MASTER'S THESIS

Relationship of mitochondrial dynamics with aggressive phenotypes of pancreatic cancer: pluripotency and migration

Relación de la dinámica mitocondrial con fenotipos agresivos de cáncer de páncreas: pluripotencia y migración

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1. ABBREVIATIONS

cDNA   Complementary DNA

CO2   Carbon dioxide

CSC   Cancer Stem Cells

CV   Crystal Violet

DFS   Disease-Free Survival

DMEM   Dulbecco's modified Eagle's media

DNM2   Dynamin 2

DRP1   Dynamin-related protein 1

EMT   Epithelial-to-Mesenchymal Transition

ER   Endoplasmic Reticulum

ERK1/2   Extracellular signal regulated kinase 1/2

FBS   Fetal Bovine Suerum

FGF   Fibroblast Growth Factor

FIS1   Mitochondiral Fission 1 Protein

GEPIA   Gen Expression Profiling Interactive Analysis

GTEx   Genotype-Tissue Expression

GTPases   Guanosine Triphosphatases

HPRT   Hypoxanthine Phosphoribosyltransferase 1

IMM   Inner Mitochondrial Membrane

IMS   Inner Mitochondrial Space

JNK   c-Jun N-terminal kinase (Jnk)

MAPK   Mitogen-Activated Protein Kinase

MCM   Macrophage-Conditioned Media

MFF	Mitochondrial Fission Factor
MFN1	Mitofusin 1
MFN2	Mitofusin 2
mDivi-1	Mitochondrial Division Inhibitor 1
MFF	Mitochondrial fission factor
Mid49	Mitochondrial Dynamic protein of 49
Mid51	Mitochondrial Dynamic protein of 51
OMM	Outer Mitocondrial Membrane
OPA1	Optic Atrophy 1
PAAD	Pancreatic Adenocarcinoma
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate-buffered saline
PC	Pancreatic Cancer
PDAC	Pancreatic Ductal Adenocarcinoma Cancer
PDX	Patient-Derived Xenografts
PGC1-α	Transcriptional coactivator peroxisome proliferator-activator receptor gamma coactivator 1 alpha
PPI	Protein-Protein Interaction
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative Real-Time Polym
RPMI	Roswell Park Memorial Institute media
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error Mean
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TCGA	The Cancer Genome Atlas Program

TME   Tumoral microenvironment

2. ABSTRACT

Pancreatic cancer is a type of cancer that acquires therapeutic resistance over time. The presence of CSCs and epithelial-to mesenchymal transition gives it a highly aggressive profile. The importance of mitochondria in all cellular processes, both physiological and pathological, leads to consider that it has an involvement in tumor processes. The overexpression of genes involved in the process of pluripotency, epithelium-mesenchymal transition and in mitochondrial dynamics indicate a worse prognosis for these patients. Our results confirmed that CSC-enriched samples present over-expression of pluripotency genes, this allowed to select them to determine the increase of mitochondrial dynamics in CSCs compared to differentiated cells, without being able to determine which mitochondrial activity was predominant. Epithelium-to-mesenchyme transition allows the tumor to spread throughout the body, ending in metastasis. The migration process was slowed down when we treated the cells with mDivi-1 whose function is to inhibit DRP1, this protein involved in mitochondrial fission acts on the progression, migration and tumor invasion.

3. RESUMEN

En cáncer de páncreas es un tipo de cancer que adquiere resistencia terapéutica con el tiempo. La presencia de CSCs y la transición epitelio a mesénquima le otorga un perfil altamente agresivo. La importancia de las mitocondrias en todos los procesos celulares, tanto fisiológicos como patológicos, lleva a considerar que tiene una implicación en los procesos tumorales. La sobreexpresión de los genes implicados el proceso de pluripotencia, transición epitelio-mesénquima y en la dinámica mitocondrial indican un peor pronóstico para estos pacientes. Nuestros resultados confirmaron que las muestras enriquecidas de CSCs presentan sobreexpresión de los genes de pluripotencia, esto permitió seleccionarlas para determinar un aumento de dinámica mitocondrial en CSCs comparado con células diferenciadas, sin poder determinar qué actividad mitocondrial predominaba. La transición epitelio a mesénquima permite que el tumor se disemine por el organismo, de manera que termina en metástasis. El proceso migratorio se vio frenado cuando tratamos las células con mDivi-1 cuya función es inhibir *DRP1*, esta proteína implicada en la fisión mitocondrial actúa sobre la progresión, migración e invasión tumoral.

4. INTRODUCTION

Based on GLOBOCAN 2018 statistics, pancreatic cancer (PC) mainly affects Asia, followed by Europe with a 28.9% incidence (1). Within the Spanish framework, PC is not the most diagnosed type of cancer, only 2.5% of tumors are diagnosed (2). However, it is the third most deadly cancer at 6.4% (2)(3).

4.1. PANCREATIC CANCER

Pancreatic ductal adenocarcinoma (PDAC) is heterogeneous and highly mutated. It begins with acinar-ductal metaplasia, and as genetic mutations accumulate, neoplastic intraepithelial lesions (PanIN) occur leading to invasive carcinoma with a largely metastatic end (4)(5)(6). 90% of cases arise sporadically, however, hereditary cases account for 10%. Older age, smoking, chronic pancreatitis, chemicals, obesity and diabetes are often the most relevant risk factors (5)(7)(8).

Symptoms progress over time, in the early stages it is usually an asymptomatic disease, only 10% of patients are diagnosed early with possible benefits of surgical resection for cure (5)(8)(9) However, in later stages the symptoms are more evident and this is when it is diagnosed, but there is no chance of a cure (5). Survival rate is low, 8% of patients achieve 5-year survival (9).

Molecular therapies are based on the activation and inhibition of molecules involved in pathways of growth, progression, invasion and metastasis (5). The first line of treatment is the combination of FOLFIRINOX (leucovorin, 5-FU, irinotecan and oxaliplatin) or gemcitabine with nab-paclitaxel, prolonging survival from 6 to 12 months (8)(9). As a second line, NAPOLI-1-2015 (nano-liposomal irinotecan with 5-FU and leucovorin) (10).

Many mutations have been seen in PDAC, hence its genetic instability. *KRAS* activation and *CDKN2A* inhibition are seen in 90-95% of cases, while TP53 inhibition is seen in 75% of cases (4). At the onset of the disease there is a low grade PanIN where *KRAS* mutation has been seen, so this suggests that there are early changes in tumor progression. However, *TP53* and *SMAD4* mutations are seen in a high degree of PanIN and pancreatic adenocarcinoma (PAAD) (11) (Figure 1).
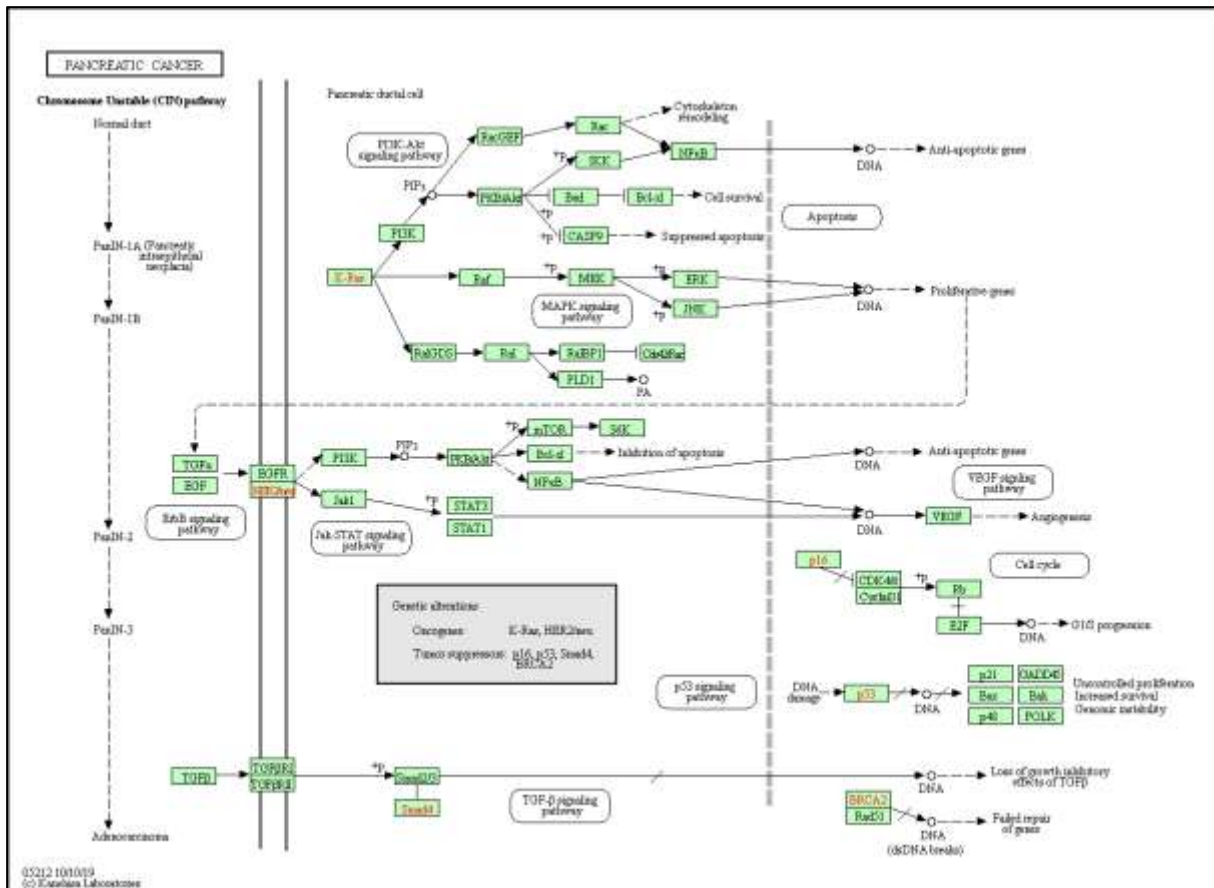
Figure 1. Molecular pathway in pancreatic cancer. *Schedule represents the different molecular pathways in each step of the disease. (KEGG, hsa05212)*

The failure of the treatment is usually due to the tumor microenvironment (TME) that favours the formation of an ideal niche that supports the tumor cells. In this environment, extracellular matrix, fibroblasts, activated pancreatic stellate cells and immune cells stand out (8). This cell group acts as a mechanical barrier where there is a decrease in vascularization and, therefore, the drugs do not reach the target cell (6). So when therapy fails, the tumor will grow back quickly and uncontrollably (4)(6).

