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THE EFFECT OF CONDITIONED MEDIA ON THE CELLULAR PROLIFERATION OF PANCREATIC CANCER CELLS, PANCREATIC STELLATE CELLS, AND MYELOID-DERIVED SUPPRESSOR CELLS

Y. P. CHONG[1], S. SAMSUDIN[1], D. A. BAHRANI[1], C. H. CHANG[1], A. MASAMUNE[2], S. H. NG[3], N. ISMAIL[4], K. L. HO[3]

[1]School of Postgraduate Studies, IMU University, Kuala Lumpur, Malaysia
[2]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan
[3]School of Pharmacy, IMU University, Kuala Lumpur, Malaysia
[4]Natural Medicines and Products Research Laboratory, Institute of Bioscience, University Putra Malaysia, Serdang, Selangor, Malaysia

Corresponding Author
Ket Li Ho, PhD; e-mail: hoketli@imu.edu.my

ABSTRACT – *Objective:* Pancreatic cancer has remained one of the most devastating diseases over the past two decades, with minimal improvements in survival rates. Its highly immunosuppressive tumour microenvironment is driven by secreted proteins, such as cytokines and growth factors, which promote the differentiation of immunosuppressive cells and influence cellular proliferation and migration. This study investigates how the secretome from pancreatic cancer and pancreatic stellate cells affects the proliferation of myeloid-derived suppressor cells and its implications for cellular proliferation.

Patients and Methods: Conditioned media from pancreatic cancer cells and pancreatic stellate cells were used to treat peripheral blood mononuclear cells, evaluating their effects on myeloid-derived suppressor cells proliferation. Additionally, pancreatic stellate cells were treated with conditioned medium from pancreatic cancer cells to assess its impact on their proliferation. Conversely, conditioned medium from pancreatic stellate cells was used to treat pancreatic cancer cells to evaluate its effects on their growth.

Results: Conditioned media from both pancreatic cancer and pancreatic stellate cells significantly enhanced the proliferation of myeloid-derived suppressor cells, although the BrdU proliferation assay revealed differing outcomes. Conditioned media from primary pancreatic cancer cells notably increased the proliferation of pancreatic stellate cells more than that from metastatic cancer cells. Similarly, primary pancreatic cancer cells exhibited greater proliferation when exposed to conditioned media from pancreatic stellate cells compared to metastatic cells.

Conclusions: The bioactive secreted proteins from pancreatic cancer and pancreatic stellate cells effectively stimulate the proliferation of myeloid-derived suppressor cells without direct cell-to-cell interactions. Factors from primary tumour cells support cancer cell survival more than those from metastatic cells, indicating potential targets for immunotherapy in early-stage cancers.

KEYWORDS: Pancreatic cancer, Pancreatic stellate cells, Myeloid-derived suppressor cells, Cells proliferation, Tumour microenvironment.

KEYWORDS: PCC, pancreatic cancer cell; TME, tumour microenvironment; PDAC, pancreatic ductal adenocarcinoma; EMT, epithelial-mesenchymal transition; MDSC, myeloid derived suppressor cell; TAM, tumour-associated macrophage; CAF, cancer-associated fibroblasts; Treg, regulatory T cell; IMC, immature myeloid cell; NK, natural killer; PSC, pancreatic stellate cell; IL-6, interleukin-6; GM-CSF, granulocyte macrophage colony-stimulating factor; SDF-1α, stromal-derived factor-1 alpha; M-CSF, macrophage colony-stimulating factor; CM, conditioned medium; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PTX3, pentraxin 3; GRN, granulin; PGRN, progranulin.

INTRODUCTION

Pancreatic cancer exerts a significant impact on overall survival rates due to its aggressive characteristics and the delayed diagnosis. Studies show that approximately 15% of individuals are diagnosed with resectable cancer at diagnosis, with the majority presenting with either locally advanced or metastatic forms, consequently resulting in unfavourable long-term prognoses[1-3]. For patients with resectable tumours, a multidisciplinary strategy involving surgical intervention followed by chemotherapy has the potential to achieve 5-year survival rates ranging from 30% to 50%, thereby emphasising the importance of early detection[4]. However, a considerable proportion of cases are in the advanced stage, where the tumours have metastasised. This substantially diminishes the likelihood of successful surgical removal and leads to dismal 5-year survival rates, highlighting the complexities associated with managing advanced stages of pancreatic cancer[5]. Despite the progression in treatment approaches and the escalating use of genomic testing and targeted therapies, pancreatic cancer endures as a condition characterised by bleak long-term survival rates, underscoring the needs for sustained research efforts and innovative methodologies to enhance outcomes.

The microenvironment of pancreatic cancer tumours is crucial in the progression of cancer, its dissemination to distant sites, and its resistance to therapeutic interventions. Consisting of diverse elements like stromal cells, immune cells, and soluble substances, this microenvironment interacts with cancerous cells and has an impact on the disease's outcomes[6,7]. The stroma, a major element of the tumour mass, acts as a key factor in promoting tumour development, assisting in cancer metastasis, and obstructing drug delivery, which consequently contribute to therapy resistance[8]. Additionally, the crosstalk between pancreatic cancer cells (PCCs) and the surrounding stroma has been identified as a contributor to an immunosuppressive tumour microenvironment (TME), impacting both T-cell proliferation and polarization[9]. Moreover, the interplay among developing tumour cells, stromal cells, and immune cells in the microenvironment of pancreatic ductal adenocarcinoma (PDAC) has repercussions on the epithelial-mesenchymal transition (EMT), ultimately impacting the cancer's aggressiveness and the patient's prognosis[10]. The microenvironment of pancreatic cancer tumours is significantly influenced by immune cells and has a pivotal role in both tumour advancement and treatment results. The immunosuppressive milieu of pancreatic cancer is characterized by the accumulation of diverse cytokines and immunosuppressive cells like myeloid derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and regulatory T cells (Tregs), which presents challenges for immunotherapy approaches that target the immune checkpoints, stromal cells, or cytokines within the TME[11].

