

AWARD NUMBER: W81XWH-17-1-0470

TITLE: Targeting Lung-Derived Proteins as a Therapeutic Strategy Against Breast Cancer Metastasis

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REPORT DATE: DECEMBER 2021

TYPE OF REPORT: Final Technical Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE DECEMBER 2021		2. REPORT TYPE Final		3. DATES COVERED 15 Aug 2017 – 14 Aug 2021	
4. TITLE AND SUBTITLE Targeting Lung-Derived Proteins as a Therapeutic Strategy Against Breast Cancer Metastasis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-17-1-0470	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Alison Allan, Dr. Raimar Loebenberg E-Mail: alison.allan@lhsc.on.ca				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UNIVERSITY OF WESTERN ONTARIO 1151 RICHMOND ST LONDON ON CANADA N6A 5B8				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Therapy for lung metastasis is often given systemically, causing significant toxicity. However, the lung holds potential for direct targeting via inhaled drugs; an approach that has shown promise in treating respiratory diseases but remains underexplored in oncology. This project tested the hypothesis that CD44-interacting proteins produced in the lung promote breast cancer metastasis and can be targeted directly using inhalable drug delivery. The major findings of the project are that the CD44-interacting proteins OPN, FGF2, and E/P/L selectins have complementary and important roles in mediating breast cancer metastatic behavior in response to the lung microenvironment. OPN and selectins (but not FGF2) were found to be necessary for metastatic colonization of the lung, particularly for more aggressive breast cancer cell lines. The pan-selectin antagonist bimosiamose was chosen for further study and subjected to drug formulation, optimization and quality assurance into an inhalable inhibitor that was tested <i>in vivo</i> for its ability to reduce breast cancer lung metastasis.					
15. SUBJECT TERMS Breast cancer; metastasis; lung microenvironment; CD44; osteopontin (OPN; basic fibroblast growth factor (FGF2; E/L/P-selectin, nanoparticles, inhalable drug delivery, pre-clinical models					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	113	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:

The lung is one of the most common and deadly sites of breast cancer metastasis, particularly in patients with aggressive triple-negative (TN) disease. Therapy for metastasis is often given systemically, causing significant toxicity. However, the lung (unlike other metastatic sites) holds potential for direct targeting via inhaled drugs; an approach that has shown promise in treating respiratory diseases but remains underexplored in oncology. We previously developed inhalable drug-loaded nanoparticles for cancer, and in the current project we aimed to apply this innovation in combination with novel biological targets to address the problem of breast cancer metastasis to lung. We previously reported that the lung microenvironment promotes metastasis of breast cancer cells, particularly those with high expression of the cell-surface receptor CD44. In order to identify therapeutic targets in the lung, we used novel *ex vivo* models and observed that the lung secretes several CD44-interacting proteins involved with metastasis, including osteopontin (OPN), E-, L-, P-selectins, and basic fibroblast growth factor (FGF2). The purpose of this project was to test the hypothesis that CD44-interacting proteins produced in the lung promote breast cancer metastasis and can be targeted directly using inhalable drug delivery. Specific Aim 1 involved elucidation of the mechanisms by which lung-derived OPN, E-, L-, P-selectins and FGF2 promote breast cancer metastasis to the lung. To assess this, we used a 2D *ex vivo* model system involving conditioned media (CM) from murine lung, as well as a 3D *ex vivo* pulmonary metastasis assay (PuMA). These assays were used in combination with human TN breast cancer cell lines and primary cells from patient-derived xenografts. Lung-derived proteins were depleted from lung-CM using functional antibodies or by using lungs from specific knockout mice. Specific Aim 2 involved assessing the therapeutic potential of directly targeting the CD44-interacting proteins E-, L-, and P-selectin in the lung. The pan-selectin antagonist bimosiamose was loaded into polybutyl cyanoacrylate nanoparticles and incorporated into inhalable effervescent carrier particles and tested in pre-clinical *in vivo* breast cancer metastasis models using an inhalable drug delivery approach with the goal of reducing breast cancer metastasis to the lung.

2. KEYWORDS:

Breast cancer, metastasis, lung microenvironment, CD44, osteopontin (OPN), basic fibroblast growth factor (FGF2), E-selectin, L-selectin, P-selectin, nanoparticles, inhalable drug delivery, pre-clinical models

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Table 1 lists the major goals of the project as stated in the approved SOW for the project, including the milestones/target dates and actual completion dates.

Table 1: Major goals of the project as stated in the approved SOW for the project

Major Task/Goal	Milestones	Target Date for Milestone Completion	Actual Completion Date or % Completion
Major Task 1: <i>In vitro</i> assessment of distinct metastatic behaviors & mechanisms	• HRPO/ACURO determination/approval • Characterization of the <i>in vitro</i> functional and mechanistic influence of lung-derived CD44-interacting proteins on breast cancer metastatic behavior	August 2018	June 2017; 100% completed
Major Task 2: 3D <i>ex vivo</i> assessment of essential lung-derived factors	• Establishment of breeding colonies of knockout mice • Characterization of the essentiality of each lung-derived factor for breast cancer metastasis. • Generation of a priority list of lung-derived factors for Major Task 4 & 5.	August 2019	November 2019; 100% completed
Major Task 3: Formulation and production of inhalable inhibitors	• Successful formulation, production and QC/QA of an inhalable drug ready for use in first <i>in vivo</i> studies	February 2019	August 2019; 100% completed
Major Task 4: <i>In vivo</i> assessment of anti-metastatic efficacy of inhalable drug using a preventative approach	• <i>In vivo</i> testing of inhalable inhibitor in the preventative setting.	December 2019	August 2021; 95% completed (<i>experiments completed, final data analysis ongoing</i>)
Major Task 5: <i>In vivo</i> assessment of anti-metastatic efficacy of inhalable drug using a therapeutic approach	• <i>In vivo</i> testing of inhalable inhibitor in the therapeutic setting.	August 2020	August 2021; 95% completed (<i>experiments completed, final data analysis ongoing</i>)
Major Task 6: Integrated data sharing plan between sites		Ongoing throughout whole funding period	August 2021; 100% completed

What was accomplished under these goals?

Major Task 1: *In vitro* assessment of distinct metastatic behaviors & mechanisms.

The proposed work for Major Task was completed and is described below. This work supported ***Specific Aim 1***, which was to determine the mechanisms by which lung-derived OPN, selectins and FGF2 support and promote breast cancer metastasis to the lung.

TASK 1.1: Local IRB/IACUC Approval; and TASK 1.2: HRPO/ACURO Approval.

This task was completed as proposed. Local IACUC approval for all animal experiments in this study was achieved on February 08, 2017. USAMRMC Animal Care and Use Review Office (ACURO) approval was granted on March 02, 2017, and has been successful with continuing annual renewal of approval since then. Local IRB determination and exemption was completed on April 07, 2017 and sent to the HRPO for review. On June 21, 2017 the HRPO confirmed that the research activities within the project do not involve human subjects, and that the research may proceed with no further requirement for review by the HRPO.

TASK 1.3: Generate lung-conditioned media (CM) for rescue and signaling experiments.

This task was completed as proposed as used for studies described below.

TASK 1.4: Characterize the functional and mechanistic influence of lung-derived CD44-interacting proteins on breast cancer metastatic behavior.

This task has been completed, with an overview of the methodology shown in **Figure 1**. Briefly, lung-CM from Task 1.3 was collected from *ex vivo* lung culture, and blocking/neutralizing antibodies against osteopontin (OPN), E-, L-, and P-selectins or FGF2 were used to immunodeplete each protein from lung-CM. Companion ELISA kits were used to quantify the presence of each protein in lung-CM and the extent of immunodepletion. Native and immunodepleted lung-CM was then used to assess (a) the functional effects of specific lung-derived CD44-interacting proteins on *in vitro* breast cancer metastatic behavior and (b) the influence on intracellular signaling.

Figures 2-6 (“A” panels) demonstrate that all 5 proteins are present in lung-CM and can be effectively immunodepleted. The functional effects of these lung-derived CD44-interacting proteins on *in vitro* breast cancer metastatic behavior were then examined using a selection of different human triple-negative (TN) breast cancer cell lines and specialized cell culture assays. As demonstrated in **Figures 2-6**, each lung-derived CD44-interacting protein examined appears to have a distinct but complementary role in supporting and promoting breast cancer metastatic behavior in the lung microenvironment. For example, OPN is involved in both migration and growth (**Figure 2**); FGF2 is only involved in growth (**Figure 3**); and E-, L-, and P-selectins are only involved in migration (**Figures 4-6**). Importantly, the specificity of each lung-derived protein at influencing specific cell behaviors was confirmed by rescue experiments that involved adding recombinant OPN, FGF2, or selectins back to depleted lung-CM at concentrations equivalent to the original depletion. These results were also highly reproducible in different TN human breast cancer cell models including MDA-MB-231 and SUM-149 immortalized cell lines, and the LRCP-BR17 cells (derived from a TN patient-derived xenograft model).

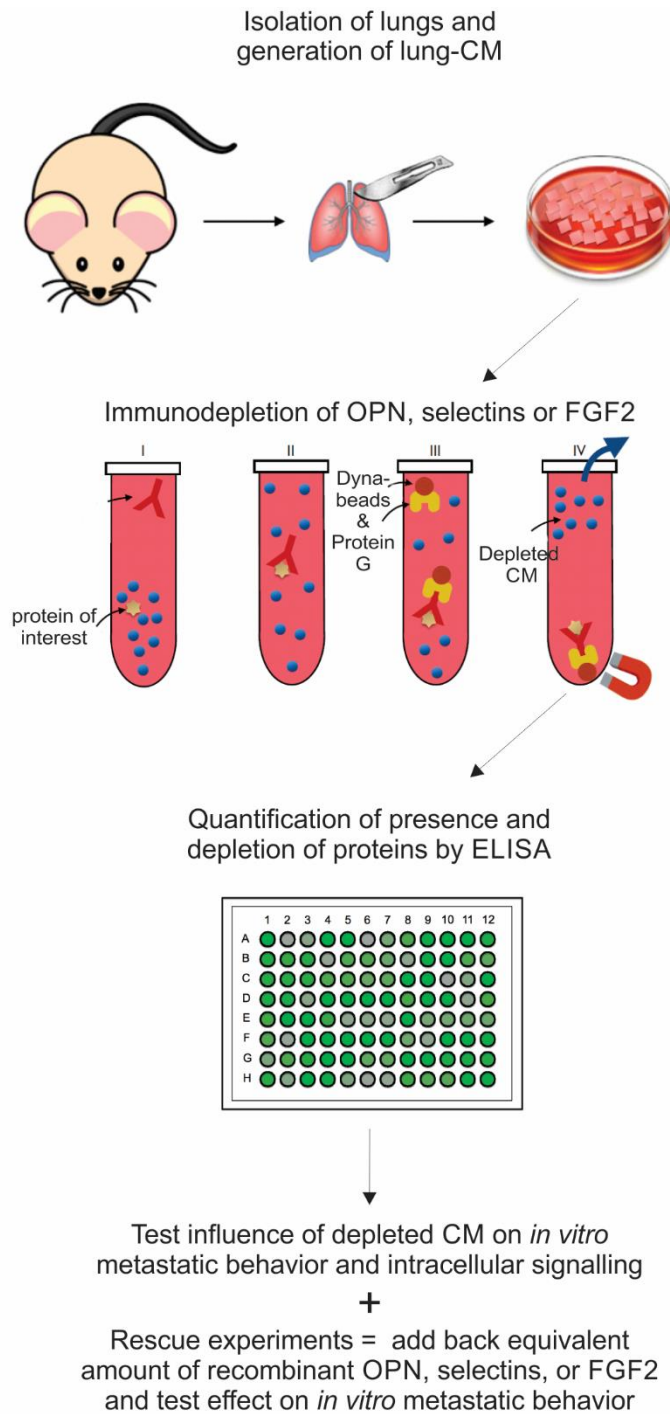


Figure 1: Methodology overview for Major Task 1.

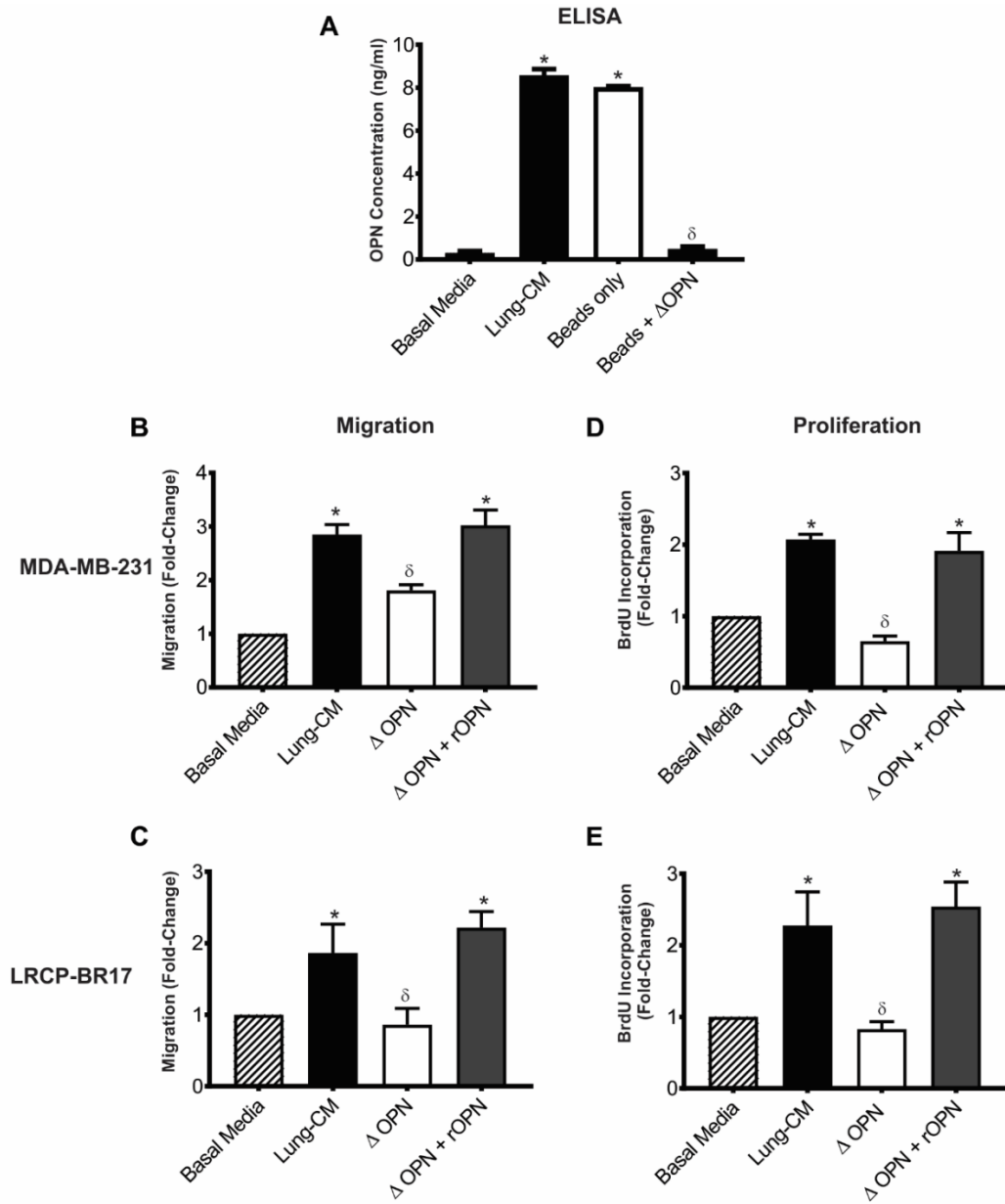


Figure 2: Depletion of osteopontin (OPN) from lung conditioned media (CM) reduces breast cancer cell migration and proliferation. **(A)** Lung-CM was generated from healthy female mouse lungs and OPN immunodepleted. Resulting OPN protein levels were determined by ELISA. Data are presented as mean [OPN] $\pm$ SEM (n = 3). **(B,C)** MDA-MB-231 and LRCP-BR17 cell migration following exposure to basal media, native lung-CM, lung-CM depleted of OPN (Δ OPN), or Δ OPN rescued by re-addition of recombinant OPN. **(D,E)** LRCP-BR17 cell proliferation following exposure to basal media, native lung-CM, lung-CM depleted of OPN (Δ OPN), or Δ OPN rescued by re-addition of recombinant OPN. Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media $\pm$ SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

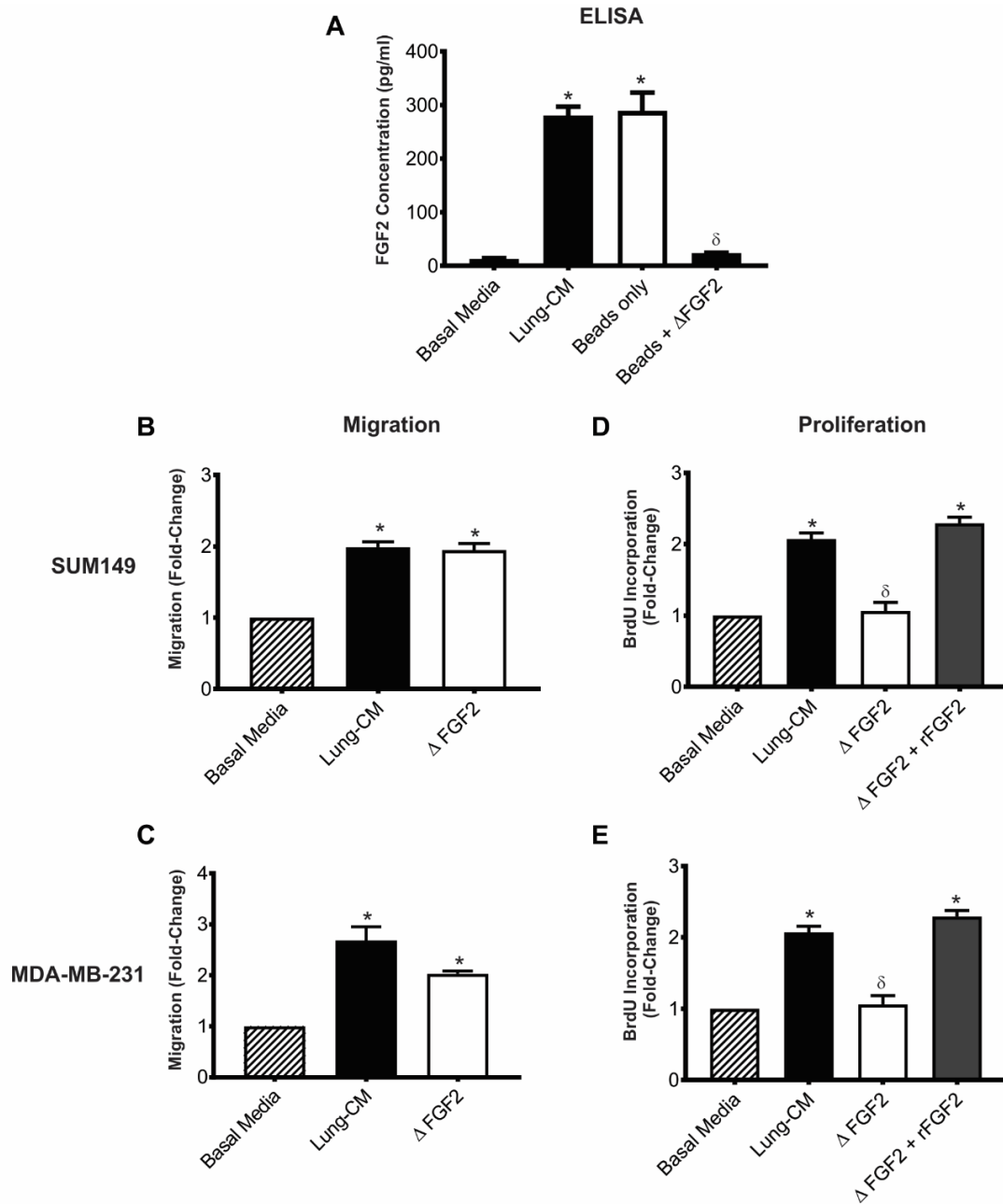


Figure 3: Depletion of basic fibroblast growth factor (FGF2) from lung conditioned media (CM) reduces breast cancer cell proliferation but not migration. **(A)** Lung-CM was generated from healthy female mouse lungs and FGF2 immunodepleted. Resulting FGF2 protein levels were determined by ELISA. Data are presented as mean [FGF2] $\pm$ SEM (n = 3). **(B,C)** SUM149 and MDA-MB-231 cell migration following exposure to basal media, native lung-CM, or lung-CM depleted of FGF2 (Δ FGF2). **(D,E)** SUM149 and MDA-MB-231 cell proliferation following exposure to basal media, native lung-CM, lung-CM depleted of FGF2 (Δ FGF2), or Δ FGF2 rescued by re-addition of recombinant FGF2. Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media $\pm$ SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

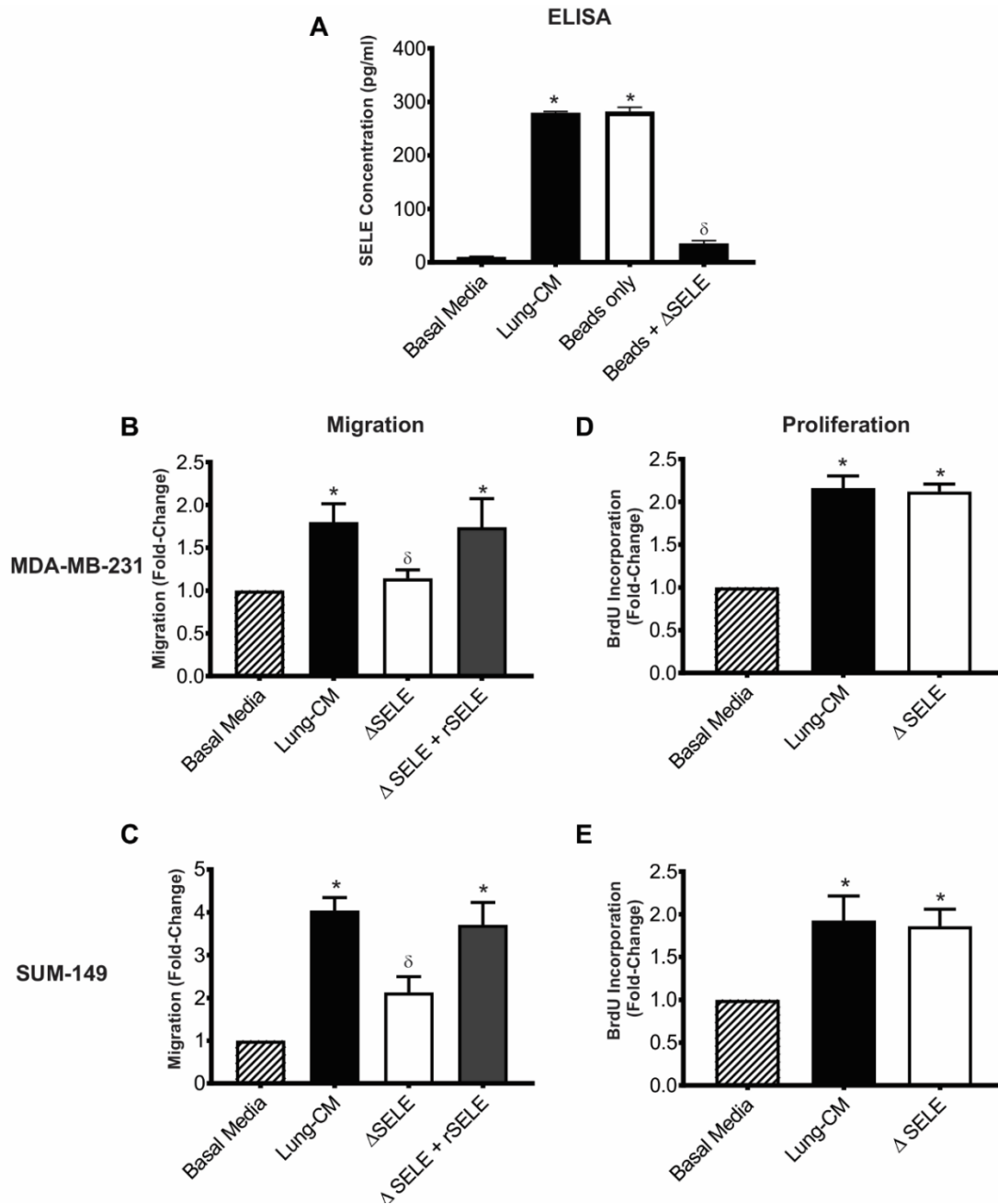


Figure 4: Depletion of E-selectin (SELE) from lung conditioned media (CM) reduces breast cancer cell migration but not proliferation. **(A)** Lung-CM was generated from healthy female mouse lungs and SELE immunodepleted. Resulting SELE protein levels were determined by ELISA. Data are presented as mean [SELE] $\pm$ SEM (n = 3). **(B,C)** MDA-MB-231 and SUM149 cell migration following exposure to basal media, native lung-CM, lung-CM depleted of SELE (Δ SELE), or Δ SELE rescued by re-addition of recombinant SELE. **(D,E)** MDA-MB-231 and SUM149 cell proliferation following exposure to basal media, native lung-CM, or lung-CM depleted of SELE (Δ SELE). Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media $\pm$ SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

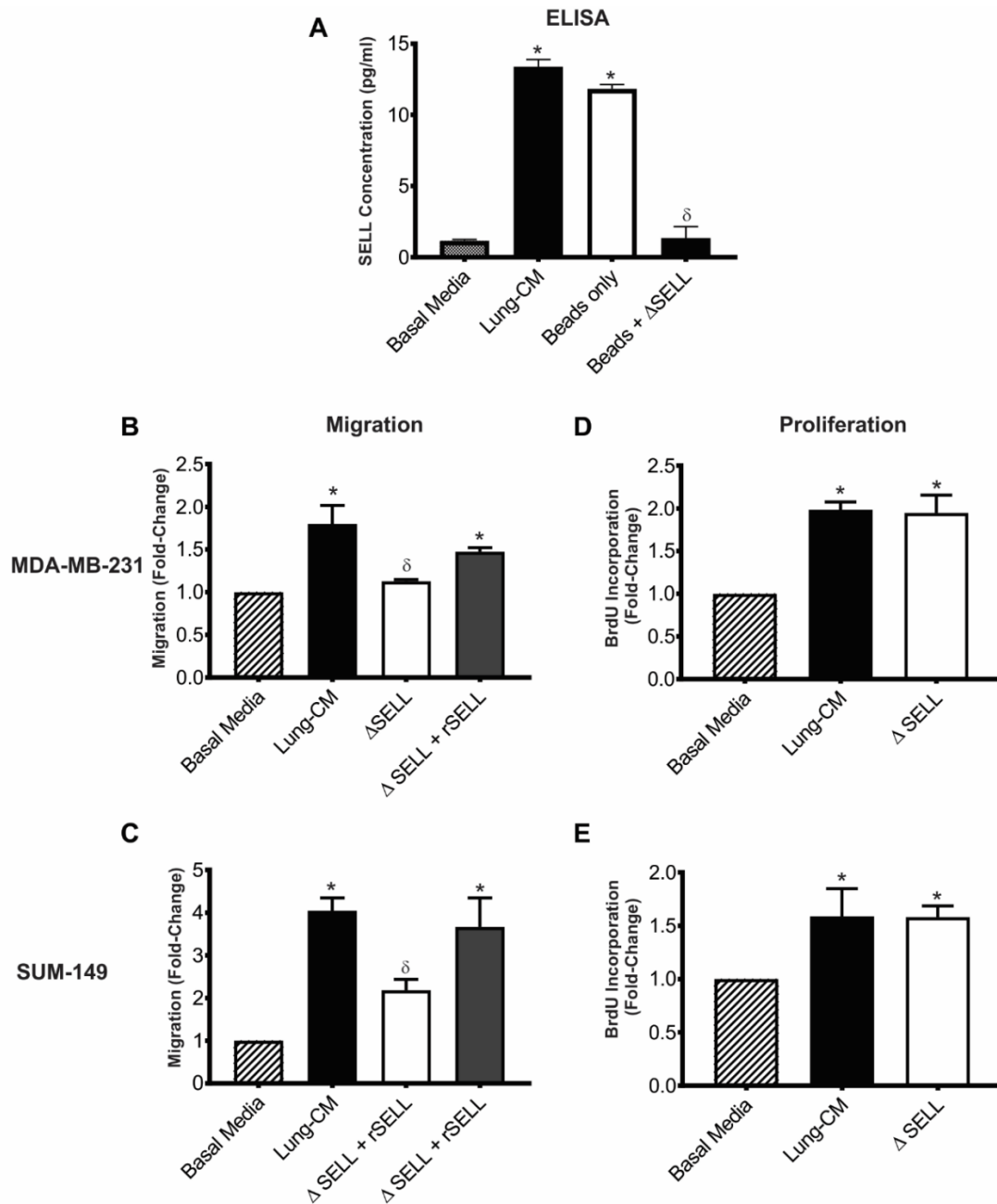


Figure 5: Depletion of *L*-selectin (*SELL*) from lung conditioned media (CM) reduces breast cancer cell migration but not proliferation. **(A)** Lung-CM was generated from healthy female mouse lungs and *SELL* immunodepleted. Resulting *SELL* protein levels were determined by ELISA. Data are presented as mean [*SELL*] $\pm$ SEM ($n = 3$). **(B,C)** MDA-MB-231 and SUM149 cell migration following exposure to basal media, native lung-CM, lung-CM depleted of *SELL* (Δ *SELL*), or Δ *SELL* rescued by re-addition of recombinant *SELL*. **(D,E)** MDA-MB-231 and SUM149 cell proliferation following exposure to basal media, native lung-CM, or lung-CM depleted of *SELL* (Δ *SELL*). Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media $\pm$ SEM ($n = 3$). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

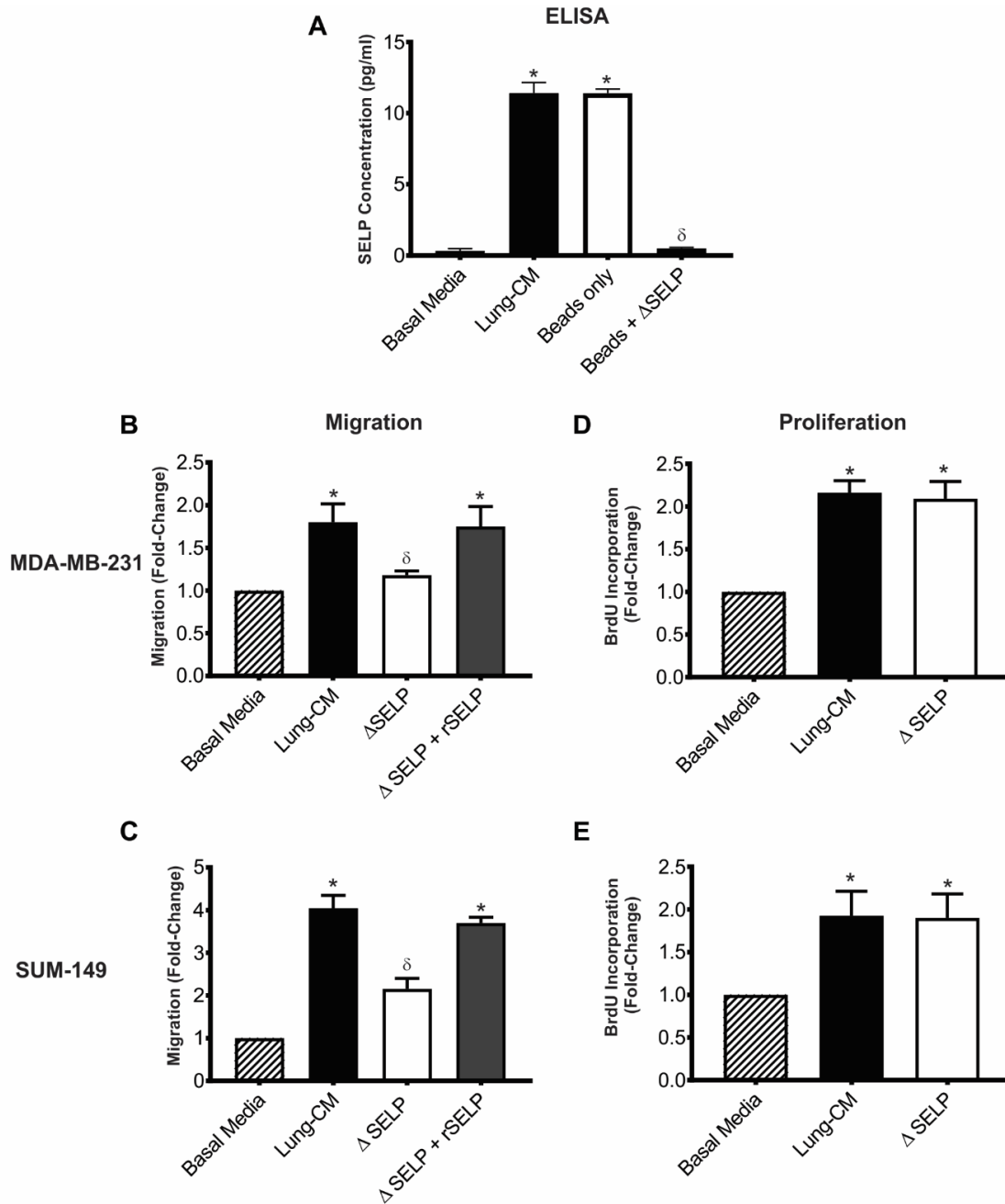


Figure 6: Depletion of P-selectin (SELP) from lung conditioned media (CM) reduces breast cancer cell migration but not proliferation. **(A)** Lung-CM was generated from healthy female mouse lungs and SELP immunodepleted. Resulting SELP protein levels were determined by ELISA. Data are presented as mean [SELP] $\pm$ SEM (n = 3). **(B,C)** MDA-MB-231 and SUM149 cell migration following exposure to basal media, native lung-CM, lung-CM depleted of SELP (Δ SELP), or Δ SELP rescued by re-addition of recombinant SELP. **(D,E)** MDA-MB-231 and SUM149 cell proliferation following exposure to basal media, native lung-CM, or lung-CM depleted of SELP (Δ SELP). Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media $\pm$ SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM (p $\leq$ 0.05).

To assess the differential activation of downstream signaling in breast cancer cells in response to lung-CM, we used a combination of protein phospho-array analysis (for broad investigation of multiple pathways) and specific investigation of the ERM (ezrin, radixin, moesin) pathway that, when phosphorylated, have been shown to serve as a linker between CD44 and the cytoskeleton to facilitate migration. Our results demonstrate that ERM is phosphorylated in breast cancer cells following exposure to the lung microenvironment (**Figure 7A**). Furthermore, phospho-array analysis demonstrates that exposure of human breast cancer cells to the lung microenvironment results in phosphorylation of several other important downstream proteins including ERK1/2, MSK1/2, CREB, Lyn, and Src (**Figure 7B**). These array results have been validated by immunoblotting (*representative data for CREB shown in Figure 7C*).

In conclusion, the major findings for this Task are that the CD44-interacting proteins OPN, FGF2, and selectins have complementary and important roles in mediating breast cancer metastatic behavior in response to the lung microenvironment, supporting the concept that these lung-derived proteins are suitable therapeutic targets for further development.

Major Task 2: 3D *ex vivo* assessment of essential lung-derived factors.

The proposed work for Major Task 2 is complete and is described below. This work also supports **Specific Aim 1**.