Another cause of therapeutic failure is the presence of cancer stem cells (CSCs) in tumor. These cells have the ability to self-renew so that they provide resistance to chemotherapy, tumor initiation and evasion of the primary tumor. Therefore, the prognosis of PDAC patients should be improve if CSCs were removed. (5)(8)(12)(13)(14).

4.2. MITOCHONDRIAL DYNAMICS

Mitochondria are highly dynamic organelles that participate in different functions such as growth, division, metabolism and apoptosis (12). They form a kind of dynamic network is

formed that varies morphologically in response to cellular stimuli (12)(13). The changes in the morphology and content of the mitochondria are given by the mitochondrial dynamics which consists of the balance between fission and fusion (14). When cells do not have enough nutrients, mitochondrial fusion protein allows mitochondria fuse together to share nutrients and maintain OXPHOS. In contrast, mitochondrial fission fragments the mitochondria for mitochondria movement to regions of high energy demand (13).
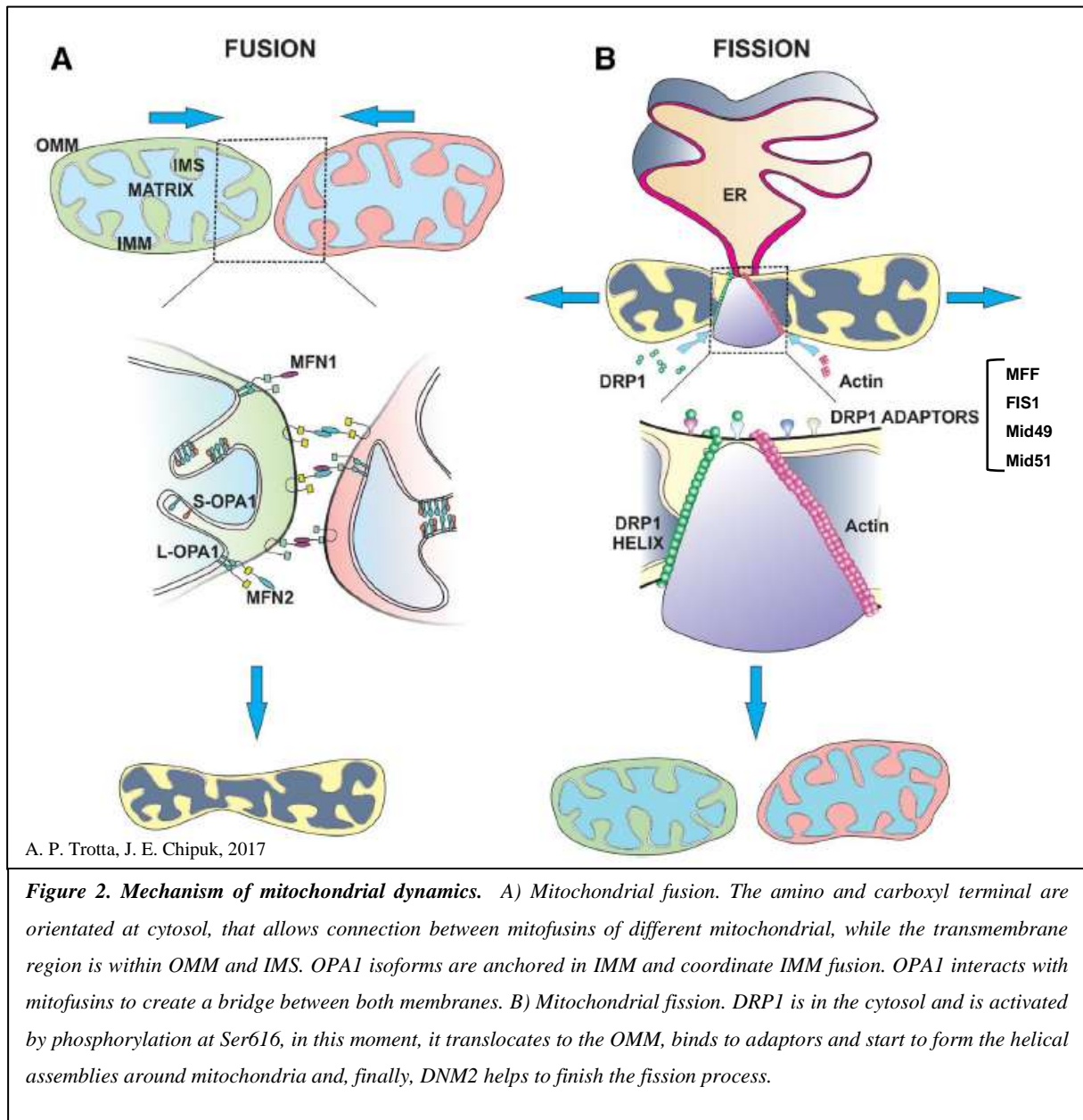
4.2.1. MOLECULAR MECHANISM

Mitochondria is composed of two membranes; outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) between both there are inner mitochondrial space (IMS) (13). In these membranes are located guanosine triphosphatases (GTPases) that regulate mitochondrial dynamics (15). GTPases for mitochondria fusion are: mitofusin 1 (*MFN1*), mitofusin 2 (*MFN2*) and optical atrophy 1 (*OPA1*), however, for mitochondrial fission is dynamine-related protein 1 (*DRP1*) with *DRP1* protein adapters: mitochondria fission factor (*MFF*), fission protein 1 (*FIS1*), mitochondrial dynamic protein 49 (*Mid49*) and 51 (*Mid51*) (16)

Mitochondrial fusion consists of the fusion of the OMM by the action of the *MFN1* and *MFN2* followed by the fusion of the IMM mediated by *OPA1* (14). This process is important for maintaining the quality of the mitochondria. In a situation where mitochondrial fusion predominates, the number of mitochondria and OXPHOS is reduced. The absence of both mitofusins means that there is no mitochondrial fusion, however in the absence of *OPA1* the OMM is fused, but not the IMM, so that the cells drift into mitochondrial fission (Figure 2A) (17).

Mitochondrial fission starts with the interaction of the endoplasmic reticulum (ER) and mitochondria, where takes place the first constriction. Likewise, *DRP1,* which is found in the cytoplasm, is recruited on the mitochondrial surface to associate with *MFF, FIS1, Mid49* and *Mid51* (14). So, *DRP1* forms helical assemblies around mitochondria, but for the last excision step, dynamin 2 (*DNM2*) is necessary for severing mitochondria (14). Fission process occurs when there are high energy demand from the cells (Figure 2B)(18).

MYC, mTOR (PI3K/Akt) and MAPK act on the signalling cascade of transcriptional coactivator peroxisome proliferator-activator receptor gamma coactivator 1 alpha (PGC1-α)

which promotes the expression of mitochondrial genes encoded in nucleus by activating transcription factors and hormone receptor to induce mitochondrial biogenesis (13)(19)(20)(21). This regulation is discussed further in the following section about mitochondrial dynamics and cancer because these three pathways are implicated in proliferation and anti-apoptotic genes, therefore, tumoral progress.

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Figure 2. Mechanism of mitochondrial dynamics. A) Mitochondrial fusion. The amino and carboxyl terminal are orientated at cytosol, that allows connection between mitofusins of different mitochondrial, while the transmembrane region is within OMM and IMS. OPA1 isoforms are anchored in IMM and coordinate IMM fusion. OPA1 interacts with mitofusins to create a bridge between both membranes. B) Mitochondrial fission. DRP1 is in the cytosol and is activated by phosphorylation at Ser616, in this moment, it translocates to the OMM, binds to adaptors and start to form the helical assemblies around mitochondria and, finally, DNM2 helps to finish the fission process.

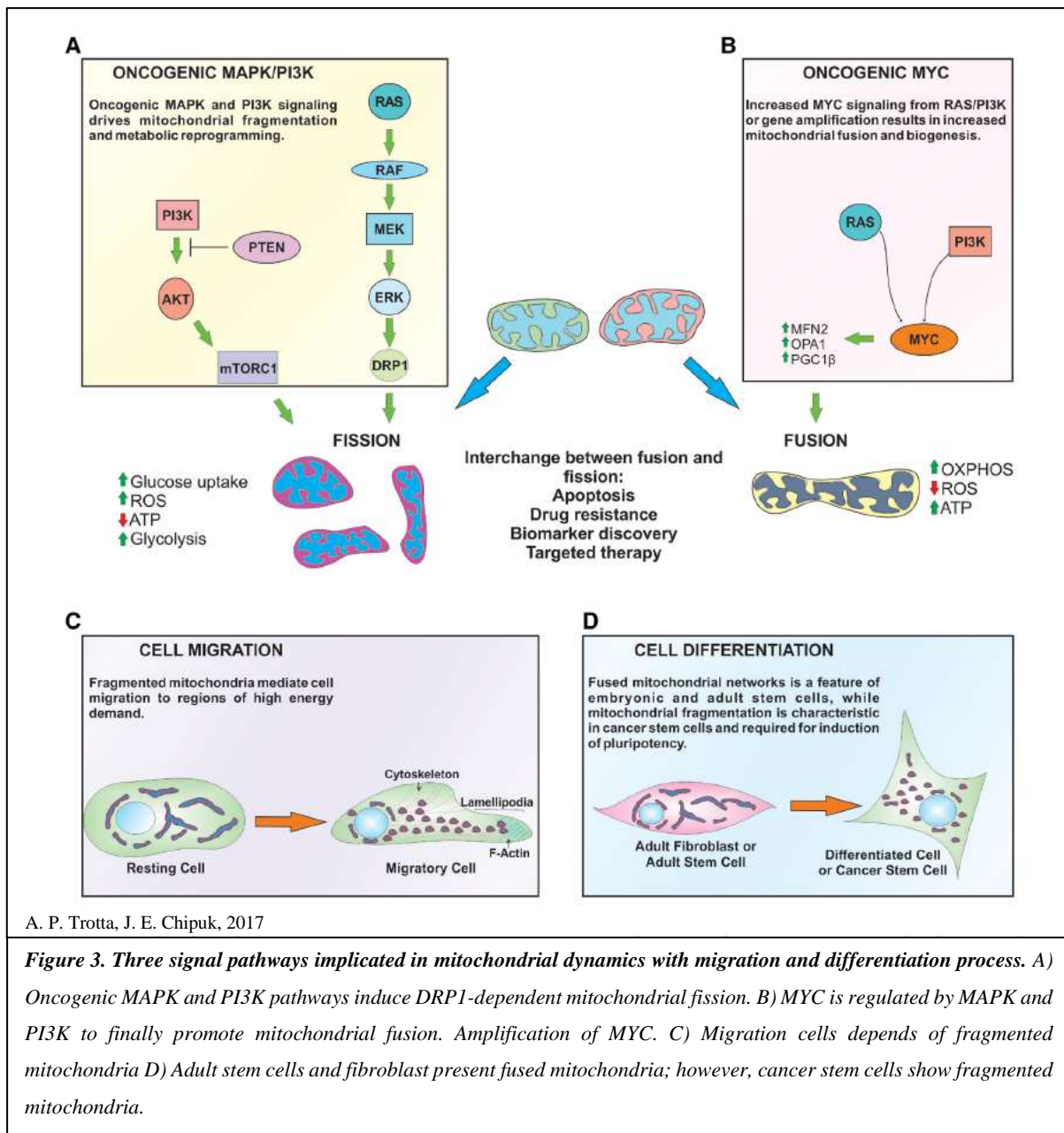### 4.2.2. MITOCHONDRIAL DYNAMICS IN CANCER

