### Original Article Heterogeneity of chemokine cell-surface receptor expression in triple-negative breast cancer

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**Abstract:** Introduction: Tumor heterogeneity is a well-established concept in cancer research. In this paper, we examine an additional type of tumor cell heterogeneity - tumor cell-surface receptor heterogeneity. Methods: We use flow cytometry to measure the frequency and numbers of cell-surface receptors on triple negative breast cancer cell lines. Results: We find two distinct populations of human triple-negative breast cancer cells MDA-MB-231 when they are grown in culture, one with low surface levels of various chemokine receptors and a second with much higher levels. The population with high surface levels of these receptors is increased in the more metastatic MDA-MB-231-luc-d3h2ln cell line. Conclusion: We hypothesize that this high cell-surface receptor population is involved in metastasis. We find that the receptor high populations can be modulated by tumor conditioned media and IL6 treatment indicating that the tumor microenvironment is important for the maintenance and sizes of these populations.

Keywords: MDA-MB-231, CCR5, CXCR3, CXCR4, stem cells, IL6

#### Introduction

Tumor heterogeneity is a well-established concept in cancer research [1-6]. Heterogeneity comes in many forms; there is tumor cell heterogeneity, such as genetic [7, 8] and epigenetic differences [9] between tumor cells within the same tumor and there is heterogeneity in the different cell types present in the tumor [10-12] such as stem cells, progenitor cells, and differentiated cells. There is also an established history of tumor microenvironment heterogeneity, for instance many cell types are recruited to the metastatic or tumor niche [13-15], such as the cells that make up the vasculature [16-18], cancer associated fibroblasts [13], and bone-marrow derived cells [15]. Even the supporting tumor vasculature is said to be heterogeneous and distinct from normal organ vasculature [19-21]. In this paper, we examine an additional type of tumor cell heterogeneity tumor cell-surface receptor heterogeneity.

Triple-negative breast cancer (TNBC) is a very aggressive cancer type in which the cancer cells lack hormone receptors rendering them

untreatable with hormone therapies and the prognosis is poor [22, 23]. One of the commonly used human cell lines to study triple-negative breast cancer in vitro and in vivo is MDA-MB-231 (we refer to it as MB231 for brevity). Another cell line significantly more metastatic that is commonly used is MDA-MB-231-luc-D3H2LN (MB231-luc). The MB231-luc cell line was derived from the MB231 cell line in multiple steps; the MDA-MB-231-luc-D3H1 (D3H1) cell line was first derived by stably transfecting the luciferase gene under the control of the SV40 promoter to facilitate imaging in live animals, an orthotopic breast cancer xenograft was generated with the D3H1 cell line, and a spontaneous metastasis to the lymph nodes was propagated to generate the MB231-luc cells [24]. Mice with orthotopic tumors generated from MB231-luc cells had more metastases in a shorter amount of time than mice with orthotopic MB231 tumors in a tumor xenograft spontaneous metastasis model [24].

Cytokine and chemokine mediated signaling are important for all aspects of tumorigenesis including proliferation, invasion, angiogenesis,

and metastasis. Their role in metastasis has been amply demonstrated in multiple studies. For instance, interleukin 6 (IL6), which influences the surface expression of chemokine receptors CCR5 and CXCR3 [25], is upregulated in TNBC with epithelial-to-mesenchymal transition-like features [26]. CXCR1 is associated with breast cancer self-renewal [27] and TNBC invasion [26]. The CCR5 cell-surface receptor activation leads to increases in cancer stem cells and increased invasion [28-30]. CXCR3 expressing cells are associated with increased motility in MB231 cells [31]. CXCR4 is expressed in breast cancer cells and is associated with metastasis [32, 33]. The hypothesis that chemokines and their receptors may have an important role in the metastatic capacity of the MB231-luc cell line is examined in the present study.

