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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 is testing the ***hypothesis*** that CD44-interacting proteins produced in the lung promote breast cancer metastasis and can be targeted directly using inhalable drug delivery. To date, the major findings for the project are that the CD44-interactiing 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 were found to be necessary for metastatic colonization of the lung, particularly for more aggressive breast cancer cell lines. The pan-selectin antagonist bimosiamose has been chosen to move forward into drug formulation and pre-clinical testing as an inhalable treatment for lung metastasis of breast cancer. Drug formulation and optimization studies are progressing well in preparation for the *in vivo* pre-clinical therapeutic studies which will be carried out in the final year of the award.

15. SUBJECT TERMS
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

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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 have previously developed inhalable drug-loaded nanoparticles for cancer, and in the current project we aim to apply this innovation in combination with novel biological targets to address the problem of breast cancer metastasis to lung. We have 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 have 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 is 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 involves 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 are using 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 are being used in combination with human TN breast cancer cell lines and primary cells from patient-derived xenografts. Lung-derived proteins are being depleted from lung-CM using functional antibodies or by using lungs from specific knockout mice. Specific Aim 2 involves assessing the therapeutic potential of directly targeting CD44-interacting proteins in the lung. Antibodies or chemical inhibitors targeting OPN, FGF2, and/or selectins are being loaded into polybutyl cyanoacrylate nanoparticles and incorporated into inhalable effervescent carrier particles. Pre-clinical *in vivo* metastasis models will be combined with inhalable drug delivery to target the lung directly rather than systemically. Mice will be subjected to single or combination treatments with inhibitors targeting the most promising lung-derived proteins from Specific Aim 1. Both preventative (before metastasis occurs) and therapeutic (after metastases are established) regimens will be tested for their ability to reduce metastatic burden.

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 or (if not completed) the percentage of completion to date. **Major Goals** in **blue font** represent those for which the target dates fell between the start of the grant and the end of the current reporting period.

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: *In vitro* assessment of distinct metastatic behaviors & mechanisms	• HRPO/ACURO determination/approval • Characterization of the *in vitro* functional and mechanistic influence of lung-derived CD44-interacting proteins on breast cancer metastatic behavior	August 2018	100% completed
Major Task 2: 3D *ex vivo* 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	75% 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 *in vivo* studies	February 2019	80% completed
Major Task 4: *In vivo* assessment of anti-metastatic efficacy of inhalable drug using a preventative approach	• *In vivo* testing of inhalable inhibitor in the preventative setting.	December 2019	0% completed
Major Task 5: *In vivo* assessment of anti-metastatic efficacy of inhalable drug using a preventative approach	• *In vivo* testing of inhalable inhibitor in the therapeutic setting. • Identification of lead candidate for future translation.	August 2020	0% completed
Major Task 6: Integrated data sharing plan between sites		Ongoing	Ongoing

➢ What was accomplished under these goals?

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

The proposed work for Major Task is complete 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 has been 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 has been completed as proposed. Lung-CM has either been used for Task 1.4 (described below), or has been banked at -80°C for use in future project-related research activities.

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

This task has been essentially completed as proposed, 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).

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

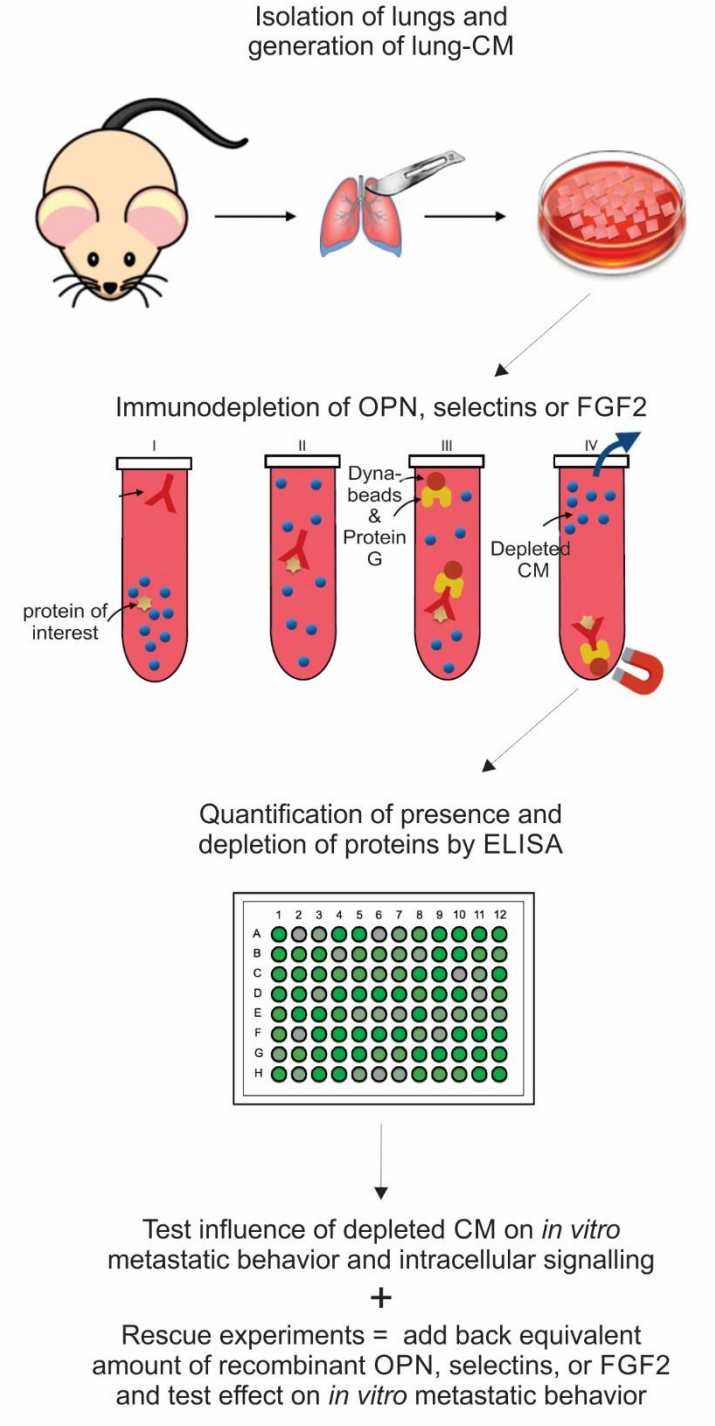


Figure 1: *Methodology overview for Major Task 1.*

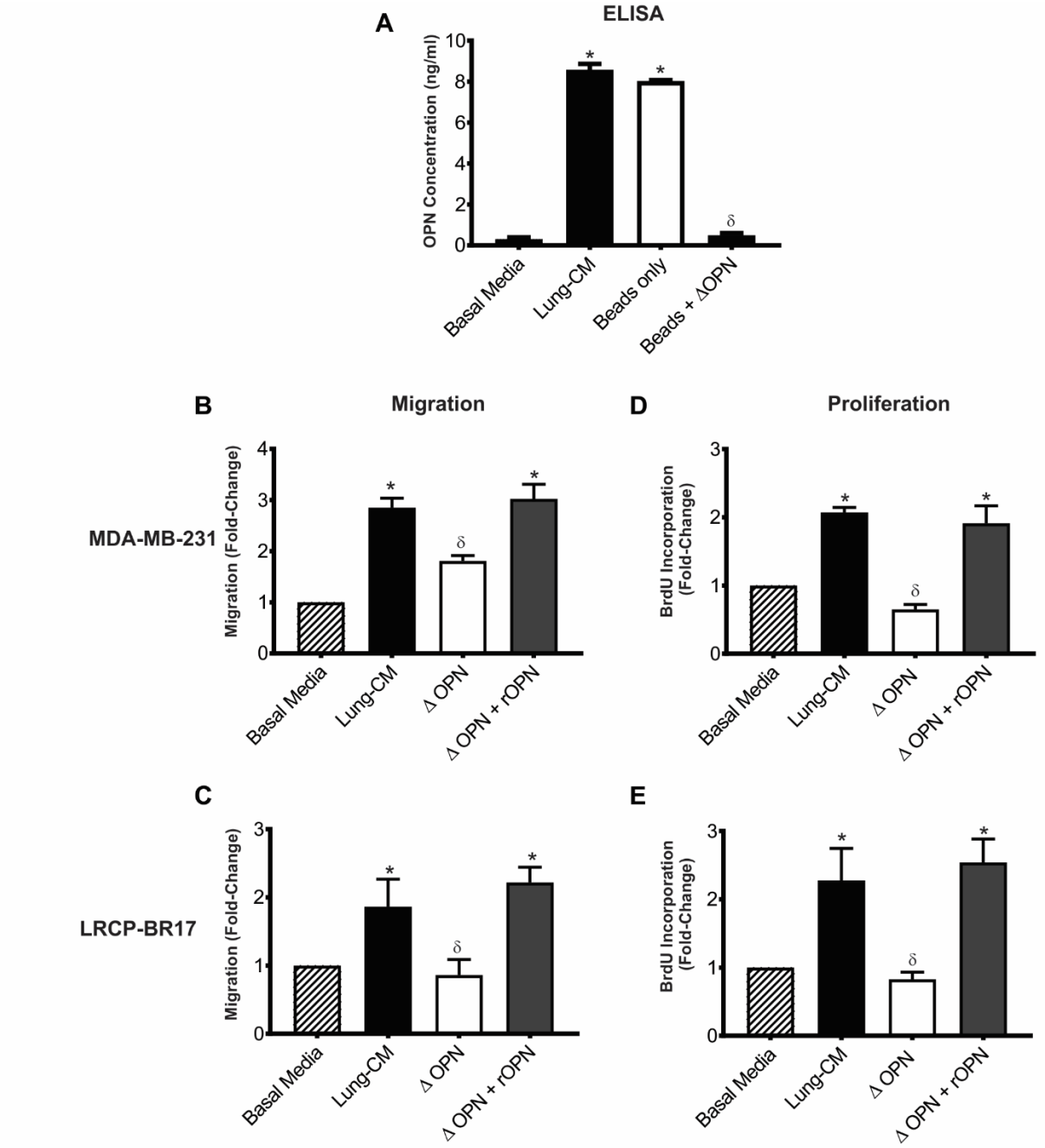


Figure 2: *Depletion of osteopontin (OPN) from lung conditioned media (CM) reduces breast cancer cell migration and proliferation*. **(A)** Lung-CM was generated from healthy female mouse lungs and OPN immunodepleted. Resulting OPN protein levels were determined by ELISA. Data are presented as mean [OPN]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

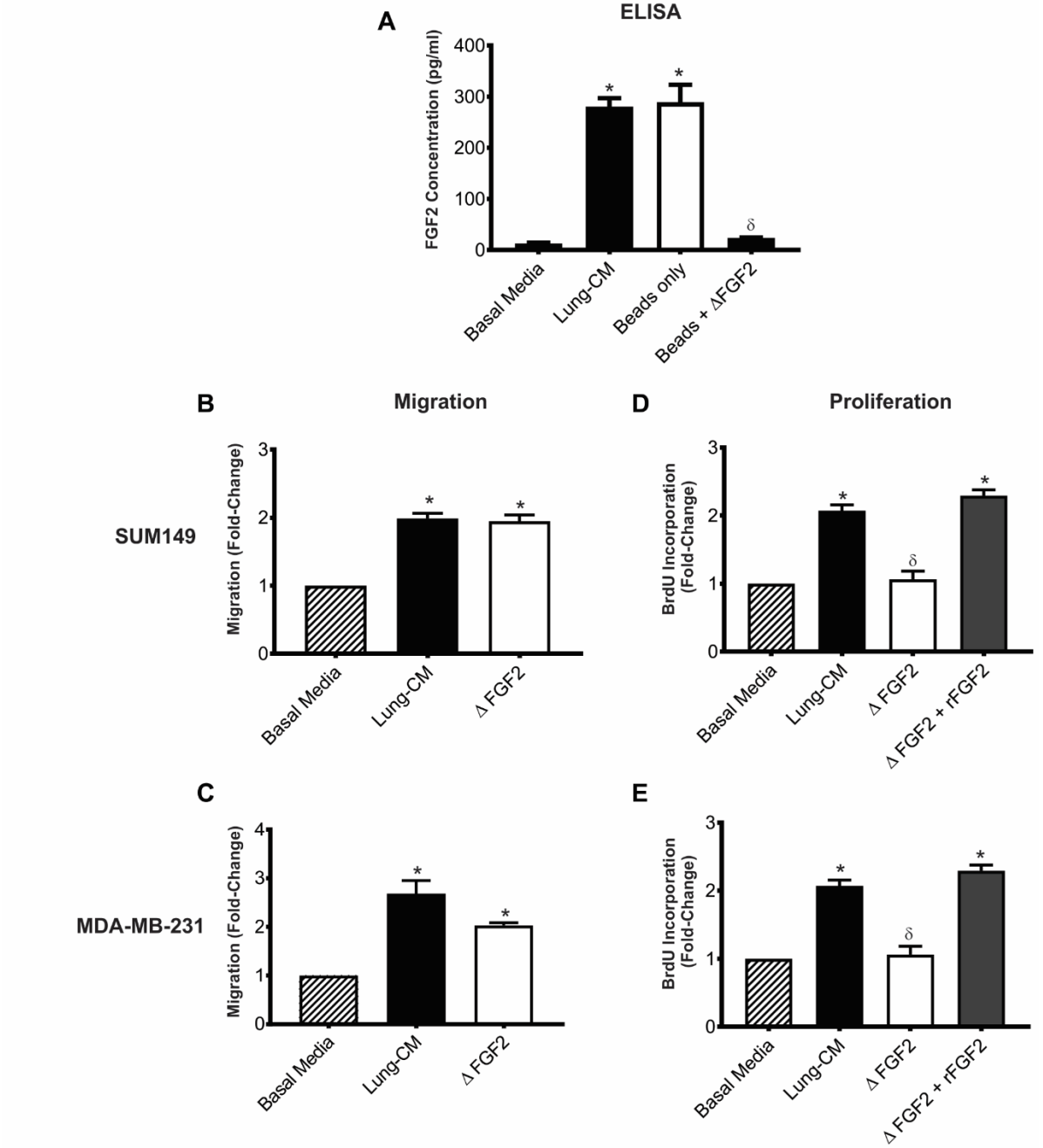


Figure 3: *Depletion of basic fibroblast growth factor (FGF2) from lung conditioned media (CM) reduces breast cancer cell proliferation but not migration*. **(A)** Lung-CM was generated from healthy female mouse lungs and FGF2 immunodepleted. Resulting FGF2 protein levels were determined by ELISA. Data are presented as mean [FGF2] ± 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 (ΔFGF2). **(D,E)** SUM149 and MDA-MB-231 cell proliferation following exposure to basal media, native lung-CM, lung-CM depleted of FGF2 (ΔFGF2), or ΔFGF2 rescued by re-addition of recombinant FGF2. Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media ± SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

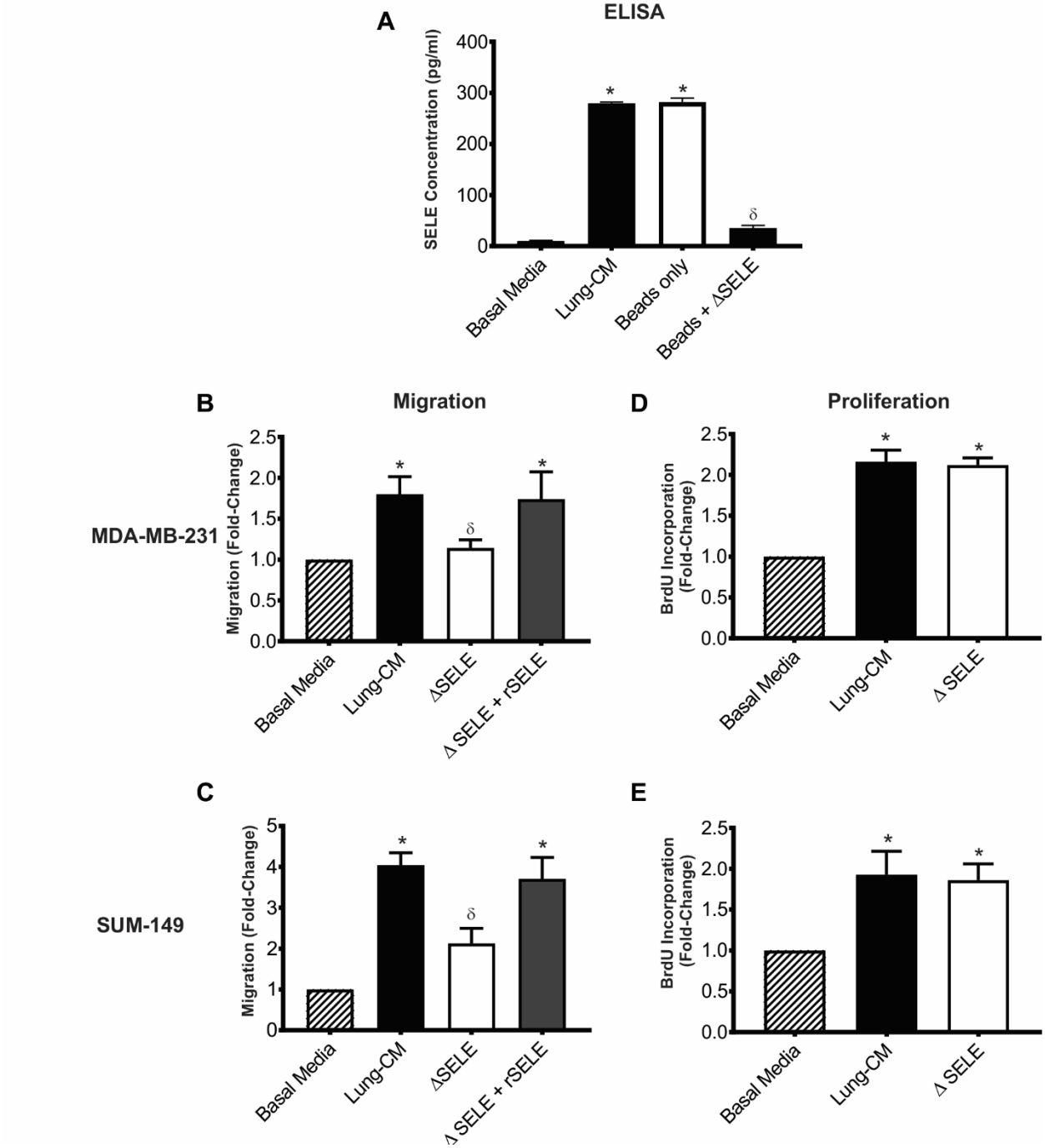


Figure 4: *Depletion of E-selectin (SELE) from lung conditioned media (CM) reduces breast cancer cell migration but not proliferation*. **(A)** Lung-CM was generated from healthy female mouse lungs and SELE immunodepleted. Resulting SELE protein levels were determined by ELISA. Data are presented as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