The energy demands of cancer cells during tumorigenesis and cancer progression induce changes in mitochondrial metabolism in order to support tumor proliferation and prevent apoptosis (13)(19). So, mitochondrial dynamics are closely related to the progression, migration

and invasion of tumor cells (12)(14)(22). Increased mitochondrial fission promotes tumor progression by inhibiting mitochondria-dependent apoptosis and activating autophagy (12). While increased fusion prevents invasion and metastasis, its deficiency favours the opposite, so it can be considered to act as a tumor suppressor (14).

The mutation in MAPK and PI3K-Akt or amplification of MYC influences the metabolic pathways by which cancer cells obtain the necessary products. MAPK pathway activate the signal cascade, finally, to phosphorylate to extracellular signal regulated kinase 1/2 (ERK1/2) and is activated. When there are KRAS mutation, activated ERK 1/2 can phosphorylate DRP1. DRP1 protein presents three residues that can be phosphorylated: S616 induces fission and S693 induces fusion, while S637 can induce both fusion and mitochondrial fission depending on cellular conditions (16)(18)(22). However, in the absence of KRAS the induction of mitochondrial fission is given by the mitogenic protein kinase (*MEK*) or the Raf (13)(22). On the other hand, mitochondrial fusion is also altered by the mutation in three pathways. The phosphorylation of MFN1 by ERK ½ at Tyr562 or c-Jun N-terminal kinase (Jnk) decrease the fusion activity, while mitochondrial fragmentation increases (Figure 3A) (16) (19).

In solid tumor such a PC, loss of PTEN, that is an inhibitor of PI3K, induces hyperactivation of PI3K-Akt pathway. PI3K mutated is similar to MAPK pathway, increase mitochondrial fragmentation, while inhibition of PI3K increase mitochondrial fusion. (Figure 3A) This pathway is important in autophagy and mitophagy where dysfunctional mitochondria is removed (13)

The two pathways described above can stimulate the expression of *MYC* (13). *MYC* participates in mitochondrial dynamics and induce the mitochondrial fusion to promote the biosynthesis of metabolites which are necessary for cell growth and proliferation (19). Oncogenic *MYC* is an activator of *PGC-1β* expression, thus is associated with increase of mitochondria mass and proliferation (13).

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Figure 3. Three signal pathways implicated in mitochondrial dynamics with migration and differentiation process. A) Oncogenic MAPK and PI3K pathways induce DRP1-dependent mitochondrial fission. B) MYC is regulated by MAPK and PI3K to finally promote mitochondrial fusion. Amplification of MYC. C) Migration cells depends of fragmented mitochondria D) Adult stem cells and fibroblast present fused mitochondria; however, cancer stem cells show fragmented mitochondria.

4.3. CANCER STEM CELLS (CSCs)

Stem cells are undifferentiated cells with unlimited capacity for proliferation and self-renewal, the latter process being important for tissue development and regeneration (8). In general, its self-renewal is kept in balance with the ability to differentiate itself through different mechanisms. A failure in this balance leads to many failures among which is the tumor process (23) The similarity between stem cells and tumor stem cells reveals that they have a similar metabolism (14). The Yamanaka factors (*OCT4, KLF4, SOX2* and c-*MYC*) allow the reprogramming of adult cells giving rise to stem cells, these are characterized by having a lower

number of mitochondria and also immature (12). CSCs reactivate the *MYC, SOX2*, and *OCT4* genes involved in self-renewal, while the *NANOG* gene is related to stem cells and CSCs (8)(24). The ability to induce tumors in other areas of the body is being shown to be due to this cell type (14).

The heterogeneity of PDAC is due to the different subpopulations present, each with different phenotypes and functions (6)(8)(25). One of them are CSCs have the ability to self-renew and provide resistance to chemotherapy, and are responsible for tumor initiation, evasion of the primary tumor, survival in anoikis conditions, and migration to other regions generating secondary tumors. (5)(8)(25)(24)(26).

Tumor cells require large amounts of energy for the synthesis of amino acids, lipids and nucleic acids, which are necessary for replication and division (12)(14). Pancreatic CSCs obtain their energy mostly from OXPHOS, while non-CSCs depend on glycolysis (25). The group discovered the importance of *PGC1α* in maintenance of OXPHOS function, self-renew and tumorigenic capacity (27). Particularly, *PGC1α* is associated with mitochondrial biogenesis, and maintains the renewal capacity of CSCs, thus favouring the spread of CSCs, tumor recurrence and metastasis (25) (28). The high levels of *PGC1α* in pancreatic CSC are due to a decrease in MYC expression, so that increase the functions performed by *PGC1α* (25).

4.4. EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

In PDAC the process of EMT favours the early spread of tumor cells inducing invasion and metastasis (29)(30). Tumor cells decreases the expression of epithelial markers while mesenchymal markers such as SNAIL1, SNAIL2 and ZEB1 increases expression level to acquire more mobility and invasiveness, and finally move to a metastatic phenotype (31). When they acquire these characteristics they are able to invade nearby tissues, and even pass into the bloodstream, reaching more distant areas (14). In cell spread process, cells synthesize cytoskeleton filaments to modify the shape of the cell and allow it to move to other regions, for the filaments synthesis is necessary large amounts of energy, so the mitochondria must be of great importance.

PDAC tumor presents a high amount of stroma that induces a hypoxic environment, so the EMT depends on the level of ROS and TME (32)(33). In a hypoxic situation the mitochondrial protein

DRP1 is able to remodel the cytoskeleton and increase the formation of lamellipodia, although in normal conditions happen too, so there is an increase in the mobility of microtubules depended on mitochondrial dynamics (Figure 3C)(19)(34).

In more detail, studies ,which compare between metastatic and non-metastatic cells in breast cancer, have allowed us to see how there is an increase in the expression of DRP1 and a decrease in mitofusins (14). In PDAC, *NF-kB* recruits and phosphorylates in S616 at *DRP1,* so this gene participates in the increase of mitochondrial fission. However, the increase in mitofusins reduces the migration process (14)(13), so there are studies try to study the inhibition of *DRP1* by mDivi-1 because increased mitochondrial fusion and thus slows down migration and tumor invasion (14).

5. OBJETIVES

In PDAC, the processes of proliferation and new tumor formation in CSCs depend on mitochondrial activity. Therefore, our study focuses on the relationship between mitochondrial dynamics and the processes of cell pluripotency and migration. We wanted to compare results and ideas about the relationship between these processes, so our objectives were the following:

1. To verify through bioinformatic tools the overexpression of pluripotency genes and see its impact on the survival of PDAC patients.
2. To study the levels of expression of the genes of mitochondrial dynamics in PDAC patients and samples enriched in CSCs.
3. To evaluate the effect of mitochondrial fission DRP1 on cell migration after its inhibition.

6. MATERIALS AND METHODS

6.1. Cell lines

Cell lines came from patient-derived xenografts (PDX) as tumor pieces. The tumor pieces were from Johns Hopkins Hospital (USA) and were isolated from the primary lesions of patient with resectable PDAC. Finally, the tumors were spread by Sancho's group under a procedure approved by the Ethics Committee from University of Zaragoza (ref. PI22/17).

6.2. Cell culture

All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 (1X) media with GlutaMAX (Gibco[TM], ThermoFisher Scientific, UK) supplemented with 10% of Fetal Bovine Serum (FBS) (Gibco[TM], ThermoFisher Scientific, UK) and 1% of penicillin/streptomycin (P/S) (Corning, USA). While Dulbecco's modified Eagle's F-12 media (DMEM) (Gibco[TM], ThermoFisher Scientific, UK) supplemented with 2% B27, 0.02% Fibroblast growth factor (FGF) (Gibco[TM], ThermoFisher Scientific, UK) and 1% P/S was used for experiments. In both cases, the cells were incubated at 37°C in a humidified incubator (Nuaire, USA) with 5% CO_2. On the other hand, sphere cultures were used to obtain a population rich in CSCs, so that can be analysed and compared with adherent cells. This process was carried out by the group following the guidelines of one of their articles (25).

