Biologicals xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Potential therapeutic effects of hAMSCs secretome on Panc1 pancreatic cancer cells through downregulation of SgK269, E-cadherin, vimentin, and snail expression

Mahsa Alidoust Saharkhiz Lahiji, Fatemeh Safari *

Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran

ARTICLE INFO

Keywords:
Panc1 cancer cells
hAMSCs
SgK269
EMT
Apoptosis

ABSTRACT

Pancreatic cancer is one of the leading causes of death from cancer worldwide. The current treatment options for pancreatic cancer are unsuccessful and thereby, finding novel and more effective therapeutic strategies is urgently required. Stem cells-based therapies are currently believed to be a potential promising option in cancer therapy. Herein, we are interested in evaluating the therapeutic effects of human amniotic mesenchymal stromal cells (hAMSCs) secretome on tumor growth suppression and EMT inhibition in Panc1 pancreatic cancer cells using 2D and 3D cell culture models. For this purpose, we employed a co-culture system using 6-well Transwell plates with a pore diameter of 0.4 μm. After 72 h treatment of Panc1 cancer cells with hAMSCs, the expression of c-Src, EGFR, SgK269, E-cadherin, Vimentin, Snail transcriptional factor, *Bax, Bcl2*, and caspase 3 was analyzed by quantitative real-time PCR (qRT-PCR) and Western blot methods. Our results showed significant reduction in tumor cell growth and motility through downregulation of c-Src, EGFR, SgK269, E-cadherin, Vimentin, and Snail transcriptional factor expression in Panc1 pancreatic cancer cells. The induction of cellular apoptosis was also found. Our finding supports the idea that the secretome from hAMSCS has therapeutic effects on cancer cells.

1. Introduction

Cancer is considered as one of the leading causes of death in human worldwide. Hormone therapy, surgery, radiation, and chemotherapy are the currently used approaches towards cancer treatment. The failure of many therapeutic options is due to drug resistance and the side effects of drugs and therefore, identifying new tools and specific platforms with the fewest side effects and high effectiveness remains a major challenge for interested researchers. At present, it is proposed that stem cells are a novel and promising platform for cancer therapy. Stem cells are a multipotent population of cells with unique biological characteristics such as self-renewal, bioactive factor secretion, low immunogenicity and differentiation capabilities. Among the various types of stem cells, mesenchymal stromal cells (MSCs) have unique properties such as easy isolation from various sources and in vitro culture and thus, they have been introduced as a potent approach for cancer therapy [1,2]. Moreover, pancreas cancer is one of the most lethal malignancies, and is considered as an unsolved health problem. In more than 60% of pancreatic cancers, c-Src activity was shown to elevate. Therefore, c-Src inhibition is established as an ideal therapeutic target in pancreatic cancer [3,4]. c-Src is a non-receptor tyrosine kinase and a member of Src family kinases (SFKs) and plays regulatory roles in various cellular signaling pathways such as cell proliferation, growth, migration, shape, differentiation and survival [5].

Atypical kinase Sugen Kinase 269 (SgK269 or PEAK1) is a member of PEAK pseudo kinase family. It seems that N-terminal region and the proline, glutamate, serine, threonine (PEST) linker are responsible for localization of SgK269 to focal adhesion and cytoskeleton rearrangements and cell migration [6,7]. The roles of SgK269 in regulating invasion and cell motility have been reported [8,9]. In pancreatic cancer, SgK269 expression level and Src activity increase. SgK269 can regulate c-Src activity and SgK269 knockdown can suppress c-Src activity [8]. Therefore, it is considered that SgK269 is a key biomarker of pancreatic cancer progression.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase. The overexpression of epidermal growth factor receptor (EGFR) in pancreatic cancer was previously reported [10–12]. Thus, EGFR is an important target in pancreatic cancer therapy [13,14]. Interestingly, several studies reported MSCs therapeutic effects in pancreatic cancer treatment [15–17]. Furthermore, c-Src is a mediator in EGFR signaling

* Corresponding author.
E-mail address: fsafari@guilan.ac.ir (F. Safari).

https://doi.org/10.1016/j.biologicals.2022.02.001
Received 8 July 2021; Received in revised form 17 January 2022; Accepted 15 February 2022

cascade. Taken together, it appears that c-Src, EGFR, and SgK269 will be important targets in pancreatic cancer therapy.