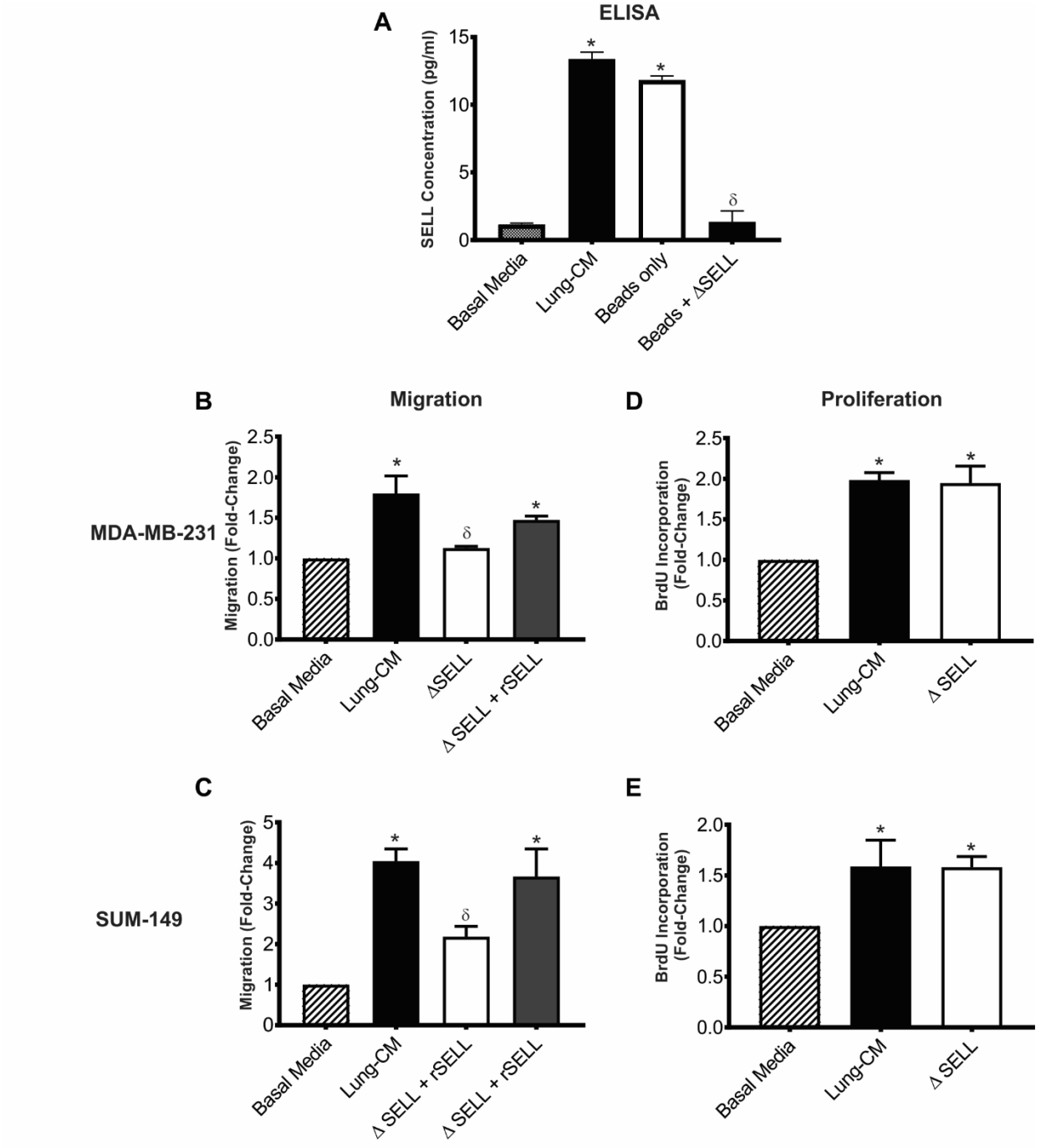


Figure 5: *Depletion of L-selectin (SELL) from lung conditioned media (CM) reduces breast cancer cell migration but not proliferation*. **(A)** Lung-CM was generated from healthy female mouse lungs and SELL immunodepleted. Resulting SELL protein levels were determined by ELISA. Data are presented as mean [SELL] ± 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 (ΔSELL), or ΔSELL 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 (ΔSELL). Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media ± SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

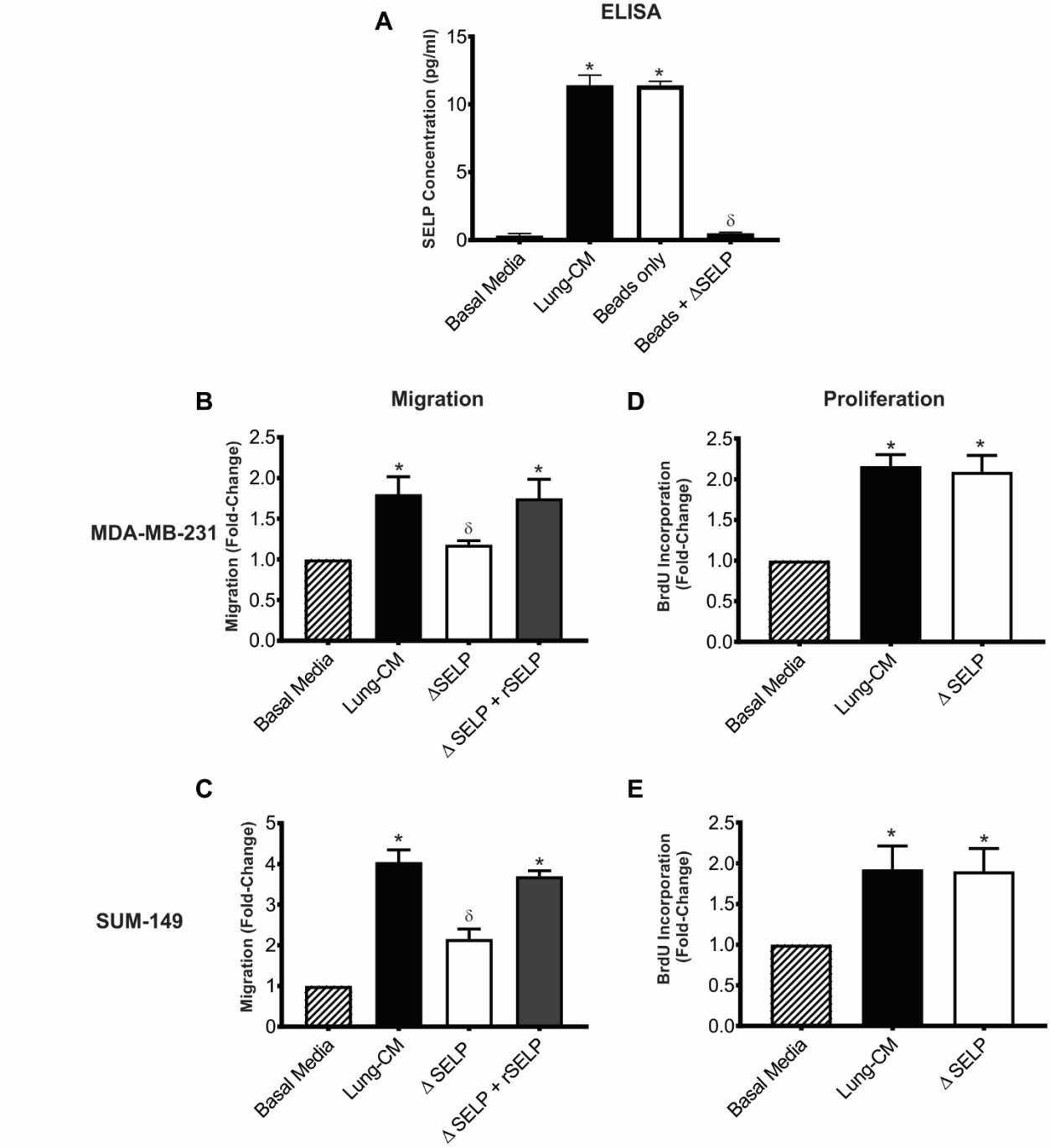


Figure 6: *Depletion of P-selectin (SELP) from lung conditioned media (CM) reduces breast cancer cell migration but not proliferation*. **(A)** Lung-CM was generated from healthy female mouse lungs and SELP immunodepleted. Resulting SELP protein levels were determined by ELISA. Data are presented as mean [SELP] ± 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 (ΔSELP), or ΔSELP 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 (ΔSELP). Data are presented as mean fold-change in migration or BrdU incorporation relative to basal media ± SEM (n = 3). * = significantly different than basal media; δ = significantly different than native lung-CM ($p \leq 0.05$).

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 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 approximately 75% complete and is described below. This work also supports ***Specific Aim 1.***

TASK 2.1: *Establish breeding colonies of knockout mice*

This task is approximately 80% complete. OPN-/- and triple-selectin-/- breeding colonies have been successfully established and used for experiments (Task 2.3, described below). The final colony (FGF2-/-) is in the process of being established in order to have sufficient animals to proceed with the planned experiments. 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.

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 approximately 70% complete. Breast cancer cells have been subjected to the *ex vivo* PuMA using wildtype versus OPN-/- or triple-selectin-/- mice. The full set of these experiments has just 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 (***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 knockout mice (***Figure 8D***). Taken together, these results suggest that OPN and selectins are individually important, but not essential, for lung metastasis of breast cancer. Detailed histological and image analysis of these samples is currently ongoing, as is the assessment of metastatic progression in the final group of FGF2 knockout mice.

TASK 2.4: *Prepare and publish manuscript(s) from Specific Aim 1.*

This task is in progress, with anticipated submission of our first primary data manuscript in November 2019.

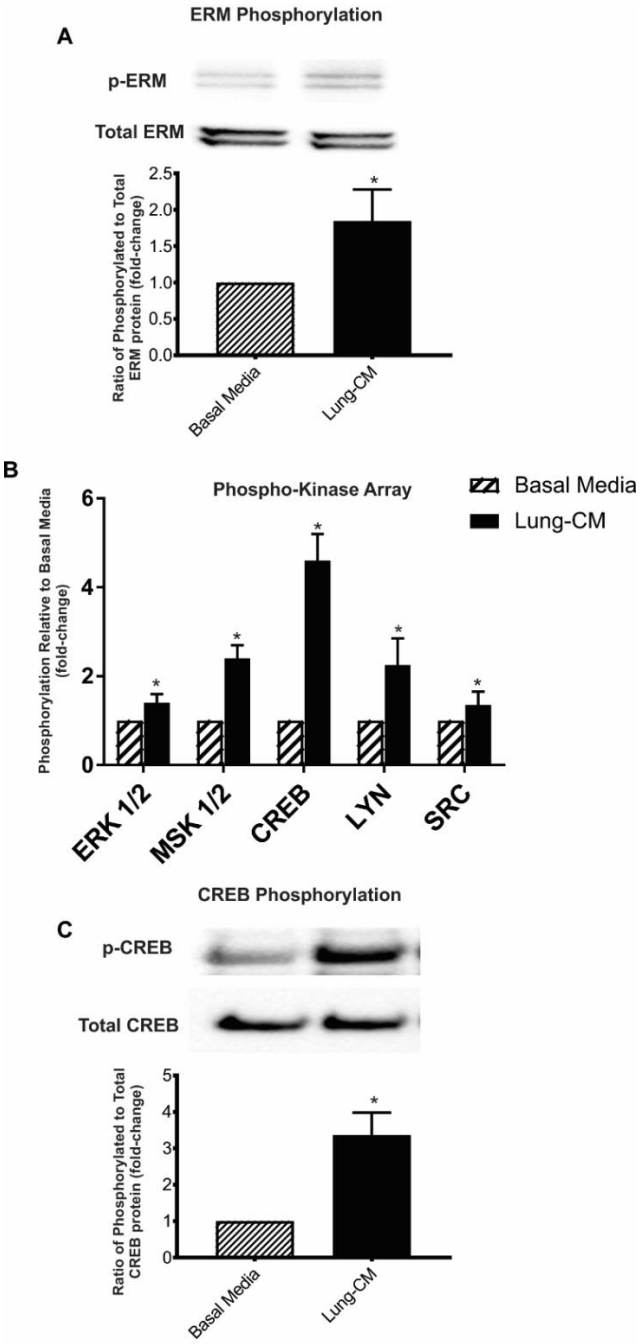


Figure 7: *Effect of lung-conditioned media (CM) on protein phosphorylation in human breast cancer cells*. MDA-MB-231 cells were cultured to 90% confluence and serum-starved for 24 h. Cells were then exposed for 15 min to basal media or native lung-CM and subjected to analysis by immunoblot or kinase 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 imunoblotting; **(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*).

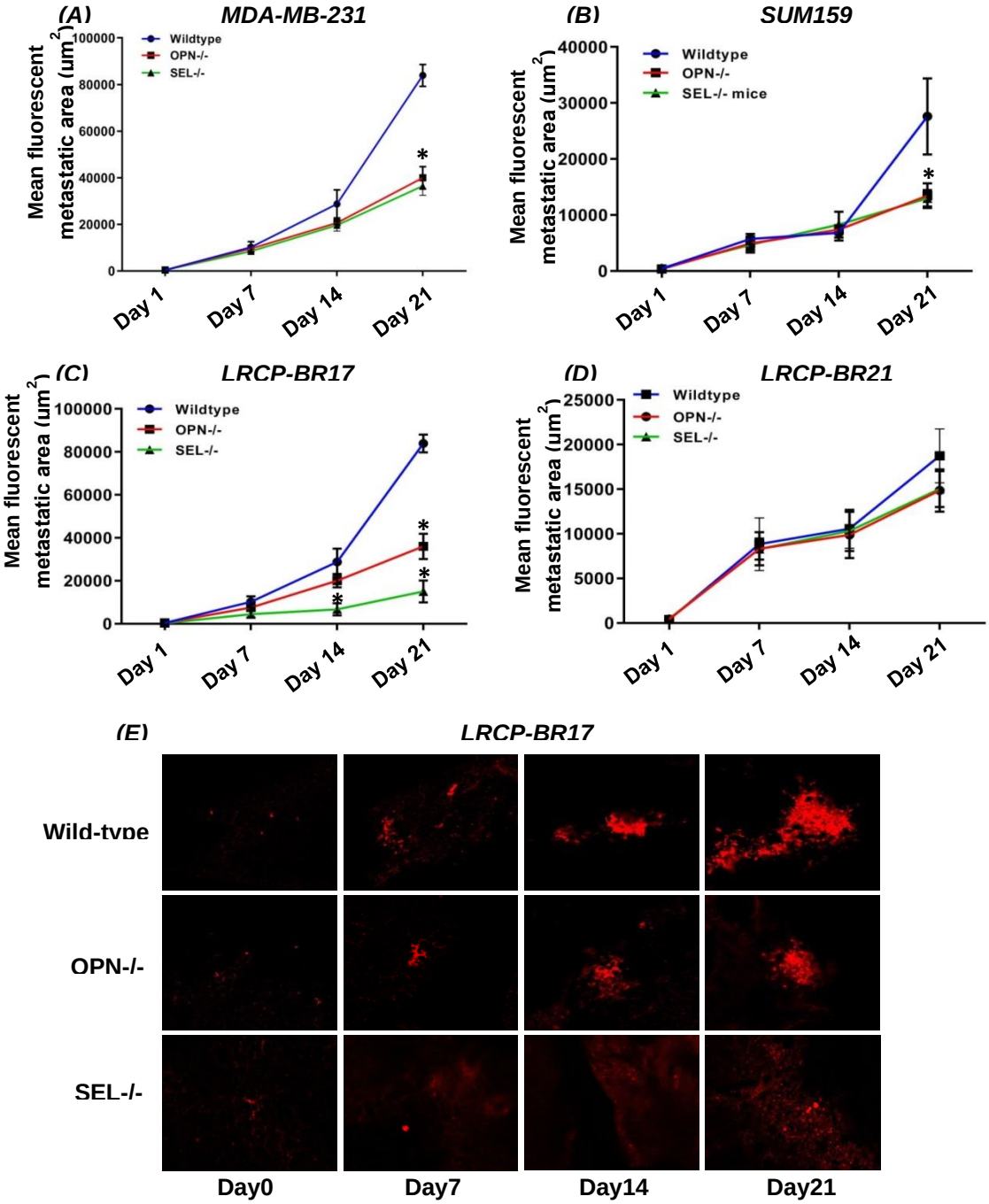


Figure 8: *Loss of OPN or E/L/P selectins in the lung reduces breast cancer metastatic progression in the pulmonary metastasis assay (PuMA)*. To assess the essentiality of OPN or selectins for metastatic progression in the *ex vivo* PuMA assay, red-fluorescent labeled MDA-MB-231 ***(A)***, SUM159 ***(B)***, LRCP-BR17 ***(C)*** and LRCP-BR21 ***(D)*** breast cancer cells were injected into wildtype (*blue datasets*), OPN-/- (*red datasets*) or triple-selectin-/- (*green 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 (μm^2) ± SEM. * = significantly different than wildtype ($p \leq 0.05$). ***(E)*** Representative images for metastatic progression in the LRCP-BR17 model for each timepoint are shown.

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

Major Task 3: Formulation and production of an inhalable inhibitor

The work to date on this Task has been 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.

Methods and Results

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.

1.1. Poly-butylcyanoacrylate nanoparticle

The poly-butylcyanoacrylate (PBCA) nanoparticles were prepared using n-butylcyanoacrylate (Loctite, Ireland) as monomer. The nanoparticles were prepared according to the following procedure: 100 µL 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 0.1µm 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 and1:100 dilutions were used. Good results were obtained for this method as well, which are shown in ***Figure 9***.

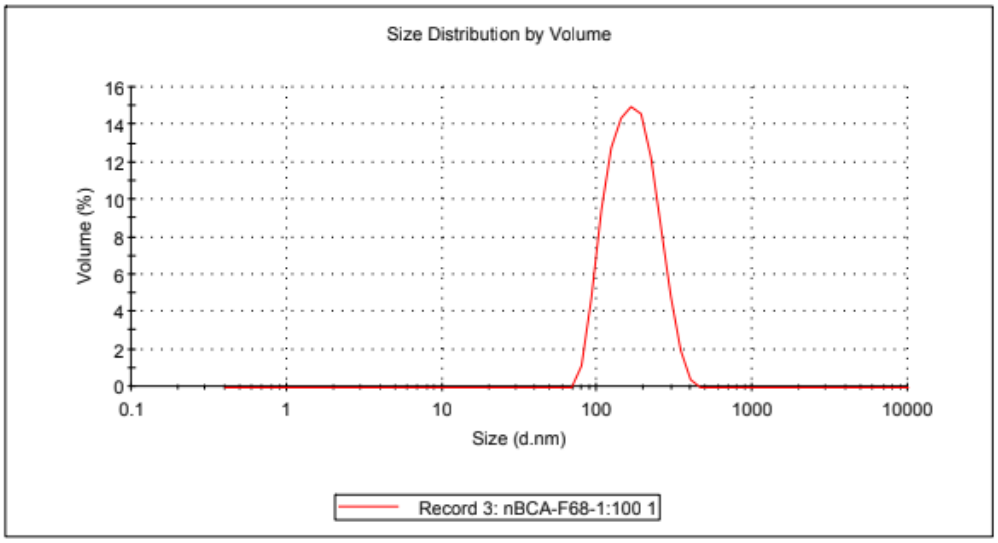
1.2. Gelatin nanoparticles

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 (40ºC) 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 dropwise for the second step of the desolvation process under constant stirring at 550 rpm and heating at 40ºC. 100 ul of 8% glutaraldehyde

A

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



B

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

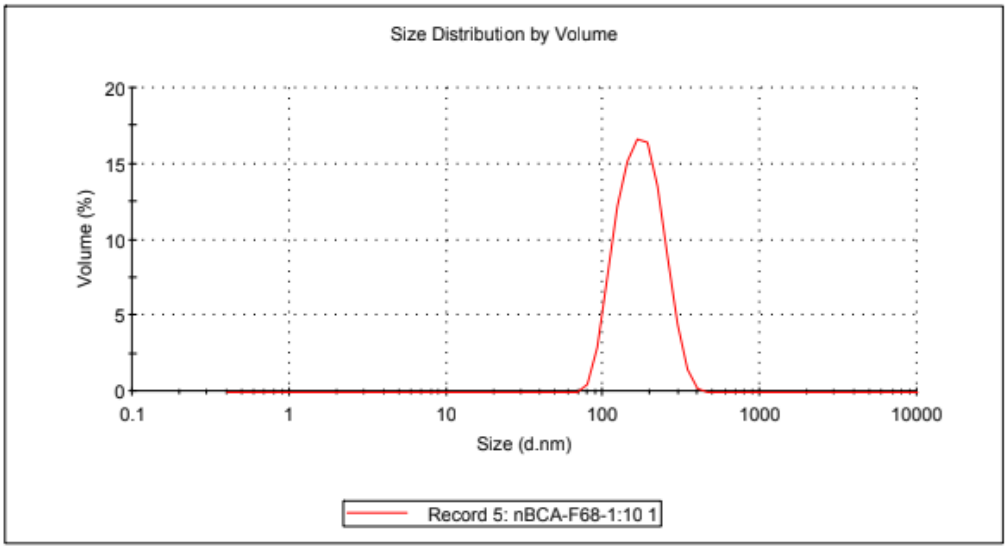
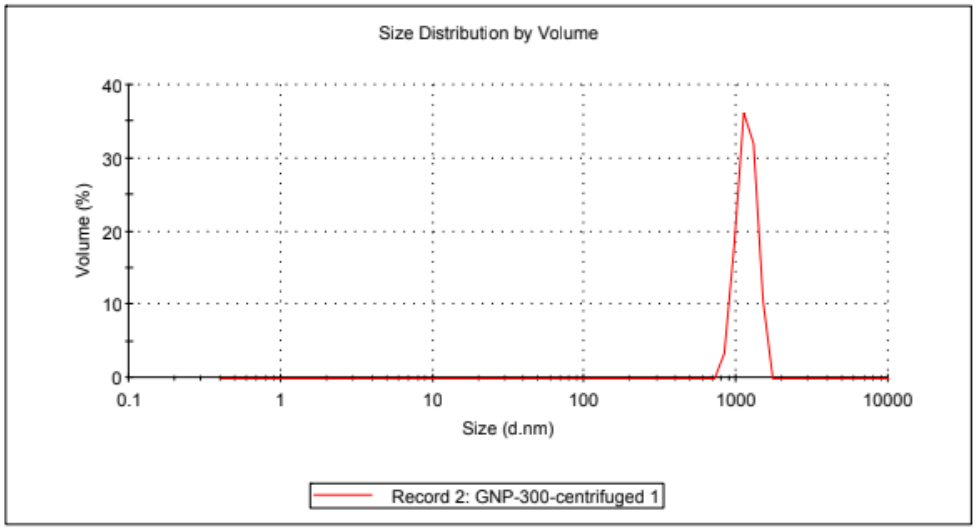


Figure 9. *Particle size (diameter, nm) and PdI of PBCA nanoparticle prepared with dextran 70 and Pluronic F68*. **(A)** Dilution factor of 100. **(B)** Dilution factor of 10.

