

Evaluation the Effect of Tumor-Associated Macrophage-Derived Factors on Pancreatic Cancer Microenvironment Cells

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ABSTRACT

Purpose: The unique tumor microenvironment (TME) of pancreatic cancer (PCa) is a critical factor contributing to its aggressive and incurable nature. Pancreatic stellate cells (PSCs) are among the most abundant stromal cells in the TME, closely associated with tumor progression, metastasis, and chemo-/radioresistance. Tumor-associated macrophages (TAMs) are another important cell type in the TME, playing a vital role in promoting tumor growth and metastasis. Limited research exists on the interaction between PSCs and TAMs, underscoring the need for more studies to better understand the interaction between these two cell types.

Methods: THP-1 monocytic cells were differentiated into macrophages, and differentiation efficiency was confirmed by morphological analysis and Western blot. PSCs were co-cultured with monocytes/macrophages, and changes in the migration and invasion abilities of PSCs were assessed using wound healing and transwell invasion assays, respectively. To investigate the effects of macrophage-derived factors, MCP-1 and Gas6, on PSC-macrophage interactions, these proteins were applied to PSCs, or their receptors (Axl and CCR2) were suppressed using siRNA technology. The effects of Gas6/Axl and MCP-1/CCR2 signaling on PSC cell viability, colony formation, and migration were then evaluated.

Results: While MCP-1 did not induce changes in colony-forming and migration abilities of PSCs, Gas6 treatment resulted an induction. This effect was reduced when the Gas6 receptor Axl was suppressed with siRNA, suggesting that Gas6/Axl signaling may play critical role in macrophage-mediated changes in PSCs.

Conclusion: Further research is needed to fully understand the interaction between PSCs and TAMs in pancreatic cancer.

Keywords: pancreatic cancer, tumor microenvironment, pancreatic stellate cells, macrophages, MCP-1, Gas6

ÖZET

Giriş: Pankreas kanserinin (PKa) karakteristik tümör mikroçevresi (TMÇ), bu kanserin agresif ve ölümcül doğasına aktif şekilde katkıda bulunmaktadır. Pankreatik stellat hücreleri (PSH'ler), TMÇ'de en bol bulunan stromal hücreler arasındadır ve tümör gelişimi, metastaz ve kemoterapi ve radyoterapi direnci ile yakından ilişkilidir. Tümörle ilişkili makrofajlar (TIM'ler), mikroçevredeki bir diğer önemli hücre türüdür ve tümör büyümesini ve metastazı desteklemede kritik rol oynamaktadır. TMÇ'nin bu iki önemli hücre türünün (PSH'ler ve TIM'ler) arasındaki etkileşime odaklanan çalışmalar oldukça sınırlıdır, bu nedenle iki hücre türü arasındaki etkileşimi daha iyi anlamak için daha fazla çalışmaya gereksinim bulunmaktadır. Bu amaçla, çalışmamızda bu iki hücre arasındaki etkileşime ve etkileşimde rol alan faktörlerin tanımlanmasına odaklandık.

Yöntem: İlk olarak THP-1 monositik hücreleri makrofajlara farklılaştırıldı ve farklılaşma etkinliği morfolojik analiz ve Western blot ile doğrulandı. PSH'ler monositler/makrofajlarla birlikte kültüre edildi ve hücrelerin göç ve invazyon yeteneklerindeki değişiklikler sırasıyla yara iyileşmesi ve invazyon analizleri ile değerlendirildi. Makrofaj kaynaklı faktörler olan MCP-1 ve Gas6'nın PSH-makrofaj etkileşimi üzerindeki etkilerini araştırmak için bu proteinler PSH'lere uygulandı veya reseptörleri (Axl ve CCR2) siRNA teknolojisi kullanılarak baskılandı. Daha sonra Gas6/Axl ve MCP-1/CCR2 sinyalizasyonunun PSH canlılığı, koloni oluşumu ve göçü üzerindeki etkileri değerlendirildi.

Bulgular: MCP-1 uygulaması PSH'lerin koloni oluşturma ve göç yeteneklerinde değişiklik yaratmazken, Gas6 hücrelerin oluşturma koloni ve migrasyon yeteneğinde bir artışa neden oldu. Ek olarak, bu etki Gas6 reseptörü Axl siRNA ile bastırıldığında azaldı. Bu bulgular Gas6/Axl sinyalizasyonunun PSH-makrofaj etkileşiminde rol alan sinyal yollarından biri olabileceğine dikkat çekmektedir.