TASK 2.1: Establish breeding colonies of knockout mice

This task is 100% complete. OPN^{-/-}, triple-selectin^{-/-}, and FGF2^{-/-} breeding colonies were successfully established and used for experiments (Task 2.3, described below). Although we had originally proposed to also look at single selectin knockouts (SELE^{-/-}, SELP^{-/-}, and SELL^{-/-}), our *in vitro* data suggests that the 3 selectins may be compensatory for each other and therefore our focus has been on the pan-selectin knockout mice for the *ex vivo* studies. This approach is also well-aligned with the use of a pan-selectin inhibitor for the therapeutic studies (Tasks 4 and 5, described below).

TASK 2.2: Transduce cell lines to stably express RFP.

This task has been completed for all breast cancer models (see example in **Figure 8E**).

TASK 2.3: Assess the essentiality of each lung-derived factor for metastatic colonization/growth within the intact lung microenvironment in the 3D *ex vivo* PuMA.

This task is 100% complete. Breast cancer cells have been subjected to the *ex vivo* PuMA using wildtype versus OPN^{-/-}, triple-selectin^{-/-} and FGF2^{-/-} mice. The full set of these experiments has been completed in MDA-MB-231, SUM159, LRCP-BR17 and LRCP-BR21 breast cancer models, and the most significant results are presented in **Figure 8**. Using breast cancer cells that are strongly metastatic to lung *in vivo* (MDA-MB-231, SUM159, LRCP-BR17), we observed that loss of OPN or E/L/P selectins significantly reduces (but doesn't completely eliminate) breast cancer progression to metastasis in the lung by Day 21 of the assay, whereas loss of FGF2 does not significantly alter breast cancer progression relative to wildtype (**Figure 8A,B,C,E**). Interestingly, in the breast cancer model that is non-metastatic to lung *in vivo* (LRCP-BR21), there is low level of metastatic growth/colonization in the PuMA overall, with no difference in wildtype versus any of the knockout mice (**Figure 8D**). Taken together, these results suggest that OPN and selectins (but not FGF2) are individually important for lung metastasis of breast cancer.

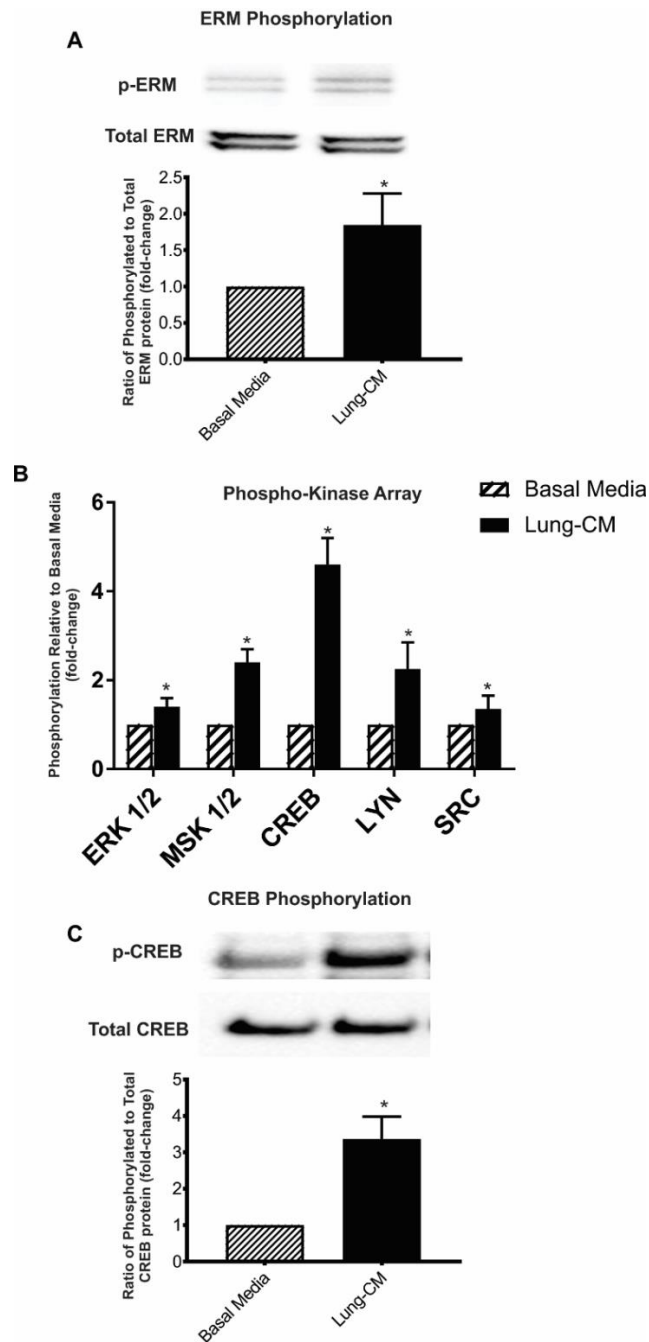


Figure 7: Effect of lung-conditioned media (CM) on protein phosphorylation in human breast cancer cells. MDA-MB-231 cells were cultured to 90% confluence and serum-starved for 24 h. Cells were then exposed for 15 min to basal media or native lung-CM and subjected to analysis by immunoblot or kinase array. Densitometry analysis was performed using Image Lab software (BioRad). Data is presented as fold-change in ratio of total:phosphorylated protein relative to basal media condition (n=3). **(A)** ERM phosphorylation as assessed by immunoblotting; **(B)** Broad investigation of protein phosphorylation using the Human Phospho-Kinase Array (R&D Systems); **(C)** Representative validation of CREB phosphorylation using immunoblot analysis. * = significantly different than basal media ($p \leq 0.05$). All other phosphorylation results were also validated by immunoblotting (*data not shown*).

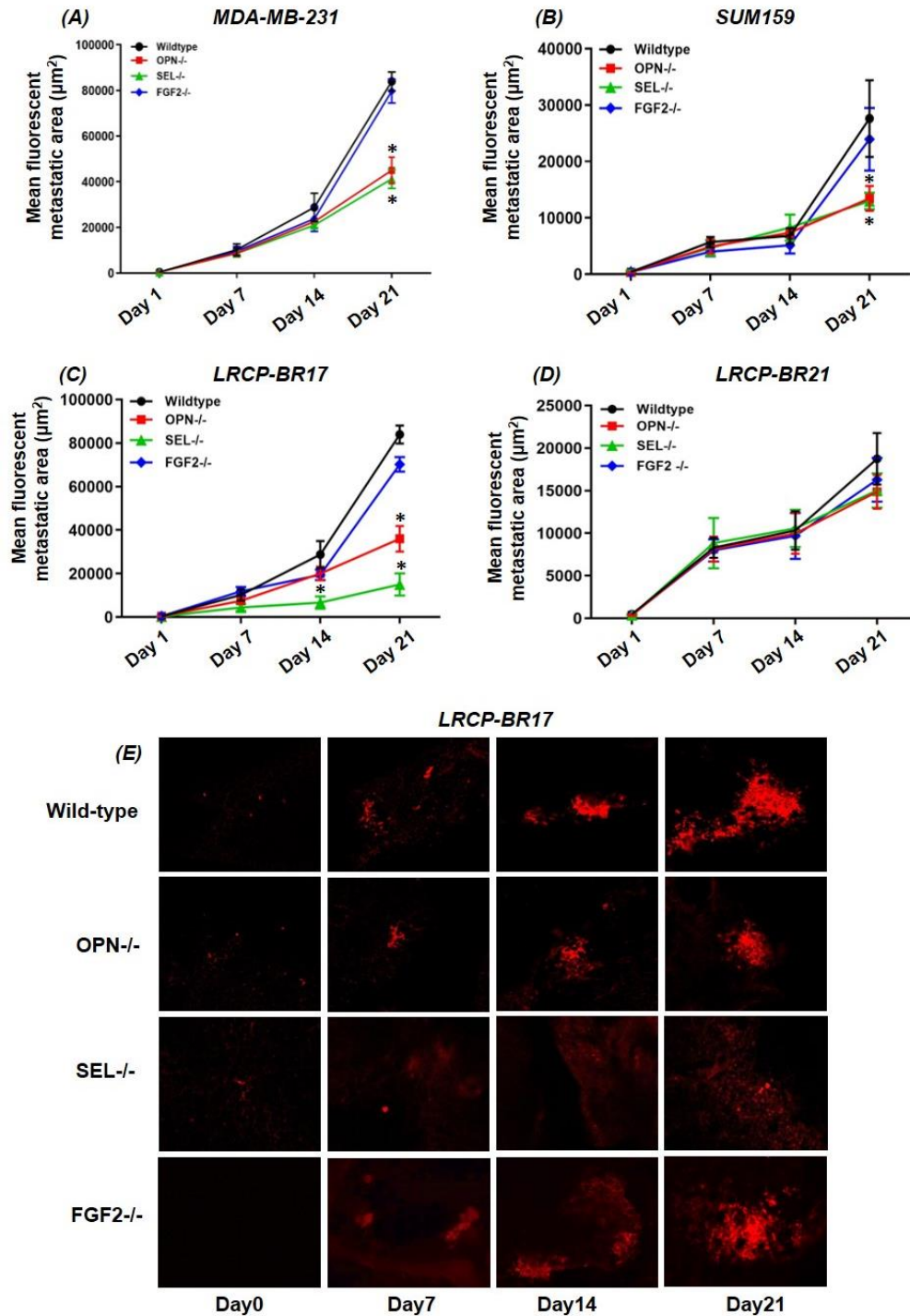


Figure 8: Loss of OPN or E/L/P selectins but not FGF2 in the lung reduces breast cancer metastatic progression in the pulmonary metastasis assay (PuMA). To assess the essentiality of OPN, selectins, or FGF2 for metastatic progression in the *ex vivo* PuMA assay, red-fluorescent labeled MDA-MB-231 (A), SUM159 (B), LRCP-BR17 (C) and LRCP-BR21 (D) breast cancer cells were injected into wildtype (black datasets), OPN^{-/-} (red datasets), triple-selectin^{-/-} (green datasets), or FGF2^{-/-} (blue datasets) mice, lungs were harvested after 15 minutes and subjected to the PuMA for 21 days (n=4 mice/dataset/cell line/timepoint). Data is presented as mean normalized fluorescent area (μm²) ± SEM. * = significantly different than wildtype (p ≤ 0.05). (E) Representative images for metastatic progression in the LRCP-BR17 model for each timepoint are shown.

TASK 2.4: Prepare and publish manuscript from Specific Aim 1.

One manuscript focused on the E-, L-, and P-selectin data has been accepted with major revisions in the journal *Biomedicines* (IF: 6.081). Revisions were resubmitted on October 05, 2021 and the final acceptance decision is pending. A copy of the submitted manuscript is included in the Appendices. We also anticipate that the data related to OPN and FGF2 will be included in secondary manuscripts in the future.

In conclusion, the major accomplishments/findings for Task 2 to date include establishment of OPN^{-/-}, triple selectin^{-/-}, and FGF2^{-/-} breeding colonies of knockout mice, which have been used to demonstrate that OPN and selectins (but not FGF2) are necessary for metastatic colonization of the lung, particularly for more aggressive breast cancer cell lines. This data provided a solid framework to move on to therapeutic application in Specific Aim 2, with a focus on pan-selectin inhibition.

Major Task 3: Formulation and production of an inhalable inhibitor

TASK 3.1: Load inhibitors into polybutyl cyanoacrylate nanoparticles; and

TASK 3.2: Assess and optimize NP drug loading efficiency/capacity.

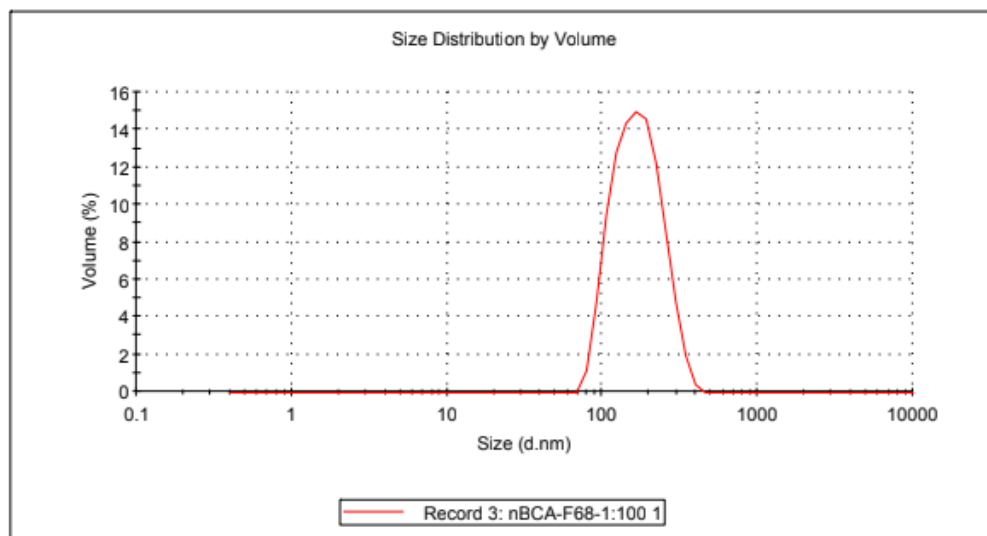
These tasks are now 100% complete. Initial work was aimed at characterizing nanoparticles obtained from different monomers and synthesis route. A platform of different conditions that can be used to efficiently synthesize nanoparticles was developed. Assessment of different nanoparticles was made using the particle size (Z-average, nm), PDI (polydispersity index) and ease of synthesis as criteria. Parameters such as type and quantity of monomer, type and quantity of surfactants, stirring time, temperature, amount of solvent and purification method were varied for optimization.

Poly-butylcyanoacrylate nanoparticles: The poly-butylcyanoacrylate (PBCA) nanoparticles were prepared using n-butylcyanoacrylate (Loctite, Ireland) as monomer. The nanoparticles were prepared according to the following procedure: 100 μ L of monomer was added to 1% dextran 70 (Sigma, Canada) solution in 10 mL of 0.01 M HCl under constant stirring at 600 rpm for 4 hours at room temperature. The particle size distribution was measured by dynamic light scattering using a photon correlation spectrometer (Zetasizer HAS 3000) from Malvern instruments. Both filtered (through a 0.1 μ m filter) and non-filtered samples were diluted 10 times in purified and degassed water and analyzed in triplicates. Good results were obtained using this method and the procedure was repeated at least in triplicate to guarantee reproducibility. PBCA nanoparticles were also prepared using a combination of 1% dextran 70 and 0.2% Pluronic F68 according to the aforementioned method. When measuring particle size, 1:10 and 1:100 dilutions were used. Good results were obtained for this method as well, which are shown in **Figure 9**.

Gelatin nanoparticles: Gelatin nanoparticles were prepared using a two-step desolvation process. Desolvation is the removal of the solvent component from the particle as a method of drying the sample in solution. In this case, the water was removed using acetone. Gelatin from two different manufacturers were tested. Three different batches from Gelita manufacturer were tested according to the following method: gelatin (1.25g) was dissolved in water (25ml) under constant heating (40°C) and stirring at 550 rpm. After completely dissolved, acetone (25ml) was added into the gelatin in solution in order to precipitate the high molecular weight gelatin of the starting material. The supernatant was taken out and the precipitate was then resuspended in water (25 ml) under heating and stirring. The solution pH was changed to 2.5 using 0.1M HCl. Acetone (75ml) was added

A

	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 166.2	Peak 1: 175.0	100.0	60.98
Pdl: 0.076	Peak 2: 0.000	0.0	0.000
Intercept: 0.948	Peak 3: 0.000	0.0	0.000
Result quality : Good			



	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 169.3	Peak 1: 177.7	100.0	56.59
Pdl: 0.058	Peak 2: 0.000	0.0	0.000
Intercept: 0.955	Peak 3: 0.000	0.0	0.000
Result quality : Good			

B

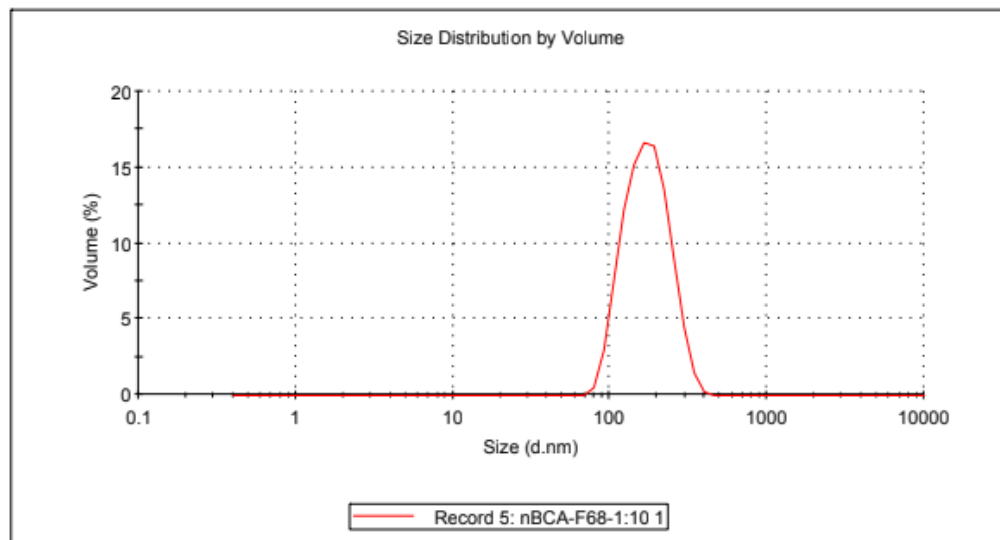


Figure 9. Particle size (diameter, nm) and Pdl of PBCA nanoparticle prepared with dextran 70 and Pluronic F68. **(A)** Dilution factor of 100. **(B)** Dilution factor of 10

dropwise for the second step of the desolvation process under constant stirring at 550 rpm and heating at 40°C. 100 μ l of 8% glutaraldehyde was added into the mixture as a crosslinker and the mixture was stirred overnight. After completion of synthesis, the solvent was removed using a rotary vapor. The particles were resuspended in water and centrifuged at 80,000 rpm for 30 minutes. The pellet was suspended in purified and degassed water for characterization.

As shown in **Figure 10A**, for the first batch, a sharp peak was obtained, nevertheless the particles were too big. For the second batch (**Figure 10B**), although the particle size was big, a good PDI was obtained as well as a sharp peak. For the third batch, no precipitation occurred in the first desolvation step. Gelatin nanoparticles using gelatin Type B from bovine skin (Sigma-Aldrich) were prepared using the aforementioned method. The method showed poor quality results and low yield. The method was then optimized varying the water/acetone ration and amounts, different amount of starting material, temperature, concentration of cross-linker, probe sonication of sample, ultrafiltration vs. centrifugation vs. filtration and different gelatin starting material. The best result obtained (**Figure 11**) was using gelatin type B from bovine skin 225 bloom (Sigma-Aldrich), using 1g of starting material, water/acetone ratio of 1:1 (20 ml); the first precipitate was dissolved in water under vigorous stirring at 10,000 rpm and 50°C. Acetone (20ml) was added dropwise and stirred for one hour. After acetone evaporation, samples were centrifuged at 10,000g for 30 minutes and the pellet was resuspended in water followed by filtration through a 0.22 μ m syringe filter.

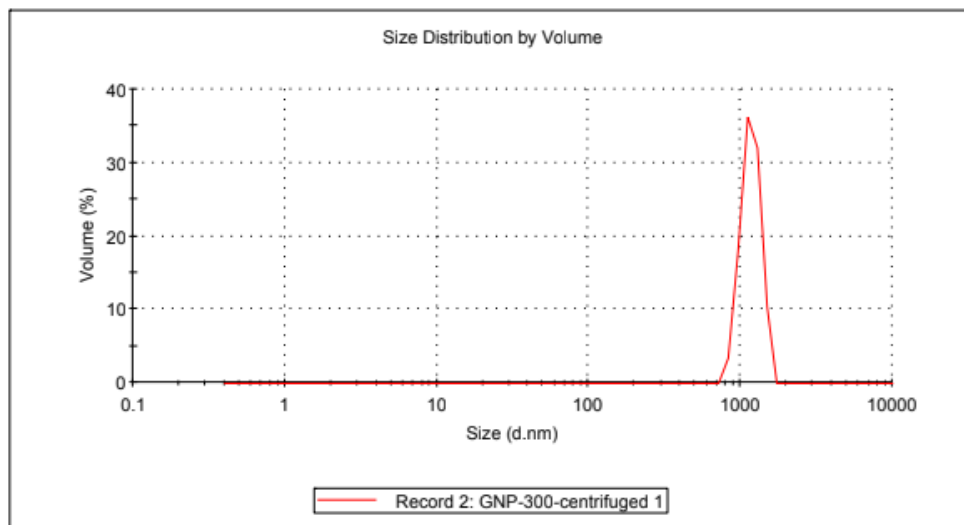
Nanoparticle properties after drug loading: PBCA nanoparticle was selected to continue with the drug loading and antibody-coating experiments due to ease of synthesis, high yield, good quality and reproducibility. Doxorubicin was used as the model drug to optimize the drug loading. The drug was added in the mixture after 30 minutes of stirring. Particles were characterized after drug loading. Results showed satisfactory particle size and PDI, 140 nm ($\pm$ 10.56) and 0.159 ($\pm$ 0.027), respectively

Antibody coating: PBCA nanoparticles prepared with dextran 70 and Pluronic F68 were used for the antibody coating due to the presence of aldehyde groups on its surface that the antibody can be adsorbed onto. MBU-monoclonal antibody was used as a model for this purpose. The nanoparticles were incubated with 100 μ g/ml of the antibody in different settings: in a beaker shaking at room temperature or at 4°C or in an Eppendorf rotating at room temperature or at 4°C (3). The samples were then centrifuged at either 12.5 rpm for 20 minutes or 24psi for 30 minutes. The supernatant was separated and used for ELISA testing. It was assumed that low supernatant antibody concentration was due to adsorption onto the particles.

ELISA procedure: Antigen solution was prepared (myclobutanil and myclobutanil-BSA conjugate) (10 μ g/mL) in coating buffer (0.01M Carbonate/bicarbonate, pH 9.4-9.6) and 100 μ L was added to each well onto the 96 well assay plate, including buffer control and negative control. The plate was covered and incubated at 2-8 °C overnight. The solution was aspirated and adherent drops were removed by tapping the inverted plate on a piece of paper towel. 300 μ l of wash buffer (PBS containing 0.05% Tween-20) was added to each well and aspirated, this procedure was repeated three times. Blocking buffer (300 μ l - PBS containing 1% BSA) was added to each well and the plate was

A

	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 2313	Peak 1: 1165	100.0	167.5
Pdl: 1.000	Peak 2: 0.000	0.0	0.000
Intercept: 0.968	Peak 3: 0.000	0.0	0.000
Result quality : Refer to quality report			



B

	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 816.2	Peak 1: 888.7	100.0	156.9
Pdl: 0.077	Peak 2: 0.000	0.0	0.000
Intercept: 0.945	Peak 3: 0.000	0.0	0.000
Result quality : Refer to quality report			

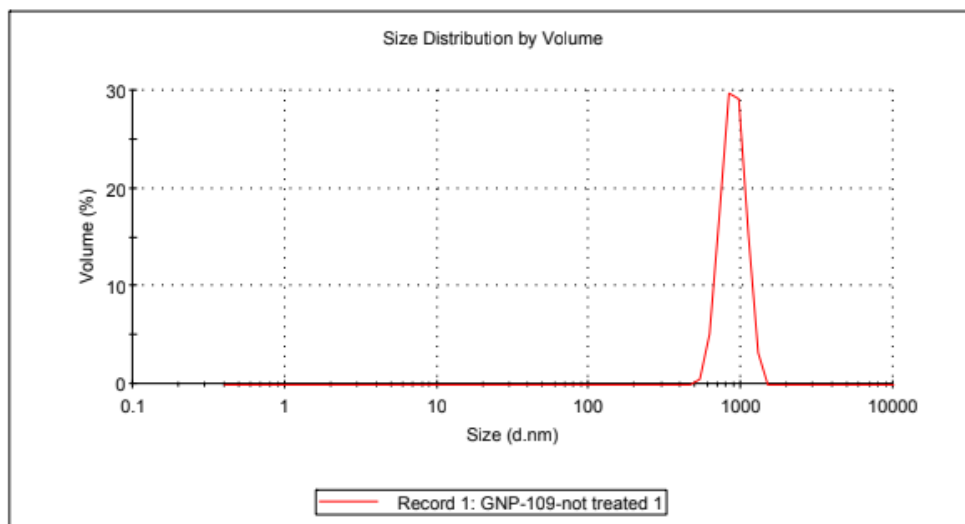


Figure 10. Particle size (diameter, nm) and Pdl of gelatin nanoparticle using different batches from Gelita manufacturer.

	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 224.4	Peak 1: 266.9	100.0	99.48
Pdl: 0.126	Peak 2: 0.000	0.0	0.000
Intercept: 0.889	Peak 3: 0.000	0.0	0.000

Result quality : Good

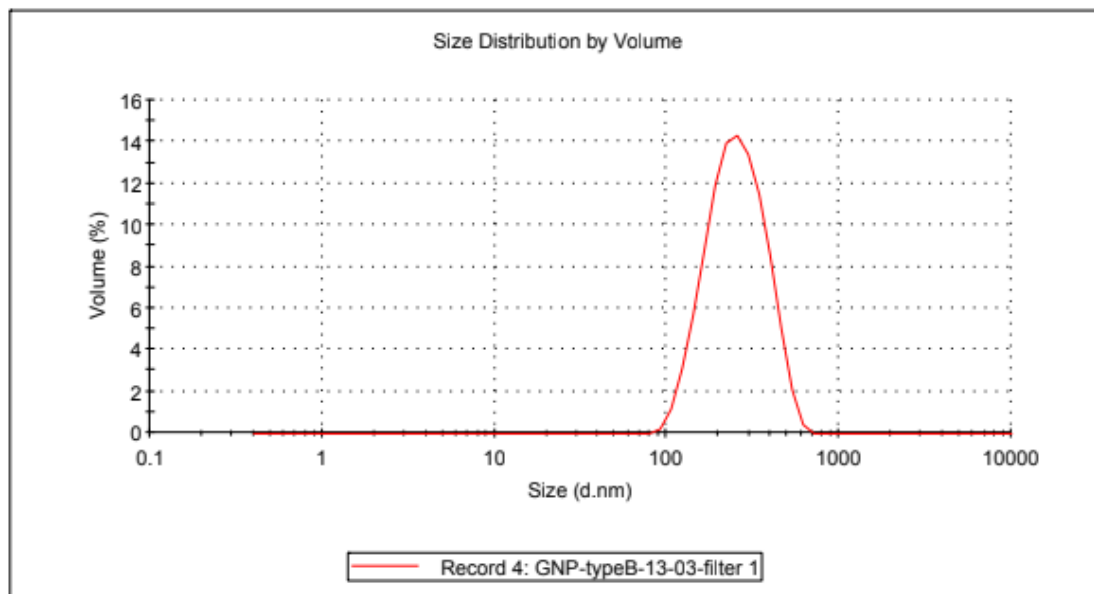


Figure 11. Particle size (diameter, nm) and Pdl of gelatin nanoparticle using gelatin type B from bovine skin.

Table 1**15 minutes incubation for color development**

	1	2	3	4	5
A		0.1685	0.2108	0.1429	0.1236
B Control		0.1151	0.0912	0.0997	0.0973
C Standard		0.2039	0.2207	0.3782	0.4015
D Standard		0.1904	0.3844	0.3786	0.3448
E		0.1402	0.1231	0.1412	0.4246
F		0.1102	0.1182	0.0777	0.3944
G		0.1422	0.1097	0.111	0.1066
H		0.0901	0.1029	0.0939	0.0819

30 minutes incubation for color development

	1	2	3	4	5
A		0.2514	0.2888	0.212	0.1818
B Control		0.1708	0.1341	0.1431	0.1245
C Standard		0.2964	0.4368	0.5243	0.547
D Standard		0.324	0.7246	0.5549	0.555
E		0.2306	0.1706	0.1772	0.6042
F		0.1641	0.1739	0.1278	0.58
G		0.2145	0.1615	0.1424	0.1469
H		0.127	0.148	0.1385	0.1208

Color code and legends:

	1	2	3	4	5
Control		CB+PBS+2AB+S	x	x	
Control		Ag+PBS+2AB+S			
Standard: 1AB		10ug/ml	25ug/ml	50ug/ml	100ug/ml
concentrations		10ug/ml	25ug/ml	50ug/ml	100ug/ml
		RTs C	RTs A		100
		Ice C	Ice A		100
		RTr C	RTr A	x	
		Ice'C	x	x	

Ag+1AB+2AB+S

kept at room temperature for 1 h. The plate was then washed three times. The supernatant of the samples previously prepared with antibody and PBCA nanoparticles were diluted with blocking buffer and pipetted to the required wells. The plate was incubated at 37°C for one hour. The plate was washed and secondary antibody (diluted HRP-conjugated goat anti- mouse IgG antibody) was added to all the sample wells except substrate control wells and the plate was incubated at 37°C for 30 minutes. After washing plate, the substrate solution was added to each well followed by incubation at 37°C for 15/30 minutes. Absorbance was measured at 405 nm on an automatic ELISA plate reader. **Table 1** shows the absorbance results.

TASK 3.3: Incorporate optimized drug-loaded NPs into inhalable effervescent carrier particles; & TASK 3.4: Quality assurance assessment of inhalable drugs

Following this optimization and the results obtained to date for Task 2, we moved forward with optimizing the use of the pan-selectin antagonist bimosiamose as an inhalable drug for lung metastasis. This choice of inhibitor was supported by both the results of Specific Aim 1, as well as additional *in vitro* studies demonstrating that bimosiamose (1 mM) effectively inhibits breast cancer proliferation and migration in response to lung-conditioned media (**Figure 12**).

The selected pan-selectin antagonist bimosiamose is an organic compound that belongs to the phenolic glycoside class. Its physicochemical properties are displayed in **Table 2**. The high lipophilicity and low water solubility of bimosiamose make it an ideal candidate for the use of nanocrystals as a drug delivery system. The reduction of the particle size into the nanometer scale is a promising approach for hydrophobic drugs in order to increase surface area which, in turn, increases the dissolution rate and solubility of poorly water-soluble compounds like bimosiamose. This is specially desired for the inhalable route of administration, since the drug has to dissolve in the low volume of pulmonary fluids.

The nanocrystal production was done using the wet bead milling process. During the stirring (high shear forces), the drug particles collide with the zirconia beads in the milling chamber, and with other drug particles, causing the drug crystals to be fractured into nanosized particles. Shear forces in the liquid phase are stronger compared to dry milling. In order to verify the best conditions and the system's suitability to reduce the particle size of bimosiamose, a compound with similar physicochemical properties was used as model (**Table 2**). Hence, this study was aimed to characterize nanocrystals obtained by wet milling process under a range of conditions with the goal to further optimize the process using the model drug erythromycin ethylsuccinate.

At an early research stage, as well as at an early stage in drug development, only small quantities of the active compound are available. Hence, using a particle size reduction method which requires only low amounts is essential. The miniaturized wet bead milling method is a valuable approach in this setting. The system is composed of an aqueous dispersion media (in which the drug has low solubility) containing stabilizers (surfactants, polymers, or both), milling pearls (zirconia beads), two magnetic stirring bars and the drug. The system is agitated by a magnetic stirring plate at a very high speed, which is the reason why stabilizers are necessary.

Preparation of nanosuspension by miniaturized wet bead milling method: Erythromycin ethylsuccinate (EE) nanosuspension was prepared by wet bead milling at a reduced scale. The system contained 3% (w/w) EE, 10 to 20% of zirconia beads (0.1 mm and density 3.7 g/cm³) and a 3% (w/w) Povacoat® (a novel PVA copolymer) aqueous solution of pH 5.7 for a total weight of 1g. The milling chamber consisted of a 2 mL glass vial containing two magnetic stir bars, polygon-shaped (8x2mm). The system was stirred at 800 rpm for up to 8 days at room temperature. Samples of 10µL were collected each day.

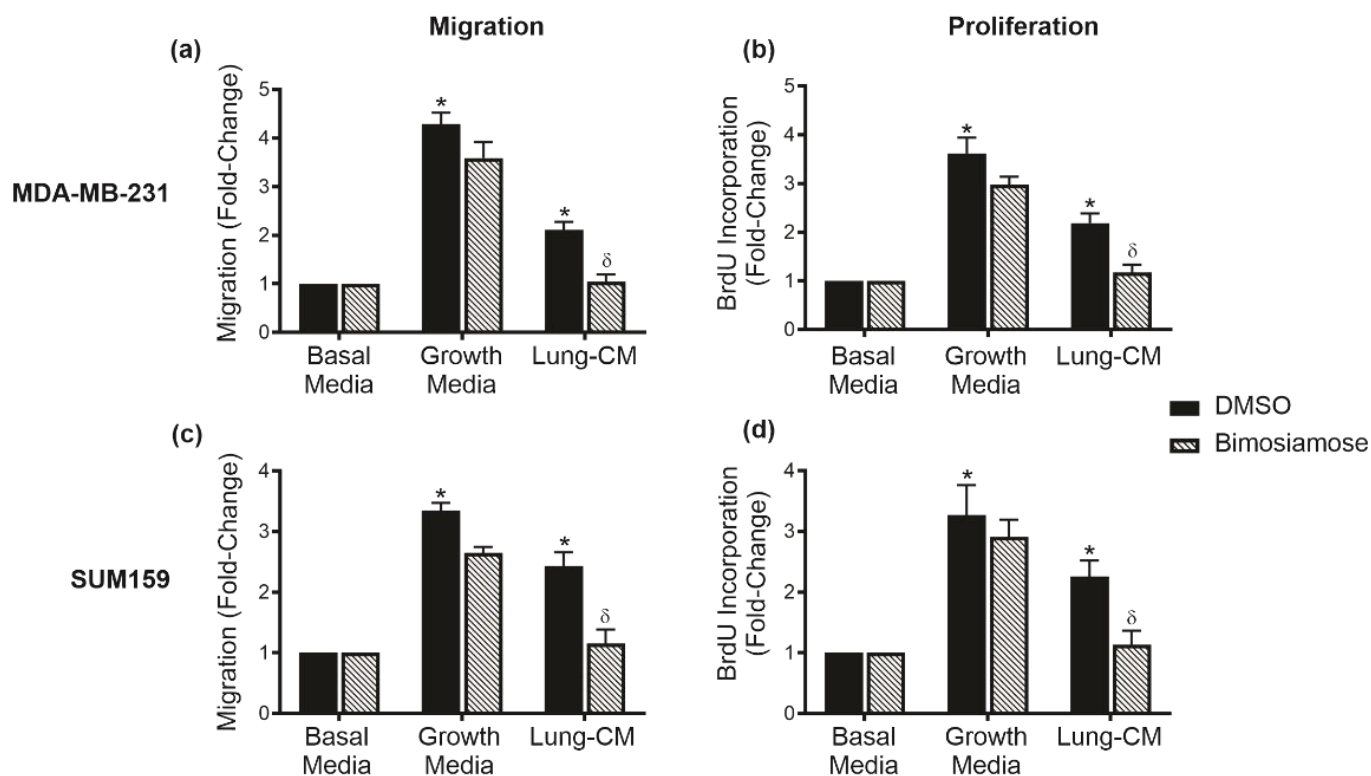
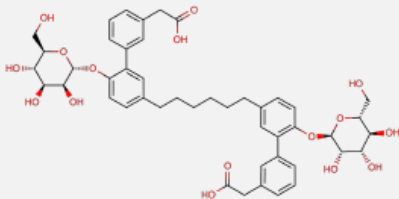
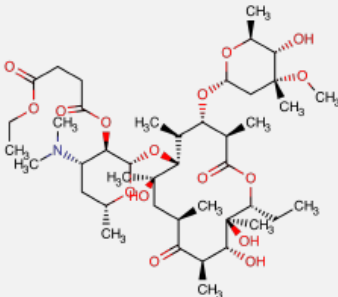


Figure 12: The pan-selectin inhibitor bimosiamose reduces *in vitro* lung-specific TN breast cancer migration and proliferation. **(a,b)** MDA-MB-231 and **(c,d)** SUM159 human breast cancer cells were treated with DMSO (vehicle control; black bars) or 1 mM bimosiamose (hatched bars) and subjected to transwell migration **(a,c)** or proliferation **(b,d)** assays in the presence of basal media, growth media (10% FBS) or lung-CM. Five HP-FOV were captured per transwell and analyzed using ImageJ. Data is presented as mean $\pm$ SEM; fold-change in relative to basal media conditions (n=3). * = significantly different from basal media. δ = significantly different from respective DMSO control.

