

Video Article

Mesenchymal Stem Cell Isolation from Pulp Tissue and Co-Culture with Cancer Cells to Study Their Interactions

Aysegül Doğan¹, Selami Demirci², Hüseyin Apdik¹, Ezgi Avşar Apdik¹, Fikretin Şahin¹

¹Genetics and Bioengineering, Yeditepe University

²National Heart, Lung, and Blood Institute (NHLBI), Sickle Cell Branch, National Institutes of Health (NIH)

Correspondence to: Selami Demirci at selami.demirci@nih.gov

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Abstract

Cancer as a multistep process and complicated disease is not only regulated by individual cell proliferation and growth but also controlled by tumor environment and cell-cell interactions. Identification of cancer and stem cell interactions, including changes in extracellular environment, physical interactions, and secreted factors, might enable the discovery of new therapy options. We combine known co-culture techniques to create a model system for mesenchymal stem cells (MSCs) and cancer cell interactions. In the current study, dental pulp stem cells (DPSCs) and PC-3 prostate cancer cell interactions were examined by direct and indirect co-culture techniques. Condition medium (CM) obtained from DPSCs and 0.4 µm pore sized trans-well membranes were used to study paracrine activity. Co-culture of different cell types together was performed to study direct cell-cell interaction. The results revealed that CM increased cell proliferation and decreased apoptosis in prostate cancer cell cultures. Both CM and trans-well system increased cell migration capacity of PC-3 cells. Cells stained with different membrane dyes were seeded into the same culture vessels, and DPSCs participated in a self-organized structure with PC-3 cells under this direct co-culture condition. Overall, the results indicated that co-culture techniques could be useful for cancer and MSC interactions as a model system.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58825/>

Introduction

Mesenchymal stem cells (MSCs), with the ability of differentiation and contribution to regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and adipose, have been isolated from almost all tissues in the adult body^{1,2}. Other than providing tissue homeostasis by producing resident cells in case of chronic inflammation or an injury, they produce vital cytokines and growth factors to orchestrate angiogenesis, immune system, and tissue remodeling³. The interaction of MSCs with cancer tissue is not well-understood, but accumulating evidence suggests that MSCs might promote tumor initiation, progression, and metastasis⁴.

The homing ability of MSCs to the injured or chronically inflamed area makes them a valuable candidate for stem cell-based therapies. However, cancer tissues, "never healing wounds", also release inflammatory cytokines, pro-angiogenic molecules, and vital growth factors, which attract MSCs to the cancerous area⁵. While there are limited reports showing inhibitory effects of MSCs on cancer growth^{6,7}, their cancer progression and metastasis promoting effects have been extensively reported⁸. MSCs directly or indirectly affect carcinogenesis in different ways including suppressing immune cells, secreting growth factors/cytokines that support cancer cell proliferation and migration, enhancing angiogenic activity, and regulating epithelial-mesenchymal transition (EMT)^{9,10}. Tumor environment consists of several cell types including cancer-associated fibroblasts (CAFs) and/or myofibroblasts, endothelial cells, adipocytes, and immune cells¹¹. Of those, CAFs are the most abundant cell type in the tumor area that secrete various chemokines promoting cancer growth and metastasis⁸. It has been shown that bone marrow-derived MSCs can differentiate into CAFs in the tumor stroma¹².

