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Project Title: The Stromal Vascular Fraction of Autologous Fat Graft Induces Proliferation of Normal and Tumour-Adjacent Breast Tissue

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SUMMARY: (no more than 250 words single spaced)

Autologous fat grafting is frequently used in breast cancer patients post mastectomy. Progenitor cells contained in Stromal Vascular Fraction, herein known as SVF, may have proliferative effects on breast parenchymal cells. To our knowledge, no study has looked at the direct effect of SVF on breast parenchymal cells outside the tumor free margins on mastectomy specimens. This study investigates the role of autogenous fat graft SVF on the growth of normal and breast cancer adjacent parenchymal cells. Methods: Samples (n=4) of abdominal fat and histologically normal breast tissue both from a tumour-free breast and from a cancerous breast outside the tumour-free margins were obtained. The presence of multipotent mesenchymal stem cells (MSCs) within the SVF cell population was assessed using established cell surface marker and differentiation assays. Next, the presence of progenitor cells in normal breast and breast tumour-adjacent parenchymal samples were quantified using a colony-forming cell (CFC) assay. SVF cells and both populations of breast parenchymal cells were cultured together in Matrigel, with a control culture lacking SVF. After 14 days, the total cell numbers and breast progenitor cell populations from each culture group were quantified using CFC assays. Results: Differentiation assays demonstrated presence of MSCs and vascular endothelial progenitor cells in SVF samples. Cultures of normal breast parenchymal cells with SVF led to a 3.35-fold expansion of breast progenitors. Cultures of breast tumour-adjacent parenchymal cells with SVF led to a 9-fold expansion of breast progenitors, compared to a 2-fold expansion when cultured alone. Conclusion: SVF is capable of increasing the proliferation of breast progenitor cells in both non-tumour and tumour-adjacent breast tissue. This increase is larger in the tumour-adjacent tissue. As a result, this study demonstrates a significant interaction between SVF within autologous fat grafts and cancer-adjacent breast tissue.

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Introduction and Background

Breast cancer accounts for one-quarter of all cancer diagnoses in women and its prevalence is known to increase with age [1]. Although many advances in therapy have been developed, including chemotherapy and hormone therapy, breast cancer is still a major health care concern in the world today as it is becoming more prevalent. Not only is it emotionally and mentally taxing for those diagnosed, but it is estimated that 5000 women in Canada will die in the year 2014 from breast cancer [1]. Upon diagnosis, these women are faced with many decisions: Mastectomy or lumpectomy? Using adjuvant therapies, including radiation or chemotherapy? Cosmetic implants or other surgical reconstruction? All of these options are given in the hopes that they can clear the body of the cancer and that the recurrence of the disease can be prevented.

Once the breast parenchyma has been surgically removed from the breast cavity, and hopefully most of the cancer cells with it, women have the option to receive silicon implants (the cosmetic route), skin or nipple-sparing reconstruction, superficial or deep inferior epigastric perforator free flap reconstruction (SIEP/DIEP procedure), additional autologous fat grafting, or simply no reconstruction at all [2]. As was aforementioned, the use of surgical reconstruction and cosmetic implants is one of the options for women undergoing mastectomy; the use of surgical reconstruction using autologous tissue paired with a mastectomy for a woman diagnosed with breast cancer has become increasingly advanced as surgical training improves. As microsurgery is becoming a more frequently used technique, so is the use of SIEP or DIEP procedure, wherein the surgeon removes the patients' abdominal fat, while being careful to maintain the blood vessels intact, removing it from the abdomen and transporting it to the breast cavity [2]. The main difference between the SIEP and DIEP procedures are the types of vessels used to supply blood flow to the new breast. The SIEP vessels are not found in all women but are usually the first choice, as they run superficially in the fatty tissue, just below the skin, whereas the DIEP vessels are as the name states-deep. These vessels run deep to the abdominal muscle layer and require a more technical surgical hand to be recovered [3]. Once the flap has been detached, surgeons anastomose the blood vessels with mammary vessels so that the flap can survive with its new blood supply. With this procedure "patients receive the dual benefit of restoration of the breast form and an improvement in contour of their lower abdomen" [2]. Figure I shows the results before and after this procedure.

The above flap reconstruction is often followed by the use of autologous fat grafting for resurfacing contour defects post-mastectomy. Fat grafting is completed by first removing fat via liposuction from the patients' abdomen or thighs. Once the lipoaspirate has been collected, it is then centrifuged to separate the oily top layer and the blood cell bottom layer from the fat cells located in the middle [2]. This middle layer is then transferred into small syringes with a beveled needle attached to it, then injected slowly into a well vascularized area of tissue in order to ensure a maximum graft take [2]. This can be done multiple times at different sites in the breast to ensure an even distribution with the newly grafted fat cells obtaining a maximum surface area. Figure II shows this technique. The use of the above fat grafting procedure has become increasingly more accepted over the last ten years as fat is a seemingly ideal filler material: it is autologous, soft, and readily available in many patients. However, it is not inert [4]. Fat tissue has been shown to harbor a rich population of both multipotent mesenchymal stem cells and vascular progenitor cells.