Sonuç: Pankreas kanserinde PSH'ler ve TIM'ler arasındaki etkileşimi net şekilde anlamak için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: pankreas kanseri, tümör mikroçevresi, pankreatik stellat hücreleri, makrofajlar MCP-1, Gas6

Pancreatic cancer (PCa) is one of the deadliest solid tumors, with a 5-year survival rate just above 10% (1). Despite recent technological advancements, PCa remains highly aggressive and lethal. Its rich tumor microenvironment (TME) significantly contributes to tumor progression, metastasis, drug resistance, and evasion of apoptosis and immune system (2-4).

Pancreatic stellate cells (PSCs), the most abundant fibroblastic cells in the PCa stroma, are quiescent under normal conditions but become activated by inflammation. During tumorigenesis, PSCs remain constantly active and play critical roles in tumor progression by creating a supportive microenvironment for cancer cells (5-8). Tumor-associated macrophages (TAMs), defined as macrophages in or around the tumor, are another important cell type in the TME and play critical roles in regulating tumor progression. In general, TAMs can be categorized into two main populations with opposite functions: M1 macrophages have anti-tumorigenic properties, whereas M2 macrophages are pro-tumorigenic (9). M2 macrophages interact bidirectionally with PCa cells and other cells in the TME, promoting tumor growth and progression (10).

Monocyte chemoattractant protein-1 (MCP-1/CCL2), a chemokine mainly released by monocytes/macrophages, regulates the migration and infiltration of monocytes/macrophages and promotes monocyte-macrophage differentiation (11, 12). Growth-arrest-specific 6 (Gas6), another protein in the PCa TME, is known to contribute to cancer cell proliferation and migration (13, 14). Gas6/Axl signaling regulates the TME by inducing TAM polarization toward M2 macrophages (15). Indeed, TAMs express high levels of Gas6 (16), suggesting the existence of a tumor-promoting cycle mediated by Gas6 signaling.

Considering all this information, we focused on investigating the interaction between two important cell types in TME, PSCs and macrophages, and evaluating the potential effects of this interaction on behaviors of PSCs, as well as identifying the factors regulating this interaction. Our results suggest that Gas6/Axl signaling may play an important role in communication between PSCs and macrophages and could serve as a therapeutic target for modulating the TME in the future.

Material and Methods

2.1. Cell Culture

THP-1 acute monocytic leukemia cells were obtained from the ATCC (Manassas, VA, USA). Human pancreatic stellate

cells (PSCs) were kindly provided by Dr. Rosa Hwank (MD Anderson Cancer Center). Both cell types were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml Penicillin/Streptomycin at 37°C in 5% CO₂, and tested for mycoplasma contamination.

2.2. Monocyte-Macrophage Differentiation and Characterization

THP-1 cells were cultured to 70-80% confluency in 75 cm² flasks. For mature (M0 type) macrophage differentiation, cells were treated with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Then, media were replaced with fresh RPMI-1640 media (without PMA) and M0 cells were cultured in this media. To polarize M0 macrophages into M2 pro-tumorigenic macrophages, IL-4 and IL-13 (25 ng/ml) were added to the flasks for 24h (17). Cells were then examined under phase-contrast microscopy, and CD68, CD163, and CD206 protein expressions were assessed by Western blot to confirm polarization.

2.3. Indirect Co-culture Experiments

Briefly, THP-1 cells were seeded in transwell inserts (0.4 µm pore size, Corning, New York, NY, USA) of 6 well plates at 2x10⁵ cells/well and cultured as monocytes or differentiated into M0/M2 macrophages. PSCs were seeded in separate 6-well plates at a density of 3x10⁵ cells/well and incubated overnight. Then, inserts containing monocytes or macrophages were transferred onto the wells and were co-cultured with PSCs for 48 h. After the co-culture period, the inserts were removed and the cells were subjected to further analyses.

2.4. MCP-1 and Gas6 Treatment

Recombinant human MCP-1/CCL2 protein (R&D Systems, Minneapolis, MN, USA) was dissolved in sterile PBS containing 0.1% bovine serum albumin at 100 µg/ml. Recombinant human Gas6 protein (R&D Systems, Minneapolis, MN, USA) was dissolved in sterile water at 500 µg/ml. PSCs were seeded in 6-well plates at 2x10⁵ cells per well and allowed to attach overnight. Before Gas6 treatment, the medium was replaced with serum-free medium, and cells were incubated overnight. Subsequently, cells were treated with Gas6 (200, 400, 600 ng/ml) for 30 min or MCP-1 (10, 25, 50 ng/ml) for 24 h. The concentration ranges and treatment periods for both Gas6 and MCP-1 were chosen based on the literature (18-20). Treated cells were used for further experiments.