Dental pulp stem cells (DPSCs), characterized as the first dental tissue-derived MSCs by Gronthos *et al.*¹³ in 2000 and then widely investigated by others^{14,15}, express pluripotency markers such as *Oct4*, *Sox2*, and *Nanog*¹⁶ and can differentiate into various cell lineages¹⁷. Gene and protein expression analysis proved that DPSCs produce comparable levels of growth factors/cytokines with other MSCs such as vascular endothelial growth factor (VEGF), angiogenin, fibroblast growth factor 2 (FGF2), interleukin-4 (IL-4), IL-6, IL-10, and stem cell factor (SCF), as well as *frms-like tyrosine kinase-3 ligand* (Flt-3L) that might promote angiogenesis, modulate immune cells, and support cancer cell proliferation and migration^{18,19,20}. While the interactions of MSCs with cancer environment have been well-documented in the literature, the relationship between DPSCs and cancer cells has not been evaluated yet. In the present study, we established co-culture and condition medium treatment strategies for a highly metastatic prostate cancer cell line, PC-3, and DPSCs to propose potential action of mechanism of dental MSCs in cancer progression and metastasis.

Protocol

Written informed consent of the patients was obtained after the approval from the Institutional Ethics Committee.

1. DPSC Isolation and Culture

1. Transfer wisdom teeth obtained from young adults aged between 17 and 20 to 15 mL tubes containing complete Dulbecco's Modified Eagle Medium (DMEM) [low glucose DMEM media, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin (PSA) solution], within 8 h after resection. Keep the tissue material cold (4 °C) during transfer to avoid potential cell death.
2. Remove the pulp tissue by sterile extraction forceps from the center of the tooth carefully, place the pulp tissue in the cold complete DMEM medium in 10 cm tissue culture dishes, and mince them into small pieces (2-3 mm) by scalpel.
NOTE: All experimental procedures should be carried out under sterile conditions in laminar flow hood. This non-enzymatic technique has been previously used^{21,22,23}.
3. Place small pulp tissues inside the tissue culture treated 6-well plates and add 200 μ L of complete DMEM media to cover each small pulp tissue pieces.
4. Incubate the tissue culture wells at 37 °C in a humidified air atmosphere (80% humidity) with 5% CO₂ for 2 h to provide tissue attachment.
NOTE: This step could be prolonged to 3-4 h by controlling the evaporation of media.
5. Add appropriate volume (2-2.5 mL) of complete DMEM medium to the wells and incubate at 37 °C in a humidified air atmosphere with 5% CO₂ for cells to spread from the tissue.
NOTE: Cells become visible after approximately 4 days and reach confluency after 8-9 days.
6. When cells reach 80% confluency, remove media from 6-well plates, wash with 2 mL of phosphate buffered saline (PBS), and add 2 mL of trypsin. Incubate for 2 min in an incubator at 37 °C with humidified air atmosphere and 5% CO₂. Then add 2 mL of complete DMEM medium followed by 2 min incubation to inhibit trypsin. Centrifuge cells at 300 x g for 5 min to pellet cells.
7. Passage cells to the flasks in complete DMEM media and store for further experiments. Add 15 mL of complete DMEM media to the T-75 flasks and transfer cells from two wells of the 6-well plate to one T-75 flasks and incubate at 37 °C with humidified air atmosphere and 5% CO₂.