Table2. Physicochemical properties of bimosiamose and erythromycin ethylsuccinate

	BIMOSIAMOSE	ERYTHROMYCIN ETHYLSUCCINATE
CHEMICAL STRUCTURE		
Molecular weight (g/mol)	862.922	862.064
Log P	3.71	3.37
Log D (@ pH 5.7)	0.603	0.652
Log S (@ pH 5.7)	-3.8	0.133

Particle size distribution: The particle size distribution (PSD) as well as the poly-dispersity index (PDI) were measured by dynamic light scattering using a photon correlation spectrometer (Zetasizer HAS 3000) from Malvern instruments. Samples were diluted to a suitable concentration, indicated by the best attenuation coefficient. Both purified degassed water and saturated EE solution were used to measure the PSD to identify if the particles would partially dissolve when prepared for the measurements with pure water, giving a false smaller particle size.

Figure 13 shows the monitoring of the PSD of a nanosuspension system with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer. Different populations can be identified during the first day of stirring, which is also reflected on the high PDI. The stirring and the PSD monitoring continued for 7 days. Not much difference was seen between days 2 and 3 (**Figure 14**). After 3 days stirring the PDI was within an acceptable range (only 1 population of nanocrystals) and the average particle size was 740 nm. After 7 days stirring a PSD of 458nm was obtained with an even lower PDI (**Figure 15**). Using water to dilute the samples did not give accurate measurements, as shown in **Figure 16**.

FINAL OPTIMIZATION OF INHALABLE BIMOSIAMOSE

Particle size reduction into the nanometer scale is a promising approach to increase the dissolution of hydrophobic drugs such as bimosiamose. The drug particles are constituted as matrix-free nanocrystals and are stabilized by surfactants, polymers, or a mixture of both. This system (stabilizer and crystals) is called a nanosuspension.

Different approaches can be taken to produce nanocrystals, such as top-down methods. Wet media milling is one example of such, in which large particles are broken into small sized particles using mechanical grinding. The miniaturized wet-bead milling system consists of a milling glass chamber filled with zirconia beads, the drug substance, a dispersion media (stabilizer in aqueous solution), and magnetic stirring bars. This system is agitated by a magnetic stirrer plate at high speed. The advantages of this is that it is a simple and low-cost method which is performed in small volumes, providing advantages in early drug development.

The stabilizing agents (surfactants and polymers) play an important role in particle size reduction and stability of nanosuspensions. In this study we screened different stabilizers (namely: Hydroxypropyl cellulose (HPC), Polyvinyl alcohol (PVA), Polyvinylpyrrolidone (PVP), Poloxamer 188 and Povacoat) to be used in the wet milling process, all of which are GRAS and have been reported to be used in inhalable therapies. Since nanocrystals, due to their small size, tend to get stuck in the upper airways, they were incorporated into microcarriers. This will enable the powder to reach the deep lung.

Preparation of nanosuspension by miniaturized wet bead milling method: A bimosiamose nanosuspension was prepared by wet bead milling at a reduced scale. The system contained bimosiamose (10 mg), zirconia beads (0.5 g) and a stabilizer aqueous solution (0.5 mL) (HPC, PVA, PVP, Poloxamer 188 or Povacoat). The milling chamber consisted of a 2 mL glass vial containing two magnetic stir bars, polygon-shaped (8x2 mm). The system was stirred at 800 rpm at room temperature up to 48hrs depending on the stabilizer used. For HPC, PVA, PVP and Povacoat, the proportion between stabilizer and drug was 4:1 and for Poloxamer 3:1.

Particle size distribution and stabilizer screening: The particle size distribution (PSD) as well as the polydispersity index (PDI) were measured by dynamic light scattering using a photon correlation spectrometer (Zetasizer HAS 3000) from Malvern instruments. Samples were diluted to a suitable concentration, indicated by the best attenuation coefficient. The PSD results are indicated as size distribution by volume.

Results

		Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 1364	Peak 1:	837.9	80.5	134.6
Pdl: 0.868	Peak 2:	87.60	19.5	13.80
Intercept: 0.946	Peak 3:	0.000	0.0	0.000
Result quality : Refer to quality report				

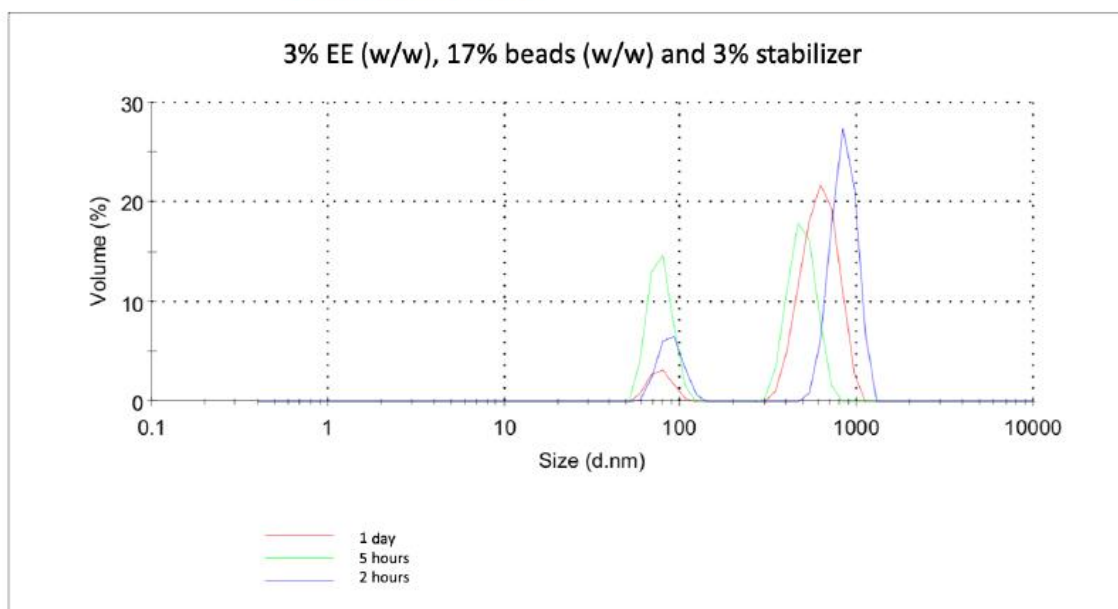


Figure 13. Particle size distribution (diameter, nm) and Pdl of EE nanocrystals prepared with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer at 2 hours, 5 hours and 24 hours.

Results

	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 740.4	Peak 1: 1164	98.8	355.7
Pdl: 0.263	Peak 2: 172.4	1.2	46.34
Intercept: 0.939	Peak 3: 0.000	0.0	0.000
Result quality : Good			

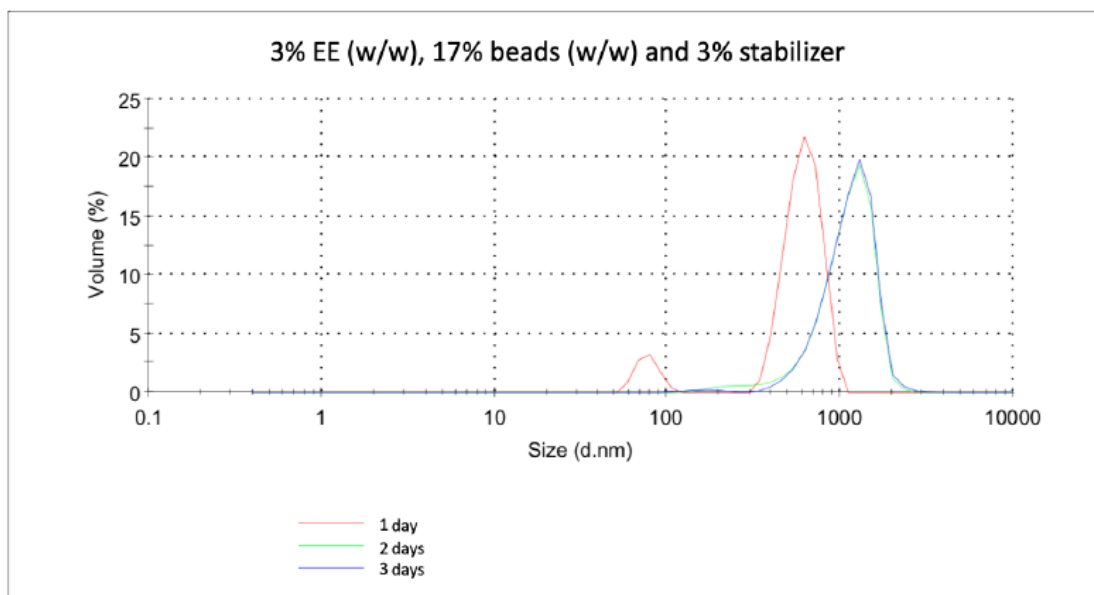


Figure 14. Particle size distribution (diameter, nm) and Pdl of EE nanocrystals prepared with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer after 3 days stirring.

Results

	Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm): 458.8	Peak 1: 738.3	100.0	304.9
Pdl: 0.195	Peak 2: 0.000	0.0	0.000
Intercept: 0.953	Peak 3: 0.000	0.0	0.000
Result quality : Good			

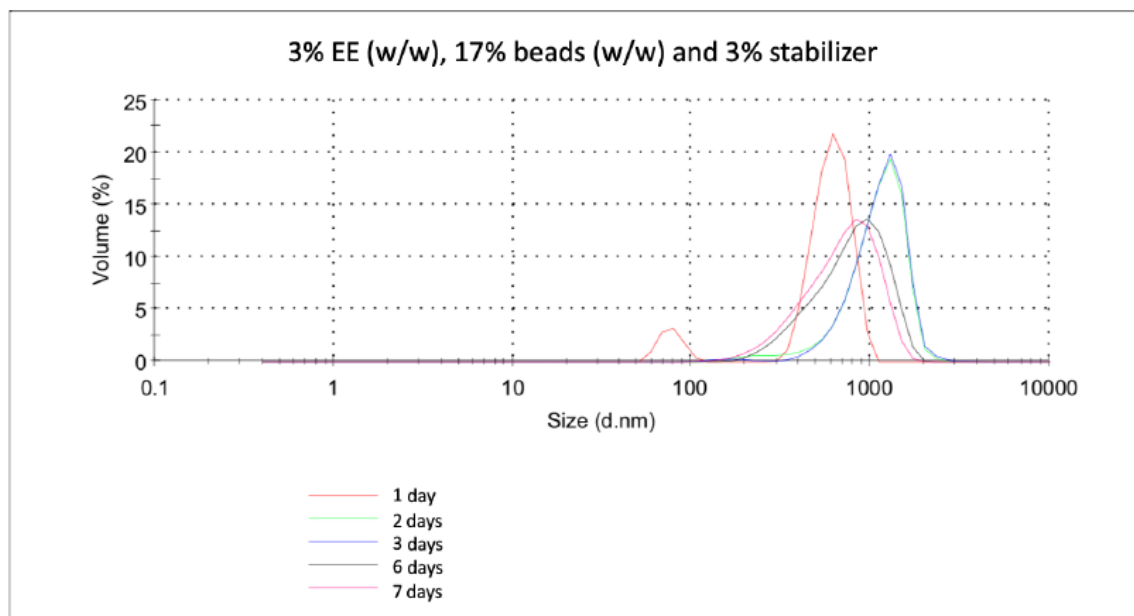


Figure 15. Particle size distribution (diameter, nm) and Pdl of EE nanocrystals prepared with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer after 7 days stirring.

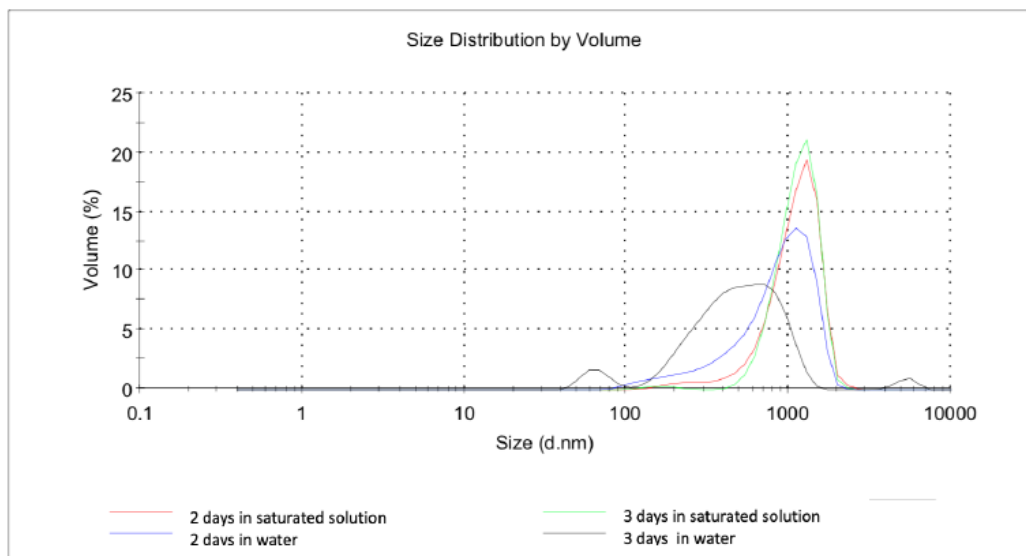


Figure 16. PSD (diameter, nm) of EE nanocrystals in water and saturated solution.

Povacoat: For Povacoat, the samples were collected for analysis at 1h, 24hrs, 36hrs and 48 hrs. **Table 3** shows the Z-average for the PSD of the nanosuspension system (done in triplicate) at the different collection time points. Different populations could be identified during the first hour of stirring. Povacoat was initially developed as a film-coating agent, wet granulation binder and solid dispersion matrix, but recent studies revealed its use as a stabilizer to prevent aggregation of nanocrystals. Because it formed a dispersion when added to water, we investigated the particle size of Povacoat alone in water (**Figure 17**). Notably, Povacoat dispersion is also in a nano scale. Hence, in order to minimize confounding factors in the PSD measurements, other stabilizers were screened: HPC, PVA, PVP and Poloxamer 188. These were completely dissolved in water, resulting in a stabilizer solution instead of a suspension.

Povacoat, HPC, PVA, PVP and Poloxamer 188: Based on the results using Povacoat, the stirring time was set to 24 hours. **Table 4** shows the Z-average for the PSD of the nanosuspension system for the different stabilizers, including a second trial using Povacoat (for comparison purposes and repeatability assessment). Based on the Z-average and Pdl results, and considering its wide use in inhalable systems, Poloxamer 188 was the stabilizer of choice.

Poloxamer 188: In order to obtain particles with bigger particle size the stirring time was reduced to 2hrs (**Table 5**). To assess the repeatability of the selected system, other batches were prepared using the 2hrs stirring time and the result was consistent (Z-avg: 117.8nm; Pdl: 0.120).

Microcarrier preparation: The optimized nanosuspension formulation was spray-freeze dried using mannitol as the carrier agent/cryoprotectant. Mannitol was dissolved in water and this solution was used to dilute the nanosuspension to a final volume of 10mL with a total solid content of 10% (w/v). The mixture was sprayed in liquid nitrogen using the following parameters: atomized air pressure: 1bar; spray rate: 0.35 ml/min; liquid nitrogen volume kept constant at 700 ml, spray nozzle positioned 10cm above the liquid nitrogen level. Once the spraying was finished, the liquid nitrogen was completely evaporated at room temperature and the samples were lyophilized overnight. The mass median aerodynamic diameter (MMAD) of the powder was measured using the Penn-Century dispersing device coupled to an aerosol diluter and an aerodynamic particle sizer (APS). The MMAD determines the deposition location of particles within the lungs. A size between 2-5 μm is usually desired to reach the deep lung. The MMAD of inhalable powder obtained was $1.81 \pm 0.64 \mu\text{m}$, Geometric standard deviation (GSD) = 2.62, which is acceptable for the intended purpose.

Table 3. Particle size distribution (diameter, nm) and Pdl of bimosiamose nanocrystals prepared with Povacoat.

Time	Z-avg (nm)	Pdl	Number of peaks
1 hour	127.7	0.234	2 (150.3 and 4779 nm)
24 hours	130.9	0.108	1
36 hours	120.5	0.104	1
48 hours	108.1	0.106	1

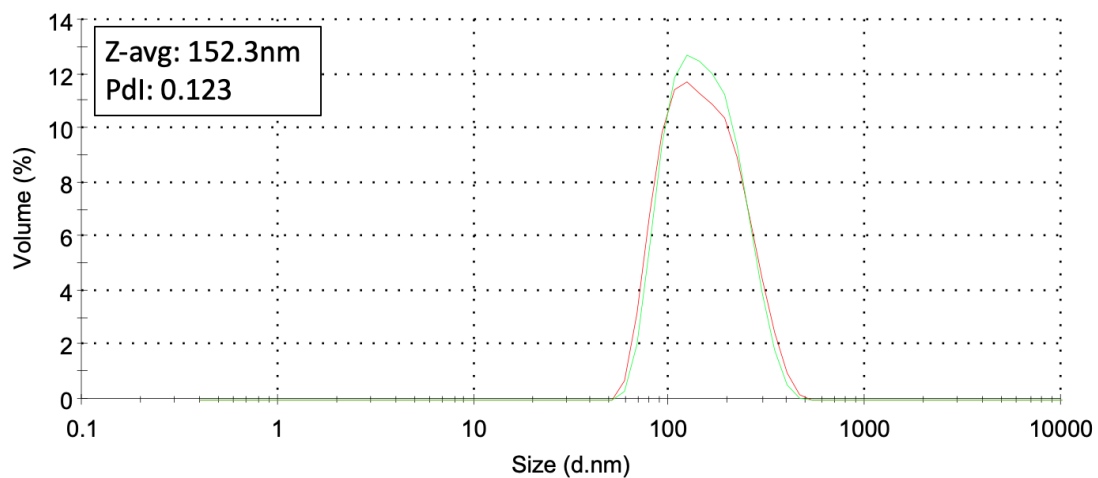


Figure 17. Particle size distribution (diameter, nm) and Pdl of Povacoat alone in water.

Table 4. Particle size distribution (diameter, nm) and Pdl of bimosiamose nanocrystals prepared with different stabilizers.

Stabilizer	Z-avg (nm)	Pdl
Povacoat	91.43	0.110
HPC	123.9	0.159
Poloxamer 188	83.72	0.102
PVP	83.76	0.102

Table 5. PSD (diameter, nm) and Pdl of bimosiamose nanocrystals prepared with Poloxamer 188.

Time	Z-avg (nm)	Pdl
2hrs	115.70	0.140
4.5hrs	99.61	0.124
5.5hrs	92.00	0.116

The nanocrystal suspension contained 20 mg of drug and stabilizer plus mannitol dissolved in water at a concentration for a total solid composition of 10% w/v. The total solid content was 1g (in 10mL), which contained 20 mg of drug, hence, 2% w/w. The final sample weight was 220 mg, giving a drug content of 4.4 mg (2% w/w), which was enough for the total amount of drug needed for the *in vivo* studies, according to the selected study design. Therefore, a formulation of inhalable nanocrystals to be used as a therapeutic strategy against breast cancer metastasis to the lungs was successfully developed, and the inhalable particles were used for the final studies in Major Tasks 4 and 5.

Major Tasks 4 and 5: *In vivo* assessment of anti-metastatic efficacy of inhalable drugs using preventative and therapeutic approaches

For the preventative approach, healthy female nude mice (n=8/group) anesthetized and inhalable bimosiamose nanocrystals (25 µg/dose/mouse) or mannitol vehicle were delivered as a pre-treatment using a DP-4M insufflator (Penn-Century Inc.). Two days after the first inhalable treatment, MDA-MB-231 cells were injected via tail vein (t.v.; 1×10^6 cells/mouse; n=8/group). Mice were then treated with inhalable bimosiamose nanocrystals (25 µg/dose/mouse) or vehicle for an additional 3 weeks post-injection to maintain pan-inhibition of lung-derived selectins. Lung metastases were allowed to develop for a further 16 weeks prior to sacrifice. For the therapeutic approach, MDA-MB-231 cells were injected via mammary fat pad (m.f.p.; 1×10^6 cells/mouse; n=8/group) and allowed to develop primary breast tumors for 4 weeks. Mice were then randomized into control or treatment groups and treated with inhalable bimosiamose nanocrystals (25 µg/dose/mouse) or vehicle for 4 weeks. Lung metastases were allowed to develop for a further 12 weeks prior to sacrifice. At endpoint, tissues (lung, liver, brain, bone, lymph nodes) were collected, formalin-fixed and are currently being sectioned (4 µm) and stained with H&E in order to assess and quantify differences in presence/extent of metastatic involvement between treatment groups.

These final two tasks were delayed due to COVID-19 (detailed in the Changes/Problems section), however with a one-year no-cost extension to the project we were able to complete the animal experiments by the end of the extended grant term in August 2021, with final data analysis ongoing. Once this is complete, it is our intent to prepare and publish a manuscript combining the drug development process and the *in vivo* drug testing results.

What opportunities for training and professional development has the project provided?

Dr. Ying Xia, Research Associate:

As part of this project, Dr. Xia provided one-on-one mentorship to 1 research undergraduate student, Gabriella Schoettle; 1 PhD student, Braeden Medeiros; and a postdoctoral fellow, Dr. Vasu Bhat, all of whom contributed to the major research activities of the project. Dr. Xia assisted these trainees in attaining greater proficiency in experimental techniques including cell culture and data analysis.

Dr. Vijay Somayaji, Research Manager:

As part of this project, Dr. Somayaji provided one-on-one mentorship to 1 PhD student, Daniela Amaral-Silva, who contributed to the major research activities of the project as described above. Dr.

Somayaji assisted Daniela in attaining greater proficiency in pharmaceutical and drug formulation techniques.

Daniela Amaral-Silva, PhD Candidate

Daniela Amaral-Silva obtained her PhD; with her doctoral thesis based partially on the drug development of inhalable bimosiamose nanocrystals.

Professional Development Opportunities

Dr. Alison Allan, PD/PI

To further her knowledge and skills in the area of breast cancer metastasis, Dr. Allan participated in the 17th International Biennial Congress of the Metastasis Research Society, which was held in Princeton, NJ in August 2018 and the Canadian Cancer Research Conference which was held in Ottawa, Canada in November 2019. Unfortunately, COVID-19 limited external professional development opportunities in 2020 and 2021.

How were the results disseminated to communities of interest?

Scientific Publications

1. Bhat, V., **Allan, AL.**, and Raouf, A. Role of the microenvironment in regulating normal and cancer stem cell activity: implications for breast cancer progression and therapy response. *Cancers*, 2019 Aug 24;11(9). pii: E1240. doi:10.3390/cancers11091240 (*see Appendix for manuscript*). Federal support acknowledged: Yes.
2. Medeiros, B and **Allan, AL**. Molecular mechanisms of breast cancer metastasis to the lung: clinical and experimental perspectives. *Int J Mol Sci*, 2019, 20, 2272; doi:10.3390/ijms20092272 (*see Appendix for manuscript*). Federal support acknowledged: Yes.
3. Bhat V, Lefebvre C, Goodale D, Rodriguez-Torres M, **Allan AL**. Isolation and functional assessment of human breast cancer stem cells from cell and tissue samples. 2020 *JoVE*, doi:10.3791/61775 (*see Appendix for manuscript*). Federal support acknowledged: Yes.
4. Khan, S.U., Xia, Y., Goodale, D., Schoettle, G., and **Allan, AL**. Lung-derived selectins as a potential therapeutic target in triple negative breast cancer metastasis. Accepted with revisions (resubmitted October 2021) to *Biomedicines* (*see Appendix for manuscript*). Federal support acknowledged: Yes.

Scientific Presentations

David Goodale, Technician:

1. Goodale, D., Xia, Y., Postenka, C., and **Allan, AL**. Knockout of lung-derived osteopontin or selectins reduces metastatic colonization in an *ex vivo* model of breast cancer lung metastasis. 2019 Canadian Cancer Research Conference, November 2019, Ottawa Canada. Federal support acknowledged: Yes.

Daniela Amaral-Silva, PhD student

2. November 2019, San Antonio TX - American Association of Pharmaceutical Sciences Annual Meeting - AAPS PHARMSCI 360. Daniela attended the conference and presented two posters titled “Delayed release drug products behave differently in physiological bicarbonate buffer” and

“In Vitro Biphasic Dissolution Test: A Study of Excipient and Manufacturing Variables”. Federal support acknowledged: Yes.

Dr. Alison Allan, PD/PI:

3. **Allan, AL.** Cellular and molecular approaches to understanding breast cancer metastasis. Multidisciplinary Breast Team Research Evening, London, ON, Canada, September 2018. Federal support acknowledged: Yes.
4. **Allan, AL.** Role of the lung microenvironment in mediating breast cancer metastasis: a balance between the “seed” and the “soil”. Cancer Research Program Seminar Series, Research Institute of the McGill University Health Centre (RI-MUHC), Montreal QC, Canada, November 2018. Federal support acknowledged: Yes.
5. **Allan, AL.** Role of the lung microenvironment in mediating breast cancer metastasis: a balance between the “seed” and the “soil”. Department of Anatomy & Cell Biology Seminar Series, Schulich School of Medicine & Dentistry, Western University, London ON, January 2020. Federal support acknowledged: Yes.

Community Outreach Activities

Dr. Alison Allan, PD/PI:

1. *Media Story*, **Allan AL.**, [Stopping breast cancer from making its way to the lungs](#), October 29, 2020. CTV London News at 6, [online, print, and TV media].
2. *Media Story*, **Allan AL.** and Hill, D. [Layoffs, lost funding and how COVID-19 may permanently hurt London's medical-research sector](#). April 6, 2020, London Free Press.
3. *Research Ambassador*, **Allan A.L.** Why is research important? Media interviews on the Weather Network to kick off Breast Cancer Awareness Month in October 2018 and October 2019 [TV media]
4. *Invited Speaker*, **Allan AL.**; Opening Ceremonies of the Breast Cancer Society of Canada Mother’s Day Walk in London, ON, Canada in May 2018 and 2019. The audience mainly consisted of members of the lay community, including breast cancer survivors and their friends and family.
5. *Featured Scientist*, **Allan, AL.**, local media stories “[Seizing the Day](#)” (September 2017) and “[Driving innovation in a world where knowledge is the new currency](#)”, (April 2019), both intended to increase interest in learning and careers in science.

What do you plan to do during the next reporting period to accomplish the goals?

Not applicable – final report

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Despite significant advances in detection and treatment, breast cancer remains a leading cause of cancer death worldwide. This is primarily a result of metastasis (the spread of cancer from the breast to distant organs), as current therapies are non-curative in the metastatic setting. The novel studies completed in this project identified 5 key factors produced by the lung that are involved in lung metastasis, one of the most common and deadly sites of breast cancer dissemination. These lung-derived factors are proteins called osteopontin, basic fibroblast growth factor, E-selectin, L-selectin, and P-selectin, and we found that they interacted with breast cancer cells to support their growth, migration/movement, and/or metastatic progression in the lung. We then developed a new inhalable drug formulation to directly target the 3 selectin proteins in the lung and tested this in pre-clinical animal models to determine its toxicity and effectiveness in reducing breast cancer metastasis to the lung. Overall, the knowledge gained in this project regarding the interaction between aggressive breast cancer cells and the lung microenvironment during lung metastasis had laid important groundwork for translation into novel, low-toxicity therapies for breast cancer in the future.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Major Task 1:

No delays experienced.

Major Task 2:

Establishment of all planned breeding colonies of knockout mice was a bit slower than expected due to problems with some breeding pairs, however we worked closely with our animal facility veterinarian and these problems were resolved. We also had a delay in finishing the final set of *ex vivo* experiments with FGF2-/- mice due to the COVID19 research shut down between March 17, 2020 and July 02, 2020. We completed these experiments as soon as research activities were allowed to resume.

Major Task 3:

There was a slight delay at the start of the project in recruiting a PhD student with the appropriate expertise to work on the drug formulation aspect of the project under Dr. Loebenberg. Daniela Amaral-Silva was successfully recruited and has been actively working on the project since January 2018. There was also a delay/problem in obtaining stocks of the pan-selectin inhibitor Rivipansel from Pfizer. Pfizer is experiencing production issues and has to prioritize drug supplies for use in an ongoing human clinical trial. They are unsure when this is going to be resolved, so in May 2018 we decided to go ahead with an alternative pan-selectin inhibitor, Bimosiamose (MedKoo Biosciences). This inhibitor required custom synthesis and QA/QC, which was a 3-4 month process. We received the drug in November 2018, at which point we began the inhalable drug development studies.

Major Tasks 4 & 5:

We were on track to begin the *in vivo* experiments in March 2020, but were delayed by the COVID-19 research shut down between March 17, 2020 and July 02, 2020. Large animal experiments were not permitted in the initial phases of re-opening, however in late August 2020 we received permission to move ahead with the first animal experiments for Major Task 4, and these experiments along with those for Major Task 5 were just completed in August 2021, with final data analysis and publication pending.

Changes that had a significant impact on expenditures

The delay in recruiting Daniela Amaral-Silva had an impact on the salary expenditures in Year 1 (only 6.5 person months worked instead of 12). The upgrades to the spray dryer equipment were purchased and delivered to Dr. Loebenberg's lab later than expected, with the final installation of spray dryer occurring in the late Fall of 2018. The equipment portion of the budget was therefore also not invoiced/recovered until installation was completed.

The COVID-19 research shut down between March 17, 2020 and July 02, 2020 resulted in a reduction in expenditures for consumables and animals, since experimental work was put on hold. However, salaries continued to be paid as per the mandates of our institutions and employees were able to carry out many research activities from home (i.e., data analysis, writing, experimental design and planning). We requested and were granted a 12-month no-cost extension (to August 14, 2021) to complete Major Tasks 4 & 5. To complete this work, the remaining funds supported consumables, animals, and salaries.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers and presentations

Scientific Publications

1. Bhat, V., **Allan, AL.**, and Raouf, A. Role of the microenvironment in regulating normal and cancer stem cell activity: implications for breast cancer progression and therapy response. *Cancers*, 2019 Aug 24;11(9). pii: E1240. doi:10.3390/cancers11091240 (see Appendix for manuscript). Federal support acknowledged: Yes.
2. Medeiros, B and **Allan, AL.** Molecular mechanisms of breast cancer metastasis to the lung: clinical and experimental perspectives. *Int J Mol Sci*, 2019, 20, 2272; doi:10.3390/ijms20092272 (see Appendix for manuscript). Federal support acknowledged: Yes.
3. Bhat V, Lefebvre C, Goodale D, Rodriguez-Torres M, **Allan AL.** Isolation and functional assessment of human breast cancer stem cells from cell and tissue samples. 2020 *JoVE*, doi:10.3791/61775 (see Appendix for manuscript). Federal support acknowledged: Yes.
4. Khan, S.U., Xia, Y., Goodale, D., Schoettle, G., and **Allan, AL.** Lung-derived selectins as a potential therapeutic target in triple negative breast cancer metastasis. Accepted with revisions (resubmitted October 2021) to *Biomedicines* (see Appendix for manuscript). Federal support acknowledged: Yes.

Scientific Conference Abstracts and Presentations

1. Goodale, D., Xia, Y., Postenka, C., and **Allan, AL.** Knockout of lung-derived osteopontin or selectins reduces metastatic colonization in an *ex vivo* model of breast cancer lung metastasis. 2019 Canadian Cancer Research Conference, November 2019, Ottawa Canada. Federal support acknowledged: Yes.
2. November 2019, San Antonio TX - American Association of Pharmaceutical Sciences Annual Meeting - AAPS PHARMSCI 360. Daniela attended the conference and presented two posters titled “Delayed release drug products behave differently in physiological bicarbonate buffer” and “In Vitro Biphasic Dissolution Test: A Study of Excipient and Manufacturing Variables”. Federal support acknowledged: Yes.
3. **Allan, AL.** Cellular and molecular approaches to understanding breast cancer metastasis. Multidisciplinary Breast Team Research Evening, London, ON, Canada, September 2018. Federal support acknowledged: Yes.
4. **Allan, AL.** Role of the lung microenvironment in mediating breast cancer metastasis: a balance between the “seed” and the “soil”. Cancer Research Program Seminar Series, Research Institute of the McGill University Health Centre (RI-MUHC), Montreal QC, Canada, November 2018. Federal support acknowledged: Yes.
5. **Allan, AL.** Role of the lung microenvironment in mediating breast cancer metastasis: a balance between the “seed” and the “soil”. Department of Anatomy & Cell Biology Seminar Series, Schulich School of Medicine & Dentistry, Western University, London ON, January 2020. Federal support acknowledged: Yes.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Alison Allan
Project Role:	PD/PI
Researcher Identifier:	Scopus Author ID: 8966957100
Nearest person month worked:	3
Contribution to Project:	Dr. Allan performed work in the area of overseeing and managing all aspects of the project.
Funding Support:	Salary support from the University of Western Ontario (65%) and the Breast Cancer Society of Canada (35%).

Name:	Ying Xia
Project Role:	Research Associate
Researcher Identifier:	Scopus Author ID: 57190123286
Nearest person month worked:	5.4
Contribution to Project:	Dr. Xia performed work in the area of <i>in vitro</i> and <i>ex vivo</i> functional and mechanistic studies of breast cancer metastatic behavior, and in daily supervision of graduate and undergraduate research students. She completed her work in the Allan lab in mid-February 2020 and moved to a different lab at Western University to take up a new position.
Funding Support:	Salary support from this project (45%) and Western University (65%)

Name:	David Goodale
Project Role:	Technician
Researcher Identifier:	Scopus Author ID: 36897019500
Nearest person month worked:	12
Contribution to Project:	Mr. Goodale performed work in the area of animal husbandry, animal studies (<i>in vivo</i> and <i>ex vivo</i>) of breast cancer metastatic behavior, and in daily management of Dr. Allan's lab.
Funding Support:	Salary support from this project (100%).

Name:	Carl Postenka
Project Role:	Technician
Researcher Identifier:	Scopus Author ID: 6506850250
Nearest person month worked:	2.4
Contribution to Project:	Mr. Postenka performed work in the area of animal husbandry, histopathology, and in daily maintenance/upkeep of Dr. Allan's lab.
Funding Support:	Salary support from this project (20%), and 80% from the Translational Breast Cancer Research Unit at the London Health Sciences Centre.

Name:	Raimar Loebenberg
Project Role:	Co-Investigator
Researcher Identifier:	Scopus Author ID: 6602898019
Nearest person month worked:	1.5
Contribution to Project:	Dr. Loebenberg performed work in the area of overseeing the drug formulation aspects of the project at the University of Alberta (Sub-Award site).
Funding Support:	Salary support from the University of Alberta (100%).