Another type of heterogeneity within the tumor population is the presence of cancer stem cells or tumor initiating cells. These cells are characterized by their ability to self-renew, to plate efficiently, proliferate without limit, and generate heterogeneous progeny [34]. In pancreatic tumors, CD133+/CXCR4+ migrating stem cells are necessary for metastasis [32]. MB231 cells have been found to have between 0-2% of stem cells [35, 36]. Breast cancer stem cells are classically identified by CD44+/CD24- or aldehyde dehydrogenase [37]. They can be regulated by their microenvironment [38]. To add further complexity, it has also been proposed that there may be two types of stem cells, a mesenchymal quiescent type and an epithelial selfrenewing type [37]. Therefore we were also interested in determining the differences in numbers of stem cell between the two cell lines.

Similarly to ecological populations, heterogeneous populations of cancer cells may increase the fitness of the overall population. If one can better understand and target the heterogeneity of the tumor, it may push the tumor to be more homogeneous. The more homogenous population might then be able to be better targeted with a single agent.

### Materials and methods

### Cell culture

MDA-MB-231 and MDA-MB-231-Iuc-D3H2LN cells were cultured in RPMI media (Gibco; Life

Technologies; Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Sigma). MB-231 were gifts from Dr Zaver Bhujwalla (JHMI, Radiology and Oncology). MB-231-luc was purchased from Caliper.

### Migration assay

50,000 MB231 and MB231-luc cells were seeded in the top chamber of a Cell Invasion/ Migration plate (CIM-plates) (ACEA Biosciences). The top chamber was filled with 40  $\mu$ l of FBS and the bottom chamber was filled with FBS. A real-time cell analyzer (RTCA) system (ACEA Bioscience) was used to monitor cell migration for 24 h. The cells migrate and attach and spread on the gold electrodes on the underside of the top chamber. The number of cells migrated is registered as a change in cell index.

### Cell collection and antibody binding for surface receptor quantitation

Cells were grown to at least 70% confluence. For harvesting, they were washed with 10ml of phosphate buffered saline (DPBS) without calcium or magnesium (Gibco, 14190) and all traces of DPBS were removed from the flasks. 3 ml of trypLE (Gibco 12604-013; Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells to detach them off the flask surface. The activity of trypLE was neutralized using 3 ml of trypsin neutralization solution (Gibco R0021-00). The cells were counted and collected by centrifugation. The cells were resuspended in stain buffer (BD Biosciences, San Jose, CA, USA) at  $4 \times 10^6$  cells/mL or  $10^5$  cells/25 µL.

Twenty-five  $\mu$ L of cells were added to polystyrene round bottom tubes (BD Biosciences 35-2008). Ten  $\mu$ L of allophycocyanin (APC)- or phycoerythrin (PE)-labeled antibodies (CCR5, CX-CR1, CXCR3, CXCR4, VEGR2, CD24, or CD44 from R&D Systems) were added to each tube. Each sample was incubated at 4°C for 45 minutes in the dark.

### Washing and flow cytometry

Following incubation with antibodies, the cells were washed with 4 ml of stain buffer (BD) in the dark to avoid photobleaching of the fluorescently labeled antibodies. Quantibrite PE (Phycoerythrin) (BD Biosciences, 340495) beads were also washed with 4 ml of stain buffer. The stain buffer was removed and the cells and beads were washed again. One hundred and fifty  $\mu$ L of stain buffer were added to the washed cells in each tube and the samples were kept on ice. The number of surface receptors was measured in a FACSCalibur flow cytometer (BD Biosciences) using the FL2 channel. The analysis was done as in Norton et. al. [39].

### Stem cell counts

To measure the number of stem cells within each MB231 cell type, we used 10  $\mu$ l of CD44 antibody labeled with APC (Allophycocyanin) and CD24 antibody labeled with PE. We used control tubes without any antibody and control tubes with just one antibody to gate receptor high and low gates for both CD24 and CD44. Then we counted the number of cells in the CD24-/CD44+ gate which gives a measure of the number of stem cells using the FL2 and FL4 channels.

