Current Biology*. https://doi.org/10.1016/j.cub.2018.01.059.

Melo, Sonia A., Linda B. Luecke, Christoph Kahlert, Agustin F. Fernandez, Seth T. Gammon, Judith Kaye, Valerie S. LeBleu, et al. 2015. "Glypican-1 Identifies Cancer Exosomes and Detects Early Pancreatic Cancer." *Nature*. https://doi.org/10.1038/nature14581.

Melo, Sonia A., Hikaru Sugimoto, Joyce T. O'Connell, Noritoshi Kato, Alberto Villanueva, August Vidal, Le Qiu, et al. 2014. "Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis." *Cancer Cell*. https://doi.org/10.1016/j.ccell.2014.09.005.

Micalizzi, Douglas S, Susan M Farabaugh, and Heide L Ford. 2010. "Epithelial-Mesenchymal Transition in Cancer: Parallels between Normal Development and Tumor Progression." *Journal of Mammary Gland Biology and Neoplasia*. https://doi.org/10.1007/s10911-010-9178-9.

Mu, Wei, Sanyukta Rana, and Margot Zöller. 2013. "Host Matrix Modulation by Tumor Exosomes Promotes Motility and Invasiveness." *Neoplasia*. https://doi.org/10.1593/neo.13786.

Nazarenko, Irina, Sanyukta Rana, Alexandra Baumann, Jessica McAlear, Andrea Hellwig, Michael Trendelenburg, Günter Lochnit, Klaus T. Preissner, and Margot Zöller. 2010. "Cell Surface Tetraspanin Tspan8 Contributes to Molecular Pathways of Exosome-Induced Endothelial Cell Activation." *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-09-2470.

Niel, Guillaume van, Gisela D'Angelo, and Graça Raposo. 2018. "Shedding Light on the Cell Biology of Extracellular Vesicles." *Nature Reviews. Molecular Cell Biology*. https://doi.org/10.1038/nrm.2017.125.

Nilsson, J., J. Skog, A. Nordstrand, V. Baranov, L. Mincheva-Nilsson, X. O. Breakefield, and A. Widmark. 2009. "Prostate Cancer-Derived Urine Exosomes: A Novel Approach to Biomarkers for Prostate Cancer." *British Journal of Cancer*. https://doi.org/10.1038/sj.bjc.6605058.

Noy, Roy, and Jeffrey W. Pollard. 2014. “Tumor-Associated Macrophages: From Mechanisms to Therapy.” *Immunity*. https://doi.org/10.1016/j.immuni.2014.06.010.

Ogawa, Yuko, Yuri Miura, Akira Harazono, Masami Kanai-Azuma, Yoshihiro Akimoto, Hayato Kawakami, Teruhide Yamaguchi, et al. 2011. “Proteomic Analysis of Two Types of Exosomes in Human Whole Saliva.” *Biological & Pharmaceutical Bulletin*.

Ojalvo, L. S., C. A. Whittaker, J. S. Condeelis, and J. W. Pollard. 2009. “Gene Expression Analysis of Macrophages That Facilitate Tumor Invasion Supports a Role for Wnt-Signaling in Mediating Their Activity in Primary Mammary Tumors.” *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.0902360.

Orimo, Akira, Piyush B. Gupta, Dennis C. Sgroi, Fernando Arenzana-Seisdedos, Thierry Delaunay, Rizwan Naeem, Vincent J. Carey, Andrea L. Richardson, and Robert A. Weinberg. 2005. “Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis through Elevated SDF-1/CXCL12 Secretion.” *Cell*. https://doi.org/10.1016/j.cell.2005.02.034.

Ostrowski, Matias, Nuno B. Carmo, Sophie Krumeich, Isabelle Fanget, Graça Raposo, Ariel Savina, Catarina F. Moita, et al. 2010. “Rab27a and Rab27b Control Different Steps of the Exosome Secretion Pathway.” *Nature Cell Biology*. https://doi.org/10.1038/ncb2000.