A

			Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm):	2313	Peak 1:	1165	100.0	167.5
PdI:	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
PdI:	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				

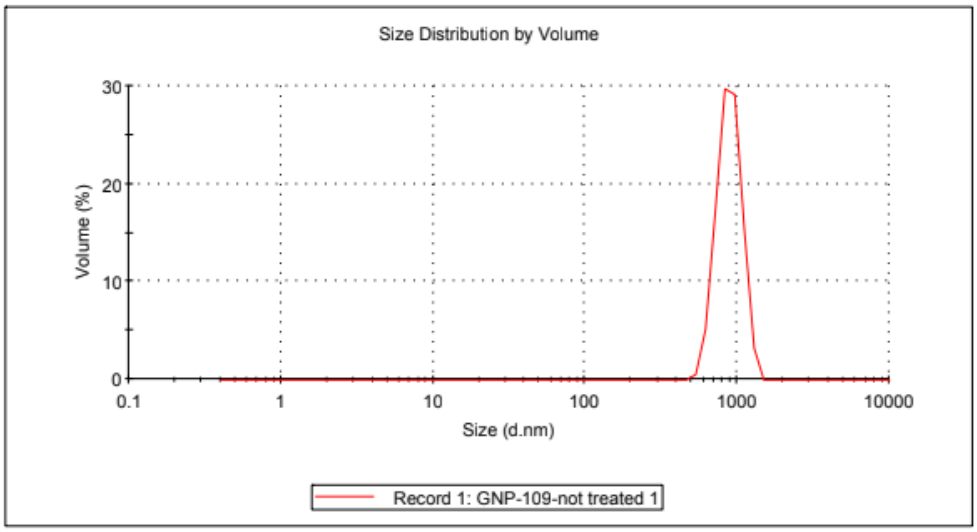


Figure 10. *Particle size (diameter, nm) and PdI of gelatin nanoparticle using different batches from Gelita manufacturer*.

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, ultrafitration 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 50ºC. 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 0.22um 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 (±10.56) and 0.159 (±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 100ug/ml of the antibody in different settings: in a beaker shaking at room temperature or at 4ºC or in an Eppendorf rotating at room temperature or at 4ºC (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 ug/mL) in coating buffer (0.01M Carbonate/bicarbonate, pH 9.4-9.6) and 100 uL 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 ul 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 ul - PBS containing 1% BSA) was added to each well

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

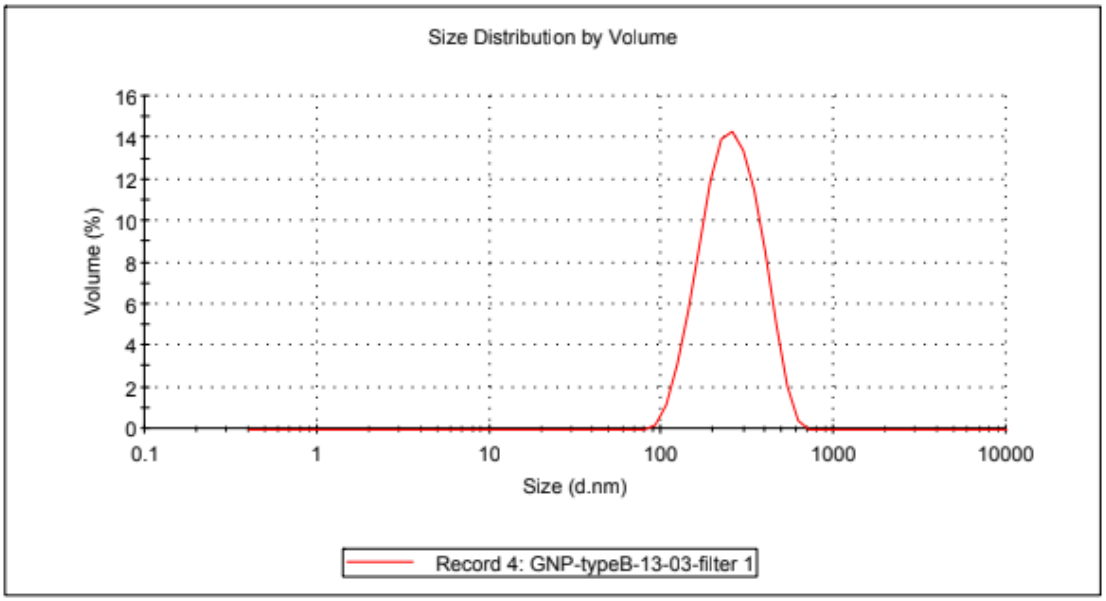


Figure 11. Particle size (diameter, nm) and PdI of gelatin nanoparticle using gelatin type B from bovine skin.

and the plate was 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.

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 concentrations	10ug/ml	**25ug/ml**	50ug/ml	**100ug/ml**	
	10ug/ml	**25ug/ml**	50ug/ml	**100ug/ml**	
	RTs C		**RTs A**	**100**	Ag+1AB+2AB+S
	Ice C		**Ice A**	**100**	
	RTr C		RTr A	x	
	Ice'C		x	x	

SPECIFIC INHIBITORS FOR INHALATION

Following this optimization and the results obtained to date for Task 2, we have 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 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.

Methods and Results

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.

1. *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.

2. *Particle size distribution*

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

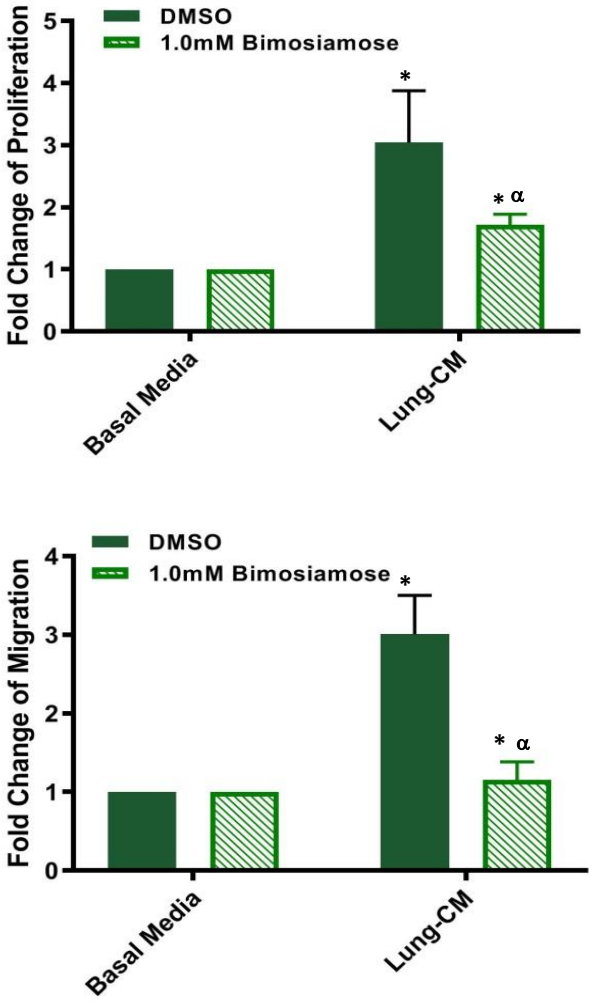
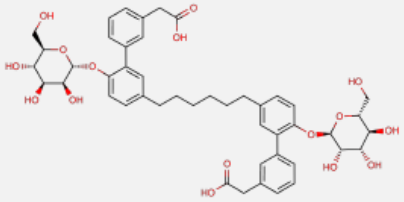
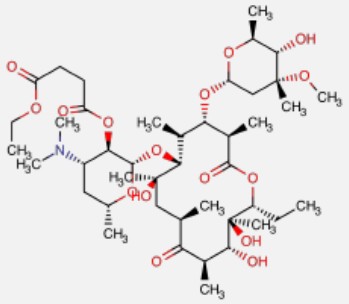


Figure 12: *The pan-selectin antagonist bimosiamose reduces breast cancer cell proliferation and migration in the presence of lung-conditioned media*. MDA-MB-231 cell proliferation (*top panel*) and migration (*bottom panel*) following exposure to basal media or native lung-CM and treatment with DMSO vehicle or bimosiamose (1 mM). Data are presented as mean fold-change in transwell migration or BrdU incorporation relative to basal media ± SEM (n = 3). * = significantly different than basal media; α = significantly different than lung-CM ($p \leq 0.05$).

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

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***.

The final work needed to complete this Task is to optimize the wet milling method using statistical analysis; make the necessary method adjustments for bimosiamose; test the nanocrystals stability after lyophilization and resuspension, and incorporate the nanocrystals into inhalable effervescent carrier particles via spray-freeze drying. This is anticipated to be completed by December 2019.

Major Task 4*: In vivo* assessment of anti-metastatic efficacy of inhalable drugs.

This task has not yet been initiated but will be upon completion of Major Task 3 for bimosiamose.

Results

			Size (d.nm):	% Volume	Width (d.nm):
Z-Average (d.nm):	1364	Peak 1:	837.9	80.5	134.6
PdI:	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				

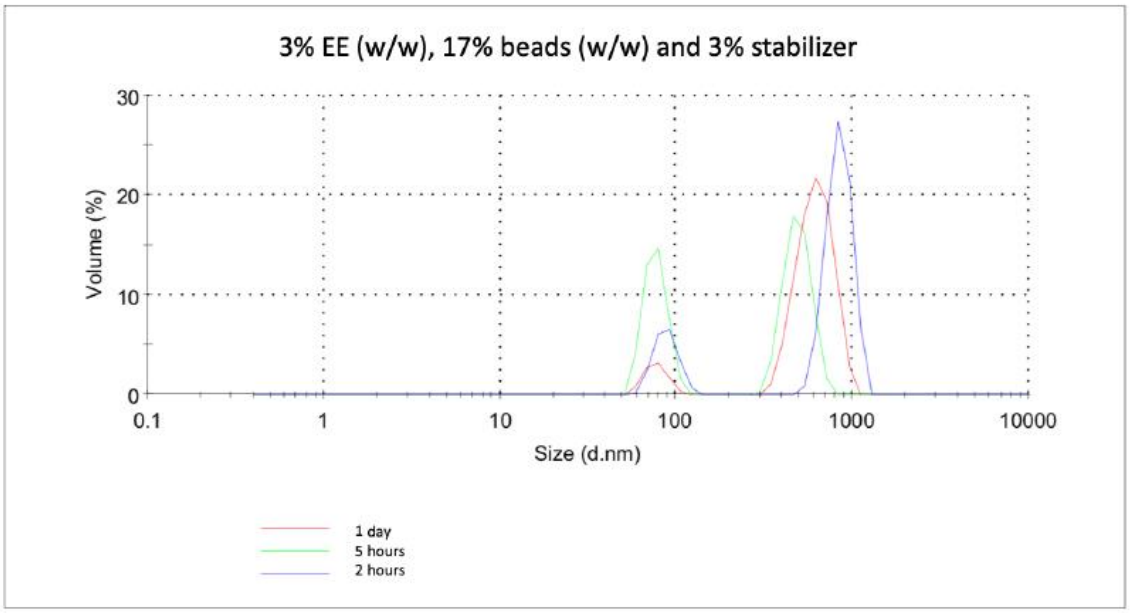


Figure 13. Particle size distribution (diameter, nm) and PdI of EE nanocrystals prepared with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer at 2 hours, 5 hours and 24 hours.

Results

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

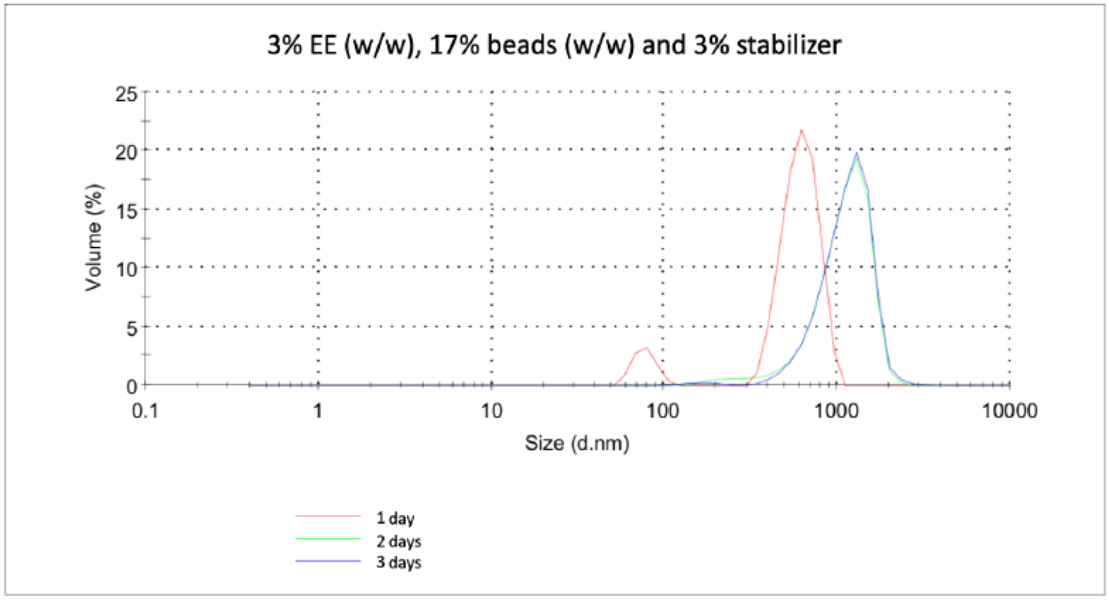


Figure 14. Particle size distribution (diameter, nm) and PdI of EE nanocrystals prepared with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer after 3 days stirring.

Results

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

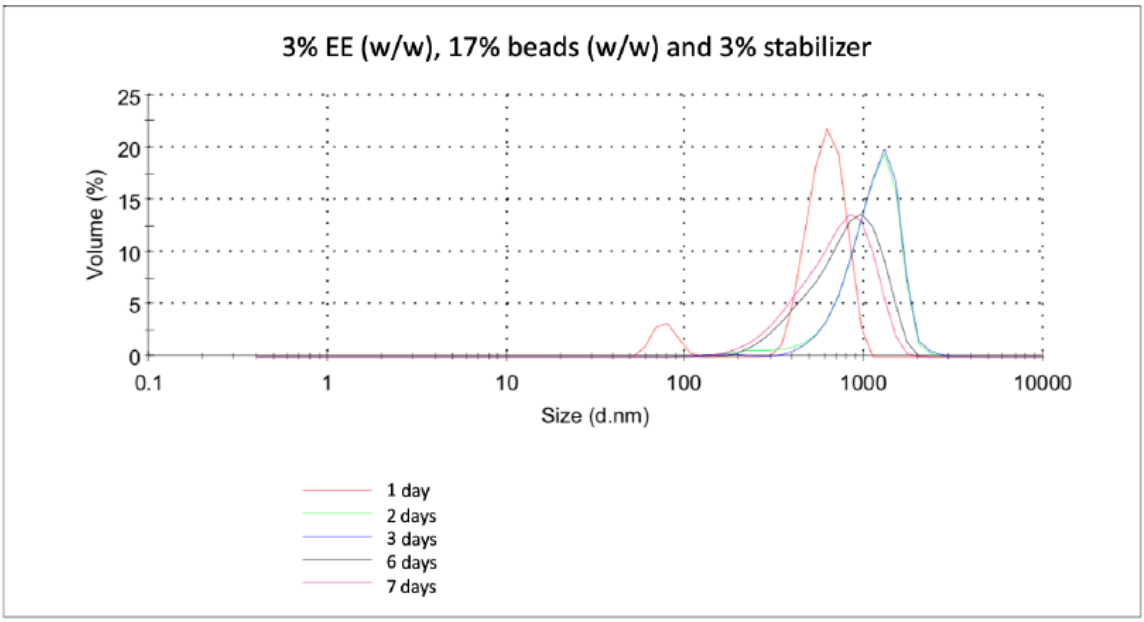


Figure 15. Particle size distribution (diameter, nm) and PdI of EE nanocrystals prepared with 3% EE (w/w), 17% beads (w/w) and 3% stabilizer after 7 days stirring.

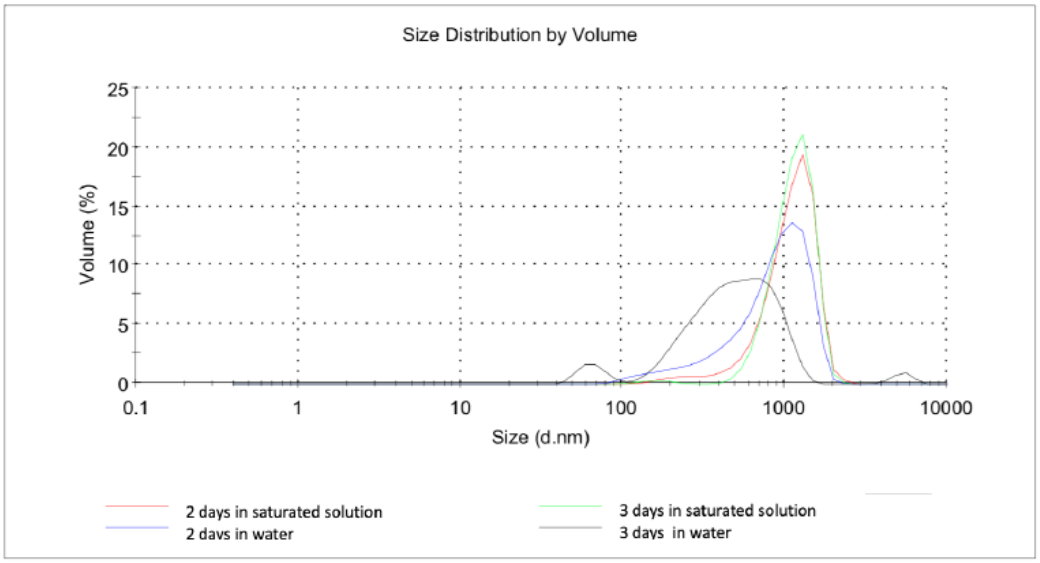


Figure 16. PSD (diameter, nm) of EE nanocrystals in water and saturated solution.

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

Training Opportunities

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 new 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 graduate 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.

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.

➢ How were the results disseminated to communities of interest?

A. Scientific Presentations

Dr. Alison Allan, PD/PI:

1. **Allan, A.L.** Cellular and molecular approaches to understanding breast cancer metastasis. Multidisciplinary Breast Team Research Evening, London, ON, Canada, September 2018.
2. **Allan, A.L.** 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.

B. Scientific Publications

1. Bhat, V., **Allan, A.L.**, 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 *(review manuscript appended).*
2. Medeiros, B and **Allan, A.L.** Molecular mechanisms of breast cancer metastasis to the lung: clinical and experimental perspectives. *Int J Mol Sci,* 2019, 20, 2272; doi:10.3390/ijms20092272 (*review manuscript appended*).

Community Outreach Activities

Dr. Alison Allan, PD/PI:

Dr. Allan was an invited speaker at the opening ceremonies of the Breast Cancer Society of Canada Mother's Day Walk in London, ON, Canada in May 2018 and May 2019. The audience mainly consisted of members of the lay community, including breast cancer survivors and their friends and family. Dr. Allan shared a lay overview of the project as an example of how breast cancer research is important to advancing treatment of breast cancer. In October of 2018, Dr. Allan gave a series of interviews on the Weather Network to kick off Breast Cancer Awareness Month.

