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"Pancreatic ductal adenocarcinoma (PDAC) and intercellular signals: from a prognostic approach to a treatment strategy"

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1. Introduction

1.1. Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC) affects ductal epithelial pancreatic cells, and it is the most common type of pancreatic neoplasm. It is characterized by a very low survival rate, with median survival of 6 months and a five-year survival for all stages of only 5%. Its high metastatic rate is responsible for such a low survival rate because it is usually diagnosed in the advanced stage to the vast majority of patients, therefore non eligible for surgery. The only strategy to improve survival rate is to diagnose PDAC at the early stages of development [1], [2] PDAC is considered as a sporadic cancer in general population since genetic mutations are limited to somatic cells. However, there are some genetic factors known to cause a higher risk for PDAC, such as BRCA1 and BRCA2 mutations responsible for familial breast-ovarian cancer syndrome or hereditary pancreatitis due to germline trypsinogen mutations. Other risk factors for PDAC are chronic pancreatitis, high-fat diet, excessive alcohol and cigarette consumption [1].

The molecular cause of PDAC lies in progressive accumulation of genetic mutations in tumour suppressor genes, oncogenes and DNA mismatch repair genes [3]. PDAC develops through many intermediary stages, known as pancreatic intraepithelial lesions (PanINs). The whole exome sequencing revealed its genetic heterogeneity mainly characterized by mutations in K-Ras, TP53, SMAD4, and CDKN2A genes [3], [4]. Every intermediary stage is associated with a specific genetic mutation or a set of mutations, all of which are found later in invasive cancer, though at a much higher frequency. The crucial mutation is the one in K-Ras gene, present in 92% of the PDAC cases [5], [6]. The wild-type K-Ras codes for a protein which binds to GTPase-activating protein (GAP) and regulates cell-cycle progression via the mitogen-activated protein kinase and AKT cascade. The mutation results in the constitutively active

protein in signal transduction. Single point activating mutations usually affect codons 12 or 13, and less frequently codons 59, 61 and 63. The mutation in codon 12 causes the conversion of glycine to aspartic acid, glutamic acid or valine. Of course, K-Ras mutation alone is not indicative enough, it has to be accompanied with other laboratory test and imaging techniques [1]–[3], [7]–[9]. Another very common mutation in PDAC affects the TP53 gene. This gene is inactivated in about 50-75% PDAC cases; it allows to the affected cells to bypass DNA damage checkpoints in the cell cycle regulation causing an uncontrolled proliferation. The mutation of TP53 is a late genetic event since the accumulation of p53 is detectable in advanced PanIN lesions. Loss of Dpc4 is another late genetic event in the pathogenesis of PDAC. Dpc4 is responsible for signalling cascades downstream of TGF-B, therefore a mutation in this protein causes decrease in growth inhibition and uncontrolled proliferation [2], [3] Somatic cells, including those of healthy exocrine pancreas, normally have embryonic signalling pathways inhibited. However, human and mouse models of PDAC show an abnormal transcriptional activation of pathways such as Hedgehog, Wnt, and Notch [10], [11]. An over-expression of Hedgehog ligands has been observed in PanIN lesions and in invasive adenocarcinomas. The studies have indicated that Hedgehog signalling might play a key role in the early PDAC pathogenesis, but also in the late maintenance. However, the exact role of Hedgehog signalling in the PDAC setting is still under research and its elucidation could lead to new diagnostic and therapeutic solutions [11], [12]Similarly, Notch pathway is activated both in early-stage PanIN lesions and in invasive carcinoma [2] [11]

The vast majority of tumours, about 70%, is located in the head of the pancreas, about 15% in the tail and only 5% in the body of pancreas. Distal cancers, located in the pancreatic body and/or tail, show a higher tendency to spread into retroperitoneal tissues, portal vein and superior mesenteric vein and artery. Other common targets of distant metastases are liver, lymph nodes, peritoneum, lungs and bones [13].

The available treatments for PDAC are chemotherapy and surgery. However, the success of surgery is very limited since only a small fraction of patients, about 15%, are actually eligible. The presence of distant metastasis and vascular involvement is an absolute contraindication for the surgery. It is important to mention that majority of patients are diagnosed only when PDAC has already developed extra-organ metastasis. However, even in the patients who have undergone the surgery the prognosis remains poor. The 5-year survival rate upon pancreatic resection does not exceed the 25% [13]

1.2. Extracellular vesicles (EVs)

EVs are known to play significant roles in the intercellular communication due to their heterogenous cargo (nucleic acids, proteins and lipids), and numerous ongoing studies are trying to identify both physiological and pathological processes in which EVs are involved [14]. It was the study of Raposo et al [15] that indicated exosomes as signalling mediators. Authors tracked by electron microscopy the vescicular shuttling of MHCII molecules in antigen presenting cells. Their work demonstrated that exosomes in antigen presenting cells were secreted by the Multivesicular body fusion (MVB) pathway. Furthermore, Valadi et al. [16], and Ratajczak et al. [17] described functional transfer of RNA molecules, found in EVs, among different cells [18].

The studies on EVs and their characterization both on class- and single-vesicle level is still ongoing. It is their size and cargo heterogeneity to pose the greatest limitations. Additionally, there is still no final agreement among scientists on the function-specific markers, with the only exception of integrins. Better experimental methods are needed in order to identify specific EV subpopulations, to examine better their biological properties and subsequently their roles [18]. Proofs of EVs role as mediators of intercellular communication are numerous and found in almost all cell types. To name a few, Ying et al. studied exosomes released by macrophages in

adipose tissue [19]. This study showed that exosomes modulated the response to insulin *in vivo*, and it was a miRNA molecule loaded in exosome responsible for the effect. Lo Cicero et al. described the process of melanogenesis control via miRNAs present in exosomes released from keratinocytes [20].

EVs have been successfully isolated from almost all bodily fluids: blood, urine, saliva, breast milk, cerebrospinal fluid, semen, ascites, amniotic fluid. As their origin and features are diverse many different terms have been used to describe them in the literature. Some were referring to their size naming them micro- or nanoparticles. Other names referred to their origin cells and they were denoted as oncosomes or proteasomes, and some names simply referred to their presence in the extracellular matrix therefore naming them exovesicles, ectosomes etc [21]. The isolation of extracellular vesicles is additionally complicated by the fact that it is usually contaminated with EV-like structures (such as apoptotic bodies, shedding vesicles etc.), and currently used methods do not allow a complete separation of non-EV structures from EVs. Thus, it is necessary to determine the EVs contribution in any study regarding molecular cargo or the functional consequences of the isolate [22]. The Executive Committee of the International Society for Extracellular Vesicles (ISEV), based on current best practices, proposed series of minimal requirements for characterization of EVs (MISEV 2014) which should be reported in the publications [22]. According to MISEV 2014, there is no gold standard isolation method, but selection criteria should be based on downstream applications of the EVs and reproducibility should be ensured [23]. Furthermore, EV characterization needs to be performed at the level of population and single vesicle with both positive and negative protein markers for 4 protein categories: (i) transmembrane EV proteins or lipid-bound proteins, (ii) EV cytosolic proteins, (iii) intracellular proteins not associated with cell membrane or endosomes and (iv) extracellular proteins not typically associated to EVs. The

minimal experimental requirements for definition of EVs have been updated last time in the 2018 [23].

Based on their biogenesis pathway, EVs are categorized generally in 2 groups: exosomes and microvesicles. However, this is not the only subdivision of the extracellular vesicles. The biogenesis of exosomes and microvesicles involves membrane-trafficking but the processes differ among these two EV types [14], [22]. Term exosome in the older literature was referred to the vesicles of unknown origin presenting 5'-nucleotidase activity. Now the term exosome denotes a membrane vesicle of 30-100 nm in diameter [22]. The biogenesis of exosomes is a highly complex process, depends on the cargo molecules and origin cell type and it is also subjected to various pathological stimuli received by cells. The cargo itself has a very dynamic composition based on differentiation and maturation cell phases. Furthermore, different subpopulations of exosomes released from the same cell my vary in their cargo composition and morphology [14]. Exosome biogenesis involves generation of the intraluminal vesicles (ILVs) in the endosome lumen during maturation into multivesicular endovesicles (MVEs), and the entire process is guided and regulated with particular sorting machinery (ESCRT). The first task of the sorting machinery is to separate cargo molecules on the domains of the limiting membrane of MVEs. This membrane then undergoes inward budding and fission, this way forming vesicles loaded with sequestered cytosol [14]. An ESCRT-independent pathway of exosome biogenesis was established upon inactivation of 4 ESCRT complexes which nevertheless did not impair formation of MVEs [24]. It has been demonstrated that ESCRT-independent pathway requires ceramide which induces membrane bending [25], [26]. The tetraspanin protein family is also involved in regulation of ESCRT-independent pathway, in particular CD81, CD63, CD82 and CD9 are enriched on the exosome surface and involved in endosomal cargo sorting [14], [27], [28]. Before being released in the extracellular space, the newly formed exosomes have to pass through a series of events of cargo sorting to MVEs, ILVs

and finally to target MVEs to the cell membrane to be secreted. The exosome release can be constitutive (e.g. cell differentiation, embryonic development) and modulated by physiological or pathological states of the cell [14].

Microvesicles range from 100 to 1000 nm in diameter, but in the case of oncosomes even up to 10um. They are released upon outward budding and fission of the cell membrane [29]. The formation of microvesicles is a consequence of multiple molecular rearrangements of lipids and proteins of plasma membrane, and calcium ions levels [14], [30]. The complex of cytosekeletal elements and their regulators is also necessary for microvesicle biogenesis. It was demonstrated that microvesicle formation in tumour cells is regulated by RHO family of GTPases and RHO-associated protein kinase (ROCK). Additionally, the metabolic changes of Warburg effect may influence the formation of oncosomes. Microvesicles simply sprout out of the membrane into extracellular space, on the contrary from the exosomes which require transport to the cell membrane prior release [14], [31].

EV cargo is highly dependent on physiological or pathological state of the producing cell, and it is also cell-type specific. Cargo molecules are also reported to be important regulators of EV biogenesis. For example, major histocompatibility complex class II (MHCII) ectopic expression leads to a biogenesis of MVEs, and finally a release of EVs [32], [33]. Molecules which are mainly recycled to the cell membrane are less probable to become enriched in exosome cargo. Due to impairment or lack of recycling regulators and retrograde transport from endosomes to the Golgi apparatus, some molecules could become enriched in exosome cargo. Membrane-associated cargo molecules are directed to the areas of membrane budding through affinity for lipid rafts or anchoring to membrane lipids. Cytosolic molecules directed to microvesicles have to bind to the membrane inner layer [14]. Nucleic acids (mRNAs, non-coding RNAs, miRNAs and DNA sequences) are an important part of exosomal cargo and their incorporation in EVs is a regulated process. In presence of specific sequence motifs, miRNAs

may be differentially sorted to exosomes. The actual contribution of passive and active incorporation of RNA molecules into EVs is not fully elucidated [14]. However, KRAS-MEK signalling pathway has been described as a regulator of miRNA sorting into exosomes [34].

The primary destiny of multivesicular endosomes is to fuse with lysosomes in order to be degraded. This process is inhibited by mechanisms which consequently allow for MVEs secretion from cells [35], [36]. It is mostly unknown how this important equilibrium between degradation and secretion is being maintained. However, there are some findings on this matter. Important role in this regulation is played by sorting machinery [35], [36]. For instance, MHCII is sorted into MVEs directed to lysosomal degradation through ubiquitylation, but MHCII sorted into MVEs through ubiquitin-independent pathway are secreted [14], [36], [37]. Also, calcium ion has been recognized as a regulator of exosome secretion in many cell types, such as cortical neurons, mast cells and K562 cell line [38]–[40]. Fission of the cell membrane, necessary for the release of microvesicles, relays upon interaction between actin and mysion with consequent ATP-dependent contractions [14].

After being released from the cell surface, EVs have to find a way to the target cells, bind to the cell membrane and activate receptors and the process of their internalization. Not all of these mechanisms are fully understood and described so far, and the study is additionally complicated since EV-cell interactions are not universal for all EVs and target cells [14], [41]. The targeting of specific cells is probably conditioned by proteins enriched on surface of both EVs and target cells. However, the effects can be also autocrine in some cases. Molecules responsible for recognition of target cells by EVs include tetraspanins, integrins, lectins, components of extracellular matrix and lipids [14], [42], [43]. It has been observed *in vivo* that integrin heterodimers directed EVs to specific recipient cells. Cancer exosomes are an excellent example, it has been observed that they are targeted to specific distant sites, based on integrin composition, where they are able to promote formation of metastasis [44]. Not only integrins

are able to regulate exosome targeting but also tetraspanins, heparan sulfate proteoglycans, lectins, glypican 1 and CD44 play important roles in exosome-cell interactions. It was reported that also a specific lipid composition of EVs might impact their docking on recipient cells [14]. EVs are internalized in target cells by clathrin-dependent or independent endocytosis, or by endocytosis via lipid rafts and caveolae. Which mechanism will be activated depends on the specific composition of the vesicles. Furthermore, presence of lipid rafts at the plasma membrane contributes to EV internalization [14]. Once internalized, EVs follow the endocytic pathway to reach MVEs and they are mostly directed for degradation in lysosomes. However, EVs manage to release their cargo in the cytoplasm by back fusion with the membrane of the MVEs avoiding lysosome degradation this way. This process is of high importance and therefore it should be studied with live-imaging methods and super-resolution in order to give responses to numerus questions in this field [14]. EVs trigger various cell responses by activating the plasma membrane receptors. An important discovery was that fibronectin, carried by cancer-derived vesicles, promoted the anchorage-independent growth of fibroblasts and so causing the acquisition of a malignant phenotype [45]. Direct fusion of EVs with the target cell membrane allows for release of intraluminal cargo molecules, such as miRNA and mRNA in the cytoplasm of target cell. Also, direct fusion is necessary for exchange of lipids and transmembrane proteins between EVs and cells [14]. Functionally active mRNAs and miRNAs released from EVs regulate gene expression of the target cells through de-novo translation and post-translation regulation of its mRNAs. In some phases of development and stress response, miRNAs exchanged between different cells via EVs play an important role. The capacity to modify transcriptome within target cells allows for phenotypic changes sometimes causing pathologies, such as cancer. By exploiting the ability of EVs to shuttle their cargo in different cells they are being developed as potential therapeutics, as nanocarriers of biologically active molecules [41].

To directly visualize EV uptake by cells, vesicles have to be labelled with fluorescent dyes. The most frequently used dyes for EV labelling are PKH26, PKH67, Dil and DiD [41]. Methods such as flow cytometry and confocal microscopy are used to measure the uptake of EVs in cells. It is very important to discriminate internalized and surface-bound dyes, for that purpose cell surface is treated with acid or trypsin. There are concerns that use of fluorescent dyes might impair the normal behaviour of EVs, therefore it is important to study their potential effects [41]. Although fluorescent microscopy is widely used for EV studies, its limitation concerns the assessment of EV uptake and dynamic localization of individual vesicles. This limitation could be overcome by fusing fluorescent protein markers with vesicular proteins, such as CD9 and CD63 tagged with GF [41].

1.3. Extracellular vesicles as mediators of cancer and metastasis development

The communication among tumour and stromal cells is a hallmark of cancer progression [46], [47]. Mediators of this communication are usually EVs loaded with various cargo molecules and secreted by both normal and tumour cells. Lately EVs have been also correlated with metastasis development, and there are numerous ongoing studies trying to shed a light on the actual mechanism by which EVs promote cancer progression [46], [48], [49].

Malignant transformation is a complex process guided by point mutations which cause the de-differentiation of a cell that grows uncontrollably and finally develops into cancer cell [46]. The study of Stefanius et al. used a two-stage *in vitro* cell transformation assay (CTA) to analyse exosome contribution to malignant cell transformation [46]. The NIH/3T3 cell line was treated for 3 days with initiator and then 2 weeks with promotor. The results showed that pancreatic cancer-derived exosomes act as initiators. Exosomes derived from normal pancreas cells had no effect whatsoever on treated cells. Furthermore, cells transformed by pancreas cancer-derived exosomes were able to form aggressive tumours once administered into mice.

Authors showed that exosomes derived from various pancreatic cancer cell lines, namely Capan-2, MIAPaCa-2 and Panc-1, function as initiators in CTA assay ultimately transforming NIH/3T3 cell line and causing tumour growth in mice [46] There are several published findings of cancer patient sera and/or isolated cancer-derived exosomes mediated transfer of malignant characteristics to primed cells causing their transformation. The term "primed cells" refers to cell with mutated oncogenes and/or oncosuppressors. Despite the fact that details on exosome-mediated transfer among studies diverge, the common point is that exosomes are important mediators of cell transformation [50]–[52].

The enzyme tissue transglutaminase and fibronectin, the component of extracellular matrix, have been identified to mediate the transforming activities of EVs in study on breast carcinoma cells and glioma cells [53]. Additionally, it has been proposed in this study that EVs are able to transform normal tumour stroma cells by transferring cancer cells' characteristics. This would be a major game changer in our current understanding of tumour progression since it suggests that aberrant proliferation of tumour stroma and epithelium cells is due to exposure to cancer EVs [45] However, it seems that EV cargo molecules have a limited lifespan after being released into cells. Authors in order to promote the transformation of NIH/3T3 fibroblasts and MCF10A mammary epithelial cells treated them repeatedly with freshly isolated EVs throughout the assay. This means that *in vivo*, in tumour setting, the continuous release of EVs by cancer cells into microenvironment would provide the constant supply of EVs to stromal and epithelium cells, this way causing and maintaining a malignant transformation [53].