2.5. siRNA Transfection

PSCs were seeded in 6-well plates at a density of 2×10^5 cells/well. After 24 h, cells were transiently transfected with 100 nM concentrations of control siRNA, eEF2K or AXL siRNA (Sigma-Aldrich, St. Louis, MO, USA) using HiPerfect transfection reagent (Qiagen, Hilden, Germany) in Opti-MEM Reduced Serum Medium (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. After 6 h of transfection, Opti-MEM media were changed with culture media containing 10% FBS, and cells were incubated for additional 48 h for Western blot analysis.

2.6. Colony Formation Assay

PSCs were seeded in 24-well plates at a density of 2×10^2 cells/well. Following overnight incubation, cells were treated with MCP-1, Gas6, or siRNAs as described above. After the treatments, media were replaced with fresh media and cells were cultured for 10-14 days until visible colonies formed. Colonies were then stained with crystal violet (0.5% w/v) and counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Wound Healing Assay

The wound healing assay was used to assess the migration ability of cells. PSCs were plated at a density of 2×10^5 cells per well in 6-well plates and incubated for 24 h. Subsequently, wounds were created using a 200- μ l sterile pipette tip, followed by gentle washing with culture media to remove cellular debris. Cells were then either co-cultured with monocytes/macrophages or treated with Gas6. Wounds were photographed immediately (0 h) and again at 24 and 48 h using a phase-contrast microscope (Nikon Eclipse TE-200-U). At least three random, non-overlapping images were taken from each well.

2.8. Matrigel Transwell Invasion Assay

PSCs were seeded in 6-well plates and co-cultured with monocytes/macrophages as previously described. After 24 h, cells were suspended in 200 μ l of serum-free media and added to Matrigel-coated Transwell inserts (8- μ m-pore size; Fisher Scientific). The lower chambers of the inserts were filled with 500 μ l of media containing 10% FBS as chemoattractant. After 24 h, invading cells were fixed and stained using a Hema 3 staining kit (Thermo Scientific, Waltham, MA, USA). Cells on the upper side of the membrane were removed by wiping with a cotton swab, and the membranes were photographed. The number of invaded cells was quantified by counting at

least six fields per membrane using ImageJ software and results were expressed as the mean number of invaded cells from triplicate measurements.

2.9. Western Blot

Cells were collected and lysed with RIPA lysis buffer. Protein concentration was then measured, and lysates were mixed with Laemmli loading buffer, heated at 95°C for 5 min, and separated by SDS-PAGE using a 4%-15% gradient gel. Proteins were transferred to PVDF membranes, blocked in 5% milk in TBS-T, and probed with primary antibodies: eEF2K, p-eEF2 (Thr56), Src, p-Src (Tyr416), MMP-2, Snail, integrin beta 1, p-Akt (Ser473), Akt, CCR2 (Cell Signaling Technology); CD68, CD163, CD206 (Abcam); MCP-1, Axl, p-Axl (Tyr702) (R&D Systems); Gas6 (Abcam). Membranes were then incubated with HRP-conjugated secondary antibodies and detected using HyGLO Chemiluminescent HRP Antibody Detection Reagent. Each membrane was exposed to film (Kodak) in a dark room for 0.5-10 min. For subsequent detections with different antibodies, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA, USA) for 15 min. β -actin (Sigma-Aldrich, St. Louis, MO, USA) and GAPDH (Cell Signaling Technology, Danvers, MA, USA) were used as loading controls.

2.10. Statistical Analyses

Data were expressed as means or fold changes \pm standard deviations (SDs). Statistical analysis was performed using the Student's t-test and one-way ANOVA to determine statistical significance by using GraphPad Prism 9.0 statistical software for Windows. *P* values indicate the probability of the means compared, being equal with **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Results

3.1. High CD68 and CD163 expression is associated with poor survival in PDAC patients

Tumor-associated macrophages are known to be associated with the aggressiveness of various cancers, including PCa. Kaplan-Meier survival curves were generated for macrophage markers, CD68 (pan-macrophage marker) and CD163 (M2-pro-tumorigenic marker), in PDAC patients with low and high expression levels. As shown in **Fig. 1A**, patients with high CD68 and CD163 expression have significantly shorter overall survival.