6.3. Wound healing assay

The cells were seeded into 24-well plates at a density of $1.5 \cdot 10^5$ cells/well and incubated 5 days with supplemented RMPI media, to finally obtain a cellular monolayer. The following step was changing media, half the wells with DMEM F-12 media, and the other half with macrophage-conditioned media (MCM), and the plate was incubated 48 hours more. After that, the wound was made with the tip of a 20-μL pipette and washed several times with Phosphate-buffered saline (PBS) (ThermoFisher Scientific, UK) until complete removal of the dead or

unattached cells. After the last wash, the cells were treated with mDivi-1 (Selleckchem, S7172) at different concentrations: 0, 20 and 40 μM. The healing status of the wound was observed with Nikon Eclipse TS100 inverted binocular microscope (BIRIDEN, Uruguay) and photographed at 0h, 24h, 48h and six days after scratching. The scratch area was calculated by ImageJ and the wound closure was calculated with the next formula: ((Area 0h – Area 48h) / Area 0h) * 100.

6.4. Proliferation assay

The cells were seeded into 96-well plates at a density of $1.0 \cdot 10^4$ cells/well and incubated for 24h with supplemented RMPI media. After incubation, the plated cells were treated with mDivi-1 (Selleckchem, S7172) in DMEM F-12 media at different concentrations: 0, 20, 40 and 80 μM. The plate was incubated for 0h, three days and seven days. After that, the cells were stained with 0.2% crystal violet (CV) (GibcoTM, ThermoFisher Scientific, USA) for 30-45 min. After that, we removed the CV with a Pasteur pipette, washed the wells with tap water and, finally, we let the plate dry. Once the plate was dried, we added 1% Sodium dodecyl sulfate (SDS) and incubated it for 1h. After that, the plate was introduced into the spectrophotometer (Synergy HT, BioTek, USA) and absorbance read at 560 nm.

6.5. Real- Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Previously, Sancho's group prepared the cDNAs of the different cell lines for the qRT-PCR. Quantitative real-time PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystem, ThermoFisher Scientific, USA) and detected by StepOnePlusTM Real-Time PCR System (Applied BiosystemsTM, ThermoFisher Scientific, USA). The results were normalised with Hypoxanthine Phosphoribosyltransferase 1 (HPRT) mRNA for mRNA measurement. All the primers were designed (Thermo Fisher Custom Primers, InvitrogenTM, ThermoFisher Scientific, USA) by the director and are shown in Table 1. Each experiment was repeated two times in three, two or one biological samples.

	HOUSEKEEPING	
PRIMERS	**REVERSE**	**FORWARD**
HPRT	CGA GCA AGA CGT TCA GTC CT	TGA CCT TGA TTT ATT TTG CAT ACC

	MITOCHONDRIAL DYNAMIC	
PRIMERS	**REVERSE**	**FORWARD**
12S	CTA TTG ACT TGG GTT AAT CGT GTG A	CCA CGG GAA ACA GCA GTG AT
DRP1	AGG CAC CTT GGT CAT TCC TG	AGA AAA TGG GGT GGA AGC AGA
FIS1	AGG CCT TAA AGT ACG TCC GC	TGC CCA CGA GTC CAT CTT TC
DNM2	TCT CCC CCT GAT TGG GGA TG	AGA CAG AGC GAA TCG TCA CC
MFN1	GCC ATT ATG CTA AGT CTC CGC	GCC TCC TCT CCG CCT TTA AC
MFN2	GTC TCA GGT TGA GGT TGG CT	GGC AGC AGA GGC GTA AGG A
OPA1	GCG AGG CTG GTA GCC ATA TT	CTG TGG CCT GTG AGG TCT G

	PLURIPOTENCY	
PRIMERS	**REVERSE**	**FORWARD**
NANOG	TGC CAC CTC TTA GAT TTC ATT CTC T	AGA ACT CTC CAA CAT CCT GAA CCT
KLF4	ATG TGT AAG GCG AGG TGG TC	ACC CAC ACA GGT GAG AAA CC

SOX2	CGG GGC CGG TAT TTA TAA TC	AGA ACC CCA AGA TGC ACA AC
OCT3/4	CTG CAG TGT GGG TTT CGG GCA	CTT GCT GCA GAA GTG GGT GGA GGA A

Table 1. Reverse and forward primers used in qRT-PCR

6.6. Bioinformatic analysis and pathways construction

For the box plots comparing gene expression between normal and tumor tissues. We used the Pancreatic Adenocarcinoma (PAAD) dataset from The Cancer Genome Atlas Program (TCGA), which was compared with normal tissue database from TCGA and Genotype-Tissue Expression (GTEx). The box-plots were generated using the GEPIA2 (Gen Expression Profiling Interactive Analysis) webtool with Expression DIY tool (http://gepia2.cancer-pku.cn). The expression data were first log2(TPM+1) transformed and log2FC was defined as median of tumor and normal tissue. The method for differential analysis was one-way ANOVA, $*p \leq 0.01$.

The correlation plots were generated using PAAD database with TCGA tumor, TCGA normal and GTEx dataset, the data were analysed with Pearson correlation coefficient using the non-log scale for calculation and use the log-scale axis for visualization by GEPIA2 webtool.

Disease free survival (DFS) plots were generated PAAD dataset with survival plot tool by GEPIA2 webtool. The method analysis was Mantel-Cox test, median was selected for splitting the high-expression (50%) and low-expression (50%) cohorts and we inclued hazard ratio and 95% confidence interval.

The pathway construction was performed using the multiple proteins tool from STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org/) webtool. The parametres were: organism = homo-sapiens; meaning of network edges = molecular action; active interaction sources = all; minimum required interaction score = medium confidence (0.400); max number of interactors to show = none; and network display mode = interactive svg. The two parameters to take into account are: the average local clustering coefficient, i.e. the closer it is to 1, the probability of interaction is greater; and the other, the protein-protein

interaction (PPI) enrichment p-value, a lower p value means the proteins are more biologically connected.

6.7. Statistical analysis

The statistical analysis was performed using GraphPad Prism 8.0. The wound healing data was analysed by Krustal-Wallis test with $\alpha = 0.05$. Two-tailed Unpaired T test was used to analyse the ratio between fission and fusion genes. Differentiate expression of pluripotency genes was assessed by two-tailed Mann Whitney test. Two way-ANOVA was used to analyse the expression of mitochondrial dynamic genes, $\alpha = 0.05$. The proliferation assay was analysed with non-linear regression test, the selected equation was the user-defined equation = absolute IC50 from normalized data. All data are presented as mean $\pm$ SEM. We considered ***p < 0.001 very significant or *p < 0.05 significant.

7. RESULTS

7.1. *Network of connection between mitochondrial dynamics and the processes of pluripotency and migration*

Tumors in PDAC are composed of cellular subpopulations, which can be subjected to epithelial-to-mesenchymal transition to acquire more mobility and invasiveness, eventually achieving an aggressive phenotype. Within these subpopulations, CSCs stand out for being undifferentiated and for their ability to self-renew. In addition, they express different genes involved in the regulation of pluripotency, *NANOG*, *KLF4, SOX2*, and *OCT4*. Like other tumor cells, CSCs can also undergo the EMT process, acquiring an invasive capacity along with the characteristics they already possessed. In the other hand, previous studies by the group suggest that the process of mitochondrial dynamics may be essential for migration in CSC. Therefore, we wanted to study the relationship between the three processes.

First of all, we wanted to correlate mitochondrial dynamics with the other two pathways. For that, we used PAAD database from the TCGA tumor, TCGA normal and GTEx dataset correlation web tool by GEPIA2. In the one hand, we tried to correlate mitochondrial dynamics (*DNM1L, FIS1, DNM2, MFN1, MFN2* and *OPA1)* with pluripotency genes (*NANOG, SOX2, KLF4* and *OCT4)*, obtaining very significant positive results ($p = 0$) (Figure 4A). In the other hand, the mitochondrial dynamics and the EMT process (*SNAIL1, SNAIL2* and *ZEB1)* were also correlated and the results were significatively positive ($p = 0$) (Figure 4B). As described by other authors, the correlation between EMT and pluripotency was positive (Figure 1C) (35).

Once the positive correlation between the processes was confirmed, their connection was studied using the multiple proteins options in homo-sapiens organism by STRING. The results
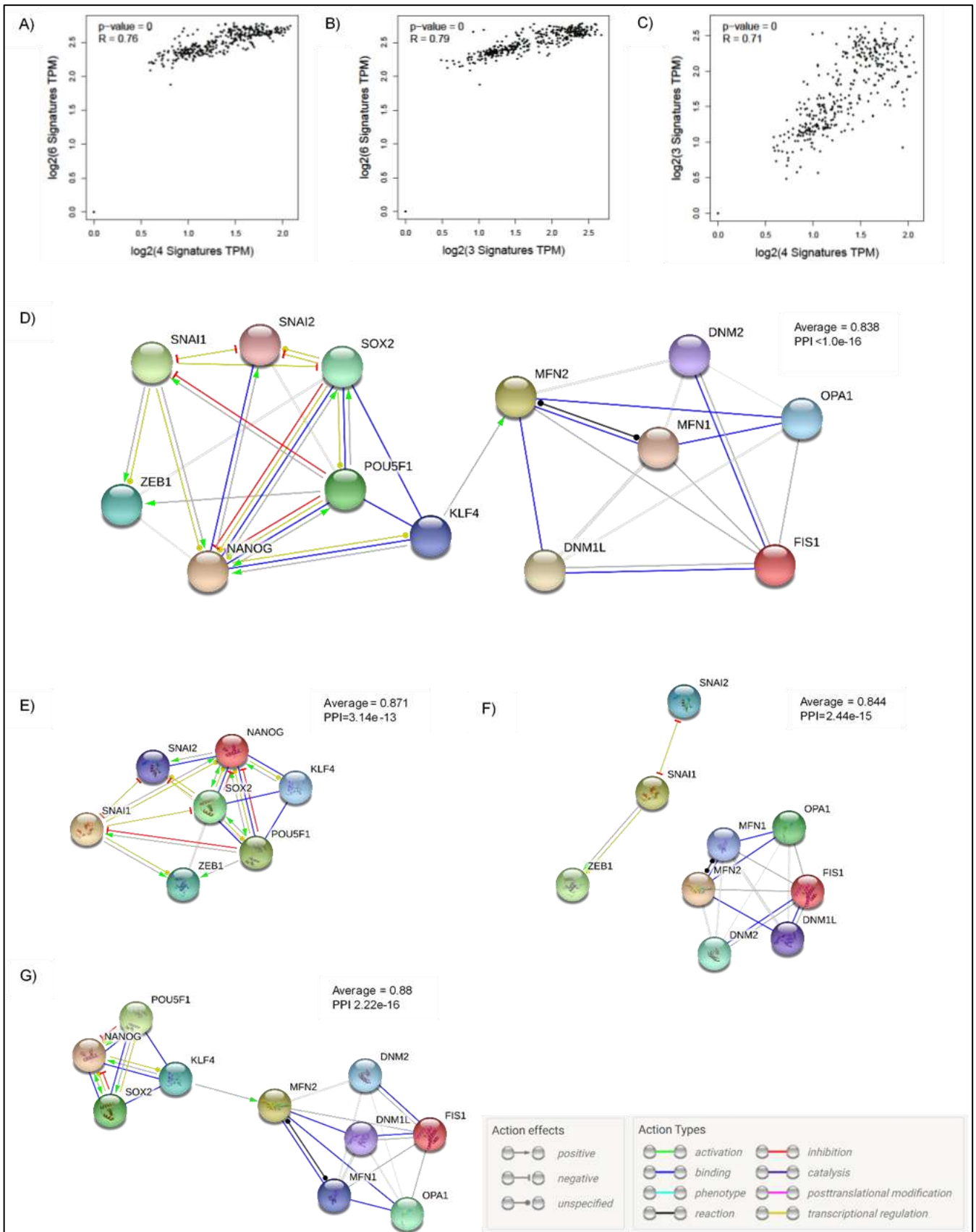
Figure 4. Representative schedule about the mitochondrial interaction with pluripotency and migration process. *Correlation graphs of A) mitochondrial dynamic and EMT. B) mitochondrial dynamic with pluripotency and C) pluripotency and EMT were obtained with Pearson coefficient by GEPIA2 (p = 0). Network connection between D) mitochondrial dynamic, pluripotency, and migration E) pluripotency and EMT F) EMT and mitochondrial dynamic and G) pluripotency and mitochondrial dynamic were created by STRING.*