1.4. Metastasis: seed-and-soil vs genometastasis theory

Paget's classical "seed-and-soil" theory of metastasis is not sufficient to fully describe progression of many tumours, including PDAC. Indeed, some metastatic patterns can be explained by the anatomical-mechanical hypothesis claiming that they are determined by the

anatomy of vascular and lymphatic drainage at primary tumour site, and the fact that the circulating cancer cells' arrest mainly occurs at the adjacent organ. Both hypotheses are accepted, and their validity depends on the type of tumour [54], [55]. However, the dissemination of cancer cells through circulation may not be the unique principle for metastasis formation since some phases of the metastatic development are very inefficient. Indeed, only 0.001% of circulating cancer cells can actually survive in the circulation and thus lead to metastasis [56].

The hypothesis of "genometastasis" represents another model explaining metastasis dissemination. It is based on the horizontal transfer of genetical material (DNA, RNA, miRNAs, retrotransposon elements, mutated and amplified oncogenes) carried in circulation to susceptible cells in distant organs. These genetic elements in the target cells activate and promote mitogenic signalling pathways causing the malignant transformation [56]. Circulating DNA and RNA molecules are found in several forms of molecular complexes, linked to serum proteins, or loaded into extracellular vesicles [55]. Recent studies have demonstrated that exosomes are one of the important cancer-derived factors causing formation of pre-metastatic niche and metastasis by inducing inflammation, vascular leakiness, and recruitment of bone marrow cells. Furthermore, it was possible to identify melanoma patients at a risk for metastasis using exosomal protein signature [57]. The steps leading to liver pre-metastatic niche formation were significantly mediated with pancreatic cancer-derived exosomes [49]. The study of Hoshino et al. assessed whether the surface molecules of cancer-derived exosomes are targeting them to specific organs by profiling exo-proteome of different tumour models all of them with a tendency to metastasize to a specific organ [44]. The exosomes isolated from organotropic cancer cell lines which primarily metastasize to the lung, liver or both were injected into mice and their uptake and biodistribution were assessed. Indeed, the exosomes derived from cells known to primarily metastasize in the lung have showed a significantly increased uptake in the

lung, when compared to the non-lung-metastatic cell line. The same result was observed also for liver-specific metastasis [44]. In particular, the pancreatic cancer cell-derived exosomes showed a specific targeting to the liver, thereby confirming *in vivo* the organotropic distribution of the cell line of origin. Remarkably, cancer exosomes were sufficient to redirect metastasis of cells that normally would lack the possibility to metastasize a specific organ. Authors have demonstrated the association among exosomal integrin expression profiles and tissue organotropism. Quantitative mass spectrometry and Western blot analysis confirmed that integrins alpha 6 (ITGα6), beta 4 (ITGβ4) and beta 1 (ITGβ1) are highly expressed in lung-tropic exosomes. Furthermore, the integrin beta 5 (ITGβ5) and alpha 5 (ITGαv) are specific for liver-tropic exosomes. Another important feature of the metastatic cell-derived exosomes is the presence of integrin alpha 2 beta 1 (TGα2β1) which is not present on non-tumour exosomes. This integrin could be useful as a biomarker for metastasis [44]. Recently, it was experimentally demonstrated, that cell-free cancer-derived DNA can transform non-tumour cells *in vitro* upon treatment with colorectal cancer patient plasma or by co-culturing tumour and non-tumour cells [58], [59]. Abdouh et al. suggested that mutation and/or inactivation of oncosuppressors in distant recipient cells could cause the aberrant expression of membrane proteins, this way permitting the genome integration of circulating oncogenic factors [60]. The successful integration of foreign nucleic acids into target cells is not such a new concept in biology, since numerous DNA and RNA viruses (Herpes virus, Hepatitis B and C, Human papilloma virus) are known to be able to integrate themselves into the target cell genome causing carcinogenesis. It is the lack of an adequate immune response that favours the reactivation of integrated viral genes permitting an active replication even many years after the infection [61]. It is suggested that late cancer metastasis might follow the same pattern: the integrated genetic material might remain silent for long periods and be expressed in the favourable conditions following failure of some homeostatic mechanisms [60]. Integrations of

the genetic elements, transported by extracellular vesicles, has been confirmed in the study on glioblastoma multiforme (GBM) [62]. In the malignant brain tumours activation of epithelial growth factor receptor (EGFR) is a crucial event for malignant transformation, and majority of the GBM cases have amplified EGFR, in particular a mutant version EGFRvIII. There oncogenic receptor kinases accumulate in membrane lipid rafts of cancer cells and were detected in microvesicles released from these cells. In this study cells were exposed to the microvesicles containing the mutant version of EGFR to validate the uptake and consequent expression of the EGFRvIII with downstream activation of MAPK and Akt pathways. Indeed, the successful integration of EGFRvIII was confirmed in the exposed cells and they showed an increase in related signalling pathways. Microvesicle-treated cells also showed expression of several other genes involved in tumour growth, angiogenesis and survival. It has been suggested that this mechanism could be valid in various human tumours and that outcome of oncogene incorporation into target cells could be increase tumour progression, metastasis, and angiogenesis. Therefore, inhibiting the molecules responsible for the microvesicle exchange could be a potential therapeutic strategy [62].

Liquid biopsy is a method for detection of circulating tumour cells and nucleic acids in biological fluids, but mostly blood. It can be used for diagnostics, prognostics and patients' follow up [63]. It is a non-invasive technique which allows to repeatedly sample biomarkers from small amounts of blood, or other bodily fluid, in order to follow the cancer progression. Furthermore, it is a very useful method for pancreatic cancer given the anatomical and clinical difficulties related to pancreas and the current diagnostic limitation non permitting an early disease detection [63]. The benefits of liquid biopsy have been already demonstrated in pancreatic, breast and colorectal cancer [64]. However, it is somewhat difficult to obtain pure circulating tumour cells from the sample due to their heterogeneous morphology and very limited number in the blood stream (cca. $1x10^9$). It is even harder to isolate specific

subpopulations of CTCs which may present crucial genetical and molecular features for cancer progression and metastasis [55]. Circulating nucleic acids are more abundant than circulating tumour cells in plasma and sera of the cancer patients. This fact pointed into direction of circulating nucleic acids as potential drivers of the metastasis process, a phenomenon termed as genometastasis. The association between cancer and the presence of free circulating DNA is well-known, and numerous studies have characterized cell free nucleic acids. Furthermore, it has been demonstrated that patients with malignant tumours present higher amounts of cell free DNA than those with the benign disease [65]. The free circulating DNA is present in plasma in mostly in double-stranded form, but some single-stranded forms have also been identified. Furthermore, DNA molecule has to be protected by other molecules and membranous formations from enzymatic degradation [55]. The free circulating RNA is present as mRNA and miRNA molecules. The later are known post-transcriptional regulators of expression of various target genes important for cell proliferation, differentiation, and apoptosis

[55]. Circulating DNA and RNA molecules are found in several forms of molecular complexes, linked to serum proteins, or loaded into extracellular vesicles. The vesicle role is important since they protect the cell free nucleic acids from nucleases, and they impede the immune system to recognize these molecules. Therefore, EVs have been recognized as important carriers of genetic information [55]. The genetic cargo, in particularly miRNA molecules, have been demonstrated many times as tumour suppressors or enhancers in colorectal, liver, and lung cancer. It is important to highlight that exosomes carry a heterogenous mix of miRNAs, so the effect will depend on their particular combination [66]–[69]. In a very recent study, Abdouh et al have demonstrated, for the first time, that EVs released from colorectal cancer cells transfer mutated cancer genes and several coding and non-coding RNA molecules to susceptible cells, this way impairing signalling pathways involved in cancer growth and progression [70] . The microarray identified presence of various mRNA and non-coding RNA

molecules in BRCA1 KO fibroblasts treated with cancer derived EVs. Several miRNAs identified in this study have been previously associated with progression and invasion of colorectal cancer [71]–[73]. These results, together with the previously published ones on BRCA1 KO fibroblast treatments with patient sera and conditioned cancer cell medium, propose new insights in mechanisms underlying the malignant cell transformation and the role of horizontal gene transfer of malignant traits [50], [51], [56], [72]. Furthermore, this study reported that cancer EV cargo contains genomic DNA and that some mutated cancer genes were successfully transferred to target cells.

In the future, lineage tracing studies and extensive sequencing studies will be necessary in order to give the ultimate proof of genometastasis' scientific validity. Moreover, it is important to give unambiguous evidence that observed phenotypical changes are caused by EVs, and to determine the influence of epigenetic and somatic mutations. Finally, the genometastasis validity *in vivo* should be assessed [70].

2. The aim of research project

The majority of PDAC patients present with an advanced disease and, even when operable, most patients will develop shortly after a metastatic disease. Therefore, the challenge is to identify patients not in risk from disease relapse due to presence of micro-metastasis, therefore actually benefiting from surgery. The genometastasis hypothesis could help to overcome this limitation since it suggests that tumour-derived extracellular vesicles might be responsible for transformation of distant cells, and therefore metastases. So, if this hypothesis is to be confirmed valid in PDAC together with identification of molecules responsible for transformation they could be employed as biomarkers for micro-metastasis.

Our aim is to provide supporting results for genometastasis theory in the setting of PDAC, since the results available in literature are rather scarce. To achieve the project goals we will do following:

1. assess if the sera of PDAC patients is able to induce cell transformation *in vitro* and which genetic changes occur in the recipient cells;
2. assess if the pancreatic tumour cell lines are able to transform non-tumour cells *in vitro* and identify the phenotypical changes in treated cells*;*
3. assess the genometastasis *in vivo* using animal models who received transformed cells from the point 2;
4. characterize the tumours formed in animal models using immunohistochemistry.

The project could have an innovative scientific impact, leading to define a better knowledge of the metastatic process in PDAC. Through the evaluation of the potential transforming effect of PDAC sera on non-tumour cell lines and co-cultures of pancreatic cancer and normal cells, the study aims at demonstrating a new possible paradigm of cancer dissemination, which relies on inter-cellular signalling systems, rather than direct cancer cell migration to the metastatic site.

This model was already described in colorectal cancer, but it has never been evaluated extensively in PDAC. The results obtained in this pilot phase should be followed in the future with identification and characterization of key mediators of cell-to-cell communication leading to metastasis. As a consequence, results of this study might have relevant potential applications in clinical practice. Once identified the molecules responsible for genometastasis, they could be used for development of effective and reliable assays to predict disease recurrence or progression. This could distinguish patients that, despite radical surgical resection of primary tumour and an apparent metastasis-free clinical picture, exhibit a higher risk of relapse. From this clinical perspective, the identified plasmatic mediators could serve as useful tools in defining better treatment strategies for resectable PDAC patients, avoiding useless and ineffective surgical and/or radiotherapeutic approaches. This study could pave the way for future analyses aiming to reveal new possible molecular targets in the early phases of the metastatic process, prompting further development of more specific and effective therapies.

3. Materials and methods

3.1. Cell lines and culture conditions

As target cells for malignant transformation, we used hTERT-HPNE (CRL-4023, ATCC, USA), HEK293T XPack CMV-XP-GFP-EF1Puro (XPAK530CL-1, System Biosciences, USA).

The hTERT-HPNE cell line represents intermediary stage during acinar-to-ductal metaplasia in pancreas. They have an undifferentiated phenotype and active Notch signalling pathway. The HEK293T cell line is a highly transfectable derivative of human embryonic kidney 293 cells and contains the SV40 T-antigen. Both hTERT-HPNE and HEK293T cell lines were maintained in Dulbecco's Modified Eagle Medium (ECM0749L, EuroClone, IT) supplemented with 10% of exosome depleted fetal bovine serum (FBS) for co-culture experiments and for PDAC serum treatments with 10% patients' sera, 1mM L-glutamine (BE17, 605E, Lonza, BE), 1mM penicilline-streptomycin (ECB3001D, EuroClone, IT) and 1% of MEM non-essential amino acids for HEK293T cell line.

We used cell lines AsPC-1 (BS TCL 171, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Biobanking of Veterinary Resources, IT), Capan-2 (BS TCL 10, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Biobanking of Veterinary Resources, IT) and Panc-1 (courtesy of Dott. Malavolta, INRCA Ancona) as a donor cell lines for co-culture experiements.

The AsPC-1 is a cell line re-established from a tumoral xenograft in nude mouse which expressed characteristics of the primary adenocarcinoma. It was maintained as per suppliers' recommendations in RPMI 1640 medium (ECB9006L, EuroClone, IT) supplemented with 1mM sodium pyruvate, 20% FBS and 1mM penicilline-streptomycin. The Capan-2 cell line derives from pancreatic adenocarcinoma and it was maintained in McCoy 5A medium

(ECM0210L, EuroClone, IT) with 10% FBS and 1mM penicilline-streptomycin. The Panc-1 cell line is a model of pancreatic epitheloid carcinoma and derives from pancreatic duct tissue, and it was maintained in DMEM medium with 10% FBS, 1mM L-glutamine and 1mM penicilin-streptomycin.

All cell lines were maintained at 37 °C in humidified atmosphere containing 95% air and 5% CO2. When cells reached 80–90% confluence, they were passaged using 0.05% Trypsin-EDTA (ECB3052D, EuroClone, IT).

3.2. Cell co-cultures

For cell co-culture assays we used ThinCerts^{TM} with pore size of 1 um for 6-well plates (657610, Greiner bio-one, CH). The pore size of 1um allows only for the small extracellular vesicles to pass through the membrane, maintaining all other cell components, cells and bigger molecules. In the 6-well plates were seeded 50.000 cells per well of recipient cell lines in 3ml of exosome depleted cell culture medium. In ThinCerts was seeded the same quantity of pancreatic cancer cell lines in 2ml of exosome depleted cell culture medium. The cultures were maintained for 20 days at 37 °C in humidified atmosphere containing 95% air and 5% CO2. When cells reached confluence, they were passaged using 0.05% Trypsin-EDTA.

3.3. Serum treatments

We obtained pooled PDAC patients' serum from Tissue Biobank of the 1st Surgical Clinic, University Hospital of Padova, Italy. The patients' sera were drawn before the chemotherapy therefore they were free from any drugs. We have pooled sera from 25 patients with medium age of 69 years, both male and female. The pooled control serum was obtained from 25 healthy volunteers of medium age of 60 years. Both healthy donor and PDAC patients' sera were filtered with 0.22 um sterile filters. The recipient cell cultures were supplemented with 10% of

PDAC or healthy donor serum, and the medium was changed every 2 days for 3 weeks, as previously described [74].

3.4. Soft agar assay

At the end of the co-culture and serum treatments, we assessed cell transformation in vitro using soft agar assay. The test was done in triplicate for each condition. The soft agar assay is based on the anchorage-independent growth of tumour cells whereas non-tumour cells are unable to proliferate. Sterile solutions of agar at concentrations of 1% and 0.6% were prepared in deionized water. Cell culture medium was prepared at 2X concentration and sterilized. The 6-well dishes were used for the assay. The bottom layer of plate was made of 1% agar and 2X cell culture medium in the ratio 1:1, upon solidification the upper layer was prepared of 0.6% agar and cell suspension in medium in a ratio 1:1. The agar was let to solidify at room temperature in cell culture hood for 30 minutes before placing it into incubator at 37°C and 5% CO2. The 6-well plate was incubated for 21 days, with addition of 100ul of fresh medium twice a week to prevent desiccation. Colonies were visualized and photographed under the Eclipse Ti2E microscope (Nikon, Tokyo, Japan) and we counted only those larger than 50um.

3.5. Western Blot

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Proteins were run on 8% or 12% polyacrylamide gel and transferred to a nitrocellulose membrane (10600006, GE Helathcare Life science, DE). Membranes were blocked in TBS buffer (20 mM TRIS, 150mM NaCl, pH 7.4) containing 5% Bovine Serum Albumine and exposed to rabbit-anti-mTOR (2983, Cell signalling, MA, USA), rabbit-anti-phospho-mTOR (5536, Cell signalling, MA, USA), rabbit-anti-FN1 (26836, Cell signalling, MA, USA), rabbit-anti-phospho-Stat3 (9145, Cell signalling, MA, USA), rabbit-anti-Stat3 (4904, Cell signalling, MA, USA), rabbit-

anti-Vimentin (3932, Cell signalling, MA, USA), rabbit-anti-Bcl-xL (2764, Cell signalling, MA, USA), rabbit-anti-GAPDH (2118, Cell signalling, MA, USA), rabbit-anti-PD-L1 (13684, Cell signalling, MA, USA), rabbit-anti-N-Cadherin (4061, Cell signalling, MA, USA) and rabbit-anti-E-Cadherin (3195, Cell signalling, MA, USA) overnight at 4°C. Membranes were washed in TBST (TBS-0.05% Tween-20) and incubated with either anti-rabbit HRP-conjugated secondary antibody for 1 h at room temperature. After several washes in TBST, the blots were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (34580, Thermo Fisher Scientific, MA, USA). All western blots were done in triplicate.

3.6. Chemoresistance

To assess whether the cell lines treated with PDAC serum and co-cultured with pancreatic cell lines have acquired chemoresistance we selected anticancer drugs. In particular, we assessed cellular viability upon treatments with Gemcitabine (G6423, Sigma, DE), Doxorubicin (CAS number 23214-92-8), 5-fluorouracil (F6627, Sigma, DE) and Paclitaxel (CAS number 33069-62-4). Treated and control cells were seeded 20.000 per well and incubated with drugs at their IC50 concentration for 72h. Subsequently their vitality was assessed by MTT assay. The MTT assay is based on the reduction of a yellow tetrazolium salt (A2231,0001, Applichem GmbH, DE) to purple formazan crystals by metabolically active cells. The insoluble formazan crystals are dissolved using DMSO (EMR385100, EuroClone, IT) and the resulting coloured solution was quantified by measuring absorbance at 570 nm using a multi-well spectrophotometer. All tests were done in triplicate for each condition. Finally, we compared the cell viability between treated and control cell lines.