MDSCs are recognised for their significant involvement as a predominant subset of immunosuppressive cells within pancreatic cancer[12]. Immature myeloid cells (IMCs) representing myeloid progenitor cells are typically present in the bodies of healthy individuals and do not have immunosuppressive functions. The development of MDSCs is facilitated by chronic inflammatory conditions commonly associated with conditions such as cancers, chronic infections, and autoimmune diseases[13-15]. In instances of chronic infection or cancer, a reduction in the number of peripheral myeloid cells leads to enhanced myelopoiesis and cell migration prior to their full differentiation, resulting in the accumulation of myeloid cells exhibiting potent immunosuppressive properties[16,17]. Owing to their myeloid lineage and functions, this heterogeneous cell population has been termed MDSCs[18].

MDSCs have a pivotal role in the advancement of pancreatic cancer through the establishment of an immunosuppressive environment within the tumour, consequently facilitating immune evasion and cancer progression[19]. They carry out immunosuppressive functions that promote tumour growth and dissemination, eventually leading to unfavourable clinical consequences through the creation of an immunosuppressive TME. The distinguishing characteristic of MDSCs lies in their capacity to hinder the cytotoxic functions of various immune cells, such as T cells and natural killer (NK) cells, thereby contributing to unfavourable clinical consequences in pancreatic cancer patients[20-22].

Several factors play a role in the immunosuppression observed in the TME, facilitating tumour progression, and significantly reducing treatment efficacy and patient prognosis. For instance, ligands like Galectin-8 that bind to receptors such as LILRB4 can stimulate the proliferation of MDSCs through specific signalling pathways like STAT3 while suppressing others like NF-κB[23]. Research studies have shown that factors derived from pancreatic cancer or pancreatic stellate cells (PSCs) promote the differentiation of neutrophils into MDSCs, ultimately resulting in the establishment of an immunosuppressive microenvironment[12,24,25]. These factors include interleukin-6 (IL-6), granulocyte macrophage colony-stimulating factor (GM-CSF), stromal-derived factor-1 alpha (SDF-1α), and macrophage colony-stimulating factor (M-CSF) derived from PCC line[26]. In addition, the presence of these factors in PSC-conditioned media has been linked to the activation of the Nrf2 pathway, leading to the elevation of metabolic genes associated with cell proliferation and ROS detoxification, thereby enhancing MDSCs differentiation in pancreatic ductal adenocarcinoma cell lines[24].

Collectively, the enhancement of MDSCs differentiation and functionality is facilitated by the secreted molecules originating from PCCs and PSCs. Nevertheless, the precise impact of MDSCs proliferation and the reciprocal influence between PCCs and PSCs on MDSCs proliferation have not been definitively elucidated. Therefore, the current investigation aims to explore the effects of conditioned media derived from PCCs and PSCs on MDSCs viability and proliferation, as well as on the viability and proliferation of PCCs and PSCs when subjected to the secreted molecules from one another.

PATIENTS AND METHODS

Cell line and cell culture

The human PCC cell lines PANC10.05 and SW1990 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, and 1% penicillin and streptomycin (Kyoto, Nacalai Tesque, Japan). The immortalised human PSC line, hPSC21-S/T was derived from a resected pancreas from a patient that was undergoing surgery for pancreatic cancer[27]. They were maintained in Dulbecco's Modified Eagle medium/Ham's F-12 supplemented with 10% heat-inactivated FBS, and 1% penicillin and streptomycin. During the experiment, PCCs and PSCs were cultured in 1:1 ratio of DMEM: DMEM/Ham's F12.

Conditioned medium (CM) collection

PCCs and PSCs were seeded at a final density of 1.5 x 10^5 cells per well in 6-well culture plates (Eppendorf, Hamburg, Germany). Cells were incubated for 3 days, and the CM was collected and stored at -80°C.

The viability and proliferation of MDSCs

Ethical approval

Written informed consent was obtained from all volunteers that have donated blood for peripheral blood mononuclear cells (PBMCs) isolation and the following experiments. The protocol of this study was approved by Joint Committee on Research Ethics, International Medical University, Malaysia.

PBMCs isolation

Whole blood was donated by volunteers and collected in Vacutainer® blood collection tubes with anticoagulant (EDTA or heparin). The blood was then layered on top of histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) in a 1:1 ratio and centrifuged for 30 minutes at 400 x g. After centrifugation, the opaque interface containing the mononuclear cells was aspirated and washed with phosphate buffered saline solution (PBS) thrice. After the last wash, supernatant was discarded, and the pellet was resuspended with 1 mL of culture medium.

Treatment of PBMCs with CM

PBMCs (2 x 10^6 cells per well) were seeded in 6-well culture plates, and CM collected from PCCs, and PSCs were added to achieve a concentration of 10% (total volume per well = 3 mL). As each cell line had a different growth rate, normalisation was performed (formula shown below) to adjust the final volume of CM used to treat the PBMCs. This would avoid potential bias due to the difference in concentration of secreted proteins in CM (CM from groups with a lower cell number will have lower concentration of secreted proteins from PCCs and/or PSCs).

$$Normalised\ CM\ volume = \frac{standard\ cell\ number * \times 300\ \mu L}{Number\ of\ cells}$$

$$* standard\ cell\ number = cell\ number\ for\ the\ group\ with\ the\ highest\ number\ of\ cells$$

Cells were cultured for 7 days with medium changed on day 3. After 7 days, the cells were harvested for the subsequent assays.