### Tumor conditioned media

Tumor conditioned media (TCM) was prepared as in Lee et al. [40]. Briefly, MB231 and MB231luc cells were grown in T175 tissue culture flasks in complete media until they were confluent. After removing the complete media and washing with 1×DPBS, 8 ml of serum free media (RPMI media) was added to each flask and incubated for 24 h. The 8 ml of tumor conditioned media containing factors secreted by the tumor cells was then removed from each flask, filtered, aliquoted, and stored at -20°C.

### TCM and IL6 treatment

TCM was thawed and MB231 and MB231-luc cells were treated for 1.5 h with 2 ml of TCM. The cells were treated with 10 ng/ml of IL6 for 1.5 h. This step was done after washing the cells with PBS and before trypsinizing the cells.

### Clone isolation protocol

MB231-luc cells were diluted to 1 cell per ml in growth media. Ten ml of the diluted cells were placed in 10 cm cell culture dishes and the cells were allowed to grow. Once colonies were visible, individual colonies were detached in isolation with trypLE and expanded separately in 6 well plates and the cells were grown until the wells were confluent.

# Counting surface receptors on endothelial cells from mb231 tumors

*Tumor xenograft models:* The animal protocols were approved by the Institutional Care and Use Committee at the Johns Hopkins University. Two million MB231-luc cells combined with 100  $\mu$ l of 50% matrigel solution were injected into the upper inguinal mammary fat pad of the mice under anesthesia. After 5 weeks, the tumors were collected.

*Tumor digestion:* Orthotopic tumors generated from MB231 cells in nude mice were excised and weighed. The tumors were minced with razor blades and digested with 9 ml of collagenase (Worthington Biochemicals, MA, USA) and 1 ml of dispase (Worthington Biochemicals, MA, USA) per gram of tumor. The tumors were placed in a 37°C water bath for 30 minutes to facilitate digestion after which 75 µl per DNAse was added and the incubation at 37°C was continued for another 30 minutes. Five ml of endothelial cell (EGM2-MV made from singlequots EBM2 base - Gibco) media with 1% sodium nitrate on ice was added to the tumor digest and the cells were sieved through a 70 µm cell strainer. The cells are collected by centrifugation at 1400 rpm for 4 min.

Endothelial cell isolation: The cells collected from the tumors were re-suspended in 6ml of media for each gram of starting tumor. One µl of biotinylated anti-mouse CD34 antibody (Biolegend, San Diego) was added and the cells were allowed to rotate for 10 minutes at 4°C in a tumbler. The cells were washed with 2 ml of stain buffer (2% FBS in DPBS from BD), centrifuged for 5 min at 1400 rpm, and re-suspended in stain buffer. Twenty-five µl of pre-washed Dynabeads (Invitrogen) were added to the cells and rocked at 4°C for 20 min. A magnet was used to remove the bound cells, which were then re-suspended in stain buffer.

Surface receptor counts: The isolated endothelial cells were incubated with 10  $\mu$ l of CD31-APC and VEGFR2-PE antibodies (R&D Systems) for 45 min, covered, and on ice. The cells were washed with 4 ml of stain buffer twice with a 5 min, 1400 rpm centrifugation step at 4°C in between. The cells are gated and surface receptors were counted in a FACSCalibur flow cytometer (BD Biosciences).



Figure 1. MB231 and cell MB231-luc ACEA motility assay. A. Plot of cell index over time for 24 hours. B. The average motility index for MB231 and MB231-luc cells at 24 h.

### Results

## MB231-luc cells have a higher motility rate than MB231 cells

Since MB231-luc cells are known to be more metastatic than MB231 cells we wanted to test if they are more motile than MB231 cells in culture. We measured the relative motilities of MB231 and MB231-luc cells in an ACEA motility assay using CIM plates. Although the cells move from one compartment to another we refer to this as a motility assay because no gradient of chemotactic factors exists between the 2 chambers. We performed 6 replicates for each cell line, Figure 1A. The two cell lines are clearly delineated and have different motility rates. The replicates were averaged at time 24 h (Figure 1B), and MB231-luc cells are more than two times as motile as the MB231 cells. Using a Kolmogorov-Smirnov one-tailed test, MB231luc cells were statistically more motile than MB231 cells. This result is consistent with MB231-luc cells having a more metastatic phenotype.