The aim of the present study was to evaluate the therapeutic effects of human amniotic mesenchymal stromal cells based on tumor growth suppression and inhibition of invasion in Panc1 pancreatic cancer cells by analyzing SgK269, c-Src, EGFR, Csk, E-cadherin, Vimentin and Snail transcriptional factor expression using 2D and 3D cell culture models. By using quantitative real time PCR (qRT-PCR) and Western blot methods, we evaluated the expression of related target molecules in Panc1 pancreatic cancer cells. Moreover, the induction of cellular apoptosis in Panc1 cancer cells after treatment with stem cells was analyzed using DAPI staining. For confirmation, the expression of *Bax, Bcl2*, and Caspase 3 were evaluated. Our results support the idea that MSCs may be a novel and effective therapeutic approach to treat pancreatic cancer cells by targeting specific gene expression.

2. Materials and methods

2.1. Cell lines and culture condition

The hAMSCs were obtained from Iranian Biological Resource Center (IBRC, Cat No: C10893, male, newborn, fibroblast-like) and pancreatic cancer cell (Panc1) was provided from Pasture Institute (Tehran, Iran). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Bio idea BI201, Iran), 100 μg/ml penicillin G/streptomycin and 2% L-glutamine. The condition of cell culture was according to our previous report [17].

2.2. Indirect co-culture condition

Panc1 pancreatic cancer cells (1.5×10^5) were first seeded on the lower surface of a 6-well plate. On the next day, MSCs were seeded at the same density (Panc1: MSCs of 1:1) on the upper surface of a polycarbonate transmembrane filter in a Transwell filter system (pore size 0.4 μm; BD Falcon, Bedford, MA, USA). DMEM supplemented with 10% FBS, 100 μg/ml penicillin G/streptomycin and 1% L-glutamine was used as a basal culture medium. In addition, Panc1 and hAMSCs were co-cultured for 72 h, along with culturing Panc1 cells alone as control. After 72 h, Panc1 cells (control and co-cultured) were applied for MTT assay, qRT-PCR and Western blot experiments [17].

2.3. MTT assay

The effect of MSC-CM on the viability of Panc1 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (MTT assay kit, Bio IDEA, CatNo:BI1017, Iran), as previously explained [17].

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

In order to perform quantitative real-time RT-PCR analysis, Panc1 cells were lysed after 72 h of co-culturing with hAMSCs. In addition, total RNA was extracted using 500 μL Trizol® reagent based on the manufacturer-provided protocol (Invitrogen Life Technologies, Carlsbad, CA, USA), followed by reverse transcription into cDNA based on the manufacture's protocol (ReveretAid M-Mulv reverse transcriptase kit, Thermo Fisher Scientific, MA, USA). Furthermore, real-time RT-PCR was implemented to amplify cDNA using SYBR Green dye universal master mix (Bioron GmbH, Germany) in a LightCycler 480 (Roche) using the primers for GAPDH: (F) 5′-CAA GGT CAT CCA TGA CAA CTTTG-3′, (R) 5′-GTCCACCACCCTGTTGCTGTAG-3′; Bax: (F) 5′-GTCGCCCTTTTCT ACTTTGCC -3′, (R) 5′-CTCCCGCCACAAAGATGGTCA-3′, and Bcl-2: (F) 5′-CCCCTCGTCCAAGAATGCAA-3′, (R) 5′- TCTCCCGGTTATCGTACC CTG-3′ [17], as well as SGK269: (F) 5′- CAACATACAGCAAC TTAGGGC -3′, (R) 5′-GCTCTGTATTGGCTCTTATGATC-3′, and CSK (F) 5′-CACTACACCTCAGACGCAGA-3′, (R) 5′- GCGACTTTGTTCCCTCGG TA-3′ for forty cycles. The data represented the average copy number normalized to the *GAPDH* housekeeping gene. Furthermore, the primers were synthesized by Pishgam Biotech Co. (Tehran, Iran). The negative control reaction was set similar to the above-mentioned one using deionized water instead of cDNA. The thermal conditions of the PCR were primary denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s for 45 cycles, annealing at 59 °C (SGK269) and 58 °C (CSK) for 30 s, and amplification at 72 °C for 30 s. The primers used for EGFR were: (F) 5′-GTTGGGCACTTTTGAAGATC-3′, (R) 5′- CAATGAGGACATAACCAG CC-3′, and c-Src: (F) 5′-AGGAACCAACAATTCGTCGGA-3′, (R) 5′-GCACCTTTTACCCTGATTCCC-3′ under the conditions of 95 °C for 90 s, followed by 45 cycles at 95 °C for 30 s, 62 °C for 30 s (EGFR) and 60 °C for 30 s (c-Src), and 72 °C for 30 s [17]. Primers used for E-cadherin were: (F) 5′-GCCGAGAGCTACACGTTCAC-3′, (R) 5′- CAGGCGTAGA CCAAGAAATG-3′ and for Vimentin-F: 5′-CTACGTCCACCCGCACC TAC-3′; R: 5′- CCAGCGAGAAGTCCACCGAG-3′ with the following conditions: 95 °C for 90 s; followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s (E-cadherin) and 57 °C for 30 s (Vimentin) and 72 °C for 30 s. All of the reactions were repeated three times.