Multipotent mesenchymal stem cells (MSCs), collectively and herein referred to as adipose-derived stem cells (ASCs), are progenitor cell populations that can be enriched in the stromal vascular fraction (SVF), found in the infranate resulting from high force centrifugation of fat

tissue. Recent studies by Mandel *et al.* have shown an interaction between multipotent mesenchymal stem cells (MSCs) found in SVF, and breast tumour cells that may result in growth stimulation of breast cancer cells *in vitro* [5]. These results raise concern about the possibility of interaction between ASCs in grafted fat and remnant breast cancer cells in the parenchyma outside of the surgical margins. These interactions could also have a part in wound healing or may contribute to an increase in the proliferation of the remaining cancer cells; to our knowledge, no study has looked at the direct effect of ASCs and breast parenchymal cells derived from tissue surrounding a known cancer. A comparison of the effect of ASCs on tumour-free and tumour-adjacent breast parenchymal cell proliferation needs to be done alongside looking at the effect of ASCs on tumour-adjacent parenchymal growth in order to observe the effects of these stem cells.

As fat grafting is a widely practiced procedure performed by many plastic surgeons, and the interaction between the fat tissue transplanted and the cells in the environment into which it is being transplanted is not well studied, no one knows the true risks or benefits of these interactions. Could there be a negative clinical effect to this practice, and if so, could this change the amount of fat grafting procedures done? Safety of the patient is of number one concern for all physicians alike and knowledge of these interactions could contribute to that safety. Our study investigates whether ASCs can influence the behavior of breast progenitor cells derived from tumour-free and tumour-adjacent breast tissue *in vitro*. We want to know if the ASCs interact in a negative way with the breast parenchyma containing microscopic cancer cells left behind post-mastectomy; interactions such as increasing the risk of recurrence or altering the length of remission that the patient experiences are examples of the negative effects that we want to prevent. The role of wound healing is also a considered interaction in both normal and cancerous breast tissue. In order to do this, the purpose of our study will be to determine the effect of abdominal tissue-derived ASCs on the number and characteristics of breast progenitor cells isolated from both tumour-free and tumour-adjacent breast parenchyma and then relate these results to the breast tissue *in vivo*. From here the study will be continued in order to expand our study patient population and continue researching the effects of ASCs on human breast tissue, including why this proliferation is occurring and if there is a way to stop it.

Material and Methods

Tissue Sample Collection

Samples of abdominal fat and 'histologically normal' breast tissue (8cm beyond the tumour-free surgical margins) were obtained from four (n=4) consented patients. These samples were obtained from the operating room at the time of surgery: the adipose was obtained directly from the patients' abdomen and the breast was delivered to pathology as a whole specimen. In the pathology department, the head pathologist, Dr. Janice Safneck, sectioned the breast using sterile technique, ensuring that a new blade was used when slicing tumour versus non-tumour invaded parenchyma. Sections of breast parenchyma were collected termed *Near Surrounding Breast Parenchyma (Near SBP)*, indicating that it was collected approximately 3cm from the tumour outside the tumour margins, and *Far SBP*, indicating that it was at least 5-8cm from the tumour. See Figure III. A section of the tumour itself was also obtained, however due to the limitation in knowledge of tumor growth *in vitro* I did not handle the tumour beyond the digestion stage. A non-tumour containing breast was collected in the same manor from reduction mammoplasty procedures and the breast parenchyma from these samples was used as a control or *Normal Surrounding Breast Parenchyma (NSBP)*.

Isolation of Adipose-Derived Stem Cells

Samples of adipose tissue were digested using an established protocol [6-modified, 7]. Figure IV correlates with the following description. Briefly, tissue was washed with PBS solution and then minced finely using a scalpel. Next, the now minced sample was digested in a mixture of collagenase and hyaluronidase dissolved in DMEM for approximately 17 hours at 37 Celsius while being agitated constantly. The remaining suspension was then removed from the incubator and centrifuged at 300g (1400rpm) to separate the stromal cells from adipocytes. The oily supernatant consisting of adipocyte products was discarded. The infranate, referred to as the *Stromal Vascular Fraction (SVF)*, was re-suspended in red blood cell lysis buffer (160 mM NH₄Cl). Next it was serially centrifuged at 300g and re-suspended in Hank's buffered salt solution (HBSS) supplemented with 2% v/v fetal bovine serum (FBS). The final cell product contained in the infranate was re-suspended in growth media (10% v/v FBS in DMEM + 1% v/v penicillin-streptomycin) and cultured in 10cm plates (see below).

Isolation of Breast Parenchymal Cells

Samples of breast parenchyma were obtained 3cm and 5-8cm beyond the surgical margins from the same patient as that of the abdominal tissue [7,8]. These breast parenchyma samples were minced and digested using the same protocol as that for fat described above. Post-digestion, the suspension was centrifuged at 750 rpm. The resulting pellet contained epithelial cells, stromal cells, and red blood cells. This pellet was re-suspended and centrifuged again at 1100 rpm. The resulting supernatant (containing breast epithelial cells) was collected and re-suspended. The infranate (containing stromal cells and red blood cells) was discarded. These epithelial cells were considered to be *breast parenchymal cells* and were re-suspended and cultured as described below. Once the tumour-adjacent and normal breast tissue were isolated and grown in culture, Figure V shows the differences between the two populations via immunofluorescent images.