Dr. Allan was also a featured scientist in a local media story in September 2017 called "Seizing the Day", and another in April 2019 called "Driving innovation in a world where knowledge is the new currency", 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?**

To date the research activities between Dr. Allan's lab and Dr. Loebenberg continue to progress separately, as was originally proposed and expected for the first year or two of the project. Once the drug formulation studies are completed and we move into the final pre-clinical *in vivo* studies, there will be a much greater integration of the two labs for the planned studies in the final Major Task of the project, including trainees visiting both labs to carry out joint experiments. We expect that this will contribute greatly to accomplishing our goals as laid out in the SOW.

4. IMPACT:

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

Nothing to Report.

- **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 been a bit slower than expected due to problems with some breeding pairs, however we worked closely with our animal facility veterinarian and these problems seem to now be resolved.

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 initial studies presented in this report.

➢ 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, in Year 2 of the project.

➢ 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

A. Scientific Presentations

Dr. Alison Allan, PD/PI:

1. **Allan, A.L.** Cellular and molecular approaches to understanding breast cancer metastasis. Multidisciplinary Breast Team Research Evening, London, ON, Canada, September 2018. Federal support acknowledged: Yes.

2. **Allan, A.L.** 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.

B. Scientific Publications

1. Bhat, V., **Allan, A.L.**, 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 *(review manuscript appended).* Federal support acknowledged: Yes.

3. Medeiros, B and **Allan, A.L.** Molecular mechanisms of breast cancer metastasis to the lung: clinical and experimental perspectives. *Int J Mol Sci,* 2019, 20, 2272; doi:10.3390/ijms20092272 (*review manuscript appended*). 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 has 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:	12
Contribution to Project:	Dr. Xia has performed work in the area of *in vitro* and *ex vivo* functional and mechanistic studies of breast cancer metastatic behavior, and in daily supervision of graduate and undergraduate research students.
Funding Support:	Salary support from this project (100%).

Name:	David Goodale
Project Role:	Technician
Researcher Identifier:	Scopus Author ID: 36897019500
Nearest person month worked:	6
Contribution to Project:	Mr. Goodale has performed work in the area of animal husbandry, animal studies (*in vivo* and *ex vivo*) of breast cancer metastatic behavior, and in daily management of Dr. Allan's lab.
Funding Support:	Salary support from this project (50%), and 50% from the Cancer Research Society.

Name:	Carl Postenka
Project Role:	Technician
Researcher Identifier:	Scopus Author ID: 6506850250
Nearest person month worked:	2.4
Contribution to Project:	Mr. Goodale has 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:	Gabriella Schoettle
Project Role:	Undergraduate Summer Research Student
Researcher Identifier:	Not applicable
Nearest person month worked:	3.5
Contribution to Project:	Ms. Shoettle has performed work in the area of *in vitro* assessment of drug candidates in preparation for the drug formulation studies.
Funding Support:	Salary support from this project from the Ontario Ministry of Education (50%), and the University of Western Ontario (50%).

Name:	Raimar Loebenberg
Project Role:	Co-Investigator
Researcher Identifier:	Scopus Author ID: 6602898019
Nearest person month worked:	1.5
Contribution to Project:	Dr. Loebenberg has 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 has 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 has performed work in the area of drug formulation studies.
Funding Support:	Salary support from this project (100%).

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

  Nothing to Report.

- **What other organizations were involved as partners?**

  Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **Collaborative Awards:**

  Not Applicable.

- **Quad Charts:**

  Not Applicable.

9. APPENDICES:

Bhat, V., **Allan, A.L.**, 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 *(review manuscript appended).*

Medeiros, B and **Allan, A.L.** Molecular mechanisms of breast cancer metastasis to the lung: clinical and experimental perspectives. *Int J Mol Sci,* 2019, 20, 2272; doi:10.3390/ijms20092272 (*review manuscript appended*).

cancers

MDPI

Review

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

Vasudeva Bhat [1,2], Alison L. Allan [3,4] and Afshin Raouf [1,2,*]

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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 β1 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 heterogenous 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 $CD49f^{high}EpCAM^{low/-}$ 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].

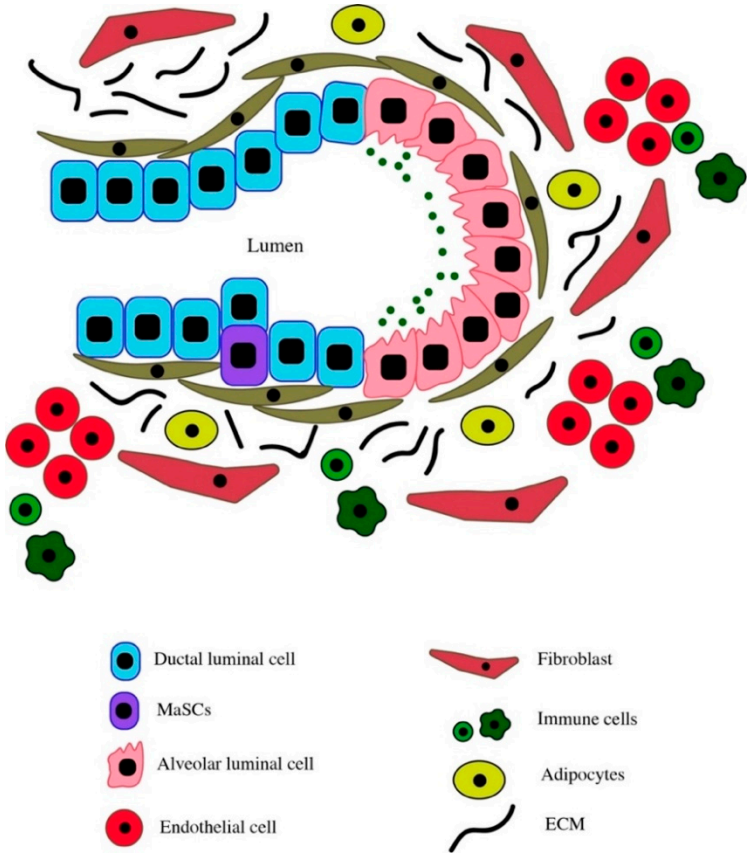

Figure 1. Schematic representation of the breast tissue microenvironment showing bilayered arrangement of ducts and alveolar structures consisting of luminal and myoepithelial cells. These structures are embedded in fibrous stroma consisting of fibroblasts, adipocytes, and immune cells. The luminal cells 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 α6 and α1 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 α1 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 α6 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 Boulander 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	[125]
	• Activation of JAK1/STAT3 signaling pathway in TNBC cell lines	[126]
	• Activation of JAG1-NOTCH3 signaling pathway in ER^{+} breast cancer cell lines	[127]
Interleukin-8	• Enhances bCSC activity and induction of chemoresistance in TNBC cells	[128]
	• Regulation of bCSCs in HER2+ breast cancers via activation of IL-8-CXCR1 signaling axis	[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	[86]
	• Enhances tumor forming ability of TNBC cells	[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 ◦ **Pre-adipocytes** ◦ **Adipocytes** ◦ **MSCs** ◦ **CAFs**	• Enhances bCSC self-renewal via exosome secretion	[133]
	• Secretes adipsin and enhances bCSC activity through activation of C3a-C3aR signaling	
	• Increases mammosphere-forming ability of breast cancer cell via activation of P2 purinergic pathway	[134]
	• Secretes IL-6 and CXCL7 and enhances bCSC self-renewal and proliferation in mouse xenograft model	[135]
	• Secretes prostaglandin and enhances bCSC expansion	[136]
	• Promotes bCSC self-renewal via CCL2 secretion	[137,138]
	• Secretes IL-6 and IL-8 thereby protects bCSCs from chemotherapeutic agents	[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 (TGFβ) 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 TGFβ 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 TGFβ 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 TGFβ 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 (TNFα) 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 TNFα 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 $ER\alpha^+$ breast cancer tumors and CD90 and Ephrin 4A (Eph4A) expressed on bCSCs results in activation of NFκB-mediated secretion of cytokines such 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 HIF1α mediated increase in bCSCs [161]. In addition to this, it has been shown that under chronic hypoxic conditions, the expression of HIF-2α 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-2α 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/EBPδ 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 $ER\alpha^+$ breast cancer cells was capable of elevating both $ER\alpha^+$ and $ER\alpha^-$ 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 secret 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 IL1-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, αSMA, fibroblast-specific protein1 (FSP1), PDGF receptor beta (PDGFRβ), and CAV1. Intriguingly, one of the CAF subsets ($FAP^{high}CD29^{high}\alpha SMA^{high}FSP1^{high}PDGFR\beta^{high}CAV1^{low}$) 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 $CD10^{+}GPR77^{+}$ TAFs. 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 TGFβ 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 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 are 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 show 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.

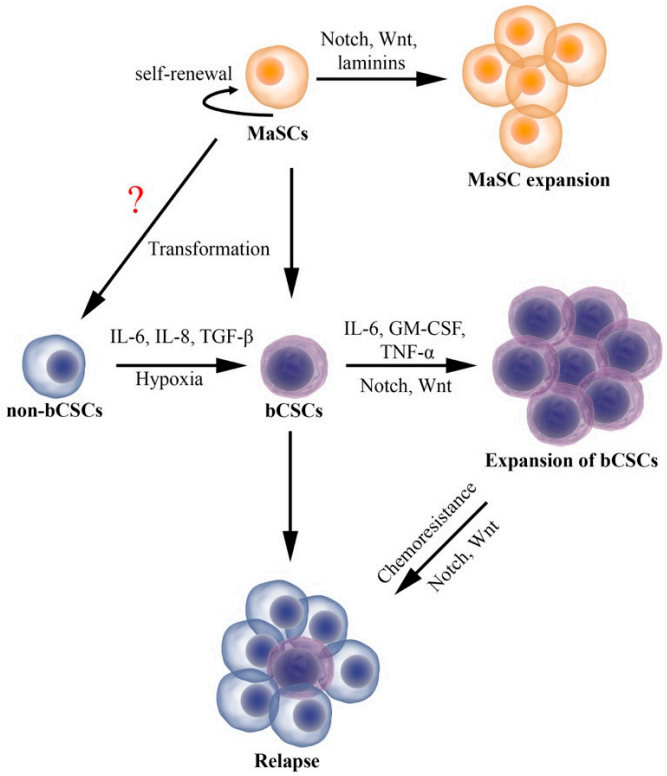

Figure 2. Schematic representation of mammary stem cell (MaSC) expansion, breast cancer stem cell (bCSC) plasticity, development of chemoresistance and relapse. Different cytokines released by immune cells, fibroblasts, adipocytes along with tumor cells in the tumor microenvironment regulate both MaSC 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.

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References

1. Bissell, M.J. The differentiated state of normal and malignant cells or how to define a "normal" cell in culture. *Int. Rev. Cytol.* **1981**, *70*, 27–100.
2. Bissell, M.J.; Hall, H.G.; Parry, G. How does the extracellular matrix direct gene expression? *J. Theor. Biol.* **1982**, *99*, 31–68. [CrossRef]

3. Bissell, M.J.; Labarge, M.A. Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment? *Cancer Cell* **2005**, *7*, 17–23. [PubMed]
4. Bissell, M.J.; Rizki, A.; Mian, I.S. Tissue architecture: The ultimate regulator of breast epithelial function. *Curr. Opin. Cell Biol.* **2003**, *15*, 753–762. [CrossRef] [PubMed]
5. Mintz, B.; Illmensee, K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 3585–3589. [CrossRef] [PubMed]
6. Kenny, P.A.; Bissell, M.J. Tumor reversion: Correction of malignant behavior by microenvironmental cues. *Int. J. Cancer* **2003**, *107*, 688–695. [CrossRef] [PubMed]
7. Martin, G.S. The hunting of the src. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 467–475. [CrossRef]
8. Martin, G.S. The road to src. *Oncogene* **2004**, *23*, 7910–7917. [CrossRef] [PubMed]
9. Scadden, D.T. The stem-cell niche as an entity of action. *Nature* **2006**, *441*, 1075–1079. [CrossRef]
10. Jensen, U.B.; Lowell, S.; Watt, F.M. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: A new view based on whole-mount labelling and lineage analysis. *Development* **1999**, *126*, 2409–2418.
11. Jones, P.H.; Watt, F.M. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* **1993**, *73*, 713–724. [CrossRef]
12. Nilsson, S.K.; Johnston, H.M.; Whitty, G.A.; Williams, B.; Webb, R.J.; Denhardt, D.T.; Bertoncello, I.; Bendall, L.J.; Simmons, P.J.; Haylock, D.N. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* **2005**, *106*, 1232–1239. [CrossRef] [PubMed]
13. Stier, S.; Ko, Y.; Forkert, R.; Lutz, C.; Neuhaus, T.; Grunewald, E.; Cheng, T.; Dombkowski, D.; Calvi, L.M.; Rittling, S.R.; et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J. Exp. Med.* **2005**, *201*, 1781–1791. [CrossRef] [PubMed]
14. Garcion, E.; Halilagic, A.; Faissner, A.; ffrench-Constant, C. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin c. *Development* **2004**, *131*, 3423–3432. [CrossRef] [PubMed]
15. Ohta, M.; Sakai, T.; Saga, Y.; Aizawa, S.; Saito, M. Suppression of hematopoietic activity in tenascin-c-deficient mice. *Blood* **1998**, *91*, 4074–4083. [PubMed]
16. Deugnier, M.A.; Teuliere, J.; Faraldo, M.M.; Thiery, J.P.; Glukhova, M.A. The importance of being a myoepithelial cell. *Breast Cancer Res.* **2002**, *4*, 224–230. [CrossRef] [PubMed]
17. Petersen, O.W.; Polyak, K. Stem cells in the human breast. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a003160. [CrossRef] [PubMed]
18. Gimpl, G.; Fahrenholz, F. The oxytocin receptor system: Structure, function, and regulation. *Physiol. Rev.* **2001**, *81*, 629–683. [CrossRef]
19. Wagner, K.U.; Young, W.S., 3rd; Liu, X.; Ginns, E.I.; Li, M.; Furth, P.A.; Hennighausen, L. Oxytocin and milk removal are required for post-partum mammary-gland development. *Genes Funct.* **1997**, *1*, 233–244. [CrossRef]
20. Haaksma, C.J.; Schwartz, R.J.; Tomasek, J.J. Myoepithelial cell contraction and milk ejection are impaired in mammary glands of mice lacking smooth muscle alpha-actin. *Biol. Reprod.* **2011**, *85*, 13–21. [CrossRef]
21. Potten, C.S.; Watson, R.J.; Williams, G.T.; Tickle, S.; Roberts, S.A.; Harris, M.; Howell, A. The effect of age and menstrual cycle upon proliferative activity of the normal human breast. *Br. J. Cancer* **1988**, *58*, 163–170. [CrossRef]
22. Soderqvist, G.; Isaksson, E.; von Schoultz, B.; Carlstrom, K.; Tani, E.; Skoog, L. Proliferation of breast epithelial cells in healthy women during the menstrual cycle. *Am. J. Obstet. Gynecol.* **1997**, *176*, 123–128. [CrossRef]
23. Vogel, P.M.; Georgiade, N.G.; Fetter, B.F.; Vogel, F.S.; McCarty, K.S., Jr. The correlation of histologic changes in the human breast with the menstrual cycle. *Am. J. Pathol.* **1981**, *104*, 23–34.
24. Suzuki, R.; Atherton, A.J.; O'Hare, M.J.; Entwistle, A.; Lakhani, S.R.; Clarke, C. Proliferation and differentiation in the human breast during pregnancy. *Differentiation* **2000**, *66*, 106–115. [CrossRef]
25. Russo, J.; Russo, I.H. Development pattern of human breast and susceptibility to carcinogenesis. *Eur. J. Cancer Prev.* **1993**, *2*, 85–100. [CrossRef]
26. Russo, J.; Russo, I.H. Development of the human breast. *Maturitas* **2004**, *49*, 2–15. [CrossRef]

27. Deome, K.B.; Faulkin, L.J., Jr.; Bern, H.A.; Blair, P.B. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female c3h mice. *Cancer Res.* **1959**, *19*, 515–520.
28. Hoshino, K. Morphogenesis and growth potentiality of mammary glands in mice. I. Transplantability and growth potentiality of mammary tissue of virgin mice. *J. Natl. Cancer Inst.* **1962**, *29*, 835–851.
29. Daniel, C.W.; Deome, K.B.; Young, J.T.; Blair, P.B.; Faulkin, L.J., Jr. The in vivo life span of normal and preneoplastic mouse mammary glands: A serial transplantation study. 1968. *J. Mammary Gland Biol. Neoplasia* **2009**, *14*, 355–362. [CrossRef]
30. Smith, G.H.; Medina, D. A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. *J. Cell Sci.* **1988**, *90*, 173–183.
31. Stingl, J.; Eirew, P.; Ricketson, I.; Shackleton, M.; Vaillant, F.; Choi, D.; Li, H.I.; Eaves, C.J. Purification and unique properties of mammary epithelial stem cells. *Nature* **2006**, *439*, 993–997. [CrossRef]
32. Shackleton, M.; Vaillant, F.; Simpson, K.J.; Stingl, J.; Smyth, G.K.; Asselin-Labat, M.L.; Wu, L.; Lindeman, G.J.; Visvader, J.E. Generation of a functional mammary gland from a single stem cell. *Nature* **2006**, *439*, 84–88. [CrossRef]
33. Lloyd-Lewis, B.; Harris, O.B.; Watson, C.J.; Davis, F.M. Mammary Stem Cells: Premise, Properties, and Perspectives. *Tr. Cell Biol.* **2017**, *27*, 556–567. [CrossRef]
34. Wuidart, A.; Sifrim, A.; Fioramonti, M.; Matsumura, S.; Brisebarre, A.; Brown, D.; Centonze, A.; Dannau, A.; Dubois, C.; Van Keymeulen, A.; et al. Publisher Correction: Early lineage segregation of multipotent embryonic mammary gland progenitors. *Nat. Cell Biol.* **2018**, *20*, 1099. [CrossRef]
35. Lilja, A.M.; Rodilla, V.; Huyghe, M.; Hannezo, E.; Landragin, C.; Renaud, O.; Leroy, O.; Rulands, S.; Simons, B.D.; Fre, S. Clonal analysis of Notch1-expressing cells reveals the existence of unipotent stem cells that retain long-term plasticity in the embryonic mammary gland. *Nat. Cell Biol.* **2018**, *20*, 677–687. [CrossRef]
36. Villadsen, R.; Fridriksdottir, A.J.; Ronnov-Jessen, L.; Gudjonsson, T.; Rank, F.; LaBarge, M.A.; Bissell, M.J.; Petersen, O.W. Evidence for a stem cell hierarchy in the adult human breast. *J. Cell Biol.* **2007**, *177*, 87–101. [CrossRef]
37. Eirew, P.; Stingl, J.; Raouf, A.; Turashvili, G.; Aparicio, S.; Emerman, J.T.; Eaves, C.J. A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. *Nat. Med.* **2008**, *14*, 1384–1389. [CrossRef]
38. Dontu, G.; Ince, T.A. Of mice and women: A comparative tissue biology perspective of breast stem cells and differentiation. *J. Mammary Gland Biol. Neoplasia* **2015**, *20*, 51–62. [CrossRef]
39. Wang, J.; Xia, T.S.; Liu, X.A.; Ding, Q.; Du, Q.; Yin, H.; Wang, S. A novel orthotopic and metastatic mouse model of breast cancer in human mammary microenvironment. *Breast Cancer Res. Treat.* **2010**, *120*, 337–344. [CrossRef]
40. Xia, T.S.; Wang, J.; Yin, H.; Ding, Q.; Zhang, Y.F.; Yang, H.W.; Liu, X.A.; Dong, M.; Du, Q.; Ling, L.J.; et al. Human tissue-specific microenvironment: An essential requirement for mouse models of breast cancer. *Oncol. Rep.* **2010**, *24*, 203–211.
41. Silberstein, G.B. Tumour-stromal interactions. Role of the stroma in mammary development. *Breast Cancer Res.* **2001**, *3*, 218–223. [CrossRef] [PubMed]
42. Wiseman, B.S.; Werb, Z. Stromal effects on mammary gland development and breast cancer. *Science* **2002**, *296*, 1046–1049. [CrossRef] [PubMed]
43. Parmar, H.; Cunha, G.R. Epithelial-stromal interactions in the mouse and human mammary gland in vivo. *Endocr. Relat. Cancer* **2004**, *11*, 437–458. [CrossRef] [PubMed]
44. Williams, J.M.; Daniel, C.W. Mammary ductal elongation: Differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev. Biol.* **1983**, *97*, 274–290. [CrossRef]
45. Varner, V.D.; Nelson, C.M. Cellular and physical mechanisms of branching morphogenesis. *Development* **2014**, *141*, 2750–2759. [CrossRef] [PubMed]
46. Potten, C.S.; Loeffler, M. Stem cells: Attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **1990**, *110*, 1001–1020. [PubMed]
47. LaBarge, M.A.; Petersen, O.W.; Bissell, M.J. Of microenvironments and mammary stem cells. *Stem Cell Rev.* **2007**, *3*, 137–146. [CrossRef]

