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RESEARCH ARTICLE

In vitro interaction between mouse breast cancer cells and mouse mesenchymal stem cells during adipocyte differentiation

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Abstract

Surgical treatment following breast cancer, i.e. lumpectomy and mastectomy, may not efficiently remove all cancerous cells. As such, when mesenchymal stem cells (MSCs) are incorporated into the breast reconstruction process, it is likely that those MSCs will encounter remnant cancerous cells after transplantation into the defect site. The potential interaction between breast cancer cells and MSCs remains unclear. We hypothesized that paracrine interactions might occur between cells and various proteinases, growth factors and other cytokine molecules in the local microenvironment. Conditioned media (CM) from two mouse mammary cancer cell lines (4T1 and 4T07) and one mouse mammary epithelial cell line (NMuMG) were studied in the experimental model. Post-confluent mouse MSCs (D1 cells) were differentiated with an adipogenic hormonal cocktail. Conditioned media from the three cell types did not have an inhibitory effect on D1 cell viability; however, triglyceride (TG) and Oil red O (ORO) analysis results showed that 4T1-CM significantly inhibited D1 adipocyte differentiation and reduced lipid vesicle accumulation in the differentiating D1 cells. Preliminary analysis of the conditioned media revealed that a higher presence of matrix metalloprotease-9 (MMP-9) and urokinase plasminogen activator (uPA) was present in the 4T1-CM as compared to the levels found in 4T07-CM and NMuMG-CM, which were below the detection limit. Additionally, the conditioned medium of differentiated D1 cells on day 12 had a negative effect on 4T1 and 4T07 cell viability but no effect on NMuMG cell viability. The results suggest that mouse breast cancer cells modulate mouse MSC adipogenic differentiation, the level of modulation specific to the metastatic level. Copyright © 2009 John Wiley & Sons, Ltd.

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1. Introduction

Breast cancer is the most frequently diagnosed cancer in women, and ranks second, after lung cancer, among cancer deaths in women (Jemal *et al*., 2008). Surgical treatment for breast cancer includes lumpectomy or mastectomy, which are often followed by plastic and reconstructive surgery to repair the soft tissue defects resulting from tumour resection (Patrick, 2001). Common breast reconstruction strategies include collagen injections, autologous tissue transfers and breast implants (Ashinoff, 2000; Billings and May, 1989; von Heimburg *et al*., 2003); however, there is no one option that will satisfy all clinical needs. Fat tissue is often found in excess on the human body, and is easily obtained by excision or liposuction. Transplantation of fat tissue, however, has not been consistently successful in patients (Patrick, 2000). Transplanted autologous fat tissue often significantly resorbs over time, resulting in 40–60% loss of graft volume (Patrick, 2004). Tissue volume loss is attributed to insufficient tissue vascularization that limits the supply of oxygen and nutrients to the tissue and, therefore, the long-term tissue survival (von Heimburg *et al*., 2005).

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Tissue-engineering strategies are thus being developed for the generation of adipose tissue. Stem cells offer an attractive cell source for this application (Gomillion and Burg, 2006), where these unique cells are able to provide replacement cells for a specific differentiated cell type (Ballas *et al*., 2002). Stem cells are able to divide and renew over long periods of time and can differentiate into specialized cells (Conrad and Huss, 2005; Spangrude, 2003). Various sources for stem cells include embryonic tissue, bone marrow, adipose tissue, spleen and placenta (Goessler *et al*., 2006; Minguell *et al*., 2001).

Bone marrow is the source of haematopoietic stem cells and mesenchymal stem cells (MSCs) (Arai *et al*., 2005; Vats *et al*., 2002). MSCs are multipotent cells that are easily isolated, easily cultured and readily expanded in the laboratory setting. The D1 mouse bone marrow mesenchymal stem cell line is a useful model that can provide foundational information about the differentiation process *in vitro* (Yang, 2007; Cui *et al*., 1997, 2006; Diduch *et al*., 1993; Li *et al*., 2003). Induced by an adipogenic hormonal cocktail comprised of insulin, isobutylmethylxanthine (IBMX) and dexamethasone, D1 cells will differentiate into mature adipocytes capable of producing triglyceride-containing vesicles and enhanced expression of aP2 (fatty acid bind protein 422/aP2) (Hunt *et al*., 1986; MacDougald and Lane, 1995). The 4T1 and 4T07 mouse mammary cancer cell lines share a common origin, but differ in metastatic behaviour (Gao *et al*., 2004; Mi *et al*., 2004). 4T1 cells metastasize to the lung, liver, bone and brain, while 4T07 cells are highly tumourigenic but fail to metastasize.