MB231 and MB231-luc cells in culture have two distinct sub-populations

Since MB231-luc cells are more motile than MB231 cells in vitro, we wanted to understand the possible mechanisms for these phenomena. One possible reason for these differences is that the two cell lines have different numbers of cellsurface receptors involved in motility. We examined several types of surface receptors: CXCR1, associated with breast cancer stem ce-Il self-renewal and breast cancer invasion [27], CX-CR3, associated with increased motility in breast cancer [31], CXCR4, which is associated with breast cancer metastasis, and CC-R5 which promotes breast cancer metastasis to the lymph nodes through inter-

leukin 6 (IL6) [40] and cancer invasion [28-30].

When investigating the dot plot of the FL2 (Phycoerythrin channel) and FL1 channel channel (overlaps with FL2 channel) the FACSCalibur flow cytometer it became clear that there are two distinct populations, one small population with very high receptor levels and another bulk population with lower numbers of surface receptors in cultures of both cell lines. In Figure 2, it is clear that both MB231 and MB231-luc cultures have a sub-population with a high number of CXCR3 and CXCR1 receptors. The MB-231-luc cells (Figure 2C, 2D) have more cells in this population than the MB231 cells (Figure **2A**, **2B**) for both CXCR3 and CXCR1. In both MB231 and MB231-luc cells, the cells in 'high' sub-population have more than 10 times as many surface receptors as cells in the 'bulk' population.



**Figure 2.** The receptor high populations in MB231 and MB231-luc cells measured by flow cytometry. A. The CXCR3 receptor high population in MB231 cells. B. The CXCR1 receptor high population in MB231 cells. C. The CXCR3 receptor high population in MB231-luc cells. D. The CXCR1 receptor high population for MB231-luc cells.

### Two distinct populations exist only for certain receptors

We determined the fraction of MB231 and MB231-luc cells in culture expressing high numbers of CCR5, CXCR3, CXCR1, and CXCR4 surface receptors. The percent of cells with a high receptor population for each cell line is shown in Figure 3A, 3B. Receptor high populations, whose percentages are > 1% of the total, exist for CCR5, CXCR3, and CXCR1. Neither MB231 nor MB231-luc cells appear to have an appreciable population of cells expressing high numbers of surface CXCR4 (less than 1%) (Figure 3B). The MB231-luc cultures have significantly higher percentages of cells with high numbers of surface CCR5 compared to MB231 cells (Figure 3A), 2.9% for MB231-luc compared to 1.6% for MB231. Also the sub-population of MB231-luc cultures expressing CXCR1 (Figure 3B) is statistically higher than in MB231 cultures, 5.92% compared to 4.42%. CXCR3 is inconclusive due to the large standard deviation in the MB231-luc cultures, see **Figure 3A**. The relative sizes of the sub-population of MB231 and MB231-luc cultures expressing high numbers of CXCR4 are statistically different, but the fractions are probably too small to have an impact on the population dynamics. We also measured VEGFR2 receptor levels and found that MB231-luc cells have higher surface receptor numbers than MB231 cells and that MB231-luc cells have a VEGFR2 receptor high populations, see <u>Supplemental Figure 1</u>.

### CCR5 and CXCR3 high populations overlap

It is unclear whether the receptor high populations for different receptors are distinct subpopulations or whether they appear in the same small population of cells. Therefore we wanted to determine whether there was overlap in the two different receptor high populations (CCR5 and CXCR3). We used a CXCR3



**Figure 4.** The percentages of receptor high populations of MB231 and MB231-luc cells treated with control and tumor-conditioned media (TCM). A. The CXCR3 receptor high population in MB231 treated with regular media. B. The CXCR3 receptor high population in MB231 treated with tumor-conditioned media. C. The percentages of CCR5 and CXCR3 receptor high populations in MB231 cells treated with control media or TCM. D. The percentages of CCR5 and CXCR3 receptor high populations in MB231-luc cells treated with control media or TCM.