2. Characterization of DPSCs

1. Perform morphological analyses.
 1. Seed cells (step 1.6) in tissue culture coated flasks (or 6-well plates) in complete DMEM medium for at least 8 passages to observe cell morphology.
 2. Visualize cells by light microscope and define fibroblast-like cell morphology. Cells should attach to the culture dishes and have spindle-like cell morphology.
NOTE: Alternatively, cells can be cultured as single cells for up to 14 days in well-plates to observe colony formation capacity that is a specific characteristic of fibroblasts and MSCs.
2. Perform surface marker analyses.
 1. Trypsinize the cells from step 1.7. Remove media from the T-75 tissue culture flask, wash with 2 mL of PBS, and add 2 mL of trypsin. Incubate for 2 min in an incubator at 37 °C with humidified air atmosphere and 5% CO₂. Then add 2 mL of complete DMEM medium followed by 2 min incubation to inhibit trypsin. Centrifuge cells at 300 x g for 5 min to pellet cells.
 2. Fix the cells with 4% paraformaldehyde for 20 min at room temperature in 1.5 mL tubes and then wash them with 500 μ L of PBS 3 times to remove paraformaldehyde.
 3. Incubate fixed cells with the antibodies against CD29, CD34, CD14, CD45, CD90, CD105, CD166, and CD73 for 1 h at 4 °C in 100 μ L of PBS.
NOTE: The concentration of antibody used is 0.5 μ g/mL. CD34, CD14, and CD45 are used as negative markers, while CD29, CD90, CD105, CD166, and CD73 are used as positive cell surface markers for MSCs.
 4. Wash cells 3 times with PBS and use respective secondary antibodies such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), etc. for labelling. Incubate cells with 1:500 diluted secondary antibodies in 100 μ L of PBS for 30 min at 4 °C and wash 3 times with PBS.
 5. Keep samples in the dark for flow cytometry analysis and detect positive and negative staining by flow cytometry.
NOTE: Use unstained control cells to arrange forward and side scatter. Arrange gating of positively stained populations by excluding dead cells, debris, and un-stained population. Use 100 μ m nozzle with 45 psi sheath pressure and collect 10,000 events to determine positive DPSCs by arranging channels.
3. Perform differentiation of DPSCs.
 1. Seed 1×10^4 cells onto 24-well plates in complete DMEM media and incubate for 24 h at 37 °C in a humidified air atmosphere with 5% CO₂.
 2. Formulate differentiation media using complete DMEM medium as base media. Prepare osteogenic media by mixing 100 nM dexamethasone, 10 mM β -glycerophosphate, and 0.2 mM ascorbic acid. Prepare chondrogenic media by mixing $1 \times$ insulin-transferrin-selenium (ITS-G), 100 nM dexamethasone, 100 ng/mL transforming growth factor beta (TGF- β), 14 μ g/mL ascorbic acid, and 1 mg/mL bovine serum albumin (BSA). Prepare adipogenic media by mixing 100 nM dexamethasone, 5 μ g/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 60 μ M indomethacin.
NOTE: Differentiation media can be kept at 4 °C for at least one week.
 3. Change growth media to osteo-, chondro-, or adipo-genic differentiation media and refresh media twice a week for two weeks.
 4. Confirm differentiation by von Kossa and Alcian blue staining, enzyme activity (alkaline phosphatase activity), immunocytochemistry, and quantitative polymerase chain reaction (qPCR) analyses according to the protocols previously described²¹.

1. Perform von Kossa and Alcian blue stainings on cells that are fixed with 4% paraformaldehyde at room temperature for 10 min. Wash the fixed cells with PBS and stain them with the vonKossa kit according to the manufacturer's recommendations to observe calcium deposits.
2. Prepare the Alcian blue staining solution by dissolving 1.00 g of Alcian blue dye in 100 mL of 3% (v/v) acetic acid for further staining. Wash the fixed cells with PBS and stain cells for 30 min with Alcian blue solution. Visualize the stained samples by a light microscope.

3. Preparation of Condition Medium (CM)

1. Replace media of cells from step 1.7 with fresh complete DMEM 24 h before CM collection.
NOTE: Passage 2-4 is recommended.
2. Collect condition medium (CM) from cultured DPSCs when cells reach 80% confluency. Centrifuge collected media at 300 x g for 5 min to remove waste tissue material and cell debris.
NOTE: Alternatively, use 0.2 µm syringe filters to remove debris from the condition medium.
3. Collect supernatant and store at -20 °C for further experiments.
NOTE: Keep the supernatant at -80 °C for long-term storage.