Name:	Vijay Somayaji
Project Role:	Manager
Researcher Identifier:	Scopus Author ID: 6603256858
Nearest person month worked:	3.6
Contribution to Project:	Dr. Somayaji performed work in the area of drug formulation studies, daily supervision of graduate students, and in daily management of Dr. Loebenberg's lab.
Funding Support:	Salary support from this project (30%) and from the University of Alberta (70%).

Name:	Daniela Amaral-Silva
Project Role:	Graduate Student
Researcher Identifier:	Not applicable
Nearest person month worked:	6.5
Contribution to Project:	Ms. Amaral-Silva performed work in the area of drug formulation studies.
Funding Support:	Salary support from this project (0%); supported by external graduate student awards (100%).

Name:	Gabriella Schoettle
Project Role:	Undergraduate Summer Research Student
Researcher Identifier:	Not applicable
Nearest person month worked:	0.5
Contribution to Project:	Ms. Schoettle performed work in the area of <i>in vitro</i> drug studies.
Funding Support:	Salary support from this project from the Ontario Ministry of Education (50%), and the University of Western Ontario (50%).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Two new grants during granting period (0% overlap with this project):

1. *Title of Grant:* Influence of breast cancer-derived extracellular vesicles (BCEVs) on lung metastasis of triple negative breast cancer
Source: Canadian Institutes of Health Research (CIHR); Project Grant
Amount: \$872,100 total (CAD)
Dates of Project: 2021-2026
Name of P.I.: **Alison L. Allan**
Co-Applicants: Karla Williams, David Litchfield
2. *Title of Grant:* A phase III randomized controlled trial and economic evaluation of Stereotactic Ablative Radiotherapy (SABR) for Comprehensive treatment of OligoMETastatic (1-3 metastases) cancer: SABR-COMET-3
Source: Canadian Institutes of Health Research; Project Grant Program
Amount: \$699,380 total (CAD); *Subcontract to my lab:* \$295,775 total (CAD)
Dates of Project: 2020-2026
Name of P.I.: Robert Olson (NPI); David Palma, **Alison L. Allan**, Stuart Peacock, Scott Tyldesley (Co-PIs)
Co-Applicants: Alanah Bergman, Mitchell Liu, Alex Louie, Liam Mulroy, Helen McTaggart-Cowan, Devin Schellenberg, Senth Sashendra, Andrew Warner

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable.

9. APPENDICES

Four manuscripts attached.

Review

Role of the Microenvironment in Regulating Normal and Cancer Stem Cell Activity: Implications for Breast Cancer Progression and Therapy Response

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Received: 26 July 2019; Accepted: 19 August 2019; Published: 24 August 2019



Abstract: The epithelial cells in an adult woman's breast tissue are continuously replaced throughout their reproductive life during pregnancy and estrus cycles. Such extensive epithelial cell turnover is governed by the primitive mammary stem cells (MaSCs) that proliferate and differentiate into bipotential and lineage-restricted progenitors that ultimately generate the mature breast epithelial cells. These cellular processes are orchestrated by tightly-regulated paracrine signals and crosstalk between breast epithelial cells and their tissue microenvironment. However, current evidence suggests that alterations to the communication between MaSCs, epithelial progenitors and their microenvironment plays an important role in breast carcinogenesis. In this article, we review the current knowledge regarding the role of the breast tissue microenvironment in regulating the special functions of normal and cancer stem cells. Understanding the crosstalk between MaSCs and their microenvironment will provide new insights into how an altered breast tissue microenvironment could contribute to breast cancer development, progression and therapy response and the implications of this for the development of novel therapeutic strategies to target cancer stem cells.

Keywords: microenvironment; mammary stem cells; breast cancer stem cells; hypoxia; immune cells; cytokines

1. Introduction

Adult tissue regeneration and maintenance are mainly regulated by continual turnover of mature cells. This process is mediated by the presence of tissue-specific stem cells, the functions of which depend on both intrinsic and extrinsic molecular signals. Extrinsic signals from the extracellular environment can activate intracellular signaling required for the expression of genes related to self-renewal, proliferation, differentiation and cell-fate commitment of stem cells. Adult stem cells reside in a specific tissue microenvironment composed of cellular components such as stromal fibroblasts, tissue-specific mature cells, immune cells, adipose and endothelial cells. The non-cellular portion of the stem cell microenvironment includes extracellular matrix components, growth factors and cytokines. Interactions with their surrounding tissue microenvironment provides stem cells with favorable conditions to either self-renew, proliferate, or differentiate into progenitor cells [1,2]. Such a notion is supported by observations indicating that primary cells, once isolated from their native microenvironment, exhibit altered proliferation and differentiation potentials that can be reinstated by controlling their microenvironment in ex vivo cultures [3,4].

Studies performed using mouse models have also provided data reinforcing the observations made using primary human cells. For example, while the injection of carcinoma cells into blastocysts resulted in the generation of genetically normal mice, the subcutaneous injection of the same cells resulted in the development of teratomas [5]. Subsequent studies using Rous sarcoma virus that contained the oncogene pp60src demonstrated that injection of the virus in the wing of a chick resulted in a tumor, while injection of the virus into the chick embryo failed to form tumors [6–8]. These studies suggest that the tissue microenvironment can play either a tumor-suppressive or a tumor-promoting role depending on the physiological context.

The stem cell microenvironment, also known as the stem cell ‘niche’, has been extensively studied with respect to its critical role in regulating hematopoietic stem cell self-renewal and differentiation leading to the maintenance of the human hematopoietic system. In addition, the role of the non-cellular components of the stem cell microenvironment has also been extensively studied using animal models [9]. For example, the role of extracellular matrix proteins such as β 1 integrins in skin [10,11]; osteopontin in the hematopoietic system [12,13] and tenascin C in the nervous system [14,15] have all been shown to play an essential role in regulating tissue-specific stem cell functions.

2. Normal Mammary Tissue Microenvironment and Function

2.1. Cellular Composition of the Normal Mammary Gland

The mammary gland is an intricate network of interconnected ducts and alveolar structures. These structures are composed of both luminal and myoepithelial cells. In ducts, luminal cells are organized to form hollow tubes that are surrounded by a continuous layer of myoepithelial cells, whereas in alveoli, luminal cells are arranged to form clusters of grape-like structures that are surrounded by non-contiguous myoepithelial cells, allowing luminal cells to be in constant contact with the surrounding stroma [16,17]. In contrast to the mouse mammary gland where the alveolar structures are surrounded mostly by adipose cells, in human breast tissue the bilayered ducts and alveolar structures are surrounded by a basement membrane composed of laminin and collagen. During pregnancy, luminal cells within the alveoli can further differentiate into milk-producing cells under the influence of prolactin. The myoepithelial cells are essential for milk ejection into the ducts by contracting in the presence of oxytocin [18–20]. These epithelial cells double in number during each estrous cycle [21–23]. Interestingly, during pregnancy and lactation, the epithelial content of the breast tissue also expands by up to nine times the original cell numbers. During pregnancy in mice, a 27-fold increase in epithelial cell number has been reported [24]. Post weaning, epithelial cells undergo apoptosis and the gland reverts back to a non-pregnant state through a process known as involution [25,26]. This dynamic process of expansion and regression makes the gland highly regenerative and allows the female breast to support multiple pregnancies.

This extensive regenerative potential of the mammary gland is due to the presence of the primitive mammary stem cells (MaSCs), which can give rise to both luminal and myoepithelial cells that make up the ductal and alveolar structures. For the sake of simplicity, both mouse mammary and human breast stem cells will be referred to as MaSCs in this article. Experimental evidence has demonstrated the highly regenerative capacity of the mammary gland, whereby even a small fragment of mouse mammary structure transplanted into de-epithelized (cleared) mammary fat pads can regenerate the entire mammary gland [27]. In support of this observation, subsequent studies showed that any part of the mammary epithelial tree can produce successful engraftment [28–30], suggesting that cells with regenerative capacity are dispersed throughout the mammary tree. Isolation of mouse MaSCs has been made possible through the identification of cell surface markers enabling the study of their proliferation, differentiation, and self-renewal potentials in vitro and in vivo [31,32]. These studies also demonstrate that MaSCs obtained from mouse mammary glands are able to generate bilayered mammary structures containing both luminal and myoepithelial cells [31,32]. However, current evidence suggests that in postnatal mammary gland, the MaSCs are heterogeneous in nature and consist of unipotent stem

cells capable of generating luminal or myoepithelial cells [33–35]. This finding challenges the bipotent property of MaSCs in the postnatal mouse mammary gland.

Current evidence suggests that human breast MaSCs reside in the ductal structures of the mammary gland [36], although the presence of human MaSCs in other locations in the gland remains unexplored. Xenotransplantation of CD49^{high}EpCAM^{low/-} human breast epithelial cells into mouse renal capsules resulted in generation of mammary structures, albeit at a low frequency [37]. This low regeneration of mammary structures could be either due to the lack of unique markers that provide further enrichment of MaSCs, or due to the lack of an appropriate/favorable microenvironment to facilitate the regenerative ability of human MaSCs in the renal capsule. Notably, the mouse mammary gland and human breast tissue microenvironments are different in their composition. Human breast tissue consists of collagen-rich inter- and intra-lobular stroma which is absent in the mouse mammary gland. In contrast, the mouse mammary gland is mainly made up of an adipose-rich stroma that surrounds the ducts [38]. Generation of humanized mammary mouse models is possible and subcutaneous implantation of human breast tissue in this model can accurately recapitulate the microenvironment of the human breast [39,40].

2.2. Components of the Normal Breast Tissue Microenvironment

MaSCs, like other tissue-specific stem cells, reside in a niche (microenvironment) in the breast that consists of different cell types including epithelial cells, fibroblasts, adipocytes, vascular endothelial cells, and immune cells (Figure 1). The breast tissue niche also includes non-cellular components such as basement membrane (BM) and extracellular matrix (ECM) components, growth factors, and cytokines that are vital for cell function. MaSCs have the ability to self-renew, proliferate and/or differentiate to generate mature luminal and the myoepithelial cells of the breast tissue. To this end, the autocrine and paracrine signals initiated by growth factors and cytokines of the niche, the regulatory signals initiated by the matrix components (laminin and collagen in particular), as well as cell-cell interactions within the niche are essential to the regulation of MaSC function [41–48].

In the ducts, the luminal cells are surrounded by a continuous layer of myoepithelial cells. However, in the alveolar structures, the luminal cells are surrounded by discontinuous layer of myoepithelial cells [49,50] allowing the luminal cells of the alveolar structures to interact with and receive signals from the different microenvironment components. Such interactions facilitate the further differentiation of alveolar luminal cells into milk-producing cells. Myoepithelial cells and fibroblasts are capable of secreting important ECM components including fibronectins, laminins, collagens, and proteoglycans that in turn help provide a defined ECM architecture and the necessary signals for highly regulated functions of mature cells, progenitors, and MaSCs within the mammary gland [51]. During the lactation and involution phases of pregnancy, this complex ECM is disrupted and then re-formed based on the action of different proteases such as matrix metalloproteinases (MMPs), as well as the action of immune cells such as eosinophils and mast cells [52–54]. During mouse mammary gland development at puberty, these immune cells are recruited near the terminal end buds (similar to TDLUs found in the human breast tissue) and mediate ductal outgrowth and branching morphogenesis by remodeling surrounding matrix [55].

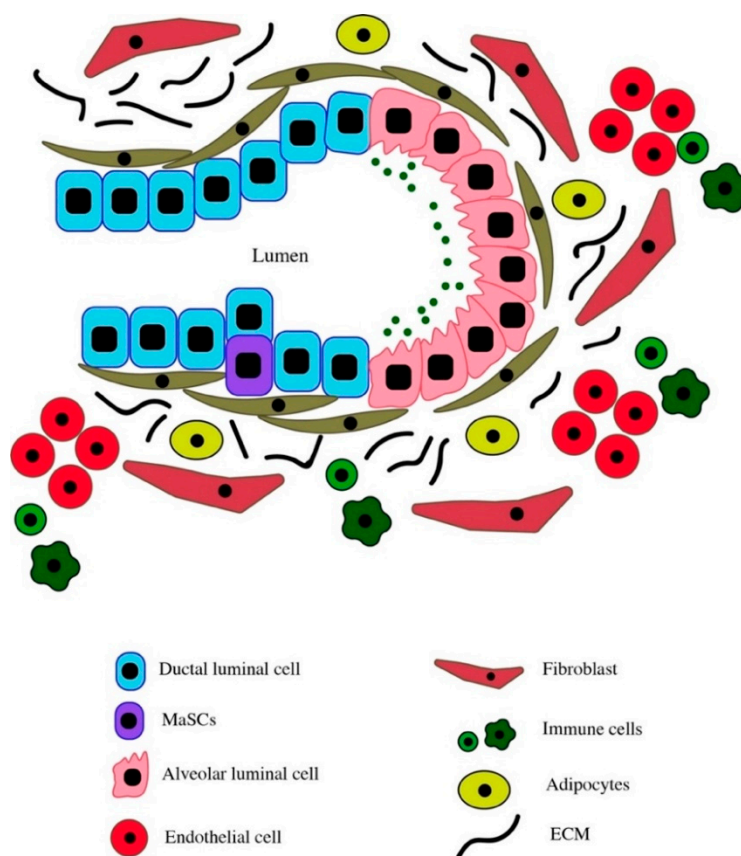


Figure 1. Schematic representation of the breast tissue microenvironment showing bilayered arrangement of ducts and alveolar structures consisting of luminal and myoepithelial cells. These structures are embedded in fibrous stroma consisting of fibroblasts, adipocytes, and immune cells. The luminal cells in the ducts are surrounded by a continuous lining of myoepithelial cells, while the myoepithelial cells lining the luminal cells in alveoli are discontinuous, which allows luminal cells to interact with surrounding stroma.

2.2.1. Immune Cells

Immune cells such as macrophages, eosinophils, neutrophils, and mast cells have been shown to play an important role in normal mammary gland development [55–60]. Macrophages in particular have been demonstrated to play a vital role in mammary gland development. Transplantation of MaSCs into macrophage-deficient mouse mammary fat pads showed defective mammary reconstitution ability [61], suggesting that the presence of macrophages throughout mammary gland development process is required for the normal functioning of MaSCs. Additional roles of macrophages in regulating mammary gland development via their direct interaction with MaSCs was reported recently [62]. The expression of the Notch receptor ligand Dll1 on MaSCs was shown to interact with Notch3 receptor (Nr3) expressed on adjacent macrophages, resulting in activation of intracellular Notch signaling. This interaction was shown to be necessary for the maintenance of macrophage numbers in the mammary gland, as well as secretion of Wnt ligands such as Wnt3a, Wnt10 and Wnt16 into the MaSC niche. These macrophage-secreted Wnt ligands then utilize positive feedback mechanisms to regulate MaSC activity [62]. Observations by Zeng and Nusse also demonstrated that in mice, Wnt3A-responsive cells were enriched in MaSCs, and that MaSCs exposed to Wnt3A displayed enhanced regenerative ability in vivo [63].

2.2.2. Extracellular Matrix

Extracellular matrix components such as laminin are also known to interact with integrin receptors expressed by stem and progenitor cells, and these interactions transduce signals required for the normal

functioning of undifferentiated cells [64–66]. It therefore comes as little surprise that cells expressing $\alpha 6$ and $\alpha 1$ integrins display mammary regenerative abilities in vivo [31,32,36,37]. Alpha-1 integrins have been shown to be involved in the proliferation of alveolar progenitors [67] as well as maintenance and regulation of regenerative ability of MaSCs [68]. ECM components have also been shown to regulate the expression of $\alpha 1$ integrins on both human and mouse mammary epithelial cells [69]. Moreover, protein microarray analysis of the ECM revealed that laminin 1 is required for maintenance of bipotential progenitors in a quiescent state, while P-Cadherin is required for myoepithelial cell differentiation [70]. These observations are particularly interesting in light of additional findings that the presence of $\alpha 6$ integrin-expressing bipotent progenitors in laminin-enriched Matrigel results in their proliferation without differentiation, while placing the same cells on collagen-coated plates results in their differentiation into mature luminal and myoepithelial cells [70]. In contrast, luminal cell differentiation appears to be instead dependent on cell-cell contact [70]. These observations identify the ECM as a strong modulator of MaSC and progenitor cell functions during normal mammary gland development.

2.2.3. Stroma

Studies in mouse models have also demonstrated the influence of stromal cells on mammary gland development. Epithelial-stromal cross-talk is necessary for the proper development and maintenance of the mammary gland [43]. A recent study has demonstrated that Gli2 expressing stromal cells secreted paracrine factors (Igf1, Fgf7, Hgf, Wnt2B, and Bmp7) to promote MaSC self-renewal and ductal outgrowth [71]. It has been observed that when mammary epithelium is recombined with salivary gland mesenchyme, the epithelium differentiates into salivary gland structures [45]. Interestingly, xenotransplantation of mouse embryonic skin epidermal cells into the mouse renal capsule along with embryonic mammary mesenchyme of either the rat- or mouse resulted in the generation of bilayered mammary ductal structures. These structures consisted of epithelial cells capable of responding both to estrogen and lactogenic hormones by differentiating into milk producing cells [72]. Additional studies by Boulanger et al. demonstrated that non-mammary epithelial stem cells exposed to the mammary gland microenvironment were capable of generating a functional mammary gland that contained cells capable of reconstituting mammary fat pads in serial transplantations [73–75]. In vitro models have shown that genes such as HDAC7 that regulate breast epithelial cell proliferation are also capable of reprogramming the extracellular microenvironment [76]. Furthermore, an in vitro 3D Matrigel culture system demonstrated that the regenerative ability of MaSCs was enhanced in presence of fibroblasts, a major stromal component of the breast tissue [77], suggesting the importance of mammary fibroblast in regulating MaSC activity. Taken together, these studies highlight the importance of the stromal microenvironment in defining cell fate and tissue function.

3. Breast Tumor Microenvironment

Just as the normal tissue environment plays a critical role in regulating mammary stem/progenitor cell functions, accumulating evidence suggest that the tumor microenvironment (TME) also plays an essential role in regulating cancer stem cell (CSC) activity [78–89] and tumor progression. Current evidence indicates that similar to normal breast tissue, breast tumor growth and progression is regulated through hierarchically organized cancer cell populations which are maintained by CSCs that exhibit self-renewal and proliferation potentials [90,91]. The direct experimental evidence demonstrating the transformation of MaSCs into bCSC remains elusive. Interestingly, both normal and cancer stem cells express common markers such as CD44 and ALDH [92,93]. In addition, conserved signaling pathways such as Notch and Wnt that regulate MaSC function (i.e. self-renewal, proliferation, and cell fate determination) are also active in bCSCs [94–96]. Molyneux et al, showed that deletion of *BRCA1* in human breast luminal progenitors resulted in basal-like breast cancers on *P53* mutant background [97]. These findings suggest that both normal MaSCs and/or mammary progenitors may have the potential to transform into bCSCs.

These CSCs are thought to be responsible for tumor recurrence and therapy resistance [98–100]. Previously, it was believed that resistance to chemotherapeutic drugs was acquired through accumulation of genetic alterations that generate a heterogeneous population of tumor cells with diverse phenotypes [101,102]. However, the cancer stem cell hypothesis suggests that since CSCs are responsible for maintaining tumor cells, the lack of therapies for specifically targeting these CSCs is responsible for tumor recurrence [103–110]. This issue can be addressed, at least in part, by advances in next generation sequencing (NGS) platforms that have enabled the examination of genomic and transcriptomic changes of tumors at the single cell level [111–115]. Such powerful technology has revealed that tumors (including breast tumors), can undergo a clonal evolution process which is a driving force behind tumor heterogeneity [116,117]. Moreover, comparing therapy-resistant metastatic tumors to matched primary tumors using single-cell genomics has revealed the existence of therapy-resistant clonal cells in the primary tumors; further supporting the role of CSCs in therapy resistance and tumor progression [118].

Breast cancer stem cell (bCSC) functions can be influenced by different cytokines and cell types present in the TME, including mesenchymal stem cells (MSCs), cancer associated fibroblasts (CAFs), and tumor associated leukocytes (TILs) (summarized in Table 1) [119]. Interestingly, in addition to the role of the primary TME in regulating bCSC activity, organ-specific microenvironments play an important role in the metastatic process. Previously, Chu et al demonstrated that soluble factors from the lung microenvironment induced chemotactic migration of CD44⁺ALDH^{high} bCSCs, suggesting an interaction between bCSCs and the microenvironment in regulating tissue-specific metastasis [120]. Furthermore, bone-derived osteopontin has been shown to maintain the bCSC phenotype and promote bone metastasis [121]. These observations strongly suggest that the microenvironment is an important modulator of bCSC function including therapy resistance, recurrence and metastasis. Therefore, understanding the interaction between bCSCs and their microenvironment will help in the identification of new therapeutic targets for improved treatment of breast cancer.

3.1. Cytokines

In addition to matrix components, the TME contains several non-cellular components including cytokines, chemokines, and growth factors that are secreted by the various cell types that make up the TME. These cytokines can create a chronic inflammatory environment that favors tumor cell survival and disease progression [122–124] while at the same time suppressing immune cell functions.

Table 1. Summary of the role of cytokines, immune cells, and stromal cells in regulating breast cancer stem cell (bCSC) activity in the tumor microenvironment.

Stimulant	Action	References
Interleukin-6	• Dedifferentiation of CD44^{low} MCF10A to CD44^{high} cells • Activation of JAK1/STAT3 signaling pathway in TNBC cell lines • Activation of JAG1-NOTCH3 signaling pathway in ER⁺ breast cancer cell lines	[125] [126] [127]
Interleukin-8	• Enhances bCSC activity and induction of chemoresistance in TNBC cells • Regulation of bCSCs in HER2+ breast cancers via activation of IL-8-CXCR1 signaling axis	[128] [129]
TGFβ	• Increases the number of CD44^{high} CD24^{low} cell population	[130]
TNFα	• Enriches the CD44⁺CD29⁺ bCSC population in Luminal-A breast cancer cells	[83]
Oncostatin-M	• Upregulation of SNAIL and CD44 expression in TNBC cell lines • Enhances tumor forming ability of TNBC cells	[86] [86]
CD8 ⁺ T cells	• Promotes bCSCs expansion and EMT	[131]

Table 1. Cont.

Stimulant	Action	References
TAMs	Promotes secretion of cytokine such as IL-6, IL-8 and GM-CSF and maintenance of bCSCs	[132]
Stromal Cells	Enhances bCSC self-renewal via exosome secretion	[133]
- Pre-adipocytes - Adipocytes - MSCs - CAFs	- Secretes adiponin and enhances bCSC activity through activation of C3a-C3aR signaling - Increases mammosphere-forming ability of breast cancer cell via activation of P2 purinergic pathway - Secretes IL-6 and CXCL7 and enhances bCSC self-renewal and proliferation in mouse xenograft model - Secretes prostaglandin and enhances bCSC expansion - Promotes bCSC self-renewal via CCL2 secretion - Secretes IL-6 and IL-8 thereby protects bCSCs from chemotherapeutic agents	[134] [135] [136] [137,138] [139]

3.1.1. Interleukins

The cytokine interleukin-6 (IL-6) has been shown to increase the expression of a CD44, a known marker of bCSCs [140], in MCF10A cells expressing tamoxifen induced Src kinase oncoprotein (MCF10A-Scr) [125]. In addition, CD44^{high} MCF10A-Scr cells generated tumors at a higher frequency as compared to CD44^{low} cells in mouse xenografts. When breast cancer cells derived from invasive ductal carcinoma tissues were treated with transformed MCF10A conditioned media, there was a conversion or dedifferentiation of CD44^{low} non-bCSCs to CD44^{high} bCSCs [125]. These observations suggest that extracellular factors present in the TME can play an important role in promoting stemness in breast cancer cells. Interestingly, IL-6-induced stemness in breast cancer cells has been shown to occur by activating the expression of OCT4 gene via the janus kinase/signal transducer and activator of transcription protein 3 (JAK1/STAT3) pathway [126]. In addition, IL-6 has been shown to upregulate jagged 1 (JAG1) and activate JAG1-NOTCH3 signaling, which ultimately results in higher secretion of IL-6 [127]. Thus, autocrine IL-6 signaling can then increase the proliferation and self-renewal potentials of CSCs that exhibit higher expression of NOTCH3 [127]. A recent study showed that presence of the IL-6 superfamily member, oncostatin-M (OM) in the TME upregulates genes related to the CSC phenotype such as SNAIL and CD44 in TNBC cells lines, leading to enhanced tumor formation in vivo. However, this effect of oncostatin-M was inhibited in the presence of IFN- β [86], suggesting that IFN- β could be an effective therapeutic agent against CSCs in TNBC.

Previous reports indicate that ALDH1⁺ bCSCs showed a higher expression of the IL-8 receptor and the CXCR gene [141]. To this end, IL-8 signaling has been associated with enhanced CSC activity and chemoresistance in triple negative breast cancers (TNBCs) [128]. Interestingly, the IL-8-CXCR1 signaling axis has been shown to be important in regulating bCSC function in HER2-positive breast cancers [129]. Patient-derived breast cancer cells demonstrated enhanced mammosphere formation in the presence of IL-8, while inhibition of IL-8-CXCR1 and HER2 signaling impaired mammosphere-forming activity [129]. These findings suggest that the IL-8-CXCR1 signaling axis could be a useful therapeutic target in treating HER2-positive breast cancer patients. Furthermore, inhibition of CXCL1 by reparixin reduced bCSC activity and prevented metastatic spread of breast cancer cells in mouse xenograft models [142].

3.1.2. Transforming Growth Factor β and Tumor Necrosis Factor α

The role of transforming growth factor β (TGF β) in regulating tumor cell proliferation, metastasis, and remodeling of the TME has also been well documented [143]. Very recently, Katsuno et al. demonstrated that prolonged exposure of human breast epithelial cells to TGF β enhanced the epithelial-to-mesenchymal transition (EMT) phenotype and increased the number of CD44^{high}CD24^{low} cell population [130]. Using a mouse breast cancer model, it was also recently demonstrated that TGF β mediated homing of human bone-marrow derived stem cells to breast cancer tumors, thereby

enhancing tumor growth and bone metastasis [144]. Moreover, using a mathematical model, Bocci et al. demonstrated that autocrine and paracrine TGF β signaling in combination with cell-cell communication activated Notch signaling to give rise to a heterogeneous population of bCSCs, and that IL-6-enhanced Notch-Jagged1 signaling was necessary for the maintenance of bCSCs [80].

Other cytokines, such as tumor necrosis factor alpha (TNF α) have been shown to regulate bCSC activity. When tumor cells from the Luminal-A breast cancer subtype were exposed to TME-enriched conditions consisting of TNF α and endothelial growth factor (EGF), the breast cancer cell population became enriched for a CD44⁺CD29⁺ CSC phenotype with increased metastatic properties [83].

3.2. Immune Cells

Tumor-associated immune cells, such as natural killer cells, macrophages, neutrophils, dendritic cells, and T and B lymphocytes, relay signals to their neighboring cells through secreted cytokines. These cytokines in the TME play an important role in the development of multiple cancers. Cytokines act directly on the tumor cells, fibroblasts, and adipocytes in the TME in an autocrine or paracrine fashion regulating important cell functions. This inflamed environment in the tumor niche also effects CSC activity [84,145–148]. While CD8⁺ T cells normally play a key role in eliminating tumor cells, Santisteban et al. showed that CD8⁺ T cells can promote bCSC expansion and EMT in vivo [131]. Tumor associated macrophages (TAMs) have also been shown to play an important role in tumorigenesis [149,150]. The interaction between CD11b and Ephrin expressed by TAMs found in ER α ⁺ breast cancer tumors and CD90 and Ephrin 4A (Eph4A) expressed on bCSCs results in activation of NF κ B-mediated secretion of cytokines such as IL-6, IL-8, and GM-CSF. These cytokines in turn play important roles in the maintenance of CSCs (i.e., self-renewal) and their proliferation and differentiation to generate new cancer cells [132].

3.3. Hypoxia

As solid tumors grow, due to decreased nutrient and oxygen supply, hypoxic regions develop within the TME where oxygen tension drops down to ~1%. Current evidence now indicates that this hypoxic microenvironment has the potential to regulate both normal stem cell function as well as CSC function [151–158]. It was recently demonstrated that breast cancer cells exposed to hypoxic conditions in vitro can activate the PI3K/AKT signaling pathway and promote enrichment of CD24[−]CD44⁺ CSC characteristics in xenotransplantation models [85]. *In vitro* studies demonstrated that repetitive cyclic exposure of normoxic and hypoxic conditions selectively enriched for breast cancer cells with a CSC phenotype. This subpopulation of cells was found to display EMT features and highly metastatic behavior in xenograft models [159]. Another study showed that a hypoxic TME resulted in hypoxia-inducible factor 1 (HIF1)-mediated expression of adenosine receptor 2B (A2BR) in human breast cancer cells. This increase in A2BR was sufficient to increase expression of CSC phenotype mediators, IL-6 and NANOG [160]. Moreover, Conley et al. reported that the use of anti-angiogenic agents such as sunitinib and bevacizumab in tumor-bearing mice created a hypoxic environment that facilitated a HIF1 α mediated increase in bCSCs [161]. In addition to this, it has been shown that under chronic hypoxic conditions, the expression of HIF-2 α is elevated in breast cancer cells, which in turn display a CSC phenotype by inducing the expression of stem cell markers, such as c-Myc, OCT4, and Nanog. In the same study, in vivo experiments demonstrated that increased expression of HIF-2 α in breast cancer cells promotes tumorigenicity and resistance to paclitaxel via activation of Wnt and Notch signaling pathways [162]. IL-6 signaling was shown to cooperate with a hypoxic TME conditions to induce expression of C/EBP δ and other “stemness” promoting factors such as Nanog, Sox2 and Klf4 in breast cancer stem cells [163]. Lastly, a recent study demonstrated that hypoxia-induced secretion of IL6 specifically by ER α ⁺ breast cancer cells was capable of elevating both ER α ⁺ and ER α [−] bCSC self-renewal and proliferation in a JAK-STAT pathway-dependent manner in vitro [164]. Based on such evidence, it is rational to hypothesize that the hypoxic areas of TME would foster the maintenance of bCSCs and that the decreased concentration of therapeutic drugs in these hypoxic areas could contribute to CSC survival and tumor recurrence.

3.4. Tumor Stroma

Different cell types of the stroma have been shown to play a key role in regulating CSC activity. Adipocytes secrete different growth factors, cytokines, and chemokines necessary for regulation of different cellular processes such as self-renewal and proliferation [165–168]. Subcutaneous co-injection of mouse mammary adenocarcinoma cells with adipose tissue from mouse mammary fat pads resulted in increased tumor volume compared to the xenografts initiated with breast cancer cells alone [169], suggesting the importance of adipose tissue in promoting tumor growth. Furthermore, Iyengar et al., demonstrated that conditioned media from adipocytes was sufficient to enhance breast cancer cell proliferation in vitro. In addition, subcutaneous injection of breast cancer cells with murine adipocytes enhanced their tumorigenic and metastatic activity in vivo [170]. Subsequent studies demonstrated that exosomes secreted from pre-adipocytes enhanced bCSC self-renewal and breast tumorigenesis via activation of the SOX9/miR-140 signaling pathway [133], and that exosomes from mesenchymal stem cell derived adipocytes enhanced proliferation of breast cancer cells through activation of the Hippo signaling pathway both in vitro and in vivo [171]. Goto et al., also recently demonstrated that mammary gland adipocytes secrete a serine protease, Adipsin, which triggers cleavage of complement C3 and activated C3 receptor (C3aR) signaling in breast cancer cells. Inhibition of the C3a-C3aR signaling axis results in decreased proliferation and maintenance of CSC properties in human breast cancer patient derived xenograft cells [134], suggesting that the adipsin-C3a-C3aR signaling is an important component of TME and bCSC activity.

Mesenchymal stem cells (MSCs) make up another small but important cell type present in the stroma of the mammary gland. Recently, crosstalk between MSCs and breast tumor cells has been shown [172], and accumulating evidence suggest that MSCs promote tumor growth, metastasis and development of resistance to therapy [173–175]. MSCs were also shown to activate P2 purinergic receptor signaling in breast cancer cells, which in turn increases their mammosphere forming ability [135]. Another study demonstrated that that ALDH1-expressing MSCs have the ability to infiltrate breast tumors and regulate bCSC self-renewal and proliferation, resulting in enhanced tumor growth in mouse xenograft models. In this model, the increase in MSC-induced self-renewal and proliferation of bCSCs was triggered by a positive feedback loop of IL-6 and CXCL7 cytokines secreted by MSCs [136].

In addition to adipocytes and MSCs, fibroblasts make up the majority of cells present in the stroma. Although the role of normal fibroblasts in promoting breast cancer tumor progression has been controversial, recent studies now provide evidence that both normal and activated cancer-associated fibroblasts (CAFs) can promote breast cancer cell growth in vitro and in animal model systems [176]. Indeed, these studies reveal that constitutively secreted cytokines, such as CCL7, IL-6, and IL-8, can activate the release of platelet-derived growth factor BB (PDGF-BB) from breast cancer cells that stimulates release of IL-1 β by the fibroblasts and in turn induces breast cancer cell proliferation [176]. Interestingly, IL-6 and IL-8 also promote bCSC self-renewal [177,178]. Moreover, CAF-secreted prostaglandins have been shown to promote secretion of IL-6 that results in bCSC expansion [137,138]. Interestingly, senescent primary normal breast luminal cells activate breast stromal fibroblasts in an IL-8-STAT3 pathway-dependent manner. These activated fibroblasts displayed pro-carcinogenic features and promote a CSC-like phenotype by increasing expression of stem cell markers, such as CD44, ALDH, SOX2, OCT4, NANOG, and KLF4. These activated fibroblasts also induced EMT in breast cancer cells both in vitro and in vivo [179]. A recent study demonstrated that Sonic Hedgehog ligand secreted by TNBC cells confers and activates normal stromal fibroblasts. These activated fibroblasts in turn secreted FGF5 and produced fibrillar collagen-rich ECM essential for maintenance of the CSC phenotype and development of chemoresistance [180]. Another study showed that breast cancer cells activate fibroblasts and induced secretion of chemokine ligand 2 (CCL2). Fibroblast-derived CCL2 plays a key role in promoting bCSC self-renewal and tumorigenesis in a Notch1-dependent manner both in vitro and in vivo [139]. These observations indicate that the stromal fibroblasts (and in particular their activated derivatives) contribute to bCSC activity and tumorigenesis.

Interestingly, single cell RNA-Seq analysis shows that CAFs in the TME are heterogeneous in nature and can be classified into three functionally distinct subsets based on their gene expression profiles and associated with different origins [181]. Thus, it is important to identify and characterize the subpopulation of CAFs that play a critical role in promoting bCSC activity. This will further help in using CAFs as prognostic or predictive biomarkers. Costa et al. identified four different subsets of CAFs in human breast tumors based on cell surface protein expression of fibroblast-associated protein (FAP), CD29, α SMA, fibroblast-specific protein1 (FSP1), PDGF receptor beta (PDGFR β), and CAV1. Intriguingly, one of the CAF subsets (FAP^{high}CD29^{high} α SMA^{high}FSP1^{high}PDGFR β ^{high}CAV1^{low}) enhanced T-regulator cell activity in order to inhibit effector T cell proliferation, thus playing an important role in creating an immunosuppressive microenvironment in TNBCs [182]. Furthermore, Su et al. demonstrated that CAFs expressing CD10 and GPR77 were highly potent in remodeling the TME [183]. These CAFs also secrete IL6 and IL8 which can induce bCSC enrichment and chemoresistance [183]. Breast cancer cells treated with chemotherapeutic agents, such as docetaxel or cisplatin, displayed enhanced survival in the presence of CD10⁺GPR77⁺ CAFs. Furthermore, in vitro co-culturing of breast cancer cells with CD10⁺GPR77⁺ CAFs resulted in an increase in the proportion of CD24⁻CD44⁺ALDH1⁺ bCSCs and enhanced mammosphere formation. In addition, co-injection of patient derived breast cancer cells with CD10⁺GPR77⁺ CAFs promoted tumor formation as well as the proportion of bCSCs upon serial translation of breast cancer cells [183], and binding of the ECM protein hyaluronan (HA) to the stem cell receptor CD44 resulted in Nanog mediated activation of stem cell specific genes such as *Sox2* and *Rex1* in breast cancer cells. This interaction was essential in Stat3-mediated activation of multi-drug resistance (MDR1) gene expression which in turn resulted in the development of resistance to doxorubicin and paclitaxel [184]. Taken together, this evidence demonstrates the crucial role of the stromal component of the TME in bCSC maintenance and development of chemoresistance.