Cancer cells in stage II or III breast cancer patients have invaded surrounding epithelial tissue of mammary glands and even metastasized distally; consequently, a lumpectomy or mastectomy for treatment will likely not remove all cancer cells. Therefore, mesenchymal stem cells which are used in breast tissue reconstruction may interact with remnant breast cancer cells and resident epithelial cells. The interaction of adipocytes and other cells has important scientific implications in the differentiation process. Stockdale and Levine (1984, 1985), while evaluating the interaction of 3T3-L1 adipocytes and NMuMG mammary epithelial cells, first documented that the interaction of mammary epithelium with adipocytes may be mediated by extracellular components. Many secretory proteins have been evaluated as potential adipogenic inhibitors including multiple pro-inflammatory cytokines (IL-1, IFNγ, TGFβ and TNFα) (Button *et al*., 2001; Gregoire *et al*., 1992; Richardson *et al*., 1992; Sparks *et al*., 1994). Because reports concerning adipogenic inhibition by vascular endothelial growth factor (VEGF) and MMPs are lacking, we selected these markers for evaluation. Specifically, we examined the effect of mouse cancer cells on D1 cell viability and cell differentiation, and we examined the effect of D1 cells on the viability of mouse cancer cells and epithelial cells.

2. Materials and methods

2.1. Cell culture

Mouse bone marrow MSCs (D1), mouse metastatic mammary cancer cells (4T1), mouse non-metastatic mammary cancer cells (4T07) and mouse mammary epithelial cells (NMuMG) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; ATCC) containing 10% heat-inactivated fetal bovine serum (FBS; Mediatech Inc., Herndon, VA, USA), with 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Gibco/Invitrogen Corp., Grand Island, NY, USA) in a humidified, 5% CO_2 incubator at 37 °C. Insulin was added to the culture medium of the NMuMG cells according to the ATCC product specifications, for a final insulin concentration of 10 μg/ml. Every 2–3 days, cell culture medium (CCM) was replaced with supplemented DMEM.

2.2. Conditioned medium collection

The 4T1, 4T07 and NMuMG cells were seeded at 1.3×10^4 cells/cm^2 in individual 75 cm^2 culture flasks and grown to confluence. After the cells reached confluence, the cells were cultured with DMEM for an additional 24 h. The conditioned medium was subsequently collected and termed 4T1-CM, 4T07-CM, NMuMG-CM, respectively. The cells were trypsinized and counted. The volume of conditioned medium was adjusted with DMEM according to cell number. The conditioned medium was stored at – 80 °C and warmed to 37 °C before use. Prior to application, the conditioned media were mixed with an equal volume of cell culture medium containing 20% FBS, in order to obtain a final FBS concentration of 10%.

D1 cells were seeded at the same density in 75 cm^2 culture flasks and grown to confluence (where the time at which the cell culture reached confluence was denoted as day 0). The post-confluent D1 cells were further cultured with an adipogenic hormonal cocktail that contained 0.5 mM 3-isobutyl-1-methylxanthine, 100 μg/ml bovine insulin and 0.5 μM dexamethasone (Pittenger *et al*., 1999) for 2 days, followed by 2 days of culture with insulin additive only (cell culture medium containing 100 μg/ml bovine insulin), then 8 days with cell culture medium only (Figure 1). The day before the conditioned medium was collected, the medium on the D1 cells was changed and refreshed with DMEM. The conditioned media collected on days 6 and 12 were termed D1-d6-CM and D1-d12-CM, respectively. The conditioned media were stored at – 80 °C and warmed to 37 °C before use.

2.3. Cell viability assay

Cell viability was measured quantitatively using an alamarBlue assay (BioSource International Inc., Camarillo,

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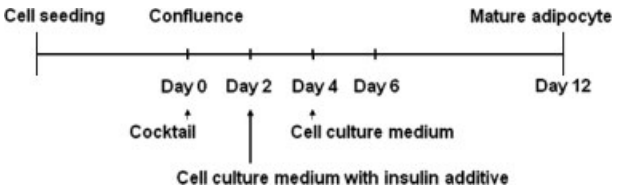

Figure 1. Experimental flowchart detailing the induction of D1 cells to adipocytes

CA, USA) (Ahmed *et al.*, 1994). Briefly, 100 µl water-soluble alamarBlue reagent was added to each well and the samples were incubated for 2 h on an orbital shaker. A volume of 150 µl of each sample was pipetted into individual wells of a clear, flat-bottomed 96-well microplate. The fluorescence was read with excitation wavelength of 544 nm and emission wavelength of 590 nm using an ELISA reader. All samples were assayed in triplicate. The results were reported as the cell viability percentage (average OD/average negative control OD) ± standard deviation (SD).

Three cell viability experiments were conducted as follows. First, the effect of 4T1-CM, 4T07-CM and NMuMG on pre-confluent D1 cell viability was investigated. D1s were seeded in 24-well plates at a density of 1.3×10^4 cells/cm^2/well with 1 ml culture medium and then incubated overnight at 37 °C and 5% CO_2. D1 cells were first cultured with DMEM cell culture medium, 4T1-CM, 4T07-CM or NMuMG-CM, respectively, for 4 days, when confluence (approximately 1.2×10^5 cells) was reached. D1 cells cultured with cell culture medium were defined as the controls. Cell viability was determined after 2 and 4 days of culture, using an alamarBlue assay (BioSource International, Inc., Camarillo, CA, USA) (Ahmed *et al.*, 1994).