4. Treatment of Cancer Cells with CM

1. Perform cell viability analyses.
 1. Seed PC-3 cells (human prostate cancer cells) onto 96-well plates at a cell density of 5×10^3 cells/well in complete DMEM and incubate in a humidified chamber at 37 °C and 5% CO₂ for 24 h.
 2. Treat cells with 10, 20, 30, 40, and 50% of CM (v/v) mixed with complete DMEM for 24 h.
 3. Measure cell viability by using 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay as described previously²⁴.
2. Perform terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analyses.
 1. Seed PC-3 cells onto 6-well cell culture plates at a cell density of 2×10^5 cells/well and incubate in a humidified chamber at 37 °C and 5% CO₂ overnight.
 2. Mix 20% CM (v/v) with complete DMEM medium and apply to the cells for 24 h.
 3. Trypsinize cells and suspend in 50 µL of TUNEL reaction mixture (labeling solution + enzyme solution, supplied with the kit) and incubate at 37 °C for 60 min in a humidified and 5% CO₂ atmosphere.
NOTE: For trypsinization, remove media from 6-well cell culture plates, wash with 1 mL of PBS and add 500 µL of trypsin. Incubate for 2 min in an incubator at 37 °C with humidified air atmosphere and 5% CO₂. Add 1 mL of complete DMEM medium followed by 2 min incubation to inhibit trypsin. Centrifuge cells at 300 x g for 5 min to pellet cells.
 4. Rinse with PBS and analyze cells in PBS by using flow cytometry.
3. Perform qPCR analyses.
 1. Seed PC-3 cells onto 6-well cell culture plates at a cell density of 2×10^5 cells/well and incubate in a humidified incubator at 37 °C and 5% CO₂ overnight.
 2. Mix 20% CM (v/v) with complete DMEM medium and apply to the cells for 24 h.
 3. Trypsinize cells and collect cell pellet for RNA isolation and cDNA synthesis.
NOTE: Remove media from 6-well cell culture plates, wash with 1 mL of PBS, and add 500 µL of trypsin. Incubate 2 min in an incubator at 37 °C with humidified air atmosphere and 5% CO₂. Add 1 mL of complete DMEM medium followed by 2 min incubation to inhibit trypsin. Centrifuge cells at 300 x g for 5 min to pellet cells.
 4. Perform qPCR experiments according to the previously described protocol²⁵.
4. Perform cell migration of cancer cells.
 1. Seed 1×10^5 PC-3 cells onto 12-well plates and incubate in a humidified incubator overnight at 37 °C and 5% CO₂.
 2. Scratch cells with a sterile 200 µL tip and change medium immediately with fresh medium containing various concentrations of CM [e.g., 10, 20, 30, 40, and 50% of CM (v/v) mixed with complete DMEM].
 3. Observe scratches under an inverted microscope and take pictures at different time intervals (0 and 24 h).
 4. Measure the scratch closure by using Image J software using the formula:

$$\text{Scratch Closure (\%)} = \frac{\text{Scratch area (0 h)} - \text{Scratch area (24 h)}}{\text{Scratch area (0 h)}} \times 100$$

NOTE: Open the scratch image with Image J software. Draw a line that has the same magnitude as the scale bar that already exists in the image. Click analyze, set scale, and observe distance in pixels as the magnification of the drawn line. Write the size of the scale bar to the known distance part, arrange unit (pixels, cm, etc.), and click ok. Go to the analyze section again and click on measurements. This will first give the size of the scale bar as the selected unit. Click on one edge of scratch and drag until reaching the other end of the scratch. Note the value for each time point (0 h and 24 h). Plug these values into the formula above and calculate the scratch closure.

5. Cell Migration by Indirect Contact of Cancer Cells and DPSCs

1. Seed 3×10^4 DPSCs onto 24-well plate inserts with 0.4 µm pore and incubate in a humidified incubator overnight at 37 °C.

2. Seed PC-3 cells onto 24-well plates at a cell density of 5×10^4 and incubate in a humidified incubator overnight at 37 °C and 5% CO₂.
3. Scratch PC-3 cells with a sterile 200 µL tip, change medium with fresh medium, and place inserts carrying DPSCs onto PC-3 cells.
4. Observe cells under an inverted microscope and take pictures at different time intervals (0 and 24 h) to analyze cell migration.