4. Clinical Implications

Although the 10-year overall patient survival in breast cancer has dramatically improved, this disease remains the leading cause of cancer-related death in women worldwide due to tumor recurrence and therapy resistance [185]. Based on expression of receptors such as estrogen receptor (ER), progesterone receptor (PR) and HER2, breast cancers are classified clinically into luminal A (ER⁺PR⁺HER2⁻), luminal B (ER⁺PR⁺HER2^{+/-} and/or Ki67^{high}), HER2 positive (ER⁻), and triple negative tumors lacking expression of all three receptors [186]. With no effective targeted therapy options currently available, triple negative breast cancer (TNBC) constitutes the most aggressive type of breast cancer, with poor overall survival. Growing evidence suggests that the aggressive nature of TNBC tumors could be due to the presence of a higher frequency of bCSCs (CD44^{high}CD24^{low/-}) as compared to other breast cancer subtypes [187–190]. In contrast, luminal and HER2⁺ breast cancer subtypes are thought to be ALDH⁺ (CD44⁺CD24^{low/-}ALDH1⁺) [191,192]. These observations suggest that the bCSC subset within tumors is heterogeneous in nature with respect to the phenotype and possibly function among the different breast cancer subtypes. Single-cell transcriptomic analysis of primary and metastatic tumors of different breast cancer subtypes could certainly provide very interesting information about the heterogeneity of the bCSCs. Such information could then provide a framework to hypothesize as to how heterogeneity in the bCSC compartment of the different breast cancer subtypes could be predictive of therapy response and therapy resistance.

Until recently, research efforts were mainly focused on identifying genes and genetic alterations that regulate tumor growth and progression while viewing tumors as consisting of fairly homogenous cell populations [193,194]. Such studies have led to the development of successful therapies to block signaling pathways essential to tumor growth such as estrogen receptor blockers (e.g., Tamoxifen, Fulvestrant), and the HER2 receptor blocker, Herceptin. However, the current clinical challenge in the management of breast cancer is the development of therapy resistance, relapse, and metastasis. To this end, bCSCs have now been established to be the cells responsible for maintaining and regenerating

tumors. However, targeting bCSCs has proven to be challenging, as most of the current therapy options fail to target these cells, which remain protected in their niche and contribute to therapy resistance, relapse, and ultimately metastasis. To this end, regulation of bCSC function and induction of chemoresistance by external factors such as cytokines, chemokine and hypoxia is becoming apparent as potential strategies that could target the interaction of bCSCs with cellular and non-cellular components of the TME as more effective therapeutic approaches. For example, the CXCR1 inhibitor repertaxin in combination with lapatinib significantly abrogated bCSC activity in both HER2-positive and negative tumors in preclinical animal models [129,195]. Treatment with an IL6-neutralizing antibody completely eliminated chemoresistance of ovarian and lung cancer stem cells in vivo [196,197], suggesting that targeting IL-6 could be an effective strategy in eliminating bCSCs as well. In glioma, the TGF β inhibitor SB431542 promoted differentiation of CSCs in vitro [198]. These observations suggest that targeting the secreted factors of the TME could represent an effective therapeutic strategy in eliminating bCSCs. It is, however, important to take into consideration the subtle differences between mouse and human mammary gland. For preclinical trials, it would be important to use an orthotopic mouse model whose tissue environment has been altered (humanized) to resemble the human breast. Moreover, reconstruction of the tumor microenvironment in 3D Matrigel in patient-derived organoid cultures (PDOCs) would be another way of assessing the potential action of these neutralizing antibodies or inhibitors on bCSCs. The strength of the PDOC system is that it enables the study of tumor-infiltrating leukocytes in bCSC function which is not easy to model in the mouse.

5. Conclusions

The postnatal adult mammary gland is maintained by the most primitive self-renewing population of MaSCs. Depending on microenvironmental cues, MaSCs differentiate to lineage-restricted progenitors, which eventually generate mature luminal and myoepithelial cells of the mammary gland. This defines the hierarchical organization of mammary epithelial cells and ultimately the normal mammary gland. Studies performed on mouse models have demonstrated that both intrinsic factors (effectors of Notch and Wnt signaling pathways) and extrinsic factors (tissue microenvironment) regulate MaSC function. Multiple studies have identified surface markers to isolate and characterize the murine MaSC population; however, there is still a lack of defined unique stem cell markers to definitively identify MaSCs in the human breast. As a result of this, the activity of MaSCs and their interactions with the microenvironment in human breast tissue remains poorly understood. Importantly, bCSCs share many of the specific characteristics and functions of normal MaSCs (summarized in Figure 2), including their dependence on the tissue or tumor microenvironment for regulating their proliferative and self-renewal potentials. Accumulating evidence shows that molecular mechanisms that regulate normal MaSCs (such as Notch and Wnt signaling pathways) can also help in maintenance of bCSCs' phenotype, resulting in breast tumorigenesis, progression, and metastasis. This suggests that understanding the role of normal breast MaSCs and their tissue environment would provide some insights into understanding the role of TME in regulating breast CSC activity.

Despite early detection and therapy options, the majority of deaths in breast cancer patients occur due to resistance to therapy and metastasis. bCSCs represent a small number of cells within the heterogeneous tumor cell populations that have the potential to regenerate the tumor and are thought to be responsible for tumor recurrence, therapy resistance and ultimately metastasis. Our previous notion that breast cancer manifestation occurs solely by cell intrinsic factors (mutations, gene amplification) has been challenged by more recent studies described in this review and elsewhere. Extensive research combined with clinical trials has been conducted with the goal of eliminating CSCs, however due to CSC plasticity, these attempts have not been very successful. There is compelling evidence that the tumor microenvironment plays a critical role in regulating CSC plasticity that drives the ability of a non-CSC to dedifferentiate into a CSC, thereby contributing to tumor initiation, progression, therapy resistance, and metastasis. Studies have shown that a bCSC-supportive niche consisting of activated fibroblasts, immune cells and adipocytes alter bCSC activity either by direct interaction

or through secreted factors. Several preclinical trials targeting cytokines have shown promising results in inhibiting tumor growth. Taken together, these studies suggest that targeting the cellular and non-cellular components of the tumor microenvironment could serve as an effective therapeutic strategy for both reducing tumor growth and also sensitization of the therapy resistant bCSCs.

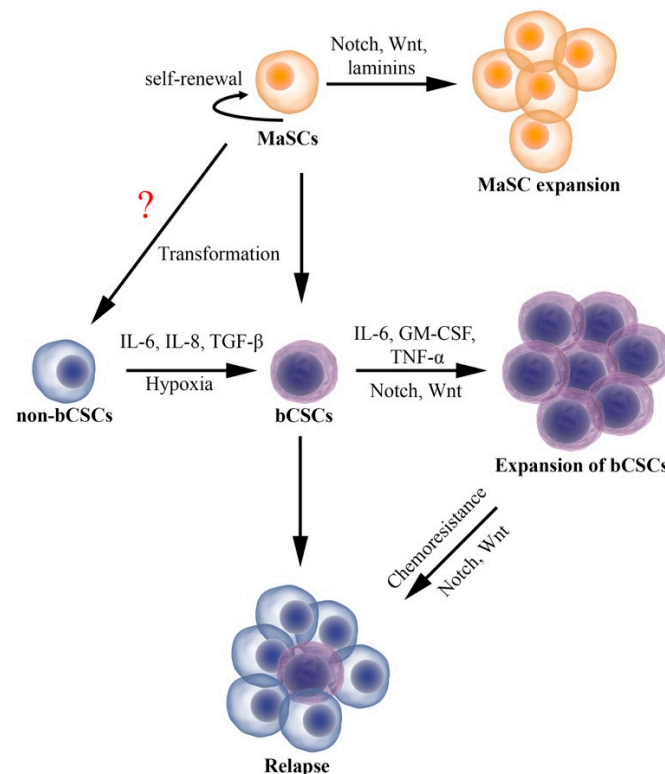


Figure 2. Schematic representation of mammary stem cell (MaSC) expansion, breast cancer stem cell (bCSC) plasticity, development of chemoresistance and relapse. Different cytokines released by immune cells, fibroblasts, adipocytes along with tumor cells in the tumor microenvironment regulate both MaSC and bCSC activity. It is possible that MaSCs could acquire sufficient genetic changes that allow them to transform directly into malignant cancer cells devoid of stem cell properties (non-bCSCs). However, this hypothesis requires further experimental evidence.

Author Contributions: Conceptualization, A.R. and A.L.A.; Literature review, V.B.; Writing-Original draft preparation, V.B.; Writing-Review and Editing, V.B., A.R. and A.L.A.

Funding: Research in the lab of A.R. on normal breast development is funded by National Sciences and Engineering Research Council of Canada. Research in the lab of A.L.A. on breast cancer stem cells and the tumor microenvironment is funded by grants from the Canadian Cancer Research Society Research Institute and the U.S. Army Department of Defense Breast Cancer Program (Grant # BC160912). A.L.A. is supported by the Breast Cancer Society of Canada.

Acknowledgments: We thank members of our laboratories for their helpful discussions and support. Although we sought to summarize a broad body of work on normal and cancer stem cells, due to space limitations we sincerely apologize to those authors whose work is not discussed/cited.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Molecular Mechanisms of Breast Cancer Metastasis to the Lung: Clinical and Experimental Perspectives

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Received: 31 March 2019; Accepted: 6 May 2019; Published: 8 May 2019



Abstract: Breast cancer is the most commonly diagnosed cancer in women worldwide, and >90% of breast cancer-related deaths are associated with metastasis. Breast cancer spreads preferentially to the lung, brain, bone and liver; termed organ tropism. Current treatment methods for metastatic breast cancer have been ineffective, compounded by the lack of early prognostic/predictive methods to determine which organs are most susceptible to developing metastases. A better understanding of the mechanisms that drive breast cancer metastasis is crucial for identifying novel biomarkers and therapeutic targets. Lung metastasis is of particular concern as it is associated with significant patient morbidity and a mortality rate of 60–70%. This review highlights the current understanding of breast cancer metastasis to the lung, including discussion of potential new treatment approaches for development.

Keywords: breast cancer; lung metastasis; pre-metastatic niche; exosomes; tumor secreted factors; targeted therapies

1. Introduction

Globally breast cancer is the most common malignancy in women, and 626,679 deaths worldwide in 2018 were attributed to it [1]. In the past, breast cancer has been a higher burden in developed nations due to risk factors associated with lifestyle [1]. However, in developing nations the incidence rates of breast cancer have increased in recent years due to advancements in health infrastructure and the adoption of a ‘westernized’ lifestyle [1]. In Canada, 1 in 8 women will develop breast cancer over their lifetime while 1 in 31 will die from their disease [2]. Of the deaths caused by breast cancer, over 90% are attributed to metastasis-related complications [3]. Metastasis is a poorly understood process that begins with the detachment of tumor cells from the primary tumor and their intravasation into the blood stream [4]. These circulating tumor cells (CTCs) eventually arrest in the capillary beds of distant organs and extravasate through the vascular wall into the parenchyma, resulting in the generation of metastatic colonies in the secondary site [4].

Breast cancer has a tendency to target the bone, brain, liver and lung; known as organ tropism [5]. For breast cancer patients with metastases; 30–60% have lesions in the bone, 4–10% in the brain, 15–32% in the liver, and 21–32% in the lung [6]. Lung metastases in particular tend to occur within 5 years of initial breast cancer diagnosis and have a significant impact on patient morbidity and mortality. Physiologically, these metastases disrupt normal lung function, resulting in coughing, labored breathing, hemoptysis, and eventual death. Lung metastasis remains difficult to treat, with an estimated 60–70% of patients who die of breast cancer having lung metastasis [7]. For patients with metastases confined solely to the lung, the prognosis is exceedingly poor with a median survival

of only 25 months [8]. This poor outcome is attributed to the limited number of treatment options associated with inoperable lesions [9].

The underlying mechanisms that dictate which organ(s) become colonized by breast cancer are complex and influenced by many factors, one of which is molecular subtype. First described by Perou et al. (2000), breast cancer can be subdivided into four main clinical subtypes on the basis of gene expression profiles and receptor status (estrogen receptor [ER], progesterone receptor [PR], human epidermal growth factor receptor 2 [HER2]) and proliferation status as assessed by Ki67 [10]. These clinical subtypes (in order of increasing aggressiveness) include: luminal A (ER⁺/PR⁺), luminal B (ER⁺/PR⁺/HER2⁻/Ki67⁺), HER2 overexpressing (ER⁻/PR⁻/HER2⁺) and basal-like/triple-negative (TN) (ER⁻/PR⁻/HER2⁻). While bone is the most common site for metastasis across all subtypes, TN breast cancer has the greatest tendency to metastasize to the lung; occurring in ~32% of patients compared to ~21% of luminal A/B and ~25% of HER2+ patients [6]. However, the timing and mechanisms by which breast cancer molecular subtype may influence metastasis to the lung is not yet understood.

In this review, we summarize current advancements in the understanding of molecular mechanisms that drive breast cancer metastasis to the lung. By integrating the complex body of work that surrounds this topic, we highlight key therapeutic targets and potential/emerging treatment approaches.

2. The Lung Metastatic Niche

The process of metastasis is highly inefficient, with less than 0.01% of primary tumor cells successfully completing the metastatic cascade to develop macrometastases at the secondary site [11]. Clinically established patterns of organ-specific metastasis suggest that the site in which the cancer grows successfully is not random, but rather influenced by the microenvironment in the secondary organ. This phenomenon was first described by Stephen Paget in 1889, who hypothesized that cancer cells (the “seed”) grew preferentially in the microenvironment of select organs (the “soil”) only if the conditions at that site were permissive for growth [12]. Supporting this theory, our research group has shown that in the presence of organ-conditioned media from common sites of breast cancer metastasis (lymph node, lung, liver, bone, brain), breast cancer cells demonstrate organ-specific responses in proliferation and migration; indicating certain organs produce soluble components that support metastatic behavior [13]. However, in the early 1900s, James Ewing suggested a competing theory that organ-specific metastasis was regulated solely by physiological blood flow patterns [14]. Certainly the physical characteristics of organs such as the lung lends weight to Ewing’s theory, particularly for breast cancer. The lung is the first major capillary bed that a breast cancer cell encounters after escaping into the bloodstream. As tumor cells circulate through the lung, they may come into contact with as much as 100 m² of surface vasculature. Since these tumor cells are approximately five times larger than the exceedingly narrow pulmonary capillaries, the likelihood of breast cancer cell arrest in these capillary beds and subsequent extravasation into the lung tissue is high [15,16]. The lung capillaries are comprised of endothelial cells that are encapsulated by a basement membrane and adjacent alveolar cells. To facilitate transendothelial migration and extravasation, tumor must express cell surface markers specific for the lung microenvironment [15,16]. However, although extravasation may occur fairly easily via these physical processes, the ability of individual metastatic cells to successfully transition to micrometastases and subsequently progress to macrometastases is quite rare, and thus these final events represent rate-limiting steps in metastasis that rely on the optimal collaboration of “seed” and “soil”. Therefore, it is likely that Paget’s and Ewing’s theories are highly complementary in the development of metastasis to the lung [17].

More recently, growing evidence that the primary tumor has the potential to “prime” or augment distant organ microenvironments in preparation for metastasis has added a further level of complexity to Paget’s seed and soil theory [18–21]. The generation of this “pre-metastatic niche” is hypothesized to be critical for the process of metastasis, and can be divided into four phases consisting of priming, licensing, initiation and progression [22]. Priming is initiated by the secretion of tumor-derived soluble factors (TDSFs) and/or exosomes by the primary tumor as it undergoes uncontrolled proliferation and

becomes hypoxic. These molecules target the bone marrow for recruitment and initiate the remodeling of the target secondary organ, generating a pre-mature metastatic niche. Bone marrow-derived cells (BMDCs) and immune regulatory/suppressive cells are then progressively recruited to the secondary site by the continual secretion of factors from the primary tumor. These processes facilitate the licensing phase, generating an immune-suppressed environment and an extracellular matrix (ECM) conducive for cancer colonization. Disseminated cancer cells that enter this fertile metastatic niche may stay in a dormant state until the conditions at the secondary site can support tumor outgrowth into micrometastases; termed initiation. In the final progression stage, the growth of micrometastases is regulated by tumor secreted factors and other regulatory cells that infiltrate the secondary site, thus enabling transition to macrometastases [22].

In order for these processes to occur successfully, there must be a well-choreographed sequence of molecular and cellular events that enable the generation of a fertile pre-metastatic niche, and this is regulated by a variety of tumor-secreted factors, exosomes and stromal components. In particular, the close interplay between primary tumors and lung priming was first highlighted by Lyden and colleagues (2005). They demonstrated that bone marrow derived hematopoietic progenitor cells (HPCs) expressing vascular endothelial growth factor receptor-1 (VEGFR1) and very late antigen-4 (VLA-4) targeted areas of the lung with increased fibronectin deposition [23]. Upon binding, VLA-4⁺VEGFR1⁺ HPCs secrete MMP9 to induce pro-metastatic changes in the lung ECM [23]. Subsequent studies have elucidated how the BMDCs are recruited to the pre-metastatic niche and the underlying mechanisms of increased fibronectin deposition in the lung; believed to be regulated by tumor-derived exosomes and a variety of tumor-secreted and stromal-derived factors. These are summarized in Tables 1 and 2 and described in greater detail below.

Table 1. Molecular and cellular components secreted by breast cancer primary tumors that are associated with the promotion of lung metastasis.

Secreted Component	Molecule	Mobilized/Target Cell Type	Mechanism(s)	Reference(s)
Tumor-Derived Exosomes (TDEs)	PD-L1	T cells	Blunts T-cell activation and killing activities	[24]
	miR-122	Fibroblasts	Reprograms metabolic activity, resulting in decreased glucose need at pre-metastatic site by inhibiting pyruvate kinase	[25]
	miR-105	Endothelial cells	Uptake reduces expression of the gap junction protein ZO-1, promoting metastasis at the pre-metastatic site	[26]
	ITG $\alpha_6\beta_1$	Lung SPC ⁺ Epithelial Cells/ Lung S100A4 ⁺ Fibroblasts	Targets exosomes to lung to induce pre-metastatic niche formation	[27]
Tumor-Derived Soluble Factors (TDSFs)	P2Y2R	CD11b ⁺ BMDCs	Mediates LOX expression, causing collagen cross linking in the lung recruiting CD11b ⁺ BMDCs	[28]
	TGF β	Cancer cells	Primes breast cancer cells by inducing ANGPTL4 which disrupts endothelial tight junctions at distant sites	[29]
	VCAM1	Endothelial cells	Facilitates transendothelial migration of tumor cells into the lung	[30]
	CSF-1	Macrophages	Recruits macrophages to the primary tumor, inducing an aggressive phenotype with a propensity to metastasize to the lung	[31]
	CXCR4/ CCR7	SDF-1/CCL21 ⁺ endothelial cells	Enables tumor cell adhesion to lung endothelium	[32,33]
	LOX		Leads to collagen crosslinking and recruitment of CD11b ⁺ BMDCs	[34]

PD-L1, Programmed Death—Ligand 1; miR, microRNA; ITG $\alpha_6\beta_1$, Integrin alpha 6 beta 1; SPC, Surfactant Protein C; P2Y2R, Purinergic Receptor; TGF β , Transforming Growth Factor beta; ANGPTL4, Angiopoietin-like 4; VCAM1, Vascular Cell Adhesion Molecule 1; CSF-1, Colony Stimulating Factor-1; CXCR4, C-X-C Chemokine Receptor 4; CCR7, C-C Chemokine Receptor Type 7; SDF-1, Stromal Cell Derived Factor-1; CCL21, C-C Motif Chemokine Ligand 21; LOX, Lysyl Oxidase.

Table 2. Molecular and cellular components secreted by stromal cells associated with promoting breast cancer metastasis to the lung.

Secreted Component	Molecules	Mobilized/Target Cell Type	Mechanism(s)	Reference(s)
Stromal-Derived Factors (SDFs)	PGE2	BMDCs/Cancer Cells	Recruits BMDCs to the lung and enhances CTC adhesion	[35]
	ANG-2	CCR2 ⁺ Tie2 [−] Macrophages	Recruits macrophages, which cause endothelial cells to release proinflammatory and angiogenic factors	[36]
	FN, TN-C, PSTN, VCAN	BMDCs and cancer cells	Promotes the adhesion of BMDCs and CTCs	[37–42]
	CCL2	Cancer cells	Produced by CCR2 ⁺ inflammatory monocytes, increases vasculature permeability	[43]
	MMP2	BMDCs	Remodels lung ECM	[44]
	HSF1	CAFs/cancer cells	Reprograms CAFs and cancer cells to promote metastasis into the niche	[45]
	Id3	VEGFR1 ⁺ BMDCS	BMDC-derived, required for recruitment of VEGFR1 ⁺ BMDCs to areas of increased fibronectin deposition in the lung	[23]
	IL-32	Cancer cells	CAF-derived, increases the metastatic potential of breast cancer cells	[46]

PGE2, Prostaglandin E2; ANG-2, Angiopoietin-2; CCR2, C-C Chemokine Receptor Type 2; FN, Fibronectin; TN-C, Tenascin-C; PSTN, Periostin; VCAN, Versican; BMDCs, Bone Marrow-Derived Cells; CCL2, C-C Motif Chemokine Ligand 2; CCR2, C-C Chemokine Receptor 2; MMP2, Matrix Metalloproteinase 2; ECM, Extracellular Matrix; HSF1, Heat Shock Factor 1; Id3, Inhibitor of Differentiation 3; VEGFR1, Vascular Endothelial Growth Factor Receptor 1; IL-32, Interleukin-32; CAF, Cancer Associated Fibroblast.

3. Tumor-Derived Exosomes

Tumor-derived extracellular vesicles can be subdivided on the basis of size including apoptotic bodies (1000–5000 nm), microvesicles (200–1000 nm) and exosomes (30–150 nm) [47]. Exosomes are formed through the endosomal pathway and predominantly released from cells through fusion with the plasma membrane [48,49]. Tumor-derived exosomes (TDEs) have been shown to play a significant role in modifying the lung microenvironment. This is highlighted by recent studies involving pre-treatment of mouse models with TDEs from lung-seeking breast cancer cell lines that showed the potential to “educate” the lung, inducing changes that made it more susceptible to metastasis [50]. Exosomes secreted by the primary tumor have the ability to target the lung through use of integrins such as ITG $\alpha_6\beta_1$ [27]. Upon targeting specific organs, breast cancer-derived exosomes can deliver their cargo of RNA, DNA and proteins to induce pro-metastatic changes in the lung [51]. Exosome production and packaging is not static but is instead regulated by several factors including environmental stimuli, such as hypoxia [52,53]. It has been demonstrated that breast cancer exosome production is increased substantially in hypoxic conditions in a HIF-1 α dependent manner [54]. Furthermore, exosomes have the potential to translate properties such as chemotherapy resistance and increased invasiveness to recipient breast cancer cells [55,56].

3.1. Exosomes and Immune Suppression

The process of generating the pre-metastatic niche in lung is highly reliant on immune-suppression to ensure that CD8⁺ T cells, natural killer (NK) cells and patrolling monocytes are masked from the presence of tumor cells trying to establish themselves as metastatic lesions in the lung [57,58]. Interestingly, Yang and colleagues (2018) demonstrated that breast cancer-derived exosomes expressing programmed cell death-1 (PD-L1) on their surface have the ability to blunt T-cell activation and killing activities, effectively protecting tumor cells from immune surveillance. By stunting the immune

response, tumor cells have the potential to successfully seed and colonize distant organ sites, such as the lung [24]. While cancer cells in the bloodstream try to avoid circulating immune elements, there is growing evidence to suggest that there is a connection between immune cell dysregulation and chronic inflammation at the pre-metastatic site [59–61]. Macrophages (key regulators of the immune response and part of the innate immune system) phagocytose invading cells in order to induce the expression of cytokines and chemokines [62]. Chow et al. (2014) demonstrated that exosomes derived from MDA-MB-231 and MCF7 breast cancer cell lines have the ability to hijack lung macrophage activity by activating the NF- κ B pathway, resulting in the expression of the pro-inflammatory markers IL-6, TNF α , G-CSF and CCL2 and promoting lung metastasis in vivo [63].

Precipitated by the work of Lyden and colleagues, BMDCs have emerged as a significant contributor to establishing a pre-metastatic niche [64–66]. Furthermore, although it is well-established that myeloid-derived suppressor cells (MDSCs) enable tumor progression, their development during tumor growth was unknown [67–70]. Xiang and colleagues (2009) demonstrated that bone marrow myeloid cells can be forced to differentiate into myeloid-derived suppressor cells (MDSCs, CD11b⁺Gr[−]11⁺) by breast cancer derived exosomes [71]. The resulting change induces the accumulation of MDSCs expressing Cox2, IL-6, VEGF and arginase-1 at the lung, generating a pro-inflammatory and immune suppressed environment permissive for metastasis [71]. In a similar study, Peinado et al. (2012) demonstrated that exosomes from highly metastatic melanoma cells had the ability to “educate” BMDCs from non-tumor bearing mice, pushing towards a pro-vasculogenic and pro-metastatic phenotype via the upregulation of MET [72]. The relevance of this process to breast cancer lung metastasis has yet to be investigated.

3.2. Exosomes and Stromal Cells

Beyond interactions with immune cells, breast cancer-derived exosomes have the ability to influence the status of the lung microenvironment by modulating the function of stromal cells. Fong et al. (2015) demonstrated that exosomes isolated from the MDA-MB-231 breast cancer cell line contained miR-122, and once applied to lung fibroblasts these exosomes were able to reprogram glucose metabolism, reducing glucose uptake by inhibiting pyruvate dehydrogenase activity [25]. This suggests that prior to colonization of the lung, secreted exosomes from the primary tumor can reduce glucose uptake, allowing for newly arrived cancer cells to have enough energy to facilitate rapid proliferation. Complementary to this data, Zhou and colleagues (2014) demonstrated that exosomes released from MD-MB-231 cells were also enriched with miR-105 [26]. Tail vein injection of miR-105 containing exosomes resulted in modulation of the vasculature of common sites of metastasis such as the lung such that it became “leaky” [26]. It was determined that exosomal delivery of miR-105 to endothelial cells resulted in the downregulation of the tight junction protein ZO-1 [26]. Taken together, these studies provide further evidence to support the concept that breast cancer derived exosomes play a critical role in establishing a permissive niche in the lung required for metastatic colonization, and highlights the possibility of considering exosomes in the clinical setting.

3.3. Exosomes as Clinical Biomarkers

Tumors secrete a number of factors into peripheral circulation (Table 1) that have the potential to serve as a method of clinical monitoring of disease progression. Increased attention has thus been put towards developing non-invasive blood-based biomarker approaches [73–75]. Many current methods have focused on enumeration and characterization of circulating tumor cells (CTCs), but this has proved to be a difficult task due to their sparse concentrations in blood [76,77]. In patients with early stage breast cancer, the CTC detection rate ranges between 23–37%, and currently no effective strategy exists to leverage CTCs analysis in order to predict which organs might be affected by metastasis [78]. In comparison, tumor-derived exosomes may provide an attractive alternative as they are stable in blood, have the ability to be isolated from most bodily fluids (blood, urine, semen, milk) and are present in circulation at similar quantities as soluble proteins (10⁵·mL^{−1}) [79,80]. The innate issue

with exosomes is differentiating their origin from normal or cancerous tissue. Etayash and colleagues (2016) demonstrated that by utilizing the characteristics of tumor-derived exosomes (such as the overexpression of CD24, CD63 and EGFR), they were able to isolate exosomes of breast cancer origin by a multiplexed cantilever array sensor [81]. By isolating breast cancer exosomes, proteomic analysis may provide the opportunity to identify sites susceptible to metastasis by identifying the presence of specific organotropic integrins. Additionally, RNA analysis coupled with such proteomic data may provide insight into how the exosomes are altering the secondary site, providing a targeted approach to circumvent potential metastases. An alternative method developed by Zhai et al. (2018) demonstrated that isolating patient plasma and incubating the sample with Au nanoflare probes specific to the pro-metastatic exosomal miR-1246 was able to identify 100% of breast cancer patients with metastatic disease [82]. These methods of early detection have great promise but must be refined, validated in the clinical setting, and ideally coupled with new treatment approaches to have clinical applicability.

3.4. Therapeutic Implications of Exosomes

Beyond biomarkers, exosomes could provide an effective strategy to treat inoperable metastatic lung lesions. In the majority of cases, lung metastases are multidrug resistant and efforts to change treatments is thwarted by most chemotherapeutics having low aqueous solubility requiring alternative delivery methods [83–85]. Exosomes, compared to other proposed delivery methods, have the potential for organ-specificity and privileged immune status that results in reduced drug clearance [86]. Applying this, Kim et al. (2016) demonstrated that exosomes released by macrophages can be loaded with paclitaxel using ultrasound treatment and used to induce cytotoxicity in multidrug resistant lung cancer cells [87]. Future work in the context of breast cancer lung metastasis is crucial to move towards translation of this potential therapeutic approach to the clinic.

Interestingly, while tumor-secreted vesicles provide hope for earlier detection of lung metastasis and treatment, there is emerging evidence to suggest that they may actually further complicate treatment outcomes. Keklikoglou et al. (2019) recently demonstrated that the administration of taxanes and anthracyclines to mice bearing breast tumors induced the release of extracellular vesicles with an increased pro-metastatic capability [88]. These extracellular vesicles contained elevated amounts of annexin A6 which targets lung endothelial cells to induce NF- κ B activation, resulting in CCL2 release that caused Ly6C⁺CCR2⁺ expansion at the lung that enables the establishment of a fertile pre-metastatic niche [88].

In addition to chemotherapy, immunotherapy has emerged as a revolutionary approach to cancer treatment and management. Several recent clinical trials have focused on determining the efficacy of PD-L1 inhibitors in the context of metastatic breast cancer [89,90]. Interestingly, in metastatic melanoma, Chen et al. (2018) were able to demonstrate that the amount of PD-L1 expressed on tumor-derived exosomes was a predictor for response to anti-PD-L1 therapy [91], whereby responders had lower baseline levels of PD-L1 expressed on exosomes, and after 3–6 weeks of treatment the expression was more pronounced for responders [91]. These observations indicate the importance of considering the role of exosomes when designing treatment regimens for patients with metastatic disease, from the perspective of drug delivery and response.

4. Tumor-Derived Secreted Factors

In addition to the secretion of exosomes, breast cancer primary tumors release a variety of other factors that have the potential to prime or augment the lung microenvironment, known as tumor derived secreted factors (TDSFs) (Table 1). An aspect of the niche that is critical for successful metastatic colonization is the status of the ECM. In a pro-metastatic state, secondary organs such as the lung upregulate the expression of several ECM components including versican, tenascin-c, periostin and fibronectin [37–41,92]. As with exosome production, the effect that primary tumor-secreted factors have on the secondary site is regulated by both environmental stimuli and interactions with stromal cells that comprise the tumor microenvironment. Hypoxia within the primary tumor influences

a wide variety of processes, including the increased expression of lysyl oxidase (LOX). LOX is an amine oxidase that crosslinks collagen and elastins in the ECM and is associated with a variety of pro-metastatic processes [42]. Erler et al. (2009) demonstrated that under hypoxic conditions breast cancer primary tumors increase LOX expression, inducing crosslinking of collagen in the lung [34]. This change in the ECM enables the adhesion of CD11b⁺ myeloid cells, and upon binding produces MMP2 resulting in the cleavage of collagen that is required for recruitment of BMDCs and cancer cells to the lung microenvironment [34]. This highlights an interesting observation that the status of the ECM determines which cells are recruited to the lung and can be changed by these recruited cells to promote metastatic seeding.

5. Stromal-Derived Influences

In addition to hypoxia, the primary tumor may be influenced by cells that comprise the tumor microenvironment which includes immune cells, endothelial cells, adipocytes and several other cell types [93] that produce factors that influence the metastatic process (Table 2). Emerging evidence suggests that a subset of activated stromal cells termed cancer-associated fibroblasts (CAFs) cause a multitude of changes in the behavior of breast cancer cells [94–96]. These CAFs are a heterogeneous population of cells that vary in origin and are characterized by the expression of several markers including α SMA and PDGFR- β [97]. In the context of breast cancer, CAFs drive the progression of metastasis by paracrine signaling and mechanical pressure on the cancer tissue [98]. In relation to paracrine signaling, CAFs in the tumor microenvironment have been shown to secrete IL-32 and, once bound to integrin β 3 on the cell surface of breast cancer cells, results in the activation of p38 MAPK signaling that causes increased expression of EMT markers such as fibronectin, N-cadherin and vimentin [99]. In order to determine the influence of CAFs on metastasis *in vivo*, BT549 breast cancer cells were co-cultured with CAFs and injected subcutaneously in mice, resulting in increased lung metastases [46].

While the primary tumor has the ability to influence the immune response directly, this is also achieved by the help of lung stromal cells. Breast primary tumors have the ability to up-regulate the expression of S100A8 and S100A9 in the lung microenvironment [46]. These proteins are part of the S100 family which are characterized as calcium-binding cytosolic proteins. The expression of these elements act as strong chemoattractants for both neutrophils and macrophages and promote the metastatic potential of the breast primary tumors [46]. Specifically in the context of lung metastasis, S100A8 and S100A9 have been shown to induce the recruitment of Mac-1⁺ myeloid cells to the lung that results in the secretion of migration stimulating factors (TNF α , MIP2 and TGF) and ECM remodeling [100].

The primary tumor must influence several molecular and cellular processes in order to ensure that metastasis to the lung is successful. One important example of this is the interplay between BMDCs and the primary tumor, a relationship first highlighted by Lyden and colleagues [23] that has gained increasing traction in recent years. Interestingly, previous research has demonstrated that bone marrow derived mesenchymal stromal cells (MSCs) have the potential to differentiate into CAFs upon co-culture with tumor cells [101,102]. Building off this observation, Raz et al. (2018) used collagen- α 1 tracking of transplanted bone marrow cells to demonstrate that MSCs make up a substantial proportion of CAFs that are present in the breast primary tumor and lung lesions [103]. These MSC-derived CAFs generate a unique inflammatory profile depending on the site they are recruited to and promote pro-metastatic features such as angiogenesis, enabling breast cancer metastasis to the lung [103]. Interestingly, the interaction between breast cancer cells and bone marrow cells is also crucial for cancer dormancy. Breast cancer dormancy is a significant clinical concern, as cancer cells in this state remain in mitotic arrest, making them resistant to drugs targeting highly proliferative cells. Upon recurrence, these senescent cells have the potential to resurge as progressive metastatic disease. It is widely accepted that the bone marrow serves as a sanctuary site for dormant breast cancer cells, and although this process is believed to be mediated by MSCs, the molecular underpinning of this relationship remains poorly understood [104–108]. Expanding on this interaction using a transwell model to recapitulate

communication between MSCs and breast cancer cells, Bliss et al. (2016) showed that breast cancer cells prime MSCs to secrete exosomes containing miR-222/223 which induce senescence in the recipient cancer cells [109].