48. Loeffler, M.; Roeder, I. Tissue stem cells: Definition, plasticity, heterogeneity, self-organization and models–a conceptual approach. *Cells Tissues Organs* **2002**, *171*, 8–26. [CrossRef]
49. Stirling, J.W.; Chandler, J.A. The fine structure of the normal, resting terminal ductal-lobular unit of the female breast. *Virchows Arch. A Pathol. Anat. Histol.* **1976**, *372*, 205–226. [CrossRef]
50. Stirling, J.W.; Chandler, J.A. The fine structure of ducts and subareolar ducts in the resting gland of the female breast. *Virchows Arch. A Pathol. Anat. Histol.* **1977**, *373*, 119–132. [CrossRef]
51. Inman, J.L.; Robertson, C.; Mott, J.D.; Bissell, M.J. Mammary gland development: Cell fate specification, stem cells and the microenvironment. *Development* **2015**, *142*, 1028–1042. [CrossRef] [PubMed]
52. Talhouk, R.S.; Chin, J.R.; Unemori, E.N.; Werb, Z.; Bissell, M.J. Proteinases of the mammary gland: Developmental regulation in vivo and vectorial secretion in culture. *Development* **1991**, *112*, 439–449. [PubMed]
53. Correia, A.L.; Mori, H.; Chen, E.I.; Schmitt, F.C.; Bissell, M.J. The hemopexin domain of mmp3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with hsp90beta. *Genes Dev.* **2013**, *27*, 805–817. [CrossRef] [PubMed]
54. Kessenbrock, K.; Dijkgraaf, G.J.; Lawson, D.A.; Littlepage, L.E.; Shahi, P.; Pieper, U.; Werb, Z. A role for matrix metalloproteinases in regulating mammary stem cell function via the wnt signaling pathway. *Cell Stem Cell* **2013**, *13*, 300–313. [CrossRef] [PubMed]
55. Gouon-Evans, V.; Rothenberg, M.E.; Pollard, J.W. Postnatal mammary gland development requires macrophages and eosinophils. *Development* **2000**, *127*, 2269–2282.
56. Monks, J.; Geske, F.J.; Lehman, L.; Fadok, V.A. Do inflammatory cells participate in mammary gland involution? *J. Mammary Gland Biol. Neoplasia* **2002**, *7*, 163–176. [CrossRef] [PubMed]
57. Sferruzzi-Perri, A.N.; Robertson, S.A.; Dent, L.A. Interleukin-5 transgene expression and eosinophilia are associated with retarded mammary gland development in mice. *Biol. Reprod.* **2003**, *69*, 224–233. [CrossRef]
58. Lilla, J.N.; Werb, Z. Mast cells contribute to the stromal microenvironment in mammary gland branching morphogenesis. *Dev. Biol.* **2010**, *337*, 124–133. [CrossRef]
59. Pollard, J.W.; Hennighausen, L. Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 9312–9316. [CrossRef]
60. O'Brien, J.; Lyons, T.; Monks, J.; Lucia, M.S.; Wilson, R.S.; Hines, L.; Man, Y.G.; Borges, V.; Schedin, P. Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am. J. Pathol.* **2010**, *176*, 1241–1255. [CrossRef]
61. Gyorki, D.E.; Asselin-Labat, M.L.; van Rooijen, N.; Lindeman, G.J.; Visvader, J.E. Resident macrophages influence stem cell activity in the mammary gland. *Breast Cancer Res.* **2009**, *11*, R62. [CrossRef]
62. Chakrabarti, R.; Celia-Terrassa, T.; Kumar, S.; Hang, X.; Wei, Y.; Choudhury, A.; Hwang, J.; Peng, J.; Nixon, B.; Grady, J.J.; et al. Notch ligand dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. *Science* **2018**, *360*. [CrossRef]
63. Zeng, Y.A.; Nusse, R. Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. *Cell Stem Cell* **2010**, *6*, 568–577. [CrossRef]
64. Wang, H.; Luo, X.; Leighton, J. Extracellular matrix and integrins in embryonic stem cell differentiation. *Biochem. Insights* **2015**, *8*, 15–21. [CrossRef]
65. Boudreau, N.J.; Jones, P.L. Extracellular matrix and integrin signalling: The shape of things to come. *Biochem. J.* **1999**, *339*, 481–488. [CrossRef]
66. Schwartz, M.A. Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a005066. [CrossRef]
67. Li, N.; Zhang, Y.; Naylor, M.J.; Schatzmann, F.; Maurer, F.; Wintermantel, T.; Schuetz, G.; Mueller, U.; Streuli, C.H.; Hynes, N.E. Beta1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli. *EMBO J.* **2005**, *24*, 1942–1953. [CrossRef]
68. Taddei, I.; Deugnier, M.A.; Faraldo, M.M.; Petit, V.; Bouvard, D.; Medina, D.; Fassler, R.; Thiery, J.P.; Glukhova, M.A. Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. *Nat. Cell Biol.* **2008**, *10*, 716–722. [CrossRef]
69. Delcommenne, M.; Streuli, C.H. Control of integrin expression by extracellular matrix. *J. Biol. Chem.* **1995**, *270*, 26794–26801. [CrossRef]

70. LaBarge, M.A.; Nelson, C.M.; Villadsen, R.; Fridriksdottir, A.; Ruth, J.R.; Stampfer, M.R.; Petersen, O.W.; Bissell, M.J. Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. *Integr. Biol. (Camb.)* **2009**, *1*, 70–79. [CrossRef]
71. Zhao, C.; Cai, S.; Shin, K.; Lim, A.; Kalisky, T.; Lu, W.J.; Clarke, M.F.; Beachy, P.A. Stromal gli2 activity coordinates a niche signaling program for mammary epithelial stem cells. *Science* **2017**, *356*. [CrossRef]
72. Cunha, G.R.; Young, P.; Christov, K.; Guzman, R.; Nandi, S.; Talamantes, F.; Thordarson, G. Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat. (Basel)* **1995**, *152*, 195–204. [CrossRef]
73. Boulanger, C.A.; Bruno, R.D.; Mack, D.L.; Gonzales, M.; Castro, N.P.; Salomon, D.S.; Smith, G.H. Embryonic stem cells are redirected to non-tumorigenic epithelial cell fate by interaction with the mammary microenvironment. *PLoS ONE* **2013**, *8*, e62019. [CrossRef]
74. Boulanger, C.A.; Bruno, R.D.; Rosu-Myles, M.; Smith, G.H. The mouse mammary microenvironment redirects mesoderm-derived bone marrow cells to a mammary epithelial progenitor cell fate. *Stem Cells Dev.* **2012**, *21*, 948–954. [CrossRef]
75. Boulanger, C.A.; Mack, D.L.; Booth, B.W.; Smith, G.H. Interaction with the mammary microenvironment redirects spermatogenic cell fate in vivo. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3871–3876. [CrossRef]
76. Cutano, V.; Di Giorgio, E.; Minisini, M.; Picco, R.; Dalla, E.; Brancolini, C. Hdac7-mediated control of tumor microenvironment maintains proliferative and stemness competence of human mammary epithelial cells. *Mol. Oncol.* **2019**, *13*, 1651–1668.
77. Makarem, M.; Kannan, N.; Nguyen, L.V.; Knapp, D.J.; Balani, S.; Prater, M.D.; Stingl, J.; Raouf, A.; Nemirovsky, O.; Eirew, P.; et al. Developmental changes in the in vitro activated regenerative activity of primitive mammary epithelial cells. *PLoS Biol.* **2013**, *11*, e1001630. [CrossRef]
78. Roato, I.; Ferracini, R. Cancer stem cells, bone and tumor microenvironment: Key players in bone metastases. *Cancers* **2018**, *10*, 56. [CrossRef]
79. Prager, B.C.; Xie, Q.; Bao, S.; Rich, J.N. Cancer stem cells: The architects of the tumor ecosystem. *Cell Stem Cell* **2019**, *24*, 41–53. [CrossRef]
80. Bocci, F.; Gearhart-Serna, L.; Boareto, M.; Ribeiro, M.; Ben-Jacob, E.; Devi, G.R.; Levine, H.; Onuchic, J.N.; Jolly, M.K. Toward understanding cancer stem cell heterogeneity in the tumor microenvironment. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 148–157. [CrossRef]
81. Liubomirski, Y.; Lerrer, S.; Meshel, T.; Rubinstein-Achiasaf, L.; Morein, D.; Wiemann, S.; Korner, C.; Ben-Baruch, A. Tumor-stroma-inflammation networks promote pro-metastatic chemokines and aggressiveness characteristics in triple-negative breast cancer. *Front. Immunol.* **2019**, *10*, 757. [CrossRef]
82. Chen, W.; Qin, Y.; Liu, S. Cytokines, breast cancer stem cells (bcscs) and chemoresistance. *Clin. Transl. Med.* **2018**, *7*, 27. [CrossRef]
83. Weitzenfeld, P.; Meshel, T.; Ben-Baruch, A. Microenvironmental networks promote tumor heterogeneity and enrich for metastatic cancer stem-like cells in luminal-a breast tumor cells. *Oncotarget* **2016**, *7*, 81123–81143. [CrossRef]
84. Korkaya, H.; Liu, S.; Wicha, M.S. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *J. Clin. Investig.* **2011**, *121*, 3804–3809. [CrossRef]
85. Kim, H.; Lin, Q.; Glazer, P.M.; Yun, Z. The hypoxic tumor microenvironment in vivo selects the cancer stem cell fate of breast cancer cells. *Breast Cancer Res.* **2018**, *20*, 16. [CrossRef]
86. Doherty, M.R.; Parvani, J.G.; Tamagno, I.; Junk, D.J.; Bryson, B.L.; Cheon, H.J.; Stark, G.R.; Jackson, M.W. The opposing effects of interferon-beta and oncostatin-m as regulators of cancer stem cell plasticity in triple-negative breast cancer. *Breast Cancer Res.* **2019**, *21*, 54. [CrossRef]
87. Zhou, X.; Li, T.; Chen, Y.; Zhang, N.; Wang, P.; Liang, Y.; Long, M.; Liu, H.; Mao, J.; Liu, Q.; et al. Mesenchymal stem cellderived extracellular vesicles promote the in vitro proliferation and migration of breast cancer cells through the activation of the erk pathway. *Int. J. Oncol.* **2019**, *54*, 1843–1852.
88. Brown, Y.; Hua, S.; Tanwar, P.S. Extracellular matrix-mediated regulation of cancer stem cells and chemoresistance. *Int. J. Biochem. Cell Biol.* **2019**, *109*, 90–104. [CrossRef]
89. Ciardiello, C.; Leone, A.; Budillon, A. The crosstalk between cancer stem cells and microenvironment is critical for solid tumor progression: The significant contribution of extracellular vesicles. *Stem Cells Int.* **2018**, *2018*, 6392198. [CrossRef]

90. Kakarala, M.; Wicha, M.S. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. *J. Clin. Oncol.* **2008**, *26*, 2813–2820. [CrossRef]
91. Brooks, M.D.; Wicha, M.S. Tumor twitter: Cellular communication in the breast cancer stem cell niche. *Cancer Discov.* **2015**, *5*, 469–471. [CrossRef]
92. Liu, S.; Cong, Y.; Wang, D.; Sun, Y.; Deng, L.; Liu, Y.; Martin-Trevino, R.; Shang, L.; McDermott, S.P.; Landis, M.D.; et al. Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem Cell Rep.* **2014**, *2*, 78–91. [CrossRef]
93. Kim, W.T.; Ryu, C.J. Cancer stem cell surface markers on normal stem cells. *BMB Rep.* **2017**, *50*, 285–298. [CrossRef]
94. Zhou, W.; Wang, G.; Guo, S. Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells. *Biochim. Biophys. Acta.* **2013**, *1836*, 304–320. [CrossRef]
95. Zhang, Z.M.; Wu, J.F.; Luo, Q.C.; Liu, Q.F.; Wu, Q.W.; Ye, G.D.; She, H.Q.; Li, B.A. Pygo2 activates MDR1 expression and mediates chemoresistance in breast cancer via the Wnt/beta-catenin pathway. *Oncogene* **2016**, *35*, 4787–4797. [CrossRef]
96. Stylianou, S.; Clarke, R.B.; Brennan, K. Aberrant activation of notch signaling in human breast cancer. *Cancer Rec.* **2006**, *66*, 1517–1525. [CrossRef]
97. Molyneux, G.; Geyer, F.C.; Magnay, F.A.; McCarthy, A.; Kendrick, H.; Natrajan, R.; Mackay, A.; Grigoriadis, A.; Tutt, A.; Ashworth, A.; et al. BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. *Cell Stem Cell* **2010**, *7*, 403–417. [CrossRef]
98. Korkaya, H.; Kim, G.I.; Davis, A.; Malik, F.; Henry, N.L.; Ithimakin, S.; Quraishi, A.A.; Tawakkol, N.; D'Angelo, R.; Paulson, A.K.; et al. Activation of an il6 inflammatory loop mediates trastuzumab resistance in her2+ breast cancer by expanding the cancer stem cell population. *Mol. Cell* **2012**, *47*, 570–584. [CrossRef]
99. Oskarsson, T.; Batlle, E.; Massague, J. Metastatic stem cells: Sources, niches, and vital pathways. *Cell Stem Cell* **2014**, *14*, 306–321. [CrossRef]
100. Yu, F.; Yao, H.; Zhu, P.; Zhang, X.; Pan, Q.; Gong, C.; Huang, Y.; Hu, X.; Su, F.; Lieberman, J.; et al. Let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell* **2007**, *131*, 1109–1123. [CrossRef]
101. Lonning, P.E.; Knappskog, S. Mapping genetic alterations causing chemoresistance in cancer: Identifying the roads by tracking the drivers. *Oncogene* **2013**, *32*, 5315–5330. [CrossRef]
102. Makhija, S.; Sit, A.; Edwards, R.; Aufman, K.; Weiss, H.; Kanbour-Shakir, A.; Gooding, W.; D'Angelo, G.; Ferrell, R.; Raja, S.; et al. Identification of genetic alterations related to chemoresistance in epithelial ovarian cancer. *Gynecol. Oncol.* **2003**, *90*, 3–9. [CrossRef]
103. Shackleton, M. Normal stem cells and cancer stem cells: Similar and different. *Semin. Cancer Biol.* **2010**, *20*, 85–92. [CrossRef]
104. Ebben, J.D.; Treisman, D.M.; Zorniak, M.; Kutty, R.G.; Clark, P.A.; Kuo, J.S. The cancer stem cell paradigm: A new understanding of tumor development and treatment. *Expert Opin. Ther. Targets* **2010**, *14*, 621–632. [CrossRef]
105. Visvader, J.E. Cells of origin in cancer. *Nature* **2011**, *469*, 314–322. [CrossRef]
106. Clevers, H. The cancer stem cell: Premises, promises and challenges. *Nat. Med.* **2011**, *17*, 313–319. [CrossRef]
107. Visvader, J.E.; Lindeman, G.J. Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. *Nat. Rev. Cancer* **2008**, *8*, 755–768. [CrossRef]
108. O'Brien, C.A.; Kreso, A.; Jamieson, C.H. Cancer stem cells and self-renewal. *Clin. Cancer Res.* **2010**, *16*, 3113–3120. [CrossRef]
109. Trumpp, A.; Wiestler, O.D. Mechanisms of disease: Cancer stem cells—targeting the evil twin. *Nat. Clin. Pract. Oncol.* **2008**, *5*, 337–347. [CrossRef]
110. Moitra, K.; Lou, H.; Dean, M. Multidrug efflux pumps and cancer stem cells: Insights into multidrug resistance and therapeutic development. *Clin. Pharmacol. Ther.* **2011**, *89*, 491–502. [CrossRef]
111. Sun, Z.; Wang, C.Y.; Lawson, D.A.; Kwek, S.; Velozo, H.G.; Owyong, M.; Lai, M.D.; Fong, L.; Wilson, M.; Su, H.; et al. Single-cell rna sequencing reveals gene expression signatures of breast cancer-associated endothelial cells. *Oncotarget* **2018**, *9*, 10945–10961. [CrossRef]
112. Zhu, D.; Zhao, Z.; Cui, G.; Chang, S.; Hu, L.; See, Y.X.; Liang Lim, M.G.; Guo, D.; Chen, X.; Poudel, B.; et al. Single-cell transcriptome analysis reveals estrogen signaling coordinately augments one-carbon, polyamine, and purine synthesis in breast cancer. *Cell Rep.* **2019**, *27*, 2285–2798. [CrossRef]

113. Navin, N.E. The first five years of single-cell cancer genomics and beyond. *Genome Res.* **2015**, *25*, 1499–1507. [CrossRef]
114. Saadatpour, A.; Lai, S.; Guo, G.; Yuan, G.C. Single-cell analysis in cancer genomics. *Trends Genet.* **2015**, *31*, 576–586. [CrossRef]
115. Zhao, Y.; Carter, R.; Natarajan, S.; Varn, F.S.; Compton, D.A.; Gawad, C.; Cheng, C.; Godek, K.M. Single-cell rna sequencing reveals the impact of chromosomal instability on glioblastoma cancer stem cells. *BMC Med. Genom.* **2019**, *12*, 79. [CrossRef]
116. Gupta, R.G.; Somer, R.A. Intratumor heterogeneity: Novel approaches for resolving genomic architecture and clonal evolution. *Mol. Cancer Res.* **2017**, *15*, 1127–1137. [CrossRef]
117. Wang, Y.; Waters, J.; Leung, M.L.; Unruh, A.; Roh, W.; Shi, X.; Chen, K.; Scheet, P.; Vattathil, S.; Liang, H.; et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. *Nature* **2014**, *512*, 155–160. [CrossRef]
118. Vareslija, D.; Priedigkeit, N.; Fagan, A.; Purcell, S.; Cosgrove, N.; O'Halloran, P.J.; Ward, E.; Cocchiglia, S.; Hartmaier, R.; Castro, C.A.; et al. Transcriptome characterization of matched primary breast and brain metastatic tumors to detect novel actionable targets. *J. Natl. Cancer Inst.* **2019**, *111*, 388–398. [CrossRef]
119. Ma, X.J.; Dahiya, S.; Richardson, E.; Erlander, M.; Sgroi, D.C. Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res.* **2009**, *11*, R7. [CrossRef]
120. Chu, J.E.; Xia, Y.; Chin-Yee, B.; Goodale, D.; Croker, A.K.; Allan, A.L. Lung-derived factors mediate breast cancer cell migration through cd44 receptor-ligand interactions in a novel ex vivo system for analysis of organ-specific soluble proteins. *Neoplasia* **2014**, *16*, 180–191. [CrossRef]
121. Pio, G.M.; Xia, Y.; Piaseczny, M.M.; Chu, J.E.; Allan, A.L. Soluble bone-derived osteopontin promotes migration and stem-like behavior of breast cancer cells. *PLoS ONE* **2017**, *12*, e0177640. [CrossRef]
122. Lu, H.; Ouyang, W.; Huang, C. Inflammation, a key event in cancer development. *Mol. Cancer Res.* **2006**, *4*, 221–233. [CrossRef]
123. Ribatti, D. The concept of immune surveillance against tumors. The first theories. *Oncotarget* **2017**, *8*, 7175–7180. [CrossRef]
124. Jarnicki, A.; Putoczki, T.; Ernst, M. Stat3: Linking inflammation to epithelial cancer—more than a "gut" feeling? *Cell Div.* **2010**, *5*, 14. [CrossRef]
125. Iliopoulos, D.; Hirsch, H.A.; Wang, G.; Struhl, K. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via il6 secretion. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 1397–1402. [CrossRef]
126. Kim, S.Y.; Kang, J.W.; Song, X.; Kim, B.K.; Yoo, Y.D.; Kwon, Y.T.; Lee, Y.J. Role of the il-6-jak1-stat3-oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cell Signal.* **2013**, *25*, 961–969. [CrossRef]
127. Sansone, P.; Storci, G.; Tavolari, S.; Guarnieri, T.; Giovannini, C.; Taffurelli, M.; Ceccarelli, C.; Santini, D.; Paterini, P.; Marcu, K.B.; et al. Il-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J. Clin. Investig.* **2007**, *117*, 3988–4002. [CrossRef]
128. Dominguez, C.; McCampbell, K.K.; David, J.M.; Palena, C. Neutralization of il-8 decreases tumor pmn-mdscs and reduces mesenchymalization of claudin-low triple-negative breast cancer. *JCI Insight* **2017**, *2*. [CrossRef]
129. Singh, J.K.; Farnie, G.; Bundred, N.J.; Simoes, B.M.; Shergill, A.; Landberg, G.; Howell, S.J.; Clarke, R.B. Targeting cxcr1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting her2 via her2-dependent and -independent mechanisms. *Clin. Cancer Res.* **2013**, *19*, 643–656. [CrossRef]
130. Katsuno, Y.; Meyer, D.S.; Zhang, Z.; Shokat, K.M.; Akhurst, R.J.; Miyazono, K.; Derynck, R. Chronic tgf-beta exposure drives stabilized emt, tumor stemness, and cancer drug resistance with vulnerability to bitopic mtor inhibition. *Sci. Signal.* **2019**. [CrossRef]
131. Santisteban, M.; Reiman, J.M.; Asiedu, M.K.; Behrens, M.D.; Nassar, A.; Kalli, K.R.; Haluska, P.; Ingle, J.N.; Hartmann, L.C.; Manjili, M.H.; et al. Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells. *Cancer Res.* **2009**, *69*, 2887–2895. [CrossRef]
132. Lu, H.; Clauser, K.R.; Tam, W.L.; Frose, J.; Ye, X.; Eaton, E.N.; Reinhardt, F.; Donnenberg, V.S.; Bhargava, R.; Carr, S.A.; et al. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. *Nat. Cell Biol.* **2014**, *16*, 1105–1117. [CrossRef]