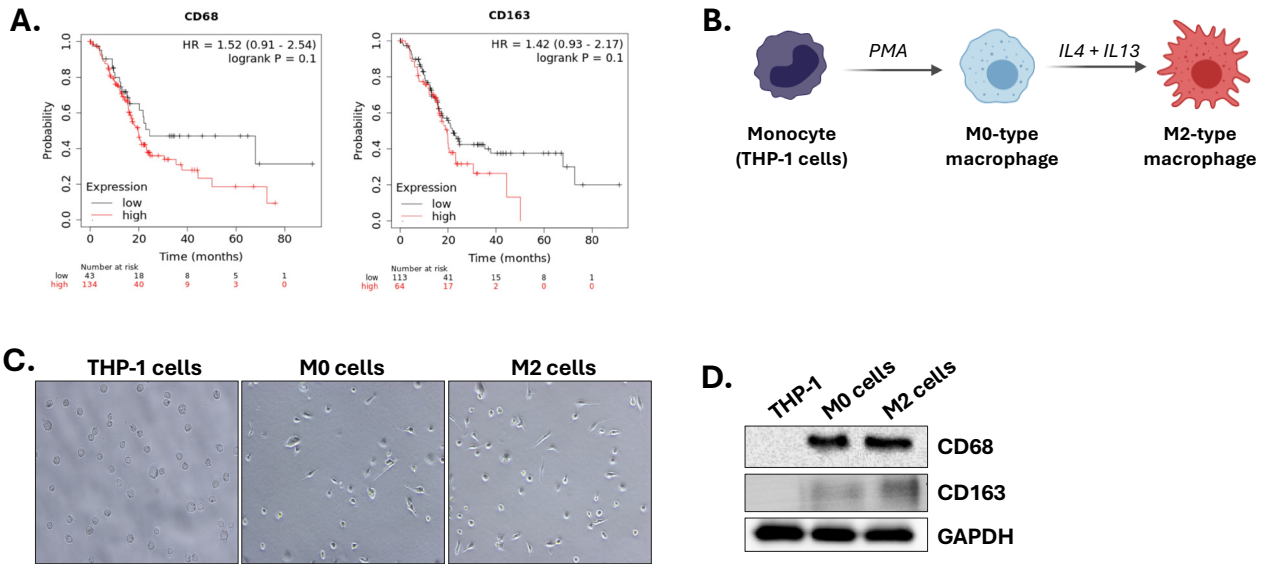


Figure 1: The survival analyses were performed in KM plotter using publicly available pancreatic ductal adenocarcinoma mRNA data that is available within the database. The presence of macrophages in tumors is associated with poor overall survival in PDAC patients. (A) High expression of CD68 and CD163 is correlated with poorer overall survival (OS) in PDAC patients as determined by Kaplan-Meier survival analysis ($p=0.1$). The numbers of patients with low and high eEF2K expressions are presented at the bottom of the graph. Mo, months. (B) Macrophages used in the study were differentiated from THP-1 monocytic cell lines. The differentiated process were performed as illustrated. (C) The differentiation efficacy was first evaluated by morphological changes. (D) The changes in the expression of differentiation-related markers were assessed by Western blot.