was 0.838 and the PPI enrichment p-value was <1.0e-16, thus the probability that the interaction represented (Figure 4D) between the different proteins was close to maximum confidence.

As expected, the proteins encoded by the pluripotency genes presented a high number of interactions with those encoded by the EMT genes, the average of the resulting clustering coefficient was 0.871 and the PPI enrichment p-value was 3.14e-13 (Figure 4E). The interaction between mitochondrial dynamics and EMT did not present any point of interaction. However the values obtained were really good, because the average of coefficient was 0.844 and the PPI enrichment p-value was 2.44e-16 (Figure 1F). In the other hand, mitochondrial dynamic proteins presented one point of conection with the proteins encoded by plurpotency genes, the average of the resulting clustering coefficient was 0.88 and the PPI enrichment p-value was 2.22e-16 (Figure 4G).

Therefore, stemness may be interacting both with the pathway that regulates the EMT and with the proteins involved in mitochondrial dynamics. Moreover, with these connections it could be said that mitochondrial dynamics and the EMT process are indirectly related.

7.2. Increased expression of pluripotent genes indicates poorer survival in PDAC patients

In PDAC, the presence of CSCs has been associated with ineffective chemotherapy treatments, and therefore represents a worse prognosis for patients with chemoresistant tumors. The CSCs have different levels of pluripotency gene expression that allow them to be identified. Thus, the aim was to evaluate the level of expression of pluripotency genes (*NANOG*, *KLF4*, *SOX2* and *OCT4)* using expression tool by GEPIA2. The box-plot graphs showed increased expression in the tumor tissue with respect to normal tissue. In particular, *KLF4* gene was the only one that showed a significant increase ($p \leq 0.01$) (Figure 5A). However, although the other three genes did not show significative results, we were able to see the same tendency in the four genes. In addition, analysis of the expression signature of the four genes showed significant overexpression in the tumor tissue ($p \leq 0.01$) (Figure 5B).

In addition, we wanted to see if the expression of these genes had an impact on patient survival. For these analyses, we used the PAAD database from TCGA, the Kaplan-Meier was obtained with the survival plot tool by GEPIA2. The DFS graph allowed see how the level of expression

of pluripotency genes influences the survival rate, in other words, patients with low levels of expression survived longer than those with higher levels (Figure 5C).

As described by Heeschen's group, growth in spheres increases the proportion of CSCs (24). Therefore, we wanted to see if our enriched-CSC cultures from PDAC PDX showed an increase in the expression of the pluripotency genes with respect to differentiated cells.

The results obtained by qRT-PCR revealed an increase in the expression of all the genes in enriched-CSCs samples, and in particular, *NANOG* presented a very significant increase in the level of expression (p = 0.0006) and *OCT4* gene showed a significant increase as well (p = 0.0291) (Figure 5D).
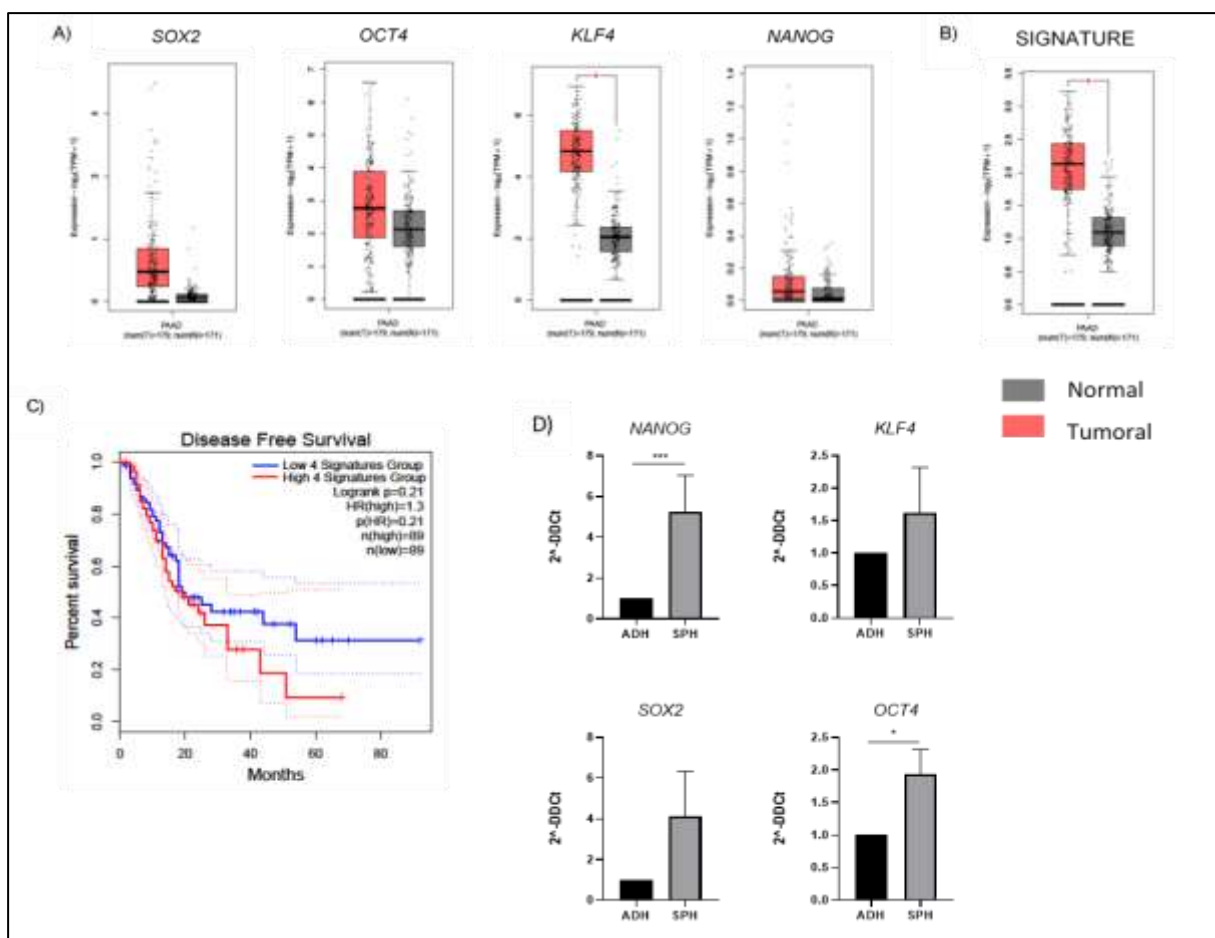

*Figure 5. The expression of pluripotency genes is increased in enriched CSCs, that shows worse survival patients. A) Differentiate expression of pluripotency genes and B) signature of pluripotency genes between tumour and normal tissue were obtained with one-way ANOVA by GEPIA2. *p < 0.01 C) Kaplan-Meier graph of disease-free survival in PAAD showed worse prognosis when there are high level expression of pluripotency genes and was created with Mantel-cox test by GEPIA2. D) Expression of pluripotency genes was increased in CSC-enriched samples PDAC, it was analysed by Mann Whitney test, ***p < 0.001, *p < 0.05. Data are presented as mean ± SEM. n=7*

In conclusion, expression of pluripotency genes was increased in PDAC, which was associated with reduced survival in patients. On the other hand, sphere culture was a useful model for CSC enrichment and because of that, we will use it in the next analysis.

7.3. *Increased expression of mitochondrial dynamics genes reveals a poor prognosis in PDAC*

Other studies have revealed that mitochondrial dynamics are involved in tumor progression and invasion due to a disturbance in the balance between mitochondrial fusion and fission (12). As with the pluripotency genes, their expression was analysed in both normal and tumor tissue with Expression DIY tool in PDAC patients by GEPIA2. The mitochondrial fission genes (*DRP1, FIS1* and *DNM2*) were significantly (p ≤ 0.01) increased in the tumor tissue with respect to normal (Figure 6A). However, while one of the genes involved in mitochondrial fusion (*MFN1*) exhibited low levels of expression in tumor tissue, the other fusion genes, *MFN2* and *OPA1*, were expressed significantly more in tumor (Figure 6B).