MDSCs isolation

CM collected from PCCs and PSCs were used to treat isolated PBMCs for 7 days to induce MDSCs differentiation. As a control, PBMCs were also seeded without CM treatment to access the suppressive properties of uninduced MDSCs. On day 7, the uninduced and CM-induced MDSCs were isolated using an immunomagnetic positive selection isolation kit (Stemcell Technologies, Vancouver, Canada). Isolated MDSCs were then seeded in 96-well plate at a density of 0.25 x 10^4 cells per well and incubated overnight.

Cell viability assay for MDSCs

The isolated CM-induced MDSCs were treated with CM at the concentrations of 10%, 20% and 30%. Both the uninduced MDSCs and untreated MDSCs were seeded as control. The cell viability was then assessed using CellTiter-Glo® Luminescent Cell Viability Assay at 48 hours (Promega, Madison, VI, USA).

BrdU proliferation assay for MDSCs

The isolated CM-induced MDSCs were treated with CM at the final concentrations of 30%. Both the uninduced MDSCs and untreated MDSCs were seeded as control. After 24 hours, BrdU reagent was added and incubated for another 24 hours. The cell proliferation was then assessed using the BrdU Cell Proliferation Kit according to the manufacturer's instructions (Millipore, Darmstadt, Germany), and the results were obtained by reading the plate using a spectrophotometer microplate reader set at dual wavelength of 450/550 nm (Infinite® M Plex, Tecan, Zurich, Switzerland).

Cell viability assay for PCCs

PCCs were seeded at a final density of 1.5 x 10^5 cells per well in 6-well culture plates and incubated overnight (Eppendorf, Hamburg, Germany). After incubation, the seeded PCCs were treated with the CM collected from PSCs at the concentrations of 10%, 20%, and 30% for a total duration of 24- and 48 hours. To access the cell viability, CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was added at the end of each timepoints, and further incubated for 4 hours (Promega, Madison, WI, USA). Lastly, the absorbance at 490 nm was recorded using a microplate reader (Infinite® M Plex, Tecan, Zurich, Switzerland).

Cell viability assay for PSCs

PSCs were seeded at a final density of 1.5 x 10^5 cells per well in 6-well culture plates and incubated overnight (Eppendorf, Hamburg, Germany). After incubation, the seeded PSCs were treated with the CM collected from PCCs at the concentrations of 10%, 20%, and 30% for a total duration of 24- and 48 hours. To access the cell viability, CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was added at the end of each timepoints, and further incubated for 4 hours (Promega, Madison, WI, USA). Lastly, the absorbance at 490 nm was recorded using a microplate reader (Infinite® M Plex, Tecan, Zurich, Switzerland).

Statistical analysis

All experiments were performed in triplicates and statistical analysis was performed using Statistical Package of Social Sciences (SPSS) software (version 25; IBM, Armonk, NY, USA). Analysis of Variance (ANOVA) was carried out, followed by Duncan post-hoc test to analyse the differences among groups. A *p*-value less than or equal to 0.05 was considered significant.

RESULTS

Effect of CM on the proliferation of MDSCs

For control purposes, we have included the uninduced-MDSCs, which consists of MDSCs isolated from PBMCs without CM treatment, and the untreated induced-MDSCs, which consists of MDSCs isolated from PBMCs with CM treatment. The aim of this inclusion is to conduct a comparative analysis in terms of the cellular proliferation of the MDSCs that undergo differentiation in the absence of secreted molecules originating from PCCs or PSCs, in contrast to the MDSCs that differentiate in the presence of these secreted molecules in the CM.

All induced-MDSCs exhibited higher proliferation rate in comparison to uninduced-MDSCs following exposure to CM. This observation suggests that the components present in the CM stimulate the proliferation or facilitate the survival of MDSCs, with the induction process likely involving differentiation mechanisms that enhance cell survival. The cellular proliferation of all groups of induced-MDSCs increased as the concentration of CM increased. This dose-dependent effect implies that elevated levels of CM offer a greater amount of stimulatory or supportive factor for MDSCs proliferation. As the MDSCs were exposed to 30% CM, those treated with SW1990 cells CM displayed proliferation rate that was at least twice as high as those treated with PANC10.05 cells CM, and approximately four times greater than those treated with PSCs CM. This comparison highlights that the CM derived from SW1990 cells contains factors that significantly enhance MDSCs proliferation compared to CM from PANC10.05 cells and PSCs (Figure 1a).

As we stained the MDSCs with BrdU after CM treatment, both treated and untreated induced MDSCs exhibited an increased rate of cell proliferation in comparison to uninduced MDSCs (Figure 1b). This observation suggests that the components present in CM have the capability to stimulate or enhance the proliferation of MDSCs, thereby facilitating their growth and proliferation. MDSCs that were induced by CM and exposed to 30% CM displayed a decreased proliferation rate when compared to induced-MDSCs that did not undergo CM treatment. This implies that although CM initially fosters the proliferation of MDSCs at lower concentrations, higher concentrations or prolonged incubation could potentially result in a threshold. Notably, at a concentration of 30% CM, induced-MDSCs treated with CM from PANC10.05 cells and PSCs showed notably reduced proliferation rates in contrast to induced-MDSCs without CM exposure. Conversely, induced-MDSCs treated with CM from SW1990 cells at 30% CM displayed a proliferation rate that was akin to induced-MDSCs that did not receive CM treatment. This discrepancy indicated that SW1990 cells CM may have factors that better sustain or enhance MDSC proliferation compared to PANC10.05 cells CM and PSCs CM even with further CM exposure.