133. Gernapudi, R.; Yao, Y.; Zhang, Y.; Wolfson, B.; Roy, S.; Duru, N.; Eades, G.; Yang, P.; Zhou, Q. Targeting exosomes from preadipocytes inhibits preadipocyte to cancer stem cell signaling in early-stage breast cancer. *Breast Cancer Res. Treat.* **2015**, *150*, 685–695. [CrossRef]
134. Goto, H.; Shimono, Y.; Funakoshi, Y.; Imamura, Y.; Toyoda, M.; Kiyota, N.; Kono, S.; Takao, S.; Mukohara, T.; Minami, H. Adipose-derived stem cells enhance human breast cancer growth and cancer stem cell-like properties through adipsin. *Oncogene* **2019**, *38*, 767–779. [CrossRef]
135. Maffey, A.; Storini, C.; Diceglie, C.; Martelli, C.; Sironi, L.; Calzarossa, C.; Tonna, N.; Lovchik, R.; Delamarche, E.; Ottobrini, L.; et al. Mesenchymal stem cells from tumor microenvironment favour breast cancer stem cell proliferation, cancerogenic and metastatic potential, via ionotropic purinergic signalling. *Sci. Rep.* **2017**, *7*, 13162. [CrossRef]
136. Liu, S.; Ginestier, C.; Ou, S.J.; Clouthier, S.G.; Patel, S.H.; Monville, F.; Korkaya, H.; Heath, A.; Dutcher, J.; Kleer, C.G.; et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res.* **2011**, *71*, 614–624. [CrossRef]
137. Kalluri, R. The biology and function of fibroblasts in cancer. *Nat. Rev. Cancer* **2016**, *16*, 582–598. [CrossRef]
138. Rudnick, J.A.; Arendt, L.M.; Klebba, I.; Hinds, J.W.; Iyer, V.; Gupta, P.B.; Naber, S.P.; Kuperwasser, C. Functional heterogeneity of breast fibroblasts is defined by a prostaglandin secretory phenotype that promotes expansion of cancer-stem like cells. *PLoS ONE* **2011**, *6*, e24605. [CrossRef]
139. Tsuyada, A.; Chow, A.; Wu, J.; Somlo, G.; Chu, P.; Loera, S.; Luu, T.; Li, A.X.; Wu, X.; Ye, W.; et al. Ccl2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Cancer Res.* **2012**, *72*, 2768–2779. [CrossRef]
140. Al-Hajj, M.; Wicha, M.S.; Benito-Hernandez, A.; Morrison, S.J.; Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3983–3988. [CrossRef]
141. Charafe-Jauffret, E.; Ginestier, C.; Iovino, F.; Wicinski, J.; Cervera, N.; Finetti, P.; Hur, M.H.; Diebel, M.E.; Monville, F.; Dutcher, J.; et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res.* **2009**, *69*, 1302–1313. [CrossRef]
142. Schott, A.F.; Goldstein, L.J.; Cristofanilli, M.; Ruffini, P.A.; McCanna, S.; Reuben, J.M.; Perez, R.P.; Kato, G.; Wicha, M. Phase ib pilot study to evaluate reparixin in combination with weekly paclitaxel in patients with her-2-negative metastatic breast cancer. *Clin. Cancer Res.* **2017**, *23*, 5358–5365. [CrossRef]
143. Papageorgis, P.; Stylianopoulos, T. Role of tgfbeta in regulation of the tumor microenvironment and drug delivery (review). *Int. J. Oncol.* **2015**, *46*, 933–943. [CrossRef]
144. Goldstein, R.H.; Reagan, M.R.; Anderson, K.; Kaplan, D.L.; Rosenblatt, M. Human bone marrow-derived mscs can home to orthotopic breast cancer tumors and promote bone metastasis. *Cancer Res.* **2010**, *70*, 10044–10050. [CrossRef]
145. Shigdar, S.; Li, Y.; Bhattacharya, S.; O'Connor, M.; Pu, C.; Lin, J.; Wang, T.; Xiang, D.; Kong, L.; Wei, M.Q.; et al. Inflammation and cancer stem cells. *Cancer Lett.* **2014**, *345*, 271–278. [CrossRef]
146. Chen, Y.; Tan, W.; Wang, C. Tumor-associated macrophage-derived cytokines enhance cancer stem-like characteristics through epithelial-mesenchymal transition. *Onco Targets Ther.* **2018**, *11*, 3817–3826. [CrossRef]
147. Sullivan, N.J.; Sasser, A.K.; Axel, A.E.; Vesuna, F.; Raman, V.; Ramirez, N.; Oberyszyn, T.M.; Hall, B.M. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. *Oncogene* **2009**, *28*, 2940–2947. [CrossRef]
148. Kulbe, H.; Chakravarty, P.; Leinster, D.A.; Charles, K.A.; Kwong, J.; Thompson, R.G.; Coward, J.I.; Schioppa, T.; Robinson, S.C.; Gallagher, W.M.; et al. A dynamic inflammatory cytokine network in the human ovarian cancer microenvironment. *Cancer Res.* **2012**, *72*, 66–75. [CrossRef]
149. Wynn, T.A.; Chawla, A.; Pollard, J.W. Macrophage biology in development, homeostasis and disease. *Nature* **2013**, *496*, 445–455. [CrossRef]
150. Coussens, L.M.; Zitvogel, L.; Palucka, A.K. Neutralizing tumor-promoting chronic inflammation: A magic bullet? *Science* **2013**, *339*, 286–291. [CrossRef]
151. Gilkes, D.M.; Semenza, G.L.; Wirtz, D. Hypoxia and the extracellular matrix: Drivers of tumour metastasis. *Nat. Rev. Cancer* **2014**, *14*, 430–439. [CrossRef]
152. Spill, F.; Reynolds, D.S.; Kamm, R.D.; Zaman, M.H. Impact of the physical microenvironment on tumor progression and metastasis. *Curr. Opin. Biotechnol.* **2016**, *40*, 41–48. [CrossRef]
153. Lin, Q.; Yun, Z. Impact of the hypoxic tumor microenvironment on the regulation of cancer stem cell characteristics. *Cancer Biol. Ther.* **2010**, *9*, 949–956. [CrossRef]

154. Simon, M.C.; Keith, B. The role of oxygen availability in embryonic development and stem cell function. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 285–296. [CrossRef]
155. Yun, Z.; Lin, Q. Hypoxia and regulation of cancer cell stemness. *Adv. Exp. Med. Biol.* **2014**, *772*, 41–53.
156. Mohyeldin, A.; Garzon-Muvdi, T.; Quinones-Hinojosa, A. Oxygen in stem cell biology: A critical component of the stem cell niche. *Cell Stem Cell* **2010**, *7*, 150–161. [CrossRef]
157. Lin, Q.; Lee, Y.J.; Yun, Z. Differentiation arrest by hypoxia. *J. Biol. Chem.* **2006**, *281*, 30678–30683. [CrossRef]
158. Carcereri de Prati, A.; Butturini, E.; Rigo, A.; Oppici, E.; Rossin, M.; Boriero, D.; Mariotto, S. Metastatic breast cancer cells enter into dormant state and express cancer stem cells phenotype under chronic hypoxia. *J. Cell. Biochem.* **2017**, *118*, 3237–3248. [CrossRef]
159. Louie, E.; Nik, S.; Chen, J.S.; Schmidt, M.; Song, B.; Pacson, C.; Chen, X.F.; Park, S.; Ju, J.; Chen, E.I. Identification of a stem-like cell population by exposing metastatic breast cancer cell lines to repetitive cycles of hypoxia and reoxygenation. *Breast Cancer Res.* **2010**, *12*, R94. [CrossRef]
160. Lan, J.; Lu, H.; Samanta, D.; Salman, S.; Lu, Y.; Semenza, G.L. Hypoxia-inducible factor 1-dependent expression of adenosine receptor 2b promotes breast cancer stem cell enrichment. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E9640–E9648. [CrossRef]
161. Conley, S.J.; Gheordunescu, E.; Kakarala, P.; Newman, B.; Korkaya, H.; Heath, A.N.; Clouthier, S.G.; Wicha, M.S. Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 2784–2789. [CrossRef]
162. Yan, Y.; Liu, F.; Han, L.; Zhao, L.; Chen, J.; Olopade, O.I.; He, M.; Wei, M. Hif-2alpha promotes conversion to a stem cell phenotype and induces chemoresistance in breast cancer cells by activating wnt and notch pathways. *J. Exp. Clin. Cancer Res.* **2018**, *37*, 256. [CrossRef]
163. Balamurugan, K.; Mendoza-Villanueva, D.; Sharan, S.; Summers, G.H.; Dobrolecki, L.E.; Lewis, M.T.; Sterneck, E. C/ebpdelta links il-6 and hif-1 signaling to promote breast cancer stem cell-associated phenotypes. *Oncogene* **2019**, *38*, 3765–3780. [CrossRef]
164. Jacobsson, H.; Harrison, H.; Hughes, E.; Persson, E.; Rhost, S.; Fitzpatrick, P.; Gustafsson, A.; Andersson, D.; Gregersson, P.; Magnusson, Y.; et al. Hypoxia-induced secretion stimulates breast cancer stem cell regulatory signalling pathways. *Mol. Oncol.* **2019**. [CrossRef]
165. Divella, R.; De Luca, R.; Abbate, I.; Naglieri, E.; Daniele, A. Obesity and cancer: The role of adipose tissue and adipo-cytokines-induced chronic inflammation. *J. Cancer* **2016**, *7*, 2346–2359. [CrossRef]
166. Seo, B.R.; Bhardwaj, P.; Choi, S.; Gonzalez, J.; Andresen Eguiluz, R.C.; Wang, K.; Mohanan, S.; Morris, P.G.; Du, B.; Zhou, X.K.; et al. Obesity-dependent changes in interstitial ecm mechanics promote breast tumorigenesis. *Sci. Transl. Med.* **2015**, *7*, 301ra130. [CrossRef]
167. Massa, M.; Gasparini, S.; Baldelli, I.; Scarabelli, L.; Santi, P.; Quarto, R.; Repaci, E. Interaction between breast cancer cells and adipose tissue cells derived from fat grafting. *Aesthet. Surg. J.* **2016**, *36*, 358–363. [CrossRef]
168. Tang, K.D.; Liu, J.; Jovanovic, L.; An, J.; Hill, M.M.; Vela, I.; Lee, T.K.; Ma, S.; Nelson, C.; Russell, P.J.; et al. Adipocytes promote prostate cancer stem cell self-renewal through amplification of the cholecystokinin autocrine loop. *Oncotarget* **2016**, *7*, 4939–4948. [CrossRef]
169. Elliott, B.E.; Tam, S.P.; Dexter, D.; Chen, Z.Q. Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: Effect of estrogen and progesterone. *Int. J. Cancer* **1992**, *51*, 416–424. [CrossRef]
170. Iyengar, P.; Combs, T.P.; Shah, S.J.; Gouon-Evans, V.; Pollard, J.W.; Albanese, C.; Flanagan, L.; Tenniswood, M.P.; Guha, C.; Lisanti, M.P.; et al. Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* **2003**, *22*, 6408–6423. [CrossRef]
171. Wang, S.; Su, X.; Xu, M.; Xiao, X.; Li, X.; Li, H.; Keating, A.; Zhao, R.C. Exosomes secreted by mesenchymal stromal/stem cell-derived adipocytes promote breast cancer cell growth via activation of hippo signaling pathway. *Stem Cell Res. Ther.* **2019**, *10*, 117. [CrossRef]
172. Worner, P.M.; Schachtele, D.J.; Barabadi, Z.; Srivastav, S.; Chandrasekar, B.; Izadpanah, R.; Alt, E.U. Breast tumor microenvironment can transform naive mesenchymal stem cells into tumor-forming cells in nude mice. *Stem Cells Dev.* **2019**, *28*, 341–352. [CrossRef]
173. Muehlberg, F.L.; Song, Y.H.; Krohn, A.; Pinilla, S.P.; Droll, L.H.; Leng, X.; Seidensticker, M.; Ricke, J.; Altman, A.M.; Devarajan, E.; et al. Tissue-resident stem cells promote breast cancer growth and metastasis. *Carcinogenesis* **2009**, *30*, 589–597. [CrossRef]

174. Mandel, K.; Yang, Y.; Schambach, A.; Glage, S.; Otte, A.; Hass, R. Mesenchymal stem cells directly interact with breast cancer cells and promote tumor cell growth in vitro and in vivo. *Stem Cells Dev.* **2013**, *22*, 3114–3127. [CrossRef]
175. Chen, D.R.; Lu, D.Y.; Lin, H.Y.; Yeh, W.L. Mesenchymal stem cell-induced doxorubicin resistance in triple negative breast cancer. *Biomed Res. Int.* **2014**, *2014*, 532161. [CrossRef]
176. Chatterjee, S.; Basak, P.; Buchel, E.; Safneck, J.; Murphy, L.C.; Mowat, M.; Kung, S.K.; Eirew, P.; Eaves, C.J.; Raouf, A. Breast cancers activate stromal fibroblast-induced suppression of progenitors in adjacent normal tissue. *Stem Cell Rep.* **2018**, *10*, 196–211. [CrossRef]
177. Ohlund, D.; Handly-Santana, A.; Biffi, G.; Elyada, E.; Almeida, A.S.; Ponz-Sarvise, M.; Corbo, V.; Oni, T.E.; Hearn, S.A.; Lee, E.J.; et al. Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J. Exp. Med.* **2017**, *214*, 579–596.
178. Sugimoto, H.; Mundel, T.M.; Kieran, M.W.; Kalluri, R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol. Ther.* **2006**, *5*, 1640–1646. [CrossRef]
179. Al-Khalaf, H.H.; Ghebeh, H.; Inass, R.; Aboussekhra, A. Senescent breast luminal cells promote carcinogenesis through interleukin-8-dependent activation of stromal fibroblasts. *Mol. Cell. Biol.* **2019**, *39*. [CrossRef]
180. Cazet, A.S.; Hui, M.N.; Elsworth, B.L.; Wu, S.Z.; Roden, D.; Chan, C.L.; Skhinas, J.N.; Collot, R.; Yang, J.; Harvey, K.; et al. Targeting stromal remodeling and cancer stem cell plasticity overcomes chemoresistance in triple negative breast cancer. *Nat. Commun.* **2018**, *9*, 2897. [CrossRef]
181. Bartoschek, M.; Oskolkov, N.; Bocci, M.; Lovrot, J.; Larsson, C.; Sommarin, M.; Madsen, C.D.; Lindgren, D.; Pekar, G.; Karlsson, G.; et al. Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell rna sequencing. *Nat. Commun.* **2018**, *9*, 5150. [CrossRef]
182. Costa, A.; Kieffer, Y.; Scholer-Dahirel, A.; Pelon, F.; Bourachot, B.; Cardon, M.; Sirven, P.; Magagna, I.; Fuhrmann, L.; Bernard, C.; et al. Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. *Cancer Cell* **2018**, *33*, 463–479.e410. [CrossRef]
183. Su, S.; Chen, J.; Yao, H.; Liu, J.; Yu, S.; Lao, L.; Wang, M.; Luo, M.; Xing, Y.; Chen, F.; et al. Cd10(+)gpr77(+) cancer-associated fibroblasts promote cancer formation and chemoresistance by sustaining cancer stemness. *Cell* **2018**, *172*, 841–856.e816. [CrossRef]
184. Bourguignon, L.Y.; Peyrollier, K.; Xia, W.; Gilad, E. Hyaluronan-cd44 interaction activates stem cell marker nanog, stat-3-mediated mdr1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. *J. Biol. Chem.* **2008**, *283*, 17635–17651. [CrossRef]
185. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2018. *CA Cancer J. Clin.* **2018**, *68*, 7–30. [CrossRef]
186. Pusztai, L.; Mazouni, C.; Anderson, K.; Wu, Y.; Symmans, W.F. Molecular classification of breast cancer: Limitations and potential. *Oncologist* **2006**, *11*, 868–877. [CrossRef]
187. Bianchini, G.; Balko, J.M.; Mayer, I.A.; Sanders, M.E.; Gianni, L. Triple-negative breast cancer: Challenges and opportunities of a heterogeneous disease. *Nat. Rev. Clin. Oncol.* **2016**, *13*, 674–690. [CrossRef]
188. Honeth, G.; Bendahl, P.O.; Ringner, M.; Saal, L.H.; Gruvberger-Saal, S.K.; Lovgren, K.; Grabau, D.; Ferno, M.; Borg, A.; Hegardt, C. The cd44+/cd24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res.* **2008**, *10*, R53. [CrossRef]
189. O'Conor, C.J.; Chen, T.; Gonzalez, I.; Cao, D.; Peng, Y. Cancer stem cells in triple-negative breast cancer: A potential target and prognostic marker. *Biomark Med.* **2018**, *12*, 813–820. [CrossRef]
190. Ma, F.; Li, H.; Wang, H.; Shi, X.; Fan, Y.; Ding, X.; Lin, C.; Zhan, Q.; Qian, H.; Xu, B. Enriched cd44(+)/cd24(−) population drives the aggressive phenotypes presented in triple-negative breast cancer (tnbc). *Cancer Lett.* **2014**, *353*, 153–159. [CrossRef]
191. Ithimakin, S.; Day, K.C.; Malik, F.; Zen, Q.; Dawsey, S.J.; Bersano-Begey, T.F.; Quraishi, A.A.; Ignatoski, K.W.; Daignault, S.; Davis, A.; et al. Her2 drives luminal breast cancer stem cells in the absence of her2 amplification: Implications for efficacy of adjuvant trastuzumab. *Cancer Res.* **2013**, *73*, 1635–1646. [CrossRef]
192. Korkaya, H.; Paulson, A.; Iovino, F.; Wicha, M.S. Her2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene* **2008**, *27*, 6120–6130. [CrossRef]
193. Benoit, Y.D. Identification of novel molecules targeting cancer stem cells. *Methods Mol. Biol.* **2018**, *1765*, 333–347.
194. Stockler, M.; Wilcken, N.R.; Ghersi, D.; Simes, R.J. Systematic reviews of chemotherapy and endocrine therapy in metastatic breast cancer. *Cancer Treat. Rev.* **2000**, *26*, 151–168. [CrossRef]

195. Ginestier, C.; Liu, S.; Diebel, M.E.; Korkaya, H.; Luo, M.; Brown, M.; Wicinski, J.; Cabaud, O.; Charafe-Jauffret, E.; Birnbaum, D.; et al. Cxcr1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J. Clin. Investig.* **2010**, *120*, 485–497. [CrossRef]
196. Wang, Y.; Zong, X.; Mitra, S.; Mitra, A.K.; Matei, D.; Nephew, K.P. Il-6 mediates platinum-induced enrichment of ovarian cancer stem cells. *JCI Insight* **2018**, *3*. [CrossRef]
197. Ogawa, H.; Koyanagi-Aoi, M.; Otani, K.; Zen, Y.; Maniwa, Y.; Aoi, T. Interleukin-6 blockade attenuates lung cancer tissue construction integrated by cancer stem cells. *Sci. Rep.* **2017**, *7*, 12317. [CrossRef]
198. Ikushima, H.; Todo, T.; Ino, Y.; Takahashi, M.; Miyazawa, K.; Miyazono, K. Autocrine tgf-beta signaling maintains tumorigenicity of glioma-initiating cells through sry-related hmg-box factors. *Cell Stem Cell* **2009**, *5*, 504–514. [CrossRef]

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Review

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

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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 ($ER^-/PR^-/HER^+$) 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 m^2 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 macrometasases 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]
	TGFβ	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; TGFβ, Transforming Growth Factor beta; ANGPTL4, Angiopoiten-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 Metallopeptidase 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-1α 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-κB pathway, resulting in the expression of the pro-inflammatory markers IL-6, TNFα, 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 ($10^{5}{\cdot}mL^{-1}$) [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-κB 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 heterogenous population of cells that vary in origin and are characterized by the expression of several markers including αSMA 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 β3 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 (TNFα, 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-α1 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 Carbonin Anyhydrase 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.