6. Co-culture Assay and Flow Cytometry Analysis

1. Label PC-3 cells and DPSCs by using PKH67 (green) and PKH26 (red) fluorescent cell linker dyes, respectively²⁶.
2. Trypsinize PC-3 and DPSCs cells, respectively. Remove media from T-75 tissue culture flask, wash with 2 mL of PBS, and add 2 mL of trypsin. Incubate for 2 min in an incubator at 37 °C with humidified air atmosphere and 5% CO₂. Add 2 mL of complete DMEM medium followed by 2 min incubation to inhibit trypsin.
3. Centrifuge cells at 300 x g for 5 min, discard supernatant, and resuspend cell pellets in the dye solution prepared in diluent-C buffer supplied by the kit (see **Table of Materials**).
4. Incubate cells in the dye solution for 10 min and terminate the staining reaction by adding 100 µL of FBS. Centrifuge cells at 300 x g for 5 min, discard the supernatant, and wash the cells with complete growth medium before co-culturing.
5. Plate labeled cells (5×10^4 /well) onto 6-well plates at 1:1 ratio (DPSCs:PC3). Maintain co-cultured cells in complete DMEM.
6. Collect cells after 24 h or 48 h incubation periods by centrifugation of cells at 300 x g for 5 min and washing with PBS.
7. Resuspend cells in 300 µL of fluorescence-activated cell sorting (FACS) buffer in 5 mL round bottom flow cytometry tubes. Vortex to disperse cell aggregates right before the sample analysis.
8. Use a 100 µm nozzle with 45 psi sheath pressure.
NOTE: Extremely high flow rate might decrease the sensitivity of fluorescence detection.
9. Use unstained control cells and single colored cells to adjust appropriate forward and side scatter laser voltage for cell types and compensation as mentioned previously²⁷.
NOTE: Use gating for live cells to exclude cell debris, dead cells, or aggregates.
10. Collect 10,000 events (100,000 is preferable) to determine percent positive green DPSCs and red PC-3 cells by arranging FL-1 (green) and FL-2 (red) channels.

Representative Results

Figure 1 depicts the general MSC characteristics of DPSCs under culture conditions. DPSCs exert fibroblast-like cell morphology after plating (**Figure 1B**). MSC surface antigens (CD29, CD73, CD90, CD105, and CD166) are highly expressed while hematopoietic markers (CD34, CD45, and CD14) are negative (**Figure 1C**). Changes at the morphological and molecular level related to osteo-, chondro-, and adipo-genic differentiation are observed in DPSCs culture followed by differentiation cocktail application (**Figure 1D**).

To determine the activity of secreted molecules from DPSCs on prostate cancer cell proliferation and migration, we applied CM that is collected from cultured dental stem cells and analyzed cancer cell proliferation and migratory behavior. CM treatment (20% v/v) was selected based on MTS cell viability analyses. PC-3 cells treated with stem cell CM were subjected to the TUNEL assay and qPCR analyses to determine cell death and apoptotic regulation under control and experimental conditions. We concluded that CM treatment increased cell viability and reduced cell death in PC-3 cell culture. *Ex vivo* cell migration assay (scratch assay) was performed to evaluate whether CM of DPSCs affects PC-3 cancer cell migration. Treatment with the concentrations of 10% and 20% CM (v/v) increased scratch closure significantly in comparison to the control group at 24 h (**Figure 2**). Similarly, 20% CM (v/v) treatment induced upregulation of extracellular matrix protein gene expressions such as collagen I, fibronectin, and laminin, which play significant roles in cell migration.