Second, the effect of 4T1-CM, 4T07-CM and NMuMG-CM on post-confluent D1 cell viability was investigated. D1 cells were seeded in 24-well plates at a density of 1.3×10^4 cells/cm^2/well with 1 ml culture medium, then incubated overnight at 37 °C and 5% CO_2 and grown to confluence in 3–4 days. Post-confluent D1 cells (day 0 designated at confluence) were induced to differentiation with an adipogenic hormonal cocktail and grouped into four treatments, including cells cultured with DMEM cell culture medium, 4T1-CM, 4T07-CM or NMuMG-CM for 12 days. D1 cells cultured with DMEM cell culture medium were designated as controls. alamarBlue assays were conducted at days 6 and 12 post-confluence.

Third, the effect of conditioned medium from D1 cells on the viability of 4T1, 4T07 and NmuMG cells was investigated. Three cell lines, i.e. 4T1, 4T07 and NMuMG cells, were seeded in 24-well plates at 1.3×10^4 cells/cm^2/well with 1 ml culture medium, and then incubated overnight at 37 °C with 5% CO_2. The cells were subsequently cultured with DMEM cell culture medium, D1-d6-CM or D1-d12-CM, respectively, for 4 days at which time cell viability assays were conducted.

2.4. Induction of D1 cell adipocyte differentiation

The effects of three different conditioned media on adiopogenic differentiation of D1 cells were investigated. D1 cells were seeded in 24-well plates at a density of 1.3×10^4 cells/cm^2 in each well with 1 ml culture medium, then incubated overnight at 37 °C and 5% CO_2, and grown to confluence in 3–4 days. Post-confluent D1 cells (day 0 designated at confluence) were induced to differentiation with an adipogenic hormonal cocktail and grouped into four treatments, including cells cultured with DMEM cell culture medium, 4T1-CM, 4T07-CM or NMuMG-CM, respectively, for 12 days. Oil Red O (ORO) and triglyceride (TG) analyses were conducted at post-confluence days 3, 6, 9 and 12 to evaluate adipogenesis.

The effect of 4T1-CM on lipid accumulation of differentiating D1 cells was also investigated. D1 cells were seeded in 24-well plates at a density of 1.3×10^4 cells/cm^2 in each well with 1 ml culture medium, then incubated overnight at 37 °C and 5% CO_2 and grown to confluence in 3–4 days. Post-confluent D1 cells were cultured in cell culture medium with adipogenic hormonal cocktail for 2 days, followed by a 2 day period with insulin additive in the cell culture medium, then an 8 day culture period with normal cell culture medium in order to induce adipocyte differentiation (Figure 1). At day 6 post-confluence, an experimental group was tested in which the medium was replaced with 4T1-CM for the remaining 6 days. At day 12, ORO and TG levels were measured to evaluate lipid accumulation in the differentiating D1 cells.

2.5. Triglyceride measurement

The amount of triglyceride (TG) produced by the differentiating D1 cells was determined using a Human Adipocyte Differentiation Assay Kit (Zen-Bio Inc., Research Triangle Park, NC, USA). Briefly, the D1 cells were rinsed with Dulbecco's PBS and the liquid aspirated. The cells were subjected to three freeze–thaw cycles and then incubated in 1 ml 1% Triton®-X-100 solution (Sigma-Aldrich) at room temperature for 30 min. After incubation, the wells were scraped with a cell scraper and the Triton solution and cell debris were placed in microtubes to be centrifuged at 3000 r.p.m. for 10 min. Triplicate mixtures of 100 µl cellular lysate and 100 µl triglyceride reagent (Thermo Electron Corp., Melbourne, Australia) were prepared. Meanwhile, glycerol standard solution (Sigma-Aldrich) was used to make a standard curve in the range 0–200 µM. The 96-well plate was incubated for 15 min at room temperature and the absorbances of all samples and standards were detected at 490 nm (Dynex Technology, Chantilly, VA, USA).

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2.6. Oil red O measurements

The lipid produced by differentiating D1 cells was stained with Oil red O (ORO). Briefly, cells were rinsed first with PBS and fixed with 2 ml 10% neutral formalin buffer for 4 h. The fixed cells were washed with distilled water, and then stained with ORO working solution for 20 min at room temperature, using 500 µl in each well of a 12-well plate. The wells were rinsed three times with water and soaked with 500 µl isopropanol/well for 15 min. The red stain, indicating lipid produced by the cells, was extracted by pipetting 500 µl isopropanol in each well for 15 min. Finally 200 µl of the solution in each well was pipetted into a well of a 96-well plate and the absorbance at 530 nm was read.

The ORO working solution was prepared as follows. 0.5 g ORO was dissolved in 100 ml isopropanol and filtered through a 0.22 µm filter membrane to produce an ORO stock solution. Then, ORO stock solution was mixed with distilled water (6 : 4) and filtered again to produce the ORO working solution.

2.7. Cell morphology

Images of the D1 cells were captured using an Axiovert 135 inverted microscope (Zeiss, Germany), Image-Pro 5.0 software (Media Cybernetics, Silver Spring, MD, USA) and a ProRes™ C10^{Plus} digital camera (Chori Imaging Corporation, Yokohama, Japan). All cellular images were photographed at a total magnification of ×320.