Peinado, Héctor, Maša Alečković, Simon Lavotshkin, Irina Matei, Bruno Costa-Silva, Gema Moreno-Bueno, Marta Hergueta-Redondo, et al. 2012. “Melanoma Exosomes Educate Bone Marrow Progenitor Cells toward a Pro-Metastatic Phenotype through MET.” *Nature Medicine*. https://doi.org/10.1038/nm.2753.

Peinado, Héctor, Simon Lavotshkin, and David Lyden. 2011. “The Secreted Factors Responsible for Pre-Metastatic Niche Formation: Old Sayings and New Thoughts.” *Seminars in Cancer Biology*. https://doi.org/10.1016/j.semcancer.2011.01.002.

Pisitkun, T., R.-F. Shen, and M. A. Knepper. 2004. “Identification and Proteomic Profiling of Exosomes in Human Urine.” *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.0403453101.

Pollard, Jeffrey W. 2004. “Tumour-Educated Macrophages Promote Tumour Progression and Metastasis.” *Nature Reviews Cancer*. https://doi.org/10.1038/nrc1256.

Pols, Maaike S., and Judith Klumperman. 2009. “Trafficking and Function of the Tetraspanin CD63.” *Experimental Cell Research*. https://doi.org/10.1016/j.yexcr.2008.09.020.

Qian, Bin-Zhi, and Jeffrey W Pollard. 2010. “Macrophage Diversity Enhances Tumor Progression and Metastasis.” *Cell*. https://doi.org/10.1016/j.cell.2010.03.014.

Radford, K J, R Thome, and P Hersey. 1997. “Regulation of Tumour Cell Motility and Migration by CD63 in a Human Melanoma Cell Line.” *Melanoma Research*. https://doi.org/10.1097/00008390-199706001-00095.

Rahib, Lola, Benjamin D. Smith, Rhonda Aizenberg, Allison B. Rosenzweig, Julie M. Fleshman, and Lynn M. Matrisian. 2014. “Projecting Cancer Incidence and Deaths to 2030: The Unexpected Burden of Thyroid, Liver, and Pancreas Cancers in the United States.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-14-0155.

Rahman, Mohammad A., Jennifer F. Barger, Francesca Lovat, Min Gao, Gregory A. Otterson, and Patrick Nana-Sinkam. 2016. “Lung Cancer Exosomes as Drivers of Epithelial Mesenchymal Transition.” *Oncotarget*. https://doi.org/10.18632/oncotarget.10243.

Raimondi, Sara, Albert B. Lowenfels, Antonio M. Morselli-Labate, Patrick Maisonneuve, and Raffaele Pezzilli. 2010. “Pancreatic Cancer in Chronic Pancreatitis; Aetiology, Incidence, and Early Detection.” *Best Practice & Research Clinical Gastroenterology*. https://doi.org/10.1016/j.bpg.2010.02.007.

Raposo, G, H W Nijman, W Stoorvogel, R Liejendekker, C V Harding, C J Melief, and H J Geuze. 1996. “B Lymphocytes Secrete Antigen-Presenting Vesicles.” *The Journal of Experimental Medicine*.

Raposo, Graça, and Willem Stoorvogel. 2013. “Extracellular Vesicles: Exosomes, Microvesicles, and Friends.” *Journal of Cell Biology*. https://doi.org/10.1083/jcb.201211138.

Rawla, Prashanth, Tagore Sunkara, and Vinaya Gaduputi. 2019. “Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors.” *World Journal of Oncology*. https://doi.org/10.14740/wjon1166.

Robbins, Paul D., and Adrian E. Morelli. 2014. “Regulation of Immune Responses by

Extracellular Vesicles." *Nature Reviews Immunology*. https://doi.org/10.1038/nri3622.