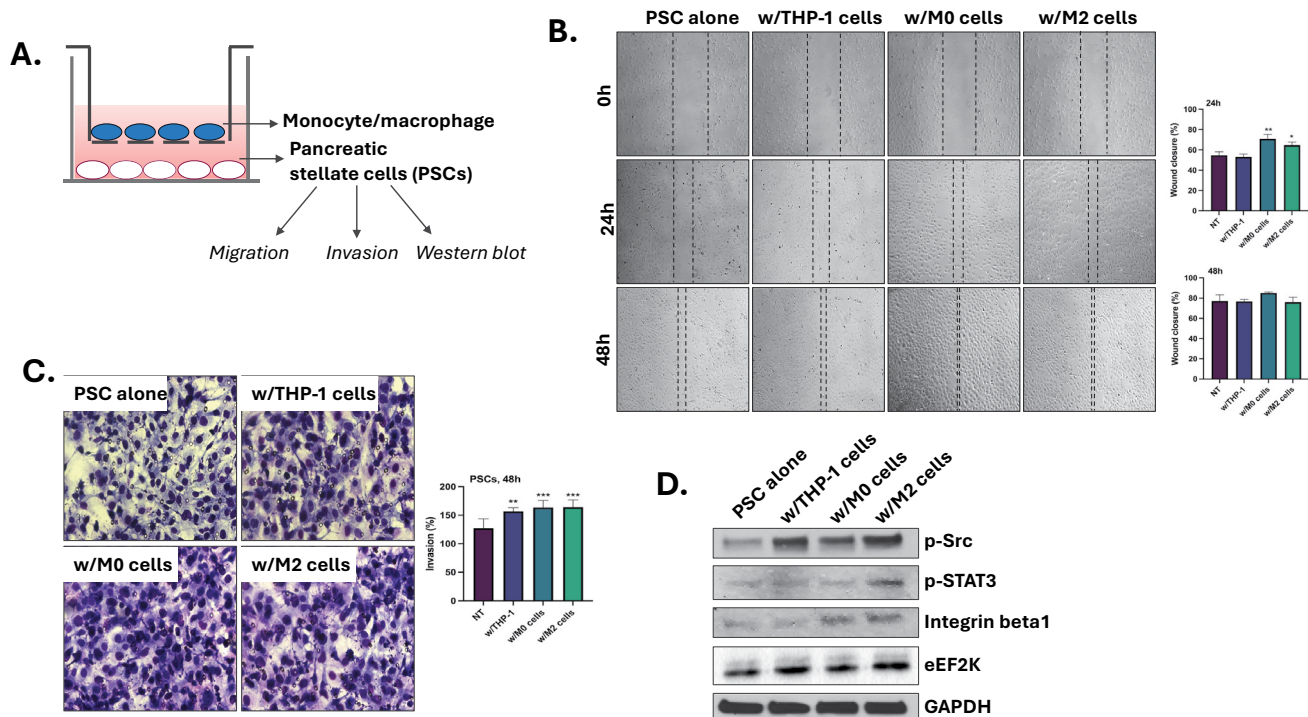


Figure 2: Macrophages increased the migration and invasion abilities and the expression of metastasis-related proteins of pancreatic stellate cells (PSCs). (A) PSCs were indirectly co-cultured with monocytes or macrophages for 48 hours as illustrated. (B, C) The effects of macrophages on cell migration and invasion were evaluated by wound healing assay and transwell invasion assay, respectively. (* $p<0.05$, ** $p<0.01$, * $p<0.001$, relative to PSC alone). (D) The changes in the expression of migration- and invasion-related proteins were assessed by Western blot.**