Although there were differences in expression individually, the joint signature of the six genes indicated a significant increase in PDAC (Figure 6C). DFS graph obtained by GEPIA2 showed that expression levels of mitochondrial dynamics genes had an impact on survival. The survival graphs for the signature of mitochondrial dynamics (p = 0.44) indicated that patients tended to have a higher survival when the expression levels of mitochondrial dynamics were low, while when its expression was high it had a negative impact on survival (Figure 6D). This was also observed significantly in the mitochondrial fusion graph (p = 0.013) (Figure 6F). However, the fission gene survival graph (p = 0.22) showed that patients tended to have better survival when expression levels were increased, while at low levels, patients had a worse prognosis (Figure 6E).

In conclusion, the genes of mitochondrial dynamics were, for the most part, overexpressed in PDAC. In addition, this increased expression was related to a poor prognosis in patients.
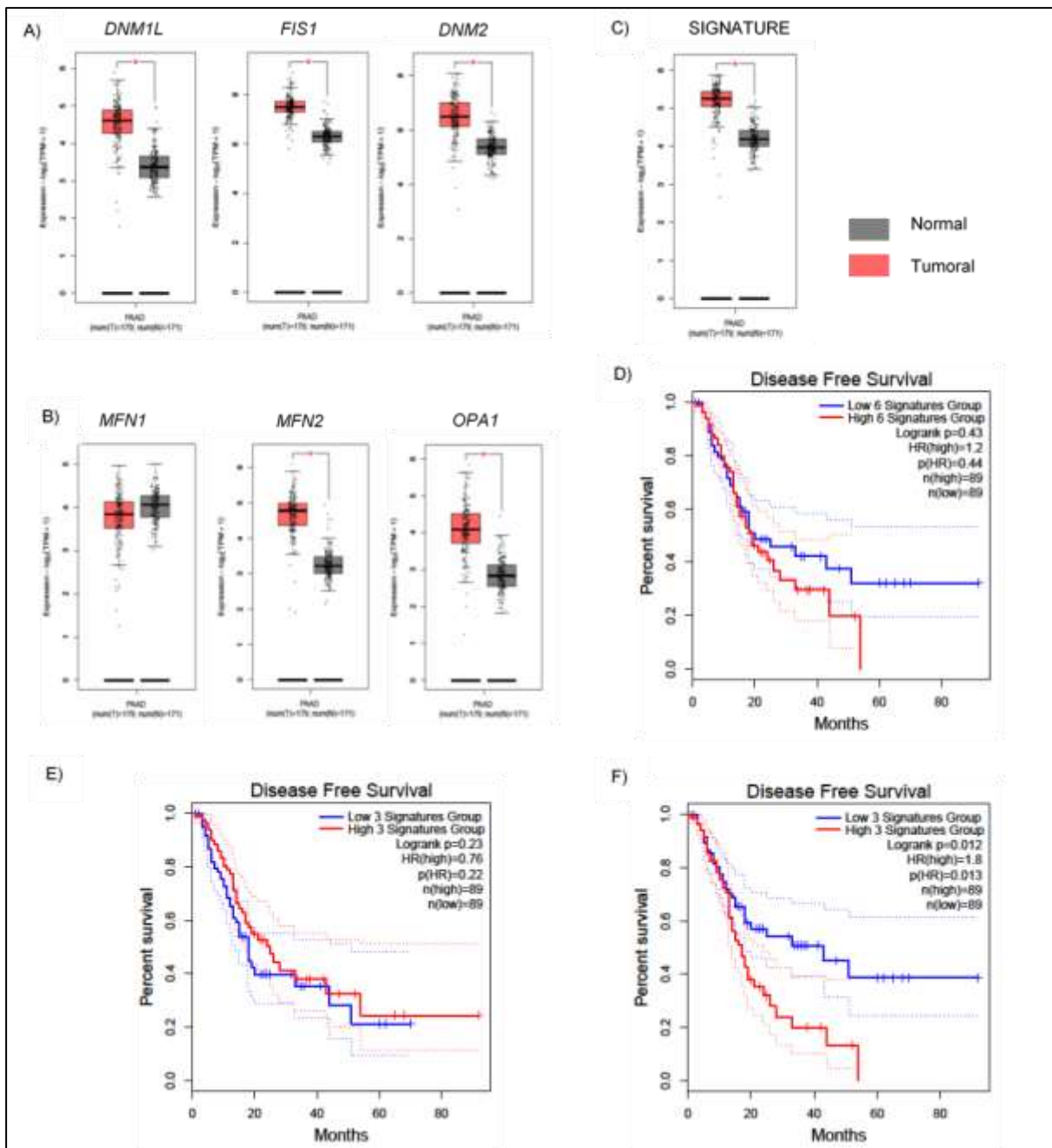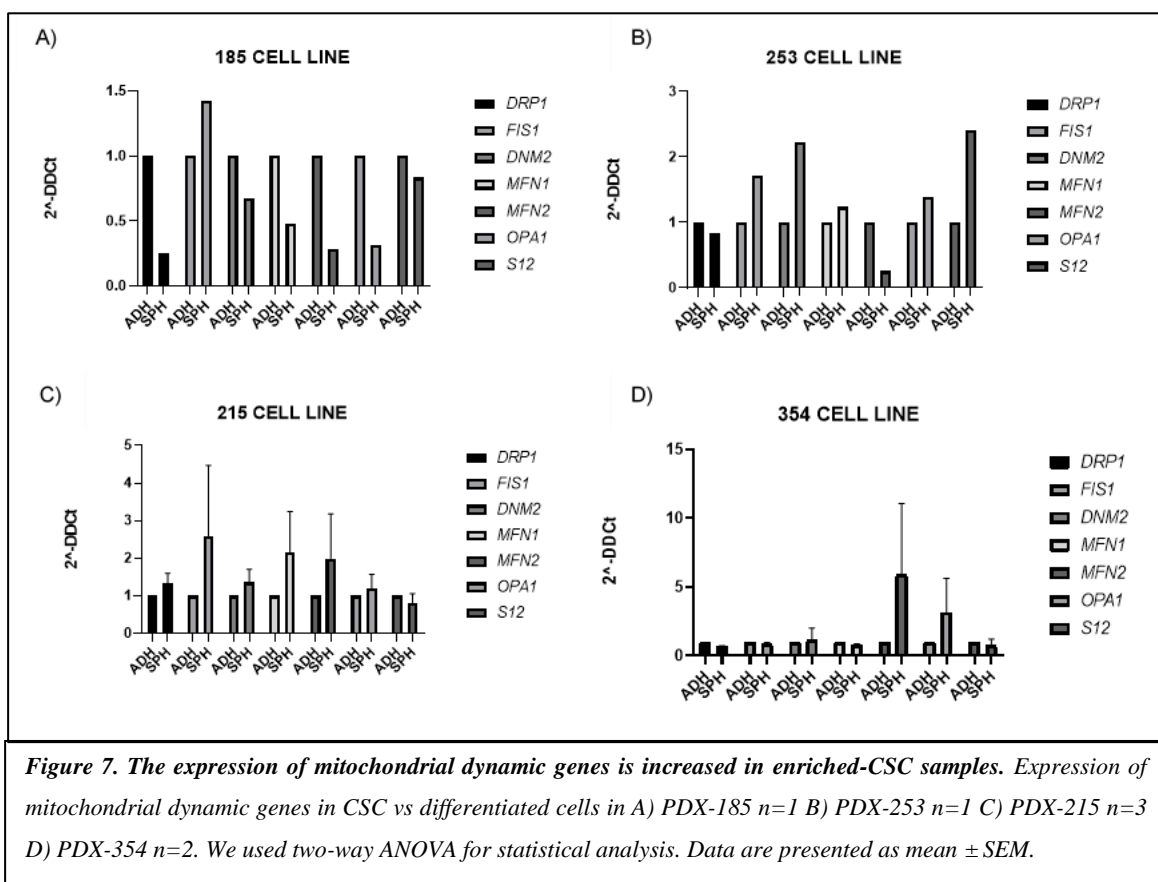
Figure 6. Mitochondrial dynamics gene expression is increased in tumoral tissue and predicts worse survival in patients.
A) expression of individual mitochondrial fission genes B) individual mitochondrial fusion genes C) signature of mitochondrial genes. Differences between tumour and normal tissue were analysed with one-way ANOVA by GEPIA2. *p < 0.01 D-F) Kaplan-Meier graph of disease-free survival in PAAD analysed with Mantel-cox Test by GEPIA2. D) Survival graphs of signature of mitochondrial genes and F) mitochondrial fusion genes (MFN1, MFN2 AND OPA1) showed worse prognosis when they are more expressed. E) Survival graph of fission genes (DNM1L, FIS1 and DNM2) showed worse prognosis when they are less expressed.

7.4. The ratio of mitochondrial fission to fusion is increased in CSC-enriched conditions.
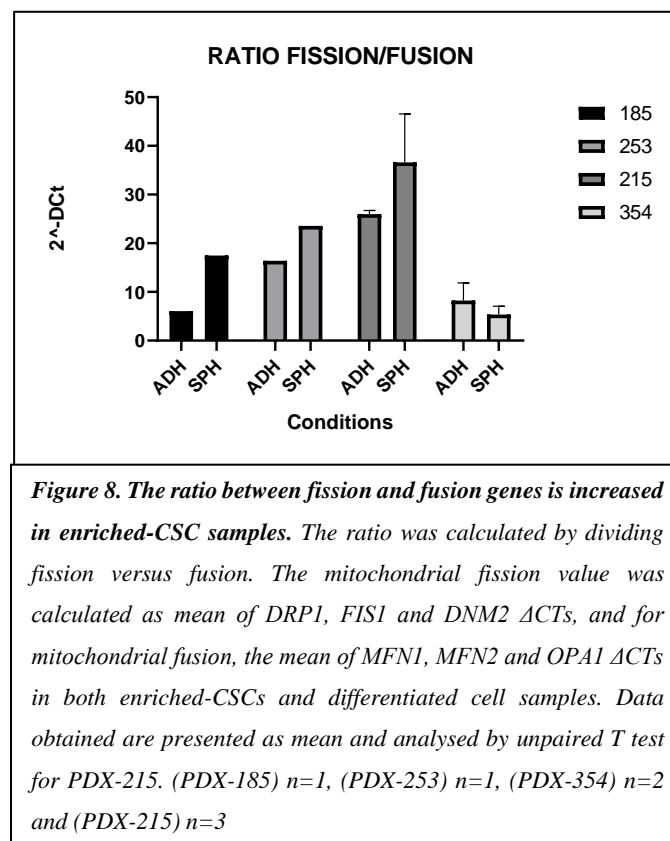
Studies have shown that mitochondrial fission is involved in cell reprogramming, and the *DRP1* gene is crucial for the development and maintenance of pluripotency. Particularly, *DRP1* maintains the self-renewal capacity of CSC (14). Our first aim was to test whether the increased expression of the mitochondrial dynamic genes seen in the previous bioinformatic analysis in PDAC is observed in both differentiated cells and CSC-enriched samples. The samples had been previously selected for their overexpression of pluripotency.