Cell Culture and Maintenance [6, modified]

Once raw tissues were isolated through the digestion process described above, the cells were cultured aseptically in 10 cm culture plates with 10ml of 1X Dulbecco's Modified Eagle's Medium (DMEM) supplements with 10% FBS and 1% penicillin/streptomycin. The number of cells per culture dish is dependent upon the initial cell count. The plates containing cells were then incubated in 5% CO₂ at 37 Celsius and maintained in order to increase cell number. Media changes were done every 3-5 days as needed by aspirating all initial medium and replacing it with 10ml of fresh medium. Once the confluence of the cells reached approximately 80%, we passaged the cells in order to expand our colonies. The plates were washed using PBS to remove the media from the cell surface and then trypsin was added to the plates. Plates were then incubated in 37 Celsius for 10-15 minutes and, once cells were detached, Hanks solution (HBSS) was added to stop the trypsinization process. Next, cells were centrifuged and the remaining pellet was re-plated using DMEM. Passaging and feeding of the cells was continued in order to maintain the cells in culture until an optimal number was reached to move forward with our experiments. Figure VI shows ASCs in culture.

Adipose-Derived Stem Cell Differentiation

Once the ASCs were isolated from tissue and cultured through 3-4 passages, we had to prove their ability to act like stem cells – to differentiate into different cell types. In order to do this we used a differentiation kit and protocol from Lonza and Bourin et al [7] that allowed us to differentiate the cells into 3 different cell lines: (i) adipocytes, (ii) osteocytes, and (iii) chondrocytes. After preparing the media provided by the kit for each cell line, the cells plated at a density of 2.5×10^5 cells/well and then incubated at 37 Celsius and 5% CO₂. Cells were treated according to the protocol provided by the kit; media was changed every 3-4 days to induce the differentiation process and maintain the cells. The differentiation took approximately 21 days

and was done on 3 separate stromal vascular fraction samples in one set, and on 2 stromal vascular fractions in a second set. After 21 days, the cells were harvested for analysis and stained as follows: for adipocytes, the intracellular fat droplets were stained by Oil Red O, for osteoblasts, the calcium within the cells was stained by Alizarin Red and for the chondrogenic pellet differentiation, pellets were formalin fixed and paraffin embedded for histological processing or frozen sectioning. Thin sections were slide-mounted and immunostained for collagens by toluidine blue to identify the chondrocytes.

Co-Culture of Adipose-Derived Stem Cells and Tumour-adjacent Breast Parenchymal Cells

Single cell suspensions of ASCs, tumour-free breast parenchymal cells, and tumour-adjacent breast parenchymal cells were inoculated into wells of a 96-well plate pre-treated with *Matrigel*TM, a commercially available three-dimensional basement membrane matrix. Cultures were maintained for 14 days, after which, cells were harvested with 0.05% trypsin-EDTA and enumerated [9]. The number of breast progenitor cells within the resulting parenchymal cell population was then enumerated using established colony forming cell (CFC) assays (below). Control cultures consisted of (i) tumour-adjacent breast parenchymal cells alone and (ii) tumour-adjacent breast parenchymal cells with culture plates pre-treated with *Matrigel*TM. Note that a co-culture of normal breast tissue and adipose derived stem cells was also preformed the same as above, and further experimentation discussed below was also done on our normal breast tissue.

CFC Assay [7]

Initially we performed a cell count by taking 10uL of cells and combining them with 90uL trypan blue. 10uL of the previous mixture was then placed on a slide and counted by the automatic hemocytometer. Based on these numbers we knew how many cells were present and they were added to 6-well plates in 4 densities: at 5, 2, 1 and 0.5×10^3 cells/well. Note cells could also be plated at the aforementioned densities in 10cm plates. 2ml of complete MesenCult media was added to each well. The cells were incubated for 14 days in the 37 Celsius, 5% CO₂ incubator in order to obtain a maximum colony size for counting. Once the 14 days was completed, the cells were then stained and scored as described below.

Staining and Enumeration of CFU-F Derived Colonies

Medium was removed from the culture wells and then the wells were washed with PBS. The cells were then fixed to the surface of the plates with 0.5ml of methanol. Methanol was removed after 5 minutes and the dishes were left to dry at room temperature overnight. Next, a mixture of 0.25mL water and 0.25 of crystal violet stain was added to the culture wells, swirled around to evenly coat, and left for 3-5 minutes. The crystal violet stain was then removed and the plates were rinsed with distilled water to remove excess stain. The plates were then rinsed until the water ran clear and left to dry. Once dry, we observed the plates under the microscope and manually scored the colonies. One mesenchymal breast tissue progenitor will provide one colony, so by counting the colonies we are able to count the number of mesenchymal stem cells. A colony must contain > 50 cells to be scored. Once plates were scored a comparison among samples was done.

