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Project Title: To examine the mechanism through which adipose-derived stem cell affect healthy and malignant breast epithelial cell proliferation

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Summary (250 words max single spaced):

Mastectomies are commonly performed procedures to increase the life expectancy of women suffering from breast cancer. Stromal Vascular Fraction (SVF) from patient's abdominal fat is often utilized to supplement fat grafts used in breast reconstructions. Unfortunately, very little is known about the interaction of SVF with residual cancer cells as well as surrounding breast tissue. This study investigates if secreted factors from the SVF cells affect proliferation of residual breast cancer cells and the surrounding breast cells. **Methods:** The effects of patient-derived SVF samples on the proliferation of estrogen receptor positive (ER+) MCF-7 breast cancer cells, tissue adjacent to ER+ breast tumours (TAT), and healthy breast cells from reduction mammoplasty samples (HBT) was examined. The secretion profile of 41 different cytokines in the conditioned media (CM) of SVF samples grown in co-cultures with the MCF-7, TAT, or HBT cells was compared to the CM of each cell type grown alone. **Results:** Placing MCF-7 cells in co-cultures with SVF cells led to a 1.53-fold ($p<0.005$) increase in their proliferation. Interestingly, CM obtained from the co-cultures of SVF+MCF-7 cells was sufficient to increase MCF-7 cell proliferation by 1.5 fold ($p<0.05$), indicating that secreted factors in this CM have pro-proliferative properties on cancer cells. The cytokine array identified increased secretion of a number of cytokines that were uniquely elevated in the co-cultures of SVF with MCF, TAT or HBT cells but not in the individual cell cultures. From the cytokine array data, we confirmed that IL-1β, MDC and RANTES cytokines were able to increase MCF-7 cell proliferation independently. **Conclusion:** Secreted factors from SVF+MCF-7 co-cultures have pro-proliferative effects on MCF-7 cells. This study is a proof of concept that examining secretion profiles of SVF co-cultures can lead to the discovery of cytokines which are able to modulate breast cancer cell proliferation.

Student Signature

Primary Supervisor Signature

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Introduction and Background

Approximately 22,000 breast cancer patients have mastectomies performed each year as one of the many steps to help cure their disease[1]. Unfortunately, the mastectomy procedure can be debilitating cosmetically as well as psychologically. Therefore, reconstructive surgical techniques have been developed to help reduce such burden for breast cancer patients. Although mastectomy surgeries significantly improve overall patient survival, they often lead to distortion of breast volume and shape, and the follow-up radiation therapy often results in breast tissue fibrosis and poor wound healing[2-4]. Both autologous tissue flaps and alloplastic implants are used in a variety of breast reconstruction cases. While the use of silicone implants has been well established and provides the patient with a quicker recovery time, 10-20% of patients are prone to developing capsular contraction and/or other long-term complications[5-8]. Recently, refinement of mastectomy reconstruction has focused on the use of autogenous fat grafts along-side autologous tissue free flaps[9-10]. In such procedures, typically, autologous fat tissue from the patient's abdomen is used as filler since it has shown promising results in repairing soft tissue defects caused by tumor resection, and local tissue deformities caused by surgical incision procedures compared to silicone implants[11-14].

Autogenous fat graft tissue is heterogeneous in composition with one component of interest being adipose derived mesenchymal stem cells (Ad-MSCs). Ad-MSCs have been shown to have the ability to differentiate into different specialized cell types including adipocytes and endothelial cells, secrete pro-angiogenic cytokines, and possess self-renewal capacity[15-23]. Recent observations suggest that the mesenchymal stem cells are important for tissue regeneration and homeostasis due to their ability to proliferate and differentiate into various specialized cell types in vitro[17-18]. These characteristics have made Ad-MSCs ideal candidates in providing better wound healing and graft maintenance in reconstructive surgeries[24,25]. Recent experiments have demonstrated that isolation of the stromal vascular fraction (SVF) from abdominal fat contains Ad-MSCs, as well as other cell types such as fibroblasts and vascular endothelial cells[26,27]. Due to the relative concentration of Ad-MSC's in SVF, as well as the properties of these cells, autologous fat grafts infused with SVF are currently being used in reconstructive surgeries. Intraoperatively, SVF is isolated from the infranate of centrifuged lipoaspirate, it is recombined with the fat graft, and is then injected into the tissue defect. On the other hand, in the laboratory SVF is isolated through an established procedure of enzymatic digestion, mechanical dissociation and centrifugation. However, the potential effects of SVF cells and/or Ad-MSCs on the proliferation and differentiation of remnant breast cancer cells as well as progenitors and stem cells that are present in the tissue adjacent to breast tumors (TAT) have only been partially studied. It is important to note that mastectomy procedures do not remove all breast tissue and therefore the interaction of the remaining tissue with SVF is of interest and concern[28,29]. Our lab previously showed that placing SVF cells in co-culture with TAT lead to a 7-fold increase in proliferation of breast epithelial progenitor cells, whereas healthy breast progenitors, obtained from reduction mammoplasties, only expanded 3 fold in similar growth conditions. In addition, mesenchymal stem cells have been shown to stimulate the growth of breast cancer cells in vitro and in vivo which could facilitate the development or recurrence of breast cancer tumors[30-35]. These observations are interesting because the TAT represent breast tissue at high risk of developing future malignancies, and therefore the use of SVF cells and Ad-MSCs in reconstruction surgeries following mastectomy procedures may not yield a desirable long-term outcome. It is interesting to study the mechanisms through which SVF enhances the expansion of remnant breast cancer cells and TAT progenitors as this knowledge could be used to understand how such factors may play a role in breast tumor recurrence. Moreover, the effects of SVF cells on the tumor microenvironment and how they influence breast cancer cells proliferation or possibly de novo tumor formation remains elusive

and highly controversial[28,29]. What is known thus far is that SVF has been shown to support tumorigeneis and promote aggressiveness by supporting Epithelial-Mesenchymal transition (EMT), enabling cells to gain invasive properties. Furthermore, studies have implicated the expression hepatocyte growth factor receptor (HGFR or c-met), a protein that is implicated in organogenesis and wound healing, as a predictive factor of cancer recurrence after autologous fat grafting[36]. SVF has also been shown to stimulate metastasis of triple negative MDA-MB232 cells in mouse models and has been shown to secrete pro-inflammatory cytokines, such as IL-1β, in the presence of MDA-MB232 cells[37,38]. Non-the-less, detailed mechanisms through which these phenomena occur has not yet been described, particularly in regards to the most prevalent form of breast cancer, the estrogen-responsive tumors.