Figure 5. Quantification of surface receptor low and high populations of cells treated with control media and tumorconditioned media. A. Quantification of MB231 cell surface receptors. B. Quantification of MB231-luc cell surface receptors.

antibody tagged with PE and a CCR5 antibody tagged with APC to determine whether these populations had significant overlap. First we verified that the receptor high population fractions were similar to our previous experiments with a single antibody. The fraction of cells expressing high numbers of surface CCR5 in MB231 was 1.8% and 2.8% in MB231-luc cells in agreement with our previous results. As shown in Figure 3C, there are extremely low levels of surface CCR5 in the CXCR3 low population in both MB231-luc and MB231 cultures. In contrast, there are high levels of surface CXCR3 in the high CCR5 population in both MB231-luc and MB231 cells. In addition there are high levels of surface CCR5 in the CXCR3 high population. These results suggest that the sub-population with high receptor numbers has high numbers of multiple receptors. In both cases the average number of these chemokine receptors in this population was higher in MB231-luc cells than in MB231 cultures. This suggests that there may be a small population of cells in triple-negative breast cancer with high levels of chemokine and cytokine receptors, which may cause them to be a more metastatic subpopulation.

### TCM treatment

Since tumor conditioned media (TCM) increased the number of lung metastases in a triple-neg-

ative breast cancer xenograft model [40-42]. we were interested in how TCM affected surface cytokine receptors on triple negative breast cancer cells. We hypothesized that since TCM mediated increased metastasis using the CCL5/CCR5 pathway [40], that TCM would increase the number of surface CCR5 receptors. We determined if treating MB231 and MB231-luc cultures with TCM would change the size of the sub-population with high numbers of surface CCR5 and CXCR3 chemokine receptors. It is clear from Figure 4A, 4B that TCM treatment increases the size of the sub-population with a high level of CXCR3 surface receptors. We found that treatment with TCM significantly increases the fractions with high numbers of CCR5 and CXCR3 surface receptors in both MB231 and MB231-luc cultures (Figure 4C). In MB231 cells, TCM treatment increases the receptor high population by 2-fold, Figure 4C. In MB231-luc cells, the receptor high population for both CCR5 and CXCR3 are significantly increased but the changes are smaller (Figure 4D).

We found that in MB231 cultures, TCM decreases the surface receptor numbers of CXCR3 and CCR5 in the high population (hi pop) and increases their numbers in the bulk population (low pop), **Figure 5A**. In MB231-luc cultures, TCM treatment does not have much effect on the surface receptor numbers, except for



**Figure 6.** The effects of IL6 treatment on CCR5 and CXCR3 cell surface receptors on MB231 cells. A. The percentages of receptor high populations on MB231 cells treated with control or IL6. B. Quantification of the number of surface receptors in the high and low populations of MB231 cells treated with control or IL6.



CXCR3, **Figure 5B**. In the sub-population with the high number of surface receptors (hi pop), TCM decreases the number of surface CXCR3 receptors and in the bulk population (low pop) it slightly increases the number of surface CXCR3 receptors (**Figure 5B**). Therefore, TCM seems to have a greater effect on MB231 surface CCR5 and CXCR3 receptors than on MB231-luc.

#### IL6 treatment

In previous work from this laboratory, Lee et al. found that IL6 was one of the important factors in the TCM that leads to breast cancer metastasis to the lymph nodes [34]. Therefore we hypothesized that IL6 would have similar effects on the surface receptors as the TCM. In contrast, we found that in MB231 cells IL6 does not increase the percentage of cells with high receptor numbers of CCR5 surface receptors (**Figure 6A**) and actually decreases the size of the sub-population of cells with a high number of surface CXCR3 receptors (**Figure 6A**). We also found that IL6 does not increase the number of CCR5 surface receptors (**Figure 6B**) and decreases the number of CXCR3 surface receptors (**Figure 6B**). Therefore, we conclude that IL6 is not the factor that is causing the receptor changes seen upon TCM treatment and that other factors in the TCM must be responsible for those effects.