Results

Results of Co-Culture of Adipose-Derived Stem Cells and Tumour-Adjacent Breast Parenchymal Cells, and Adipose-Derived Stem Cells and Tumour-Free Breast Parenchymal Cells

After being grown in *Matrigel*TM for 14 days, a functional CFC was performed on (i) tumour-adjacent breast tissue as well as on (ii) normal healthy breast tissue. Upon completion we were able to reveal that the tumour-adjacent breast tissue had less progenitors than that of the tumour-free tissue, with 5.56×10^2 versus 2.876×10^4 progenitors respectively. We then cultured

both populations with ASCs. The addition of ASCs to the normal tumour-free breast parenchyma was shown to induce epithelial breast cell proliferation; a 3.35-fold increase in the number of breast progenitor cells was observed. The tumour-adjacent breast parenchymal cell population was also cultured in the presence of ASCs, which gave an unsuspected result – a 9-fold increase in the number of breast progenitor cells was observed in the co-cultured population compared to a 2-fold increase in the co-cultured control population containing no SVF stem cells and the 3.35-fold increase mentioned above for the normal tissue (Figure VII). It is evident that the addition of ASCs to breast parenchymal tissue causes an increased proliferation of the breast cells with increased number of progenitors. This also indicates that in the presence of tumour-adjacent tissue, the breast cell proliferation is increased compared to the normal breast tissue. (Figure VIII).

Images of the primary cell cultures of both human adipose-derived stem cells (ASCs) alone and with tumour-adjacent breast parenchymal cells are shown in Figure IX (a) and (b), respectively.

Results of CFC Assay:

ASCs generate colonies that resemble fibroblasts and were scored manually by microscope obtaining the following data: 500, 1000, 2000 and 5000 Ad-MSCs have generated 11.3 ± 3.5 , 22.3 ± 2.08 , 33.3 ± 0.57 and 55 ± 6.55 CFUs respectively. From this we can show that the colony forming frequency for ASCs is between 1.1-2.2% (n=3). As the number of cells seeded onto the plate increases, so does the number of CFCs (Figure X). This data is in well correlation with recently published study which identifies the presence of >1% of CFU forming cells in adipose derived cellular fraction.

Results of Differentiation Assay:

The adipogenic and osteogenic cell lines were successfully achieved in the differentiation process of the adipose-derived stem cells (see Figure XI a and b). Chondrogenic differentiation assay is still underway as sectioning has not yet occurred.

Discussion:

Multiple studies have been performed to investigate the influence of ASCs on breast cancer cell growth. To our knowledge, no studies have looked at the effect of ASCs on the breast parenchyma *adjacent* to a tumour. We have found that human mesenchymal stem cells derived from adipose tissue (ASCs) promote progenitor cell proliferation in both normal breast tissue as well as tumour-adjacent breast tissue. We examined the effect of the ASCs on both the aforementioned sample groups compared to each other, and also compared to groups with no stem cells present. In our study, greater expansion of breast progenitor cells was achieved in the presence of ASCs than when cultured alone. Upon comparing a normal breast reduction sample, known to be tumour-free, to a tumour-adjacent sample, we found that there was a greater increase in the breast parenchymal cell proliferation in the tumour-adjacent sample than in the normal, about a 2-fold increase. From this we concluded that compared to the normal breast progenitor cells, the tissue in the tumor-adjacent breast showed enhanced expansion potential of the cells. The presence of *Matrigel*TM did not appear to influence the growth of breast progenitor cells. A precise mechanism explaining greater proliferation of breast progenitor cells in the presence of ASCs is unclear, however suspicion of either direct cell-to-cell contact or the secretion of paracrine factors is noted.

Our results therefore demonstrate that ASCs present in grafted fat derived from abdominal tissue *can* increase cell turnover in breast parenchyma adjacent to a known malignancy, as well as in breast parenchyma deemed histologically normal. The fact that there is a greater increase in the proliferation in a breast with malignant cells present could have clinical implications regarding tumor regrowth or increased cancer cell proliferation. This could play a role in cancer

recurrence rates, and if this increase is of clinical significance, it could limit the use of fat grafting post-mastectomy. The influence of ASCs in breast epithelial progenitor cell proliferation could also have implications towards breast tissue regeneration or wound healing taking place post-mastectomy. Further investigation with higher number of samples is needed before concrete conclusions can be drawn, however it is evident that the presence of stem cells derived from fat grafted material increase the growth in both normal breast parenchymal cells *and* tumour-adjacent breast parenchymal cells, with the growth in parenchyma exposed to tumour being significantly larger.