The central hypothesis of this project is that secreted factors from SVF and Ad-MSC could modulate the biological function of healthy breast stem cells and progenitors, as well as breast cancer cells. Through this study we aim to identify secreted factors that can enhance the proliferation and differentiation of breast stem cells without any effect on the proliferation of breast cancer cells. The overall goal of this project is identify factors that can be used clinically to suppress remnant breast cancer cell growth as well as to enhance breast stem and progenitor cell functions post-mastectomy and breast reconstruction in a regulated manner. We aim to mimic the effects of SVF on grafts which includes increased fat graft maintenance, faster healing post breast reconstruction and improved breast aesthetics. Our overreaching objective is to identify factors that can be used clinically to enhance breast stem and progenitor cell functions in a regulated manner.

Material and Methods

Tissue Sample Collection

All samples used in this study were collected through informed written patient consent to use their tissues samples in compliance with Health Research Ethics Board approval (Ethics #HS14919 (H2012:020) of the University of Manitoba. Tissue adjacent to breast tumor (TAT) samples were obtained from >3 cm away from the distal margin of the primary invasive ductal tumors, positive for the expression of Estrogen Receptor (ER) and Progesterone Receptor (PR). All TAT samples were deemed histologically normal by a pathologist. Lipoaspirate samples of abdominal fat from patients undergoing mastectomy/reconstructive breast surgery were obtained and the stromal vascular fraction was isolated as described below[41]. Michigan Cancer Foundation-7 (MCF-7) cells are a breast cancer cell line that express estrogen receptor (ER) and progesterone receptor (PR) and were used to mimic invasive ductal cell carcinoma cells. Discarded tissue from reduction mammoplasty surgeries were collected and used as the source of healthy breast tissue (HBT). All tissue samples were transported from the operating room to the laboratory in transport media (DMEM-F12 supplemented with 5% Bovine serum, Insulin (5µg/ml; Sigma-Aldrich), and antibiotics (Invitrogen). The HBT and TAT samples were enzymatically and mechanically dissociated and made into single cell suspension as described below[39].

Isolation of SVF from Abdominal Adipose Tissue

All collected SVF samples were isolated from abdominal adipose tissue using established protocols by my laboratory[35,41]. Adipose tissue was finely minced with a scalpel and washed thoroughly with a PBS solution containing Penicillin/Streptomycin (5%). The samples were then digested and continuously agitated for approximately 4hrs at 37°C in Ham's DMEM F-12 media which was supplemented with 2% Bovine Serum Albumin (BSA), 300 U/ml collagenase, 100

U/ml hyaluronidase, 10 ng/ml epidermal growth factor, 1 mg/ml insulin, 0.5 mg/ml hydrocortisone. The samples were then mechanically dissociated using a pipette for 1-2 minutes to further break down the samples. The liquid portion of the sample was then transferred to a 50mL falcon tube, paying careful attention to avoid transferring any solid aggregates. The samples were then centrifuged at 1200 rpm for 5 minutes in order to separate stromal cells from primary adipocytes. The supernatant, containing adipocytes, was discarded and the infranate, containing SVF, was washed with (Hank's balance salt solution) HBSS supplemented with 5% Fetal Bovine Serum (FBS) and suspended red blood cell lysis buffer (Sigma-Aldrich). The samples were subsequently centrifuged at 1200 rpm for 5 minutes, the supernatant was removed and the remaining SVF pellet was re-suspended in 5mL of cyro-preservation media containing 6%DMSO, 44% fetal calf serum and 50% Ham's DMEM F12 media with HEPES(10mM). These samples were then cryogenically stored in liquid nitrogen tanks[35,41].

Isolation of Breast Parenchymal Cells from TAT and HBT

Breast Parenchymal cells were dissociated based on protocols established by Raouf and Sun[39]. In summary, Breast tissue samples were minced, and transferred to sterile dissociation flasks containing Ham's DMEM F-12 media which was supplemented with HEPES (10mM), 2% Bovine Serum Albumin (BSA), 300 U/ml collagenase, 100 U/ml hyaluronidase, 10 ng/ml epidermal growth factor, 1 mg/ml insulin, 0.5 mg/ml hydrocortisone, and solution of Penicillin ($1x10^4$ units/mL) and Streptomycin (10mg/ML). The mixture was then shaken at about 109 rpm for 16-18 hour at 37°C. The next day fibrotic tissue was discarded from the flasks. The solution was then centrifuged at 800 rpm for 40 seconds forming an organoid-enriched (alveolar structure enriched) pellet containing primarily breast epithelial cells. The organoid-enriched pellet was then re-suspended in 5mL of cryo-preservation media and cryogenically stored[39].

Cell Culture Maintenance and Passaging

Cell lines and primary cell samples were cultured in 10cm cell tissue culture plates. 10mL of DMEM F12 media supplemented with 5% FBS was used to maintain healthy and proliferating cell cultures. All cells were grown in a humidified incubator at 37°C with 5% CO_2 with growth media changes every 3-4 days or as needed. Cultures were passaged when cells were at 80-85% confluence. Firstly, each plate was washed with 4-5mL of PBS to remove any excess FBS. Next, 5mL of Trypsin (0.25% trypsin/EDTA) was added to cell cultures and incubated for 5 minutes at 37°C with 5% CO_2 to break adherent bonds between the cells and the cell culture plate. Trypsin was consequently inhibited by addition of equal volume of HBSS supplemented with 5% FBS and cells were then centrifuged at 1200 rpm for 5 minutes. The supernatant was afterwards removed and a single cell suspension was created using 1mL of HBSS supplement with 5% FBS. The cells were then re-plated at various cell densities as required.

Initial Cell Counts

In order to accurately culture the correct number of cells, initial cell counts were determined by the following procedure. Cell counts were obtained by sampling 10µL of an initial 1mL cell suspension. This sample was mixed with 90µL of trypan blue and 10µL of this solution was placed on a counter plate and cell numbers were determined via an automatic hemocytometer (Bio-Rad ®). Only live cell (cells that excluded trypan blue dye) counts were used for experiments. Cell counts were performed in triplicates. Cell counts were performed in triplicates and average cell counts were used to set up experiment.