Cellular proliferation of PCCs and PSCs

To examine the impact of secreted proteins on PCCs and PSCs regarding cellular proliferation, the CM of PCCs were collected and employed as a treatment on PSCs, and reciprocally.

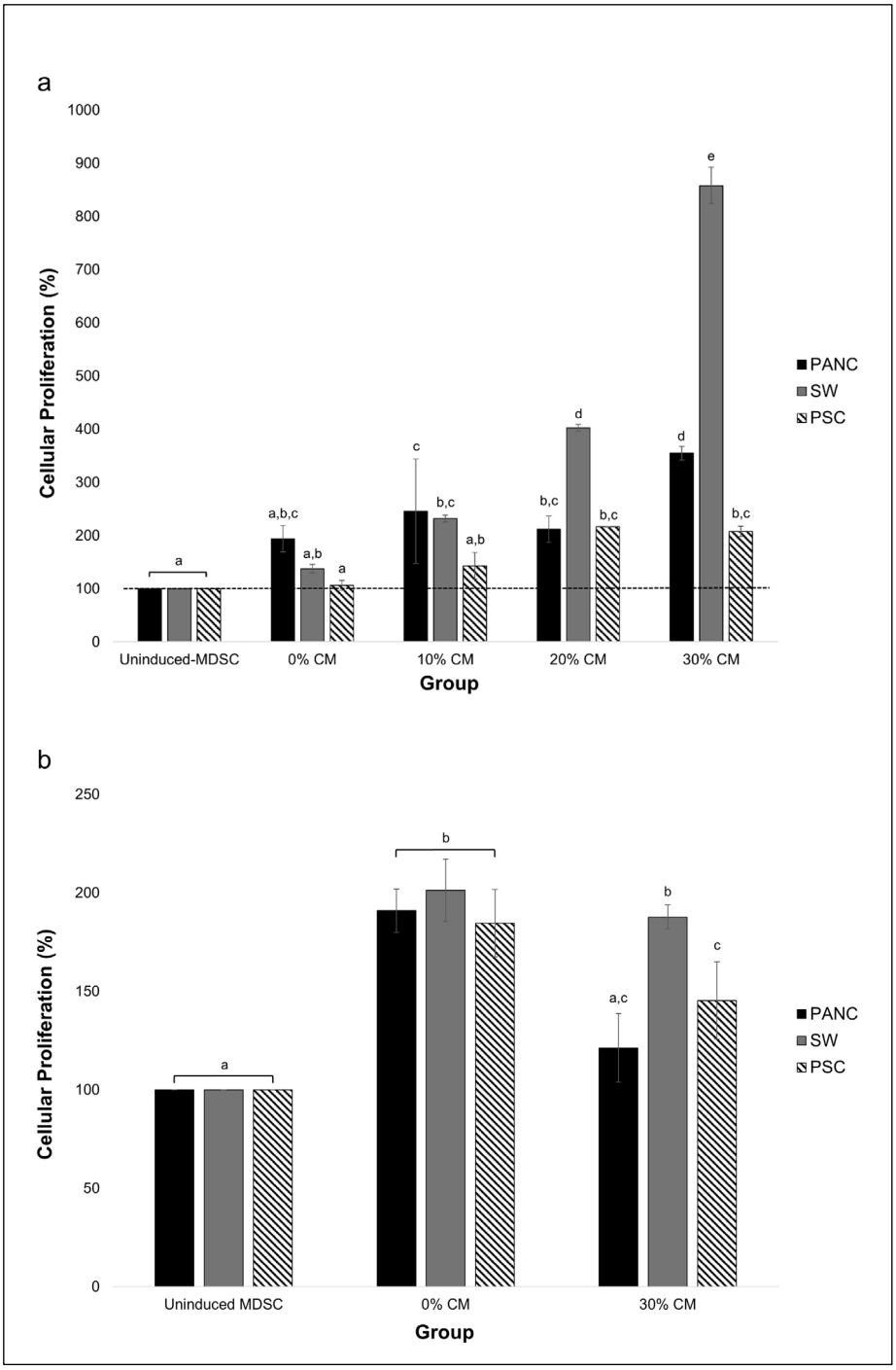

Figure 1. The cell proliferation rate of MDSCs. PBMCs were treated with CM for 7 days to induce the differentiation of MDSCs, and the uninduced- and induced-MDSCs were isolated and treated with CM for a total duration of 48 hours. A, CellTiter-Glo® Luminescent Cell Viability reagent was added after 48 hours of CM treatment, and the cell viability was measured. B, BrdU reagent was added 24 hours post CM treatment and further incubated for another 24 hours. At 48 hours post CM treatment, the BrdU cellular proliferation was measured. Statistical significance is indicated by the letters above each column, in which the columns that do not share a common letter have a significance of $p \leq 0.05$.

The data presented in Figure 2a demonstrates a significant increase in the proliferation rate of PSCs following exposure to 30% of media conditioned by PANC10.05 cells after a 48-hour incubation period. This observation implies that the components within the CM derived from PANC10.05 cells may exert a stimulatory influence on the proliferation of PSCs. In contrast to the outcomes associated with PANC10.05 cells CM, PSCs treated with SW1990 cells CM exhibited a gradual decline in proliferation rate, evident at both the 24-hour and 48-hour intervals. The diminishing viabilities suggest that SW1990 cells CM does not sustain or facilitate the proliferation of PSCs (Figure 2b).