Figure 7. The expression of mitochondrial dynamic genes is increased in enriched-CSC samples. *Expression of mitochondrial dynamic genes in CSC vs differentiated cells in A) PDX-185 n=1 B) PDX-253 n=1 C) PDX-215 n=3 D) PDX-354 n=2. We used two-way ANOVA for statistical analysis. Data are presented as mean ± SEM.*

The results obtained were as follows: the PDX-185 line didn´t show an overexpression of the genes in the CSC sample, except for *FIS1* (Figure 7A); the PDX-253 line showed overexpressed genes such as *FIS1, DNM2, MFN1* and *OPA1* and others such as *DRP1* and *MFN2* that were expressed to a lesser extent (Figure 7B); the PDX-215 line showed an overexpression of all genes so that CSC cells had higher levels of expression of mitochondrial dynamics (Figure 7C); the last line, PDX-354, presented an over-expression of the fusion genes while the fission genes were expressed less than in adherents cells (Figure 7D). Of the analyses performed for each

line, those obtained in PDX-215 are the most reliable since it was tripled, however, the PDX-354 line was duplicated and PDX-185 and 253 were only analysed once.
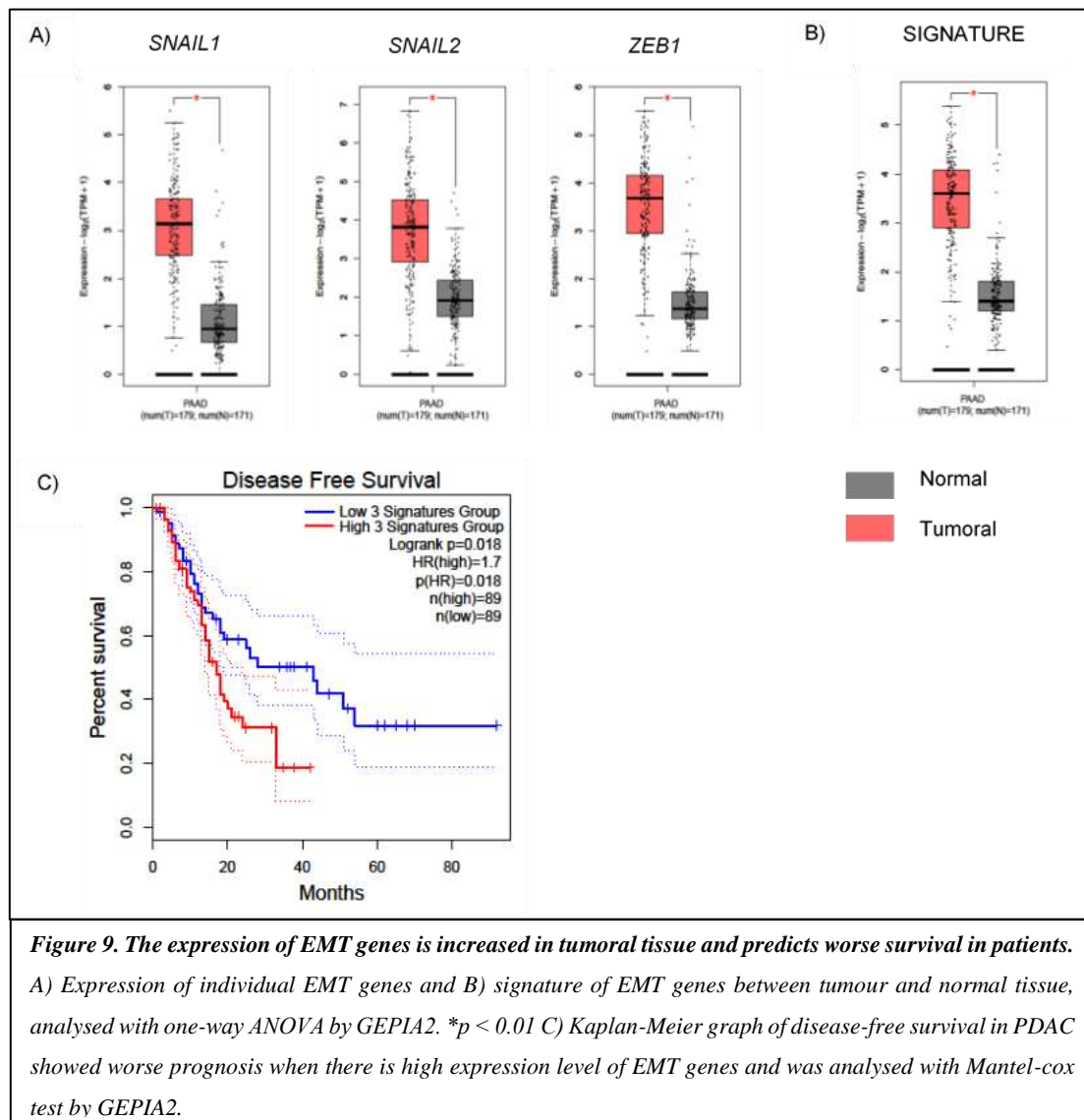
The lack of biological replicates in some lines and the different levels of expression in each cell line did not indicate anything conclusive. Due to that, a second analysis was proposed, aiming to analyse the relationship between fusion and mitochondrial fission. The results showed that PDX-185, 253 and 215 lines tended to have higher fission in CSC-enriched conditions. In fact, as mentioned above, mitochondrial fission is present in reprogramming processes of CSC, therefore, it was expected to be increased (Figure 8). Statistical analyses were not significant due to the low number of replicates.

Figure 8. The ratio between fission and fusion genes is increased in enriched-CSC samples. The ratio was calculated by dividing fission versus fusion. The mitochondrial fission value was calculated as mean of DRP1, FIS1 and DNM2 ΔCTs, and for mitochondrial fusion, the mean of MFN1, MFN2 and OPA1 ΔCTs in both enriched-CSCs and differentiated cell samples. Data obtained are presented as mean and analysed by unpaired T test for PDX-215. (PDX-185) n=1, (PDX-253) n=1, (PDX-354) n=2 and (PDX-215) n=3

7.5. Overexpression of genes involved in the epithelial-to-mesenchymal transition in PDAC reveals a worse prognosis.

Invasion and migration out of the primary tumor is seen in many cancers, in PDAC it is usually due to the EMT process, although some studies reveal that the process of metastasis is not necessarily due to EMT (29). In this process, the expression of epithelial markers decreases and

mesenchymal markers such as *SNAIL1, SNAIL2* and *ZEB1* increase their level of expression. For that, first of all, we wanted to compare the levels of expression of the genes *SNAIL1, SNAIL2* and *ZEB1* between tumor tissue and normal tissue using the bioinformatics tool GEPIA2. For that analysis, we used the same parameters that we have been described in pluripotency analyses.

Figure 9. The expression of EMT genes is increased in tumoral tissue and predicts worse survival in patients. A) Expression of individual EMT genes and B) signature of EMT genes between tumour and normal tissue, analysed with one-way ANOVA by GEPIA2. *p < 0.01 C) Kaplan-Meier graph of disease-free survival in PDAC showed worse prognosis when there is high expression level of EMT genes and was analysed with Mantel-cox test by GEPIA2.

The results showed a significant increase ($p \leq 0.01$) in the expression of the three genes in tumor tissue (Figure 9A), and analysis of the expression signature of the three genes also showed a significant increase (Figure 9B). The survival graph (p = 0.018) indicated that at higher levels of expression patients had a worse prognosis, but when the levels of expression were low the survival was better (Figure 9C).

In conclusion, PDAC tumors presented higher levels of mesenchymal markers, and its increased expression indicated worse survival.

7.6. Inhibition of DRP1 by mDivi1 induces a decrease in cell migration in PDAC

The interaction network of Figure 4D showed that there was no direct interaction between mitochondrial dynamics and the process of EMT. However, several studies indicate that the mitochondrial fragmentation is associated with cell invasion: the researchers have seen that high levels of *DRP1* increase migration. Moreover, when *DRP1* is inhibited cellular migration decreased (14).

With this idea, we wanted to see, first, if there was a correlation between mitochondrial fission with EMT (Figure 10A), especially with the *DRP1* gene (Figure 10B). The correlation analysis was performed with TCGA and GTEx database with PDAC database by GEPIA. The results showed a positive correlation in both cases.

The next experiment was a proliferation assay to determine the percentage of celular death or celular proliferation with respect to the different doses of an inhibitor of *DRP1*, called mDivi-1. The graph showed how the percentage of proliferation decreased when the dose was increased, however, this also revealed the suitable non-lethal dose and treatment time for future trials (Figure 10C).

Cellular migration was evaluated by wound healing assay. For this, the cells were in two different conditions: on the one hand, they were in MCM, which was rich in cytokines to promote the activation of the EMT process and, on the other hand, without this enrichment. In the magnified image of this control we was be able to see the stellate morphology that the cells acquired during this process (Figure 10D). The images showed how the wound remained open after 48h, when mDivi-1 was added. However, in the control of enrichment media we was able to see how the wound had closed almost completely (Figure 10E). The percentage of wound closure decreased when the concentration of mDivi-1 increased, especially in the medium enriched with MCM (Figure 10F).