Effects of SVF on MCF-7 cells in co-culture experiment

In order to determine if SVF has pro-proliferative properties on MCF-7 cells, $1.0x10^5$ SVF cells were co-cultured with $1.0x10^5$ MCF-7 cells. As controls, $1.0x10^5$ MCF-7 cells were cultured alone in triplicates. Three different patient derived SVF samples were used for co-cultures. Cell numbers were determined using the automatic hemocytometer as described below. All cells were cultured in a 96 well plate using 50µL of growth factor reduced 3-dimentional matrix, Matrigel. Matrigel was used instead of 2-dimentional media since it is a better representation of cells *in vivo*. Epithelial Cell adhesion molecule (EpCAM) is a transmembrane glycoprotein present on the surface of the majority of MCF-7 cells and absent on the surfaces of SVF cells. EpCAM expression was used as a marker for counting MCF-7 cell numbers and differentiating them from SVF cells. Cell counts were obtaining on culture days 3, 5 and 10. All cell cultures were grown in 200µL of SF-7 media supplemented with 1µL of Bovine Pituitary Extract (BPE) (100µg/mL) with media changes ensuing on culture days 3, 5 and 8. SF-7 media was prepared using Dulbecco's Modified Eagles Media (DMEM): Nutrient Mixture F-12 supplemented with 5% Fetal Bovine Serum, 0.1% w/v bovine serum albumin (BSA), 10 ng/ml Epidermal Growth Factor (EGF), 10 ng/ml cholera toxin, 1 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 U/ml penicillin, 100 µg/ml streptomycin. Matrigel cultures were enzymatically and mechanically digested as described below. In order to determine the number of MCF-7 cells in culture with SVF, all cells were stained with an anti EpCAM antibody tagged with a florescent molecule and the number of EpCAM+ cells were obtained via fluorescent-activated cell sorting (FACS). In order to prepare the cells, we first added 1µL of EpCAM antibody (1µg/1µL, Stem Cell technologies - 01420) and placed cell cultures on ice for 10 minutes. Next, cells were centrifuged at 1200RPM for 5 minutes and the supernatant was discarded. The remaining cell pellet was mixed with 300µL of HBSS supplemented with 5% FBS. Subsequently, 1µL of PE goat anti-mouse IgG (0.2mg/mL, Biolegend - 405307) was added to the cell suspension as a source of secondary fluorescing antibody. The cells were then placed on ice of 10 minutes and subsequently centrifuged as before. The remaining cell pellet was re-suspended in 300µL of HBSS supplemented with 5% FBS and 0.35µL of propidium iodine (PI, 1mg/mL). The number of viable $PI^{Negative}$ EpCAM positive cells in each Matrigel culture was ascertained using FACS.

Conditioned SF-7 Media Collection from SVF cultures and SVF+MCF-7 co-cultures

In order to study if secreted factors from SVF can affect MCF-7 cells growth, conditioned media was collected from SVF cells alone as well as SVF and MCF-7 co-cultures (SVF+MCF-7). For this purpose, three different patient-derived SVF samples were used for co-culture with MCF-7 cells as well as culture alone. 50,000 SVF cells and 50,000 MCF-7 cells were co-cultured in wells of a 12 well cell culture plate using DMEM F12 media supplemented with 5% FBS in order to collect sufficient media and better mimic cell numbers from the cytokine array experiment. Cultures involving only SVF were placed in a 10cm cell culture dish using DMEM F12 media supplemented with 5% FBS. All cultures were incubated in a humidified incubator at 37°C with 5% CO_2. Media was changed every 3-5 days as needed to maintain healthy proliferating cell cultures. All cultures were monitored until 70% confluency when 1mL of DMEM F12 was added to SVF+MCF-7 co-cultures and 5ml of DMEM F12 was added to SVF cultures. Small amount of media was added to cultures in order concentrate the secreted factors. A proportionate amount of media was added to cultures depending on the size of the culture plate. After 48hr, conditioned media was aspirated out of culture and centrifuged at 1200 rpm for 5 min to remove debris. The supernatant was removed and supplemented with 5% Fetal Bovine Serum (FBS), 0.1% w/v bovine serum albumin (BSA), 10 ng/ml Epidermal Growth Factor (EGF), 10 ng/ml cholera toxin, 1 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 U/ml penicillin, and 100 µg/ml

streptomycin to create SF-7 media. This media was subsequently used for our study. Conditioned SF-7 media was frozen in -80°C until used.

Matrigel Culture Dissociation

In order to measure cell proliferation, cell numbers need to be quantified in Matrigel on several cell culture days. For this purpose, Matrigel cultures were enzymatically and mechanically digested into single cell suspensions to ascertain accurate counts. Media from cultures was aspirated and replaced with 200µL of dispase (5mg/mL) to digest the 3-dimentional matrix. After 1 hour, all contents from all wells was removed and mixed with 400µL of HBSS supplemented with 5% FBS. The wells were then further washed with 200µL of HBSS supplemented with 5% FBS in order to collect any residual cells. The contents were then centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and cells were further digested with 500µL of Trypsin (0.25% trypsin/EDTA) for 5 minutes to create a single cell suspension. Cells were subsequently mixed with 600µL of HBSS supplemented with 5% FBS to stop the trypsinization process, and were then centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and the infranate was mixed with 300µL of HBSS supplemented with 5% FBS to create a single cell suspension. Each single cell suspension was mixed with 0.35µL of propidium iodine (PI, 1mg/mL) in-order to differentiate live cells from dead cells. The number of viable cells in each culture condition was ascertained using the propidium Iodide dye exclusion via fluorescent-activated cell sorting (FACS).

Flow Cytometry

After cells were made into a single cell suspension as per the protocol described above, the number of cells in each culture was determined using flow cytometry (Guava easycyte®). The number of viable cells in each culture condition was ascertained using propidium Iodide dye exclusion via fluorescent-activated cell sorting (FACS). 10,000 events were measured as a sample of the total single cell suspension volume, as running the entire mixture was impractical. PI negative cells were then gated and the total number of live cells in each culture was then calculated using the available data. For some experiments MCF-7 cell number was quantified using the EpCAM expression (expressed on >90% of cells) and PI negative cells as marker for live cells.

Conditioned Media Experiment – SVF and SVF+MCF-7 conditioned media

Conditioned SF-7 media from SVF cultures alone or from SVF+MCF-7 co-cultures was used to culture and examine the effects on MCF-7 cell proliferation. MCF-7 cells were cultured at a concentration of $5.0x10^4$ cell per well in 50µL of Matrigel in a 96 well plate for 10 days. Total cell counts were obtained on days 3, 5 and 10. Matrigels were allowed to solidify for 1 hour before cells were plated. Cells were allowed to settle overnight into the 3-dimentional matrix before conditioned media was added to the cultures. 200µL of regular SF-7 media supplemented with 1µL of BPE (100µg/mL) per well was used. Initial media was subsequently removed and replaced with SF-7 conditioned media from either SVF samples cultured alone or in co-culture with MCF-7 and supplemented with 1µL of BPE (100µg/mL). Each of the three SVF samples were cultured in triplicates. As controls, $5.0x10^4$ MCF-7 cells were cultured in regular SF-7 media supplemented with 1µL of BPE (100µg/mL) per well. Media from all controls was replaced with fresh SF-7 media supplemented with 1µL of BPE (100µg/mL) per well after cells were allowed to settle in the 3-dimentional matrix overnight. Media changes were done on days 3, 5 and 8 due to growth factor exhaustion. Freshly aliquoted conditioned media from either SVF or SVF+MCF-7 cultures was thawed from -80°C and supplemented with 1µL of BPE (100µg/mL)

per well. 200µL of this media was used to replenish each culture. For controls, 200µL of regular SF-7 media supplemented with 1µL of BPE (100µg/mL) was used to replenish each culture. Cultures were digested and cell counts obtained on days 3, 5 and 10 of the experiment as per the protocol described in this paper.