3.7. Wound healing assay

Upon serum and co-culture exposure, migration assay has been performed as Cecati et al. [75]. Briefly, cells were plated in 24-well plates and let to attach for 24h. When cells have reached the confluence, the scratch is applied with a 1000ul pipette tip. Then, the wells are washed with PBS buffer to take away the detached cells, and fresh cell culture medium is added. The assay for each condition was done in triplicate. The images were taken at different time points until the gap was closed, by using the Eclipse Ti2E microscope (Nikon, Tokyo, Japan).

3.8. NGS-CNV analysis

The genomic DNA was isolated from HEK293T cells exposed to PDAC patients' sera and healthy donor sera using Exgene^{TM} Clinic SV mini extraction kit (Cat. No. 108-101, GeneAll biotechnology Ltd, Korea). DNA was treated with RNase, eluted in 40µl of deionized H_2O and run on 1% agarose gel for integrity evaluation. We submitted the samples for quality control and WGS to external service provider Biodiversa Srl (Rovereto, Italy). The total sample quantity was determined on Qubit fluorometer (Cat. No. Q33226, Invitrogen). The WGS was done on NovaSeq^{TM} 6000 platform (Cat. No. 20012850, Illumina, USA) in a Pair-End 2 x 150 bp setup.

After quality control (FASTQC), the files containing Whole Genome Sequencing (WGS) paired-end reads have been aligned against the human genome hg38 by using BWA-MEM tool (ver 0.7.17), using default parameters. The produced BAM files (treated and the matched normal control samples) have been submitted to the well-known copy number caller tool Control-FREEC (ver. v11.0) [76], using default parameters. This tool is based on read-depth alignment and automatically computes, normalizes, segments copy number profiles, calculate significance (Wilcoxon test and Kolmogorov-Smirnov test p-values) for the detection of CNAs.

3.9. Tumour xenografts

As animal models we used female 4-5 week-old athymic nude mice (nu/nu Swiss; ENVIGO RMS, Udine, Italy). The mice were housed under a 12-h light–dark cycle at 23 ± 1 °C, and 50 ± 5% humidity; ad libitum diet and water were provided. We injected 200 µl of mixture (volume ratio = 1:1) of matrigel Matrigel (Corning Costar) and culture medium with the suspension of cells HEK293T+Panc-1, HEK293T+ASPC-1, and respective controls (3.0×10^6 cells per mouse) subcutaneously into the interscapular region of each mouse. We used 5 animals per experimental group. Xenograft tumor growth was monitored twice a week by caliper measurements, and tumor volumes (mm^3) were estimated using the following formula: $TV=a \times (b^2)/2$, where a and b are tumor length and width, respectively, in millimeters. At d 30, the animals were euthanized following standard protocols. The tumors were dissected from the neighboring connective tissue, frozen in nitrogen, and stored at 280°C for further analyses. All animals were maintained and handled in accordance with the recommendation of the Guidelines for the Care and Use of Laboratory Animals and experiments were approved by the Animal Care Committee of University of Calabria (OPBA), Italy.

3.10. Histological analysis

Analysis of tissue sections was performed using tissue fixed in 4% paraformaldehyde for 24 h, dehydrated and embedded in paraffin. Tissue blocks were sectioned at 5 µm and were stained using Mayer's hematoxylin and eosin to facilitate histology and morphology evaluation. The stained tissues were visualized using Olympus BX51 microscope with a 20X objective.

3.11. Immunohistochemistry

For immunohistochemistry, antigen retrieval was performed on 5 µm paraffin sections in 0.01 mol/L citrate buffer (pH 6) in microwave at low setting. Incubations with primary antibodies were performed at room temperature overnight in a humidified chamber. As primary antibodies

we used anti-Ki-67 antibody (Clone MIB-1, Dako Italia Spa, Milan, Italy, M7240), anti-cyt-18 (Clone DC 10, Dako Italia Spa, Milan, Italy, M7010), and anti-Vimentin (Clone v9, Dako Italia Spa, Milan, Italy, M0725).

Normal horse or goat serum was used as blocking agent. Biotinylated horse anti-mouse/rabbit (1:100) or biotinylated goat anti-rat (1:100) was used as the secondary antibody and revealed with a Vectastain ABC Kit Elite (Vector Laboratories, Burlingame, CA, USA, PK-6200) and a Peroxidase Substrate Kit DAB (Vector Laboratories, Burlingame, CA, USA, SK-4100). All stained slides were visualized using Olympus BX41 microscope and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition. The results for Ki-67 expression were expressed in Allred score. In Allred system of scoring, score 0-5 is given to the cells depending on the proportion of cells which are stained (proportion score PS) and score 0-3 is given depending on the intensity of staining (intensity score IS). The final Allred score is a sum of PS and IS values.

3.12. Statistical analysis

For wound healing, MTT assays, soft agar colony measurements and western blot protein expressions significant differences between the treated and untreated cells were determined using the t-test. P values less than 0.05 were considered statistically significant. All statistical analyses for abovementioned assays were performed by using the Stat6 Software for Windows (Stat6 Software, San Diego, CA, USA). Wilcoxon test and Kolmogorov-Smirnov test were used for analysis of WGS-CNV data. *In vivo* results were analysed by Student's t-test or 1-way ANOVA with Bonferroni post hoc testing performed by GraphPad-Prism7 software program (GraphPad Inc., San Diego, CA, USA). All data are reported as mean ± Standard deviations (SD) and p value < 0.05 was considered statistically significant.

4. Results

4.1. In vitro transformation upon co-culturing and PDAC patients' serum treatments

We have used co-cultures followed by soft agar assay to validate the ability of pancreatic cancer cell lines to transform non-tumour primed cells. We used non-tumour hTERT-HPNE and HEK293T as a recipient cell lines, and pancreatic cancer cell lines as donor cells, in particular Panc-1, ASPC-1 and Capan-2. All combinations have been maintained for 3 weeks in the appropriate cell medium with addition of exosome depleted FBS. We used exosome depleted serum since EVs present in FBS may significantly influence behaviour of cultured cells, including release of EVs [77]–[79].

After 3 weeks we validated the transformation by soft agar assay. The assay was done in triplicate for each co-culture condition. The Figures 1-2 show some representative colonies formed in various co-culture combinations with respective controls. The average colony number formed by HEK293T cell line was highest in combination with ASPC-1, 13 colonies (±1.69 SD) with a medium dimension of 85 μm (±21.02 SD), and those co-cultured with Panc-1 cells have formed on average 5 colonies (±0.94 SD) with a medium dimension of 65.74 μm (±9.75 SD).

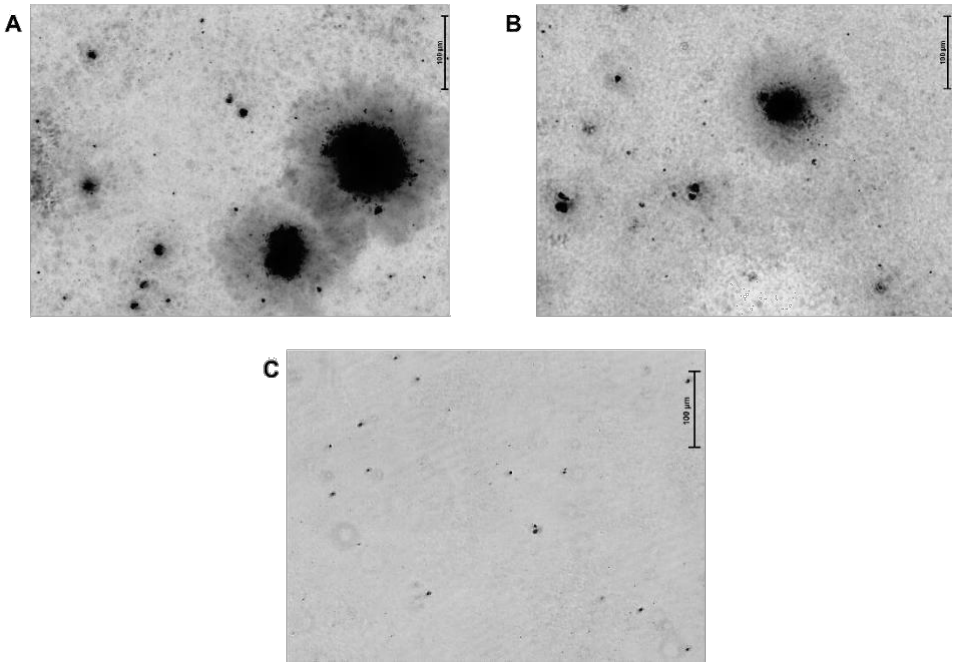

Figure 1. The representative colonies in soft agar assay for HEK293T co-culture with (A) ASPC-1; (B) Panc-1; (C) control.

The hTERT-HPNE cells in co-culture with Panc-1 hTERT-HPNE formed only one colony with dimension of 50 µm. The similar result has been observed in ASPC-1 co-culture setting with one colony of 70 µm diameter. In the combination with Capan-2 cell line we have observed 2 colonies with a medium dimension of 64.5 µm (±0.5 SD). Both control HEK293T and hTERT-HPNE cell lines did not form colonies larger than 50 µm.

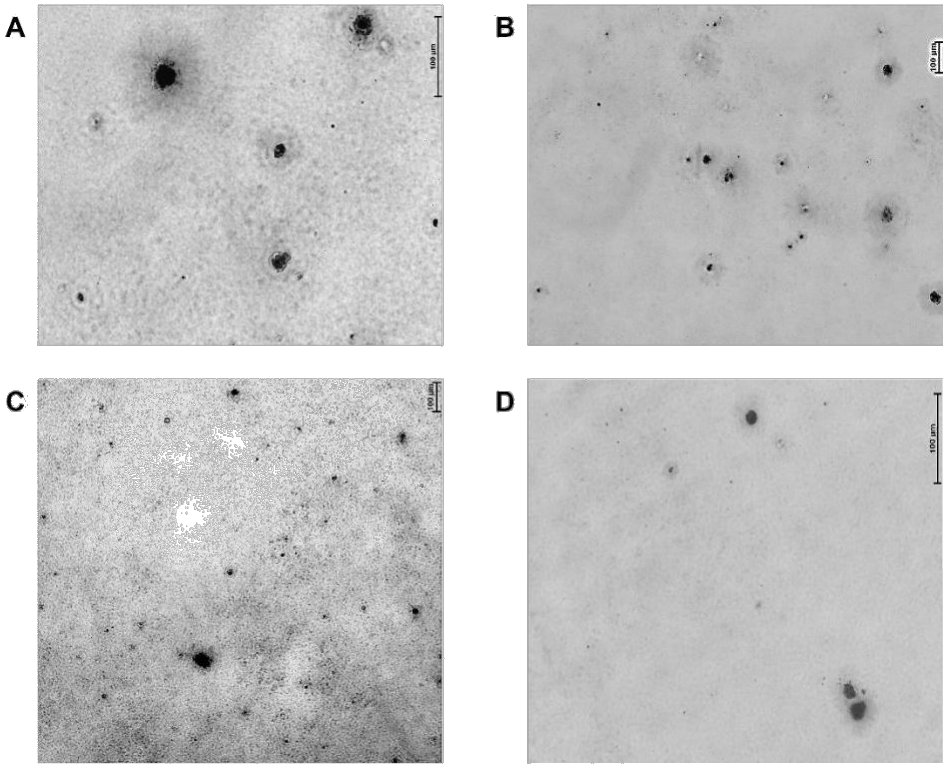

Figure 2. The representative colonies in soft agar assay for hTERT-HPNE co-culture with (A) Capan-2; (B) Panc-1; (C) ASPC-1; (D) control.

The obtained *in vitro* results indicate that HEK293T cell line was transformed with major success upon co-culturing with different pancreatic cancer cell lines, whereas hTERT-HPNE line showed less significative results.

In order to assess *in vitro* the transforming potential of PDAC patient serum, we treated hTERT-HPNE and HEK293T cell lines with pooled sera of non-metastatic PDAC patients and healthy donors for 3 weeks. The soft agar assay was done in triplicate for each condition, and Figure 3 shows some representative colonies of both serum treated cell lines and their respective controls. The average colony number formed by HEK293T cell line treated with PDAC plasma was 54 (±0.81 DS) with a medium colony size of 136 um (±69.9 DS), whereas the same cell line treated with healthy sera did not form colonies larger than 50um. Similarly, for the healthy sera treatment of hTERT-HPNE line, colonies larger than 50um have not been

observed. Moreover, hTERT-HPNE cells treated with PDAC serum formed an average of 6 (± 0.47 DS) colonies with a medium size of 64 um (±18.5 DS).

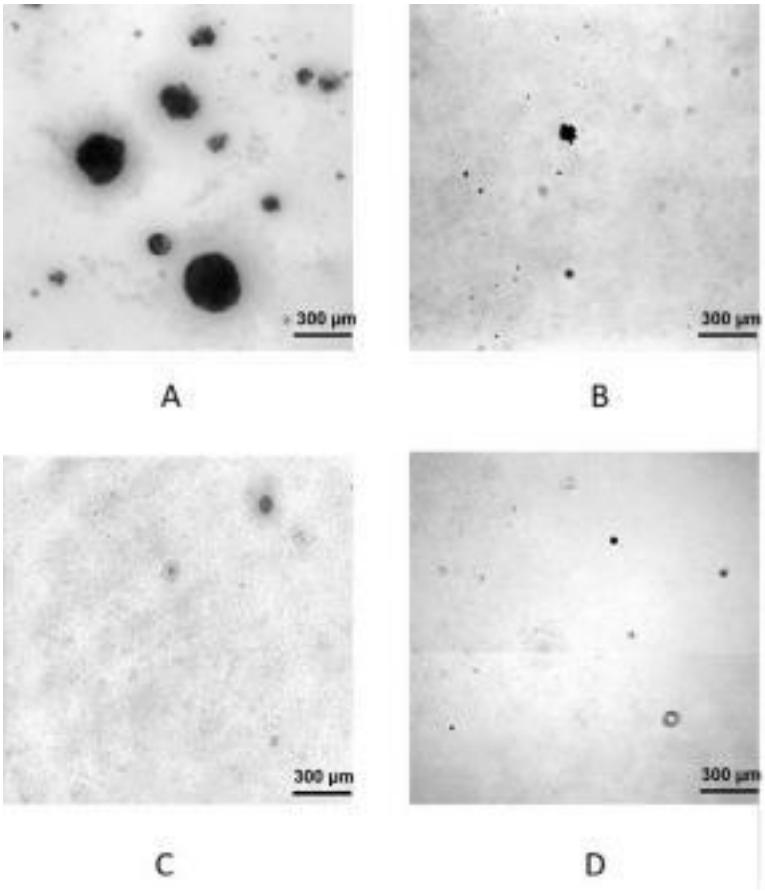

Figure 3. The representative colonies in soft agar assay for: (A) HEK293T + PDAC serum; (B) hTERT-HPNE + PDAC serum; (C) HEK293T + healthy donor serum; (D) hTERT-HPNE + healthy donor serum.

These results indicate that PDAC plasma has the potential to transform non-tumour cell lines, and that HEK293T cells seem to be more prone to malignant transformation than hTERT-HPNE cells.

In both treatment types we have observed a major transformation of HEK293T cell line in comparison with hTERT-HPNE, and the colonies had greater dimensions. However, based on *in vitro* assays, it seems that PDAC patients' serum treatments has higher potential to transform cells than co-culture assay.

4.2. Cell migration assay

We used the wound healing assay to assess variations in migration velocity and cell–cell interaction. It was completed by making a scratch on a cell monolayer and observing the closure of the gap under microscope. Firstly, using different approaches we tried to isolate the colonies from soft agar, but it was not successful since cells were not able to attach to surface, or they were lost during the procedure. After not being able to avoid the limitation of classical soft agar assay, we plated all recipient cells from co-culture for wound healing assay. Therefore, the following results are to be considered as an average of both successfully transformed cells and non-transformed ones. Also, we have seeded the non-treated cells at the same passage number as a negative control. All conditions were done in triplicate and Figures 4-10 show a representative image of all co-culture combinations, their respective controls and graphical representation of results.

The hTERT-HPNE cells co-cultured with Panc-1 after 30 hours showed an average gap closure of 74.22% (±6.03 SD), with Capan-2 an average of 78.13% (±2.08 SD), and with ASPC-1 an average closure of 70.53% (±13.21 SD). The control cells had a 30-hour average gap closure of 56.39% (±1.18 SD), 60.78% (±4.80 SD), and 40.26% (±8.05 SD) respectively.

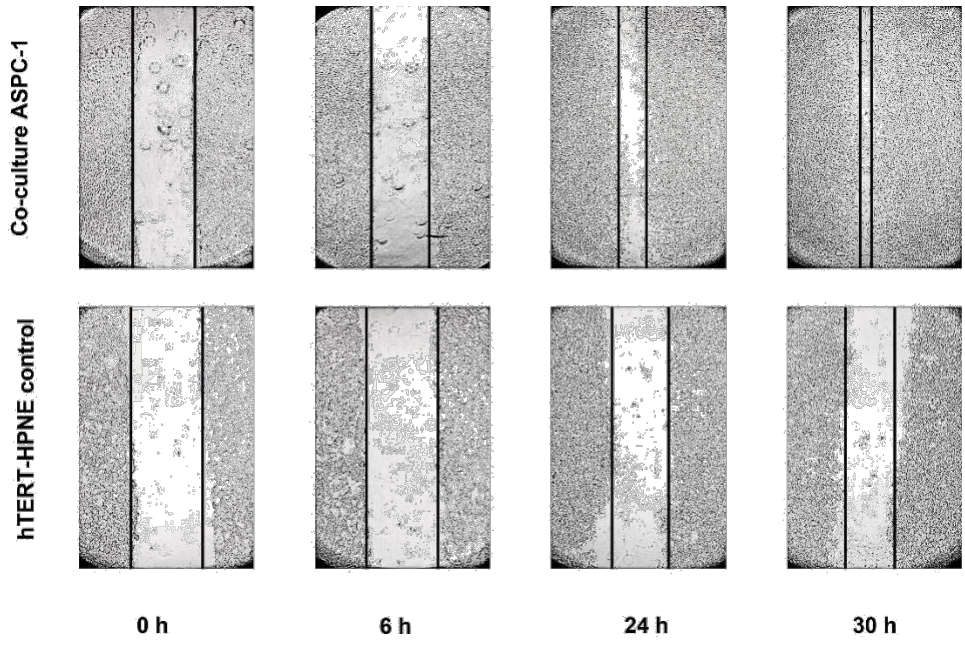

Figure 4. Wound healing assay for hTERT-HPNE co-culture with ASPC-1 and control.