Following a 48-hour incubation period with media that had been conditioned by PSCs, the PANC10.05 cells demonstrated a significant elevation of proliferation rate in all concentrations. This observation implies that factors found in the CM originating from PSCs have the ability to prompt or sustain the proliferation of PANC10.05 cells. The observed increment suggests a potential beneficial impact of factors derived from PSCs on the growth of PANC10.05 cells throughout the duration of the

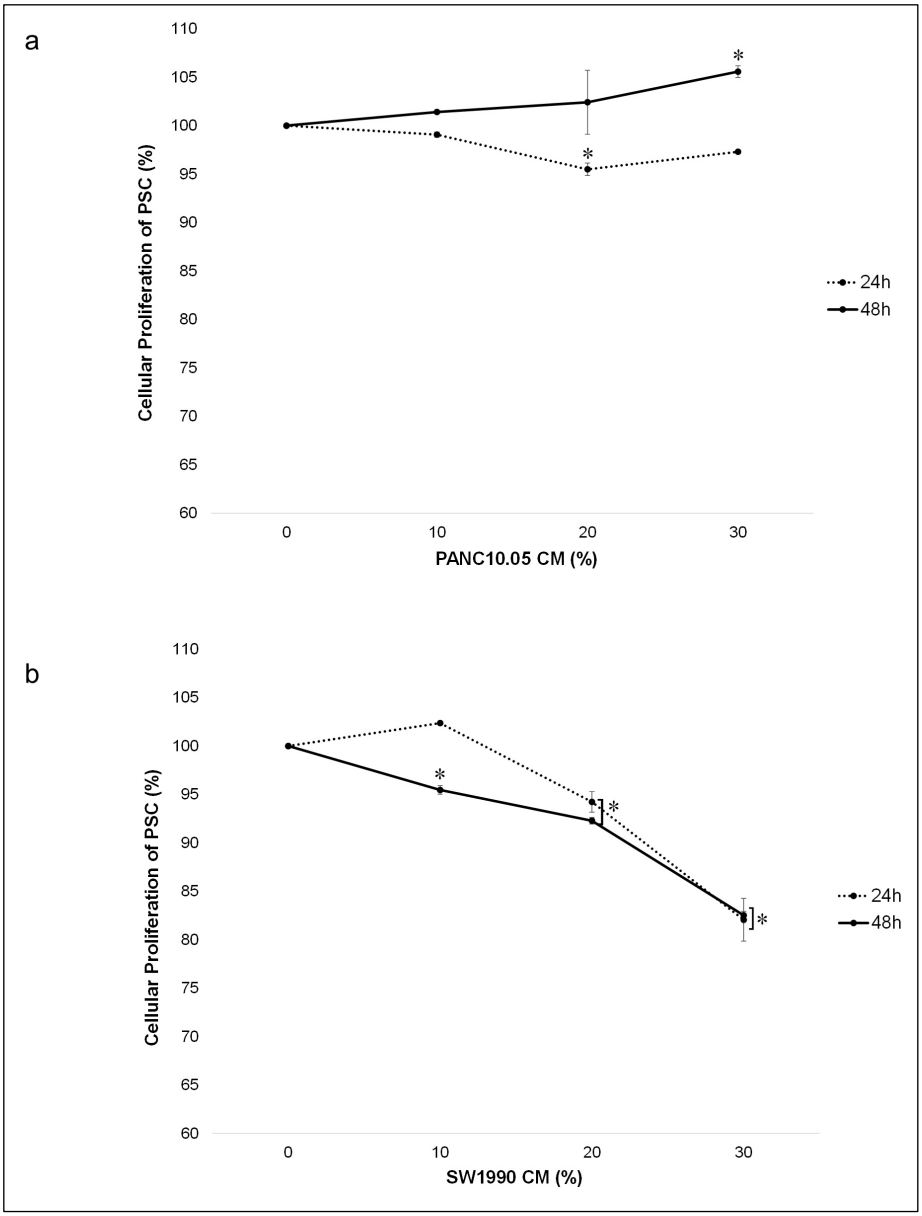

Figure 2. The cellular proliferation of PSCs at 24 and 48 hours. CM were harvested from 2 PCC lines and used to treat PSCs for 24 and 48 hours, and their cell viability was accessed. Statistical significance is indicated by the asterisk above each data point, in which the data point with an asterisk has a significance of $p \leq 0.05$.

incubation (Figure 3a). When exposed to a 10% concentration of PSCs CM, SW1990 cells initially displayed an enhancement in cellular proliferation following a 48-hour period. Nevertheless, with an increase in the concentration of PSCs CM beyond 10%, a noticeable decline in the cellular proliferation of SW1990 cells was observed. This highlights a concentration-dependent influence of PSCs CM on SW1990 cells, wherein lower concentrations initially promote cellular proliferation while higher concentrations could prove to be detrimental (Figure 3b).

DISCUSSION

In this investigation, the impact of PCC-CM and PSC-CM on the stimulation of MDSCs differentiation and their proliferative characteristics was examined, alongside exploring the influence of the secreted molecules from PCCs and PSCs on each other's proliferation rates.

In this study, two assays were employed to examine MDSCs proliferation from various aspects. The CellTiter-Glo® Luminescent Cell Viability Assay measures ATP levels to indicate viable cells with active metabolism. Conversely, the BrdU labelling method incorporates BrdU into newly synthesized DNA, allowing for the quantification of actively proliferating cells. As shown in Figure 1a, media conditioned by PCCs and PSCs exhibited a notable impact on enhancing the proliferation of induced MDSCs. MDSCs