To recruit BMDCs to the lung, previous literature has demonstrated that the state of the secondary site must be augmented to promote adhesion. Angiogenic factors such as VEGF released from lung-seeking breast cancer cells can activate the Src-FAK pathway in lung endothelial cells, resulting in increased expression of lung adhesion molecules and enhanced integrity of vascular permeability. Lung endothelial cells also express PGE2 in response to VEGF, which acts as a powerful chemoattractant to recruit BMDCs and tumor cells to the lung [110]. Under hypoxic conditions the expression profile of the primary tumor changes, causing variations in downstream targets. Previous literature has demonstrated that HIF-1 induces increased expression of Carbonic Anhydrase IX (CAIX) which has been shown to be required for breast cancer metastasis [35,111–113]. Taking this information, Chafe et al. (2015) were able to demonstrate that CAIX is required for the production of G-CSF by breast cancer cells, which in turn is responsible for the recruitment of granulocytic myeloid-derived suppressor cells to the lung for generation of a lung pre-metastatic niche [114].

6. Potential for Clinical Translation

Successful prevention of breast cancer metastasis to the lung will be dependent on identifying and treating not only the key characteristics of the “seed” (lung-seeking cancer cells), but also the “soil”; including the lung microenvironment and the pre-metastatic niche. In particular, the lung pre-metastatic niche has the potential to have significant implications in determining the risk that a particular patient may have for developing lung metastases. However, thus far most studies pertaining to the pre-metastatic niche have been limited to mouse models, although this is beginning to evolve towards the clinic. For example, knowledge that S100A8 and S100A9 are crucial for the generation of the pre-metastatic niche in the lung led to the development of S100A9 specific single photon emission computed tomography (SPECT) whole body imaging which has been tested in a pre-clinical breast cancer metastasis model [31]. In addition, using the knowledge that exosomes have the propensity to target specific organs, Nikolopoulou and colleagues (2016) isolated exosomes from the breast cancer cell line 4175-LuT which has a propensity to metastasize to the lung. The isolated exosomes were then labeled and injected into tumor-naïve female nude mice. The tissues from the mice were harvested, indicating a high accumulation of exosomes in the lung [115]. This method opens the potential to use high resolution, non-invasive imaging such as SPECT to identify pre-metastatic niche formation. Similar to this work, Soodgupta, et al. (2013) targeted VLA-4 expressed on BMDCs localized to the lung with a radiopharmaceutical. PET was subsequently performed to provide an effective method of detecting BMDCs in the pre-metastatic niche [116].

Upon identifying the presence of a pre-metastatic niche in the lung, the next step would involve preventing lung metastasis. To date, potential therapeutics targeting the pre-metastatic niche have mostly been assessed in the pre-clinical setting. However, the LSD1-specific inhibitor INCB059872 which is currently at phase 1 clinical trial for relapse of Ewing Sarcoma has been shown to reshape the myeloid compartment in a spontaneous lung metastasis model. Lee et al. (2018) demonstrated that INCB059872 reduced the migration of TN breast cancer cells, significantly reduced MSDC infiltration of the primary tumor and lung associated with a reduction in circulating CCL2, corresponding to a decrease in metastatic lung foci [117]. Alternatively, myeloid cells can be recruited to the lung by exosomes containing CSF-1 which are produced under hypoxic conditions. CSF-1 is associated with myeloid cell survival, proliferation and differentiation and has been previously been shown to be inhibited by GW2580. Pretreatment of mice with GW2580 prior to tumor implantation resulted in a significant decrease in myeloid cell recruitment to the lung and increase in anti-tumorigenic M1-macrophages [118]. Finally, as with exosomes, hypoxia has the potential to promote the generation of a pre-metastatic niche in the lung. Under hypoxic conditions hypoxia-inducible factors (HIFs) become activated, resulting in the induction of LOX and lysyl oxidase like (LOXL) proteins which

are responsible for collagen remodeling and BMDCs recruitment [119–121]. Wong et al. (2012) demonstrated that administration of two chemically different HIF inhibitors (digoxin and acriflavine) could prevent lung metastasis in an orthotropic breast cancer model. This inhibition was attributed to stunted LOX and LOXL expression which ultimately prevented collagen remodeling and BMDC recruitment [122].

Collectively, the results of these pre-clinical studies suggest that the effects of the pre-metastatic niche have the potential to be attenuated, preventing colonization. While these results are promising, the clinical benefit of these approaches for patients with an increased risk of lung metastasis has yet to be delineated. Efforts to elucidate the complicated underlying mechanisms that drive lung metastasis and pre-metastatic niche formation in the clinical setting have been hindered due to a limited number of patient samples derived from metastatic lesions. However, the implementation of rapid autopsy programs has the potential to provide these necessary samples [123]. In particular, the development of robust biobanks of matched primary tumors and metastatic lesions (from lung and other organs) would provide important research tools to advance this field. Profiling these samples could provide insightful information into the underlying mechanisms that drive metastasis to specific organs, uncovering potential biomarkers that could help predict and/or prevent organ-specific metastasis. Further research should also be focused on determining the length of time in which the metastatic niche within the lung lasts post-surgery, radiation and/or chemotherapy. This will allow for personalized treatment regimens that will ensure that even after the eradication of cancer, the recurrence of metastatic disease in the lung will be reduced or prevented altogether.

7. Conclusions

Breast cancer remains a significant burden in modern society, requiring further research to understand the underlying mechanisms that drive metastasis and how to target it. Metastasis to the lung is of particular concern as it is associated with high patient morbidity and mortality with no current effective strategies for early detection or eradication. Colonization of the lung is facilitated by a complex web of interactions with the tumor microenvironment, lung stroma, immune cells and BMDCs; and crosstalk between these components is mediated by exosomes and tumor/stroma-derived factors (summarized in Figure 1). These secreted elements are dynamic and vary based on environmental stimuli and interaction with stromal cells that infiltrate the tumor microenvironment and secondary site. Together these interactions transition the lung microenvironment into a fertile niche susceptible for cancer cell colonization. The therapeutic approaches described above hold promise for preventing lung metastasis, but have currently only been investigated in a pre-clinical setting, highlighting the need for further development and research in this area. Concurrently, a major limitation in this area of research is the lack of clinically relevant biomarkers to determine if a patient has an increased risk for lung metastasis. To address this, further research must focus on the use of exosomes as a predictor of organ-specific metastasis including validation of this in patient samples. These gaps in our current understanding demonstrate the complexity of metastasis, indicating that to have a significant impact on this disease we must consider the consolidated effect of all factors together rather than just investigating them individually. In summary, developing a deeper understanding of the processes that enable breast cancer metastasis to the lung will lead to the development of therapeutic targets and biomarkers with the ultimate goal of preventing metastatic disease.

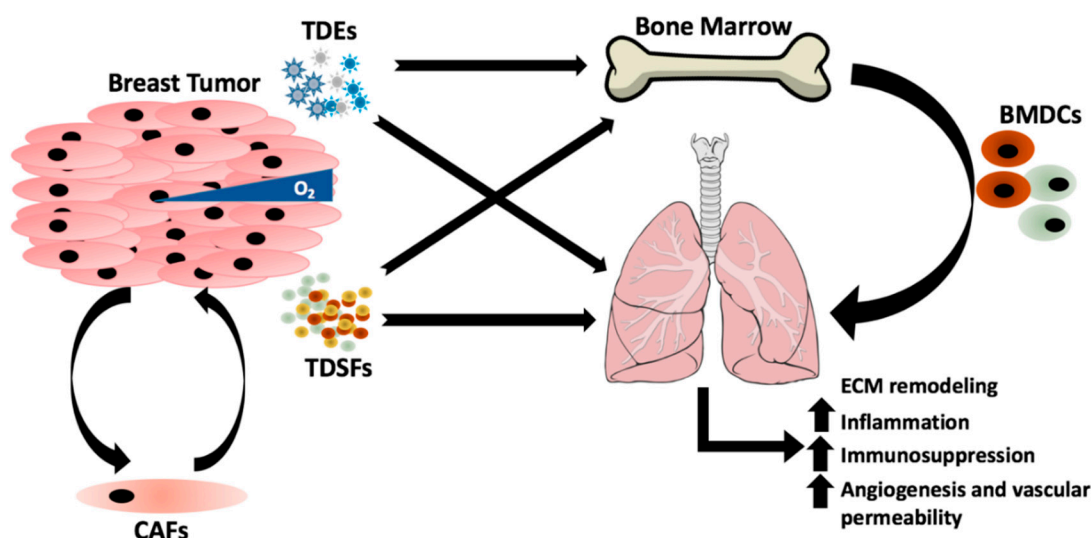


Figure 1. The underlying mechanisms that dictate the organ colonized by breast cancer are complex and influenced by many factors. Breast primary tumors regulate and prime the lung for metastasis by the secretion of tumor-derived exosomes (TDEs) and tumor-derived secreted factors (TDSFs), which target the bone marrow for recruitment of bone marrow-derived cells (BMDs) to lung in order to induce changes in the extracellular matrix (ECM) that are conducive for metastasis. Release of TDSFs from the primary tumor are often regulated by stromal cells that compose the tumor microenvironment including cancer associated fibroblasts (CAFs) or environmental stimuli such as hypoxia.

Author Contributions: Manuscript conception and writing was carried out by B.M. and A.L.A.

Funding: The authors' current work on breast cancer lung metastasis is funded by grants from the U.S. Department of Defense Breast Cancer Research Program (grant #BC160912), the Cancer Research Society (grant# 22386), a London Regional Cancer Program Catalyst Grant, and support from S. and R. Shaftoe through the London Health Sciences Foundation. Both B. Medeiros and A. Allan are supported by the Breast Cancer Society of Canada.

Acknowledgments: The authors thank the members of their laboratory for their helpful discussions and support.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

α SMA	Alpha Smooth Muscle Actin
BMDs	Bone Marrow Derived Cells
CAF	Cancer Associated Fibroblast
CCL2	Chemokine (C-C motif) Ligand 2
CCL21	Chemokine (C-C motif) Ligand 21
CCR7	Chemokine (C-C motif) Receptor Type 7
COX2	Cyclooxygenase-2
CSF-1	Colony Stimulating Factor 1
CTC	Circulating Tumor Cell
CXCR4	Chemokine (C-X motif) receptor 4
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
G-CSF	Granulocyte-Colony Stimulating Factor
HIF-1 α	Hypoxia Inducible Factor-1 Alpha
HPCs	Hematopoietic Progenitor Cells
IL-6/32	Interleukin-6 or 32
LOX	Lysyl Oxidase

LOXL	Lysyl Oxidase-Like
Ly6C	Lymphocyte Antigen 6 Complex
p38 MAPK	p38 Mitogen-Activated Protein Kinase
MDSCs	Myeloid Derived Suppressor Cells
MET	Mesenchymal-Epithelial Transition
MIP2	Macrophage Inflammatory Protein-2
MMP2/9	Matrix Metalloproteinase 2 or 9
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PDGFR-β	Platelet Derived Growth Factor Receptor Beta
PD-L1	Programmed Cell Death Ligand 1
PGE2	Prostaglandin E2
SDF-1	Stromal Derived Factor 1
S100A8/9	S100 Calcium Binding Protein A8/9
Src-FAK	Steroid Receptor Co-activator-Focal Adhesion Kinase
TDSFs	Tumor Derived Soluble Factors
TDEs	Tumor Derived Exosomes
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor Alpha
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1
VLA-4	Very Late Antigen-4
ZO-1	Zonula Occludens-1

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TITLE:

Isolation and functional assessment of human breast cancer stem cells from cell and tissue samples

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KEYWORDS:

Breast cancer stem cell (BCSC), fluorescence-activated cell sorting (FACS), colony forming assay, mammosphere assay, 3D culture model, *in vivo* tumor model

SUMMARY:

This experimental protocol describes the isolation of BCSCs from breast cancer cell and tissue samples as well as the *in vitro* and *in vivo* assays that can be used to assess BCSC phenotype and function.

ABSTRACT:

Breast cancer stem cells (BCSCs) are cancer cells with inherited or acquired stem cell-like characteristics. Despite their low frequency, they are major contributors to breast cancer initiation, relapse, metastasis and therapy resistance. It is imperative to understand the biology of breast cancer stem cells in order to identify novel therapeutic targets to treat breast cancer. Breast cancer stem cells are isolated and characterized based on expression of unique cell surface markers such as CD44, CD24 and enzymatic activity of aldehyde dehydrogenase (ALDH). These ALDH^{high}CD44⁺CD24⁻ cells constitute the BCSC population and can be isolated by fluorescence-activated cell sorting (FACS) for downstream functional studies. Depending on the scientific question, different *in vitro* and *in vivo* methods can be used to assess the functional characteristics of BCSCs. Here we provide a detailed experimental protocol for isolation of human BCSCs from both heterogenous populations of breast cancer cells as well as primary tumor tissue obtained from breast cancer patients. In addition, we highlight downstream *in vitro* and *in vivo*

functional assays including colony forming assays, mammosphere assays, 3D culture models and tumor xenograft assays that can be used to assess BCSC function.

INTRODUCTION:

Understanding the cellular and molecular mechanisms of human breast cancer stem cells (BCSCs) is crucial for addressing the challenges encountered in breast cancer treatment. The emergence of the BCSC concept dates back to the early 21st century, where a small population of CD44⁺CD24⁻/_{low} breast cancer cells were found to be capable of generating heterogeneous tumors in mice^{1,2}. Subsequently, it was observed that human breast cancer cells with high enzymatic activity of aldehyde dehydrogenase (ALDH^{high}) also displayed similar stem cell-like properties³. These BCSCs represent a small population of cells capable of self-renewal and differentiation, contributing to the heterogeneous nature of bulk tumors¹⁻³. Accumulating evidence suggests that alterations in evolutionarily conserved signaling pathways drive BCSC survival and maintenance⁴⁻¹⁴. In addition, the cell extrinsic microenvironment has been shown to play a pivotal role in dictating different BCSC functions¹⁵⁻¹⁷. These molecular pathways and the external factors regulating BCSC function contribute to breast cancer relapse, metastasis¹⁸ and development of resistance to therapies¹⁹⁻²¹, with the residual existence of BCSCs post-treatment posing a major challenge to the overall survival of breast cancer patients^{22,23}. Pre-clinical evaluation of these factors is therefore very important for identifying BCSC-targeting therapies that could be beneficial for achieving better treatment outcomes and improved overall survival in breast cancer patients.

Several *in vitro* human breast cancer cell line models and *in vivo* human xenograft models have been used to characterize BCSCs²⁴⁻²⁹. The ability of cell lines to continuously repopulate after every successive passage makes these an ideal model system to perform omics-based and pharmacogenomic studies. However, cell lines often fail to recapitulate the heterogeneity observed in patient samples. Hence, it is important to complement cell line data with patient-derived samples. Isolation of BCSCs in their purest form is important for enabling detailed characterization of BCSCs. Achieving this purity depends on the selection of phenotypic markers that are specific to BCSCs. Currently, the ALDH^{high}CD44⁺CD24⁻ cell phenotype is most commonly used to distinguish and isolate human BCSCs from bulk breast cancer cell populations using fluorescence activated cell sorting (FACS) for maximum purity^{1,3,26}. Furthermore, the properties of isolated BCSCs such as self-renewal, proliferation, and differentiation can be evaluated using *in vitro* and *in vivo* techniques.

For example, *in vitro* colony forming assays can be used to assess the ability of a single cell to self-renew to form a colony of 50 cells or more in presence of different treatment conditions³⁰. Mammosphere assays can also be used to assess the self-renewal potential of breast cancer cells under anchorage-independent conditions. This assay measures the ability of single cells to generate and grow as spheres (mixture of BCSCs and non-BCSCs) at each successive passage in serum-free non-adherent culture conditions³¹. Additionally, 3-Dimensional (3D) culture models can be used to assess BCSC function, including cell-cell and cell-matrix interactions that closely recapitulate the *in vivo* microenvironment and allow investigation of the activity of potential BCSC-targeted therapies³². Despite the diverse applications of *in vitro* models, it is difficult to model the complexity of *in vivo* conditions using only *in vitro* assays. This challenge can be

overcome by use of mouse xenograft models to evaluate BCSC behavior *in vivo*. In particular, such models serve as an ideal system for assessing breast cancer metastasis³³, investigating interactions with the microenvironment during disease progression³⁴, *in vivo* imaging³⁵, and for predicting patient-specific toxicity and efficacy of antitumor agents³⁴.

This protocol provides a detailed description for the isolation of human ALDH^{high}CD44⁺CD24⁻ BCSCs at maximum purity from bulk populations of heterogenous breast cancer cells. We also provide a detailed description of three *in vitro* techniques (colony forming assay, mammosphere assay, and 3D culture model) and an *in vivo* tumor xenograft assay that can be used to assess different functions of BCSCs. These methods would be appropriate for use by investigators interested in isolating and characterizing BCSCs from human breast cancer cell lines or primary-patient derived breast cancer cells and tumor tissue for the purposes of understanding BCSC biology and/or investigating novel BCSC-targeting therapies.

PROTOCOL:

1. Preparation- cell lines

- 1.1 Perform all cell culture and staining procedures under sterile conditions in a biosafety cabinet. Use sterile cell culture dishes/flasks and reagents.
- 1.2 Maintain human breast cancer cells at 37 °C with 5% CO₂ in defined media supplemented with fetal bovine serum (FBS) and necessary growth factors specific to each cell line.
- 1.3 Maintain mouse NIH3T3 fibroblast cell cultures (for use in colony forming assays) at 37 °C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS.
- 1.4 For all cultures, replenish the old media every 2-3 days with fresh media. Once the cultures reach 75-80% confluency, subculture into multiple sterile cell culture flasks.

2. Preparation- breast cancer tumor tissue

- 2.1 Collect the patient-derived surgical or biopsy samples directly from consenting breast cancer patients under a human ethics protocol approved by the institutional ethics board.
- 2.2 Subsequently, collect and generate tumor tissue from patient-derived xenograft models using mice under an animal ethics protocol approved by the institutional animal care committee.
- 2.3 Collect all tumor tissues under sterile conditions into a 50 mL sterile conical tube containing 30 mL DMEM:F12 media, keep on ice, and process the samples as described below within 2 hr of collection.

3. Generation of single cell suspensions of breast cancer cells

- 3.1 Aspirate media from the flask containing a monolayer of breast cancer cells that is 60-80% confluent (cell lines of your choice). Wash the cells with 1x phosphate buffered saline (PBS). Aspirate PBS and add appropriate cell dissociation solution (e.g. Trypsin:EDTA; just enough to cover the monolayer of cells) and incubate for 5 min at room temperature (recommended) or at 37 °C.
- 3.2 Add 5 mL of culture media to neutralize the activity of cell dissociation solution.
- 3.3 Transfer the resulting dissociated cell solution to a 50 ml conical tube and centrifuge at 1000 x g for 5 min.

- 3.4 Discard supernatant and resuspended the cell pellet in 5 mL of 1x PBS. Count the cells using hemocytometer and microscope.

Note1: observe for cell clumping in the hemocytometer. Repeat cell dissociation step if single cell suspension has not formed.

- 3.5 After cell counting, re-centrifuge the cell suspension at 1000 x *g* for 5 min, discard supernatant, and resuspend the cell pellet in ALDH substrate buffer at a concentration of 1 x 10⁶ cells/mL.

4. Generation of single cell suspension from tissue samples

- 4.1 Mince the tumor tissue with surgical blades using a crisscross technique to obtain smaller pieces of approximately 1 mm in size. Transfer the tissue pieces into a fresh 50 mL conical tube containing 10 mL dissociation buffer. Seal the conical tube with parafilm and incubate at 37 °C in a shaker incubator for 40 min.

Note 2: If you don't have a shaker incubator, place the tube in a 37 °C water bath and mix the tube by vortexing every 5-10 min.

- 4.2 Pellet the digested tissue by centrifuging sample at 530 x *g* for 5 min. Discard the supernatant and add 5 mL of trypsin. Pipette up and down using 1 mL pipette (set to 750 µL mark) to disrupt the pellet and incubate in a 37°C water bath for 5 min. After incubation, pipette up and down vigorously to release single cells.
- 4.3 Top up the total volume in the tube to 25 mL with DMEM:F12 media and centrifuge at 1000 x *g* for 5 min. Discard the supernatant and resuspend the pellet in 1 mL of dispase + DNase. Incubate in a 37 °C water bath for 5 min.
- 4.4 Top up the total volume in the tube to 10 mL with PBS. Mix by pipetting up and down, pass the resulting cell suspension through a 40 µm cell strainer attached to a fresh 50 mL conical tube. Centrifuge at 1000 x *g* for 5 min.
- 4.5 Discard supernatant and resuspend the cell pellet in 5 mL of 1x PBS. Count the cells and complete preparation of the cell suspension as described in steps 3.4 and 3.5.

5. Isolation of breast cancer stem cells (BCSCs)

- 5.1 Label flow tubes for the unstained control, single cell staining controls (DEAB control, ALDH, CD44-PE, CD24-PE-Cy7, 7AAD), the negative control tube (stained with DEAB, CD44-PE, CD24-PE-Cy7 and 7AAD), fluorescent minus one (FMO) control and the 'sort' tube (stained with ALDH, CD44, CD24 and 7AAD).
- 5.2 Transfer 500 µL (0.5 x 10⁶ cells) of the cell suspensions from step 3.5 or step 4.5 to each tube that is labelled cells only, CD44, CD24 and 7AAD. Place the tubes on ice until use.
- 5.3 Transfer 2 mL of sample (2 x 10⁶ cells) to respective 'ALDH' tube. Add 5 µL of DEAB to the 'DEAB control' and 'negative control' tubes and cap it tightly. Add 10 µL of ALDH substrate to the 'ALDH' tube, mix well by vortexing, and immediately transfer 500 µL to corresponding 'DEAB control' and 'negative control' tube. Recap the 'DEAB control', 'negative control' and 'ALDH tubes' and incubate at 37 °C for 30-60 min (do not exceed 60 min).
- Note 3: The optimal incubation time may require optimization depending on the cell line. Always protect the ALDH substrate and the tubes containing stained cells from light.
- 5.4 Following incubation, centrifuge all samples for 5 min at 250 x *g*. Resuspend the cells in 500

μL of ALDH substrate buffer. Add manufacturer-recommended or user-optimized concentration of anti-CD44-PE and anti-CD24-PE-Cy7 antibody cocktail and incubate at 4°C for 30 min. Add anti-CD44-PE and anti-CD24-PE-Cy7 antibodies to respective 'CD44' and 'CD24' labelled tubes.

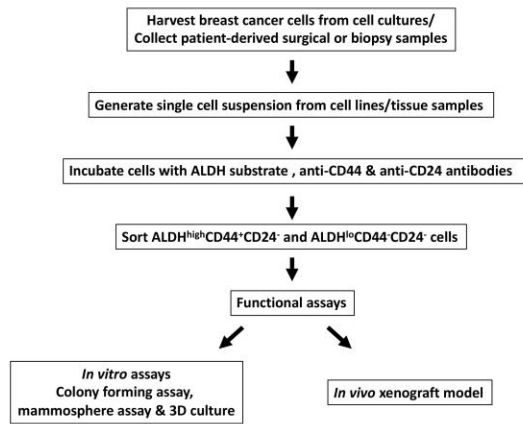
- 5.5 Following incubation, centrifuge all samples at 250 x *g* for 5 min. Resuspend the cells in 500 μL of ALDH substrate buffer. Incubate the 'negative control' tube, 'Sort tube' and the '7AAD' tube with 7AAD (suggested concentration: 0.25 μg/1 x 10⁶ cells) for 10 min on ice.

Note 4: The ALDH activity is detected in the green fluorescent channel, therefore a fluorochrome with a different compatible emission spectrum should be used. Where spectral overlap is observed during multi-parameter flow cytometry, single color controls and FMO control should be used as a guide to allow compensation between fluorochromes to minimize the spill over of fluorescent signal into other channels.

- 5.6 Set up the analysis protocol on the FACS instrument in preparation for sample analysis. Create scatter plots (forward vs side scatter, forward scatter vs fluorescent channels).
- 5.7 Using the unstained control, adjust the photomultiplier to separate debris from whole cell population and adjust the fluorescent voltage to move the whole cell population around the first log scale (10¹). Using the DEAB control, move the whole cell population within the second log scale (10²) by adjusting the green fluorescent voltage channel.
- 5.8 Analyze all the single staining controls first (ALDH, CD44-PE, CD24-PE-Cy7) and 7AAD and FMO control, adjusting the voltage to separate stained from unstained cells and to minimize the spillage of fluorescent signals into other channels.
- 5.9 Gate on the positive population for each single stained cell sample. Using the negative control tube, gate for viability (7AAD negative), ALDH^{low} and ALDH^{high} cell populations (representative gating strategy shown in **Figure 1B**).
- 5.10 Analyze multiparameter stained samples of interest to isolate BCSCs. Using the viable ALDH^{low} and ALDH^{high} gates, select for CD44⁺CD24⁻ (BCSC) and CD44⁺CD24⁻ (non-BCSC) cell population respectively (**Figure 1B**).
- 5.11 Collect viable BCSCs and non-BCSCs in collection media in sterile collection tubes (populations from two representative cell lines shown in **Figure 2A&B**). Use sorted cells for downstream *in vitro* and *in vivo* assays as described below.

Note 5: In addition to *in vitro* and *in vivo* assays described below, BCSCs can be validated by measuring the expression of pluripotent markers such as SOX2, OCT4 and NANOG via standard immunoblotting techniques.

A



B

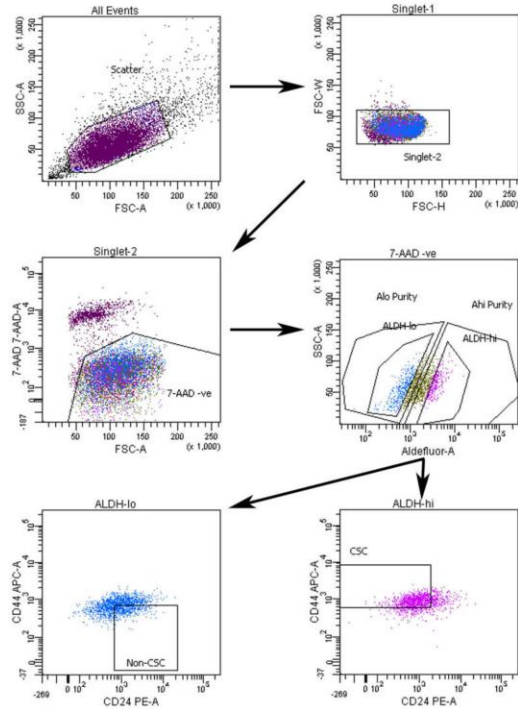


Figure 1: FACS gating strategy for isolation of BCSCs from breast cancer cell lines and tissue samples. (A) Flowchart describing the procedure of BCSC isolation. (B) Representative FACS plots showing the sort strategy used to isolate viable BCSCs and non-BCSCs from a heterogenous pool of cells. MDA-MB-231 human breast cancer cells are concurrently labeled with 7-AAD, CD44-APC, CD24-PE and the ALDH substrate. Cell subsets were isolated using a four-color protocol on a FACS ARIA III (Becton Dickinson). Cells are selected based on expected light scatter, then for singlets, and viability based on 7-AAD exclusion. Cells are then analyzed for ALDH activity and the top 20% most positive are selected as the ALDH^{high} population, while the bottom 20% of cells with the lowest ALDH activity were deemed to be ALDH^{low}. Finally, 50% of the ALDH^{low} cells are further selected based on a CD44^{low/-}CD24⁺ phenotype, and 50% of the ALDH^{high} cells are selected based on CD44⁺CD24⁻ phenotype. This figure has been adapted from Chu et al, 2014¹⁷.

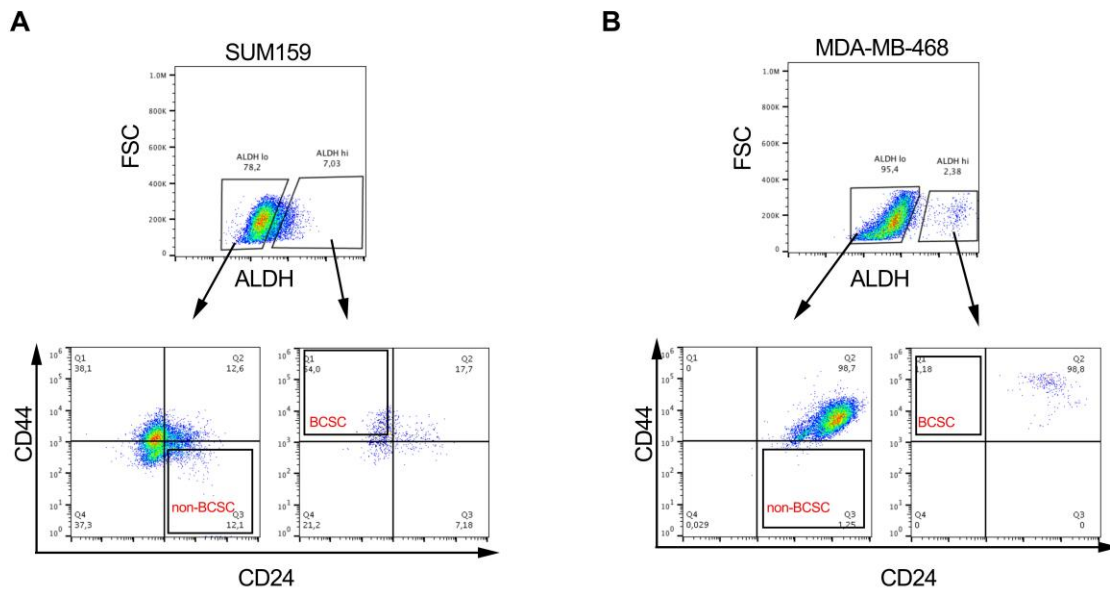


Figure 2: BCSCs proportions are variable in different breast cancer cell lines. Representative image showing the differential proportion of BCSCs and non-BCSCs in (A) SUM159 and (B) MDA-MB-468 triple negative breast cancer cell lines following labelling and sorting as described in Figure 1.

6. Colony forming assay

- 6.1 Resuspend the cells of interest (sorted cells from step 5.11 or unsorted cells from steps 3.5 or 4.5) in complete media.
- 6.2 Label three flow tubes for 1×10^2 , 2×10^2 and 5×10^2 cells. Add 2 mL of complete media and transfer the appropriate cell number (sorted from step 5.11 or unsorted cells from steps 3.5 or 4.5) in respective tubes. Mix the cell solutions thoroughly by pipetting it up and down 5 times.
- 6.3 Plate the cells in a 6-well plate and distribute the cell suspension by gently swirling the plates to obtain uniform distribution of cells.
- 6.4 Incubate the plates in a 37 °C, 5% CO₂ incubator until colonies appear (where colonies = ≥ 50 cells per colony). Carefully replenish media twice a week without disturbing colony formation.
- 6.5 Aspirate media and wash once with 1 mL PBS. Add 0.5 mL of 0.05% crystal violet solution into each well and incubate the plate for 30 minutes. Remove excess crystal violet stain by washing with 2 mL of water. Repeat the washing step until background staining has been removed.
- 6.6 Using a microscope at 4x and 10x magnification, count and record the total number of colonies generated (representative images shown in **Figure 3A**).
- 6.7 Calculate the frequency of colony formation as follows: Frequency (%) = (# of colonies formed/number of cells seeded) x 100. For example, if 25 colonies are generated from 1×10^2 cells, then the Frequency of colony formation is, Frequency = $(25/100) \times 100 = 25\%$.

- 4.1 Alternatively, replace steps 6.1 to 6.4 with an alternate method involving co-culture with fibroblasts, which provide a microenvironmental support for BCSCs through production of necessary growth and survival factors.
- 6.9 Pre-coat cell 60mm culture dishes with type I bovine collagen (1 in 30 dilution of 3 mg/mL collagen). Allow collagen to polymerize for 30 min in a 37 °C incubator. Aspirate the unpolymerized collagen and wash the plate twice with 1x PBS. Cover the collagen-coated plate with 1 mL PBS and set it aside at room temperature until use.
- 6.10 Label three flow tubes for 1×10^3 , 5×10^3 and 1×10^4 cells. Add 4 mL of colony forming assay media and transfer the appropriate number of cells (sorted from step 5.11 or unsorted cells from steps 3.5 or 4.5) into the respective tubes. Add irradiated mouse NIH3T3 fibroblasts (4×10^4 cells/mL of media). Mix cell solutions thoroughly by pipetting it up and down 5 times.
- 6.11 Aspirate the PBS from the collagen-coated culture dish from step 6.1 and plate the cell mixture onto each of the cell culture plates as described in step 6.3.
- 6.12 Incubate the plates in a 37 °C, 5% CO₂ incubator and leave them undisturbed for 7-10 days or until colonies form, without replenishing the media. Count and record the total number of colonies generated as described in steps 6.6 and 6.7.

7. Mammosphere assay

- 7.1 Resuspend the cells of interest (sorted cells from step 5.11 or unsorted cells from steps 3.5 or 4.5) in complete mammosphere media and plate cells at a seeding density of 5×10^2 cells/cm² area in a 96 well ultra-low attachment cell culture plate.
Note 6: Cell seeding density should be optimized for different cell lines.
- 7.2 Incubate the culture plates for 5-10 days in a 37 °C incubator with 5% CO₂. Carefully replenish media twice a week without disturbing mammosphere formation.
- 7.3 After incubation, count the number of mammospheres generated in each well using a microscope; where mammospheres are defined as breast cancer cell clusters greater than 100 µm in diameter (representative images shown in **Figure 3B**).
- 7.4 Calculate the mammosphere formation efficiency (MEF) as follows: $MFE (\%) = (\text{number of mammospheres per well}) / (\text{number of cells seeded per well}) \times 100$ (i.e. if 5 mammospheres are generated by 1×10^2 cells in a well, then $MFE = (5/100) \times 100 = 5\%$).
- 7.5 To subculture mammospheres, carefully transfer the media containing mammospheres content into a fresh 50 mL conical tube and centrifuge media at $1000 \times g$ for 5 min. Carefully remove supernatant, resuspend cell pellet in 500 µL of trypsin, and incubate for 5 min at room temperature.
- 7.6 Discard the supernatant and resuspend the pellet in 1 mL of complete mammosphere media. Count the cells using a hemocytometer and re-plate the cells in an ultra-low attachment cell culture plate as described in step 7.1.
Note 7: In addition to sub-culturing, the mammosphere-derived cells can be also analyzed further by FACS to assess BCSC phenotype and/or obtain pure populations of BCSCs for other downstream assays.
- 7.7 To determine the number of mammosphere-initiating cells contained within your cell populations, use an alternate method involving sphere limiting dilution analysis (SLDA). Plate cells in serial dilutions of high to low cell numbers in a 96 well ultra-low attachment cell culture plate, with the highest dilution resulting in less than one cell per well.

- 7.8 Incubate the culture plate for 10-14 days in a 37 °C incubator with 5% CO₂ and leave them undisturbed to avoid cell aggregation.
- 7.9 After incubation, count the number of mammospheres generated in each well using a microscope; where mammospheres are defined as breast cancer cell clusters greater than 100 µm in diameter. Calculate the sphere-initiating frequency and significance using Extreme Limiting Dilution Analysis (ELDA) online software (<http://bioinf.wehi.edu.au/software/elda/>).