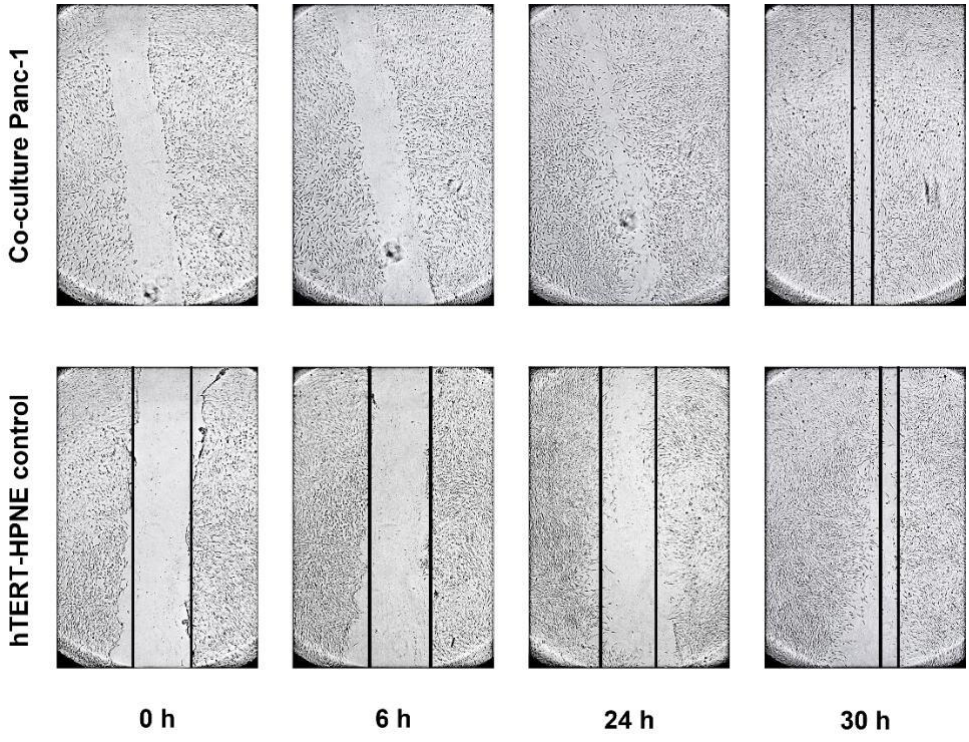

Figure 5. Wound healing assay for hTERT-HPNE co-culture with Panc-1 and control.

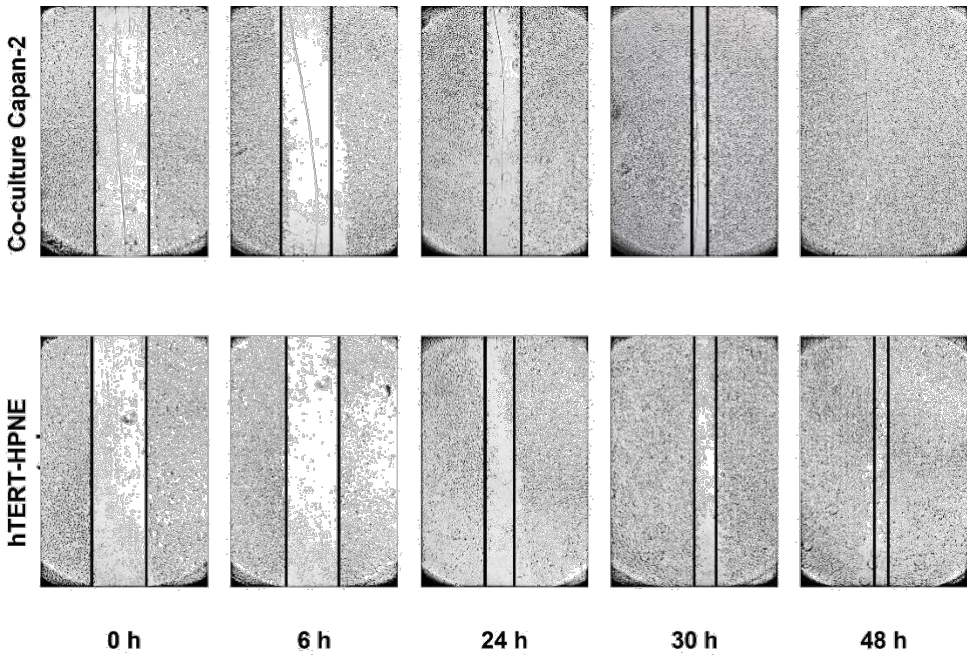

Figure 6. Wound healing assay for hTERT-HPNE co-culture with Capan-2 and control.

The HEK293T cells co-cultured with Panc-1 after 30 hours had an average gap closure of 82.68% (±8.82) compared to the 61% (±8.05 SD) of the control cells, and the ASPC-1 combination showed an average of 64.23% (±17.74) whereas the control cells closed gap at an average of 56.43% (±13.93).

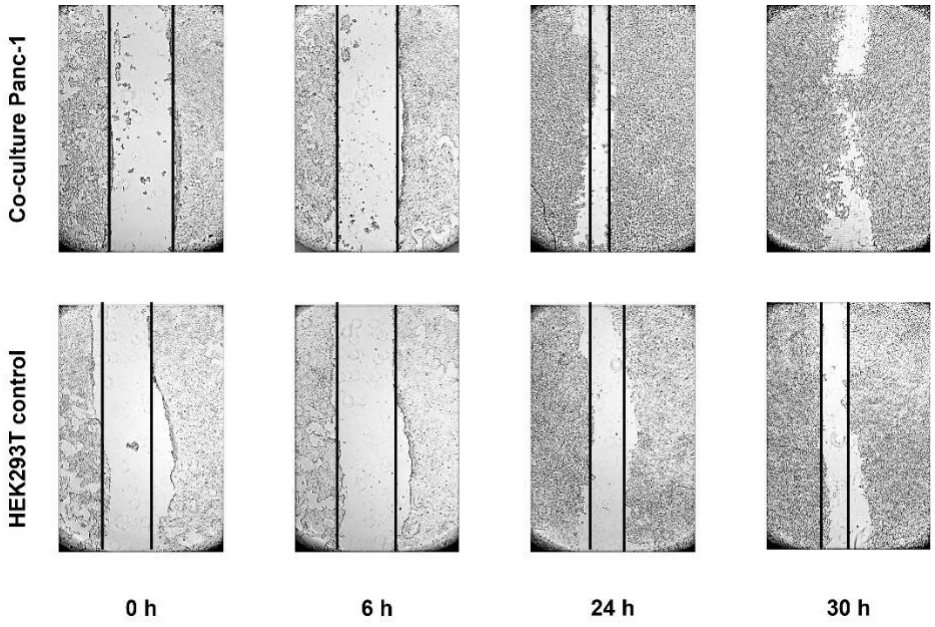

Figure 7. Wound healing assay for HEK293T co-culture with Panc-1 and control.

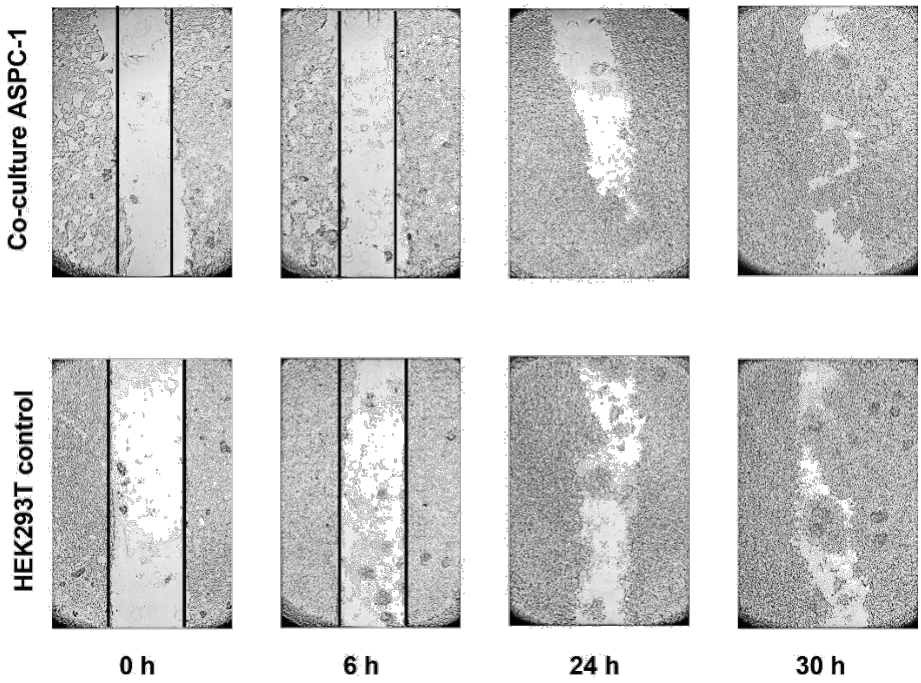

Figure 8. Wound healing assay for HEK293T co-culture with ASPC-1 and control.

Overall, we observed at least slight gain of migration ability in all co-cultured cells regardless of the tumour cell line used. However, the hTERT-HPNE treated with ASPC-1 was the most successful combination for this cell line, whereas for HEK293T it was the co-culture with Panc-1.

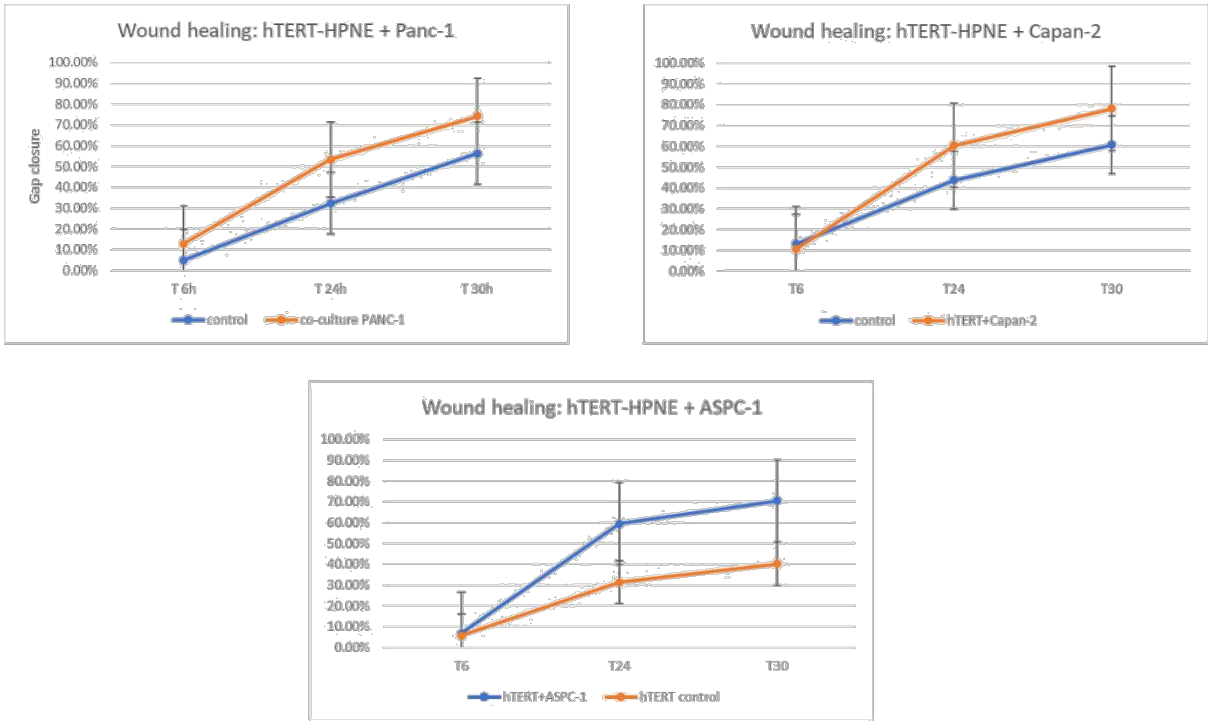

Figure 9. Wound healing assay. Graphical representation of mean values with standard deviation for hTERT-HPNE co-cultures.

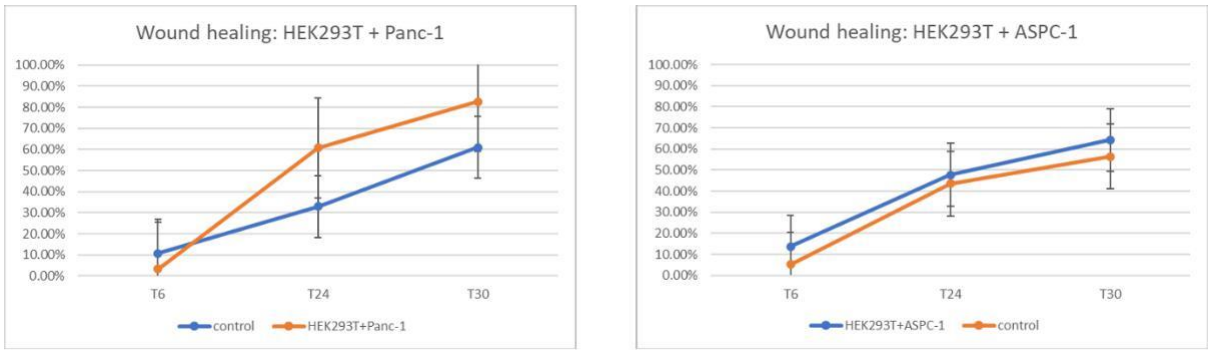

Figure 10. Wound healing assay. Graphical representation of mean values with standard deviation for HEK293T co-cultures.

We have also assessed the cell migration of cells treated with PDAC patients' sera compared to healthy control cells. Figures 11-12 show some representative images (all plates were done in triplicate) of the wound healing assay for hTERT-HPNE and HEK293T cell lines until the gap closure. The graphs in Figure 13 summarize the results for both cell lines and conditions.

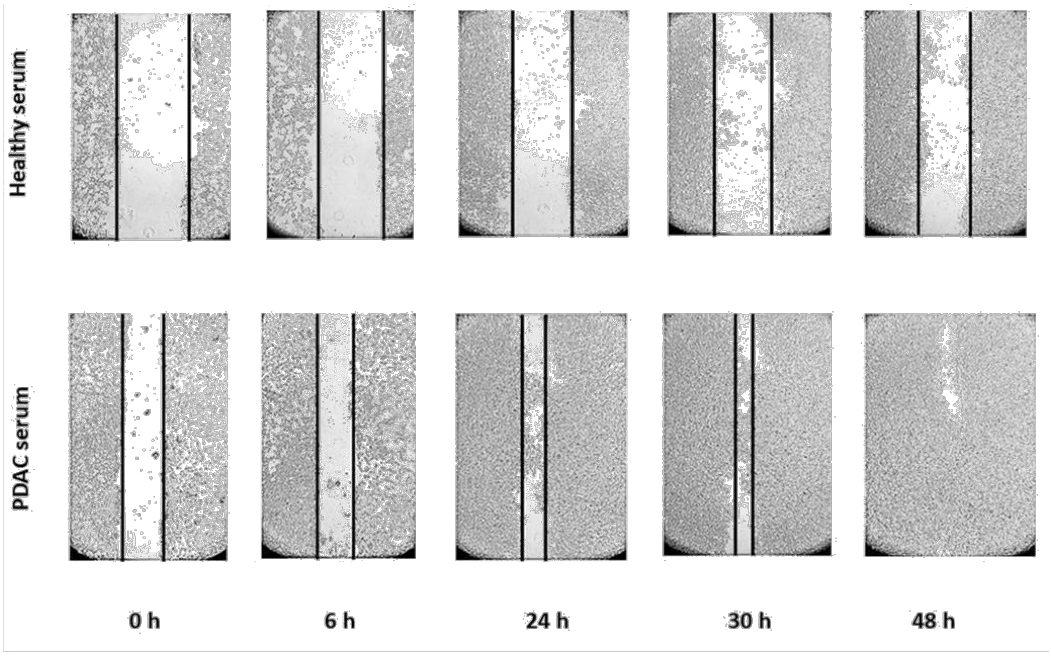

Figure 11. The representative wound healing assay for HEK293T cell line treated with healthy and PDAC serum.