References:

1. Canadian Cancer Society's Advisory Committee on Cancer Statistics. (2014). *Canadian Cancer Statistics 2014*. Toronto, ON: Canadian Cancer Society.
2. Ballard TNS, Momoh AO. Advances in Breast Reconstruction of Mastectomy and Lumpectomy Defects. *Surgical Oncology Clinics of North America*. July 2014; 23(3): 525-548
3. Janis J. Fundamentals of Perforator Flaps. In: Janis J, editor. *Essentials of Plastic Surgery*. Second Ed. USA: CRC Press; 2014. p.45-65
4. Largo RD, Tchang LAH, Mele, V, Scherberich A, Harder, Y, Wettstein R, et al. Efficacy, safety, and complications of autologous fat grafting to healthy breast tissue: A systematic review. 2014. *JPRAS*, 67(4):437-448.
5. Mandel K, Yang Y, Schambach A, Glage S, Otte A, Hass R. Mesenchymal stem cells directly interact with breast cancer cells and promote tumor cell growth *in vitro* and *in vivo*. *Stem Cells Dev*. 2013; 22(23): 3114-2127.
6. Bunnell B, Flaata M, Gagliardi C, Patel B, Ripoll C. Adipose-derived Stem Cells: Isolation, Expansion and Differentiation. National Institutes of Health-Public Access. June 2008; (45)2: 115-120. *Modified*
7. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KJ. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy*. 2013; 15(6): 641-648
8. Raouf A, Zhao Y, To K, Stingl J, Delaney A, Barbara M, Iscove N, et al. Transcriptome Analysis of the Normal Human Mammary Cell Commitment and Differentiation Process. *Cell Stem Cell* 3. July 2008: 109-118
9. Lequeuz C, Oni G, Wong C, Damour O, Rohrich R, Mojallal A, et al. Subcutaneous Fat Tissue Engineering Using Autologous Adipose-Derived Stem Cells Seeded onto a Collagen Scaffold. *Plastic and Reconstructive Surgery*. Dec 2012; 130(6): 1208-1217.

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Figure I: The right breast on the top has undergone reconstruction with an abdominal flap post-mastectomy. The bottom shows the cosmetic result of lipofilling, indicating its use in the reconstruction process



Figure II: This image shows a needle filled with centrifuged liposuction fat containing the adipose derived stem cells being injected into a breast for lipofilling.

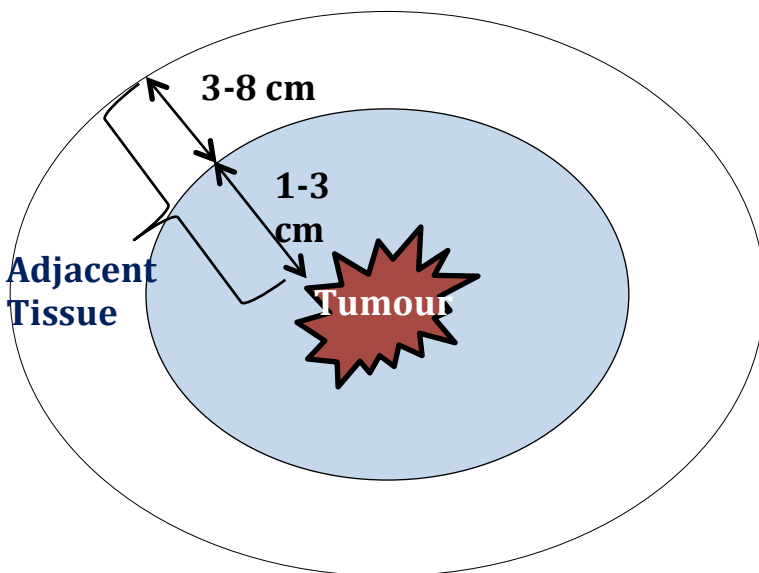


Figure III: This demonstrates a breast and the close and far sample collection sites. You will note that the close (1-3cm) as well as the far (3-8cm) are located outside the indicated tumour margins.