Seymour, Albert B., Ralph H. Hruban, Mark Redston, Carlos Caldas, Steve M. Powell, Kenneth W. Kinzler, Charles J. Yeo, and Scott E. Kern. 1994. "Allelotype of Pancreatic Adenocarcinoma." *Cancer Research*.

Siegel, Rebecca L, and et al Miller. 2018. "Cancer Statistics, 2018." *Ca Cancer J Clin*. https://doi.org/10.3322/caac.21387.

Silva, Miguel, and A Melo. 2017. "The Biology of Cancer Exosomes : Insights and New Perspectives," 1–10. https://doi.org/10.1158/0008-5472.CAN-17-0994.

Simons, Mikael, and Graça Raposo. 2009. "Exosomes - Vesicular Carriers for Intercellular Communication." *Current Opinion in Cell Biology*. https://doi.org/10.1016/j.ceb.2009.03.007.

Skog, Johan, Tom Würdinger, Sjoerd van Rijn, Dimphna H. Meijer, Laura Gainche, William T. Curry, Bob S. Carter, Anna M. Krichevsky, and Xandra O. Breakefield. 2008. "Glioblastoma Microvesicles Transport RNA and Proteins That Promote Tumour Growth and Provide Diagnostic Biomarkers." *Nature Cell Biology*. https://doi.org/10.1038/ncb1800.

Stenmark, Harald. 2009. "Rab GTPases as Coordinators of Vesicle Traffic." *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm2728.

Stoorvogel, Willem, Ger J. Strous, Hans J. Geuze, Viola Oorschot, and Alan L. Schwartzt. 1991. "Late Endosomes Derive from Early Endosomes by Maturation." *Cell*. https://doi.org/10.1016/0092-8674(91)90459-C.

Suda, Kenichi, Kenji Tomizawa, and Tetsuya Mitsudomi. 2010. "Biological and Clinical Significance of KRAS Mutations in Lung Cancer: An Oncogenic Driver That Contrasts with EGFR Mutation." *Cancer and Metastasis Reviews*. https://doi.org/10.1007/s10555-010-9209-4.

Suetsugu, Atsushi, Kimi Honma, Shigetoyo Saji, Hisataka Moriwaki, Takahiro Ochiya, and Robert M. Hoffman. 2013. "Imaging Exosome Transfer from Breast Cancer Cells to Stroma at Metastatic Sites in Orthotopic Nude-Mouse Models." *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2012.08.007.

Tadokoro, Hiroko, Tomohiro Umezu, Kazuma Ohyashiki, Toshihiko Hirano, and Junko H. Ohyashiki. 2013. “Exosomes Derived from Hypoxic Leukemia Cells Enhance Tube Formation in Endothelial Cells.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M113.480822.

Tanaka, Masao. 2014. “Thirty Years of Experience with Intraductal Papillary Mucinous Neoplasm of the Pancreas: From Discovery to International Consensus.” *Digestion*. https://doi.org/10.1159/000370111.

Taylor, Douglas D., and Cicek Gercel-Taylor. 2008. “MicroRNA Signatures of Tumor-Derived Exosomes as Diagnostic Biomarkers of Ovarian Cancer.” *Gynecologic Oncology*. https://doi.org/10.1016/j.ygyno.2008.04.033.

Théry, Clotilde, Sebastian Amigorena, Graça Raposo, and Aled Clayton. 2006. “Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids.” *Current Protocols in Cell Biology*. https://doi.org/10.1002/0471143030.cb0322s30.

Théry, Clotilde, Laurence Zitvogel, and Sebastian Amigorena. 2002. “Exosomes: Composition, Biogenesis and Function.” *Nature Reviews Immunology*. https://doi.org/10.1038/nri855.

Toro, Julieta De, Leticia Herschlik, Claudia Waldner, and Claudia Mongini. 2015. “Emerging Roles of Exosomes in Normal and Pathological Conditions: New Insights for Diagnosis and Therapeutic Applications.” *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2015.00203.