Cytokine Array Experiments

In order to investigate the interaction of SVF with TAT, breast cancer cells, and HBT, we examined the secretion levels of 41 cytokines in Matrigel. All tissues samples were made into single cell suspensions. HBT and TAT were depleted of contaminating endothelial and hematopoietic lineage cells using magnetic cell separation. CD31 and CD45 were two protein markers used to remove the endothelial and hematopoietic cells respectively. $1x10^5$ SVF cells were placed in co-cultures with $1x10^5$ TAT, MCF-7, or HBT individually in different wells of a 96-well plate. Each cell type was also cultured alone at a concentration of $1x10^5$ cells/well. All cultures were plated in triplicates. Matrigel cultures were grown in SF-7 media (200µL) supplemented with 1µL of BPE (100µg/mL) and incubated in a humidified incubator at 37°C with 5% CO_2 for 10 days. All cultures were maintained and handled in sterile conditions along with penicillin/streptomycin added to the media. Fresh SF-7 media (100µL) was added to each culture on day 7 due to the exhaustion of growth factors by cells. After 10 days, the growth media from each Matrigel culture was collected, centrifuged at 1200 rpm for 5 minutes and the supernatant was sent for cytokine array analysis. The presence of 41 different cytokines, chemokines and growth factors in each sample was measured and quantified using a high throughput enzyme-linked immunosorbent assay (ELISA) performed by a commercial source (Eve Technologies).

Cytokine-supplemented in vitro cultures

From the cytokine array experiments, a number of factors were identified to have a statistically significant change in their secretion profile when SVF was co-cultured with TAT, HBT or MCF-7 cells, compared to cultures containing single cell types. For this project, we first focused on cytokines that may regulate MCF-7 breast cancer cell growth. For this purpose, Matrigel cultures were set up containing MCF-7 cells. To start with, the effects of MDC, IL-1β and RANTES on MCF-7 cells were examined. For this purpose, $5.0x10^4$ MCF-7 cells were placed in Matrigel cultures (50µL) for 10 days and their growth media was supplemented with different cytokines at a concentration of 5ng/200mL. This experimental concentration was based on previous in vitro experiments[41]. SF-7 media supplemented with 1µL of BPE (100µg/mL) was used as growth media for all cultures. As controls, $5.0x10^4$ MCF-7 cells where placed in Matrigel cultures and grown in SF-7 media supplemented with Phosphate Buffer Saline (PBS), the vehicle for all three aforementioned cytokines. The growth media was changed on days 3, 5 and 8 due to growth factor exhaustion and the short half-life of cytokines at 37°C. All cell cultures were done in triplicates. Cultures were dissociated on days 3, 5 and 10 and counted according to the 'Matrigel Culture Dissociation' and 'Flow cytometry' protocols mentioned below.

Statistical Methodology

One-way analysis of variance with Tukey's multiple comparisons test were performed for multiple pair-wise comparisons. Single pair-wise comparisons were performed using the two-tail student t-test. Statistical significance was considered to be $p \leq 0.05$.

Results

SVF cells Increase Breast Cancer Cell Proliferation in Matrigel

Previous experiments in our lab have shown that SVF increases the proliferation of breast epithelial progenitor cells in TAT and HBT. We wanted to further investigate the properties of SVF and how it affects the proliferation of MCF-7 cells in co-culture. For this purpose, $1.0x10^5$ MCF-7 cells were co-cultured with $1.0x10^5$ SVF cells. Antibody staining for EpCAM, a transmembrane glycoprotein present on the majority of MCF-7 cells, was used as a marker to identify MCF-7 cells when counting cells using FACS. The MCF-7 cell numbers were determined on days 3, 5 and 10 of culture (Figure 1). When MCF-7 cells were placed in Matrigel cultures, the cell numbers steadily increased over the course of 10 days (Figure 1., P-values for days 3, 5 and 10 were 0.0071, 0.024 and 0.00012 respectively). Interestingly, we observed that by day 10 there was a 1.53-fold increase in the number of MCF-7 cells when co-cultured with SVF, compared to MCF-7 cells cultured alone. These findings are in keeping with other experiments that have examined the interaction of SVF and breast cancer cells[30-35]. These observations suggest that either secreted factors and/or contact with the SVF cells is responsible for the expansion of MCF-7 cells in the Matrigel cultures.

Conditioned Media from SVF and MCF-7 cells Significantly Enhance Breast Cancer Cell Growth

We examined if culturing MCF-7 cell with conditioned media collected from SVF cultures and SVF+MCF-7 co-culture would increase the proliferation of MCF-7 cells. For this purpose, MCF-7 cells were cultured in conditioned media collected from both aforementioned cell cultures. $5.0x10^4$ MCF-7 Cells were placed in 50μL of Matrigel and cell numbers were quantified on days 3, 5 and 10 using FACS. By measuring cell numbers on day 3, 5 and 10 we were able to ascertain that conditioned media form both SVF cultures and SVF+MCF-7 co-cultures significantly ($p<0.05$) increased the proliferation of MCF-7 cells, compared to cells grown in SF-7 control media (Figure 2). Culturing MCF-7 cells in conditioned media from SVF+MCF-7 co-cultures led to a 1.55-fold increase ($p<0.0005$) in MCF-7 cell numbers by day 10. This is interesting because culturing MCF-7 cells in conditioned media from SVF cultures only led to a 1.30-fold increase ($p<0.005$) in MCF-7 cell numbers by day 10. It is evident that SVF cells secrete factors which have statistically significant pro-proliferative properties on MCF-7 cells. In addition to this finding, when the conditioned media from SVF+MCF-7 co-culture was used, statistically significant amounts of either additional or different factors where secreted which have greater pro-proliferative effect on MCF-7 cells, than SVF cells on their own. It is important to note that we found a statistically significant change between the SVF conditioned media group and SVF+MCF-7 conditioned media group on all three days of the experiment ($p<0.05$). When comparing cell numbers in the SVF conditioned media group against cells grown in SF-7 control media we noticed p-values of >0.05, <0.0005 and <0.0005 on days 3, 5 and 10 respectively. When comparing cell numbers in the SVF+MCF-7 co-culture conditioned media group against cells grown in SF-7 control media, we noticed p-values of <0.0005 on days 3, 5 and 10 (Figure 2).