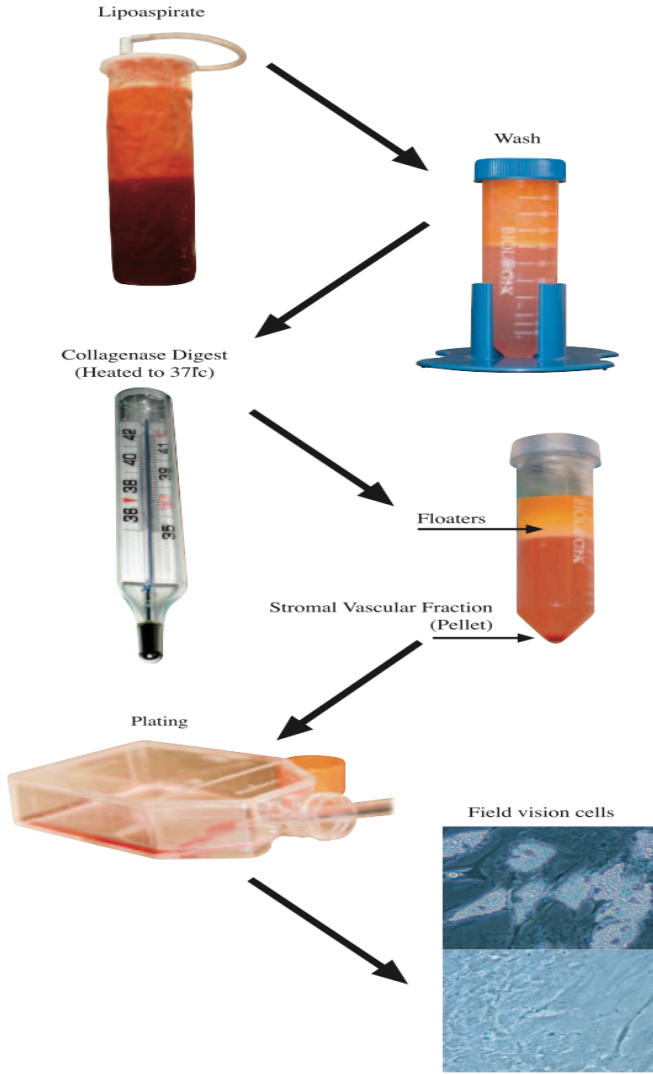
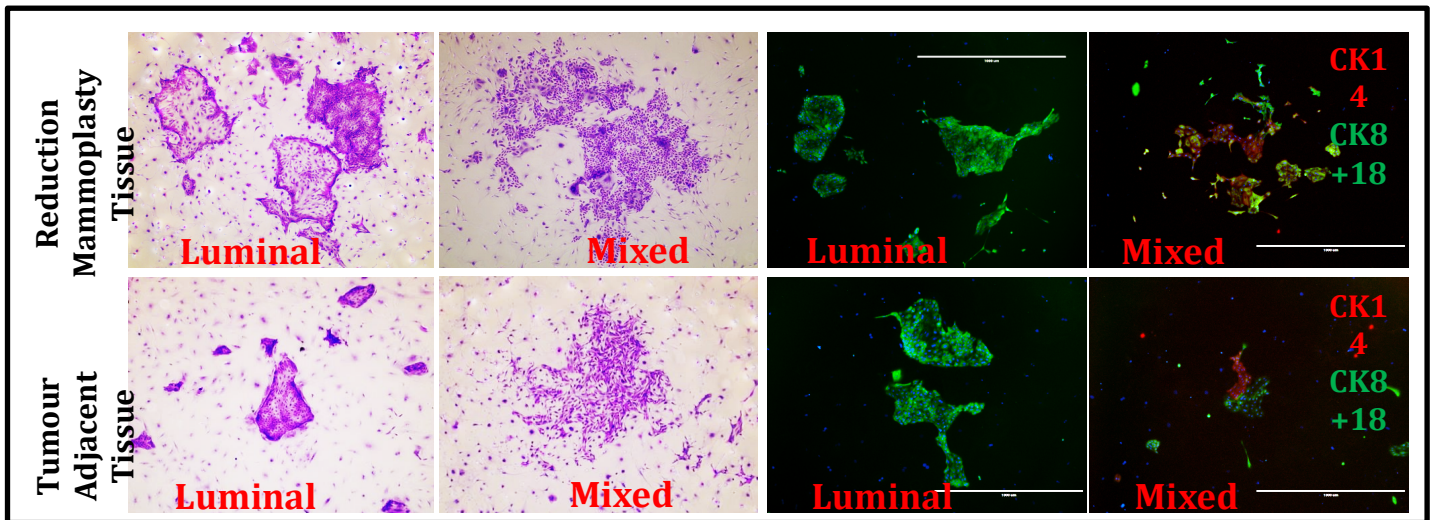


Figure IV: This is a representation of the process used to maintain our cells for culture. The sample could be a lipoaspirate (as shown in the figure) or raw adipose tissue. The sample is then washed with PBS solution and then placed in a shaking 37 degree Celsius incubator with the collagenase digest. The digested sample is then washed and centrifuged, isolated the SVF pellet as shown. The SVF was then plated and allowed to grow, as shown in the final image.

Figure V: (Below) Shows microscopic imaging as well as immunofluorescence tissue surface marker images for reduction mammoplasty tissue (containing no tumour as the control) compared to the tumour-adjacent tissue.