Tricarico, Christopher, James Clancy, and Crislyn D’Souza-Schorey. 2017. “Biology and Biogenesis of Shed Microvesicles.” *Small GTPases*. https://doi.org/10.1080/21541248.2016.1215283.

Turati, F., V. Edefonti, C. Bosetti, M. Ferraroni, M. Malvezzi, S. Franceschi, R. Talamini, et al. 2013. “Family History of Cancer and the Risk of Cancer: A Network of Case-Control Studies.” *Annals of Oncology*. https://doi.org/10.1093/annonc/mdt280.

Valadi, Hadi, Karin Ekström, Apostolos Bossios, Margareta Sjöstrand, James J. Lee, and Jan O. Lötvall. 2007. “Exosome-Mediated Transfer of MRNAs and MicroRNAs Is a Novel Mechanism of Genetic Exchange between Cells.” *Nature Cell Biology* 9 (6): 654–59. https://doi.org/10.1038/ncb1596.

Vanlandingham, Phillip A., and Brian P. Ceresa. 2009. “Rab7 Regulates Late Endocytic Trafficking Downstream of Multivesicular Body Biogenesis and Cargo Sequestration.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M809277200.

Vella, L. J., R. A. Sharples, V. A. Lawson, C. L. Masters, R. Cappai, and A. F. Hill. 2007. “Packaging of Prions into Exosomes Is Associated with a Novel Pathway of PrP Processing.” *Journal of Pathology*. https://doi.org/10.1002/path.2145.

Vong, Sylvia, and Raghu Kalluri. 2011. “The Role of Stromal Myofibroblast and Extracellular Matrix in Tumor Angiogenesis.” *Genes and Cancer*. https://doi.org/10.1177/1947601911423940.

Wasmuth, Elizabeth V., Kurt Januszyk, and Christopher D. Lima. 2014. “Structure of an Rrp6-RNA Exosome Complex Bound to Poly(A) RNA.” *Nature*. https://doi.org/10.1038/nature13406.

Webber, J. P., L. K. Spary, A. J. Sanders, R. Chowdhury, W. G. Jiang, R. Steadman, J. Wymant, et al. 2015. “Differentiation of Tumour-Promoting Stromal Myofibroblasts by Cancer Exosomes.” *Oncogene*. https://doi.org/10.1038/onc.2013.560.

Webber, Jason, Robert Steadman, Malcolm D. Mason, Zsuzsanna Tabi, and Aled Clayton. 2010. “Cancer Exosomes Trigger Fibroblast to Myofibroblast Differentiation.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-10-1722.

Wells, Alan, Clayton Yates, and Christopher R. Shepard. 2008. “E-Cadherin as an Indicator of Mesenchymal to Epithelial Reverting Transitions during the Metastatic Seeding of Disseminated Carcinomas.” *Clinical and Experimental Metastasis*. https://doi.org/10.1007/s10585-008-9167-1.

Westphalen, Christoph Benedikt, and Kenneth P. Olive. 2012. “Genetically Engineered Mouse Models of Pancreatic Cancer.” *Cancer Journal (United States)*. https://doi.org/10.1097/PPO.0b013e31827ab4c4.

Whiteside, Theresa L. 2013. “Immune Modulation of T-Cell and NK (Natural Killer) Cell Activities by TEXs (Tumour-Derived Exosomes).” *Biochemical Society Transactions*. https://doi.org/10.1042/bst20120265.

Wieckowski, E. U., C. Visus, M. Szajnik, M. J. Szczepanski, W. J. Storkus, and T. L.

Whiteside. 2009. “Tumor-Derived Microvesicles Promote Regulatory T Cell Expansion and Induce Apoptosis in Tumor-Reactive Activated CD8+ T Lymphocytes.” *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.0900970.