2.8. RNA isolation and real time quantitative RT–PCR analysis

Adipocyte differentiation of post-confluent D1 cells was induced with a hormonal adipogenic cocktail as previously described (Figure 1). Post-confluent adipogenic-induced cells were cultured for 12 days, either with or without 4T1-CM. The cells cultured in DMEM cell culture medium containing adipogenic hormonal cocktail were designated as the positive controls. At days 3 and 12 post-confluence, the cells were harvested for total RNA isolation and real-time quantitative RT–PCR was performed. The total RNA in the D1 cells was isolated according to the manufacturer's protocol, using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Briefly, the cells were disrupted by the addition of buffer RLT. The lysate was pipetted onto a QIAshredder spin column housed in a 2 ml collection tube, and was centrifuged for 2 min at maximum speed to homogenize the sample. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The sample mix was applied to an RNeasy mini-column housed in a 2 ml collection tube, and was centrifuged for 15 s at $\geq$8000 × *g*. The sample was washed with buffer RW1 and buffer RPE, respectively. Finally, the RNeasy column was transferred to a new 1.5 ml collection tube and RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and centrifuged to elute. The RNA concentration was measured with an Agilent RNA 6000 Nano Kit (Agilent Technologies Inc., Hewlett-Packard-Strabe, Waldbrone, Germany) and an Agilent 2100 Bioanalyser (Agilent Technologies Inc.).

Real-time quantitative RT–PCR was conducted according to the manufacturer's protocol, using a QuantiTect SYBR Green RT–PCR kit (Qiagen Inc., Valencia, CA, USA). Briefly, a master mix was prepared and appropriate volumes were dispensed into 0.2 ml PCR tubes. Template RNA was added to the individual PCR tubes containing the reaction. The PCR tubes were placed in the thermal cycler and the cycling programme, using Rotor-Gene (Corbette Life Science, Sydney, Australia), was started. The real-time cycler conditions were as follows: reverse transcription at 50 °C for 30 min, PCR initial activation at 95 °C for 15 min. The three-step cycling consisted of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The number of cycles performed was 35. The mRNA expression was normalized using the β-actin control $\Delta\Delta$Ct value. The primers for the control and target genes were as follows: β-actin, forward 5′-CTGACAGACTACCTCATGAAGA-3′, reverse 5′-ATGTCAACGTCACACYYCATGA-3′; PPARγ, forward 5′-ACCACTCCCATGCCTTTGAC-3′, reverse 5′-AACCATCGGGTCAGCTCGTG-3′; aP-2, forward 5′-ATGTGTGATGCCTTTGTGGGA-3′; reverse 5′-TGCCCTTTCATAAACTCTTGT-3′.

2.9. Western blot analysis

On post-confluent days 1, 3, 6 and 12, the D1s were collected and lysed in sample buffer (Bio-Rad, Hercules, CA, USA) in order to extract protein. The protein concentration was quantified using a BCA Total Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein extracts from each sample were loaded on SDS–PAGE gels for electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The various proteins were analysed using a chemiluminescence (Roche Diagnostics, IN, USA) analysis kit. Primary antibodies included: C/EBPβ, PPARγ, adipsin, aP-2 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.10. MMP-2, MMP-9, uPA and VEGF measurement

Each conditioned medium was centrifuged at 1000 × *g* at 4 °C for 10 min to pellet the cellular debris. The supernatants were collected and the level of MMP-2 and MMP-9 were assayed by ELISA according to the manufacturer's protocol. Briefly, 50 µl of the detector antibody was pipetted into each well of a 24-well plate. A volume of 50 µl of sample was added to each well and incubated at 37 °C for 4 h (for MMP-2) or 2 h (for MMP-9). The wells were washed three times with wash

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buffer and then 100 µl conjugate was pipetted into each well and incubated at room temperature for 30 min. The wells were washed three times with wash buffer, and the contents of each well were removed by inverting the plates and tapping them on paper towels. A volume of 100 µl substrate solution was added to each well and incubated in the dark at room temperature for 30 min. Finally, 100 µl stop solution was added to each sample and the absorbance in each well was measured, using a spectrophotometric plate reader at dual wavelength of 450/530 nm.

The urokinase-type plasminogen activator (uPA) levels were assayed by ELISA according to the manufacturer's protocol. Briefly, 100 µl blocking solution was pipetted into each well of a 96-well plate, and the plate was shaken at 300 r.p.m. for 30 min. The wells were washed three times with wash buffer and the excess buffer was removed by gently tapping the plate on a paper towel. A volume of 100 µl of each sample was added to each well and the plate was shaken at 300 r.p.m. for 30 min. The wells were washed and excess wash removed. A volume of 100 µl primary antibody was pipetted into all the wells and the plate was shaken at 300 r.p.m. for 30 min. The wells were washed and removed excess wash as above. A volume of 100 µl substrate was added to all wells and the plate was shaken for 10 min. The reaction was quenched by the addition of 50 µl stop solution, and the final absorbance was read immediately at 450 nm on a microplate reader.