2.5. Antibodies, SDS-PAGE and Western blot

The primary antibodies for immunoblotting included anti-B-actin (C4: sc-47778), anti-EGFR (A10), anti-Caspase 3 (sc-7272), anti-Csk (E−3: sc-166,560), anti-SgK269 (EB-8: sc-100403), Anti-E-cadherin 67A4 (Santa Cruz Biotechnology), Anti-Snail G7 (sc-271,977) which were obtained from Santa Cruz Biotechnology. Anti-Vimentin V9 and anti-*c*-Src polyclonal antibody were purchased from Invitrogen and Elabscience. hAMSCs and Panc1 cancer cells were co-cultured to conduct Western blot. As already mentioned, Panc1 cancer cells were harvested and lysed in lysis buffer (50 mM Tris -HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM Na3VO4, 1 mM PMSF) after 24 h and 72 h. Additionally, total cell lysates (TCLs) were subjected to SDS-PAGE. Furthermore, the proteins transferred to PVDF membrane filters (Millipore) were soaked in the solutions containing primary (1:300, 90 min at room temperature) and secondary antibody (1:1000, 45 min at room temperature). Furthermore, bands were visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences). Finally, a luminescence image analyzer was applied to quantify the intensities of chemiluminescence on the immunoblotted filters (LAS-4000, Fuji Film) [17].

2.6. DAPI staining assay

DAPI (4,6-Diamidino-2-phenylindole dihydrochloride) staining assay was used to determine the changes in the chromatin of Panc1 cells in a co-culture system with hAMSCs cells for 24 h. DAPI was purchased from Sigma-Aldrich (USA). Briefly, Panc1 cells were seeded in the 6-well plates (5×10^4 cells per well) containing 12 mm cover slips, and consequently treated with hAMSCs in the sample (co-culture system) and control (absence of hAMSCs) for 24 h. Then, the cells were fixed with 3.7% paraformaldehyde, permeabilized in 0.5% (w/v) Triton X-100 and 1% BSA (w/v) for 5 min, washed in PBS, and stained through DAPI. All images were taken by an inverted fluorescent microscope (Nikon Eclipse Ti-E) [17].

2.7. Hanging drop formation

The hanging drop method was performed to create a 3D cell culture model and spheroid formation. In this regard, Panc1 cells were cultured when were about 90% confluence, trypsinized and counted. In addition, ten 20-μL drops including 20×10^3 cells were pipetted into the lid of tissue culture dish (60 mm) and 5 ml of PBS was placed at its bottom [18]. The experiment was conducted on the control (cells + medium) and sample (cells + stem cell-conditioned medium). In the control

experiment, the cell culture medium containing 10% FBS, 100 μg/ml pen/strep, and 1% L-glutamine were used. However, a stem cell culture medium similar to the control one, which was previously used for culturing stem cells and reused for the sample in 3D cell culture system, was applied on samples. The media were changed every two days. Furthermore, the size and number of spheroids were determined after their formation (around three days). Briefly, spheroid formation was monitored using a phase contrast inverted microscope (INV100, BEL Engineering, Italy). Finally, spheroid number was calculated on days three and eight post-seeding [17].

2.8. Wound-healing migration assay

The cells were seeded in a culture medium onto 6-well plates at a density of 4×10^5 cells per well. The confluent monolayer of cells was scratched with a fine pipette tip, and cell migration into the wound was visualized and scored by measuring the size of the initial wound and comparing it to the size of the wound after 48 h by microscopy [19].

2.9. Statistical analysis

The data were analyzed using SPSS 22 (Chicago, IL, USA) and graphs were drawn using Graph Pad Prism 7 software. Additionally, the data were expressed as means ± standard deviation (SD). Furthermore, the experiments were performed three times, and the groups were compared using independent sample *t*-test. Finally, P value less than 0.05 was considered as statistically significant [17].