41-Plex Cytokine Array Assay Revealed Unique Secretion of Cytokines in SVF co-cultures with MCF-7, HBT and TAT cells

We hypothesized that co-culturing SVF cells with TAT, HBT or MCF-7 cells would lead to increased secretion of pro-proliferative cytokines. To this end, we examined the expression of 41 different cytokines and growth factors in Matrigel cultures initiated with SVF cells co-cultured with TAT, HBT, and MCF-7 cells or each cell type individually. Using this array, we were able to

identify cytokines whose levels were significantly ($p<0.05$) increased only in SVF co-cultures (Figure 3), but not in cultures containing single cell types. Interestingly, when SVF cells were placed in co-culture with cells from TAT samples, we observed a statistically significant decrease in TGF-α secretion suggesting that TGF-α has an anti-proliferative effect on TAT tissue (Figure 4a). In the HBT+SVF cultures, we observed a statistically significant increase in the secretion levels of GRO but a significant decrease in the secretion levels of VEGF when compared to SVF cultures (Figure 4b-c). Secreted factors that were found to be significantly ($p<0.05$) changed in the SVF+MCF-7 cell co-cultures included MDC (Macrophage Derived chemokine or CCL22), IL-1β, and RANTES (Figure 3d-f). Secretion of MDC, IL-1β and RANTES were observed to be increased in the SVF+MCF-7 co-cultures. These findings are interesting as the role of RANTES in promoting breast cancer cell growth has been described before which provides some validity to our findings and acts as a positive control[39]. Of note, the role of TGF-α and VEGF in promoting the growth of healthy breast epithelial cells has not been discussed before

IL-1β, RANTES and MDC increase breast cancer cell growth independent of SVF

To understand if the differentially secreted cytokines are capable of enhancing the proliferation of TAT, HBT, or MCF-7 cells individually, Matrigel assays were set up where the growth media was supplemented with different cytokines. The effects of RANTES, MDC, and IL-1β on the MCF-7 breast cancer cell lines were examined. Each cytokine was added at a dose of 5ng/200mL to MCF-7 cells, cultured in Matrigel. The number of live cells was ascertained using propidium die exclusion via FACS on days 3, 5 and 10. RANTES has previously been shown to increase proliferation of breast cancer cells and acted as our positive control[39]. After quantifying cell numbers we were able to determine that IL-1β, RANTES and MDC were all able to significantly ($P<0.05$) increase the proliferation of MCF-7 cells as compared to the controls (Figure 5). In the presence of IL-1β, MCF-7 cells showed a modest and yet significant ($P<0.05$) 1.3-fold increase in cell proliferation compared to the control cells after 10 days in cell culture. Similarly, RANTES and MDC cytokines also resulted in small but a significant ($p<0.05$) 1.2-fold increase in cell proliferation after 10 days in cell culture. These findings suggest that all three tested cytokines, IL-1β, RANTES and MDC have statistically significant pro-proliferative properties on MCF-7 cells at a concentration of 5ng/200mL. These findings are of interest because the role of IL-1β and MDC on MCF-7 cells proliferation has not yet been described.

Discussion

Although multiple studies have implicated Ad-MSC's in the promotion and progression of breast cancer in both in-vivo and in-vitro models, very few studies have investigated the effects of secreted factors from SVF/Ad-MSC and how they modulate breast cancer growth. [27,30-34]. In this study we hypothesized that breast cancer cells or healthy breast epithelial cells would show a different secretome profile when placed in co-cultures with SVF compared to cells grown by themselves. To our knowledge the effects of IL-1β and MDC on breast cancer cell growth have not yet been reported. Our results demonstrate that not only is the secretome of SVF capable of promoting breast cancer cell proliferation, but also when SVF is in direct contact with breast cancer cells it secretes additional pro-proliferative factors, such as IL-1β, that further stimulate its own growth. Although we determined that SVF has a unique secretome when in contact with breast cancer cells, the secretome profile of TAT and HTB with SVF were very similar to the SVF secretome profile by itself. Previous studies have shown that levels of IL-1β, and RANTES are low or undetectable in SVF samples, which is consistent with our data[42]. It is interesting that the level of IL-1β, MDC and RANTES were increased when in co-culture with SVF. These three

cytokines were further tested and determined to have pro-proliferative effects on breast cancer cells. This observation demonstrates the importance of the tumor microenvironment in tumor progression. It is noteworthy that neither cytokine on their own, at the concentrations used, were able to recapitulate, to the same degree, the effect of SVF on MCF-7 cell growth when in co-culture. Interestingly, conditioned media from SVF+MCF-7 co-cultures was able to increase MCF-7 cells proliferation to the same extent as SVF when placed in co-cultures with MCF-7 cells. This observation suggests that one of the main mechanisms through which SVF increases proliferation of MCF-7 cells is the secretion of pro-proliferative factors. This observation further suggests that in future experiments we should try these 3 cytokines together and examine their potential synergistic effects. Also, the effects of each cytokine on the proliferation of MCF-7 and other breast cancer cells should be test using various doses to obtain the smallest dose that confers the largest increase in cell proliferation. The pro-proliferative breast cancer specific cytokines that were identified through this project represent potential clinical targets for modulating remnant breast cancer cells post mastectomy, while trying to enhance breast stem and progenitor cell functions in a regulated manner. In this way, it is hoped that breast reconstruction surgeries would still benefit from the use of SVF without its pro-tumor cell growth properties. It would be fascinating to see the effect that blocking the IL-1β, MDC and RANTES receptors on breast cancer cells would have on their proliferation. Also, in this study we only examined the effects of SVF on the ER+/PR+ breast cancer cells. Future considerations should be given to different breast cancer cell lines including the triple negative MD-MB231 cells.

Breast cancer tumors display pronounced heterogeneity in its microenvrionement. Cellular types include but are not limited to, breast cancer cells, breast cancer stem cells, adipocytes, immune cells, endothelial cells and cancer associated fibroblasts (CAF). All components produce abundant cytokines, chemokines and growth factors which affect the tumor's microenvironment composition and facilitates cancer progression. CAF represent one of the most abundant type of cells in breast carcinoma and share many similarities with non-oncogenic fibroblasts such as participating in wound healing or the inflammatory response[43]. CAF play an important role in tumorigeneis, pro-metastatic signaling and metastatic growth. Interestingly enough, one of the components of SVF are fibrobroblasts alongside adipocytes, endothelial cells and hematopoietic cells[41]. This is important due the similarities in the microenvironment of breast tumors and SVF. The SVF-fibroblasts could be one of the potential cell types that secrete the majority of the proproliferative cytokines and thus are important to the tumor microenvironment. It would be important to try to separate the components of SVF in future studies to determine which cell type is responsible for secreting certain pro-proliferative cytokines and to determine their effects on breast cancer cells. This provides one possible mechanism for maintaining the positive effects of SVF while removing its deleterious effects as menitoned previously. Our study results can potentially be translatable to in-vivo models of breast cancer cells to control the progression of the disease and provide potential targets for treatment.