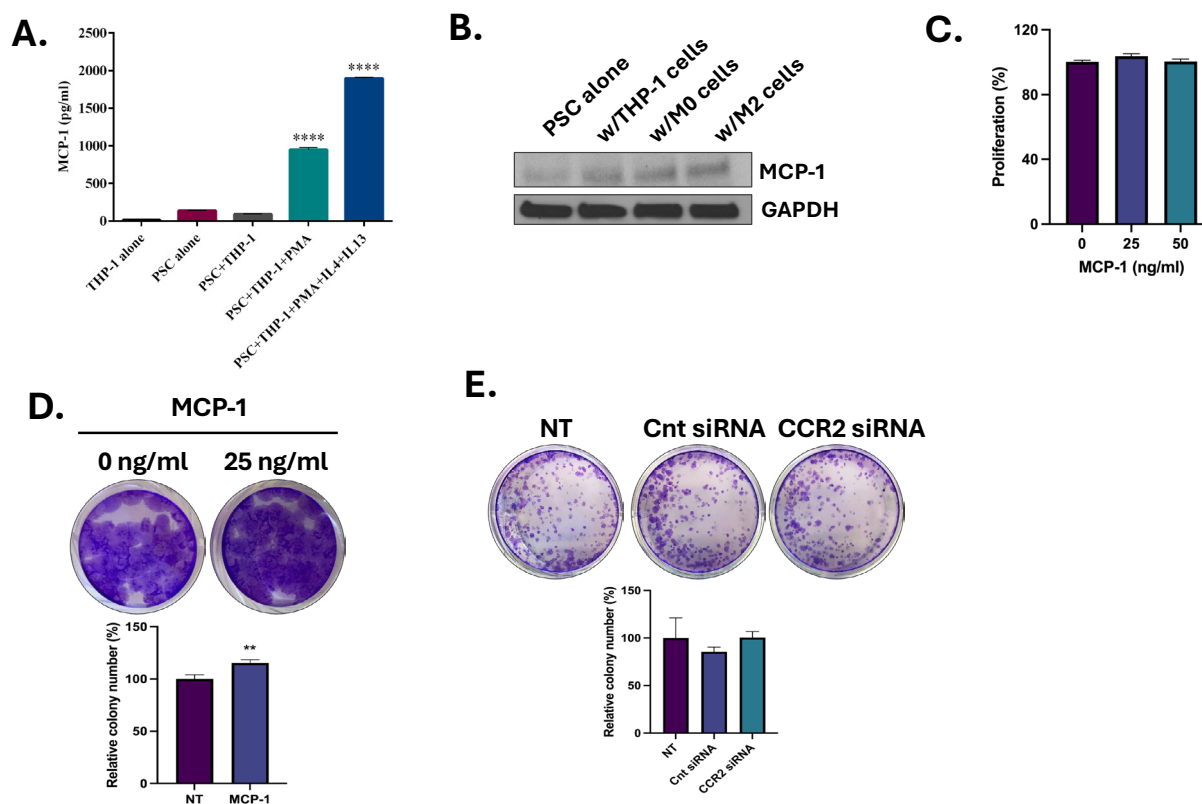


Figure 3: MCP-1 protein did not alter the proliferation and but slightly increased colony formation in PSC cells. (A) Increased levels of MCP-1 and eEF2K were detected in PSCs in the presence of macrophages. **(B)** Released MCP-1 levels in monocytes were measured by ELISA (**** $p < 0.01$, relative to PSC alone). **(C)** While MCP-1 treatment did not alter PSCs' proliferation, **(D)** it slightly induced colony-forming ability of cells. **(E)** Silencing of MCP-1 receptor, CCR2, did not change colony-forming ability of PSCs.

To investigate the interaction between PSCs and macrophages, we differentiated THP-1 monocytic cells into M0 mature macrophages or M2 pro-tumorigenic macrophages. The differentiation process is illustrated in **Fig. 1B**. The differentiation efficacy was first evaluated by morphological changes (**Fig. 1C**). Monocytes tend to grow in suspension, whereas when they differentiate into macrophages, they acquire the ability to attach to surfaces (**Fig. 1C and 1D**). The changes in the expression of differentiation-related markers were assessed by Western blot. The expression of the pan-macrophage marker, CD68, was detected in both M0- and M2-macrophages, while pro-tumorigenic macrophage marker, CD163, was expressed only in M2-macrophages (**Fig. 1D**).

3.2. Indirect co-culture of PSCs with monocytes/macrophages increased their migration and invasion abilities

To evaluate the effect of paracrine interactions between macrophages and PSCs on migration, invasion, and protein expression, indirect co-culture experiments were performed (**Fig. 2A**). Briefly, monocytes/macrophages were seeded into inserts (0.4 micron pore size), while PSC cells were seeded at the bottom of the wells, and the two cell types were co-cultured for 48 hours. Notably,

24 h of co-culture of PSCs significantly increased their migration ability (**Fig. 2B**). Besides, the presence of macrophages was shown to induce invasion of PSCs (**Fig. 2C**). Consistently, macrophages promoted the expression of p-Src, p-STAT-3, and Integrin- β 1, which are related to migration and invasion abilities of cells. Furthermore, the expression of eukaryotic elongation factor 2 kinase (eEF2K), an oncogenic protein overexpressed in PCa, was also shown to elevate in co-culture conditions (**Fig. 2D**).

3.3. MCP-1 levels increased in the presence of monocytes/macrophages

Following 48 hours interaction between PSCs and monocytes/macrophages, released MCP-1 levels, which play a critical role in monocyte recruitment and macrophage differentiation, were measured via ELISA. As shown in the graph, while monocytes and PSCs in monoculture conditions released limited levels of MCP-1, the presence of macrophages significantly increased released MCP-1 levels (**Fig. 3A**). The highest levels of MCP-1 release were detected in M2-pro-tumorigenic macrophage-PSC co-culture group (**Fig. 3A**). Consistently, MCP-1 protein expression was also found to be elevated when PSCs co-cultured with macrophages (**Fig. 3B**).

3.4. MCP-1 administration induced colony formation but not proliferation of PSCs

We then treated PSCs with recombinant MCP-1 protein to investigate changes in PSC proliferation and colony formation. MCP-1 treatment did not affect PSC proliferation but slightly increased colony formation (Fig. 3C, D). Moreover, targeting MCP-1 signaling by suppressing its receptor, CCR2, did not significantly reduce the colony-forming ability of the cells (Fig. 3E).

3.5. Gas6 expression increased in the presence of M2 macrophages

In addition to MCP-1, changes in the expression of another important chemokine, Gas6, which plays a role in monocyte-macrophage differentiation, were assessed. After 48 hours of co-culture, Gas6 expression was found to be elevated in the presence of macrophages, particularly M2 macrophages (Fig. 4A). Additionally, the expression of the active form of the Gas6 receptor, p-Axl, was also increased in macrophage-interacted groups (Fig. 4A).