We used two different culture techniques to establish direct and indirect co-cultures of PC-3 cells and DPSCs under *ex vivo* conditions. Trans-well system was selected to create an indirect interaction environment for PC-3 cancer cells. PC-3 cells were seeded onto the bottom of the well plate and inserts carrying DPSCs were placed on the top. Because we aimed to generate an in-direct interaction, trans-well membranes with 0.4 µm pore size were used to prevent physical cell movement of DPSCs from the upper part to bottom part through the membrane. Secreted molecules from DPSCs increased scratch closure of PC-3 cells significantly compared to control cells. PC-3 cells co-cultured with DPSCs demonstrated a 51% scratch closure while control cells had a 38% closure after 24 h (**Figure 3A**). Direct co-cultures containing 1:1 ratio of DPSCs and PC-3 cells were used to analyze self-organization of stem cells and cancer cells. Cells were stained with red and green membrane dyes to distinguish different cell types under microscope. PC-3 cells stained with red fluorescence dye were surrounded by DPSCs in a tube-like structure after a 24 h incubation period. Co-cultured cells stained with fluorescent dyes were analyzed by flow cytometry based on fluorescence staining, and cell ratios were detected. DPSCs and PC-3 cells created a well-organized structure in which PC-3 cells proliferated rapidly after 48 h (**Figure 3B**). Although cells were seeded at an equal ratio (1:1) for direct co-culture, 62.22% of PC-3 cells were determined after 48 h incubation, indicating the higher proliferation rate of PC-3 cells.

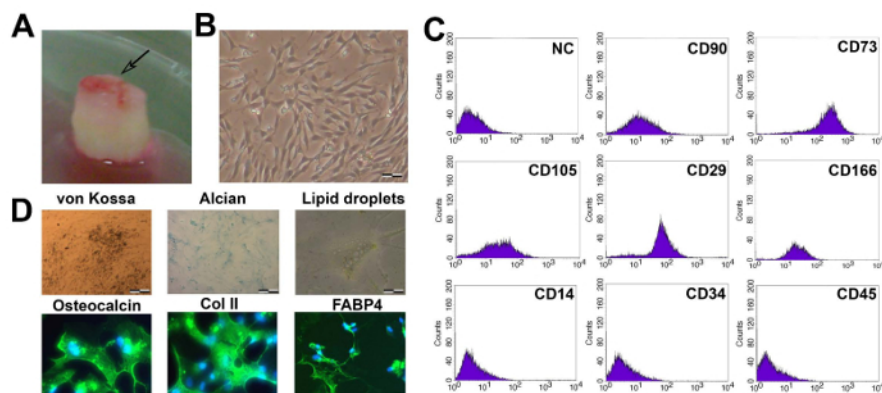


Figure 1: Characterization of dental pulp stem cells (DPSCs). (A) Pulp tissue obtained from the center of tooth. (B) Fibroblast-like cell morphology of DPSCs. Scale bar: 100 μ m. (C) Flow cytometry analyses of DPSCs. CD29, CD73, CD90, CD105, and CD 166 are positive surface markers, while CD14, CD34, and CD45 are negative surface markers. NC: negative control (growth medium treated cells). (D) Differentiation of DPSCs to mesenchymal cell types was confirmed with von Kossa staining, Alcian Blue staining, and lipid droplets (scale bar: 200 μ m). Osteocalcin, collagen type II (Col II), and fatty acid binding protein 4 (FABP4) immunostainings showed the osteo-, chondro-, and adipogenic differentiation of DPSCs. Scale bar: 200 μ m. This figure is adapted from Dogan *et al.*³⁴. [Please click here to view a larger version of this figure.](#)