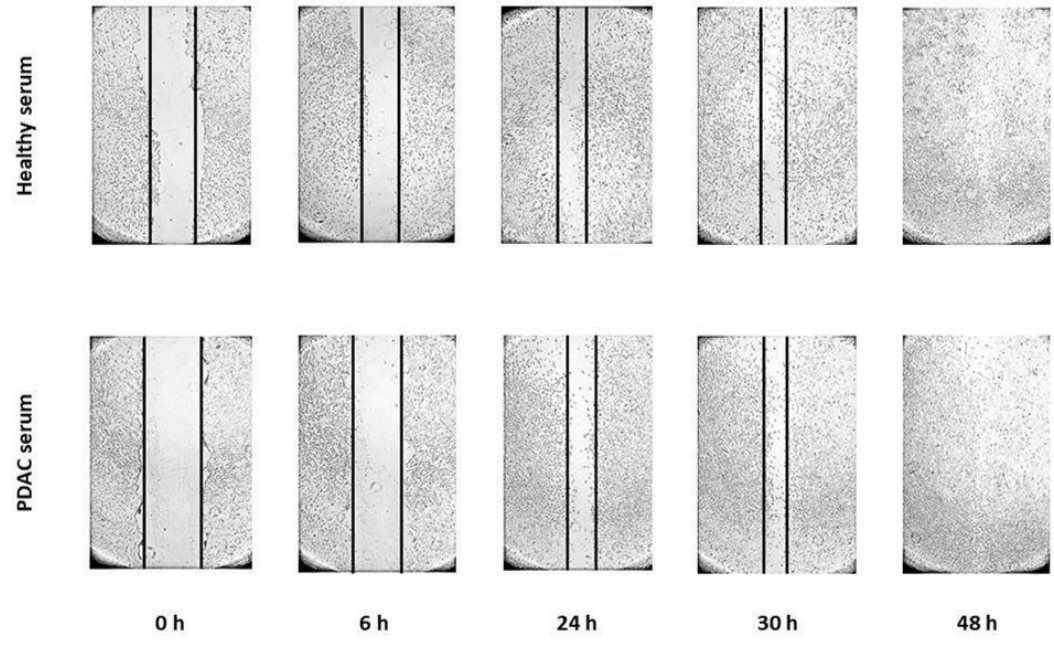

Figure 12. The representative wound healing assay for hTERT-HPNE cell line treated with healthy and PDAC serum.

The HEK293T cell line treated with PDAC serum after 48 hours had an average gap closure of 92% (± 7.13 SD) whereas the control cells at the same time point closed the gap only at 55% (± 7.27 SD). The migration of hTERT-HPNE cells treated with PDAC serum was not much different from the control group, in particular, after 48 hours treated cells showed an average of 95% of the gap closure (± 4.63 SD) and the control cells an average of 87% (± 15.63).

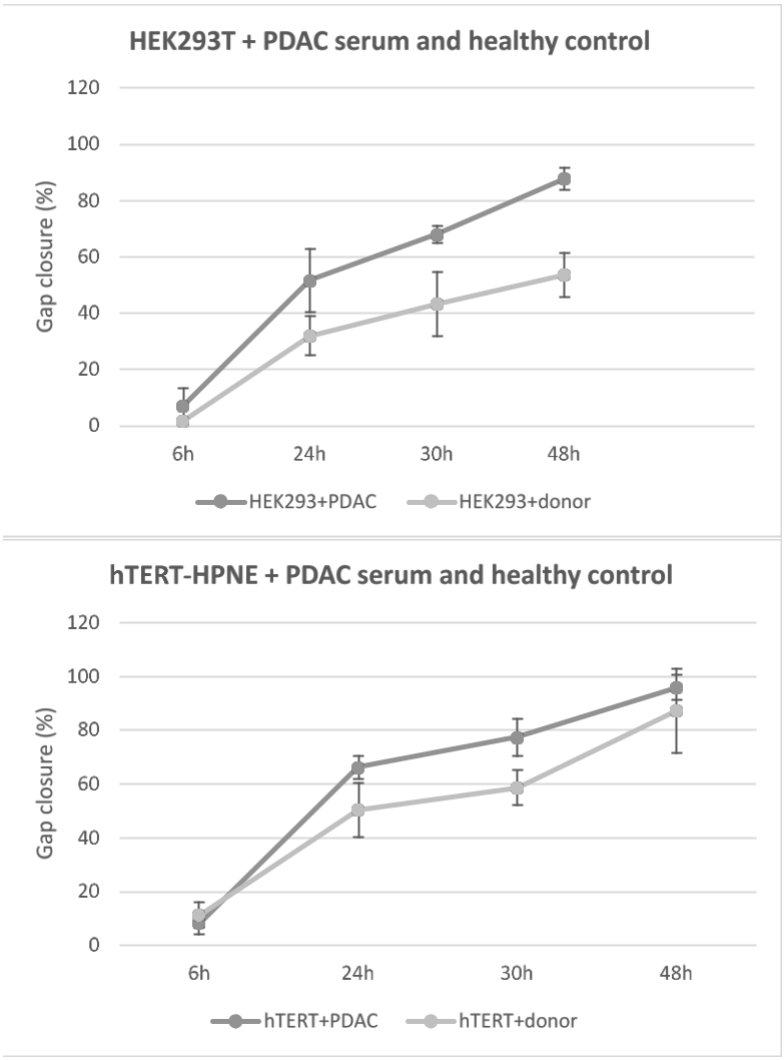

Figure 13. Graphical representation of mean values with standard deviation for HEK293T and hTERT-HPNE cell lines treated with PDAC serum compared to the healthy serum.

The results indicate that the treatment with PDAC serum greatly increased the migration of HEK293T cells, whereas hTERT-HPNE cells did not show a significant difference.

4.3. Assessment of resistance to common chemotherapeutics

As drug resistance is a hallmark of PDAC, we exposed co-cultured, and serum treated cells to common chemotherapeutics in order to validate if treatments caused gain of drug resistance. At the end of the incubation time, we assessed the IC50 based on cell viability using the MTT assay. The MTT assay was done in triplicate for each drug treatment and control condition. The results for each test group are summarized in Tables 1-3.

As the HEK293T cell line showed greater transformation in soft agar assay than hTERT-HPNE upon PDAC sera treatments, we assessed its chemoresistance as previously described for co-cultures. The PDAC serum treated HEK293T cell line has developed resistance to paclitaxel (P=0.00002). Upon exposure to 100 nM paclitaxel the control HEK293T cells had a viability of about 50% whereas the PDAC serum treated cells had a 91% of viability. Gemcitabine, doxorubicin and 5-FU caused an opposite effect reducing the viability of PDAC serum treated cells. The results are summarized in the Table 1.

Drug (concentration)	**HEK293T + healthy serum**	**HEK293T + PDAC serum**	**P-value**
Gemcitabine (13 uM)	42%	16%	0.009
Doxorubicin (100 nM)	53%	30%	0.001
Paclitaxel (100 nM)	51%	91%	0.001
5-FU (5 uM)	46%	17%	0.001

Table 1. The MTT assay results for cell lines treated with chemotherapeutics expressed as average percentage of cell viability.

As for co-cultured cells, we have observed the gain of resistance only to doxorubicin, in the hTERT-HPNE co-culture with Capan-2 (P=0.015), and HEK293T co-culture with Panc-1 (P=0.0002). We have observed that in several co-culture settings treated cells become more sensitive to the anti-tumour drugs. In particular, hTERT-HPNE+Panc-1 is more sensitive to

cisplatin and gemcitabine, HEK293T+ASPC-1 is more sensitive to cisplatin, and hTERT-HPNE+ASPC-1 is more sensitive to 5-FU treatment.

Drug (concentration)	hTERT-HPNE cell line co-culture								
	Panc-1	**Control**	**P value**	**Capan-2**	**Control**	**P value**	**ASPC-1**	**Control**	**P value**
Cisplatin (5uM)	53%	70%	0.03	79%	81%	0.40	79%	80%	0.87
Doxorubicin (400 nM)	55%	64%	0.10	76%	54%	0.015	45%	68%	0.06
Gemcitabine (20 uM)	46%	55%	0.01	66%	68%	0.32	60%	62%	0.79
5-FU (400 uM)	65%	71%	0.12	73%	72%	0.26	32%	90%	0.01
Paclitaxel (80 nM)	83%	83%	0.99	71%	64%	0.14	47%	70%	0.008

Table 2: The MTT assay results for hTERT-HPNE cell line co-cultured with Panc-1, Capan-2 and ASPC-1 treated with chemotherapeutics expressed as average percentage of cell viability.

Drug (concentration)	**HEK293T + ASPC-1**	**HEK293T control**	**P-value**
Cisplatin (5 uM)	51%	75%	0.03
Doxorubicin (400 nM)	41%	40%	0.007
Gemcitabine (20 uM)	42%	41%	0.025
5-FU (400 uM)	40%	42%	0.87
Paclitaxel (80 nM)	41%	39%	0.07
Drug (concentration)	**HEK293T + Panc-1**	**HEK293T control**	**P-value**
Cisplatin (2 uM)	43%	44%	0.89
Doxorubicin (50 nM)	74%	33%	0.0002
Gemcitabine (10 uM)	55%	48%	0.21
5-FU (50 nM)	52%	49%	0.45

Table 3: The MTT assay results for HEK293T cell line co-cultured with Panc-1 and ASPC-1 treated with chemotherapeutics expressed as average percentage of cell viability.

4.4. Western blot analysis of protein expression

Total proteins were extracted from all co-cultured cells, PDAC serum treated cell lines and their respective controls in order to analyse the expression of typically up- and down-regulated proteins in PDAC tumour cells. These chosen proteins play key roles in some signalling pathways, in epithelial-to-mesenchymal transition (EMT), enable immuno-escape of tumour cells or have anti-apoptotic effects. The protein expression results are summarized in Tables 4 -5 as average ratio among treated and control cells.

Protein	HEK293T	hTERT-HPNE
STAT3	11	0.85
p-STAT3	N/A	N/A
PD-L1	2.11	0.61
Bcl-xL	1.62	1.28
mTOR	30.2	0.02
p-mTOR	N/A	0
Vimentin	0.16	1.66
FN1	N/A	N/A
E-cadherin	N/A	N/A
N-cadherin	N/A	N/A

Table 4. Protein expression for serum treated cells. Summarized results of up and down regulated protein expressed as average ratio between PDAC and healthy serum treatments in hTERT-HPNE and HEK293T cell lines. N/A: not applied, see text.

The HEK293T cell line exposed to PDAC patients' sera showed an up-regulation of proteins responsible for increased growth, proliferation (STAT3, mTOR), and survival (Bcl-xL). The anti-apoptotic protein Bcl-xL expression is only 1.62-fold higher, and the PDL-1 responsible for immuno-escape of tumour cells is 2.11-fold higher. A very high fold change has been observed for mTOR (30-fold) and STAT3 (11-fold), moreover active, phosphorylated, p-mTOR goes from 0% (healthy serum treated) to 25% (PDAC treated serum), whereas p-STAT3 is absent in both conditions. The only down-regulated protein in HEK293T cell line is vimentin (6.25-fold). Interestingly, in the hTERT-HPNE cell line treated with PDAC sera we have observed the opposite situation. In particular, the vimentin expression was slightly up-regulated (1.66-fold). The mTOR expression was greatly reduced (50-fold), and also PD-L1 is down-regulated (1.64-fold). BCL-xL and STAT3 did not show a significant expression change in this cell line. The common feature for both cell lines was a complete absence of fibronectin, E-cadherin and N-cadherin. The representative protein bands are shown in Figure 14.

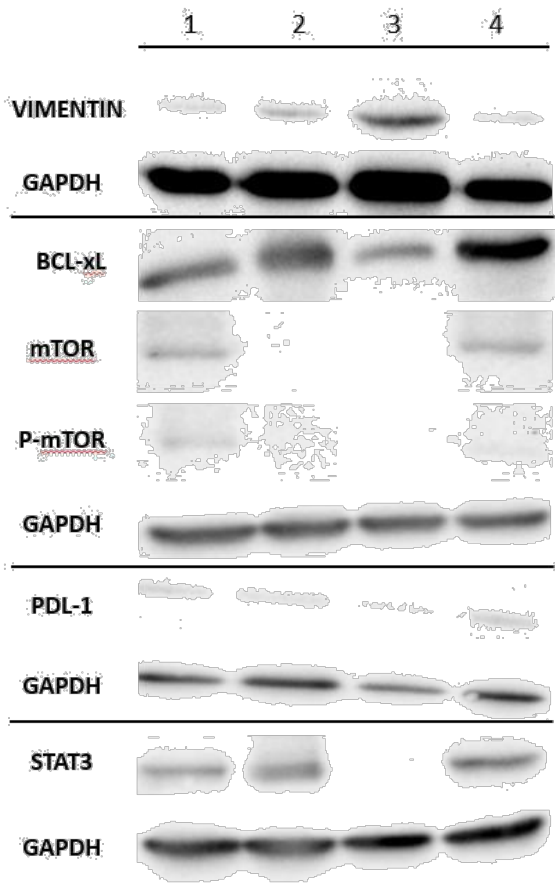

Figure 14. The representative western blot for vimentin, Bcl-xL, mTOR, P-mTOR, PDL-1 and STAT3 each with the corresponding GAPDH protein used for data normalization. The lanes correspond to the following samples: (1) hTERT-HPNE + healthy serum; (2) hTERT-HPNE + PDAC serum; (3) HEK293T + healthy serum; and (4) HEK293T + PDAC serum

The up-regulation of Bcl-xL is a common feature for almost all co-cultured cells, in particular the highest fold change of 18.99 has been observed in hTERT-HPNE+ASPC-1 co-culture. The hTERT-HPNE+Panc-1 co-culture had a 2.81-fold change, whereas a very similar fold change of 1.88 and 1.86 has been observed in HEK293T+Panc-1 and HEK293T+ASPC1 respectively. Only in the hTERT-HPNE+Capan-2 co-culture we did not observe a significant increase of Bcl-xL expression (1.35-fold change). The representative western blot membranes for Bcl-xL are presented in Figure 15.

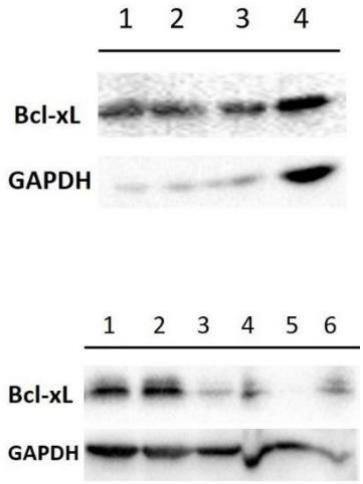

Figure 15. Western blot membranes of Bcl-xL and GAPDH used for normalization. The lanes of the upper figure correspond to: (1) HEK293T+Panc-1; (2) HEK293T control; (3) hTERT-HPNE+Panc-1; (4) hTERT-HPNE control. The lanes of the lower figure correspond to: (1) hTERT-HPNE control; (2) hTERT-HPNE+Capan-2; (3) HEK293T control; (4) HEK293T+ASPC-1; (5) hTERT-HPNE control; (6) hTERT-HPNE+ASPC-1

We found STAT3 to be over-expressed only in hTERT-HPNE+Panc-1 (4.9-fold), however it was not associated with an increased expression of its phosphorylated form. Indeed, the p-STAT3 was reduced 1-75-fold in hTERT+Panc-1. Also in HEK293T+Panc-1 we have observed a slight down-regulation of pSTAT3 (1.51-fold). The p-STAT3 band was absent in hTERT-HPNE+ASPC-1 and HEK293T+ASPC-1. There was no significant change in STAT3 expression in HEK293T+Panc-1, hTERT-HPNE+Capan-2 and hTERT-HPNE+ASPC-1. In

HEK293T+ASPC-1 we observed an unsignificant down-regulation of 1.29-fold change in STAT3 (Figure 16).

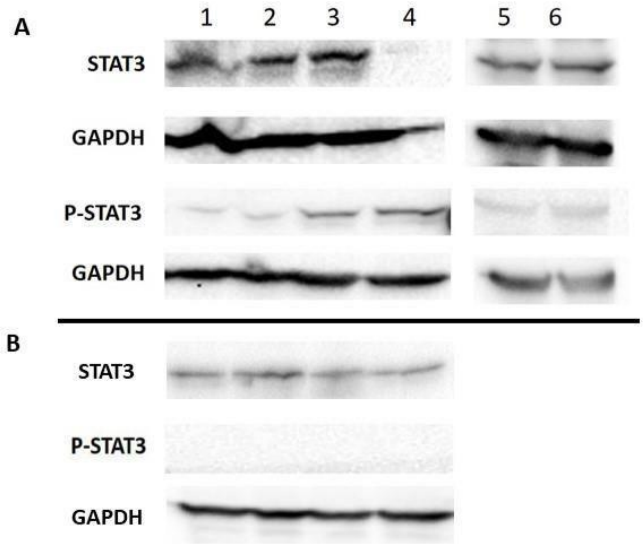

Figure 16. Western blot membranes for STAT3, p-STAT3 and respective GAPDH used for data normalization. The lanes of the figure A correspond to: (1) HEK293T+Panc-1; (2) HEK293T control; (3) hTERT-HPNE+Panc-1; (4) hTERT-HPNE control; (5) hTERT-HPNE control; (6) hTERT-HPNE+Capan-2. The lanes of the figure B correspond to: (1) hTERT-HPNE+ASPC-1; (2) hTERT-HPNE control; (3) HEK293T+ASPC-1; (4) HEK293T control

The mTOR expression was significantly increased only in co-culture hTERT-HPNE+ASPC-1 (1.82-fold) together with a 1.55-fold up-regulation of its active, phosphorylated form. A more sustained change in mTOR expression of 1.58-fold was observed in hTERT-HPNE+Panc-1 and hTERT-HPNE+Capan-2, and 1.49-fold change in co-culture HEK293T+Panc-1. In all three combinations the phosphorylated form was also slightly increased, in particular 1.56-fold, 1.39-fold, and 1.37-fold respectively.