Wolfers, Joseph, Anne Lozier, Graça Raposo, Armelle Regnault, Clotilde Théry, Carole Masurier, Caroline Flament, et al. 2001. “Tumor-Derived Exosomes Are a Source of Shared Tumor Rejection Antigens for CTL Cross-Priming.” *Nature Medicine*. https://doi.org/10.1038/85438.

Xu, Zhihong, Alain Vonlaufen, Phoebe A. Phillips, Eva Fiala-Beer, Xuguo Zhang, Lu Yang, Andrew V. Biankin, et al. 2010. “Role of Pancreatic Stellate Cells in Pancreatic Cancer Metastasis.” *The American Journal of Pathology*. https://doi.org/10.2353/ajpath.2010.090899.

Ye, Yun, Su-Liang Li, Yue-Yun Ma, Yan-Jun Diao, Liu Yang, Ming-Quan Su, Zhuo Li, et al. 2017. “Exosomal MiR-141-3p Regulates Osteoblast Activity to Promote the Osteoblastic Metastasis of Prostate Cancer.” *Oncotarget*. https://doi.org/10.18632/oncotarget.22014.

Zhang, Lin, Siyuan Zhang, Jun Yao, Frank J. Lowery, Qingling Zhang, Wen Chien Huang, Ping Li, et al. 2015. “Microenvironment-Induced PTEN Loss by Exosomal MicroRNA Primes Brain Metastasis Outgrowth.” *Nature*. https://doi.org/10.1038/nature15376.

Zheng, Peiming, Qin Luo, Weiwei Wang, Junhua Li, Tingting Wang, Ping Wang, Lei Chen, et al. 2018. “Tumor-Associated Macrophages-Derived Exosomes Promote the Migration of Gastric Cancer Cells by Transfer of Functional Apolipoprotein E.” *Cell Death and Disease*. https://doi.org/10.1038/s41419-018-0465-5.

Zhou, Weiying, Miranda Y. Fong, Yongfen Min, George Somlo, Liang Liu, Melanie R. Palomares, Yang Yu, et al. 2014. “Cancer-Secreted MiR-105 Destroys Vascular Endothelial Barriers to Promote Metastasis.” *Cancer Cell*. https://doi.org/10.1016/j.ccr.2014.03.007.

Zhu, Hongyan, and Guo-Chang Fan. 2011. “Extracellular/Circulating MicroRNAs and Their Potential Role in Cardiovascular Disease.” *American Journal of Cardiovascular Disease*.

Zhuang, Guanglei, Xiumin Wu, Zhaoshi Jiang, Ian Kasman, Jenny Yao, Yinghui Guan,

Jason Oeh, et al. 2012. “Tumour-Secreted MiR-9 Promotes Endothelial Cell Migration and Angiogenesis by Activating the JAK-STAT Pathway.” *EMBO Journal*. https://doi.org/10.1038/emboj.2012.183.

Zitvogel, Laurence, Armelle Regnault, Anne Lozier, Joseph Wolfers, Caroline Flament, Danielle Tenza, Paola Ricciardi-Castagnoli, Graça Raposo, and Sebastian Amigorena. 1998. “Eradication of Established Murine Tumors Using a Novel Cell-Free Vaccine: Dendritic Cell-Derived Exosomes.” *Nature Medicine*. https://doi.org/10.1038/nm0598-594.

Zomer, Anoek, Carrie Maynard, Frederik Johannes Verweij, Alwin Kamermans, Ronny Schäfer, Evelyne Beerling, Raymond Michel Schiffelers, et al. 2015. “In Vivo Imaging Reveals Extracellular Vesicle-Mediated Phenocopying of Metastatic Behavior.” *Cell*. https://doi.org/10.1016/j.cell.2015.04.042.

Zomer, Anoek, Sander Christiaan Steenbeek, Carrie Maynard, and Jacco Van Rheenen. 2016. “Studying Extracellular Vesicle Transfer by a Cre-LoxP Method.” *Nature Protocols*. https://doi.org/10.1038/nprot.2015.138.