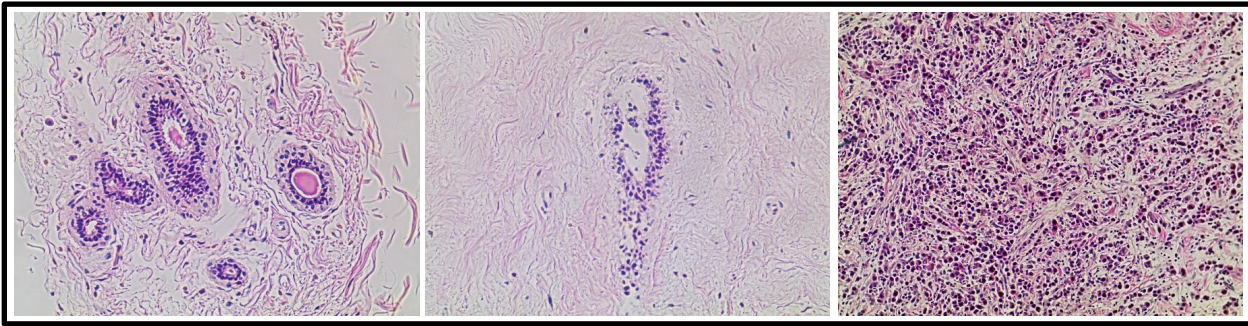


Figure VI: Show the fibroblastic nature of the ASC

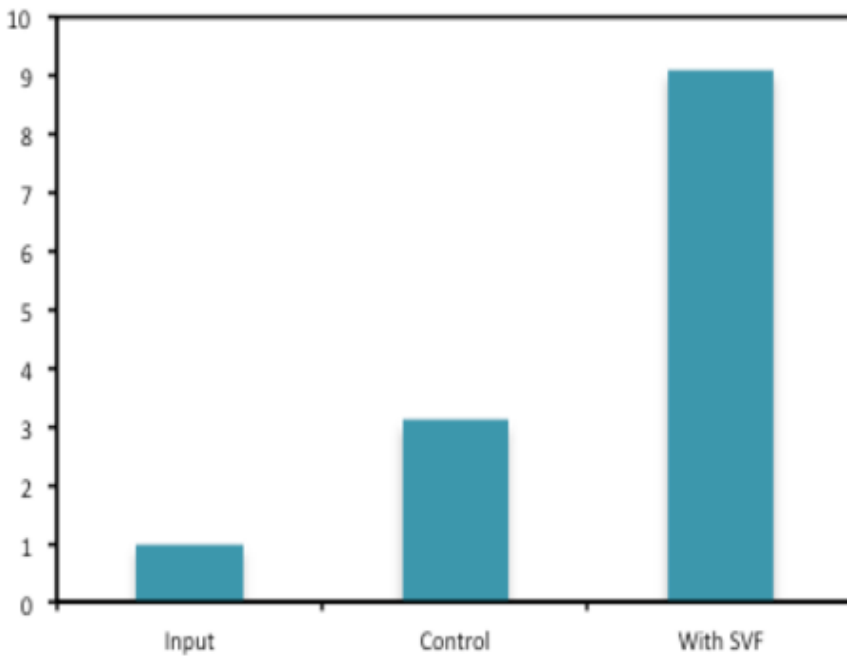


Figure VII: Number of colony forming cells (CFCs) obtained from cultures in (i) control conditions consisting of tumour-adjacent breast parenchymal cells in *Matrigel* alone and (ii) with SVF.

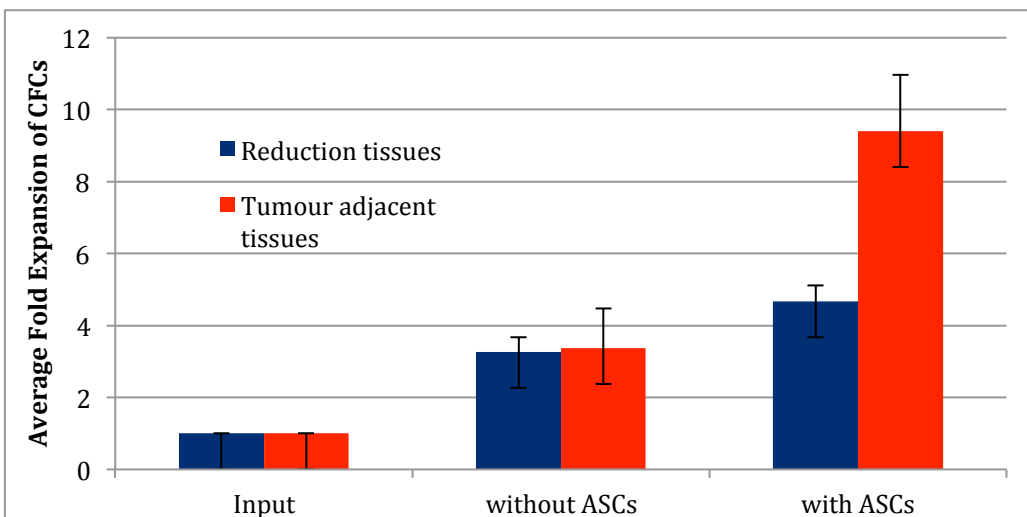


Figure VIII: This shows a comparison of the reduction breast tissue, considered histologically normal, and the tumour-adjacent tissue with and without the addition of adipose-derived stem cells on the effect of colony forming units (breast progenitor cells responsible for proliferation). As you can see, there is an increase in the number of progenitor cells with ASCs added, with a greater expansion in tumour-adjacent tissue.