VEGF was also assayed by ELISA according to the manufacturer's protocol. Briefly, 100 µl each sample was added to each well of a 96-well plate and 50 µl biotinylated anti-VEGF solution was pipetted into each well. The plate was gently tapped on the side and then incubated for 2 h at room temperature. The contents of the wells were thoroughly aspirated and washed four times. A volume of 100 µl streptavidin–HRP working solution was added to each well and incubated for 30 min at room temperature. The contents of the wells were thoroughly aspirated and the wells were washed four times. A volume of 100 µl of chromogen was added to each well, and the plate was incubated for 30 min at room temperature in the dark. Finally, 100 µl stop solution was added to each well. The absorbance of each well was read immediately at 450 nm on a microplate reader.

2.11. Statistical analysis

All results were obtained from at least three independent experiments. Results were analysed by Student's t test. $p < 0.05$ was considered significant.

3. Results

3.1. Effect of conditioned media on D1 cell viability

The viability results showed that the three conditioned media did not have an inhibitory effect on D1 cell viability pre-confluency (Figure 2) and that the three conditioned media did not have a negative effect on the viability of differentiating D1 cells (at day 6) and differentiated D1 cells (at day 12) (Figure 2).

3.2. Effect of conditioned medium on D1 adipogenic differentiation

Measurements of ORO and TG at post-confluence days 3, 6, 9 and 12 (Tables 1, 2) showed that by day 12 the presence of 4T1-CM significantly reduced ORO by 50% ($p < 0.05$) and TG by 79% ($p < 0.01$); 4T07-CM reduced ORO by 40% ($p > 0.05$) and TG by 63% ($p < 0.05$), meanwhile NMuMG-CM reduced ORO by 40% ($p > 0.05$) and TG by 38% ($p > 0.05$).

Visual observation with light microscopy showed that 4T1-CM treated cells contained fewer lipid vesicles in the perinuclear zone than the other three groups of cells, which were cultured with DMEM cell culture medium, 4T07-CM, and NMuMG-CM. On day 12 the D1 cells differentiated into mature adipocytes with visible lipid deposits (Figure 3A). Lipid vesicles were rarely observed in the D1 cells treated with 4T1-CM (Figure 3B). However, lipid vesicles were observed in

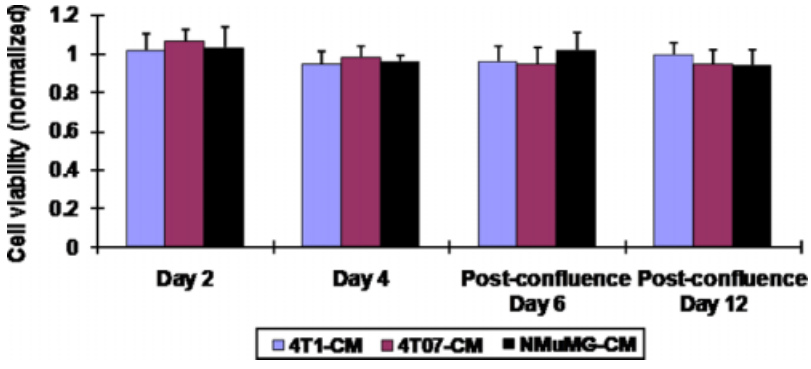

Figure 2. Relative Alamar blue values are plotted to show the effect of 4T1, 4T07 and NMuMG conditioned media on the viability of D1 cells. Alamar blue fluorescence values from the experimental groups were normalized to those of the control group, which received cell culture medium only

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Table 1. Effect of various media on ORO staining (absorbance at 530 nm) of D1 cells during adipocyte differentiation, induced by hormonal cocktail (mean ± SD, $n = 3$)

Various medium	Day 3	Day 6	Day 9	Day 12
CCM	0.90 ± 0.08	1.06 ± 0.28	1.24 ± 0.34	1.57 ± 0.94
CCM + 4T1-CM	0.80 ± 0.08	0.84 ± 0.06	0.81 ± 0.10*	0.77 ± 0.23*
CCM + 4T07-CM	0.91 ± 0.08	0.92 ± 0.11	0.99 ± 0.22	0.94 ± 0.13
CCM + NMuMG-CM	0.73 ± 0.11	0.93 ± 0.11	0.94 ± 0.29	0.94 ± 0.30

*$p < 0.05$ compared to CCM (cell culture medium).

the D1 cells treated with 4T07-CM and NMuMG-CM (Figure 3C, 3D). The combination of results suggests that 4T1-CM has a significant inhibitory effect on D1 adipocyte differentiation.

3.3. Effect of 4T1-CM on differentiated D1 cells

At day 12 post-confluence, following adipogenic induction and 6 day exposure to 4T1-CM, the ORO and TG levels were reduced significantly ($p < 0.01$) by 20% and 49%, respectively. These results suggest that 4T1-CM reduces lipid vesicle accumulation in the differentiating D1 cells (Table 3).