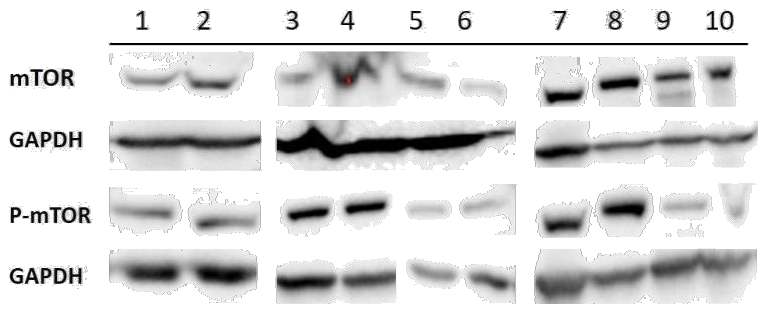

Figure 17. Western blot membranes for mTOR, p-mTOR and respective GAPDH controls. The samples in lanes are as follows: (1) hTERT-HPNE control; (2) hTERT-HPNE+Capan-2; (3) HEK293T+Panc-1; (4) HEK293T control; (5) hTERT-HPNE+Panc-1; (6) hTERT-HPNE control; (7) hTERT-HPNE control; (8) hTERT-HPNE+ASPC-1; (9) HEK293T control; (10) HEK293T+ASPC-1.

In the co-culture hTERT-HPNE+Panc-1 we have also observed a slight up-regulation of PD-L1 (1.62-fold) and vimentin (1.56-fold). A modest down-regulation of vimentin was observed in all co-cultures, beside hTERT-HPNE+Panc-1. None of the tested proteins showed a significant down-regulation in any of the co-culture settings. However, some proteins were absent, in particular PD-L1 in co-cultures hTERT-HPNE+ASPC-1 and HEK293T+Panc-1. Also, fibronectin was absent in all co-cultures beside hTERT-HPNE+Panc-1 in which it was slightly up-regulated (1.59-fold).

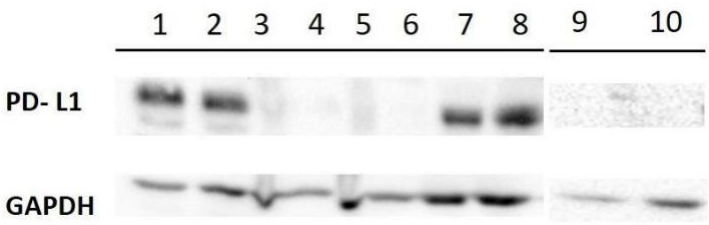

Figure 18. Western blot membranes for PD-L1 and GAPDH. The lanes correspond to: (1) hTERT-HPNE+Panc-1; (2) hTERT-HPNE control; (3) hTERT-HPNE+ASPC-1; (4) hTERT-HPNE control; (5) HEK293T+ASPC-1; (6) HEK293T control; (7) hTERT-HPNE+Capan-2; (8) hTERT-HPNE control; (9) HEK293T+Panc-1; (10) HEK293T control.

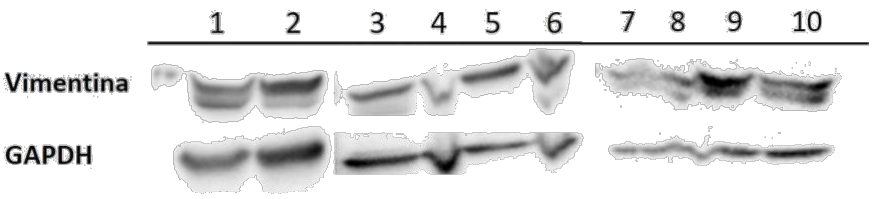

Figure 19. Western blot membranes for vimentin and GAPDH. The lanes correspond to: (1) hTERT-HPNE+Capan-2; (2) hTERT-HPNE control; (3) HEK293T control; (4) HEK293T+ASPC-1; (5) hTERT-HPNE control; (6) hTERT-HPNE+ASPC-1; (7) HEK293T+Panc-1; (8) HEK293T control; (9) hTERT-HPNE+Panc-1, (10) hTERT-HPNE control.

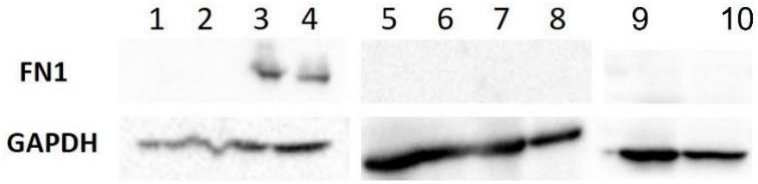

Figure 20. Western blot membranes for fibronectin and GAPDH. The lanes samples in lanes correspond to: (1) HEK293T+Panc-1; (2) HEK293T control; (3) hTERT-HPNE+Panc-1; (4) hTERT-HPNE control; (5) hTERT-HPNE control; (6) hTERT-HPNE+Capan-2; (7) hTERT-HPNE control; (8) hTERT-HPNE+ASPC-1; (9) HEK293T+ASPC-1; (10) HEK293T control.

Overall, the western blot results suggest that co-culture treatment increased expression of proteins responsible for enhanced proliferation, growth, and survival in both recipient cell lines. However, the changes in protein expression are in general modest in almost all co-culture settings.

Protein	hTERT-HPNE +Panc-1	hTERT-HPNE +ASPC-1	hTERT-HPNE +Capan-2	HEK293T +Panc-1	HEK293T +ASPC-1
STAT3	4.9	0.89	1.08	0.97	0.77
p-STAT3	0.57	N/A	N/A	0.66	N/A
PD-L1	1.62	N/A	0.74	N/A	0.93
Bcl-xL	2.81	18.99	1.35	1.88	1.86
mTOR	1.58	1.82	1.58	1.49	1.04
p-mTOR	1.56	1.55	1.39	1.37	0.61
Vimentin	1.56	0.86	0.75	0.65	0.84
FN1	1.59	N/A	N/A	N/A	N/A

Table 5. Protein expression for co-cultured cells. Summarized results of up- and down-regulated proteins expressed as average ratio between co-cultured and untreated hTERT-HPNE and HEK293T cell lines. N/A: not applied, no band.

4.5. In vivo tumour growth

Xenograft experiments were performed to evaluate, *in vivo,* the transformation capacity of co-culture treatments. Based on positive soft agar assay results we have chosen the cells combinations to inject into mice, in particular HEK293T+Panc-1 and HEK293T+ASPC-1. Since hTERT-HPNE did not show very significant results in any combination in soft agar assay, we included also other tests in selection criteria (i.e. wound healing, chemoresistance) and selected the hTERT-HPNE+Capan-2 to be injected into mice. However, the hTERT-HPNE+Capan-2 cells and hTERT-HPNE control cells did not form tumours in animals. Therefore, in the following text we will focus only on the results obtained on HEK293T co-cultures.

The cells were injected into the intrascapular region of female nude mice and we monitored tumour growth for 5 weeks. Tumour was well tolerated because no changes in body weight or

in food and water consumption were observed along with no evidence of reduced motor function (Figure 21 E). In addition, no significant differences were observed in the mean weights or histologic features of the major organs (liver, spleen and kidney) after sacrifice between control and treated mice, indicating a lack of toxic effects. The co-culture treatment both with Panc-1 and ASPC-1 significantly increased tumour growth in mice when compared to the control group.

Indeed, it was very impressive the evidence that the co-culture with Panc-1 induced an increase of tumour progression size twice as the control (Figure 21, A-D).

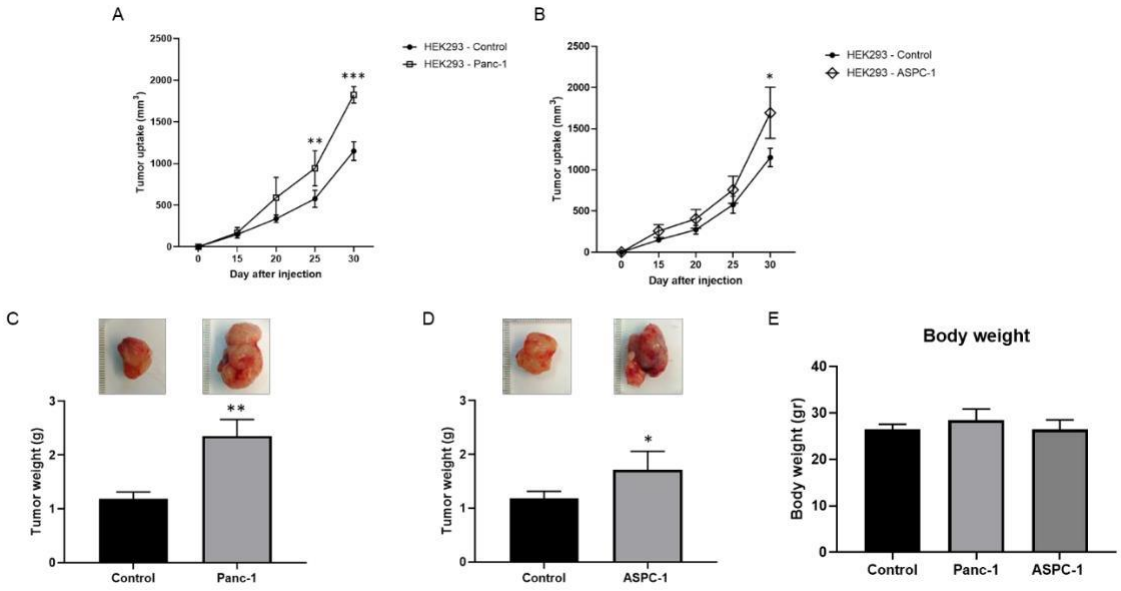

Figure 21. Impact of treatments of HEK293 cells with co-cultures from Panc-1 and ASPC-1 on tumor growth xenografts.

Hek293 cells with conditioned medium from Panc-1(A) and ASPC-1(B). Tumor growth was monitored by measuring the visible tumor sizes at the indicated time points. Tumor volume mean ± SD is shown (C-D). Images of representative individual tumors and tumor weight. (E) Body weight. For in vivo studies, data were compared using a 1way ANOVA. *p<0.05, **p<0.01 e ***p<0.001 vs. Control.

Furthermore, the HEK293T+Panc-1 tumours presented a higher intensity score of the proliferative marker Ki67 (Figure 22 D, E, F). In the same experimental conditions, similar results were observed after Cyt18 and Vimentin staining (Figure 22 G-N; Table 6).

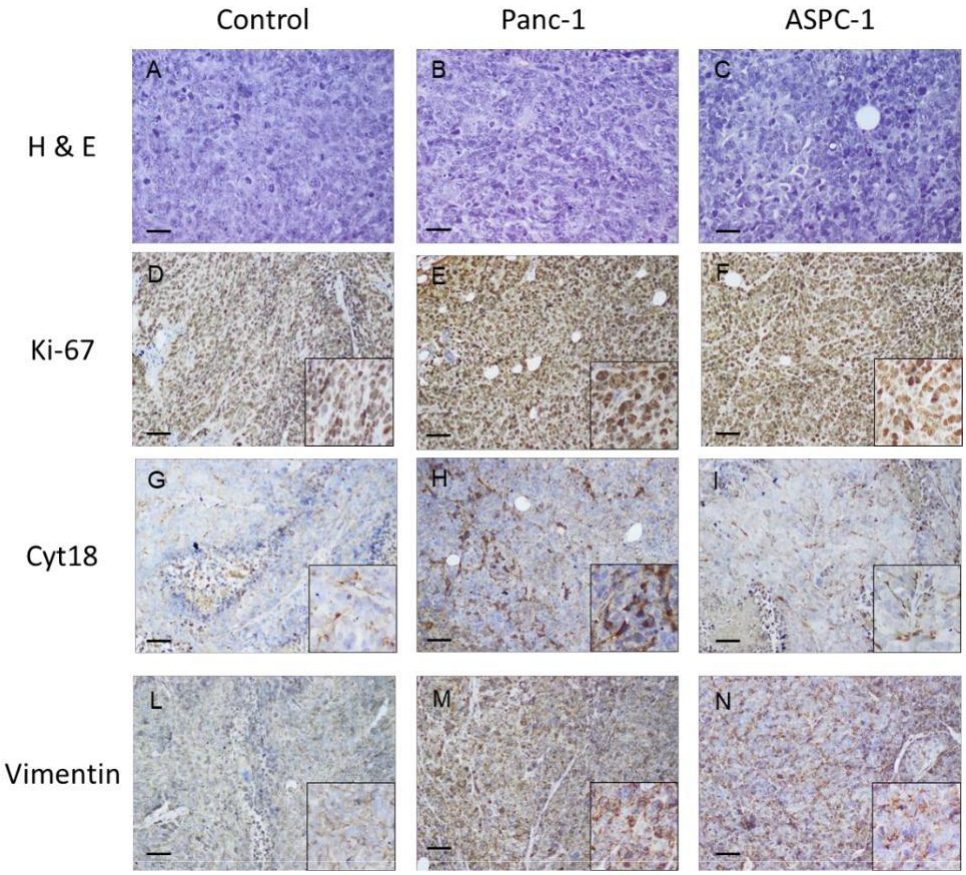

Figure 22. Influence of co-culturing HEK293 cells with Panc-1 and ASPC-1 in xenograft tumour models. (A, B, C) Haeematoxylin and Eosin (H&E) staining. Immunohistochemical staining of Ki-67, (D, E, F), Cyt-18(G, H, I) and Vimentin (L, M, N). Scale bar = 50 µm. Inserts: higher magnifications of the images

Table 6. Immunostaining scores of Ki-67 (Allred score median), Cyt18 and Vimentin in HEK293T xenograft

	Control	Panc-1	ASPC-1
Ki-67 (Allred score median)	5	8**	7**

Immunostained slides scores as follows: Total score= Proposition score+ Intensity score (range 0-8)
*P< 0,005; ** P< 0,001 (one-way ANOVA with Bonferroni Post Hoc test) control versus treated

	Control	Panc-1	ASPC-1
Cyt-18	+	+++**	++*
Vimentin	++	++++**	++++**

Staining intensity scores has been considered as it follows: - negative; + weak; ++ moderate; +++ strong
*P< 0,005; ** P< 0,001 (one-way ANOVA with Bonferroni Post Hoc test) control versus treated

4.6. Genomic imbalance in PDAC serum treated cells

After we established that HEK293T cell line were transformed upon PDAC serum treatments, we assessed the gene copy number variations using NGS approach and compared it to the control cells. The obtained paired-end sequences from genomic DNA were aligned to human genome hg38 and submitted to copy number caller software Control-FREEC (ver. v11.0) [80] for CNV detection. We have observed both gains and losses in various chromosomes. In particular, we have observed major gains in regions of chromosomes 19 and 20, and some modest changes in chromosomes 1, 4, 5 and 9. Losses have been detected in smaller number located on chromosomes 3, 4 and 7, but most of these regions did not contain important protein coding genes with roles in any tumour-related process (Figure 23 and Table 7). We have also detected gains of micro-RNAs, including miR-296 known for its role in PDAC as a tumour suppressor by targeting AKT2 [81].

Table 7. WGS-CNV analysis. List of the genes present in copy number variation in the genome of HEK293T cell line treated with PDAC patients' sera.

chr	start	end	copy number	status	Wilcoxon Rank Sum Test P-value	Kolmogorov Smirnov P-value	Genes
1	246640000	246749999	3	gain	4.34E-02	2.55E-01	CNST, LOC102724382, SCCPDH
3	33290000	33379999	1	loss	1.11E-04	9.40E-05	FBXL2
3	76610000	76719999	1	loss	6.26E-06	2.23E-06	ROBO2, LOC102724949
3	93430000	93519999	1	loss	1.40E-04	1.09E-04	None
4	49660000	49759999	1	loss	2.91E-05	1.55E-05	None
4	70690000	70779999	3	gain	1.37E-03	5.15E-04	RUFY3, UTP3
5	22290000	22379999	6	gain	2.76E-02	4.79E-03	CDH12
7	111400000	111579999	1	loss	1.46E-10	2.54E-12	IMMP2L
7	119650000	119739999	1	loss	1.15E-04	9.62E-05	LINC02476, LOC107986840
9	61000000	61509999	3	gain	2.23E-06	4.67E-06	LOC107984126, LOC105379249, LOC105379435, SPATA31A7, FAM74A4, CNTNAP3C
15	23180000	23239999	3	gain	3.63E-02	6.56E-02	LOC102723534, LOC102723564, LOC102723623, LOC105376698
19	54730000	54779999	4	gain	4.25E-04	3.21E-04	KIR3DL3, KIR2DL1, LOC101928804
19	54740000	54789999	38	gain	3.71E-04	3.21E-04	KIR3DL3, KIR2DL1, LOC101928804
19	54750000	54839999	4	gain	2.93E-06	4.53E-06	KIR3DL4, KIR3DL3, KIR2DL1, LOC101928804
19	54800000	54869999	8	gain	1.77E-03	6.15E-03	KIR2DL4, KIR3DL1, KIR2DS4, KIR3DL2
20	29120000	30079999	3	gain	8.02E-22	0.00E+00	FAM242B, FRG1EP, FRG2EP, LOC105379476, LOC105379477
20	30040000	30089999	141	gain	2.14E-03	3.09E-03	none
20	30050000	30359999	3	gain	1.01E-02	2.62E-02	FAM242A, LINC01597, LOC105372586, LOC107985433
20	51700000	52099999	3	gain	6.01E-20	0.00E+00	ATP9A, SALL4, LINC01429, LOC105372664, ZFP64
20	52100000	52989999	3	gain	1.01E-05	2.39E-08	ZFP64, LOC105372666, LOC107985427, LOC105372667, LOC105372665, LINC01524, LOC105376985, LOC105372668, TSHZ2
20	52990000	53889999	3	gain	4.21E-24	0.00E+00	TSHZ2, LOC105372669, LOC105372671, LOC101927770, ZNF217, LOC105372672, SUMO1P1
20	53890000	61649999	3	gain	7.00E-03	1.17E-03	ANKRD60, APCDD1L, APCDD1L-DT, ATP5F1E, AURKA, BCAS1, BMP7, BMP7-AS1, C20orf85, CASS4, CBLN4, CDH26, CDH4, CSTF1, CTCFL, CTSZ, CYP24A1, DOK5, EDN3, FAM209A, FAM209B, FAM210B, FAM217B, GCNT7, GNAS, GNAS-AS1, LINC01440, LINC01441, LINC01711, LINC01716, LINC01718, LINC01742, LINC02910, LOC101927932, LOC101928048, LOC105372675, LOC105372676,