Ad-MSC's have clear effects on TAT as demonstrated by previous efforts in our laboratory[35]. Published work from our laboratory showed that when SVF cells were placed in co-culture with TAT it leads to a 7-fold increase in proliferation of breast epithelial progenitor cells, whereas healthy breast progenitors only expanded 3 fold in similar growth conditions. It is unknown whether this mechanism is due to secreted factors in the secretome or via direct cell-cell contact. Our results provide a platform to test cytokines which could increase breast tumor cell proliferation. In addition, our platform will be used to identify SVF-derived cytokines responsible for enhanced proliferation of epithelial progenitors cells found in the TAT and HBT samples. It would be interesting to see how cytokines such as TGF-α could affect the proliferation of breast epithelial progenitors in TAT. Furthermore, it would be important to test how cytokines such as GRO and VEGF-A affect prolifertion of healthy breast epithelial progenitors in HBT. Ultimately,

we want to mimic the positive effects that SVF has on patients such as providing better wound healing and graft maintenance in reconstructive surgeries, and eliminate harmful effects such as uncontrolled growth of TAT and remnant breast cancer cells. This task can hopefully be accomplished through further research efforts and the discovery of additional cytokines, chemokines and other growth factors that can modulate these effects.

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Figure 1. Effect of SVF on MCF-7 cell proliferation in co-culture.
In order investigate if SVF cells could affect the proliferation of MCF-7 cells, $1.0x10^5$ MCF-7 cell were cultured with $1.0x10^5$ SVF cells in Matrigel. As a control, $1.0x10^5$ MCF-7 cell were grown alone. Cultures were grown in a growth factor reduced 3-Dimentional Matrix (Matrigel) for 10 days. The cultures were digested on the indicated days and the number of EpCAM positive MCF-7 cells was quantified using FACS. Statistical Significance on day 10 of cell culture: MCF-7 Cells alone vs MCF-7+SVF Cells ($p<0.0005$).

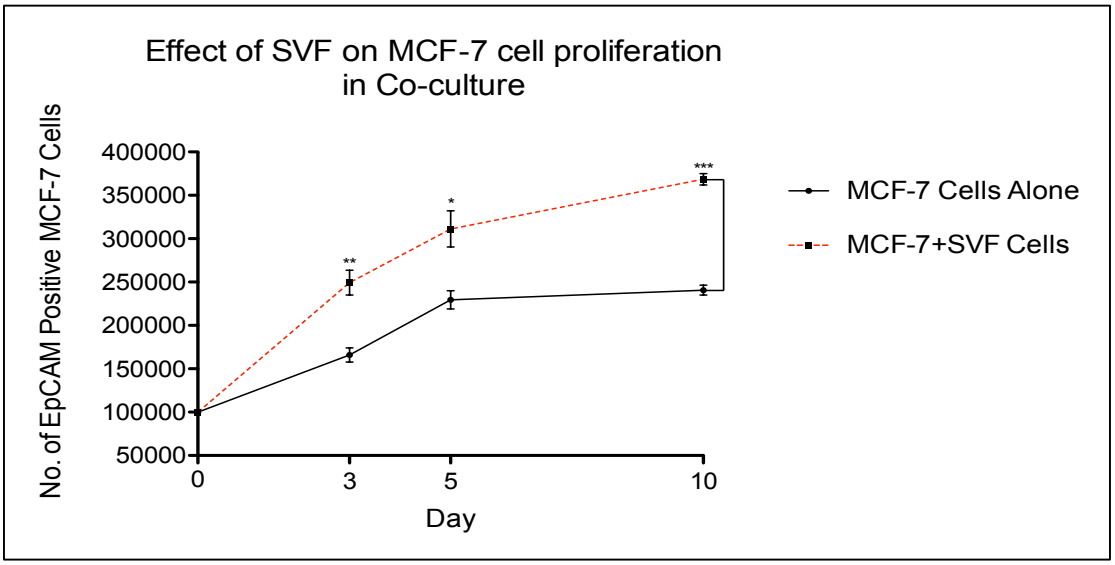


Figure 2. Effects of Conditioned Media from SVF Cultures and SVF+MCF-7 Co-cultures on MCF-7 cell growth
Conditioned media was collected from SVF cells or from SVF and MCF-7 co-cultured cells. 50,000 MCF-7 cells were grow in Matrigel in the presence of different conditioned media or SF7 growth media as a control. On indicated days, cell numbers from each gel was obtained. Average cell numbers obtained from 3 independent experiments are shown in the graph. Statistical Significance on day 10 of cell culture: SVF+MCF-7 co-culture conditioned media vs. SF-7 Control media ($P<0.0005$). SVF conditioned media vs SF-7 Control media ($P<0.05$). SVF Conditioned media vs SVF+MCF-7 Conditioned media ($p<0.05$).

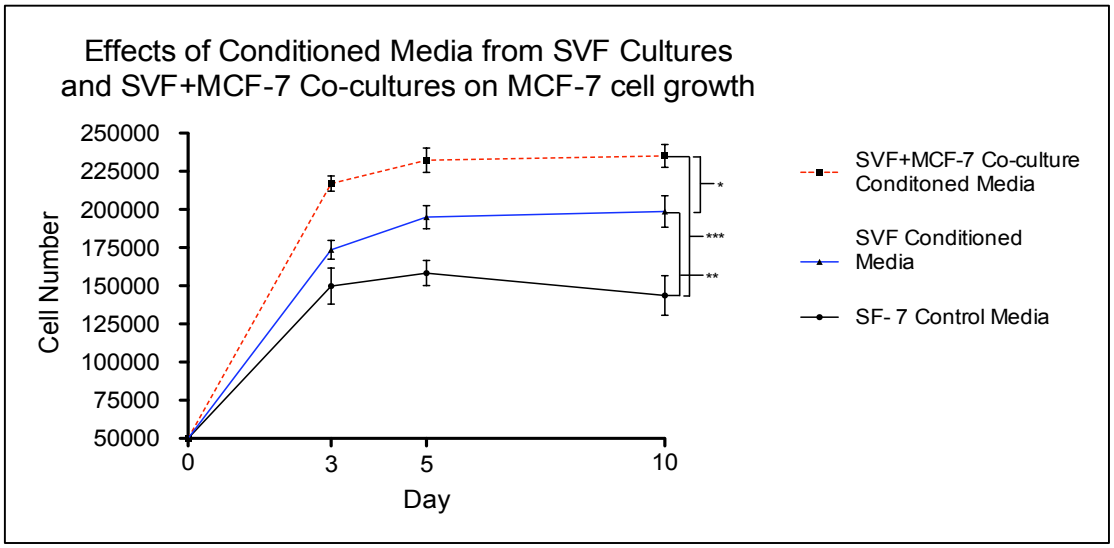


Figure 3. 41-plex cytokine array analysis reveals significant changes to the secretion of cytokines in SVF co-cultures
MCF-7 cells as well as SVF, TAT and HBT samples were made into single cell suspensions and cultured in a growth factor reduced 3-Dimentional Matrix (Matrigel) for 10 days. 41 different cytokine concentrations were analyzed and quantified in each culture using high throughput ELISA assay. One-way analysis of variance was applied to all cytokines/chemokines individually, as well as Tukey's multiple comparison test. This table summarizes the statistical significance of each pair-wise comparison. Although not statistically significant, some interesting secretion profiles warrant further investigation. Orange color represents p-values < 0.05. Red color represents p-value < 0.005.