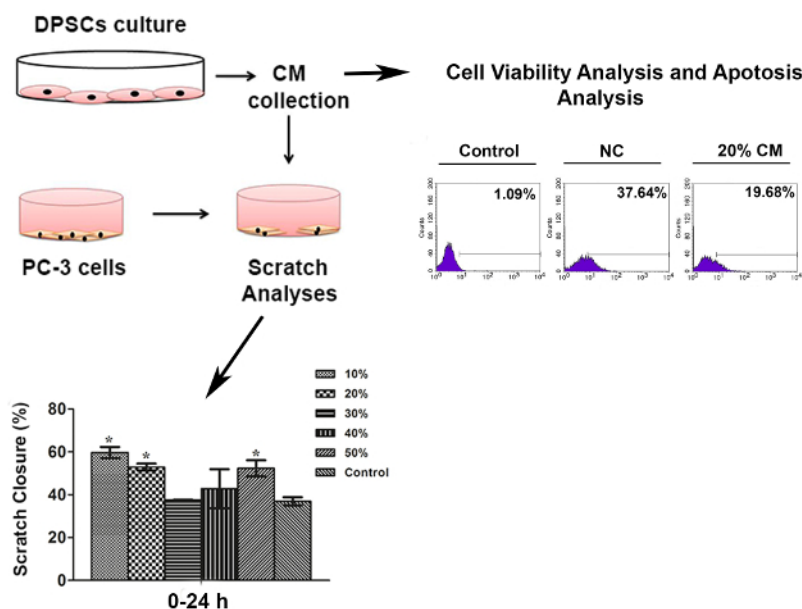


Figure 2: Collection of condition medium (CM) from DPSCs for cell viability and scratch analyses. TUNEL positive cells were decreased by 20% CM (v/v) application. Quantitative measurement of scratch closure showed that CM increased PC-3 cell migration. CM: conditioned medium; NC: negative control (growth medium treated cells). *P < 0.05. This figure is adapted from Dogan *et al.*³⁴. [Please click here to view a larger version of this figure.](#)