20	53890000	61649999	3	gain	7.00E-03	1.17E-03	ANKRD60, APCDD1L, APCDD1L-DT, ATP5F1E, AURKA, BCAS1, BMP7, BMP7-AS1, C20orf85, CASS4, CBLN4, CDH26, CDH4, CSTF1, CTCFL, CTSZ, CYP24A1, DOK5, EDN3, FAM209A, FAM209B, FAM210B, FAM217B, GCNT7, GNAS, GNAS-AS1, LINC01440, LINC01441, LINC01711, LINC01716, LINC01718, LINC01742, LINC02910, LOC101927932, LOC101928048, LOC105372675, LOC105372676, LOC105372677, LOC105372678, LOC105372679, LOC105372680, LOC105372681, LOC105372683, LOC105372685, LOC105372686, LOC105372687, LOC105372688, LOC105372690, LOC105372692, LOC105372693, LOC105372694, LOC105372695, LOC105372696, LOC105372697, LOC105372698, LOC105372699, LOC105372700, LOC105372701, LOC105372703, LOC105376989, LOC107984001, LOC107985382, LOC107985385, LOC107985389, LOC107985410, LOC107985429, LOC107985430, LOC107985434, LOC107987284, LOC112268270, LOC729296, MC3R, MIR296, MIR298, MIR4325, MIR4533, MIR4756, MIR548AG2, MIR646, MIR646HG, MTRNR2L3, NELFCD, NKILA, NPEPL1, PCK1, PFDN4, PHACTR3, PHACTR3-AS1, PMEPA1, PPP1R3D, PPP4R1L, PRELID3B, RAB22A, RAE1, RBM38, RBM38-AS1, RTF2, SLMO2-ATP5E, SPO11, STX16, SYCP2, TFAP2C, TUBB1, VAPB, ZBP1, ZNF831
20	61650000	63469999	3	gain	6.32E-37	0.00E+00	ADRM1, ARFGAP1, BHLHE23, BIRC7, CABLES2, CDH4, CHRNA4, COL20A1, COL9A3, DIDO1, FLJ16779, GATA5, GID8, HAR1A, HAR1B, HRH3, KCNQ2, KCNQ2-AS1, LAMA5, LAMA5-AS1, LINC00029, LINC00659, LINC01056, LINC01749, LOC100128310, LOC100130587, LOC101928465, LOC105369209, LOC105372704, LOC105372705, LOC105372706, LOC105372707, LOC105372708, LOC105372709, LOC105372710, LOC105372711, LOC105372716, LOC105372717, LOC105372718, LOC105372719, LOC105372721, LOC105372724, LOC105376995, LOC105376996, LOC107985428, LOC107987285, LSM14B, MIR1-1, MIR1-1HG, MIR1-1HG-AS1, MIR124-3, MIR1257, MIR133A2, MIR3195, MIR3196, MIR4326, MIR4758, MRGBP, MTG2, NKAIN4, NTSR1, OGFR, OGFR-AS1, OSBPL2, PSMA7, RBBP8NL, RPS21, SLC17A9, SLCO4A1, SLCO4A1-AS1, SNORA117, SS18L1, TAF4, TCFL5, WI2-87327B8.2, YTHDF1

20	63470000	64444167	3	gain	2.66E-04	3.03E-04	ABHD16B, ARFRP1, C20orf181, C20orf204, DNAJC5, EEF1A2, FNDC11, GMEB2, HELZ2, KCNQ2, LIME1, LINC00266-1, LKAAEAR1, LOC102723814, LOC105372725, LOC105372726, LOC105372727, LOC112268269, MHENCR, MIR1914, MIR647, MIR6813, MIR941-1, MIR941-2, MIR941-3, MIR941-4, MIR941-5, MYT1, NPBWR2, OPRL1, PCMTD2, PPDPF, PRPF6, PTK6, RGS19, RTEL1, SAMD10, SLC2A4RG, SOX18, SRMS, STMN3, TCEA2, TNFRSF6B, TPD52L2, UCKL1, UCKL1-AS1, ZBTB46, ZBTB46-AS1, ZGPAT, ZNF512B
21	10170000	10319999	3	gain	2.41E-03	7.19E-04	none
X	1	229999	7	gain	6.45E-06	2.95E-06	none
X	1800000	1979999	3	gain	3.95E-04	8.63E-05	LOC107985677

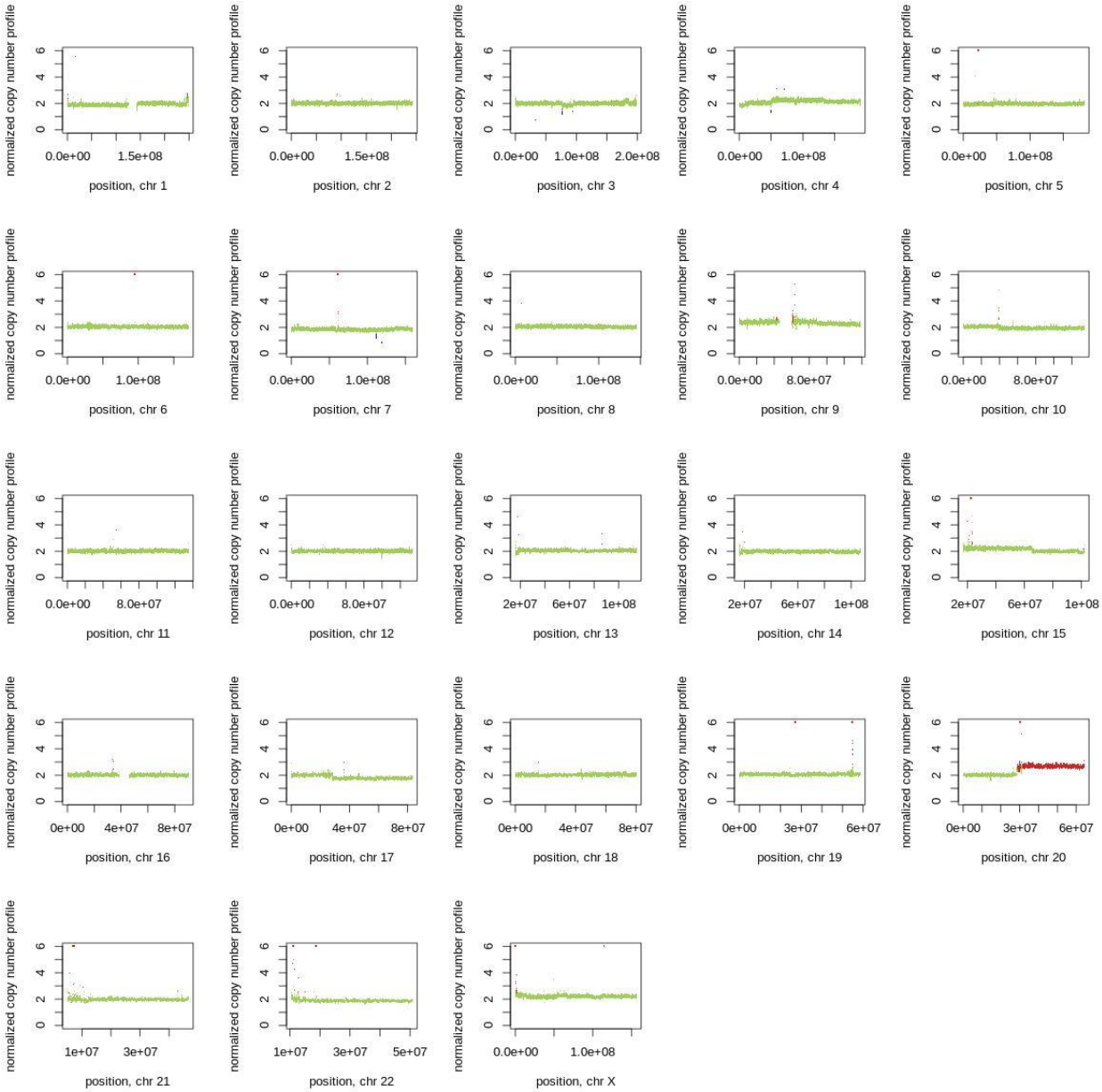

Figure 23. Graphical view of the NGS CNV results for each chromosome.

The majority of gains of gene copy number has been detected in regions enriched with genes well-known for their involvement in tumours, including PDAC. The amplified genes play roles in processes such as proliferation, tumour progression, EMT, metastasis, cell cycle regulation, cell signalling, apoptosis, immune evasion, stemness and others. Actually, the majority of amplified genes, 32 of them, are involved in proliferation and tumour progression, such as SALL4, BIRC7, SOX18 and HRH3 to name a few [82]–[84]. Furthermore, we found gain of gene copies involved in migration, invasiveness, and metastasis of PDAC, in particular RUFY3, CDH12, ZNF217, BMP7 and LAMA5 [85]–[88]. Another important feature of analysed genome was amplification of genes responsible for drug metabolism and chemoresistance. Some of them, such as AURKA, CDH4, TFAP2C, have been previously studied in PDAC, but we also found gain of CYP24A1 and ATP5F1 which have been studied in other solid tumours but their role in PDAC is not clear yet [85]–[87], [89], [90]. The ability of tumour cells to evade immune system response is crucial for the progression and metastasis, and here we have observed gain of YTHDF1 known to be overexpressed in many solid tumours including PDAC [91]. We found 2 types of killer cell immunoglobulin-like receptor genes (KIRs) that have been studied in solid tumours and correlated with immune evasion, however their role in PDAC is still unknown [92], [93]. Several important PDAC genes associated with cell cycle regulation have been found amplified, such as AURKA, OGFR and PPDPF [89], [94], [95]. Some of chromosomal regions do not contain known genetic sequences but it cannot be excluded that these regions are still able to contribute to general genomic instability in the transformed cells.

Overall, these results indicate that HEK293T cell line treated with PDAC patients' sera shows a genomic change coherent with previous findings on PDAC and other solid tumours. These findings explain and reinforce results observed in wound healing assay, western blot and chemoresistance for this cell line.

5. Discussion

Factors as late diagnosis, high resistance to chemotherapy, common relapse even after radical surgery contribute to the very high mortality of PDAC. Furthermore, PDAC has a very high metastatic potential and metastases have been observed even in patients who have undergone the complete pancreatic resection [13]. It is crucial to improve our knowledge on mechanism of metastatic development in PDAC to improve diagnostic and therapeutic approaches.

In order to contribute to the understanding of the genometastasis theory in PDAC, we have assessed the transformation ability of non-metastatic PDAC patients' sera and co-cultures of PDAC cancer cell lines and non-tumour primed cell lines hTERT-HPNE and HEK293T. Here we report the preliminary results indicating that upon both co-cultures and serum treatments the recipient cells were most likely transformed. In particular, we have observed *in vitro* and *in vivo*, that HEK293T cell line co-cultured with Panc-1 and ASPC-1 has been transformed and consequently formed tumours in mice. The HEK293T cell line treated with PDAC patients' serum has most likely been transformed based on *in vitro* tests. However, the hTERT-HPNE cell line, in both experimental treatments did not form a significant number of colonies in soft agar, and it did not form any tumours in mice. Therefore, we consider hTERT-HPNE cell line refractory to the malignant transformation due to serum and co-culture treatments.

Both recipient human cell lines are primed. In particular, hTERT-HPNE cells represent an intermediary-stage in PDAC development with K-Ras (G12D) mutation, p53 and Rb tumour suppressor genes inactivated [75]. HEK293T cell line is derived from HEK293 line upon insertion of SV40 T antigen which inhibits expression of p53 and Rb genes [96] The more efficient transformation observed in HEK293T could be explained by its high susceptibility to transfection. Indeed, this cell line is widely used in many studies for its well-known efficiency and high reproducibility in exogenous protein production. Previously Abdouh et al. have also

reported similar findings, in particular, in this study HEK293 cell line was exposed to cancer patients' sera (including one case of PDAC), and the malignant transformation was confirmed *in vivo*, whereas other normal human cell lines did not transform [50], [60]. Similar data regarding hTERT-HPNE cell line is not available for comparison since our study is, to our knowledge, the first so far reported where this cell line has been exposed to the PDAC patients' sera or co-cultured with cancer cell lines to assess the transformation *in vitro* or *in vivo*. The downstream analyses, in both experimental settings, have been done on all treated cells and not only on the transformed colonies, since colonies couldn't be isolated efficiently from the classical soft agar and propagated successfully in cell culture.

We have assessed the changes in migration ability of either PDAC serum treated cells or co-cultured cells. The HEK293T cells exposed to PDAC patients' sera gained significantly higher migratory potential when compared to control cells. Meanwhile the same type of treatment did not cause a significant change in migratory potential of hTERT-HPNE cell line (Figure 13). Furthermore, the HEK293T cell line also showed increased migration upon co-culturing with Panc-1 (82.68% vs 61%). The co-cultured hTERT-HPNE cells have increased their migration in all combinations, but the most significant group was the hTERT-HPNE+ASPC1 (70.53% vs 40.26%) (Figure 9-10). The higher cell migration ability is crucial for tumour invasiveness, angiogenesis, and metastasis development [97], [98]. Therefore, our preliminary results suggest that PDAC serum exposition and co-cultures with cancer cell lines are able to induce changes in migration ability of non-tumour cells and make them potentially more invasive. These results should be further validated with more advanced methods in order to give a stronger conclusion.

It has been reported that STAT3 protein plays a role in cell growth, cell migration, metastasis, and promotes resistance to some anti-tumour drugs [99]. STAT3 is also involved in the regulation of cancer cell survival, inflammation, and stem cell renewal. In PDAC this protein is constitutively activated by phosphorylation of Tyr705, as reported in 30-100% of human tumour tissue samples and in almost all PDAC cell lines [100]. The positive correlation

between over-expression of STAT3 and higher migratory potential was observed in our HEK293T cells treated with PDAC sera and co-culture hTERT-HPNE+Panc-1. The hTERT-HPNE cells exposed to PDAC sera showed both a slight down-regulation of STAT3 and no significant gain of migratory potential. However, the hTERT-HPNE co-cultured cells with increased migration in wound healing assay are not characterized with significant change in STAT3 expression. Despite an increase in STAT3 expression in the serum treated HEK293T cell line, we did not find its phosphorylated form in both in PDAC, and healthy sera treated cells. Similarly, all co-cultured cells showed either a complete absence of p-STAT3 or a slight down-regulation (Table 5). The similar finding on HEK293 cells lacking the p-STAT3 expression have been previously described in the literature where cells were treated with inhibitors and/or stimulators of STAT3 pathway and compared to the untreated HEK293 cells [101]. However, it has been reported the unphosphorylated STAT3 can still play a role in the transcriptional regulation of many cell-cycle regulating genes, such as CDC2, Cyclin B1, E2F1, through a different mechanism than p-STAT3 [102]. Additionally, the mechanism of STAT3 autophagy inhibition involves members of MAPK and PI3K/AKT/mTOR signalling pathways. Indeed, it has been found that p-mTOR is usually up-regulated in cells with constitutively active STAT3 expression despite unaltered basal mTOR levels [99]. We have found the correlation between overexpression of p-mTOR and STAT3 in our co-culture hTERT-HPNE+Panc-1. Interestingly, we have also observed a correlation between STAT3 and PD-L1 expression in both PDAC sera treated cell lines, and in hTERT-HPNE+Panc-1 co-culture, similarly as described previously by Song et al [103]. Indeed, we have found an overexpression of PD-L1 and STAT3 in HEK293T exposed to PDAC sera, the co-culture hTERT-HPNE+Panc-1, and their simultaneous decrease in the PDAC sera treated hTERT-HPNE cell line. Over-expression of PD-L1 has been identified in numerous human cancers. It has been correlated with T cell inactivation, and it allows for immuno-escape of cancer cells. PD-L1 is

expressed also in PDAC, and it has been associated with lower survival rates of patients [104]. The PD-L1 overexpression in HEK293T exposed to sera and in hTERT-HPNE+Panc-1 co-culture may indicate that the treatments are able to cause immuno-escape in the treated cells.

We also observed a very strong up-regulation of mTOR (30-fold) and the increase of its phosphorylated form (from 0% to 25%) in PDAC serum treated HEK293T cells. Notably, the hTERT-HPNE line showed a strong down-regulation (50-fold) of mTOR expression, almost completely shut down. Consistently with literature, p-mTOR is not expressed in the untreated HEK293 cells [105]. Regarding the co-cultured cells, we have observed the increased expression of mTOR and its active form in all combinations except for HEK293T+ASPC-1 which had unaltered basal mTOR, but reduced p-mTOR expression. The normal mTOR signalling pathway is altered in many cancers and its activity is increased in PDAC. mTOR is involved in promoting cell proliferation, growth, and survival, but it also drives the tumour cell motility and invasiveness [106], [107]. The observed expression alterations may also explain the higher migration levels of cells with up-regulated mTOR. Indeed, all co-culture combinations over-expressing mTOR have increased migration too, whereas HEK293T+ASPC-1 has unaltered expression level of mTOR and insignificant change in wound healing assay compared to control cells. The same effects have been observed in HEK293T cells exposed to PDAC sera.

We assessed some markers of epithelial-to-mesenchymal transition (EMT) and upon PDAC sera treatments we have found only vimentin to be differentially expressed in treated cells. In particular, we have observed its strong reduction in HEK293T cell line and a slight increase in hTERT-HPNE cell line whereas it was up-regulated in hTERT-HPNE+Panc-1 co-culture, but it was unaltered in all other co-culture combinations. Since vimentin up-regulation is correlated with PDAC development and metastatic behaviour [106], [108], [109], PDAC serum treatments and co-cultures might be able to induce more aggressive behaviour in treated cells.