8. 3D culture model

- 8.1 Depending on the experimental question, use basement membrane extract (BME) with or without growth factors (reduced). In order to evaluate the effect of individual growth factor on cancer cells, use growth factor reduced BME. It also helps in minimizing the non-specific effects of endogenous growth factors present in BME.

Note 8: BME solidifies above 10 °C. Always keep BME on ice even during the thawing step.

- 8.2 Carefully add 50 µL of BME per well in a 96-well plate without creating air bubbles and allow it to polymerize at 37 °C for 1 h. After 10 min of incubation, add PBS to avoid drying of the gel layer.
- 8.3 Resuspend the sorted cells from step 5.11 or unsorted cells from steps 3.5 or 4.5 at a concentration of 5×10^3 to 5×10^4 /200 µL in 3D culture media.
- 8.4 Once the BME has polymerized, remove PBS, add 200 µL of cell suspension to each well and incubate in 37 °C incubator with 5% CO₂. Add PBS to the surrounding wells to avoid evaporation of the media.
- Note 9: The optimal number of cells for plating should be determined prior to setting of the experiment. Depending on the experimental question, BCSCs can be cultured alone or with other cells types (fibroblasts/endothelial/immune cells etc.).
- 8.6 Add fresh media to the culture plates twice weekly. Maintain cultures for 10-14 days prior to analyzing the formation of organoids (representative images shown in **Figure 3C**).
- 8.7 For sub-culturing, carefully aspirate the media and add 200 µL of dispase to each well containing cells. Incubate the plate in a 37 °C incubator for 1 h. Halfway through the incubation period (30 min), take out the plate, gently pipette the dispase solution up and down 5 times, and place back in the incubator for a further 30 min.
- 8.8 After 1 h, transfer the dissociated cell solution to a flow tube. Wash the well with 1x PBS containing 2% FBS (fPBS) and transfer it to the flow tube. Centrifuge the tube at 1000 x g for 5 min. Carefully aspirate the supernatant and add 500 µL of trypsin, incubate at 37 °C for 5 min. Inactivate trypsin by adding equal amount of fPBS and centrifuge at 1000 x g for 5 min.
- 8.9 Discard the supernatant and resuspend the pellet in 1 mL of 3D culture media. Count the cells and re-plate required number of cells in the BME as in steps 8.2 to 8.4.
- Note 10: Multiple wells can be pooled to further analyze or sort the cell population of interest.

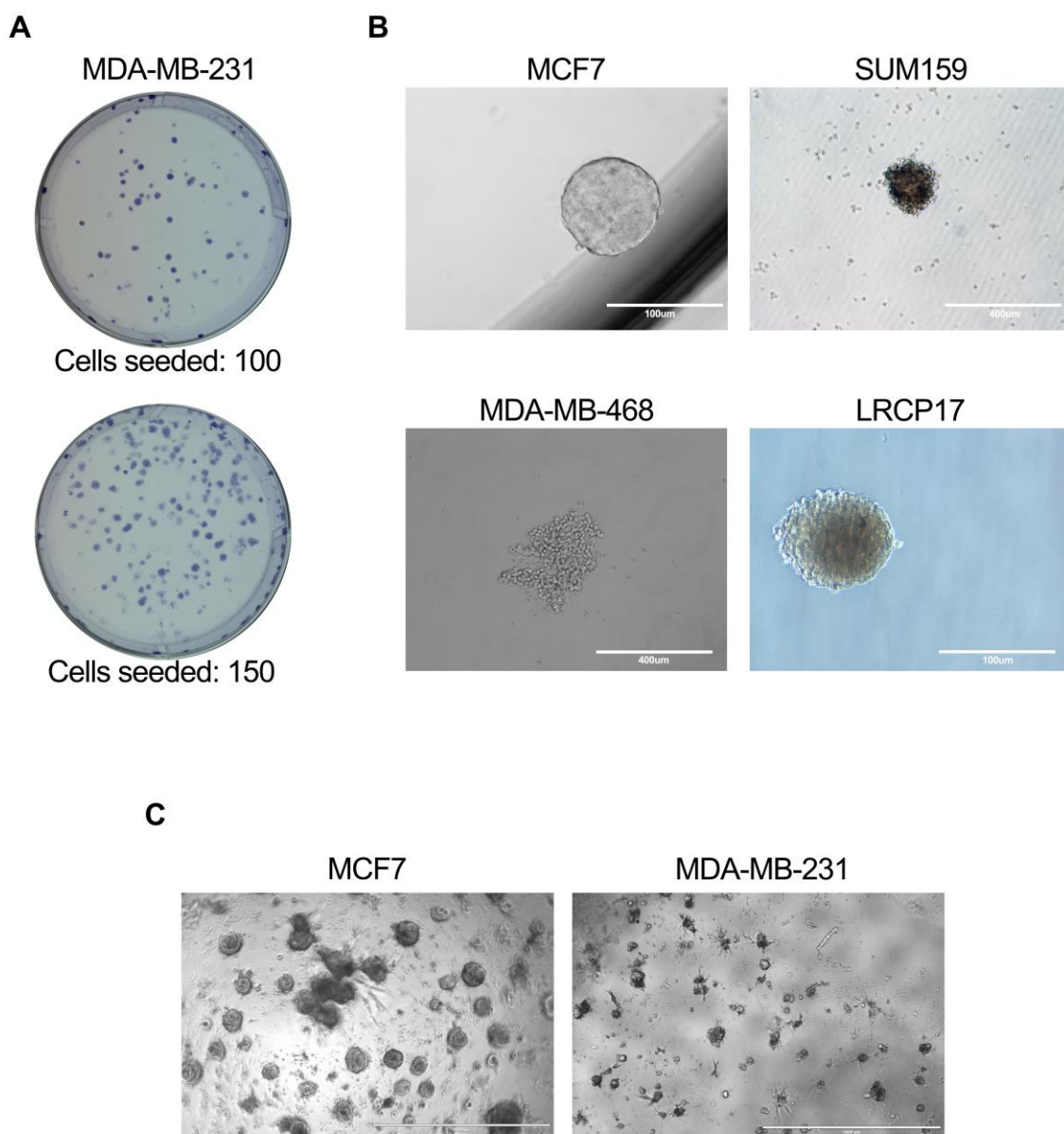


Figure 3: *In vitro* assays to assess BCSC cell function. *In vitro* assays were performed as described in protocol sections 6.1 to 6.5 (A), 7.1 to 7.4 (B), or 8.1. to 8.4 + 8.6 (C). (A) Representative image showing the colonies generated by MDA-MB-231 human breast cancer cells; (B) Representative images showing mammosphere formation by MCF7, SUM159, or MDA-MB-468 human cell lines as well as patient-derived LRCP17 breast cancer cells. (C) Representative images showing the 3D structures formed by MCF7 and MDA-MB-231 breast cancer cells in 3D cultures models.

9. *In vivo* xenograft model

9.1 Perform animal experiments under an animal ethics protocol approved by the institutional animal care committee.

9.2 In order to determine the tumor initiation capacity of breast cancer stem cells, prepare cells (sorted population from step 5.7 or unsorted populations from steps 3.5 or 4.5) using a limiting dilution approach. Serially dilute cells in PBS using between 1 and 5 different dilution groups, with doses as low as $0.01\text{--}0.2 \times 10^2$ cells/100 μL and as high as 1×10^6 cells/100 μL .

Note 11: Unsorted/whole population cells can be used as a control. The number of dilution groups used will depend on the desired scientific outcome (e.g. if only testing tumorigenicity then 1 group at a higher cell number may be used, whereas when calculating tumor-initiating capacity, it is optimal to test 5 limiting dilution doses).

9.3 To generate xenograft models from human breast cancer cells, use immunocompromised female mice (athymic nude [*nu/nu*], nonobese diabetic/severe combined immunodeficient [NOD/SCID] or NOD/SCID IL2 γ [NGS] strains).

Note 12: Although a minimum of 4 animals per group can be used, 8-12 animals per group is recommended to obtain robust results particularly for limiting dilution analysis.

9.4 Perform standard mammary fat pad (MFP) injections using 100 μL /mouse of each cell preparation, under sterile conditions in a biosafety cabinet.

Note 13: For optimal breast tumor growth and spontaneous metastasis to distant organs, the thoracic MFP is recommended. Alternatively, the inguinal MFP can also be used.

9.5 Post-injection, monitor the mice on a daily basis for general health and tumor growth at the site of injection. Upon detection of a palpable tumor, begin measuring the tumor size by calipers in two perpendicular dimensions and record weekly until endpoint.

Note 14: The experimental end point is determined based on the regulations laid out the institutional animal ethics protocol; typically, endpoint by euthanasia is usually required once tumor volumes reach 1500 mm^3 . For BCSC populations and/or higher cell doses (e.g. $>1 \times 10^4$ cells), this endpoint will likely be reached within 4-8 weeks of MFP injection. For very low cell doses and/or non-BCSC cell populations, tumor growth should be allowed to progress for up to 8 months post-injection.

9.6 From these measurements, calculate the tumor volume using the following formula: Volume in $\text{mm}^3 = 0.52 \times (\text{width})^2 \times \text{length}$. If using a limiting dilution approach, calculate tumor-initiating capacity and significance using ELDA online software (<http://bioinf.wehi.edu.au/software/elda/>).

9.7 Alternatively, to humanely extend the endpoint, surgically remove primary tumors and continue to monitor mice for health and/or development of spontaneous metastasis in distant organs. Use resected tumor tissue for the generation of serial xenotransplants.

9.8 At endpoint, harvest tissue from primary tumors and distant organs (lymph nodes, lung, liver, brain, bone) and carry out histopathological and/or immunohistochemical analysis or dissociated the tumor tissue and use in the *in vitro* assays described in sections 6-8.

REPRESENTATIVE RESULTS:

The described protocol allows isolation of human BCSCs from a heterogenous population of breast cancer cells, either from cell lines or from dissociated tumor tissue. For any given cell line or tissue sample, it is crucial to generate a uniform single cell suspension to isolate BCSCs at

maximum purity as contaminating non-BCSC populations could result in variable cellular responses, especially if the study aim is to evaluate the efficacy of therapeutic agents targeting BCSCs. Application of a stringent sorting strategy will minimize the presence of contaminating non-BCSCs and result in the ability to collect the proportion of breast cancer cells with stem cell-like characteristics that display a cellular phenotype that distinguishes them from bulk population of cancer cells. Human breast cancer cells that exhibit enhanced ALDH enzymatic activity, express high levels of the cell surface marker CD44, and low/negative expression of CD24 have an ALDH^{high}CD44⁺CD24⁻ phenotype and can be classified as BCSCs. The proportion of BCSCs within the bulk population can vary between cell lines or patients (**Figure 2**), and often depends on disease stage, with more aggressive breast cancer usually displaying a higher proportion of BCSCs^{26,36,37}.

Isolated BCSCs can be used to perform different *in vitro* and *in vivo* assays where their behavior and function can be compared to that of the bulk and/or non-BCSC populations. For example, the ability of a single breast cancer cell to self-renew and generate colonies of 50 cells can be assessed by colony-forming assays (**Figure 3A**). The ability of BCSCs to self-renew under anchorage-independent experimental conditions can be assessed by mammosphere assays, where variable sphere number, size, and sphere-initiating capacity can be analyzed and correlated with the presence and function of BCSCs (**Figure 3B**). It is important to determine the seeding cell densities for different breast cancer cell lines or breast tumor samples to obtain optimal results. This is particularly important when performing SLDA, as higher cell densities could lead to cell aggregation resulting in misinterpretation of cellular activity.

Culturing breast cancer cells in BME allows BCSCs to form 3D structures that recapitulate *in vivo* conditions (**Figure 3C**). 3D culture of breast cancer cells in the presence of other microenvironmental cell types such as fibroblasts, endothelial cells, and/or immune cells has the added capacity for investigating the role of microenvironment in 3D growth of BCSCs^{38,39}. The specific cell numbers required to generate 3D organoids may vary depending on the cell line or patient tumor source, and thus it is important to optimize the culture conditions and cell numbers prior to any large-scale experiments.

Finally, *in vivo* mouse xenograft models can be used to understand the differences in growth (**Figure 4**) self-renewal, differentiation and/or tumor-initiating ability of BCSCs *in vivo* compared to non-BCSCs or bulk cell populations. Often, the *in vitro* cellular responses observed in the presence of exogenous factors or therapeutic agents is not representative of *in vivo* setting, suggesting that *in vitro* observation should be complimented with *in vivo* studies whenever feasible. Using *in vivo* xenograft models, the cellular heterogeneity and tumor architecture is preserved and thus these models can serve as a system that closely mimics the microenvironment in human patients. *In vivo* LDA can be performed to determine the proportion of tumor-initiating cells in a given mixed population of cancer cells (BCSCs or non-BCSCs)^{40,41}. The range of cell dilutions used should be optimized and will depend on the frequency of initiating cells in the cell population of interest. Ideally these dilutions should include doses that result in 100% tumor formation, down to cell doses with no tumor formation and a reasonable range in between. The frequency of tumor-initiating cells in primary samples can be variable, and in

instances where breast tumors have very low numbers or heterogenous populations of tumor-initiating cells, performing LDA can be particularly challenging⁴². In these cases, injecting larger number of cells would be more appropriate for understanding breast cancer biology.

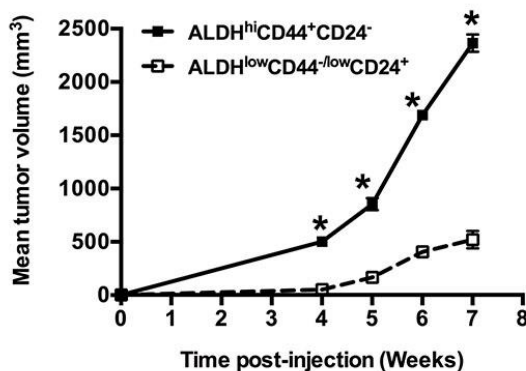


Figure 4: *In vivo* xenograft assays to assess BCSC function. MDA-MB-231 breast cancer cells were isolated by FACS as described in Figure 1 and injected into the right thoracic mammary fat pad of female NSG mice as described in protocol sections 9.1 to 9.8 (5×10^5 cells/mouse; 4 mice/cell population). Primary breast tumor growth kinetics are shown for ALDH^{hi}CD44⁺CD24⁻ (■) versus ALDH^{low}CD44^{low/-}CD24⁺ (□) populations. Data represented as the mean $\pm$ S.E.M. * = significantly different tumor size than respective ALDH^{low}CD44^{low/-} subsets at the same time-point ($P < 0.05$). This figure has been adapted from Croker et al., 2009²⁶.

DISCUSSION:

Breast cancer metastasis and resistance to therapy have become major cause of mortality in women worldwide. The existence of a sub-population of breast cancer stem cells (BCSCs) contributes to enhanced metastasis^{26,43-46} and therapy resistance^{21,47,48}. Therefore, the focus of future treatments should aim at eradicating BCSCs to achieve better treatment outcomes, and this requires accurate methods for isolating and characterizing the functional characteristics of BCSCs using both *in vitro* and *in vivo* methods.

Immortalized cell lines derived from different subtypes of breast cancer have proven to be feasible models to study breast cancer biology including the isolation and characterization of BCSCs^{26,49,50}. The high proliferative capacity and unlimited expansion ability of cell lines provides an ideal model system for performing studies that are highly reproducible and technically straightforward. However, due to the clonal origin of cell lines, they may fail to recapitulate the heterogeneity exhibited by different patients and/or by cancer cells within tumor tissue. In addition, genetic alterations can be acquired during serial passaging of cell lines and may induce genotypic or phenotypic changes that can confound experimental results⁵¹. In contrast, primary patient-derived cells, despite their limited proliferative and expansion ability, may provide a more accurate model to that observed *in vivo*. However, such samples may be more difficult to acquire and be more technically challenging to work with. All of these factors should be

considered when choosing a starting model system with which to isolate and characterize BCSCs.

FACS is a commonly used technique to isolate cells of interest based on cell surface marker expression^{52,53}. Based on cell surface antigens (CD44 and CD24) and ALDH enzymatic activity, human BCSCs can be isolated at high purity from both breast cancer cell lines and tumor tissues^{1,2}. The sorting efficiency determines the purity of sorted sample, and it is recommended that users analyze a small portion of sorted sample incubated with viability dye to check the efficiency of sorting^{53,54}. The sorting efficiency can be confounded by many factors including the presence of cell clumps, a high number of dead or dying cells, improper compensation of the fluorochromes and/or damage to cell surface antigens due to sensitivity to trypsin or collagenase during pre-sorting dissociation steps⁵³⁻⁵⁵. Therefore, generation of a proper single cell suspension and use of appropriate cell dissociation techniques will increase the sorting efficiency. While performing multiparameter cell sorting, it is important to choose fluorochromes that minimizes spectral overlap. In some cases, where spectral overlap cannot be avoided, a control that contains all the fluorochromes except one (fluorescence minus one, FMO) should be used to minimize the spillover of fluorescent signals into other channels⁵⁴. Alternatively, the spectral overlap can be reduced by immunomagnetically isolating cell populations prior to final FACS isolation of cells of interest⁵⁶.

In vitro assays such as the colony-forming and mammosphere assays described in this protocol have been extensively used to study the self-renewal and proliferative ability of BCSCs⁵⁷⁻⁶¹. Additionally, these assays can be used to assess the activity of different therapeutic drugs on BCSC function. Several evolutionarily conserved signaling pathways have been implemented in BCSC maintenance⁶², and both colony-forming⁶³⁻⁶⁵ and mammosphere assays^{63,66} have been used to assess the value of therapeutic disruption of these pathways as an intervention to block BCSC intrinsic signaling and reduce BCSC activity and disease progression. Colony forming assay using primary cells can be challenging due to low cell density, variation between samples and lack of its adaptability to isolated *in vitro* conditions. These challenges can be overcome by culturing BCSCs on a soft agar layer or by coculturing them with fibroblasts on a collagen-coated cell culture dish⁶⁷. In addition, supplementing growth factors into the culture media (such as FGF7⁶⁸) could also improve the colony-forming ability of cells isolated from tissue samples. In addition, over-digestion of tissue using collagenase or trypsin during single cell suspension generation step can result in low to zero colony-forming ability and reduce mammosphere-forming efficiency³¹. In both the assays, care should be taken to incubate the assay plates undisturbed to avoid disruption of colony or sphere structures as they are forming. It is also recommended that users extend the incubation period for primary cells (relative to cell lines) as it might take longer for these cells to form colonies or spheres.

Multiple lines of evidence have demonstrated the critical role of extracellular matrix (ECM)^{15,17,69} and stromal components, such as fibroblasts, immune cells, endothelial cells and adipocytes in influencing BCSC functions¹⁵. Thus, the 3D culture model we describe in this protocol can provide a useful experimental system for helping to recapitulate the *in vivo* tumor microenvironment in an *in vitro* setting. Although the 3D culture system closely resembles the tumor microenvironment in cancer patients, long term maintenance of cells as organoids can be

difficult. In addition, optimization of the 3D culture conditions and the ability to accurately investigate self-renewal and differentiation ability of BCSCs is challenging⁷⁰. The efficiency of organoids formed in 3D culture system depends on the growth factors supplemented in the culture media⁷¹. Absence of key components (for example, ROCK inhibitor) could lead to reduced or no organoid formation⁷¹. Media should be replenished every 3-4 days to maintain optimal cellular function and the sustainability of the culture. In order to recapitulate *in vivo* conditions and response, it is always important to allow the cells to form organoids prior to any kind of exogenous treatment⁷². Cells derived from patient samples should be giving sufficient time to form organoids, particularly if the objective is the evaluate drug response⁷².

While these *in vitro* methods are attractive and accessible experimental tools for characterizing BCSC function, tumor heterogeneity and the effect of tumor microenvironment on BCSC behavior cannot be studied with complete effectiveness. These *in vitro* assays should therefore be complemented with *in vivo* xenograft models whenever feasible in order to further validate experimental findings related to BSCS biology and/or response to novel therapeutics. Different *in vivo* models have been used study BCSC tumorigenicity and metastasis. Ectopic (subcutaneous engraftment) and orthotopic (MFP engraftment) mouse models have been used to generate breast tumors and assess longitudinal changes in tumor growth over time⁵⁰. Although both *in vivo* injection approaches can be used to study BCSC biology, the native stromal and vasculature-related components of the MFP allow more accurate recapitulation of primary breast tumor progression as observed in patients, and thus MFP injection is preferred^{73,74,75}. Finally, the use of immunocompromised mice is required for engraftment of human BCSCs and tumor growth, and this prevents incorporating the role of immune cells in tumorigenesis and metastasis studies⁷⁶. More recently, this limitation has been addressed through the use of humanized mice in which a human immune system is reconstituted via bone marrow transplantation prior to the initiation of xenograft studies⁷⁷⁻⁷⁹. However, these models are expensive and technically challenging, and thus are still not commonly used⁸⁰.

In summary, here we have provided a protocol for the isolation of human BCSCs from both breast cancer cell lines and patient-derived tumor tissue samples. We have also described *in vitro* and *in vivo* protocols for downstream assays that can be used to study BCSC function, with the ability to be optimized for different breast cancer cell sources and the flexibility to be performed under different experimental conditions. These protocols will be useful for investigators interested in cancer stem cells, breast cancer biology and therapeutic development, with the ultimate goal of improving patient outcomes in the future.

ACKNOWLEDGMENTS:

We thank members of our laboratory for their helpful discussions and support. Our research on breast cancer stem cells and the tumor microenvironment is funded by grants from the Canadian Cancer Research Society Research Institute and the U.S. Army Department of Defense Breast Cancer Program (Grant # BC160912). V.B. is supported by a Western Postdoctoral Fellowship (Western University), and both A.L.A. and V.B. are supported by the Breast Cancer Society of Canada. C.L. is supported by a Vanier Canada Graduate Scholarship from the Government of Canada.

DISCLOSURES:

The authors have nothing to disclose.

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Lung-derived selectins enhance metastatic behavior of triple negative breast cancer cells

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Abstract: Lung is one of the deadliest sites of breast cancer metastasis, particularly for triple negative breast cancer (TNBC). We have previously shown that the lung produces several soluble factors that may enhance metastatic behavior of TNBC, including E-, L- and P-selectin. Here we hypothesize that lung-derived selectins promote TNBC metastatic behavior and may serve as a potential therapeutic target. Lungs were isolated from mice and used to generate lung-conditioned media (CM). Lung-derived selectins were immunodepleted and TNBC migration and proliferation was assessed in response to native or selectin-depleted lung-CM. A 3D *ex vivo* pulmonary metastasis assay (PuMA) was used to assess metastatic progression of TNBC in the lungs of wildtype versus triple-selectin (ELP^{-/-}) knockout mice. We observed that individual lung-derived selectins enhance *in vitro* migration ($p \leq 0.05$) but not proliferation of TNBC cells, and that *ex vivo* metastatic progression is reduced in the lungs of ELP^{-/-} mice compared to wildtype ($p \leq 0.05$). Treatment with the pan-selectin inhibitor bimosiamose reduced *in vitro* lung-specific TNBC migration and proliferation ($p \leq 0.05$). Taken together, these results suggest that lung-derived selectins may present a potential therapeutic target against TNBC metastasis. Future studies are aimed at elucidating the pro-metastatic mechanisms of lung-derived selectins and developing a lung-directed therapeutic approach.

Keywords: breast cancer, metastasis, lung microenvironment, E-selectin, L-selectin, P-selectin, CD44, bimosiamose

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Biomedicines* **2021**, *9*, x. <https://doi.org/10.3390/xxxxx>

Academic Editor: Firstname Lastname

Received: date

Accepted: date

Published: date

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1. Introduction

Breast cancer is the most diagnosed cancer and a leading cause of death in women worldwide [1]. More than 90% of these deaths are due to metastasis, as current treatments are non-curative in the metastatic setting [2–4]. The lung is one of the most common and deadly sites of breast cancer metastasis, particularly in patients with aggressive triple-negative (TN) disease [5–7]. Lung metastasis often occurs within 5 years of initial diagnosis and leads to significant morbidity and mortality [5,7–10]. Patients who present with either solitary or small numbers of lung metastases (termed “oligometastatic” disease [11]) have demonstrated a survival benefit from targeted resection and/or stereotactic ablative radiation of these lesions [12,13]. However, more advanced lung metastases remain difficult to treat, with an estimated 60–70% of patients who die of breast cancer having lung metastasis [14]. This important clinical problem highlights the need to characterize the fundamental cellular and molecular drivers of this process in order to develop new therapeutic strategies.

We have previously identified several soluble factors produced by the normal lung that promote TN breast cancer metastatic behavior [15]. This work has been facilitated by the development of a novel 2D *ex vivo* model system for investigating the influence of organ-derived soluble factors on breast cancer metastatic behavior, whereby organs representing common sites of breast cancer metastasis (i.e., lung) are isolated from healthy mice and used to generate organ-conditioned media (CM) [16]. In particular, we have observed that aggressive TN breast cancer cells expressing high levels of the CD44 receptor (standard form; CD44S) have a propensity to migrate towards lung-CM *in vitro* [15] and metastasize to lung *in vivo* [17]. Previous protein array analysis of lung-CM also revealed the lung as an important source of soluble proteins known to interact with CD44, including E-, L- and P-selectins [15,18–21].

Selectins belong to a three-member family of type I cell-surface glycoproteins involved in mediating cell-cell adhesion and migration [22]. There is high sequence homology between E-, L- and P-selectin in all domains except the transmembrane and cytoplasmic domains. E-selectin is expressed by endothelial cells [22,23], L-selectin is expressed by leukocytes [24], and P-selectin is expressed by platelets and endothelial cells [25]. All three selectins have been shown to interact with CD44 in order to facilitate both normal leukocyte adhesion and rolling as well as cell-cell adhesion in pathological conditions such as cancer metastasis [21,26–29]. Selectins exist not only as membrane-bound proteins but also as cleaved soluble proteins. Cleavage is believed to be proximal to the membrane spanning domain [30–32], and *in vitro* studies suggest that soluble E-, L- and P-selectin remain functionally active after being shed from the cell surface [33–35].

Experimental mouse models deficient in one or more selectins have been developed [36–39]. Phenotypically, individual selectin deficiencies in either E-, L-, or P-selectin do not cause infertility or embryonic lethality. However, double (EP^{-/-} or PL^{-/-}) or triple (ELP^{-/-}) knockout mice experience health issues such as mucocutaneous infections due to alterations in leukocyte homeostasis and recruitment [40]. While these deficiencies may negatively affect immune cell recruitment, evidence suggests that they may also be advantageous to disruption of metastasis. Using an experimental model of breast cancer, it has been observed that lung metastasis is reduced in E^{-/-} and EP^{-/-} mice compared to wildtype [41]. Similarly, in a colon cancer model, P^{-/-}, L^{-/-} or PL^{-/-} mice demonstrate reduced lung metastasis compared to the wild-type control, with the double selectin knockouts having the lowest metastatic burden [42].

In the clinical setting, it has been reported that serum levels of E-selectin are significantly higher in patients with advanced breast cancer that have distant metastasis compared to earlier stage patients [43–45], and that high E-selectin serum concentrations are prognostic of a worse outcome [45,46]. While previous studies have not established a role for L-selectin in breast cancer, acute myeloid leukemia patients with elevated plasma L-selectin levels have poorer outcomes [47], and increased concentrations of L-selectin have also been observed in the serum of patients with metastatic versus nonmetastatic bladder cancer [48]. Soluble P-selectin has been detected in increased amounts in the sera of breast cancer and colon cancer relative to healthy controls [49,50].

While these studies provide experimental and clinical evidence supporting the importance of selectins in cancer progression, the functional role of soluble selectins in breast cancer metastatic behavior remains poorly understood. In the current study, we tested the hypothesis that lung-derived selectins promote TN breast cancer metastatic behavior and may serve as a potential therapeutic target. Using lung-conditioned media and a 3D *ex vivo* pulmonary metastasis assay (PuMA), we demonstrate that individual lung-derived selectins enhance *in vitro* migration but not proliferation of TN breast cancer cells. In addition, we demonstrate that *ex vivo* metastatic progression is reduced in the lungs of ELP^{-/-} mice compared to wildtype and that treatment with the pan-selectin inhibitor bimosi- amose reduces lung-specific TN breast cancer migration and proliferation *in vitro*. Taken together, these results suggest that lung-derived selectins may present a potential therapeutic target against TN breast cancer metastasis.

2. Materials and Methods

2.1. Cell culture and reagents

Triple negative human breast cancer cells (MDA-MB-231, SUM149, SUM159) were cultured and maintained at 37°C and 5% CO₂. MDA-MB-231 human breast cancer cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) media + 10% fetal bovine serum and passaged using 1 mM ethylene diamine tetraacetic acid (EDTA). SUM149 and SUM159 human breast cancer cells (Asterand Inc., Detroit, MI) were maintained in HAMS F-12 media supplemented with 5% FBS, 5 µg/mL insulin, 1 µg/mL hydrocortisone and 10 mM HEPES and passaged using 0.25% trypsin/EDTA in citrate saline. Additional cell lines were also engineered to express tdTomato (MDA-MB-231) or mCherry (SUM159) via lentiviral transduction. All media/supplements were from Invitrogen (Carlsbad, CA) and FBS was from Sigma-Aldrich (St Louis, MO). Cell lines were authenticated via third party testing (IDEXX BioAnalytics, Columbia, MO, USA). The pan-selectin inhibitor bimosiamose (TBC1269) [51] was obtained from MedKoo Biosciences (Morrisville, NC, USA).

2.2. Lung-conditioned media

Lung-conditioned media was generated as described previously [15]. Briefly, healthy 5-7 week old female athymic nude mice were purchased and maintained in accordance with the Canadian Council of Animal Care under a protocol approved by the University of Western Ontario Animal Care Committee (#2016-091). Mice were euthanized by CO₂ inhalation, lungs were harvested, washed 3 times in ice cold PBS, and minced into ~1 mm³ fragments. Lung fragments were weight-normalized by re-suspension in a 4:1 media to tissue (v/w) ratio in DMEM:F12 supplemented with Mito+ serum extender (1X; BD Biosciences, Mississauga, ON; basal media) + 50 U/mL penicillin/ 50 µg/mL streptomycin (Invitrogen) and incubated at 37°C, 5% CO₂ for 24 h. The resulting lung-conditioned media (CM) was further diluted 4-fold in basal media, separated from tissue fragments by centrifugation at 900 g for 15 min at 4°C and filtered through 0.22 µm filters (Corning Inc., Corning, NY). Resulting lung-CM was used immediately for experiments described below, or aliquoted and stored at -80°C for later use (Supplemental Figure S1).

2.3. Selectin enzyme linked immunosorbent assay (ELISA)

To assess the presence and concentration of selectins in lung-CM, ELISA kits for soluble mouse E-Selectin/CD62E, soluble mouse L-selectin/CD62L and soluble mouse P-selectin/CD62P (R&D Systems, Minneapolis, MN, USA) were used. Basal media, native lung-CM, and lung-CM depleted of E, L- or P-selectin (described below and Supplemental Figure S1B) were added to pre-coated microplates and ELISAs were carried out as per the manufacturer's instructions to measure the concentration of E-, L- and P-selectin in lung-CM and efficiency of immunodepletion. A four-parameter logistic (4-PL) standard curve was generated for each experiment using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA) and used to determine the exact protein concentration in each sample.

2.4. Selectin immunodepletion

E-, L- and P-selectin were individually immunodepleted from lung-CM using Dynabeads® Protein G (Life Technologies) and antibodies against specific soluble selectins as detailed in Supplemental Table S1. Dynabeads were suspended in 200 µL of wash buffer (PBS + 0.02% Tween-20) to equilibrate and then placed on the magnetic rack for 2 min for separation from solution. Wash buffer was carefully removed, replaced with wash buffer + appropriate antibody, mixed by pipetting and allowed to nutate for 20 min at RT. Following nutation, tubes were placed on the magnetic rack for 2 min to separate antibody-bound beads from solution. Supernatant was carefully removed and antibody-bound beads were washed 3x before incubation with lung-CM on the nutator for 30 min at RT.

Post-nutration, samples were placed on the magnetic rack for 2 min to separate the selectin/antibody/bead complexes from the now depleted lung-CM. A small aliquot of depleted lung-CM was used for quantification of protein levels by ELISA and the balance was stored at -80°C until used as outlined in Supplemental Figure S1.

2.5. Co-immunoprecipitation and immunoblotting

Confluent 100 mm dishes of MDA-MB-231 human breast cancer cells were washed three times with PBS and lysed with 500 µL of lysis buffer (1% NP-40, 25mM Tris-HCl pH 7.5, 100 mM NaCl, 5% Glycerol, 1x Halt™ Protease Inhibitor Cocktail [Thermo Scientific]). Cell lysates were collected and incubated on ice for 1 h prior to centrifugation for 10 min at 13,000 g and 4°C. During this period, 20 µL (0.6 µg) of Dynabeads were equilibrated in 200 µL of wash buffer (25mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol) and placed on a magnetic rack for 2 min to separate from solution. Wash buffer was carefully removed and replaced with 500 µL of MDA-MB-231 cell lysate supernatant. The remaining pellet from the cell lysates was discarded. Two µg each of recombinant mouse E-, L-, or P-selectin Fc chimera protein (R&D Systems) was added to each tube containing cell lysate/beads or beads only (control) and incubated on a rotator overnight at 4°C. Samples were then placed on a magnetic rack to separate beads/bound proteins from solution. The supernatant was carefully removed, and remaining beads were resuspended in wash buffer, washed and magnetically separated. The wash and separation steps were repeated twice and beads were suspended in 30 µL of Laemmli buffer and boiled for 5 min. Samples were placed on the magnetic rack for 2 min and the supernatant was collected. Thirty µL of each immunoprecipitated sample was subjected to separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA). Membranes were blocked using 5% skim milk in Tris-buffered saline + 0.1% Tween-20 (TBS-T; Sigma). Primary and secondary antibody concentrations are listed in Supplemental Table S2. Protein expression was visualized using Clarity Max™ Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA).

2.6. In vitro cell migration assays

Breast cancer cell migration was assessed using transwell migration assays. Fluoroblok™ transwell inserts (24-well, 8 µm pore size; Corning Inc.) were coated with 100 µL of gelatin (6 µg/well; Bioshop). Human TN breast cancer cells (MDA-MB-231, SUM149, and/or SUM159) were plated on top of transwells at 5x10⁴ cells/well. For lung-derived selectin depletion studies, the lower compartment of the transwell chambers contained either basal media (negative control), native lung-CM, or lung-CM depleted of E-, L- or P-selectin. For rescue conditions, an amount of recombinant E-, L-, or P-selectin protein (R&D Systems) equal to the calculated average protein concentration of each immunodepleted selectin was added back to depleted lung-CM (E-selectin [0.25 ng/mL], L-selectin [7.05 ng/mL]) and P-selectin [10.39 ng/mL]. For selectin inhibitor studies, the lower compartment contained basal media, basal media + 10% FBS, or native lung-CM containing 1 mM of bimosiamose or dimethyl sulfoxide (DMSO; vehicle control). Plates were incubated for 18 h at 37°C and 5% CO₂ to allow migration. After 18 h, transwell chambers were fixed with 1% glutaraldehyde in PBS for 20 min, washed, and non-migrated cells were removed from the inner portion of the membrane using a cotton swab. Membranes were carefully cut out, placed on microscope slides and mounted using ProLong® Gold Antifade Mountant with DAPI (Life Technologies). Five high powered fields of view (HP-FOV) were captured for each membrane, and mean number of migrated cells/FOV was calculated using ImageJ software (NIH, Bethesda, MD, USA).