Table 3. Effect of 4T1 conditioned media on lipid vesicle accumulation in the differentiating D1 cells, data based on ORO staining and TG values (mean ± SD, $n = 3$)

	ORO (OD at 530 nm)	TG (μM)
CCM	0.86 ± 0.11	40.24 ± 10.28
CCM + 4T1-CM	0.69 ± 0.08**	20.48 ± 10.77**

**$p < 0.01$ compared to CCM (cell culture medium).

3.4. Modulation of relevant mRNA and protein expression of D1 cells by 4T1-CM

Analysis of post-confluent, adipogenic-induced D1 cultures showed that the gene expression of *PPARγ* was reduced significantly by 54% on day 3 ($p < 0.05$) but showed no change at day 12 following exposure to 4T1-CM. The gene expression of *aP-2* was reduced to 43% ($p > 0.05$) by day 3 and to 40% ($p < 0.05$) by day 12 (Figure 4).

Table 2. Effect of various media on TG value (μM) of D1 cells during adipocyte differentiation, induced by hormonal cocktail (mean ± SD, $n = 3$)

Various medium	Day 3	Day 6	Day 9	Day 12
CCM	6.74 ± 3.50	11.35 ± 7.57	18.80 ± 13.20	32.69 ± 12.53
CCM + 4T1-CM	1.56 ± 0.80**	4.12 ± 1.53*	3.28 ± 1.48*	6.78 ± 1.82**
CCM + 4T07-CM	5.89 ± 3.57	6.42 ± 3.94	6.39 ± 4.97*	11.94 ± 4.55*
CCM + NMuMG-CM	6.54 ± 4.55	6.29 ± 1.08	8.46 ± 2.44	20.25 ± 9.18

**$p < 0.01$, *$p < 0.05$ compared to CCM (cell culture medium).

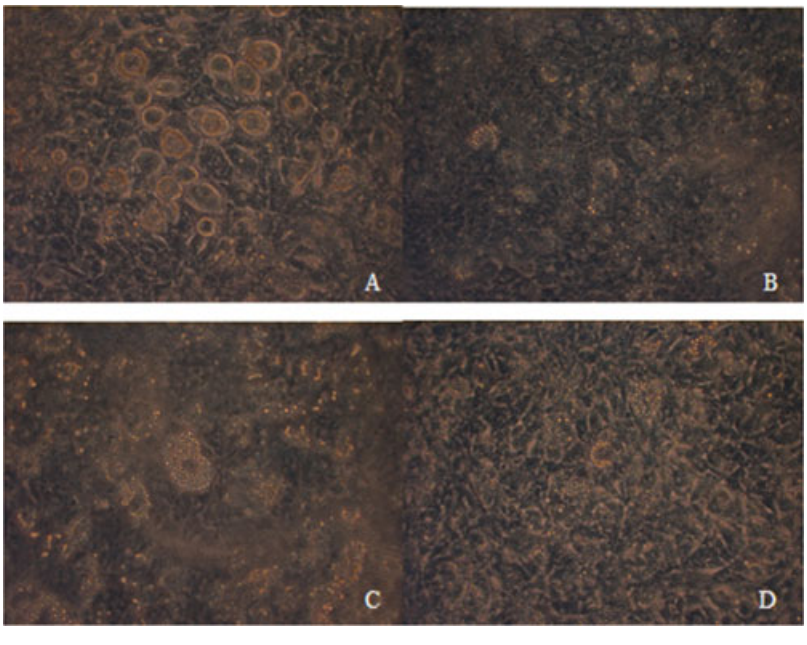

Figure 3. D1 cells were induced with adipocytic hormonal cocktails and the cell morphologies were observed. Photos were taken at ×320 total magnification and show unstained D1 cells with CCM (A) and cells treated by 4T1-CM (B), 4T07-CM (C) and NMuMG-CM (D) on day 12 post-confluence

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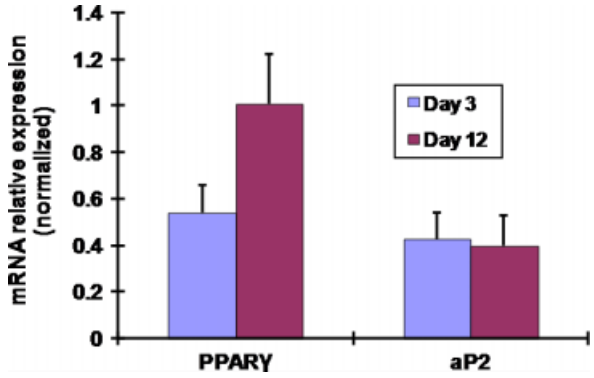

Figure 4. 4T1-CM modulates the *PPARγ* and *aP-2* gene expression of D1s on days 3 and 12 after post-confluence adipogenic induction. Relative gene expression of cells treated with 4T1-CM is shown; values were normalized to gene expression values of cells receiving cell culture medium only

As determined by qualitative Western blot analysis, C/EBPβ protein, which was expressed during early stage differentiation (days 1–3), was reduced by 4T1-CM treatment. The aP2 protein and adipsin protein, which were expressed during late stage differentiation, were also reduced by 4T1-CM treatment. 4T1-CM treatment also reduced PPARγ protein expression, which was expressed through days 6–12 (Figure 5).