Isolation of clones of cells with a high number of surface CCR5

Since cells with a high number of cell-surface CCR5 also had high CXCR3 cell-surface receptor numbers, we tried to isolate clones expressing a high number of surface receptors to better study them. We diluted MB231-luc cells into approximately 1 cell per ml and placed them in a cell culture dish. Once individual clones had grown, we expanded them separately and then measured their surface CCR5 receptor numbers. Of 30 clones we tested, 6 appeared to have a large sub-population with high surface CCR5. The sizes of the sub-population of cells with high surface CCR5 receptors were all higher than that in the original cultures and they varied among the 6 colonies; the largest receptor high expressing fraction was 20%. We also found that in a three week period, these populations with high surface receptors decreased in size (data not shown).

### In vivo tumors also exhibit receptor heterogeneity

Once we had found these sub-populations expressing a high number of surface receptors in vitro we wanted to verify that they were also present in vivo. Therefore we excised tumors from orthotopic xenografts generated in nude mice with MB231-luc cells and isolated the cells away from the rest of the tumor by a digestion protocol [43]. Using anti-human antibodies, we then measured the CXCR1, CXCR3, and CCR5 surface receptors on the human tumor cells. In all three tumors tested, we found subpopulations of cells expressing a high number of these receptors on the cell surface (**Figure 7A**). The average numbers of CCR5 and CXCR1 receptors in the sub-population expressing a low number of receptors are similar in tumors 1 and 3 but different in tumor 2 (**Figure 7B**). We found that the average number of surface receptors in the sub-populations expressing a high number of surface receptors is similar in the three tumors (**Figure 7C**).

### Cancer stem cells

Since cancer stem cells have been associated with metastasis, we wanted to compare the number of stem cells in the MB231 and MB231-luc cultures. We hypothesized that there would be more stem cells in MB231-luc cultures compared to MB231 cells. We measured the percentage of CD24-/CD44+ cells in each cell type. Surprisingly, we found that the fraction of stem cells in MB231 cultures was higher than in MB231-luc cells, **Figure 8**.

Previous work done in our laboratory showed that pre-treating mice with tumor conditioned

media (TCM) prior to establishing orthotopic MB231-luc cell xenografts resulted both in reduced time to metastasis and in a higher incidence of metastasis to the lymph nodes and the lungs. Therefore we wanted to test whether treatment with TCM increased the fraction of stem cells in MB231-luc and MB231 cultures in vitro. Interestingly, we found that TCM treatment significantly increases the number of stem cells in MB231 cultures but not in MB231-luc cultures, **Figure 8**.

### Discussion

In this paper we examined heterogeneity of triple-negative breast cancer cells in vitro and in vivo, focusing on cell-surface receptor heterogeneity. We examined in vitro cultures of two cell lines, MB231 and MB231-luc, where the MB231-luc cell line is more metastatic to investigate what receptors were candidates for the increase in metastasis. First, we verified that the MB231-luc cell line is more motile than the MB231 cell line consistent with the more metastatic phenotype of the MB231-luc cells [24, 42].

We found that there were two sub-populations of cells within cultures of both cell lines, one with baseline levels of various receptors on the cell surface and one with high levels of these receptors. Therefore we examined the number of receptors in each of these two populations. We found significant overlap within these receptor high populations. Thus many of the CCR5 high cells have high CXCR3 surface receptor expression as well. It is plausible that cells in these receptor high populations are also involved in motility or metastasis [25, 31]. This is especially so for the receptor high populations of CCR5 [29].