2.7. *In vitro* cell proliferation assays

Breast cancer cell proliferation was assessed using bromodeoxyuridine (BrdU) incorporation assays. Human TN breast cancer cells (MDA-MB-231, SUM149, and/or SUM159; 1×10^4 cells/well) were plated on 8-well Lab-tek™ chamber slides (Thermo Scientific), incubated for 24 h to allow cell adhesion, washed 1x with PBS and serum starved for 72 h. For lung-derived selectin depletion studies, media was changed to either basal media, positive control media (basal media + 10% FBS), native lung-CM, or lung-CM depleted of E-, L- or P-selectin. For selectin inhibitor studies, media was changed to either basal media, basal media + 10% FBS, or native lung-CM containing 1 mM of bimosiamose or DMSO. After 24 h, cells were exposed to BrdU (5 μ g/mL; Amersham Cell Proliferation Labelling Reagent; GE Healthcare, Piscataway, NJ, USA) for 30 min to allow incorporation into newly-synthesized DNA. Cells were washed with PBS, fixed with 10% neutral-buffered formalin (Fisher Scientific), permeabilized using 0.1% Triton X-100 (Sigma), and treated with 2N HCl to denature DNA. To minimize non-specific binding, slides were blocked for 30 min in 5% BSA + 0.1% Triton X-100 in PBS. For detection, anti-BrdU primary antibody (BD Biosciences) diluted 1:75 in 5% BSA/0.1% Triton X-100 in PBS was added and slides were incubated overnight at 4°C. A FITC-conjugated anti-mouse IgG secondary antibody (1:100 dilution; Vector Laboratories, Burlington, ON, Canada) was added for 1 h at RT. Slides were washed and mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen) and allowed to cure overnight in the dark at RT prior to analysis of five HP-FOV. Percentage of BrdU-positive cells to total nuclei were calculated using Image J.

2.8. *Ex vivo* pulmonary metastasis assay (PuMA)

An *ex vivo* pulmonary metastasis assay (PuMA) was used for real-time assessment of metastatic progression in intact lung tissue as described previously [52–54] and in Supplemental Figure S2. Wildtype or triple-selectin knockout (ELP^{-/-}) mice (B6;129S-Sele^{tm1Hyn}Sell^{tm1Hyn}Selp^{tm1Hyn}/J; JAX stock #003807; The Jackson Laboratory, Bar Harbour, ME, USA) [39] were purchased and bred/maintained in accordance with the Canadian Council of Animal Care under a protocol approved by the University of Western Ontario Animal Care Committee (#2016-091). Red fluorescent human breast cancer cells (MDA-MB-231-TdTomato or SUM159-mCherry) were harvested, suspended Hank's Balanced Salt Solution (HBSS; Invitrogen) and delivered by tail vein injection to 5-7 week old female wildtype or ELP^{-/-} mice (n=4/group; 1×10^5 cells/mouse). Within 15 min of cell injection, mice were euthanized by CO₂ inhalation. Using sterile surgical conditions, the trachea was snipped and cannulated with an 18G blunt needle. Lungs were infused under gravitational pressure with 1.2 mL of equal amounts of Lung Media 1 (Supplemental Table S3) + low melting agarose solution (0.6%, 40°C). Trachea, lungs, and heart were carefully removed en bloc and immediately placed in ice-cold PBS containing 100 U/ml penicillin and 100 μ g/ml streptomycin and incubated at 4°C for 20 min to solidify. Transverse lung sections (1–2 mm thick; 12–20 sections/lung) were generated from each lobe using a scalpel blade and sections were placed on a sterile piece of Gelfoam (~1x1 cm) that had been pre-incubated for 1-2 hours in a 6-well plate with Lung Media 2 (Supplemental Table S3). Lung sections were grown in culture for 21 days at 37°C in 5% CO₂. Media was replaced every other day and lung tissue sections turned over carefully with tweezers. At 0, 7, 14 or 21 days post-injection, seeded lung sections were removed from culture and fixed overnight in 10% buffered formalin phosphate (Fisher Scientific) + 25% sucrose (w/v) to preserve fluorescence. Sections were rinsed 3x with PBS, placed on a glass slide, and gently cover-slipped. Images were acquired using an upright Nikon A1R confocal microscope at 20X objective (Nikon), with a 591 nm emission laser (Melles Griot, Carlsbad, CA, USA). Three separate lung sections were imaged per timepoint with five FOV taken per lung section. Metastatic progression of breast cancer cell populations within the lung was determined by measuring the mean fluorescent area per FOV for each section of lung (μ m²) using

ImageJ. Data were normalized to day 0 to control for variability in cellular delivery during tail vein injection.

2.9. Data analysis

A minimum of three biological replicates were performed for each experiment, with at least three technical replicates within each experiment for transwell migration and BrdU incorporation assays. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA, USA), and data are presented as mean $\pm$ standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was used to compare means between experimental groups, followed by a Tukey's post-hoc test. In all cases, values of $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. E-, L-, and P-selectin are present in lung-CM and can interact with CD44 expressed by triple-negative breast cancer cells

Our previous studies have demonstrated that conditioned media generated from the lungs of healthy mice can be used as a model of the soluble lung microenvironment and its role in influencing breast cancer metastatic behavior [15]. Using protein array analysis, we identified more than 70 lung-derived proteins in lung-CM that are not present in basal media [15]. In particular, E-, L- and P selectin were identified as proteins for further investigation based on their previous association with cancer metastasis [18–21]. Using ELISA, we observed that E-selectin (SELE; Figure 1a), L-selectin (SELL; Figure 1b) and P-selectin (SELP; Figure 1c) are all present in lung-CM at significantly higher concentrations than in basal media ($p < 0.05$). Individual selectins could be successfully immunodepleted, resulting in lung-CM that had significantly reduced levels of E, L, or P-selectin compared to non-depleted lung-CM ($p < 0.05$). In the absence of a specific antibody, Dynabeads® Protein G alone had no significant effect on immunodepletion of selectins (Figure 1a–c).

Soluble selectins have been demonstrated to interact with CD44 in order to exert their functional effects on target cells [21,26–29]. We have previously observed that TN breast cancer cells (including the MDA-MB-231, SUM149 and SUM159 models used in this study) express high levels of the standard form of CD44 (CD44S) on their cell surface [15], suggesting a potential mechanism by which lung-derived selectins might influence breast cancer cell behavior. To confirm whether murine selectins could interact with CD44S expressed by human breast cancer cells *in vitro*, soluble forms of mouse E-, L-, and P-selectin IgG1 chimeric proteins were used for immunoprecipitation experiments. A single band was observed in each lane containing recombinant selectin protein that immunoprecipitated with the Dynabead® Protein G (Figure 1d), indicating that the recombinant chimeric selectins could interact directly with the Dynabead® Protein G without additional antibodies. In co-immunoprecipitation experiments, we observed that bands corresponding to proteins of approximately 90 kDa in weight reacted with the CD44S antibody and were observed in each of E-, L- and P-selectin immunoprecipitates (Figure 1e). The protein size detected was consistent with the band observed from the MDA-MB-231 cell lysate input control. This indicates that murine E-, L-, and P-selectin can interact with CD44S expressed by human TN breast cancer cells.

3.2. Lung-derived selectins enhance triple negative breast cancer cell migration

To determine the functional influence of individual lung-derived selectins on TN breast cancer migration, a transwell migration assay was used. MDA-MB-231 (Figure 2a–c) or SUM149 (Figure 2d–f) human TN breast cancer cells were exposed to basal media (negative control); native lung-CM; or lung-CM depleted of E-, L- or P-selectin. MDA-MB-231 and SUM149 human breast cancer cells demonstrated significantly increased migration towards native lung-CM relative to basal control media ($p < 0.05$). This increased

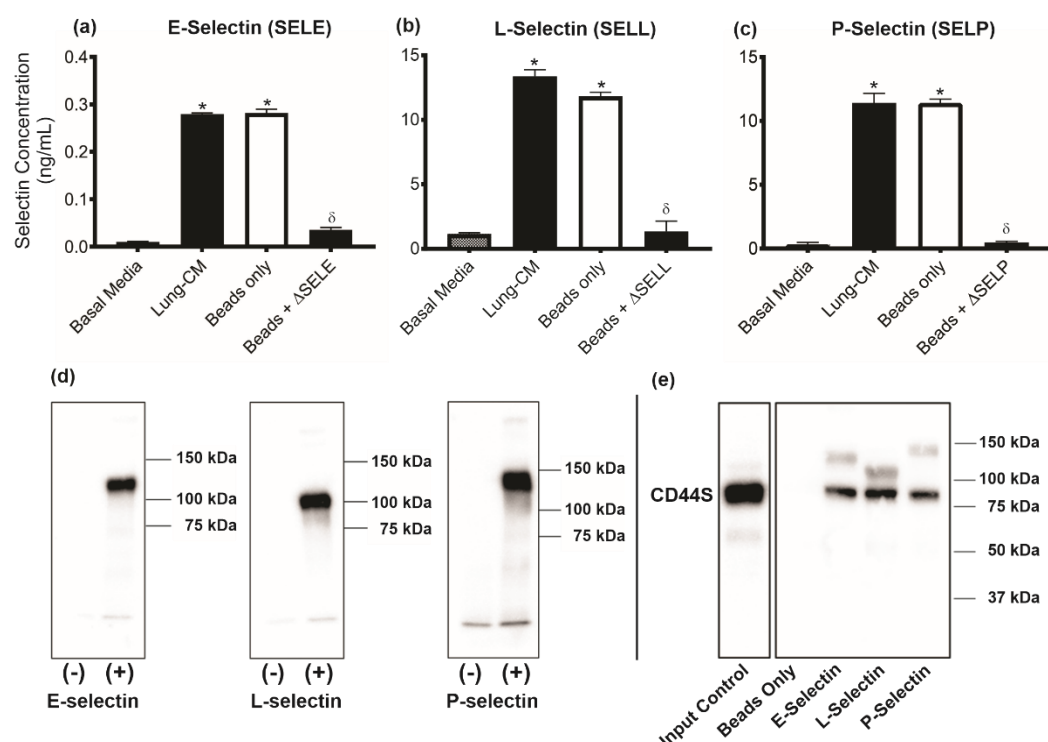


Figure 1. E-, L-, and P-selectin are present in lung-conditioned media (CM) and can interact with CD44 expressed by triple-negative breast cancer cells. Lung-CM generated from the lungs of healthy female nude mice was assessed for the presence and concentration of (a) E-selectin (SELE), (b) L-selectin (SELL), and (c) P-selectin (SELP) protein by ELISA of lung-CM. Immunodepletion (denoted by Δ) was carried out using selectin-specific antibodies coupled to Dynabeads® Protein G. Basal media and lung-CM exposed to beads only were used as controls. Data is presented as mean $\pm$ SEM (n=3). * = significantly different than basal media. δ = significantly different than respective non-depleted media. (d) Dynabead® Protein G were incubated alone or with murine E-, L- or P-selectin IgG1 chimeric protein (R&D Systems). Bound proteins were isolated and analyzed by immunoblotting. (e) MDA-MB-231 cell lysates were incubated with Dynabeads® Protein G alone or with murine E-, L- or P-selectin-human IgG1 chimeric protein. Immunoprecipitates were isolated and immunoblotted. Membranes were visualized by chemiluminescence; representative immunoblots are shown (n=3).

migration was abrogated in the presence of lung-CM depleted of either E-selectin (Figure 2a,d), L-selectin (Figure 2b,e) or P-selectin (Figure 2c,f) ($p < 0.05$). Selectin-dependent migration could be rescued by addition of individual recombinant soluble mouse selectins at a level that restored respective selectin protein levels to those detected in the native lung-CM (Figure 2a-f). Taken together, these results indicate that lung-derived E-, L-, and P-selectin enhance migration of TN breast cancer cells.

3.3. Lung-derived E-,L- or P-selectins do not individually influence breast cancer cell proliferation

To determine whether individual lung-derived selectins also have a functional role in cell proliferation, BrdU incorporation assays were used. MDA-MB-231 (Figure 3a-c) and SUM149 (Figure 3d-f) TN human breast cancer cells were exposed to basal media; native lung-CM; or lung-CM depleted of E-, L-, or P-selectin and BrdU incorporation was assessed. Consistent with our previous studies [15], we observed an increase in TN breast cancer cell proliferation in response to native lung-CM versus basal media control ($p < 0.05$) (Figure 2a-f). However, the proportion of BrdU-positive cells was unaffected by individual depletion of E-selectin (Figure 3a,d), L-selectin (Figure 3b,e) or P-selectin (Figure 3c,f) from lung-CM, suggesting that lung-derived E-,L- or P-selectins do not individually influence breast cancer cell proliferation (Figure 2a-f).

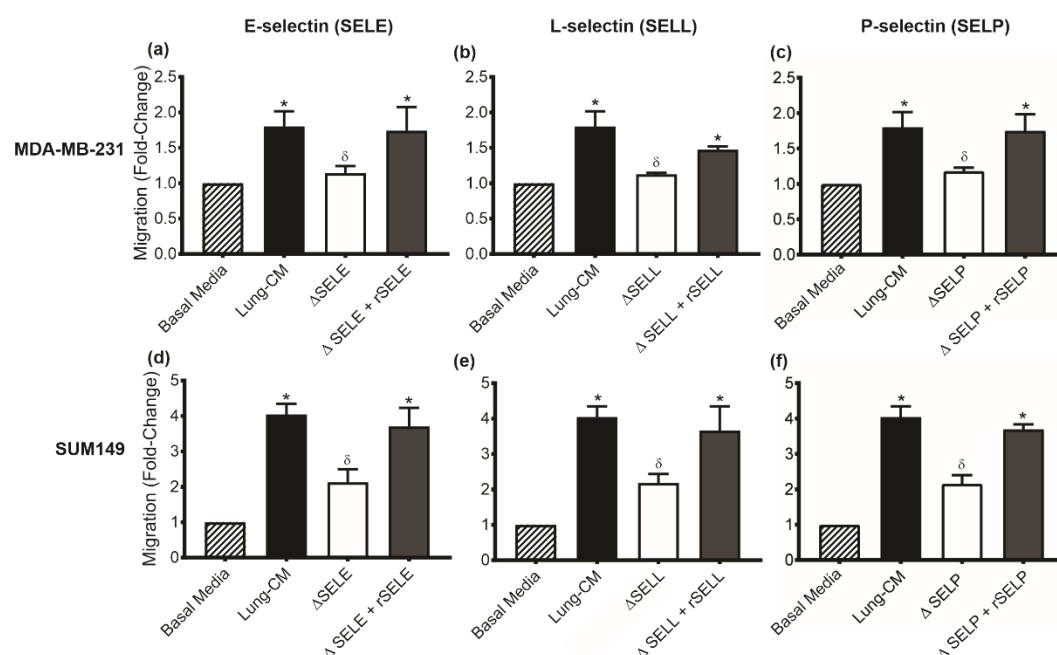


Figure 2. Lung-derived selectins enhance triple negative breast cancer cell migration. (a–c) MDA-MB-231 and (d–f) SUM149 human breast cancer cells (5×10^4 per well) were plated on top of gelatin-coated Corning® Fluroblok™ transwells (8- μ m pore size) before exposure to one of the following conditions: basal media; native lung-conditioned media (CM); lung-CM depleted of E-selectin, L-selectin or P-selectin (denoted by Δ SELE, Δ SELL, Δ SELP, respectively); or depleted lung-CM + recombinant (r) SELE, SELL, or SELP (added at the concentration needed to restore protein levels to that observed in native lung-CM). Cells were allowed to migrate for 18 h at 37°C and 5% CO₂. Transwells were fixed, stained with DAPI, and five high-powered fields of view (HP-FOV) were captured per transwell. Data is presented as mean $\pm$ SEM (standard error of the mean); fold-change in migration relative to basal media conditions (n=3). * = significantly different from basal media. δ = significantly different from non-depleted lung-CM.

3.4. Loss of E-, L- and P-selectins in the lung reduces triple negative breast cancer progression in the ex vivo pulmonary metastasis assay (PuMA)

We next wanted to assess the importance of lung-derived selectins in a more physiologically relevant model of breast cancer metastatic progression using the ex vivo pulmonary metastasis assay (PuMA; Supplemental Figure S2). Because our initial in vitro experiments demonstrated that depletion of each individual selectin effectively reduced breast cancer migration in a similar manner, we were interested in determining a concomitant deficiency in all three selectins would lead to reduced metastatic progression in the PuMA in the context of triple-selectin (ELP^{-/-}) knockout mice [39]. We have previously observed that SUM149 cells are only weakly metastatic both in vivo and in the ex vivo PuMA (unpublished data), so for these experiments we utilized the more metastatic SUM159 cell line instead of SUM149. MDA-MB-231 and SUM159 TN breast cancer cells were seeded into the lungs of wildtype or ELP^{-/-} mice. We observed that ex vivo breast cancer metastatic progression is reduced in the lungs of ELP^{-/-} mice relative to wildtype control after 21 days ($p < 0.05$) (Figure 4), suggesting that selectins are important for lung metastasis of TN breast cancer and may serve as a potential therapeutic target.

3.5. The pan-selectin inhibitor bimosiamose reduces in vitro lung-specific TN breast cancer migration and proliferation

Finally, to begin exploring this therapeutic potential, we used bimosiamose (TBC-1269), a small-molecule non-oligosaccharide inhibitor of E-, L-, and P-selectin binding [55].

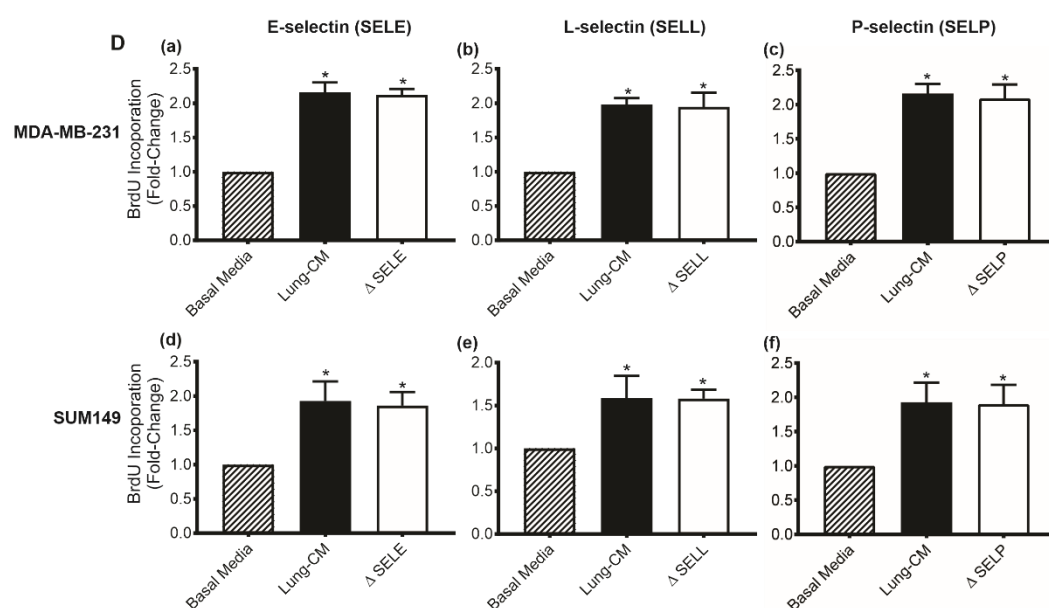


Figure 3. Lung-derived E-,L- or P-selectins do not individually influence breast cancer cell proliferation (a–c) MDA-MB-231 and (d–f) SUM149 human breast cancer cells were plated at a density of 1×10^4 cells/well on 8-well chamber slides and serum starved for 72 h. Cells were then exposed to basal media; lung-CM; and lung-CM depleted of E-selectin, P-selectin or L-selectin (denoted by Δ) for an additional 24 h before assessing proliferation based on BrdU incorporation. Five HP-FOV per well were captured and used to enumerate the percentage of BrdU-positive cells using ImageJ. Data is presented as mean $\pm$ SEM; fold-change in BrdU relative to basal media ($n=3$). * = significantly different from basal media.

MDA-MB-231 (Figure 5a,b) and SUM159 (Figure 5c,d) TN human breast cancer cells were exposed to basal media; regular growth media (10% FBS; positive control) or native lung-CM, and transwell migration and BrdU incorporation was assessed in response to DMSO (dimethyl sulfoxide; vehicle control) or 1 mM bimosiamose. Consistent with our observations in the *ex vivo* PuMA model (Figure 4), we observed that pan-selectin inhibition results in reduced *in vitro* TNBC metastatic behavior including a decrease in both migration (Figure 5a,c) and proliferation (Figure 5b,d) ($p < 0.05$) down to the level of the basal media control. Interestingly, this inhibition only occurred in the presence of lung-CM but not normal growth media, suggesting that the potential therapeutic effect of bimosiamose in reducing TNBC metastatic behavior may be lung-specific.

4. Discussion

Despite significant advances in early detection and treatment, breast cancer remains the most commonly diagnosed cancer and a leading cause of cancer-related deaths among women worldwide [1]. The majority of these deaths are due to metastasis, which remains incurable. In particular, patients with aggressive triple-negative (TN) disease demonstrate an increased propensity for lung metastasis leading to poor outcomes [5–10]. Paget's "seed and soil" hypothesis of metastasis was originally proposed in 1889 and postulates that the formation of metastatic tumors at distant sites such as the lung is related not only to the intrinsic properties of the cancer cell (the "seed") but also to factors found in the organ microenvironment (the "soil") that allow the tumor cell to survive and grow [56]. Although the presence and expression of selectins in the lung "soil" has been well-established [57–59], their specific role in TN breast cancer metastatic behavior is poorly understood. In the current study, we demonstrate that individual lung-derived selectins are necessary (but not sufficient) to promote *in vitro* migration, but do not individually have an effect on proliferation of TN breast cancer cells. In addition, *ex vivo* metastatic progression is

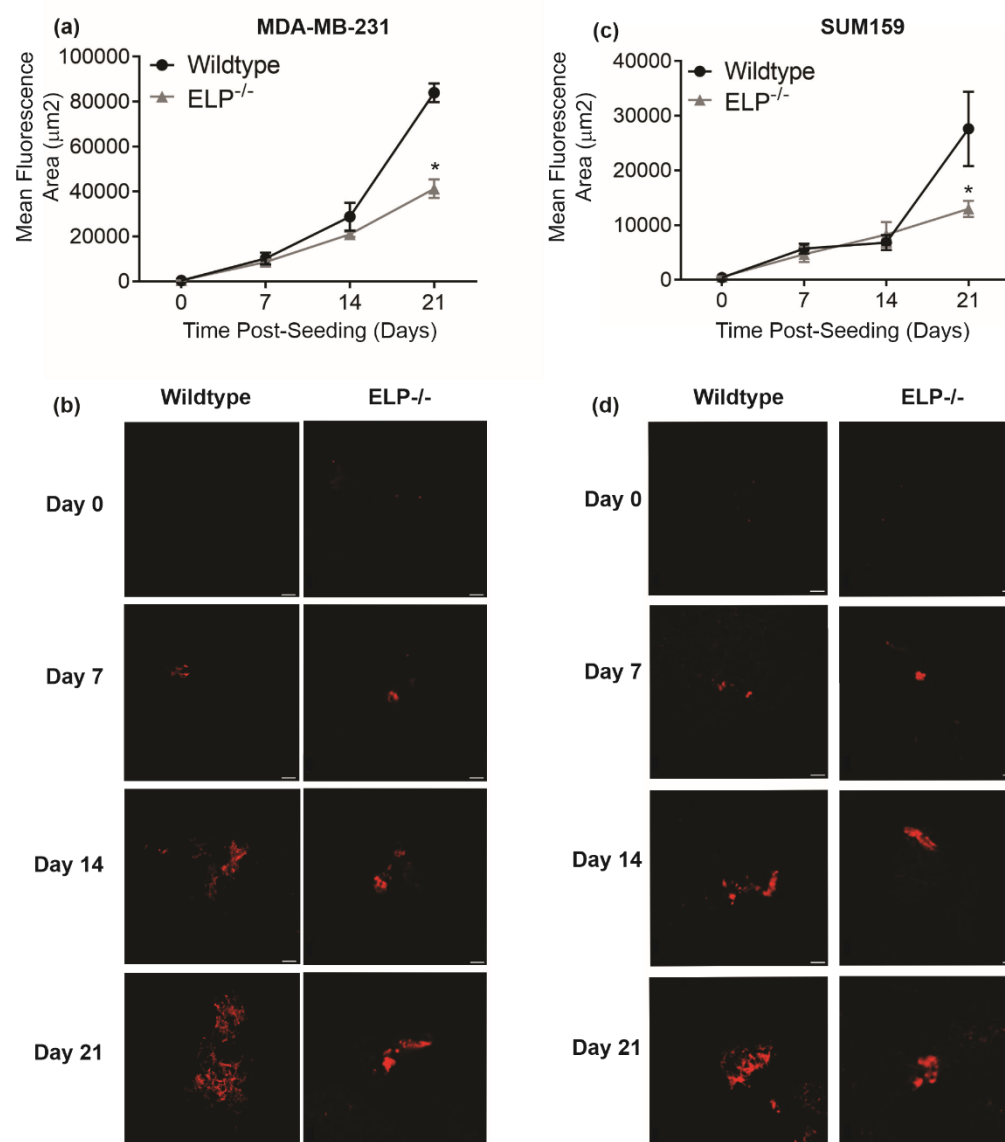


Figure 4. Loss of E-, L- and P-selectins in the lung reduces triple negative breast cancer progression in the *ex vivo* pulmonary metastasis assay (PuMA). **(a,b)** MDA-MB-231-TdTomato or **(c-d)** SUM159-mCherry human TN breast cancer cells were injected into female wildtype or ELP^{-/-} mice (n=4/group/timepoint; 1×10⁵ cells/mouse). Lungs were harvested after 15 min and subjected to the PuMA for 21 days. **(a,c)** Data are presented as mean ± SEM; fluorescent area (μm²) normalized to time 0. * = significantly different than wildtype. **(b,d)** Representative images for metastatic progression in the **(b)** MDA-MB-231 and **(d)** SUM159 models in wildtype and ELP^{-/-} mice are shown. Scale bars = 100 μm.

reduced in the lungs of ELP^{-/-} mice compared to wildtype, and treatment with a pan-selectin inhibitor reduces lung-specific TN breast cancer migration and proliferation *in vitro*. To the best of our knowledge, this study is the first in the literature to examine the interaction with and the response of breast cancer cells to soluble selectins in the context of the lung microenvironment. Our novel findings suggest that lung-derived selectins may present a potential therapeutic target against TN breast cancer metastasis.

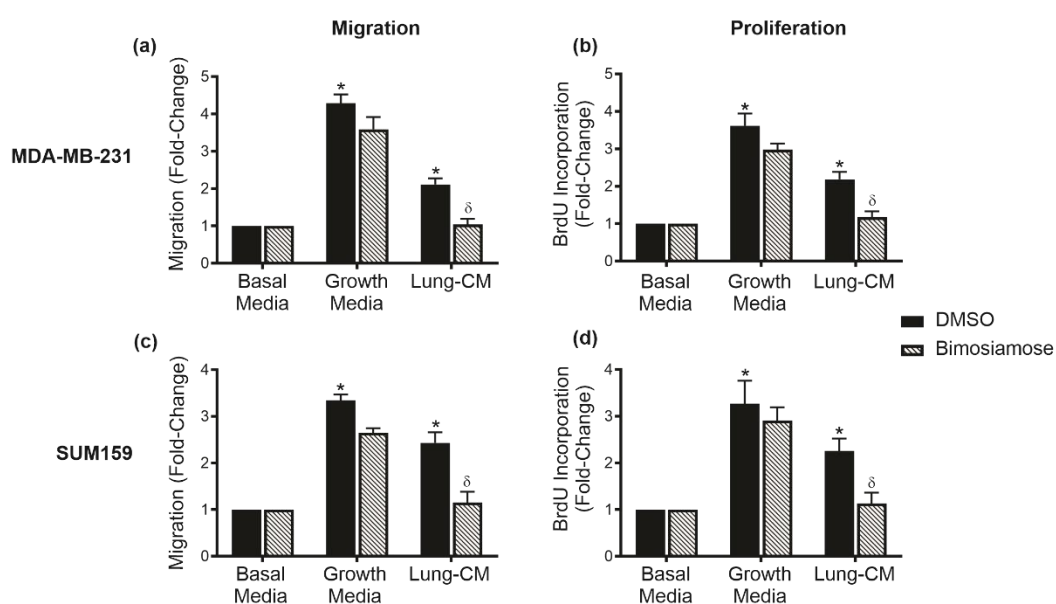


Figure 5. The pan-selectin inhibitor bimosiamose reduces *in vitro* lung-specific TN breast cancer migration and proliferation. **(a,b)** MDA-MB-231 and **(c,d)** SUM159 human breast cancer cells were treated with DMSO (vehicle control; black bars) or 1 mM bimosiamose (hatched bars) and subjected to transwell migration **(a,c)** or proliferation **(b,d)** assays in the presence of basal media, growth media (10% FBS) or lung-CM. Five HP-FOV were captured per transwell and analyzed using ImageJ. Data is presented as mean $\pm$ SEM; fold-change in relative to basal media conditions (n=3). * = significantly different from basal media. δ = significantly different from respective DMSO control.

We observed that lung-derived E-, L-, or P-selectins play a functional role in migration of TN breast cancer cells but do not influence proliferation. A previous study conducted by Kumar and colleagues was among the first to demonstrate that soluble E-selectin has a chemotactic effect on immune cells [34]. This was followed by a more recent study in which soluble E-selectin was shown to promote the migration of MDA-MB-231 human breast cancer cells potentially through interactions with CD44 [60], which supports our findings. With respect to proliferation, there is no direct evidence implicating E-selectin in the proliferation of cancer cells *in vitro*. However, soluble E-selectin has previously been shown to mediate angiogenesis *in vitro* and reduce lung metastasis *in vivo* [41]. It is difficult to extrapolate whether changes in the number of metastatic foci observed were related to migration and/or proliferation, as no comparison in the size of metastatic foci was made between wildtype and E-selectin deficient mice. Similarly, in experimental models of melanoma [61] and colon cancer [59], P-selectin deficiency results in reduced lung metastasis. At the cellular level, evidence suggests a role for P-selectin in cancer cell proliferation, with subcutaneously implanted colon cancer cells having significantly lower primary tumour growth in P-selectin deficient mice versus wildtype controls [59]. In comparison to E- and P-selectin, relatively little is known about L-selectin and its functional influence in breast cancer. Although further studies are required to determine whether each lung-derived selectin functions differently/synergistically in influencing the metastatic behavior of TN breast cancer cells, our studies with triple selectin knockout mice and the pan-selectin inhibitor bimosiamose support the possibility that E-, L- and P-selectin may be redundant/compensatory for each other in the context of breast cancer lung metastasis.

The results of this study and previous investigations by our group and others suggests that lung-derived selectins may be exerting their metastatic influence via interactions between selectins and the CD44 receptor on breast cancer cells [15,26,27,62,63]. The

interaction of E-selectin with CD44 expressed by LS174T colon carcinoma cells was originally hypothesized based on CD44 immunoreactivity with the HECA-452 antibody, which detects sialofucosylated oligosaccharides (a group that E-selectin binds to) [20]. For P- and L-selectin, studies demonstrated an interaction with CD44 by blot rolling assays [18]. However, detection of a direct interaction by co-immunoprecipitation of selectins with CD44 expressed by breast cancer cells has only been reported for E-selectin [64]. We therefore investigated this further using recombinant mouse soluble selectin-human IgG chimera proteins, which are capable of directly interacting with Dynabead® Protein G. This allowed us to avoid potential issues of immunoprecipitating CD44 or selectins using antibodies that target the ectodomains through which they may interact, since the IgG region that interacts with the Dynabead® Protein G is located near the C-terminus of the chimera, far from the ectodomains. We tested this using soluble mouse selectins, as these were the forms that we hypothesized were found in our lung-CM model. The interaction of CD44 with mouse selectins that we observe is likely facilitated by the high degree of homology (~72%) shared between human and mouse selectins [22]. It should be noted that depleting the cell lysate of CD44 would have provide additional support for the selectin/CD44 interaction (i.e. disappearance of the 90 kDa band). Finally, CD44 is also expressed by immune cells such as T lymphocytes and monocytes, so there is the potential that lung-derived selectins may also play a role in immune infiltration during lung metastasis [65,66].

The observed inhibition of breast cancer metastatic behavior in triple selectin knock-out mice or following pan-selectin chemical inhibition in our *ex vivo* models of the lung microenvironment provides promising preclinical data supporting the therapeutic value of targeting selectins for the treatment of breast cancer lung metastasis. Future follow-up *in vivo* studies could test the use of an inhalable system for delivering bimosiamose directly to the lung to treat or prevent metastasis. Drugs (ranging from chemotherapeutics to antibiotics) are frequently adapted as inhalable nanoparticles to facilitate the inhalable delivery of these drugs directly into the lung [66,67]. Inhalable delivery of drugs has many advantages including high bioavailability, rapid onset of action, and direct delivery to the lungs [68,69]. This approach has been used for a variety of respiratory diseases including cystic fibrosis, asthma, and tuberculosis in both preclinical mouse models and patients [70–73]. Additionally, a variety of inhalable drug delivery methods have been adapted to cancer treatment, particularly lung cancers (summarized in [72]). Notably, bimosiamose has previously been adapted to an inhalable delivery method for the treatment of asthma patients [74].

In summary, breast cancer lung metastasis is a major contributor to disease-related mortality, particularly in patients with TN disease. The novel findings presented here support the idea that the lung microenvironment plays an important role in TN breast cancer metastasis, specifically via lung-derived E-, L-, and P-selectin. In accordance with the “seed and soil” theory, targeting the lung microenvironment and interfering with factors such as selectins could be a potential therapeutic strategy. Future studies are therefore aimed at elucidating the pro-metastatic mechanisms of lung-derived selectins and developing a lung-directed therapeutic approach for TN breast cancer via an inhalable drug delivery model. We believe that targeting the constant composition of the lung may be more effective than trying to target the highly heterogeneous population of cells that make up TN breast cancer both within and between patients. In the future, this strategy could contribute to improved treatment of breast cancer in the metastatic setting and help reduce the burden of this disease.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Selectin immunodepletion conditions, Table S2: Antibody conditions for co-immunoprecipitation and immunoblotting, Table S3: Media formulations for lung tissue used in the PuMA, Figure S1: Lung-conditioned media (CM) generation and selectin immunodepletion schematic, Figure S2: Pulmonary metastasis assay (PuMA) schematic.

Author Contributions: Conceptualization, A.L.A.; methodology, S.U.K, D.G., Y.X., G.S.; formal analysis, S.U.K, D.G., Y.X., G.S., A.L.A.; investigation, S.U.K, D.G., Y.X., G.S.; resources, A.L.A.; data curation, A.L.A.; writing—original draft preparation, S.U.K., A.L.A.; writing—review and editing, S.U.K, D.G., Y.X., G.S., A.L.A.; supervision, A.L.A.; project administration, A.L.A.; funding acquisition, A.L.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs, through the Breast Cancer Research Program, under Award No. W81XWH-17-1-0470. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office. In addition, A.L.A. is supported by the Breast Cancer Society of Canada.

Institutional Review Board Statement: Experiments involving animals were carried out in accordance with the Canadian Council of Animal Care under an animal ethics protocol approved by the University of Western Ontario Animal Care Committee (#2016-091). No human studies were conducted.

Acknowledgments: We thank Jenny Chu and Matt Piaseczny for their optimization and validation of the lung-conditioned media model and PuMA procedure (respectively) in our laboratory as described in [15,54].

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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