41-plex cytokine array analysis

Cytokines	SVF+TAT vs SVF+MCF7	SVF+Normal vs SVF+MCF7	SVF+TAT vs SVF	SVF+TAT vs TAT	SVF+Normal vs SVF	SVF+Normal vs Normal	SVF+MCF7 vs SVF	SVF+MCF7 vs MCF7	SVF vs TAT	SVF vs Normal	SVF vs MCF7	TAT vs MCF7	Normal vs MCF7
GRO pan	0.05	0.005	0.05	1	0.005	0.05	1	1	1	1	1	1	1
TGF-a	1	1	0.05	0.05	0.05	1	1	1	0.0005	0.005	1	0.005	1
G-CSF	1	1	1	1	1	0.05	1	1	1	1	1	1	1
IFN-G	1	1	1	1	1	1	1	1	1	1	1	1	1
IL-10	1	0.05	1	1	1	0.05	1	1	1	1	1	1	1
MCP-3	1	1	1	1	1	1	1	1	1	1	1	1	1
MDC	0.0005	0.0005	0.0005	1	0.005	1	0.0005	0.0005	1	0.05	0.0005	0.0005	0.0005
IL-15	1	1	1	1	1	1	1	1	1	1	1	1	1
IL-17A	1	1	1	1	1	1	1	0.05	1	1	1	1	1
IL_1RA	1	1	1	1	1	1	0.05	0.005	1	1	1	1	1
IL-1a	1	1	1	1	0.05	1	1	0.05	0.005	0.0005	0.0005	1	1
IL-1B	0.0005	0.0005	1	1	1	1	0.0005	0.005	1	1	1	1	1
IL-3	1	1	1	1	1	1	1	1	1	1	1	1	1
IL-4	1	1	0.05	1	1	1	1	1	1	1	1	1	1
IL-6	1	1	1	1	1	1	1	1	1	1	1	1	1
IP-10	1	1	1	1	1	1	1	0.0005	1	1	0.0005	0.0005	0.0005
MIP-1a	1	1	1	1	1	1	1	1	1	1	1	1	1
MIP-1B	1	1	1	1	1	1	1	1	1	1	1	1	1
RANTES	0.0005	0.0005	1	1	1	1	0.0005	0.0005	1	1	0.0005	0.0005	0.0005
TNF-a	0.005	1	0.005	1	0.05	1	1	1	0.05	1	1	1	1
TNFB	1	1	1	1	1	1	1	1	1	1	1	1	1
VEGF-A	0.0005	0.005	0.005	1	0.05	0.05	1	1	0.005	0.0005	1	0.05	0.0005

Normal -> reduction mammoplasty sample
TAT -> tissue adjacent to breast tumor
MCF7 -> estrogen-responsive breast cancer cells
SVF -> stromal vascular fraction (source of MSCs)
Statistically significant
Not significant but interesting secretion profile

The discovery assay consisted of 41 cytokines of which, 22 were chosen for further analysis through Anova table of variance

12/22 cytokines were chosen for further studies

Numbers in each cell represent p-values

Figure 4. Co-cultures with SVF leads to secretion of unique cytokines from healthy and malignant breast cells. SVF, TAT and HBT were made into single cell suspensions and cultured in a growth factor reduced 3-Dimentional Matrix (Matrigel) for 10 days. Media from all cultures was analyzed and quantified for 41 different cytokine concentrations using high throughput ELISA assay.
(a) Secretion profiles of SVF+TAT specific cytokine TGF-α in Matrigel cultures. When SVF was co-cultured with TAT there was a significant decrease in the secretion of TGF-α when compared to TAT and SVF cultures alone. Statistical Significance: SVF+TAT vs TAT ($P<0.05$). SVF+TAT vs. SVF ($P<0.05$).
(b) Secretion profiles of SVF+HBT specific cytokine GRO in Matrigel cultures. When SVF was co-cultured with HBT there was a significant increase in the secretion of GRO pan cytokines when compared to HBT and SVF cultures alone. Statistical Significance: SVF+HBT vs HBT ($P<0.05$). SVF+HBT vs. SVF ($P<0.05$).

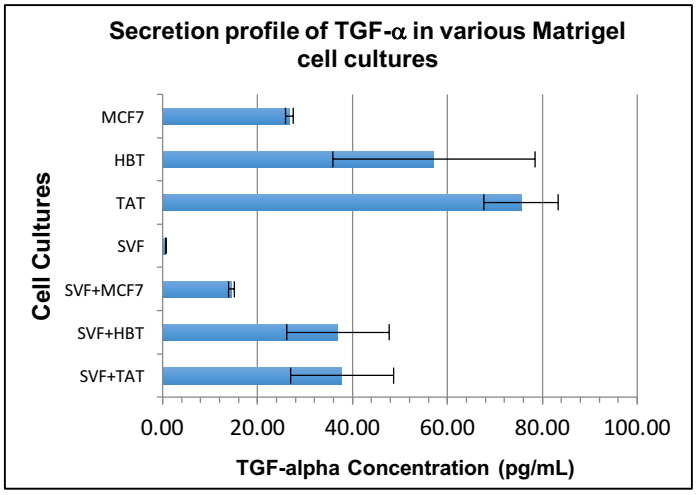
(**c) Secretion profiles of SVF+HBT specific cytokine VEGF-A in Matrigel cultures.** When SVF was co-cultured with HBT there was a significant decrease in the secretion of VEGF-A when compared to HBT and SVF cultures alone. Statistical Significance: SVF+HBT vs HBT ($P<0.05$). SVF+HBT vs. SVF ($P<0.05$).

(d) Secretion profiles of SVF+MCF-7 specific cytokine MDC in Matrigel cultures. When SVF was co-cultured with MCF-7 there was a significant increase in the secretion of MDC when compared to MCF-7 and SVF cultures alone. Statistical Significance: SVF+MCF-7 vs MCF-7 ($P<0.0005$). SVF+MCF-7 vs. SVF ($P<0.0005$).

(f) Secretion profiles of SVF+MCF-7 specific cytokine IL-1β in Matrigel cultures. When SVF was co-cultured with MCF-7 there was a significant increase in the secretion of IL-1β when compared to MCF-7 and SVF cultures alone. Statistical Significance: SVF+MCF-7 vs MCF-7 ($P<0.005$). SVF+MCF-7 vs. SVF ($P<0.005$).