It has been shown that there is altered CCL5 expression in breast cancer patients and that an increase in CCL5 increases metastasis in MB231-luc xenograft models [40]. From work done in our laboratory, it was found that lymphatic endothelial cells (LEC) in vitro secrete CCL5 in response to exposure to media conditioned by MB231 cells [40]. LEC cells resident in lymph nodes also appear to secrete CCL5 in mice harboring cells disseminated from an orthotopic tumor xenograft generated by MB-231-luc cells. In vivo the secreted factors contained in the tumor conditioned media (tumor

secretome) would be emanating from the actual tumor in the animal. In our model a gradient of CCL5 is generated and tumor cells expressing CCR5 on the cell surface follow the gradient to the lymph nodes facilitating metastasis [40]. CCL5 was found to be high in the plasma and breast tissue of patients with breast cancer [29, 40]. The CCL5/CCR5 pathway is active in triple negative breast cancers but only a subpopulation of cells express CCR5 and respond to CCL5 [29]. Velasco-Velazguez and colleagues found that about 7% of MDA-MB-231 cells expressed high levels of CCR5 and that these high cells were more invasive than cells expressing low levels of CCR5 [29]. Therefore the CCR5 high cells could be the cells that are most likely to migrate and metastasize.

It has been determined that in Ewing sarcoma, there is a sub-population of cells expressing high numbers of CXCR4 receptors on the cell surface that depends upon its microenvironment and promotes migration and invasion [44]. While CXCR4 is expressed in some breast cancers and has been found to promote metastasis, we did not find evidence that it is responsible for metastasis in these cell lines. CXCR4 was not highly expressed in either of the cell lines. We hypothesize that CCR5 or CXCR3 receptors are more likely candidates for regulators of metastasis in these cell lines and perhaps more generally in triple negative breast cancer.

CCR5 is a candidate biomarker for metastasis: The frequency of CCR5 high cells was around two times greater in the MB231-luc cell lines than in the MB231 cell line. CXCR1 frequency was greater in MB231-luc cells but by a lot less while CXCR3 frequency was not greater in the MB231-luc. These results suggest that the CC-R5 receptor may be more important for metastasis than CXCR1 and CXCR3.

Tumor conditioned media has been shown to accelerate metastasis in a tumor xenograft mouse model [42]. Specifically, IL6 in the tumor-conditioned media has been shown to promote metastasis to the lymph nodes and the lungs [40]. Since we were examining the receptors responsible for metastasis, we treated the TNBC cell lines with TCM and measured the number of surface receptors and the size of the receptor high sub-population after treatment. We found that the TCM had the most effect on the CXCR3 receptor, greatly increasing the size of the sub-population of the cells expressing a high number of cell-surface receptors in both cell lines. It also greatly influenced the number of surface CXCR3 receptors. Although TCM affected both MB231 and MB231-luc cell-surface receptors, the increases were higher in the MB231 cell line. One possible reason for this is that the MB231 cells can be pushed into having more surface receptors whereas the MB-231-lucs are already partially optimized for this.

IL6, on the other hand, had the opposite effect on CXCR3 receptors; it reduced the size of the receptor high sub-population. Therefore it is unclear from these results whether high levels of CXCR3 contribute to or reduce metastasis. It is possible that this is a very variable receptor that is not actually critical for metastasis.

The heterogeneity of the number of surface receptors, while variable, does seem to be controlled by signaling from the cell population. Even when we isolate one cell and propagate it, we find that the resulting culture has two populations, a smaller one with high numbers of cellsurface chemokine receptors and a larger, bulk one with low numbers of these same receptors. The largest high receptor sub-population we were able to capture was about 20%. We also found that over time, the cell populations reverted back to close to their normal fraction of receptor high cells. This suggests that the frequency of receptor high populations are controlled by the bulk cell population. Therefore, we suggest that the cell population changes over time to maintain its original proportion. This may be similar to how stem cell populations monitor the number of stem cells in their microenvironment and their rates of symmetric vs asymmetric divisions [45, 46]. Thus the tumor microenvironment may be very critical for the percentages of these receptor high cells in tumors.