3. Results

3.1. Downregulation of EGFR, c-Src, SgK269 and csk expression in Panc1 cancer cells after treatment with hAMSCs

It was previously demonstrated that c-Src activity and expression are elevated in pancreatic tumorigenesis [3,4,20,21]. In EGFR phosphorylation signaling pathway, c-Src is also a key mediator and it was revealed that the overexpression of c-Src and EGFR is associated with tumor formation *in vivo* [22]. Therefore, combination or individual inhibition of c-Src and EGFR are critical targets in cancer therapy. Furthermore, SgK269 is a positive regulator of c-Src [8]. So, we were interested in the evaluation of the therapeutic effect of hAMSCs on EGFR and c-Src expression in Panc1 cancer cells. To do so, Panc1 cells were co-cultured with hAMSCs cells using Transwell filter system (Fig. 1A). After 72 h, the viability of Panc1 cancer cells was first analyzed by MTT assay (Fig. 1B). Then, Panc1 cancer cells under the treatment with hAMSCs were harvested to analyze EGFR, c-Src, SgK269 and Csk expression at both gene and protein levels (Fig. 1C–H). Csk is a critical kinase to inhibit c-Src activity via Tyr530 phosphorylation [23,24] and thus, Csk expression was assessed in this experiment. Our results showed that EGFR, c-Src, Csk and Sgk26 expression was reduced in Panc1 cells under the treatment with hAMSCs.

3.2. Suppression of EMT in Panc1 cancer cells under treatment with hAMSCs by upregulation of E-cadherin and downregulation of Vimentin and Snail transcriptional factor

During EMT, loss of cell-cell adhesion occurred in epithelial cells and the cells acquired mesenchymal cell features and became more invasive and motile. At molecular levels, it was well known that the epithelial markers such as E-cadherin expression were reduced and mesenchymal markers such as Vimentin and Snail expression were elevated (Fig. 2A) [25–27]. To find the possible effect of hAMSCs on cancer cell migration, wound healing assay was first performed. In this regard, hAMSCs and Panc1 cells were co-cultured for 24 h and then, with a pipette tip, Panc1 cells were scratched and then, they were again incubated for 24 h. Migration of Panc1 cells into the wound was assessed at hour zero and after 24 h of treatment with hAMSCs (Fig. 2B). RNAs from Panc1 cells were then extracted and cDNAs were synthesized. Finally, we performed qRT-PCR to evaluate the expression of E-cadherin and Vimentin (Fig. 2C and D). Moreover, Panc1 cells under treatment of hAMSCs were lysed and total lysate of cells was applied to Western blot (Fig. 2E). Our results showed that EMT was suppressed in Panc1 cancer cells by hAMSCs and