Therefore, mDivi-1 inhibited cell migration. This migration inhibition was more pronounced in conditions where the EMT process was favored by cytokines.
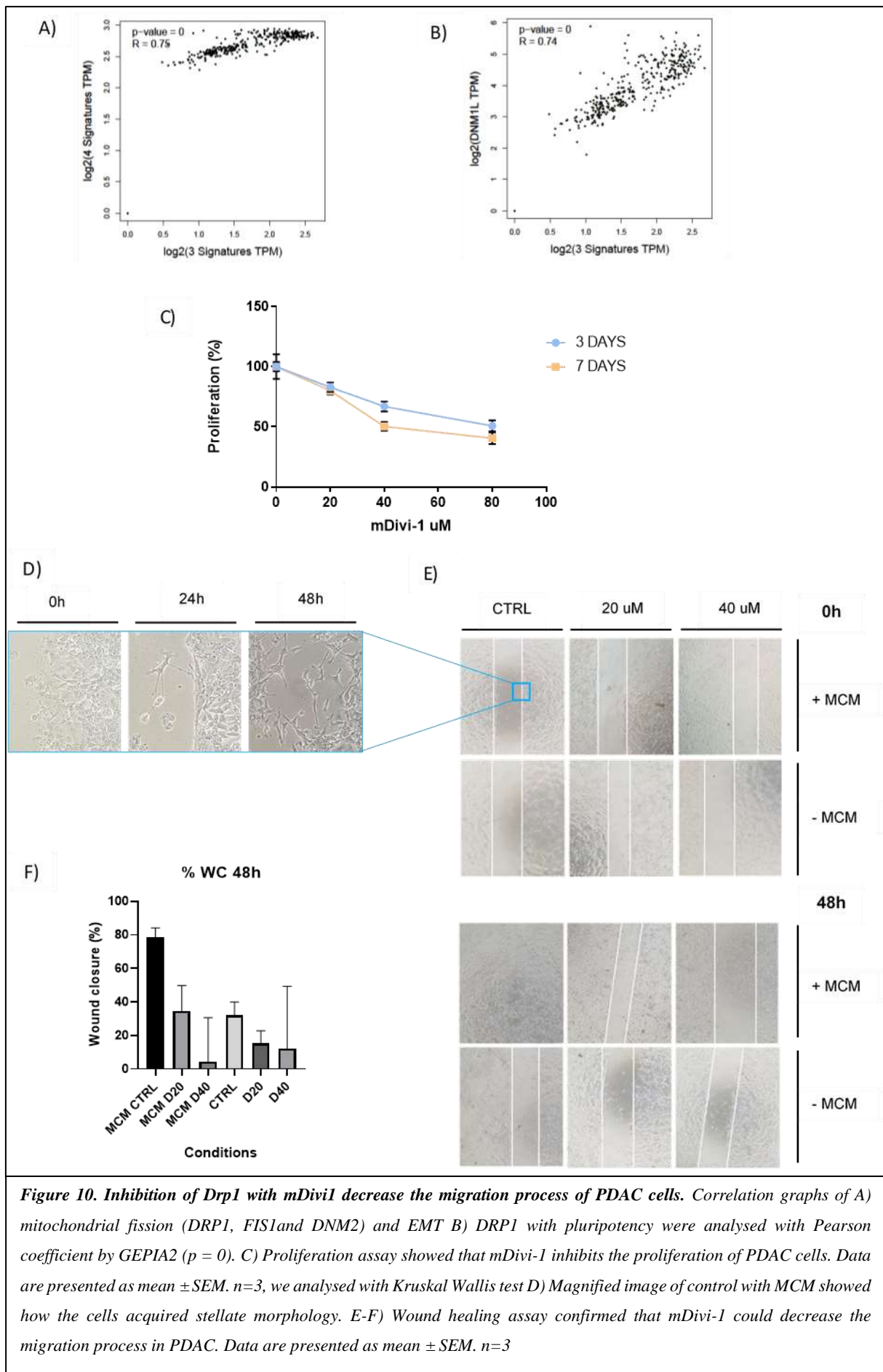
Figure 10. Inhibition of Drp1 with mDivi1 decrease the migration process of PDAC cells. Correlation graphs of A) mitochondrial fission (DRP1, FIS1and DNM2) and EMT B) DRP1 with pluripotency were analysed with Pearson coefficient by GEPIA2 (p = 0). C) Proliferation assay showed that mDivi-1 inhibits the proliferation of PDAC cells. Data are presented as mean ±SEM. n=3, we analysed with Kruskal Wallis test D) Magnified image of control with MCM showed how the cells acquired stellate morphology. E-F) Wound healing assay confirmed that mDivi-1 could decrease the migration process in PDAC. Data are presented as mean ± SEM. n=3

8. DISCUSSION

Mitochondria generate the energy needed for all cellular processes, including proliferation, migration or survival (13). In these processes, the mitochondria morphology is modified by mitochondrial fusion or fission proteins, depending on the process or cellular conditions, in which cells are at that moment (19)(23). Structural change occurs by the balanced action of mitochondrial fission and fusion, and its deregulation would lead to metabolic dysfunction resulting in disease (13)(19). During this dysfunction, the cells suffer a situation of stress that induces the process of senescence in which the cells try to recover the mitochondrial balance. However, in PDAC, due to the accumulation of mutations that it presents, the cells may be susceptible to other processes such as cell reprogramming or EMT (8)(36)(37)(38). In PDAC, the presence of CSCs is associated with therapeutic resistance, and if these cells are subjected to EMT, their invasion into other areas is facilitated. The group found that the migration of CSCs depends on the mitochondrial fission process, which is consistent with the importance of mitochondrial dynamics in tumor progression, as explained by other authors (12).

Our study shows that overexpression of the genes for mitochondrial dynamics, pluripotency and EMT is associated with a worse prognosis in patients with PDAC. The molecular connection between the three processes has not been determined yet, but our aim was to study the relationship between them. Finally, our interaction network showed that there is connection between the three processes. The association between the pluripotency process and EMT is very close, *SNAIL1* has an activating effect on *ZEB1* and *NANOG*, acting also at the level of transcriptional regulation. Studies have shown the interaction between *NANOG* with the factors *SNAIL1* and *SNAIL2* during cell reprogramming (39). On the other hand, *ZEB1* has been shown to inhibit the miR-200 family which represses the epigenetic regulator *BMI1*, therefore, the miR-200/Zeb1 axis controls the switch between stemness and differentiated cell particularly in pancreatic CSCs (31)(35)(38).

Several studies have shown the connection between mitochondrial dynamics and CSCs. Mitochondrial fission is involved in cell reprogramming, and the *DRP1* gene is crucial for the development and maintenance of pluripotency. Moreover, the *DRP1* gene plays an important role in the cell cycle, which is associated with the process of self-renewal, characteristic of these CSCs (14). On the other hand, studies on the epigenetic regulation of CSCs reveal that in normal breast tissue the repression of the miR-200 family, described previously, acts in another way

where it activates *PGC1α* which co-activates *MFN1* in charge of regulating the asymmetric cell division that allows maintaining the renewal capacity of stem cells (35). Importantly, *PGC1α* is associated in PDAC, with mitochondrial biogenesis, which regulates the self-renewal capacity of CSCs, thus favouring the spread of CSCs, tumor recurrence and metastasis (25)(28).

Our results obtained by PCR did not reveal any difference between mitochondrial fission and fusion in CSCs. Perhaps the lack of biological samples is the cause, so an analysis with a larger number of samples would have determined which mitochondrial process was predominant and could have shown a clear profile in PDAC. However, the ratio of fission to fusion revealed an increase in the mitochondrial dynamics of PDAC CSC-enriched samples. This is to be expected since, as commented above, mitochondrial dynamics are closely linked to cell reprogramming (40). To accurately determine the predominant dynamics of each cell group, further analysis must be performed.

Nevertheless, there is evidence that the difference in expression of mitochondrial dynamics affects tumor development and profile. Increased mitochondrial fission frequently occurs in cancer, and is due to overexpression of *DRP1*, this fact promotes metastasis in breast cancer and favours liver tumor cell survival (12)(13) Increased *DRP1* is also associated with actin filaments which are involved in the movement of cells to other regions (13). However, the decrease in *MFN1* or *MFN2* favours the proliferation, invasion and migration of tumor cells (12). These facts were verified, when there is overexpression of *MFN1* or *MFN2* or inhibition of *DRP1* a decrease in migration and invasive potential of tumor cells was seen (13).

Although the connection network does not show interaction between EMT and mitochondrial dynamics. Other studies reveal that pancreatic tumor cells have high mitochondrial fragmentation, associated with tumor migration and invasion (18). Our studies reveal that inhibition of *DRP1* by mDivi-1 slows down the cell migration process. Therefore, the drug inhibits the migration process, but the lack of statistical analysis does not determine anything conclusive. However, other studies show that mDivi-1 by inhibiting mitochondrial fission promotes mitochondrial fusion and affects outside the mitochondria in a way that stops the cell cycle. It would be interesting to study it under hypoxic conditions, since MSDs and ROS levels are associated with the EMT process in PDAC. mDivi1 in different studies has shown that it only affects the survival and proliferation of transformed cells, but it is still being studied because its effects depend on cell type and type of disease (18)(13).

In conclusion, although the exact molecular mechanism by which mitochondrial dynamics influences the processes of pluripotency and EMT is not known, it is known that there is a certain interaction, as has been seen so far. In PDAC, genes such as *PGC1α* are the link between mitochondrial biogenesis and CSCs, and the increase in mitochondrial dynamics in CSCs enhances the connection between both processes. On the other hand, the molecular connection between mitochondrial dynamics and EMT remains unknown. However, the inhibition of the migration process by the *DRP1* inhibitor directed to the mitochondrial dynamics of tumor cells reveals that both processes are related.

Therefore, the importance of mitochondrial biogenesis in stemness and EMT allows studying possible treatments focused on mitochondrial dynamics, so that in the future it is possible to treat cancers with an aggressive profile, as is the case with PDAC.

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