Along these lines we have measured the surface receptor numbers of cells from tumor xenografts and established that they too have receptor high populations of similar sizes as the in vitro cells. Therefore, we conclude that both the frequency of receptors and average number of surface receptor numbers were upheld in the in vivo breast cancer model.

During the clonal isolation experiments with MB231-luc cells, we found that about 30% of the isolated clones had high numbers of sur-

face receptors. Interestingly, this is much greater than we would expect by chance, which was around 3-5% of receptor high cells, so this supports the hypothesis that receptor high clones are either better able to proliferate or to form colonies. This could make them more efficient once they reach the site of metastasis.

We thought that the MB231-luc cells would contain a higher fraction of cancer stem cells than the 231 cells given that breast cancer stem cells are hypothesized to be important for the generation of primary and metastatic tumors. Surprisingly, we found that the MB231 cell cultures actually had a higher fraction of cancer stem cells than MB231-luc cells. This suggests that while stem cells might be important for metastasis they are not sufficient for metastasis and that other factors are necessary. One possibility is that the cells need to be able to migrate, which would be coordinated by other receptors, and that once the cells reach their destination, they undergo EMT and become stem cells. There is support in the literature that EMT confers a stem-like phenotype [47-49].

This work addresses the tumor receptor heterogeneity of triple-negative breast cancer cells. This heterogeneity may increase the fitness of the tumor and cause it to be able to adapt and become resistant to therapy. If one can better understand the processes involved in generating tumor heterogeneity it may be possible to push the tumor into a more homogeneous state that could be more susceptible to monotherapies.

### Conclusion

In conclusion, we have established that triplenegative breast cancer cells exhibit two distinct populations of cells expressing different numbers of cell-surface receptor numbers. We have established that CCR5 is a possible candidate receptor for influencing the differences in metastatic ability of the two similar cell lines, MDA-MB-231 and MDA-MB-231-luc. The CXCR3 receptor had variable responses to metastatic influences but may also be important in metastasis. These results have been validated by in vivo mouse xenograft models of breast cancer. While this work is not an exhaustive search of chemokine receptors, it does point to CCR5 as a potentially important receptor influencing metastasis in TNBC and could serve as a potential biomarker for cells that will actually metastasize.

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### Disclosure of conflict of interest

The authors declare that they have no competing interests.

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### Supplemental material

MB231-luc cells have higher surface VEGFR2 receptor numbers than MB231 cells

MB231-luc cells have been cultured to be metastatic to the lymph nodes but they come from the same MB231 cell culture. To understand whether these cells have differences in surface receptor numbers, we measured surface VEGFR2 on both cell lines. We found that MB231-luc cells have higher numbers of VEGFR2 cell-surface receptors than MB231 cells, **Supplemental Figure 1A**. The numbers of VEGFR2 cell surface receptors for MB231 tumor cells are on the same order of magnitude as [41].

MB231-luc cells have two distinct populations with different VEGFR2 receptor levels

When investigating the dot plot of the FL2 (PE) and FL1 channel (overlaps with FL2 channel) the FACSCalibur flow cytometer it became clear that there were two distinct receptor populations, one small population with very high receptor levels and another bulk population with lower numbers of surface VEGFR2 receptors. In both MB231 and MB231-luc cells that 'high' population (hi pop) had receptor numbers over 10 times as large as the 'bulk' population (low pop) surface receptor numbers, **Supplemental Figure 1B-D**. In the bulk population the number of VEGFR2 surface receptors are only slightly higher in MB231-luc than MB231 cells (~30% higher), **Supplemental Figure 1B**. In contrast, the receptor 'high' population was about 200% higher in MB231-luc cells than MB231 cells, **Supplemental Figure 1D**.



Supplemental Figure 1. VEGFR2 receptor levels in MB231 and MB231-luc cell lines. (A) The quantified surface receptor numbers of VEGFR2 in both cell lines. (B-D) The quantified number of surface VEGFR2 levels in the total population (B), low population (C), and high population (D) when the cells are treated with regular media and tumor-conditioned media.