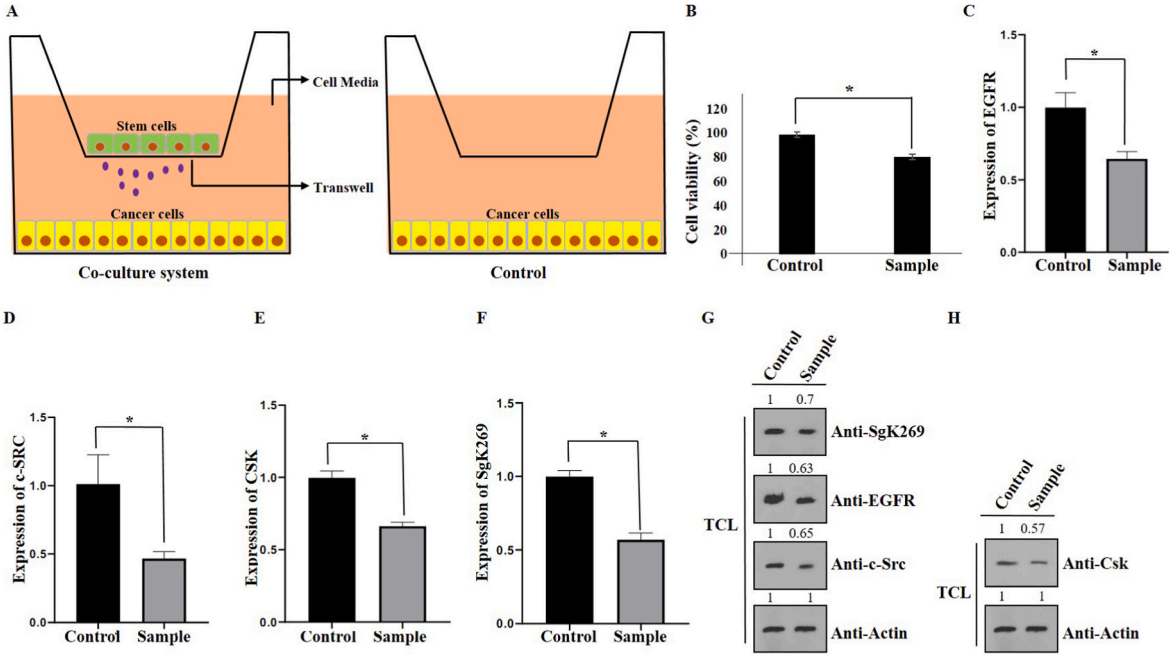

Fig. 1. Schematic model of co-culture system used in this study (A). Inhibitory effect of hAMSCs on Panc1 cancer cells using MTT assay. Data represent mean ± SD of three independent experiments. *p < 0.05 was considered to be statistically significant. (B). Relative expression of EGFR, c-Src, Csk and SgK269 of Panc1 cells after 72 h were shown. Data represent mean ± SD of three independent experiments. *p < 0.05 was considered to be statistically significant (C–F). The expression of EGFR, c-Src, SgK269 and Csk proteins by using Western blot was also shown (G and H). Actin used as an internal control (TCL: total cell lysate).

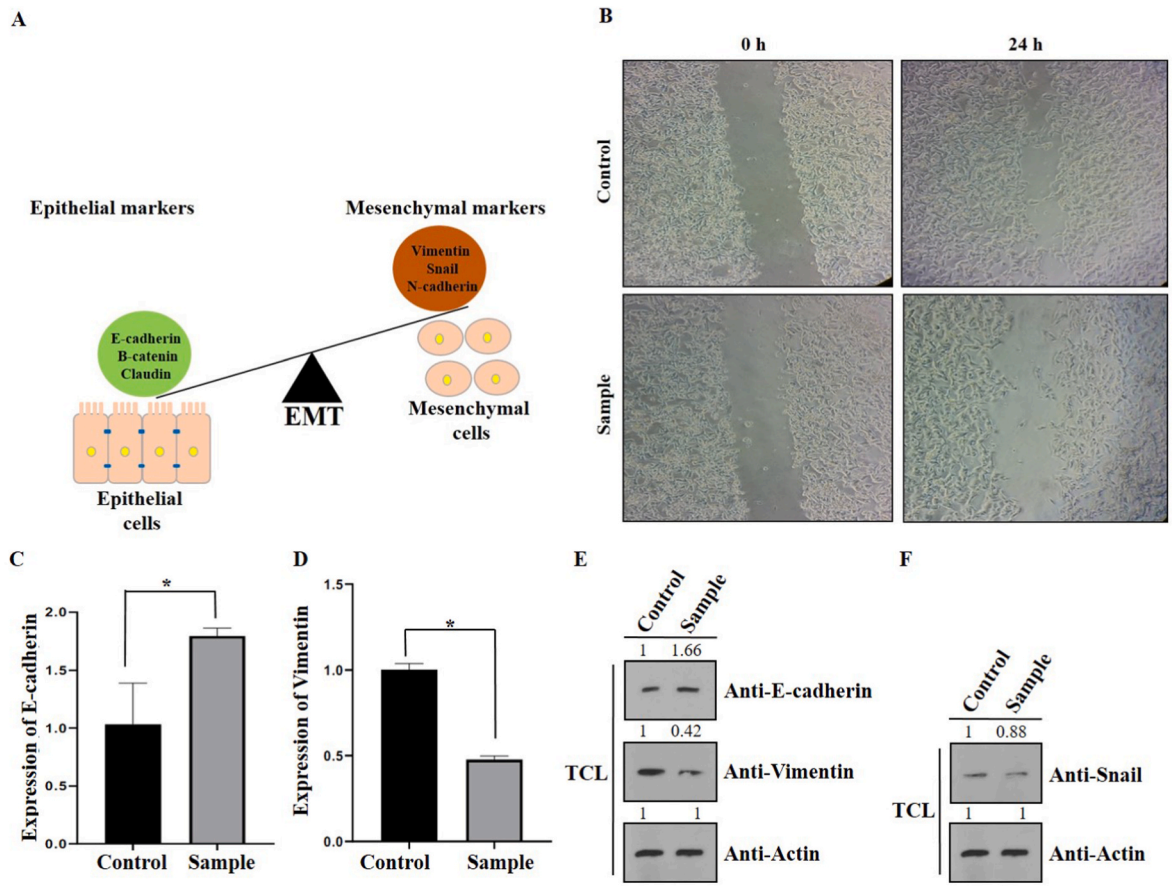

Fig. 2. Schematic model of epithelial-mesenchymal transition (EMT) (A). Effects of hAMSCs on the migration of Panc1 cells in different time (0 and 24 h after treatment). Images were obtained using phase-contrast microscopy. Scale bars represent 50 μm (B). Relative expression of E-cadherin mRNA and relative expression of Vimentin mRNA of Panc1 cells after 72 h treatment with hAMSCs were shown (C, D). Data represent mean ± SD of three independent experiments. *$p < 0.05$ was considered to be statistically significant. The expression of E-cadherin and Vimentin proteins was shown (E). The expression of Snail transcriptional factors was also detected (F). Actin used as an internal control (TCL: total cell lysate).

thereby, the expression of E-cadherin was reduced and Vimentin expression was upregulated at both gene and protein levels. We also found that the transcriptional factors such as Snail downregulated (Fig. 2F). These results indicated that hAMSCs may be a potential therapeutic approach for pancreatic cancer therapy via EMT inhibition.