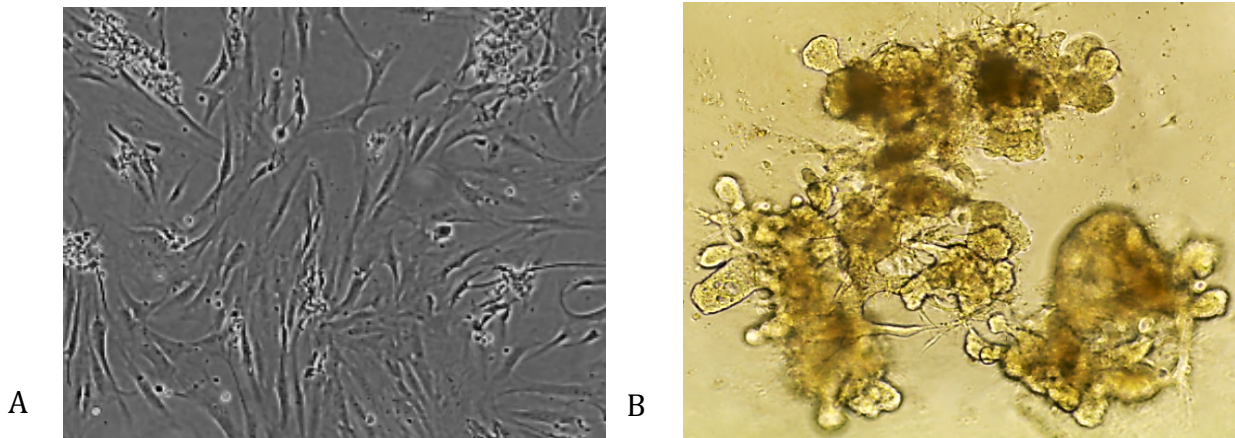


Figure IX: Photomicrographs of (a) primary adherent adipose-derived stem cells, and (b) tumour-adjacent breast parenchymal cells in culture plates pre-treated with *Matrigel™*.

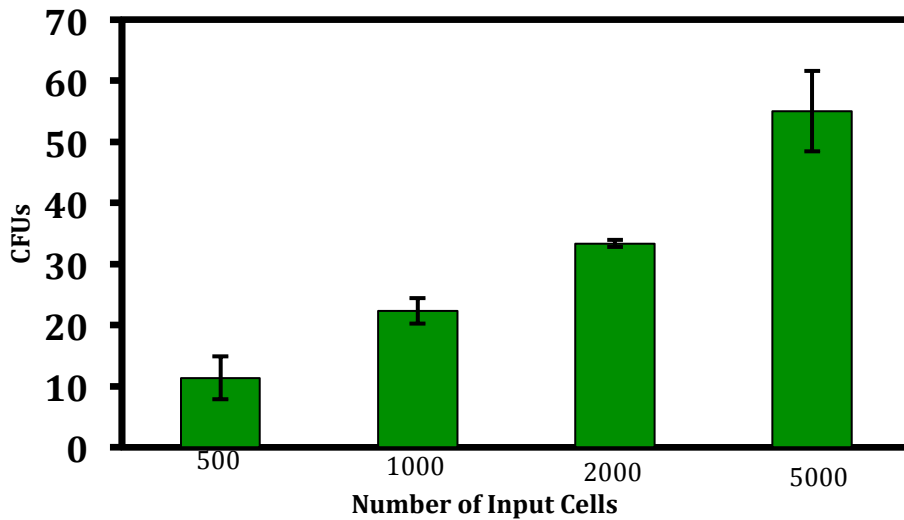


Figure X: This graph shows that the number of colonies counted, indicating the number of breast progenitor cells (CFUs), increases with the number of breast parenchymal cells initially plated. As you can see, the more cells plated, the more progenitor cells produced, leading to expansion of the breast cell population.

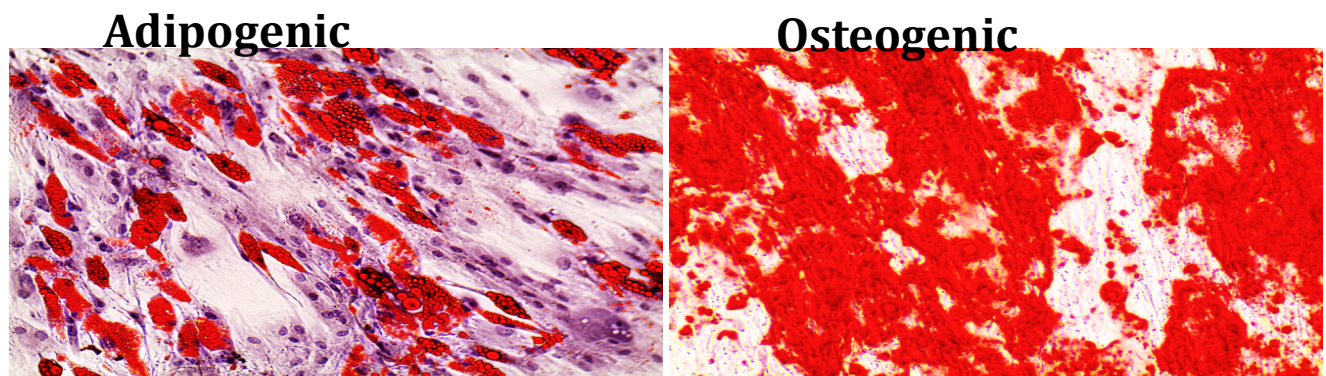


Figure XI: Above are the results of adipose-derived stem cells undergoing differentiation. The cells were able to be successfully induced into adipocytes and osteocytes, indicating that they are functioning stem cells.