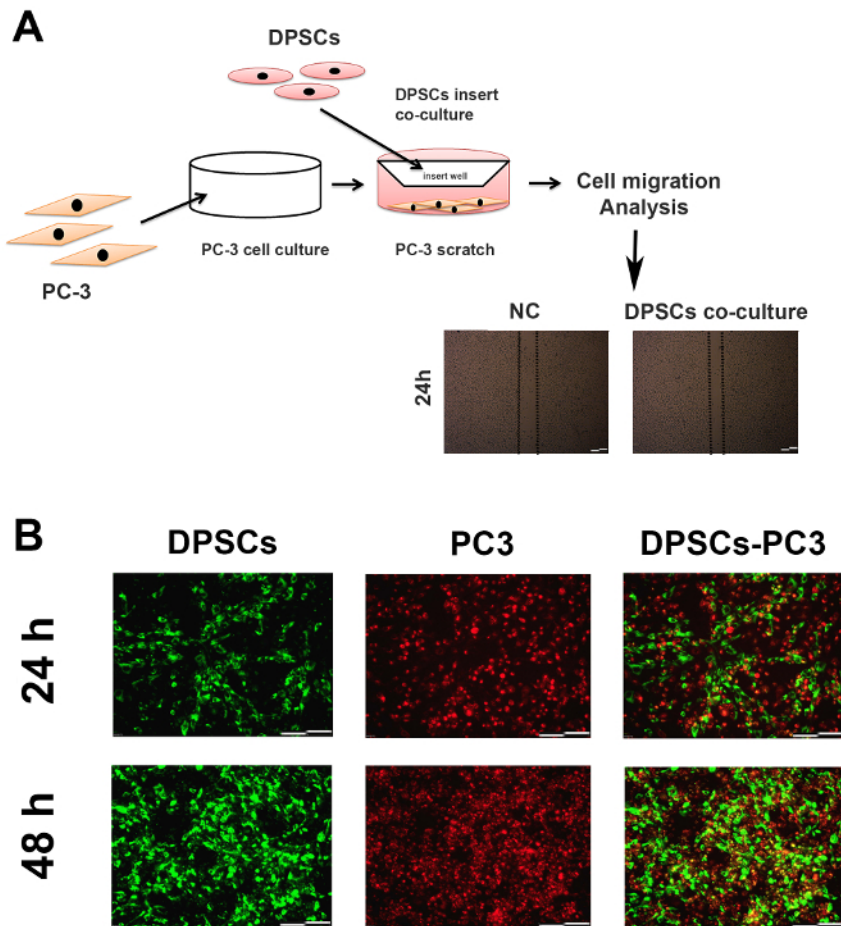


Figure 3: Methodology for trans-well cell migration assay and co-culture. (A) PC-3 cell scratch closure in the trans-well co-culture system. (B) Interaction pattern of stained DPSCs (green fluorescence) and PC-3 cells (red fluorescence) after 24 h and 48 h of co-culture (1:1 seeding ratio). Higher PC-3 cell number was detected with respect to DPSCs. Scale bar: 200 μ m. This figure is adapted from Dogan *et al.*³⁴. [Please click here to view a larger version of this figure.](#)

Discussion

Contribution of MSCs to tumor environment is regulated by several interactions including hybrid cell generation *via* cell fusions, entosis or cytokine and chemokine activities between stem cells and cancer cells²⁸. Structural organization, cell-cell interactions, and secreted factors determine cancer cell behavior in terms of tumor promotion, progression, and metastasis to surrounding tissue. Proper *ex vivo* model systems to investigate the mechanisms behind the interactions of resident cell populations are required to understand cellular communications for cancer progression and metastasis.

We used DPSCs to create a model system for prostate cancer and dental stem cell interactions. The advantage of this type of adult stem cell is the accessibility of tissue source and easy isolation steps. On the other hand, using only DPSCs without comparison with well-known adult stem cell types is a limitation of this protocol. Current co-culture methods are categorized into direct and indirect techniques which include paracrine signaling of soluble secreted molecules and direct culture of different cell populations in the same environment^{29,30,31}. CM of fully characterized DPSCs was used to evaluate paracrine signaling mediated cancer cell growth in culture. CM application is the simplest method for cell interaction studies and allows for observation of soluble mediator activities in the culture system. Although CM is not a fully adequate model system, CM application as a co-culture method is very efficient to observe cell-cell interactions *ex vivo*. CM of DPSCs increased the cell proliferation and migration of prostate cancer cells, indicating the acquisition of metastatic phenotype due to the factors secreted from DPSCs.

Another model of cell-cell interaction is the establishment of a physical barrier such as a trans-well membrane between cell populations³⁰. Trans-well systems are divided into two types: those which allow cell movement through the pores, and those enabling transfer of secreted factors while hindering cell contact through the membrane as well. We used trans-wells with 0.4 μ m pore size to analyze the closure of PC-3 scratches, revealing higher cancer cell migration rate in the DPSC group compared to the control cells.

Although CM and trans-well based systems are advantageous for simply analyzing contributions of a particular cell type³², direct culturing of different subpopulations in the same environment is necessary in parallel with indirect co-culture methods. Seeding ratio can be controlled and structural organization of distinct populations can be easily analyzed by direct co-culture of MSC with cancer cells. We used 1:1 ratio of DPSCs

and PC-3 cells stained with green and red fluorescence membrane dyes, respectively. DPSCs encircled PC-3 cells and created a tube-like structure in culture wells. PC-3 cells formed clusters in the form of islets surrounded by channel-like structures generated by DPSCs.

Recently, Brunetti *et al.* showed that DPSCs secrete TNF-related apoptosis-inducing ligand (TRAIL) during osteogenic differentiation and affect myeloma cancer cell viability, indicating the possible interactions of dental stem cells with cancer cells³³. Our study is the first report that evaluates interactions of dental derived MSCs and prostate cancer cells as an *ex vivo* model. We used three different direct/indirect approaches in our experiments. Proliferation of cancer cells and high migration rates were detected either by CM and trans-well assays or by co-culturing differentially stained cells that allows for multiple interactions.

Disclosures

The authors have nothing to disclose.

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