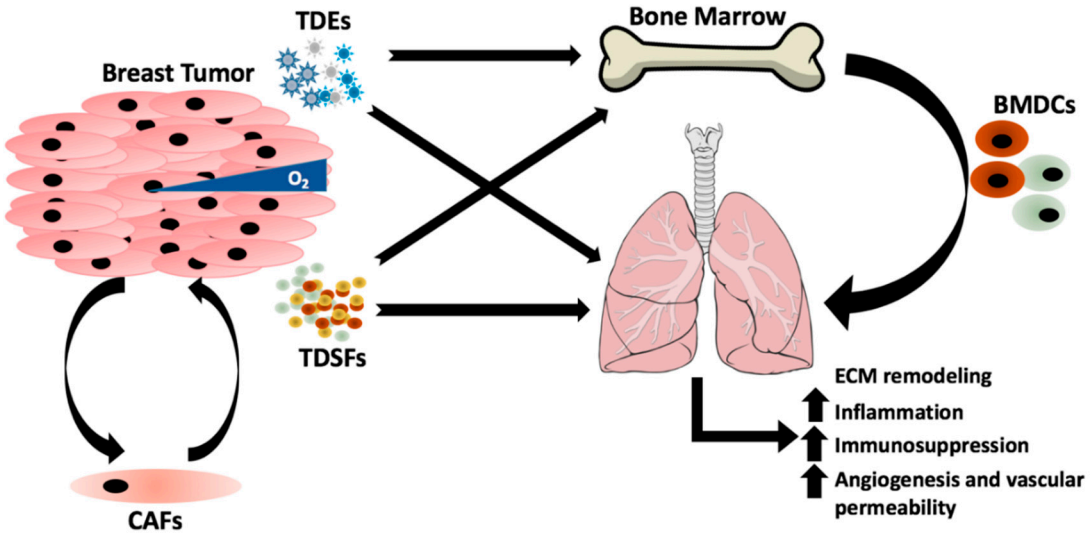

Figure 1. The underlying mechanisms that dictate the organ colonized by breast cancer are complex and influenced by many factors. Breast primary tumors regulate and prime the lung for metastasis by the secretion of tumor-derived exosomes (TDEs) and tumor-derived secreted factors (TDSFs), which target the bone marrow for recruitment of bone marrow-derived cells (BMDCs) 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.

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Abbreviations

αSMA	Alpha Smooth Muscle Actin
BMDCs	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-1α	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

References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef]
2. Canadian Cancer Society. Available online: http://www.cancer.ca/en/cancer-information/cancer-type/breast/statistics/?region=on (accessed on 2 January 2019).
3. Chaffer, C.L.; Weinberg, R.A. A Perspective on Cancer Cell Metastasis. *Science* **2011**, *331*, 1559–1564. [CrossRef]
4. Lambert, A.W.; Pattabiraman, D.R.; Weinberg, R.A. Emerging Biological Principles of Metastasis. *Cell* **2017**, *168*, 670–691. [CrossRef] [PubMed]
5. Hess, K.R.; Varadhachary, G.R.; Taylor, S.H.; Wei, W.; Raber, M.N.; Lenzi, R.; Abbruzzese, J.L. Metastatic patterns in adenocarcinoma. *Cancer* **2006**, *106*, 1624–1633. [CrossRef] [PubMed]
6. Wu, Q.; Li, J.; Zhu, S.; Wu, J.; Chen, C.; Liu, Q.; Wei, W.; Zhang, Y.; Sun, S. Breast cancer subtypes predict the preferential site of distant metastases: A SEER based study. *Oncotarget* **2017**, *8*, 27990–27996. [CrossRef]
7. Schlappack, O.K.; Baur, M.; Steger, G.; Dittrich, C.; Moser, K. The clinical course of lung metastases from breast cancer. *J. Mol. Med.* **1988**, *66*, 790–795. [CrossRef]
8. Xiao, W.; Zheng, S.; Liu, P.; Zou, Y.; Xie, X.; Yu, P.; Tang, H.; Xie, X. Risk factors and survival outcomes in patients with breast cancer and lung metastasis: A population-based study. *Cancer Med.* **2018**, *7*, 922–930. [CrossRef] [PubMed]
9. Jin, L.; Han, B.; Siegel, E.; Cui, Y.; Giuliano, A.; Cui, X. Breast cancer lung metastasis: Molecular biology and therapeutic implications. *Cancer Biol. Ther.* **2018**, *19*, 858–868. [CrossRef]
10. Perou, C.M.; Sørlie, T.; Eisen, M.B.; Van De Rijn, M.; Jeffrey, S.S.; Rees, C.A.; . Fluge, Ø. Molecular portraits of human breast tumours. *Nature* **2000**, *406*, 747. [CrossRef] [PubMed]
11. Luzzi, K.J.; MacDonald, I.C.; Schmidt, E.E.; Kerkvliet, N.; Morris, V.L.; Chambers, A.F.; Groom, A.C. Multistep nature of metastatic inefficiency: Dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am. J. Pathol.* **1998**, *153*, 865–873. [CrossRef]
12. Paget, S. The distribution of secondary growths in cancer of the breast. *Lancet* **1889**, *133*, 571–573. [CrossRef]
13. Chu, J.E.; Xia, Y.; Chin-Yee, B.; Goodale, D.; Croker, A.K.; Allan, A.L. Lung-Derived Factors Mediate Breast Cancer Cell Migration through CD44 Receptor-Ligand Interactions in a Novel Ex Vivo System for Analysis of Organ-Specific Soluble Proteins12. *Neoplasia* **2014**, *16*, 180–191. [CrossRef]
14. Ewing, J. *Neoplastics*, 3rd ed.; Saunders: Philadelphia, PA, USA, 1928; pp. 77–89.

15. Macdonald, I.C.; Groom, A.C.; Chambers, A.F. Cancer spread and micrometastasis development: Quantitative approaches for in vivo models. *BioEssays* **2002**, *24*, 885–893. [CrossRef] [PubMed]
16. Stott, S.L.; Hsu, C.-H.; Tsukrov, D.I.; Yu, M.; Miyamoto, D.T.; Waltman, B.A.; Rothenberg, S.M.; Shah, A.M.; Smas, M.E.; Korir, G.K.; et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 18392–18397. [CrossRef]
17. Koop, S.; Macdonald, I.C.; Luzzi, K.; Schmidt, E.; Morris, V.L.; Grattan, M.; Khokha, R.; Chambers, A.F.; Groom, A.C. Fate of melanoma cells entering the microcirculation: Over 80% survive and extravasate. *Cancer Res.* **1995**, *55*, 2520–2523.
18. Costa-Silva, B.; Aiello, N.M.; Ocean, A.J.; Singh, S.; Zhang, H.; Thakur, B.K.; Becker, A.; Hoshino, A.; Mark, M.T.; Molina, H.; et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat. Cell Biol.* **2015**, *17*, 816–826. [CrossRef]
19. Yu, Z.; Zhao, S.; Ren, L.; Wang, L.; Chen, Z.; Hoffman, R.M.; Zhou, J. Pancreatic cancer-derived exosomes promote tumor metastasis and liver pre-metastatic niche formation. *Oncotarget* **2017**, *8*, 63461–63483. [CrossRef]
20. Sánchez, C.A.; Andahur, E.I.; Valenzuela, R.; Castellón, E.A.; Fullá, J.A.; Ramos, C.G.; Triviño, J.C. Exosomes from bulk and stem cells from human prostate cancer have a differential microRNA content that contributes cooperatively over local and pre-metastatic niche. *Oncotarget* **2015**, *7*, 3993–4008. [CrossRef] [PubMed]
21. Ubellacker, J.M.; McAllister, S.S. The unresolved role of systemic factors in bone metastasis. *J. Bone Oncol.* **2016**, *5*, 96–99. [CrossRef]
22. Liu, Y.; Cao, X. Characteristics and Significance of the Pre-metastatic Niche. *Cancer Cell* **2016**, *30*, 668–681. [CrossRef] [PubMed]
23. Kaplan, R.N.; Riba, R.D.; Zacharoulis, S.; Bramley, A.H.; Vincent, L.; Costa, C.; Macdonald, D.D.; Jin, D.K.; Shido, K.; Kerns, S.A.; et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nat. Cell Biol.* **2005**, *438*, 820–827. [CrossRef]
24. Yang, Y.; Li, C.-W.; Chan, L.-C.; Wei, Y.; Hsu, J.-M.; Xia, W.; Cha, J.-H.; Hou, J.; Hsu, J.L.; Sun, L.; et al. Exosomal PD-L1 harbors active defense function to suppress T cell killing of breast cancer cells and promote tumor growth. *Cell Res.* **2018**, *28*, 862–864. [CrossRef]
25. Fong, M.Y.; Zhou, W.; Liu, L.; Alontaga, A.Y.; Chandra, M.; Ashby, J.; Chow, A.; O'Connor, S.T.F.; Li, S.; Chin, A.R.; et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nat. Cell Biol.* **2015**, *17*, 183–194. [CrossRef] [PubMed]
26. Zhou, W.; Fong, M.Y.; Min, Y.; Somlo, G.; Liu, L.; Palomares, M.R.; Yü, Y.; Chow, A.; O'Connor, S.T.F.; Chin, A.R.; et al. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* **2014**, *25*, 501–515. [CrossRef]
27. Hoshino, A.; Costa-Silva, B.; Shen, T.-L.; Rodrigues, G.; Hashimoto, A.; Mark, M.T.; Molina, H.; Kohsaka, S.; Di Giannatale, A.; Ceder, S.; et al. Tumour exosome integrins determine organotropic metastasis. *Nat. Cell Biol.* **2015**, *527*, 329–335. [CrossRef] [PubMed]
28. Joo, Y.N.; Jin, H.; Eun, S.Y.; Park, S.W.; Chang, K.C.; Kim, H.J. P2Y2R activation by nucleotides released from the highly metastatic breast cancer cell contributes to pre-metastatic niche formation by mediating lysyl oxidase secretion, collagen crosslinking, and monocyte recruitment. *Oncotarget* **2014**, *5*, 9322–9334. [CrossRef]
29. Padua, D.; Zhang, X.H.; Wang, Q.; Nadal, C.; Gerald, W.L.; Gomis, R.R.; Massagué, J. TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **2008**, *133*, 66–77. [CrossRef] [PubMed]
30. Chen, Q.; Zhang, X.H.F.; Massagué, J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. *Cancer Cell* **2011**, *20*, 538–549. [CrossRef]
31. Eisenblaetter, M.; Flores-Borja, F.; Lee, J.J.; Wefers, C.; Smith, H.; Hueting, R.; Cooper, M.S.; Blower, P.J.; Patel, D.; Rodríguez-Justo, M.; et al. Visualization of Tumor-Immune Interaction—Target-Specific Imaging of S100A8/A9 Reveals Pre-Metastatic Niche Establishment. *Theranostics* **2017**, *7*, 2392–2401. [CrossRef] [PubMed]
32. Müller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M.E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S.N.; et al. Involvement of chemokine receptors in breast cancer metastasis. *Nat. Cell Biol.* **2001**, *410*, 50–56. [CrossRef]
33. Furusato, B.; Mohamed, A.; Uhlén, M.; Rhim, J.S. CXCR4 and cancer. *Pathol. Int.* **2010**, *60*, 497–505. [CrossRef]

34. Erler, J.T.; Bennewith, K.L.; Cox, T.R.; Lang, G.; Bird, D.; Koong, A.; Le, Q.-T.; Giaccia, A.J. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the pre-metastatic niche. *Cancer Cell* **2009**, *15*, 35–44. [CrossRef]
35. Lock, F.; McDonald, P.; Lou, Y.; Serrano, I.; Chafe, S.; Ostlund, C.; Dedhar, S. Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene* **2013**, *32*, 5210. [CrossRef]
36. Srivastava, K.; Hu, J.; Korn, C.; Savant, S.; Teichert, M.; Kapel, S.S.; Augustin, H.G. Postsurgical adjuvant tumor therapy by combining anti-angiopoietin-2 and metronomic chemotherapy limits metastatic growth. *Cancer Cell* **2014**, *26*, 880–895. [CrossRef]
37. Oskarsson, T.; Acharyya, S.; Zhang, X.H.; Vanharanta, S.; Tavazoie, S.F.; Morris, P.G.; Massagué, J. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat. Med.* **2011**, *17*, 867. [CrossRef]
38. Calvo, A.; Catena, R.; Noble, M.S.; Carbott, D.; Gil-Bazo, I.; Gonzalez-Moreno, O.; Huh, J.-I.; Sharp, R.; Qiu, T.-H.; Anver, M.R.; et al. Identification of VEGF-regulated genes associated with increased lung metastatic potential: Functional involvement of tenascin-C in tumor growth and lung metastasis. *Oncogene* **2008**, *27*, 5373–5384. [CrossRef] [PubMed]
39. Morishita, R.; Kyutoku, M.; Taniyama, Y.; Katsuragi, N.; Shimizu, H.; Kunugiza, Y.; Iekushi, K.; Koibuchi, N.; Sanada, F.; Oshita, Y. Role of periostin in cancer progression and metastasis: Inhibition of breast cancer progression and metastasis by anti-periostin antibody in a murine model. *Int. J. Mol. Med.* **2011**, *28*, 181–186. [CrossRef] [PubMed]
40. Malik, G.; Knowles, L.M.; Dhir, R.; Xu, S.; Yang, S.; Ruoslahti, E.; Pilch, J. Plasma fibronectin promotes lung metastasis by contributions to fibrin clots and tumor cell invasion. *Cancer Res.* **2010**, *70*, 4327–4334. [CrossRef] [PubMed]
41. Abdel-Ghany, M.; Pauli, B.U.; Elble, R.C.; Cheng, H.-C. Lung Endothelial Dipeptidyl Peptidase IV Promotes Adhesion and Metastasis of Rat Breast Cancer Cells via Tumor Cell Surface-associated Fibronectin*. *J. Biol. Chem.* **1998**, *273*, 24207–24215.
42. Wang, T.-H.; Hsia, S.-M.; Shieh, T.-M.; Yang, L. Lysyl Oxidase and the Tumor Microenvironment. *Int. J. Mol. Sci.* **2016**, *18*, 62. [CrossRef]
43. Qian, B.-Z.; Li, J.; Zhang, H.; Kitamura, T.; Zhang, J.; Campion, L.R.; Kaiser, E.A.; Snyder, L.A.; Pollard, J.W. CCL2 recruits inflammatory monocytes to facilitate breast tumor metastasis. *Nat. Cell Biol.* **2011**, *475*, 222–225.
44. Bates, A.L.; Pickup, M.W.; Hallett, M.A.; Dozier, E.A.; Thomas, S.; Fingleton, B. Stromal matrix metalloproteinase 2 regulates collagen expression and promotes the outgrowth of experimental metastases. *J. Pathol.* **2015**, *235*, 773–783. [CrossRef] [PubMed]
45. Scherz-Shouval, R.; Santagata, S.; Mendillo, M.L.; Sholl, L.M.; Ben-Aharon, I.; Beck, A.H.; Dias-Santagata, D.; Koeva, M.; Stemmer, S.M.; Whitesell, L.; et al. The reprogramming of tumor stroma by HSF1 is a potent enabler of malignancy. *Cell* **2014**, *158*, 564–578. [CrossRef]
46. Gebhardt, C.; Németh, J.; Angel, P.; Hess, J. S100A8 and S100A9 in inflammation and cancer. *Biochem. Pharmacol.* **2006**, *72*, 1622–1631. [CrossRef] [PubMed]
47. Yáñez-Mó, M.; Siljander, P.R.-M.; Andreu, Z.; Zavec, A.B.; Borràs, F.E.; Buzas, E.I.; Buzas, K.; Casal, E.; Cappello, F.; Carvalho, J.; et al. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **2015**, *4*, 27066. [CrossRef]
48. Liu, Y.; Gu, Y.; Han, Y.; Zhang, Q.; Jiang, Z.; Zhang, X.; Huang, B.; Xu, X.; Zheng, J.; Cao, X. Tumor Exosomal RNAs Promote Lung Pre-metastatic Niche Formation by Activating Alveolar Epithelial TLR3 to Recruit Neutrophils. *Cancer Cell* **2016**, *30*, 243–256. [CrossRef] [PubMed]
49. Fang, T.; Lv, H.; Lv, G.; Li, T.; Wang, C.; Han, Q.; Yu, L.; Su, B.; Guo, L.; Huang, S.; et al. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nat. Commun.* **2018**, *9*, 191. [CrossRef]
50. Huang, H.; Zheng, X.; Cai, C.; Yao, Z.; Lu, S.; Meng, X.; Miao, Y.; He, Z.; Cai, C.; Zou, F. Exosomes derived from breast cancer lung metastasis subpopulations promote tumor self-seeding. *Biochem. Biophys. Commun.* **2018**, *503*, 242–248. [CrossRef] [PubMed]
51. Colombo, M.; Raposo, G.; Théry, C. Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annu. Cell Dev. Biol.* **2014**, *30*, 255–289. [CrossRef] [PubMed]