3.3. Induction of cellular apoptosis in hAMSCs-treated Panc1 cancer cells

Apoptosis (or programmed cell death) is considered to play a crucial role in maintaining homeostasis in our body. To evaluate the induction of apoptosis in Panc1 cells after treatment with hAMSCs cells, both cell lines were cultured in a co-culture system. After 72 h, Panc1 cells were stained with DAPI, and we could detect condensed chromatin and DNA fragmentation of hAMSCs-treated Panc1 cells (Fig. 3A). Furthermore, Panc1 cells were applied to extract RNA for qRT-PCR. Our results demonstrated the induction of *Bax* expression and the reduction of *Bcl2* levels (Fig. 2B and C). By using Western blot, Caspase 3 protein expression was also evaluated (Fig. 2D). Based on the results, the cellular apoptosis was induced in hAMSCs-treated Panc1 cells.

3.4. Stem cell-conditioned medium has inhibitory effects on Panc1 cancer cells growth by a 3D cell culture system

In a 3D cell culture system, the cells are able to grow in all directions and it is more reflective of *in vivo* cell behaviors. In the present study, we performed the hanging drop technique, which is a simple method to form spheroids [18]. As we already mentioned, spheroids appeared after around three days (Fig. 4A) [17]. Then, we analyzed the size and number of spheroids, which represents the lower size and number in the sample (cells + stem cells-conditioned medium) compared to the control (cells + medium) (Fig. 4B and C). Moreover, the relative expression of *c-Src*, *SgK269, Vimentin,* and *E-cadherin* were analyzed (Fig. 4D–G). The results of this part indicated the therapeutic effects of hAMSCs condition medium on Panc1 cancer cells growth in a 3D cell culture system.

4. Discussion

Pancreatic cancer is one of the leading causes of death from cancer in human worldwide. The detection of this disease in early stages is difficult and the current therapy platforms are not effective. In the present study, we are interested in exploring the therapeutic effects of hAMSCs secretome on several potential targets molecules in pancreatic cancer proliferation such as EGFR, Src, CSK, SgK269 and pancreatic cancer invasion such as E-cadherin, Vimentin and transcriptional factor Snail (as EMT markers). Based on the results, we found that hAMSCs secretome is a promising approach in pancreatic cancer therapy. Interestingly, we previously showed that EGFR, Src, CSK, and the other member of PEAK family pseudokinase, SgK223 (or Pragmin) may be potential targets in MiaPaca2 pancreatic cancer therapy via hAMSCs condition medium [17]. Due to key role of SgK269 during growth factor receptor signaling and promotion of invasion [28], we have evaluated the anti-tumor activity of hAMSCs secretome on Panc1 pancreatic cancer cells through SgK269 expression in the present study. We found that SgK269 is a critical target in pancreatic cancer cells therapy using hAMSCs