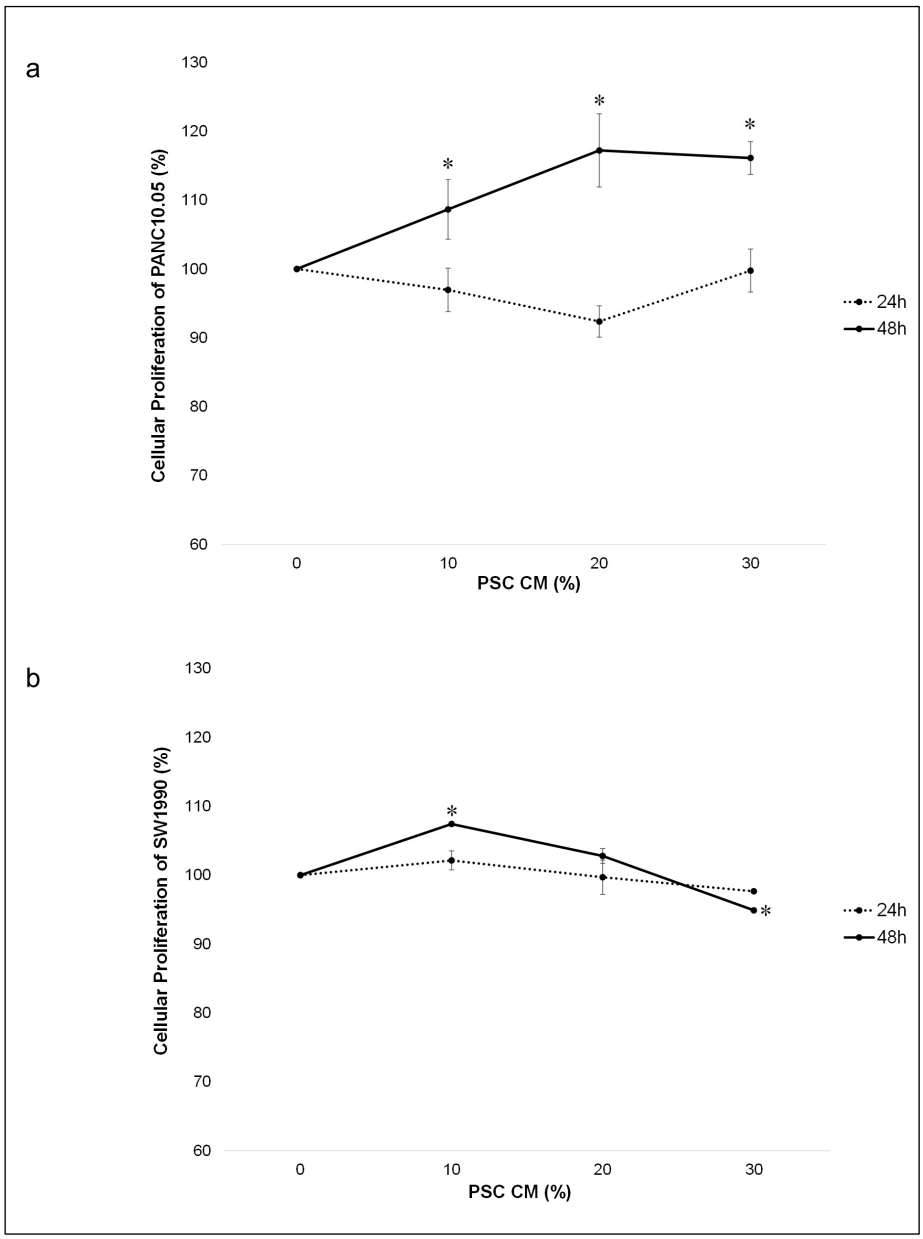

Figure 3. The cellular proliferation of PANC10.05 and SW1990 cells at 24 and 48 hours. CM were harvested from PSCs and used to treat PCCs for 24 and 48 hours, and their cell viability was accessed. Statistical significance is indicated by the asterisk above each data point, in which the data point with an asterisk has a significance of $p \leq 0.05$.

that were induced by CM demonstrated a higher proliferation rate in comparison to the non-induced MDSCs. Therefore, it was inferred that the differentiated MDSCs had a higher rate of proliferation compared to immature MDSCs. When compared between the induced MDSCs, it was found that the cellular proliferation is directly correlated with the concentration of CM. Furthermore, the CM from SW1990 cells demonstrated a better efficacy in promoting cell proliferation. This was indicated by the notably higher proliferation rate observed, in comparison to PANC10.05 cells and PSCs treated groups.

Interestingly, the groups treated with PANC10.05 cells and PSCs CM showed a considerably lower proliferation rate as compared to the untreated group in the BrdU cell proliferation assay (Figure 1b). Even the group treated by SW1990 cells CM was merely showing a proliferation rate on par with the untreated group. These results were inconsistent with the cellular proliferation assay shown in (Figure 1a) and it is noteworthy that the CellTiter-Glo® Luminescent Cell Viability Assay measures the total number of viable cells based on ATP levels, reflecting overall cell viability and metabolism. In contrast, the BrdU assay measures the rate of DNA synthesis, serving as an indicator for cellular proliferation. Thus, cells that proliferate rapidly will show higher BrdU readings compared to cells that proliferate more slowly, even if the total number of cells is the same. Additionally, the CellTiter-Glo® Luminescent Cell Viability Assay assesses cellular proliferation over a 48-hour period, whereas BrdU incorporation is limited to the final 24 hours. Consequently, the peak in cellular proliferation that may have occurred

during the initial 24 hours would not be detected by the BrdU assay. Besides, we postulate that there may be a threshold for CM-induced MDSCs proliferation, in which the proliferation will be halted once the secreted molecules in the CM have been depleted or after a certain duration.