52. Shao, C.; Yang, F.; Miao, S.; Liu, W.; Wang, C.; Shu, Y.; Shen, H. Role of hypoxia-induced exosomes in tumor biology. *Mol. Cancer Res.* **2018**, *17*, 120. [CrossRef]
53. Kore, R.A.; Edmondson, J.L.; Jenkins, S.V.; Jamshidi-Parsian, A.; Dings, R.P.; Reyna, N.S.; Griffin, R.J. Hypoxia-derived exosomes induce putative altered pathways in biosynthesis and ion regulatory channels in glioblastoma cells. *Biochem. Biophys. Rep.* **2018**, *14*, 104–113. [CrossRef]
54. King, H.W.; Michael, M.Z.; Gleadle, J.M. Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* **2012**, *12*, 421. [CrossRef]
55. Santos, J.C.; Lima, N.D.S.; Sarian, L.O.; Matheu, A.; Ribeiro, M.L.; Derchain, S.F.M. Exosome-mediated breast cancer chemoresistance via miR-155 transfer. *Sci. Rep.* **2018**, *8*, 829. [CrossRef]
56. Peng, J.; Wang, W.; Hua, S.; Liu, L. Roles of Extracellular Vesicles in Metastatic Breast Cancer. *Breast Cancer* **2018**, *12*. [CrossRef]
57. Liu, Y.; Cao, X. Immunosuppressive cells in tumor immune escape and metastasis. *J. Mol. Med.* **2016**, *94*, 509–522. [CrossRef]
58. Sangaletti, S.; Tripodo, C.; Sandri, S.; Torselli, I.; Vitali, C.; Ratti, C.; Botti, L.; Burocchi, A.; Porcasi, R.; Tomirotti, A.M.; et al. Osteopontin Shapes Immunosuppression in the Metastatic Niche. *Cancer Res.* **2014**, *74*, 4706–4719. [CrossRef]
59. Smith, H.A.; Kang, Y. The Metastasis-Promoting Roles of Tumor-Associated Immune Cells. *J. Mol. Med.* **2013**, *91*, 411–429. [CrossRef] [PubMed]
60. D'Amico, L.; Roato, I.; D' Amico, L. The Impact of Immune System in Regulating Bone Metastasis Formation by Osteotropic Tumors. *J. Immunol.* **2015**, *2015*, 1–7. [CrossRef] [PubMed]
61. Wang, D.; Dubois, R.N. Immunosuppression associated with chronic inflammation in the tumor microenvironment. *CARCIN* **2015**, *36*, 1085–1093. [CrossRef] [PubMed]
62. Gordon, S.; Martinez-Pomares, L. Physiological roles of macrophages. *Pflugers Arch* **2017**, *469*, 365–374. [CrossRef] [PubMed]
63. Chow, A.; Zhou, W.; Liu, L.; Fong, M.Y.; Champer, J.; Van Haute, D.; Chin, A.R.; Ren, X.; Gugiu, B.G.; Meng, Z.; et al. Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF-κB. *Sci. Rep.* **2014**, *4*, 5750. [CrossRef]
64. Chen, Y.; Gou, X.; Kong, D.K.; Wang, X.; Wang, J.; Chen, Z.; Huang, C.; Zhou, J. EMMPRIN regulates tumor growth and metastasis by recruiting bone marrow-derived cells through paracrine signaling of SDF-1 and VEGF. *Oncotarget* **2015**, *6*, 32575–32585. [CrossRef]
65. Xie, X.; Guo, P.; Yu, H.; Wang, Y.; Chen, G. Abstract 101: Human bone marrow-derived endothelial progenitor cells promote the transformation of periostin-positive hepatocellular carcinoma cells into CD36-positive metastasis-initialing cells in pre-metastatic niche. *Cancer Res.* **2018**, *78*, 101. [CrossRef]
66. Giles, A.J.; Reid, C.M.; Evans, J.D.; Murgai, M.; Vicioso, Y.; Highfill, S.L.; Wexler, L. Activation of hematopoietic stem/progenitor cells promotes immunosuppression within the pre–metastatic niche. *Cancer Res.* **2016**, *76*, 1335–1347. [CrossRef] [PubMed]
67. Mareel, M.; Madani, I. Tumour-Associated Host Cells Participating at Invasion and Metastasis: Targets for Therapy? *Acta Chir. Belg.* **2006**, *106*, 635–640. [CrossRef] [PubMed]
68. Gabrilovich, D.I.; Nagaraj, S. Myeloid-derived-suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* **2009**, *9*, 162–174. [CrossRef]
69. Serafini, P.; Borrello, I.; Bronte, V. Myeloid suppressor cells in cancer: Recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin. Cancer Biol.* **2006**, *16*, 53–65. [CrossRef]
70. Yang, L.; DeBusk, L.M.; Fukuda, K.; Fingleton, B.; Green-Jarvis, B.; Shyr, Y.; Lin, P.C. Expansion of myeloid immune suppressor Gr+ CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* **2004**, *6*, 409–421. [CrossRef]
71. Xiang, X.; Poliakov, A.; Liu, C.; Liu, Y.; Deng, Z.B.; Wang, J.; Grizzle, W.E. Induction of myeloid-derived suppressor cells by tumor exosomes. *Int. J. Cancer* **2009**, *124*, 2621–2633. [CrossRef]
72. Peinado, H.; Alečković, M.; Lavotshkin, S.; Matei, I.; Costa-Silva, B.; Moreno-Bueno, G.; Hergueta-Redondo, M.; Williams, C.; García-Santos, G.; Ghajar, C.M.; et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* **2012**, *18*, 883–891. [CrossRef] [PubMed]

73. Berghuis, A.M.S.; Koffijberg, H.; Prakash, J.; Terstappen, L.W.M.M.; Ijzerman, M.J. Detecting Blood-Based Biomarkers in Metastatic Breast Cancer: A Systematic Review of Their Current Status and Clinical Utility. *Int. J. Mol. Sci.* **2017**, *18*, 363. [CrossRef]
74. Cree, I.A. Liquid biopsy for cancer patients: Principles and practice. *Pathogenesis* **2015**, *2*, 1–4. [CrossRef]
75. Palmirotta, R.; Lovero, D.; Cafforio, P.; Felici, C.; Mannavola, F.; Pellè, E.; Quaresmini, D.; Tucci, M.; Silvestris, F. Liquid biopsy of cancer: A multimodal diagnostic tool in clinical oncology. *Ther. Adv. Med. Oncol.* **2018**, *10*. [CrossRef]
76. Allan, A.L.; Keeney, M. Circulating tumor cell analysis: Technical and statistical considerations for application to the clinic. *J. Oncol.* **2010**, *2010*, 426218. [CrossRef]
77. Lowes, L.E.; Bratman, S.V.; Dittamore, R.; Done, S.; Kelley, S.O.; Mai, S.; Morin, R.D.; Wyatt, A.W.; Allan, A.L.; Marchetti, D. Circulating Tumor Cells (CTC) and Cell-Free DNA (cfDNA) Workshop 2016: Scientific Opportunities and Logistics for Cancer Clinical Trial Incorporation. *Int. J. Mol. Sci.* **2016**, *17*, 1505. [CrossRef]
78. Lv, Y.; Niu, Y.; Su, H.; Feng, A.; Zhang, Y. Role of Circulating Tumor Cell (CTC) Monitoring in Evaluating Prognosis of Triple-Negative Breast Cancer Patients in China. *Med. Sci.* **2017**, *23*, 3071–3079.
79. Martins, T.S.; Catita, J.; Rosa, I.M.; Silva, O.A.B.D.C.E.; Henriques, A.G. Exosome isolation from distinct biofluids using precipitation and column-based approaches. *PLoS ONE* **2018**, *13*, e0198820. [CrossRef]
80. Ko, J.; Carpenter, E.; Issadore, D. Detection and isolation of circulating exosomes and microvesicles for cancer monitoring and diagnostics using micro-/nano-based devices. *Analyst* **2017**, *141*, 450–460. [CrossRef]
81. Etayash, H.; McGee, A.R.; Kaur, K.; Thundat, T. Nanomechanical sandwich assay for multiple cancer biomarkers in breast cancer cell-derived exosomes. *Nanoscale* **2016**, *8*, 15137–15141. [CrossRef]
82. Zhai, L.Y.; Li, M.X.; Pan, W.L.; Chen, Y.; Li, M.M.; Pang, J.X.; Duan, W.J. In Situ Detection of Plasma Exosomal MicroRNA-1246 for Breast Cancer Diagnostics by a Au Nanoflare Probe. *ACS Appl. Mater. Interfaces* **2018**, *10*, 39478–39486. [CrossRef]
83. Abu Lila, A.S.; Kiwada, H.; Ishida, T. The accelerated blood clearance (ABC) phenomenon: Clinical challenge and approaches to manage. *J. Control. Release* **2013**, *172*, 38–47. [CrossRef]
84. Hayeshi, R.; Masimirembwa, C.; Mukanganyama, S.; Ungell, A.-L.B. The potential inhibitory effect of antiparasitic drugs and natural products on P-glycoprotein mediated efflux. *Eur. J. Pharm. Sci.* **2006**, *29*, 70–81. [CrossRef] [PubMed]
85. Bauer, K.S.; Karp, J.E.; Garimella, T.S.; Wu, S.; Tan, M.; Ross, D.D. A phase I and pharmacologic study of idarubicin, cytarabine, etoposide, and the multidrug resistance protein (MDR1/Pgp) inhibitor PSC-833 in patients with refractory leukemia. *Leuk. Res.* **2005**, *29*, 263–271. [CrossRef]
86. Pidaparti, M.; Bostrom, B. Comparison of allergic reactions to pegasparaginase given intravenously versus intramuscularly. *Pediatr. Blood Cancer* **2012**, *59*, 436–439. [CrossRef] [PubMed]
87. Kim, M.S.; Haney, M.J.; Zhao, Y.; Mahajan, V.; Deygen, I.; Klyachko, N.L.; Inskoe, E.; Piroyan, A.; Sokolsky, M.; Okolie, O.; et al. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine* **2016**, *12*, 655–664. [CrossRef]
88. Keklikoglou, I.; Cianciaruso, C.; Güç, E.; Squadrito, M.L.; Spring, L.M.; Tazzyman, S.; Cassará, A. Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models. *Nat. Cell Biol.* **2019**, *21*, 190. [CrossRef] [PubMed]
89. Vikas, P.; Borcherding, N.; Zhang, W. The clinical promise of immunotherapy in triple-negative breast cancer. *Cancer Manag.* **2018**, *10*, 6823–6833. [CrossRef]
90. Solinas, C.; Gombos, A.; Latifyan, S.; Piccart-Gebhart, M.; Kok, M.; Buisseret, L. Targeting immune checkpoints in breast cancer: An update of early results. *ESMO Open* **2017**, *2*, e000255. [CrossRef]
91. Chen, G.; Huang, A.C.; Zhang, W.; Zhang, G.; Wu, M.; Xu, W.; Yu, Z.; Yang, J.; Wang, B.; Sun, H.; et al. Exosomal PD-L1 Contributes to Immunosuppression and is Associated with anti-PD-1 Response. *Nat. Cell Biol.* **2018**, *560*, 382–386. [CrossRef] [PubMed]
92. Ricciardelli, C.; Sakko, A.J.; Ween, M.P.; Russell, D.L.; Horsfall, D.J. The biological role and regulation of versican levels in cancer. *Cancer Metastasis Rev.* **2009**, *28*, 233–245. [CrossRef]
93. Wang, M.; Zhao, J.; Zhang, L.; Wei, F.; Lian, Y.; Wu, Y.; Gong, Z.; Zhang, S.; Zhou, J.; Cao, K.; et al. Role of tumor microenvironment in tumorigenesis. *J. Cancer* **2017**, *8*, 761–773. [CrossRef]
94. Donnarumma, E.; Fiore, D.; Nappa, M.; Roscigno, G.; Adamo, A.; Iaboni, M.; Russo, V.; Affinito, A.; Puoti, I.; Quintavalle, C.; et al. Cancer-associated fibroblasts release exosomal microRNAs that dictate an aggressive phenotype in breast cancer. *Oncotarget* **2017**, *8*, 19592–19608. [CrossRef] [PubMed]

95. Luo, H.; Tu, G.; Liu, Z.; Liu, M. Cancer-associated fibroblasts: A multifaceted driver of breast cancer progression. *Cancer Lett.* **2015**, *361*, 155–163. [CrossRef] [PubMed]
96. Roswall, P.; Bocci, M.; Bartoschek, M.; Li, H.; Kristiansen, G.; Jansson, S.; Lehn, S.; Sjolund, J.; Reid, S.; Larsson, C.; et al. Microenvironmental control of breast cancer subtype elicited by paracrine platelet derived growth factor-CC signaling. *Nat. Med.* **2018**, *24*, 463–473. [CrossRef] [PubMed]
97. Shiga, K.; Hara, M.; Nagasaki, T.; Sato, T.; Takahashi, H.; Takeyama, H.; Lin, H.-J. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Mol. Cell. Basis Metastasis* **2015**, *7*, 2443–2458. [CrossRef]
98. Karagiannis, G.S.; Poutahidis, T.; Erdman, S.E.; Kirsch, R.; Riddell, R.H.; Diamandis, E.P. Cancer-Associated Fibroblasts Drive the Progression of Metastasis through both Paracrine and Mechanical Pressure on Cancer Tissue. *Mol. Cancer Res.* **2012**, *10*, 1403–1418. [CrossRef] [PubMed]
99. Wen, S.; Hou, Y.; Fu, L.; Xi, L.; Yang, D.; Zhao, M.; Liu, M. Cancer-associated fibroblast (CAF)-derived IL32 promotes breast cancer cell invasion and metastasis via integrin β3–p38 MAPK signalling. *Cancer Lett.* **2019**, *442*, 320–332. [CrossRef] [PubMed]
100. Hiratsuka, S.; Watanabe, A.; Aburatani, H.; Maru, Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat. Cell Biol.* **2006**, *8*, 1369–1375. [CrossRef] [PubMed]
101. Shangguan, L.; Ti, X.; Krause, U.; Hai, B.; Zhao, Y.; Yang, Z.; Liu, F. Inhibition of TGF-β/Smad Signaling by BAMBI Blocks Differentiation of Human Mesenchymal Stem Cells to Carcinoma-Associated Fibroblasts and Abolishes their Protumor Effects. *STEM CELLS* **2012**, *30*, 2810–2819. [CrossRef] [PubMed]
102. Peng, Y.; Li, Z.; Yang, P.; Newton, I.P.; Ren, H.; Zhang, L.; Wu, H.; Li, Z. Direct contacts with colon cancer cells regulate the differentiation of bone marrow mesenchymal stem cells into tumor associated fibroblasts. *Biochem. Biophys. Commun.* **2014**, *451*, 68–73. [CrossRef] [PubMed]
103. Raz, Y.; Cohen, N.; Shani, O.; Bell, R.E.; Novitskiy, S.V.; Abramovitz, L.; Levy, C.; Milyavsky, M.; Leider-Trejo, L.; Moses, H.L.; et al. Bone marrow–derived fibroblasts are a functionally distinct stromal cell population in breast cancer. *J. Exp. Med.* **2018**, *215*, 3075–3093. [CrossRef]
104. Habeck, M. Bone-marrow analysis predicts breast-cancer recurrence. *Mol. Med. Today* **2000**, *6*, 256–257. [CrossRef]
105. Mansi, J.L.; Berger, U.; McDonnell, T.; Pople, A.; Rayter, Z.; Gazet, J.C.; Coombes, R.C. The fate of bone marrow micrometastases in patients with primary breast cancer. *J. Clin. Oncol.* **1989**, *7*, 445–449. [CrossRef]
106. Naume, B.; Zhao, X.; Synnestvedt, M.; Borgen, E.; Russnes, H.G.; Lingjaerde, O.C.; Strømberg, M.; Wiedswang, G.; Kvalheim, G.; Kåresen, R.; et al. Presence of bone marrow micrometastasis is associated with different recurrence risk within molecular subtypes of breast cancer. *Mol. Oncol.* **2007**, *1*, 160–171. [CrossRef] [PubMed]
107. Patel, S.A.; Ramkissoon, S.H.; Bryan, M.; Pliner, L.F.; Dontu, G.; Patel, P.S.; Amiri, S.; Pine, S.R.; Rameshwar, P. Delineation of breast cancer cell hierarchy identifies the subset responsible for dormancy. *Sci. Rep.* **2012**, *2*, 906. [CrossRef]
108. Aguirre-Ghiso, J.A. Models, mechanisms and clinical evidence for cancer dormancy. *Nat. Rev. Cancer* **2007**, *7*, 834–846. [CrossRef] [PubMed]
109. Bliss, S.A.; Sinha, G.; Sandiford, O.A.; Williams, L.M.; Engelberth, D.J.; Guiro, K.; Isenalumhe, L.L.; Greco, S.J.; Ayer, S.; Bryan, M.; et al. Mesenchymal Stem Cell-Derived Exosomes Stimulate Cycling Quiescence and Early Breast Cancer Dormancy in Bone Marrow. *Cancer Res.* **2016**, *76*, 5832–5844. [CrossRef]
110. Liu, S.; Jiang, M.; Zhao, Q.; Li, S.; Peng, Y.; Zhang, P.; Han, M. Vascular endothelial growth factor plays a critical role in the formation of the pre-metastatic niche via prostaglandin E2. *Oncol. Rep.* **2014**, *32*, 2477–2484. [CrossRef] [PubMed]
111. Lou, Y.; McDonald, P.C.; Oloumi, A.; Chia, S.; Ahmadi, A.; Kyle, A.; Leung, S.; Huntsman, D.; Clarke, B.; Sutherland, B.W.; et al. Targeting Tumor Hypoxia: Suppression of Breast Tumor Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors. *Cancer Res.* **2011**, *71*, 3364–3376. [CrossRef]
112. Pacchiano, F.; Carta, F.; McDonald, P.C.; Lou, Y.; Vullo, D.; Scozzafava, A.; Dedhar, S.; Supuran, C.T. Ureido-Substituted Benzenesulfonamides Potently Inhibit Carbonic Anhydrase IX and Show Antimetastatic Activity in a Model of Breast Cancer Metastasis. *J. Med. Chem.* **2011**, *54*, 1896–1902. [CrossRef] [PubMed]

113. Touisni, N.; Maresca, A.; McDonald, P.C.; Lou, Y.; Scozzafava, A.; Dedhar, S.; Winum, J.-Y.; Supuran, C.T. Glycosyl Coumarin Carbonic Anhydrase IX and XII Inhibitors Strongly Attenuate the Growth of Primary Breast Tumors. *J. Med. Chem.* **2011**, *54*, 8271–8277. [CrossRef]
114. Chafe, S.C.; Lou, Y.; Sceneay, J.; Vallejo, M.; Hamilton, M.J.; McDonald, P.C.; Bennewith, K.L.; Moller, A.; Dedhar, S. Carbonic Anhydrase IX Promotes Myeloid-Derived Suppressor Cell Mobilization and Establishment of a Metastatic Niche by Stimulating G-CSF Production. *Cancer Res.* **2015**, *75*, 996–1008. [CrossRef]
115. Nikolopoulou, A.; Amor-Coarasa, A.; Wuestemann, T.; Matei, I.; Hoshino, A.; DiMagno, S.; Babich, J. Tumor exosomes as molecular probes to detect breast cancer pre-metastatic niches: Radiolabeling with I-131 and tissue uptake studies in "naïve" nude mice. *J. Nucl. Med.* **2016**, *57*, 524.
116. Soodgupta, D.; Jiang, M.; Zheleznyak, A.; Shokeen, M.; Hurchla, M.A.; Weilbaecher, K.N.; Tomasson, M.H.; Anderson, C.J. Very late antigen-4 ($\alpha_4\beta_1$ Integrin) targeted PET imaging of multiple myeloma. *PLoS ONE* **2013**, *8*, e55841. [CrossRef]
117. Lee, S.H.; Diamond, M.; Chadderton, A.; Liu, H.; Volgina, A.; Roman, V.; Weber, M.; He, C.; Stewart, R.; Hertel, D.; et al. Abstract 3929: The FAD-directed LSD1 specific inhibitor, INCB059872, inhibits cell migration and metastasis by suppressing premetastatic niche formation in a spontaneous metastasis mouse model. *Cancer Res.* **2018**, *78*, 3929. [CrossRef]
118. Borin, T.F.; Angara, K.; Rashid, M.; Shankar, A.; Iskander, A.; Ara, R.; Jain, M.; Achyut, B.R.; Arbab, A.S. Abstract 1043: CSF-1R inhibitor prevented pre-metastatic lung niches in metastatic mammary tumor. *Cancer Res.* **2017**, *77*, 1043. [CrossRef]
119. Erler, J.T.; Bennewith, K.L.; Nicolau, M.; Dornhöfer, N.; Kong, C.; Le, Q.-T.; Chi, J.-T.A.; Jeffrey, S.S.; Giaccia, A.J. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nat. Cell Biol.* **2006**, *440*, 1222–1226. [CrossRef] [PubMed]
120. Wong, C.C.-L.; Gilkes, D.M.; Zhang, H.; Chen, J.; Wei, H.; Chaturvedi, P.; Fraley, S.I.; Wong, C.-M.; Khoo, U.-S.; Ng, I.O.-L.; et al. Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 16369–16374. [CrossRef]
121. Corle, C.; Seagroves, T.N.; Liao, D.; Johnson, R. Hypoxia-Inducible Factor-1α Is a Key Regulator of Metastasis in a Transgenic Model of Cancer Initiation and Progression. *Cancer Res.* **2007**, *67*, 563–572.
122. Wong, C.C.-L.; Zhang, H.; Gilkes, D.M.; Chen, J.; Wei, H.; Chaturvedi, P.; Hubbi, M.E.; Semenza, G.L. Inhibitors of Hypoxia-Inducible Factor 1 Block Breast Cancer Metastatic Niche Formation and Lung Metastasis. *J. Mol. Med.* **2012**, *90*, 803–815. [CrossRef] [PubMed]
123. Pisapia, D.J.; Salvatore, S.; Pauli, C.; Hissong, E.; Eng, K.; Prandi, D.; Sailer, V.-W.; Robinson, B.D.; Park, K.; Cyrta, J.; et al. Next-Generation Rapid Autopsies Enable Tumor Evolution Tracking and Generation of Preclinical Models. *JCO Precis. Oncol.* **2017**, 1–13. [CrossRef] [PubMed]