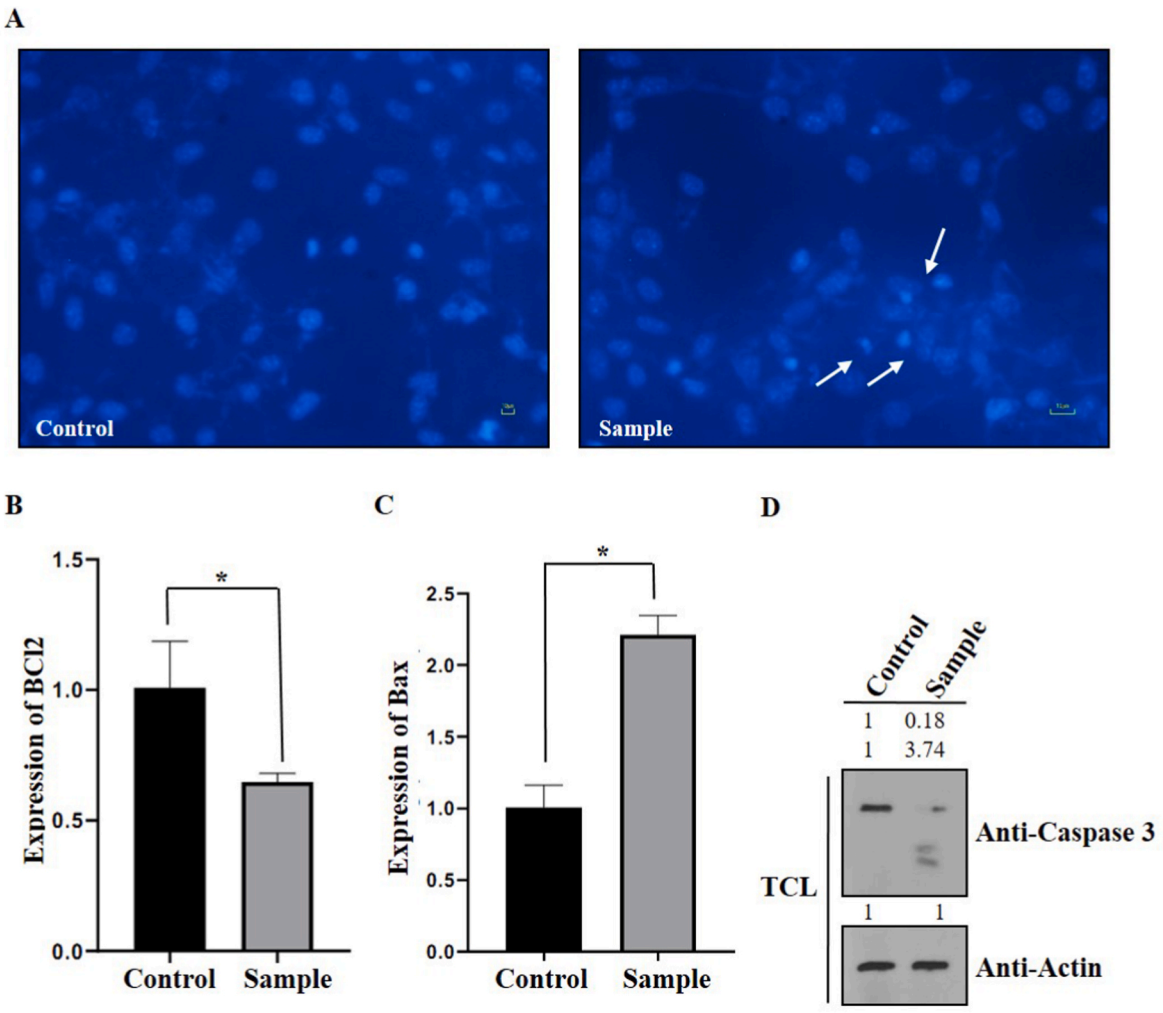

Fig. 3. DAPI staining of Panc1 cells under treatment with hAMSCs cells (Sample) compared with control (A). The experiments were performed three times (original microscope magnification, 40X, Scale bar, 10 μm). Relative expression of Bax mRNA and relative expression of Bcl2 mRNA of Panc1 cells were shown. Data represent mean ± SD of three independent experiments. *$p < 0.05$ was considered to be statistically significant (B, C). The expression of Caspase 3 proteins (pro caspase 3: upper band; cleaved caspase 3: two lower bands) by using Western blot was also shown (D). Actin used as an internal control (TCL: total cell lysate).

secretome. The oncogenic roles of SgK269 in other cancer types were previously found. In this regard, Croucher et al. showed that SgK269 was overexpressed in breast cancer and SgK269 promoted EMT. They found that phosphorylation of Y635 by Lyn tyrosine kinase was required for SgK269 to promote cell invasion through Erk and Stat 3 activation [29]. Notably, the involvement of SgK269/TGF-β signaling in EMT breast cancer was also shown [30,31]. In another study, Ding et al. demonstrated that in lung cancer, the expression of SgK269 is increased and it is mediated in lung cancer metastasis and EMT [32]. However, it was reported that the loss of SgK269 expression is associated with tumor growth and invasion in gastric cancer [33] and downregulation of SgK269 was reported in colorectal cancer, while overexpression of SgK269 resulted in the inhibition of tumor growth and metastasis [34]. Taken together, it seems that SgK269 can act as either tumor suppressor or tumor oncogenic depending on cancer type and cell context. It was also shown that SgK269 operates with the Src/p130Cas/Crk/paxillin and Erk cytoskeletal signaling pathways to regulate cell motility and tumor promotion [6]. In this regard, more experiments are required to evaluate of the effects of hAMSCs secretome on cytoskeleton regulatory proteins.

Considering the involvement of EGFR and c-Src in various cell signaling pathways and the high activity of EGFR and c-Src kinase in pancreatic cancer [3,12], EGFR and c-Src are the potential targets in the treatment of many cancers including pancreatic cancer. Similar to our previous study, EGFR and c-Src expression was suppressed in hAMSCs-treated Panc1 cells. However, the molecular mechanisms of downregulation of EGFR and c-Src in hAMSCs-treated Panc1 cells are unknown.