The proliferation of MDSCs is profoundly influenced by the tumour microenvironment, which varies across different organs and tumour types[28]. This environment is shaped by a complex interplay of cytokines, growth factors, and the unique characteristics of different MDSC subsets[29]. Consequently, the proliferation rates of MDSCs can vary significantly depending on the specific local conditions. In particular to the MDSC subsets, the polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), which account for more than 90% of the overall MDSCs, have been documented to lack proliferative capacity in comparison to the monocytic myeloid-derived suppressor cells (M-MDSCs)[30,31]. This phenomenon may elucidate why an overall increase in proliferation rate was not observed, especially if the predominant population of the CM-induced MDSCs was PMN-MDSCs, which inherently lack proliferative activity. Nonetheless, this proposition requires confirmation through further research concentrating on the specific subsets of MDSC that were elicited by the CM.

Other than that, we suggest that the discrepancy between the cellular proliferation rate exhibited by both PCC lines was due to the upregulated Pentraxin 3 (PTX3) in SW1990 CM, which support the proliferation of MDSCs[32]. PTX3 is an inflammatory molecule that plays a role in tumour progression by interacting with various signalling pathways. PTX3 was identified as being elevated not only in pancreatic adenocarcinoma but also in a variety of cancer types. Its increased levels were noted to be linked with an unfavourable prognostic factor, the metastasis of disease, and potentially serving as a distinctive indicator for early diagnosis of pancreatic cancer as well as for differentiating between malignant and benign pancreatic conditions[33-37]. In a Cell Counting Kit-8 (CCK8) assay, the growth of glioblastoma cells was suppressed when PTX3 was knocked down, in which the study has demonstrated that PTX3 negatively influenced cellular autophagy, resulting in enhanced cellular survival and proliferation in glioblastoma cells[38]. Additionally, the reduction in PTX3 expression led to a decreased proliferation rate in breast cancer and cervical cancer cells, as evidenced in the investigation[39,40]. Suppression of PTX3 activity significantly diminished the impact of metastasis-related cellular mechanisms such as cellular chemotaxis, migration, adhesion, and invasion, which play a critical role in cellular proliferation[39]. In summary, these studies suggest that PTX3 upregulation exerts a positive influence on cellular proliferation in various cancer cell lines, underscoring the therapeutic potential of targeting PTX3 to impede tumour development and progression by inhibiting the expansion of immunosuppressive MDSCs.

As there is a keen interest in investigating the interplay between PCCs and PSCs regarding cellular proliferation, with potential implications on cancer metastasis and invasion, the corresponding CM from each cell type were obtained and subjected to reciprocal treatment. When PSCs were treated with CM derived from PANC10.05 cells, the marginal increase in the proliferation rate of PSCs indicates that PANC10.05 cells may secrete molecules that have the potential to promote the proliferation of PSCs in the long term (Figure 2a). Conversely, the declining cellular proliferation in SW1990 cells CM treated group suggests that the factors released by SW1990 cells might not be conducive to PSCs proliferation, hinting at a possibly unfavourable or inhibitory effect of the components in the CM derived from SW1990 cells (Figure 2b).

As we treat the PANC10.05 cells with CM derived from PSCs, the increased cell viabilities imply that PSCs may release molecules that stimulate the proliferation of PANC10.05 cells (Figure 3a). Whereas for the SW1990 cells, the results showed an initial rise in cellular proliferation at a lower concentration of PSCs CM, followed by a decline at higher concentrations, suggesting a multifaceted interplay (Figure 3b). This indicates that certain components in PSCs CM might have a positive impact on SW1990 cells at lower levels but could become less beneficial or even detrimental at higher concentrations.

Figure 4 summarises our findings on the relationship between MDSCs, PANC10.05 cells, SW1990 cells and PSCs. In general, it was found that PANC10.05 cells and PSCs promote the proliferation of each other while SW1990 cells work synergistically with MDSCs. Our inference suggests that this variance is attributable to the particular stage of the tumour from which the cell lines were derived; specifically, the PANC10.05 cells originated from a primary tumour, while the SW1990 cells originated from a metastatic tumour. We postulate that in the initial stages of pancreatic carcinoma, the PCCs will work synergistically with PSCs to enhance the immunosuppression in the TME, including the recruitment of immature MDSCs. During this period, the PCCs and PSCs will mutually enhance their proliferation rates, which may be contributed by the upregulated Granulin (GRN) in PANC10.05 cells[32]. Granulins are smaller peptides that are cleaved from the Progranulin (PGRN) precursor. Overexpression of PGRN has been detected in various malignancies, such as lung cancer, breast cancer, and other solid tumours[41-43]. Within these specific tissues, elevated levels of PGRN facilitate the advancement of tumours by stimulating cellular