However, other mesenchymal markers (N-cadherin and fibronectin) and the epithelial marker E-cadherin were not expressed in any conditions in both cell lines exposed to PDAC patients' sera. Fibronectin was slightly up-regulated only in hTERT-HPNE+Panc-1 co-culture and it was absent in all other co-cultured cells. Previously, it was reported that E-Cadherin and fibronectin were not expressed in HEK293 cells [110], [111] and both E- and N-cadherin were not expressed in a pancreatic cancer cell line MIAPaCa-2 [112]. Taken together, these results may indicate an incomplete EMT switch in treated cell lines.

Finally, we found a moderate overexpression of Bcl-xL in both cell lines after treatment with PDAC patient serum and in all co-culture combinations. Bcl-xL is an anti-apoptotic protein crucial for the survival of pancreatic cancer cells and other solid tumours too. Its over-expression drives PDAC progression by inhibiting oncogene-induced senescence and apoptosis in PanIN lesions and promotes metastasis [112]–[114]. Furthermore, the over-expression of Bcl-xL could potentially make cells more resistant to some chemotherapeutics. In particular, the altered expression of Bcl-xL has been associated to resistance to microtubule targeting drugs, such as paclitaxel in hepatocellular and pancreatic cancer cells [115]–[117]. Interestingly, we also observed the development of paclitaxel chemoresistance in HEK293T cells after PDAC serum treatments, but not in the co-cultured cells. Moreover, the paclitaxel resistance could also be due to the observed mTOR and p-mTOR increase. Indeed, it has been reported that inactivation of mTOR increased the sensitivity of cells to paclitaxel in other solid tumours [118]. On the contrary, PDAC sera treated HEK293T resulted to be more sensitive to the other tested drugs commonly used in treatments for PDAC and other cancers, specifically gemcitabine, 5-fluorouracil and doxorubicin. While doxorubicin intercalates DNA and disrupts the DNA repair, gemcitabine and 5-FU are nucleoside analogues that, when incorporated into DNA, act as chain terminators [119]. The observed higher sensitivity of treated HEK293T may be due to the mechanism of action of these drugs, which is very efficient on fast proliferating

cells. Indeed, HEK293 cells have a higher proliferation upon treatments with cancer patient serum, as previously described by Abdouh et al [60]. This high proliferation of serum treated HEK293T is also supported by the observed up-regulation of mTOR and STAT3 expression, known to be involved in cell growth and proliferation. Additionally, it has been reported that gemcitabine sensitivity in PDAC cells is correlated with overexpression of transcription factor AP-2 gama (TFAP2C) [120]. Indeed, in the genome of our HEK293T PDAC sera treated cells we observed the gain of TFAP2C copies. In the co-cultured cells, we have observed gain of resistance only to doxorubicin in two co-culture combinations, in particular hTERT-HPNE+Capan-2 and HEK293T+Panc-1. Similarly, to serum treated cells, in some co-cultured cells we have observed the higher sensitivity to gemcitabine, 5-FU, and cisplatin, in particular HEK293T+ASPC-1 and hTERT-HPNE+Panc-1. One of the molecular mechanisms responsible for doxorubicin resistance is the MEK/ERK pathway. This signalling pathway regulates signal transmission from growth signals to transcription factors thereby promoting cell proliferation and inhibiting cell death. It has been demonstrated in hepatocellular carcinoma that ERK inhibition significantly increased sensitivity to doxorubicin by promoting apoptosis [121]. Also, in a study based on murine breast cancer models it has been reported that MAPK and Pi3K/Akt signalling pathways are involved in the doxorubicin resistance [122]. In our co-cultured cells, which developed the doxorubicin resistance, in western blot we have observed upregulation of the proteins involved in these signalling pathways, in particular mTOR. It has been previously described that p-STAT3 over-expression correlates positively with cisplatin resistance in some tumours [99]. Indeed, the p-STAT3 expression is down-regulated in both of our cisplatin-sensitive co-cultures HEK293T+ASPC-1 and hTERT-HPNE+Panc-1.

Tumour xenografts have been immunoassayed for proteins known to be associated with higher risk of metastasis and poor survival, in particular Ki-67, vimentin and cytokeratin-18 (Cyt-18).

Ki-67 is a nuclear protein exhibiting a strong correlation with cell proliferation and growth, cancer progression and metastasis development, therefore it is commonly used marker in pathological studies [123]. The Ki-67 expression as detected by immunohistochemistry is one of the most reliable indicators of the proliferative status of cancer cells. Clinically, Ki-67 has demonstrated to be associated with poorly differentiated cancer cells and to be more expressed in malignant tissues. The Ki-67 labelling is also commonly used as an independent prognostic indicator of patients' survival [123], [124]. Previous studies on PDAC have demonstrated that higher Ki-67 expression is associated with higher risk of tumour relapse and poor survival [124]. Our Ki-67 expression results on HEK293T+Panc-1 and HEK293T+ASPC-1 xenografts have been expressed as Allred score (Table 6). The obtained Allred scores in our xenografts are significantly high and this confirms the observations on their invasiveness and aggressiveness. The score for HEK293T+Panc-1 is higher than for HEK293T+ASPC-1 which is in line with greater tumour size observed in this model. Furthermore, we have observed strong intensity staining scores for vimentin and Cyt-18 in HEK293T+Panc-1. The xenograft model HEK293T+ASPC-1 also had a strong intensity score for vimentin, but moderate for Cyt-18.

Vimentin is one of the mesenchymal markers, but it is also found expressed in epithelial tumours and associated with more invasive phenotype. In PDAC context positive vimentin labelling has been usually observed in metastatic cancers and correlated clinically with poor survival [125]. Curiously in our western blot analysis of co-cultures we did not observe an up-regulation of vimentin, whereas immunolabeling of tumour xenografts showed a significant increase in both HEK293T+Panc-1 and HEK293T+ASPC-1.

The cytoskeletal protein cytokeratin-18 (CK18 or Cyt-18) is mostly found in single-layer epithelium of many organs, but also in cancers arising from these cells. Although its primary

role is structural it was also found to be involved cell cycle progression and some cancer signalling pathways such as Wnt and ERK1/2-MAPK. Also it was reported that reduced levels of Cyt-18 were correlated with a decreasing rate of cell apoptosis [126]–[131]. The antibodies against Cyt-18 are widely used as diagnostic tumour markers since its increased levels are correlated with adverse features of carcinomas. In particular, PDAC samples are usually stained positive for Cyt-18 [131]. Indeed, in our xenograft model HEK293T+Panc-1 we found a strong intensity staining score for Cyt-18, whereas it was moderate in HEK293T+ASPC-1. Overall immunostaining results of our xenograft models confirm the aggressive phenotype of formed tumours in animal models. It reinforces the hypothesis that circulating elements, and not primary cancer cells, are able to generate transformation in primed cells. In our study we have used the co-culture inserts with 1um pore size which should permit only vesicles smaller than 1um to pass to the recipient cells. The obtained results should be in future reinforced with results confirming the identity of the vesicles and cargo molecules responsible for transformation.

In the end, we have analysed genomic changes upon PDAC serum treatments in the transformed HEK293T cell line using NGS for copy number variation. Genomic imbalance is found in many cancer types, including PDAC [132]–[136]. Some events are tumour-type specific, although many of them are common amongst different types of tumours. Previous studies on breast and pancreatic cancer have found a positive correlation between gene copy number variation and change in expression. In particular, they have demonstrated that majority of amplified genes are also overexpressed [134]–[136]. Heidenblad et al. have studied genome-wide transcriptional effects of gene copy number variations on expression profiles of 29 pancreatic cancer cell lines using cDNA microarray [136]. Authors report that gains/losses of 1-2 gene copies did not cause major expression changes, but the effect is more pronounced when 3 or more gene copies are present. However, it is necessary to keep in mind that also

genes with normal copy number may be upregulated due to activating mutations or epigenetic changes this way posing a limitation for interpretation of these results and making the straightforward relationship among CNV and overexpression [136]. Previous studies on PDAC, based on FISH (fluorescent in situ hybridization), CGH (comparative genomic hybridization) and aCGH (array-CGH), have successfully identified most common genomic alterations. In particular, PDAC usually presents loss of chromosome regions 1p, 8p, 9p, 12q, 17p and 18q, together with gain of regions 1q, 3q, 7q, 8q and 20q [132]–[134]. Numerous genes known to be directly involved in PDAC are indeed localized in these chromosomal regions. Furthermore, Gutierrez et al. using SNP-array have identified and classified genomic changes into two groups and correlated them with PDAC tumour histopathology [132].

Our CNV results are coherent with previous findings on PDAC, and we report that in our HEK283T cells treated with PDAC sera are present alterations of genes directly involved in PDAC. Out of 32 amplified genes, known to be important for growth and proliferation, 20 of them have been already described to play a role in PDAC. Indeed, we have observed that HEK293T cells transformed with PDAC sera showed a significantly high growth in soft agar when compared to the control cells. Furthermore, we have also observed a major gain of migratory potential. Among genes found to be present in higher copy number we have identified genes that reinforce our in vitro obtained data. In particular, we have found 3 copies of some transcription factors, in particular SALL4, TSHZ2, CTCFL, TFAP2C, GATA5 and SOX18. The transcription factor SALL4 regulates a network of transcriptional factors involved in stemness, pluripotency and chromatin remodelling not only in normal stem cells but also in cancer cells. Indeed, SALL4 expression has been associated with several cancer types [84]. Furthermore, we found an amplification of CDH12 (Cadherin-12), a subtype of N-cadherin. It is expressed in many cell types and plays important roles also in cancer development. First studies on non-small-cell lung cancer reported its role in cancer progression and correlation

with poor prognosis [137]. Furthermore, its role has been demonstrated also in salivary adenoid cystic carcinoma, and recently colorectal cancer. It was reported that CDH12 is one of the key regulators of colorectal cancer invasiveness and metastasis by promoting EMT through activation of transcriptional factor Snail [87], [88]. BIRC7 (Baculoviral IAP repeat-containing 7) has recently been identified as one of the key apoptosis regulators in PDAC and was correlated with poor prognosis. BIRC7, also known as KIAP or Livin, is overexpressed in many solid tumours, such as hepatocellular, gastric and breast cancer. Its role was linked with prognosis, tumour differentiation grade and invasiveness [83]. In PDAC tumour tissues overexpression of BIRC7 has been demonstrated previously. Furthermore, it was reported that patients with higher BIRC7 expression survived much shorter than patients who did not express BIRC7. A significant correlation has been also observed among BIRC7 expression and tumour size, invasion and metastasis [83]. Numerous studies have suggested that histamine plays a role as an autocrine or paracrine growth factor by binding to four different G protein-coupled receptors (histamine receptors H1, H2, H3, and H4), triggering various cell signalling pathways. The presence of different histamine receptors has been confirmed in various cancer types, such as colorectal, gastric, and hepatocellular [138]. The study on pancreatic cancer cell line PANC-1 suggested the important role of all 4 types of histamine receptors in the promotion of tumour growth. In particular, histamine receptor 3 has been linked to the cell cycle regulation and tumour proliferation [82]. However, we have also observed amplifications of some genes which are well-known positive prognostic factors in PDAC due to their role as inhibitors of cell growth and proliferation. In particular, we report that OGFR and ArfGAP1 are present in 3 copies in HEK293T PDAC sera transformed cells. OGFR (opioid growth factor receptor) interacts with opioid growth factor and regulates cell growth by the cyclin-dependent kinase inhibitor (CKI) p16 and regulates transition from G1 to S phase of cell cycle. The action of OGF on the p21 pathway has been demonstrated in several pancreatic cell lines, namely PANC-

1, Capan-2 and BxPC-3 [94]. ArfGAP1 has been identified as a key controller of mTORC1 complex in the regulation of cell growth by inhibiting mTOR activity. Its role in various cancer types is not completely understood and it is under studies [139]. Curiously, in western blot we did not find active form of mTOR although we have observed an up-regulation of basal mTOR.

We have found amplification of several genes known to be linked with drug metabolism and resistance. In the MTT chemoresistance assay we have demonstrated that HEK293T cells treated with PDAC sera were resistant to treatment with paclitaxel, whereas were more sensitive to other tested drugs (gemcitabine, 5-FU and doxorubicin). Our results show an amplification of AURKA (aurora kinase A), which plays crucial roles in mitotic spindle formation and centrosome maturation, and it is one of the fundamental tumour-linked genes also in PDAC [89], [95]. Previously it has been reported that inhibition of AURKA expression using specific siRNA greatly reduced in vitro colony formation of various pancreatic cancer cell lines and improved their sensitivity to taxanes [95]. Remarkably, we have observed in our study that HEK293T cell line treated with PDAC patients' sera became resistant to paclitaxel, member of taxane group of drugs, and a copy number variation of AURKA might be one of the causal factors. Furthermore, paclitaxel biotransformation in the cell is mediated by members of cytochrome P450 protein family, which are usually found to be overexpressed or mutated in the paclitaxel-resistant clones [140], [141]. Here, we have found that CYP24A1, a gene for cytochrome P450 subunit, was amplified in PDAC serum treated cells. The paclitaxel resistance is also linked to overexpression of Bcl proteins [140] which we have also observed in the western blot for our HEK293T cell line treated with PDAC sera. However, upon treatments with gemcitabine, 5-FU and doxorubicin, we have observed increase in the sensitivity of the PDAC serum treated cells. Some genes detected in CNV analysis have been previously associated with this phenomenon. In particular, it has been reported that down-regulation of ATP synthase genes is linked to the resistance to many anticancer drugs in various

cancers, as for example the 5-FU resistance of colorectal cancer cells [142]. We have found two ATP synthase genes to be amplified, namely ATP9A and ATP5F1E, together with significant sensitivity of treated cells to 5-FU. Transcription factor AP-2 gamma (TFAP2C) is a target of miR-10a-5p, and its overexpression resensitizes pancreatic cancer cells to gemcitabine [120]. We found TFAP2C to be amplified in our HEK293T PDAC serum treated cell line and we observed a significant sensitivity to gemcitabine treatments.

In wound healing assay we demonstrated that treated HEK293T gained a significant migration ability. In fact, in CNV analysis we found several copy number variations of genes important for EMT and invasiveness in PDAC, but also other solid tumours. The most important genes linked to migratory behaviour of the cancer cells are ZNF217 (zinc finger protein 217), BMP7 (bone morphogenetic protein 7), RUFY3 (RUN And FYVE Domain Containing protein 3), LAMA5 (laminin-5), MRGBP (MORF4-related gene-binding protein) and cadherins 12 and 4. ZNF217 is defined as oncogene in many solid tumours, and its amplification and/or overexpression has been widely reported in studies, including ones on PDAC. Its action mechanism is based on binding to DNA through its zinc fingers thus targeting potential protein binding partners to specific genome positions. ZNF217 is able to promote AURKA, also amplified in our HEK293T cells, and activates PI3K/Akt pathway [143], [144]. Previous studies have reported its amplification and overexpression to be associated to metastatic behaviour in breast, colorectal and pancreatic cancer [143], [145]. It promotes cancer cell invasion by impairing expression of E-cadherin which consequently induces EMT switch and gives rise to metastasis development [143]. BMP7 is pleiotropic signalling molecule, a member of TGF-β protein superfamily, well-known for its role in cell development, growth, and invasion by altering target gene transcription. It was associated with metastasis and poor prognosis in many human tumours, and recently also with resistance to immunotherapy [85], [146], [147]. In PDAC it was reported that BMP is important for initiation of EMT in a number

of pancreatic cancer cell lines. Several BMP family members, including BMP7, enhance Slug expression, inhibit TbRIII expression and so promote EMT-associated invasion [85]. Synthesis and deposition of Laminin 5 (LAMA5) by carcinomas has been proposed as a novel mechanism of their invasion. Previous studies have offered new proofs for the role of basal membrane and function of different laminin-integrin receptors for cancer cell mobility. This feature was also studied in the PDAC setting using cell lines. The published results confirm that PDAC cells produce high quantities of laminin-5 and deposit it in the basement membrane enabling the cell migration using alpha 3 beta 1 integrin receptor recognizing laminin-5 [86]. MRGBP binds to protein components of histone acetyltransferase and deacetyltransferase complexes, and its upregulation is related to cell proliferation, migration, and invasion in many cancer types, including PDAC. It has been reported that MRGBP knockdown inhibited proliferation and migration of PDAC cells, and its overexpression had inverse effects promoting EMT. It has been proposed as a novel biomarker for PDAC for diagnostic and therapeutic approaches [148]. Overall, the results obtained for HEK293T cells treated with PDAC patients' sera confirm the malignant transformation and gain of invasive phenotype.

6. Conclusions

In this study we offered the results in the support of genometastasis theory in the setting of PDAC. We have demonstrated *in vitro* and *in vivo* that non-tumour cell lines co-cultured with pancreatic cancer cell lines have been successfully transformed. Also, we have demonstrated *in vitro* that non-tumour cell lines exposed to PDAC patients' sera have been most likely transformed. Although we have used the 1μm pore size inserts in the co-culture experiments we cannot conclude in a definitive way that extracellular vesicles are responsible for the transformation. Therefore, in the further studies it is necessary to isolate selectively extracellular vesicles and treat recipient non-tumour cell lines to reinforce the hypothesis. Furthermore, it is important to identify the type of molecules loaded into extracellular vesicles responsible for driving the malignant transformation.

Overall, this study offers interesting preliminary results and could pave the way for the future studies leading to the advancements in the clinical practice. Once identified the EV-carried molecules responsible for genometastasis they could be addressed for improvement of the prognostic assays.

7. References

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