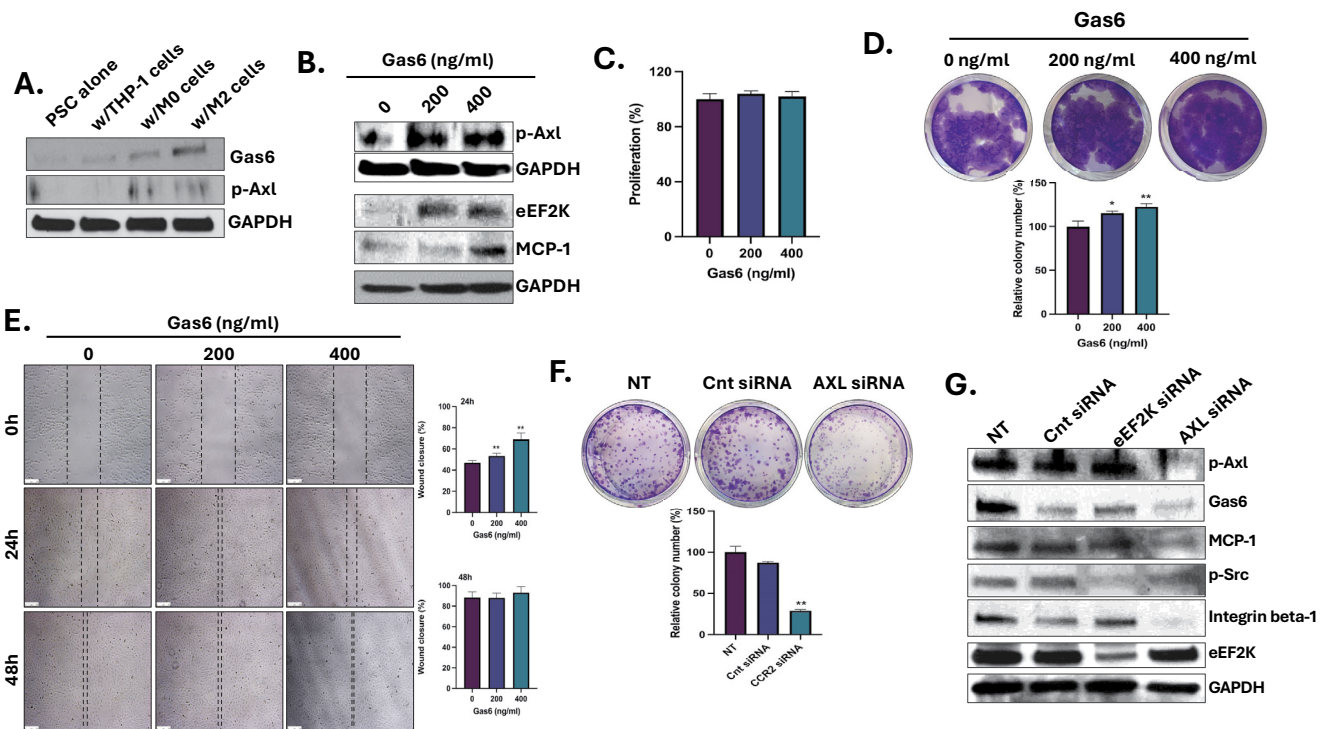


Figure 4: Increased Gas6 levels induced colony-formation and migration of PSCs and targeting Gas6/Axl signaling decreased colony-forming ability and the expression of aggressiveness-related proteins. (A) Gas6 protein and p-Axl were detected to be increased in the presence of macrophages. **(B)** Gas6 treatment induced the phosphorylation of its receptor and the expression of eEF2K and MCP-1 proteins. **(C-E)** While Gas6 exposure did not alter cell proliferation, caused an induction in colony formation and migration abilities of PSCs. **(F)** Targeting Gas6/Axl signaling by suppressing its receptor via siRNA caused a reduction in colony-forming ability of PSCs and **(G)** decreased the expression of p-Axl, Gas6, MCP-1, p-Src and Integrin-β1.

3.6. Gas6 treatment induced the expression of MCP-1 and promoted colony-formation and migration of the cells

We then applied recombinant Gas6 to PSCs to evaluate its role on PSCs colony formation, migration and protein expression. Following 30 minutes Gas6 exposure, the expression p-Axl was shown to increase (Fig. 4B). Additionally, eEF2K protein expression was increased in response to Gas6 treatment. Interestingly, 400 ng/ml of Gas6 dramatically induced MCP-1 protein expression, suggesting a potential crosstalk between these two

proteins (Fig. 4B). Although Gas6 treatment did not significantly alter PSC proliferation, it induced colony formation and cell migration (Fig. 4C-E).