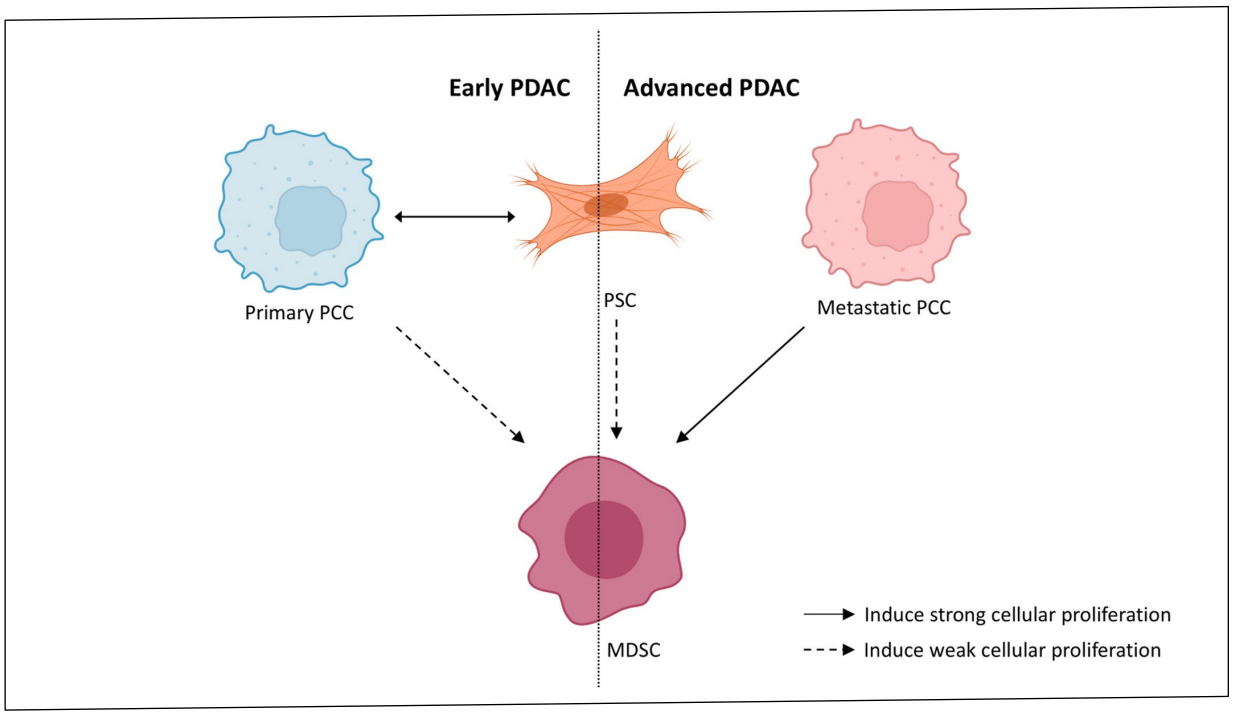

Figure 4. Summary of the cellular proliferation of PCCs, PSCs, and MDSCs.

processes including but not limited to cell proliferation, migration, invasion, angiogenesis, malignant progression, resistance to chemotherapeutic agents, and evasion of immune surveillance[44-47]. A recent investigation has demonstrated that PGRN not only has the capability to enhance proliferation in a cell line of papillary thyroid carcinoma (PTC), but it also has the ability to impede the process of apoptosis in these PTC cells. As indicated by their flow cytometry analysis, PGRN hinders apoptosis by facilitating the transition of PTC cells from G1 phase to the S phase, resulting in decreased rates of apoptosis. Overexpression of PGRN leads to increased anti-apoptotic proteins such as BCL2 and CyclinD1, while reducing the levels of pro-apoptotic proteins like BAX and BAD, subsequently restraining apoptosis. Conversely, depletion of PGRN induces the opposite outcome, characterised by decreased expression of BCL2 and CyclinD1, and increased expression of BAX and BAD, thereby enhancing the apoptosis of PTC cells. Moreover, PGRN triggers the JAK2-STAT3/4 signalling pathway, which further contributes to the inhibition of apoptosis in PTC cells[48]. According to their findings, it can be inferred that the upregulation of GRN within PANC10.05 cells might have played a role in supporting their intrinsic cellular growth and suppressing apoptotic processes, consequently leading to a progressive enhancement in cell survival rates. However, as the disease advances to the metastatic stage, the PCCs and PSCs will not stimulate each other's proliferation to the same extent as observed in the primary tumour stage due to the downregulated GRN. Instead, the PCCs will shift their focus towards promoting the proliferation of mature MDSCs to further establish an immunosuppressive microenvironment.

CONCLUSIONS

One limitation of this research pertains to the lack of investigation into the cell death mechanism, leading to uncertainty regarding whether the reduced cell viability has resulted from cell death. Ultimately, the bioactive secreted proteins from PCCs and PSCs demonstrate the capability to stimulate MDSCs with increased proliferation rates without necessitating direct cell-cell interactions. Additionally, the factors derived from the primary tumour cell were observed to sustain the proliferation or survival of cancer cells in comparison to those from the metastatic tumour cell. These discoveries shed new lights for future research endeavours focusing on the development of immunotherapeutic tailored for early-stage cancers, aiming to impede the cellular proliferation of cancer cells to prevent disease metastasis or suppress the proliferation of immunosuppressive cells for improved therapeutic interventions. Last but not least, the proposed differentially expressed proteins could serve as valuable indicators for the early detection of cancer.

Artificial Intelligence:

Artificial Intelligence was used in streamlining the search for pertinent academic papers and extracting essential insights from each article. Authors declare that there is no plagiarism in the manuscript, including figures.

Author contributions:

Ket Li Ho, Sook Han Ng, and Norsharina Ismail have contributed to the conception and design of the study. Atsushi Masamune established and provided the cell line, as well as offered valuable advice throughout the project. The material preparation, data acquisition, interpretation and analysis were performed by Yuen Ping Chong, Suriani Samsudin, Dina Athariah Bahrani, and Chung Han Chang. The first draft of the manuscript was written by Yuen Ping Chong, and all authors were included in the validation and final approval of the version of the article to be published.

Conflict of Interest:

The authors declare that they have no conflict of interest to disclose.

Data Availability Statement:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Committee Approval:

This study was conducted in accordance with the Declaration of Helsinki of 1975 (as revised in 2013), and the protocol was reviewed and approved by the Ethical Board of IMU University.

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Informed Consent:

All subjects provided written informed consent for inclusion before they participated in the study.

ORCID ID:

Ket Li Ho: https://orcid.org/0000-0002-9605-1018
Yuen Ping Chong: https://orcid.org/0009-0004-5849-168X
Atsushi Masamune: https://orcid.org/0000-0001-7184-7282
Sook Han Ng: https://orcid.org/0000-0002-9552-8523
Norsharina Ismail: https://orcid.org/0000-0002-2580-8300

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