3.5. MMP-2, MMP-9, uPA and VEGF content in conditioned medium

The ELISA results showed a significantly higher presence of MMP-9 and uPA in 4T1-CM than the amount found in both 4T07-CM and NMuMG-CM, which were both below the detection limit. However, there was a significantly lower presence of VEGF in 4T1-CM when compared to the amount of VEGF found in the other conditioned media ($p < 0.01$) (Table 4).

3.6. Effect of D1-d6-CM and D1-d12-CM on 4T1, 4T07 and NMuMG cell viability

The results showed that both D1-d6-CM and D1-d12-CM had an inhibitory effect on 4T1 cell viability. Only D1-d12-CM had an inhibitory effect on 4T07 cell viability. D1-d6-CM and D1-d12-CM did not have a negative effect on NMuMG cell viability (Figure 6).

4. Discussion

It is likely that tissue-engineering approaches toward reconstructing breast tissue following a lumpectomy or mastectomy will involve the implantation of adipogenic differentiating stem cells. These implanted cells may encounter remnant breast cancer cells that were not removed from the microenvironment by surgical treatment. Although the genetic mechanisms that control the differentiation of preadipocytes from MSCs into mature adipocytes are understood to a large extent (Bowers and Lane 2007), the interactive regulation in the adipocyte differentiation microenvironment, i.e. the implanted stem cells and the host remnant cancer cells, is largely unknown.

In the adipocyte differentiation microenvironment the interaction of adipocytes and other surrounding cells is not well understood. Pioneering studies by

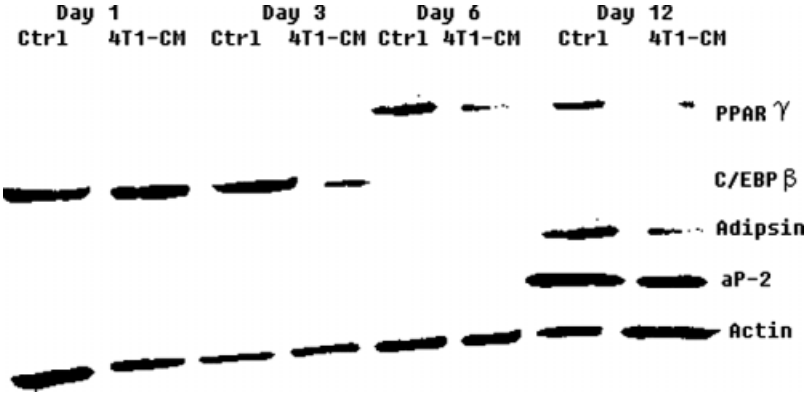

Figure 5. Western blot results show the modulation of C/EBPβ, PPARγ, adipsin and aP-2 protein expression by exposure of D1s to 4T1-CM during different stages of post-confluent adipogenic induction

Table 4. MMP-2, MMP-9, VEGF and uPA content (pg/ml) in conditioned media assayed by ELISA (mean ± SD, $n = 3$)

	MMP-2	MMP-9	uPA	VEGF
4T1-CM	BDL	67.55 ± 7.35**	285.37 ± 82.64**	781.00 ± 174.75**
4T07-CM	BDL	BDL	BDL	1698.89 ± 444.60
NmuMG-CM	BDL	BDL	BDL	1778.92 ± 465.42

**$p < 0.01$ compared to two others; BDL, below detection limit.

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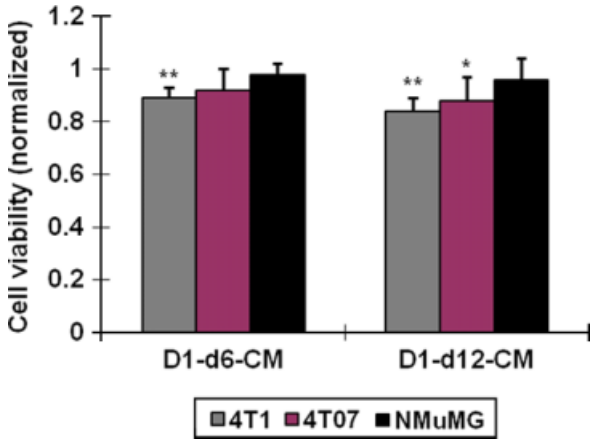

Figure 6. Effect of D1-d6-CM and D1-d12-CM on 4T1, 4T07 and NMuMG cell viability on day 4. Data are normalized to those of cell culture medium samples. *$p < 0.05$; **$p < 0.01$

Stockdale and Levine evaluated the interaction of 3T3-L1 adipocytes and NMuMG mammary epithelial cells and found that the interaction of mammary epithelium with adipocytes resulted in a marked increase in proliferation of the mammary epithelium, possibly facilitated by the extracellular components (Levine and Stockdale, 1984, 1985). In the present work we found that, on day 12, D1 cell-conditioned medium had an inhibitory effect on 4T1 and 4T07 cancer cell viability, but had no effect on the NMuMG cells. Meanwhile, 4T1, 4T07 and NMuMG cell conditioned medium did not have an inhibitory effect on D1 cell viability.