3.7. Targeting the Gas6/Axl axis decreased colony-formation and MCP-1 expression

Next, we silenced the Gas6 receptor, Axl, using siRNA technology. Axl silencing significantly reduced the colony-forming ability of PSCs (Fig. 4F). As expected, silencing of Axl decreased expression levels of Gas6 and p-Axl (Fig.

4G). Interestingly, MCP-1, p-Src, and Integrin- β 1 were also reduced in Axl-silenced groups, while there were no changes in eEF2K expression (**Fig. 4G**).

Discussion

In this study, we showed that indirect interaction between PSCs and macrophages increases the migration and invasion abilities of PSCs. Notably, the Gas6 protein, primarily released by monocytes/macrophages, was shown to play a role in regulating communication between these two cell types. Targeting Gas6 receptor, Axl, resulted in a decrease in the expression levels of Gas6, MCP-1, p-Src, and Integrin-beta1, further supporting the central role of Gas6/Axl signaling in regulating processes related to cancer aggressiveness.

The presence of macrophages, especially M2 pro-tumorigenic macrophages, has been known to correlate with tumor growth, metastasis, and resistance to therapy (21, 22). Consistently, high expression levels of macrophage markers, CD68 and CD163, are associated with poorer overall survival in PDAC patients (23). Most studies have focused on the effect of TAMs on cancer cells; however, research on their role in PSCs, the most common cell type in PCa TME, remains limited. Therefore, we indirectly co-cultured PSCs with monocytes/macrophages to evaluate the effect of this interaction on the behaviors of PSCs. The results showed that macrophages enhanced the migration and invasion abilities of PSCs via paracrine signaling mechanisms.

TAM-derived factors such as cytokines, chemokines and exosomes have been shown to support tumor progression by promoting tumor proliferation, motility and invasion (24, 25). For instance, MCP-1, a chemokine involved in macrophage recruitment and differentiation, is known to promote tumor aggressiveness by regulating the interactions between tumor cells and macrophages (26). In addition, increased levels of MCP-1 in the serum of PDAC patients are associated with poor prognosis (27). Despite studies indicating its role in cancer-macrophage communication, research focusing on its role from the perspective of PSCs is limited. Our results showed that MCP-1 levels were increased as a result of paracrine interaction between PSCs and TAMs, and MCP-1 administration enhanced colony formation of PSCs, suggesting that MCP-1 may act as a mediator in the crosstalk between macrophages and PSCs.

In addition to MCP-1, we investigated the role of Gas6, another important chemokine involved in monocyte-macrophage differentiation and tumor aggressiveness. Gas6 contributes to tumor progression by promoting macrophage differentiation and cancer cell proliferation, migration and angiogenesis (28, 29). Our results showed that Gas6 expression was increased in the presence of M2 macrophages, and recombinant Gas6 treatment enhanced colony formation and cell migration. Furthermore, Gas6 administration further elevated MCP-1 levels, and targeting the Gas6 receptor, Axl, significantly reduced colony formation and MCP-1 expression in PSCs, suggesting a potential feedback loop between Gas6 and MCP-1. Currently, no study has demonstrated the crosstalk between Gas6 and MCP-1 in the cancer progression process. The only study indicating a potential interaction between Gas6 and MCP-1 showed that Gas6 expression was related to increased infiltration of inflammatory monocytes expressing CCR2 in venous thrombosis mouse models. Additionally, *in vitro* expression of both CCR2 and CCL2 was shown to depend on Gas6 expression in monocytes and endothelial cells (30). Here, we showed for the first time the role of Gas6 in macrophage-PSC interaction and potential interaction between Gas6 and MCP-1.

Conclusion

Our study showed the role of macrophage-derived factors, MCP-1 and Gas6, in promoting aggressive behaviors of PSCs. The findings specifically highlight the significance of Gas6 signaling in mediating macrophage-PSC interactions and suggest that targeting the Gas6/Axl axis could offer a new therapeutic strategy for future studies. Further research is needed to explore the potential of this pathway as a target for therapeutic intervention and to better understand the mechanisms underlying its effects on tumor progression.

Declarations

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Conflicts of Interest

The authors declare no competing interests.

Ethics Approval

N/A

Availability of Data and Material

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

D.K. wrote the manuscript and performed all experiments. D.K. and B.O. conceived and coordinated the study. E.D. and E.U. contributed to writing the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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