(g) Secretion profiles of SVF+MCF-7specific cytokine RANTES in Matrigel cultures. When SVF was co-cultured with MCF-7 there was a significant increase in the secretion of RANTES when compared to MCF-7 and SVF cultures alone. Statistical Significance: SVF+MCF-7 vs MCF-7 ($P<0.0005$). SVF+MCF-7 vs. SVF ($P<0.0005$).

Figure 4a.



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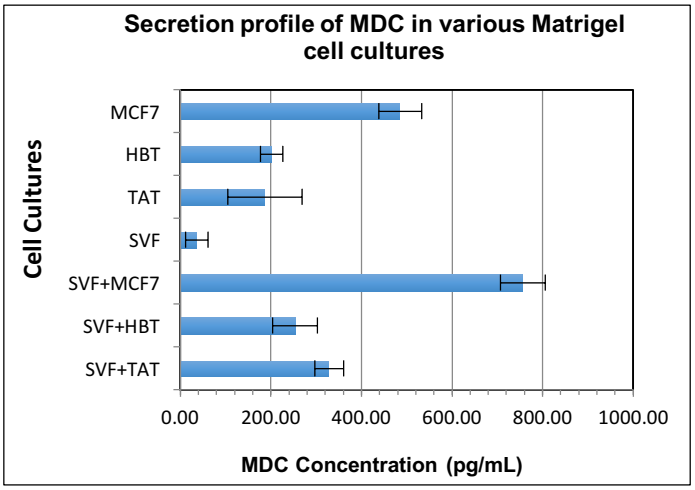

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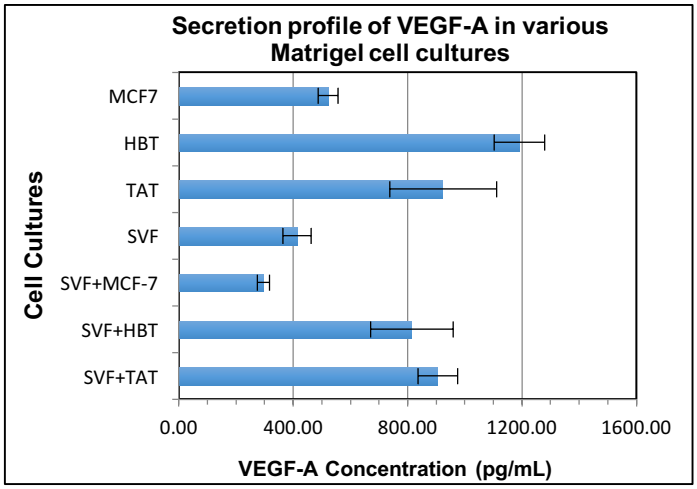
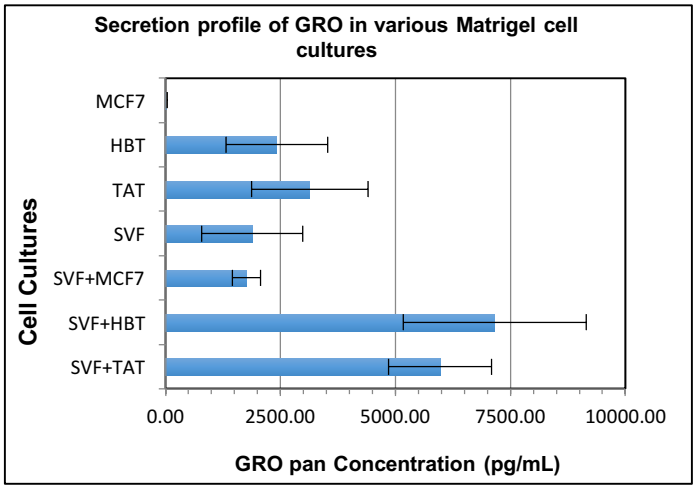


Figure 4d.



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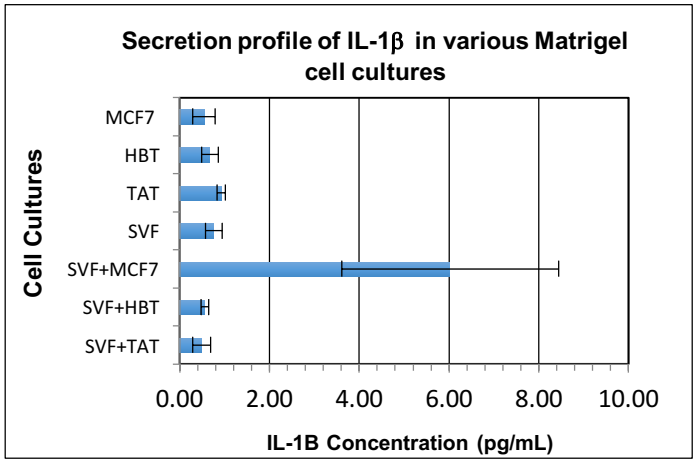

Figure 4f.

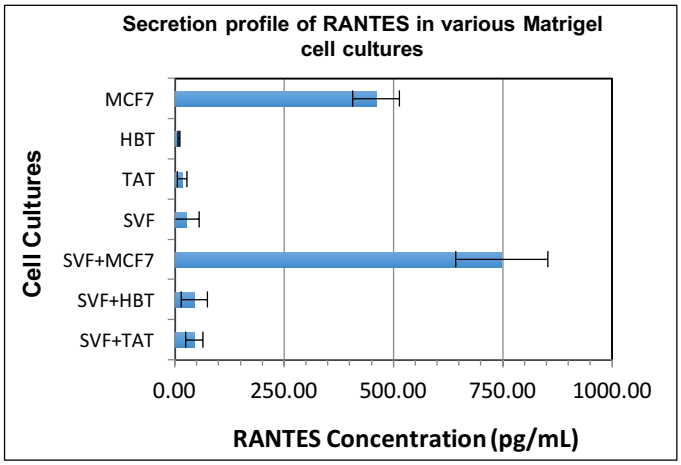

Figure 5 Effects of MCF-7+SVF specific cytokine IL-1β, MDC and RANTES on MCF-7 cell proliferation. $5.0x10^4$ MCF-7 cells were made into single cell suspensions and cultured in a growth factor reduced 3-Dimentional Matrix (Matrigel) for 10 days. IL-1β, MDC and RANTES were added at 5ng/200mL to cell cultures. The control contained only $5.0x10^4$ MCF-7 cells and PBS, the vehicle used for IL-1β, MDC and RANTES. The cultures were subsequently analysed for total cell numbers using FACS. Statistical significance: P-values for the comparisons of MCF-7+IL-1β, MCF-7+MDC and MCF-7+RANTES vs MCF-7 were 0.0081, 0.0076 and $4.47x10^{-6}$ on day 10 respectively.