C/EBPβ and PPARγ are differentiation-mediating transcription factors, while aP2 and adipsin are late markers of adipogenesis (Cao *et al*., 1991; Rosen and MacDougald, 2006; Yeh *et al*., 1995; Ito *et al*., 2007). PPARγ was significantly reduced to 54% on day 3 ($p < 0.05$), with no change on day 12. Expression of aP-2 was reduced by 43% ($p > 0.05$) by day 3 and to 40% by day 12 ($p < 0.05$), respectively (Figure 4). The protein expression analysis showed that D1 cell differentiation was, in part, mediated by 4T1-CM, through the downregulation of C/EBPβ expression at the early stages of differentiation and subsequent decrease in expression of PPARγ, aP2 and adipsin at terminal differentiation. On day 12, 4T1-CM significantly reduced both lipid and TG production, while 4T07-CM significantly reduced only the TG production. Based on the combined results, the 4T1-CMs had a significant inhibitory effect on D1 cell adipogenic differentiation and resulted in reduced lipid accumulation. Meng and co-workers (2001) found that 3T3-L1 adipocyte differentiation was completely inhibited by co-culturing 3T3-L1s with various cells derived from specific breast cancer cell lines (T47D, MCF-7, SSC202, SSC78 and SSC30) or was inhibited in a dose-dependent manner by breast cancer cell-conditioned medium. Meng and co-workers further found that 3T3-L1 adipocyte differentiation was not inhibited by co-culture with normal human primary mammary epithelial cell conditioned medium, which was similar to our findings.

There are several well-known pro-inflammatory cytokines found in cellular conditioned medium (IL-1, IFNγ, TGFβ and TNFα), which have been studied and are confirmed to inhibit adipocyte differentiation (Button *et al*., 2001; Gregoire *et al*., 1992; Richardson *et al*., 1992; Sparks *et al*., 1994). However, the other known extracellular components secreted in cell-conditioned medium, such as MMPs, uPA and VEGF, have not been studied extensively. The MMP/TIMP system is considered to play a role in the control of proteolytic events and adipogenesis (Bourlier *et al*., 2005; Chavey *et al*., 2003; Croissandeau *et al*., 2002). Urokinase-type plasminogen activator uPA was found to be one of the modulators for the differentiation of 3T3-L1 cells (Seki *et al*., 2001). Additionally, the blockade of VEGF signalling could inhibit *in vivo* adipose tissue formation (Fukumura *et al*., 2003). The literature suggests that, in addition to well-known pro-inflammatory cytokines, MMPs, uPA and VEGF may be involved in adipocyte differentiation. Hence, we selected MMPs, uPA and VEGF as the foci for component evaluation of the conditioned medium in the work presented here.

Our results demonstrate that the three types of cell conditioned medium exhibit a difference in MMP-9, uPA and VEGF expression. Mi and co-workers found that metastatic 4T1 cells exhibit significantly increased osteopontin, integrin-linked kinase, MMP-2 and uPA expression in contrast to non-metastatic 4T07 cells (Mi and others 2006), confirming that 4T1-conditioned medium and 4T07-conditioned medium contain different cytokines. Chavey and co-workers found that MMPs are differentially expressed in adipose tissue; however, they did not study the role of MMP-9 in their work (Chavey *et al*., 2003). Rehman and co-workers found that adipose stromal cells secreted VEGF, TGFβ and hepatocyte growth factor (Rehman and others 2004). Thus MMP-9, uPA and VEGF likely have an autocrine effect; however, one cannot conclude that MMP-9 and uPA in the conditioned medium affected adipocyte differentiation in a paracrine manner. One can only speculate that MMP-9, uPA and VEGF might affect adipocyte differentiation, hence substantiating the changes seen with the addition of conditioned medium.

The inhibitory effect of differentiated D1 cell conditioned medium on 4T1 and 4T07 cell viability at day 12 may be attributed to a number of factors, including proteins secreted by the adipocytes. However, since the medium conditioned by mature adipocytes was composed of many lipids, the inhibition may be lipotoxicity-related. Chamras and co-workers, for example, found that 3T3-L1 preadipocytes stimulated breast cancer cell growth but that, in contrast, after differentiation the mature adipocytes inhibited the clonal growth of breast cancer cells (Chamras *et al*., 1998). Similarly, Johnston and co-workers also found that 3T3-L1-CM inhibited the growth of MCF-7 breast cancer cells but stimulated the growth of normal breast epithelial cells (Johnston *et al*., 1992). Tirosh *et al*. (2004) studied the death mechanism of macrophages as a result of exposure to triglycerides. They

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found that exposure of J774.2 macrophages to triglyceride led to decreased apoptosis and to increased survival within the first 24 h after treatment with triglyceride. In contrast, after 48 h the triglyceride effect culminated in massive cell death of 50%. Triglycerides may directly regulate lipotoxicity by inducing oxidative stress.

Further studies of the conditioned media from other pairs of metastatic/non-metastatic cancer cell lines will need to be evaluated to define relationships beyond the 4T1 cell line. Additional studies examining the interactions between D1 cells and 4T1/4T07 cells, using a trans-well co-culture system and a direct co-culture system, are in progress.

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