Furthermore, we found that EMT was suppressed in Panc1 cancer cells through upregulation of E-cadherin and downregulation of Vimentin and Snail transcriptional factor after treatment with hAMSCs. It is worth noting that our recent study showed the inhibition effects of hAMSCs secretome on invasion of LNCaP prostate cancer cells [19]. It was well established that Snail transcriptional factor suppressed E-cadherin expression by binding to E-boxes in the *CDH1* promoter [35]. Notably, it was revealed that growth factors including EGF and TGF-β are able to induce EMT [36]. Based on the results, EGFR is also downregulated in Panc1 cancer cells by hAMSCs secretome. Therefore, it supports the idea that hAMSCs secretome inhibits EMT not only by downregulation of Snail transcriptional factor but also by EGFR downregulation. The role of TGF-β is unknown in our study and thus, finding the roles of TGF-β in hAMSCs-treated Panc1 cells is suggested.

Our results suggested that hAMSCs can be considered as a novel and safe therapeutic platform particularly for the inhibition of tumor migration in pancreatic cancer cells. EGFR, c-Src, SgK269, E-cadherin, Vimentin and Snail transcriptional factor are key targets in Panc1 pancreatic cancer cells, which were downregulated by hAMSCs secretome. There is a great need to clarify the possible mechanisms involved

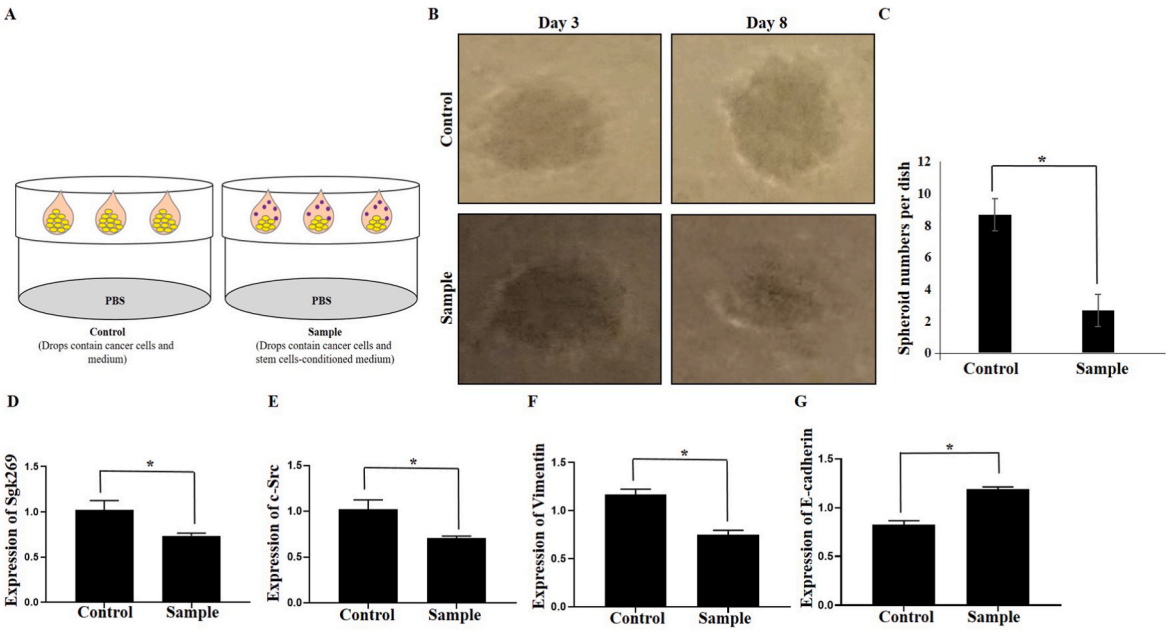

Fig. 4. Schematic model of spheroids formation by using hanging drop technique (A). Stem cell conditioned medium inhibits size and number of spheroids (Magnification: 20x, Scale bar: 100 μm; Three independent experiments were done. *p < 0.05 was considered to be statistically significant) (B, C). Relative expression of *SgK269, c-Src, Vimentin,* and *E-cadherin* mRNA of Panc1 cells after 72 h were shown. Data represent mean ± SD of three independent experiments. *p < 0.05 was considered to be statistically significant (D–G).

in this process to find the potential targets and specific therapy designs.

Author contribution

F.S. designed the research. F.S. and M.A.S.L. performed the experiments and analyzed data. F.S. and M.A.S.L. wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare no competing financial interests.

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