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Giuseppina Augimeri\textsuperscript{1,2,\#}, Maria E. Gonzalez\textsuperscript{1,3,\#}, Alessandro Paoli\textsuperscript{1,2}, Ahmad Eido\textsuperscript{1,3}, Yehyun Choi\textsuperscript{4}, Boris Burman\textsuperscript{1}, Sabra Djomehri\textsuperscript{1,3}, Santhosh Karthikeyan\textsuperscript{5}, Sooryanarayana Varambally\textsuperscript{5}, Johanna M. Buschhaus\textsuperscript{6}, Yu-Chih Chen\textsuperscript{7}, Loredana Mauro\textsuperscript{2}, Daniela Bonofiglio\textsuperscript{2}, Alexey I. Nesvizhskii\textsuperscript{1,8}, Gary D. Luker\textsuperscript{3,6}, Sebastiano Andò\textsuperscript{2}, Euisik Yoon\textsuperscript{3,4}, Celina G. Kleer\textsuperscript{1,3,*}

\textsuperscript{1} Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, 48109, USA
\textsuperscript{2} Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende, CS, Italy
\textsuperscript{3} Rogel Cancer Center, University of Michigan, Ann Arbor, MI, USA
\textsuperscript{4} Department of Electrical Engineering and Computer Science and Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA
\textsuperscript{5} Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham, Birmingham, 35233, AL, USA
\textsuperscript{5} Center for Molecular Imaging, Department of Radiology, University of Michigan, Ann Arbor, MI,
\textsuperscript{7} UPMC Hillman Cancer Center, Department of Computational and Systems Biology, Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15232, USA
\textsuperscript{8} Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, 48109, USA.

\# These authors contributed equally

\* Lead contact and corresponding author: Celina G. Kleer, MD., kleer@umich.edu, Tel. +1-734-615-3448

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Abstract

Patients with triple negative breast cancer remain at risk for metastatic disease despite treatment. The acquisition of chemoresistance is a major cause of tumor relapse and death, but the mechanisms are far from understood. We have demonstrated that breast cancer cells (BCCs) can engulf mesenchymal stem/stromal cells (MSCs), leading to enhanced dissemination. Here, we show that clinical samples of primary invasive carcinoma and chemoresistant breast cancer metastasis contain a unique hybrid cancer cell population co-expressing pan-cytokeratin and the MSC marker fibroblast activation protein-alpha. We show that hybrid cells form in primary tumors and that they promote breast cancer metastasis and chemoresistance. Using single cell microfluidics and in vivo models, we found that within the hybrid cell population are polyploid senescent cells that contribute to metastatic dissemination. Our data reveal that WNT5A plays a crucial role in supporting the chemoresistance properties of hybrid cells. Furthermore, we identify that WNT5A mediates hybrid cell formation through a phagocytosis-like mechanism that requires BCC-derived Interleukin 6 and MSC-derived C-C Motif Chemokine Ligand 2. These findings reveal hybrid cell formation as a novel mechanism of chemoresistance and suggest that interrupting this mechanism may be a potential strategy to overcome breast cancer drug resistance.
Introduction

Despite advances in breast cancer treatment, breast cancer is still a leading cause of mortality in women worldwide (1). One of the major limitations of breast cancer therapy is the establishment of resistance to chemotherapy, which leads to patients’ relapse and death (2). In particular, triple negative breast cancer (TNBC) frequently recurs after an initial response to chemotherapy (3). Elucidating the mechanisms underlying breast cancer resistance to treatment is needed to improve survival.

The crosstalk between breast cancer cells (BCCs) and the components of the tumor microenvironment has emerged as a crucial modulator of the effectiveness of drug therapy (4). BCCs communicate with stromal cells through paracrine loops and cell-cell and cell-matrix interactions, creating a permissive tumor microenvironment that facilitates cancer cell escape to drug treatment (5). Mesenchymal stem/stromal cells (MSCs) are self-renewing pluripotent cells that are recruited from the bone marrow and the adipose tissue to the primary tumor site by soluble molecules released by BCCs (6). In response to the local signals produced by BCCs, MSCs release factors that support resistance to therapy and breast cancer progression (7-9). Additionally, MSCs can contribute to the development of breast cancer chemoresistance through physical interaction which BCCs (10). Our lab and others have demonstrated that MSCs can be engulfed by BCCs, supporting breast tumorigenesis (11, 12). We have isolated and characterized patient-derived MSCs from human breast cancer metastasis, and have shown that MSC engulfment by BCCs induces heritable transcriptional changes resulting in a hybrid cell population with enhanced metastatic ability (11). However, the phenotypic features of hybrid cells and their role in breast cancer progression are not well understood.

Here, using unbiased RNA-sequencing studies we demonstrate that MSCs derived from patients’ metastasis express fibroblast activating protein-alpha (FAP), and identify and quantify hybrid cells co-expressing cytokeratin and FAP in clinical samples of primary tumors and distant...
metastasis. We provide direct evidence that MSC engulfment by BCCs generates hybrid cells in orthotopic tumors and lung metastases using two photon microscopy. We found that the hybrid cell population is enriched in senescent cells and displays a chemoresistant phenotype. We identify that MSC engulfment utilizes a phagocytosis-like mechanism that requires WNT5A-mediated IL6 and CCL2 secretion. These data provide the foundation to regulate hybrid cell formation as a potential strategy to overcome breast cancer chemoresistance.
Results

Primary breast carcinomas and distant metastasis contain a population of hybrid cancer cells expressing mesenchymal stem/stromal cell markers.

We have shown that BCCs can engulf MSCs, generating a hybrid cancer cell population with phenotypic epithelial to mesenchymal transition (EMT) and expression of EMT proteins (e.g., Zinc Finger E-Box Binding Homeobox 1, ZEB1 and smooth muscle actin, SMA), and greater metastatic ability than non-engulfing BCCs (11). To identify specific markers that allow detection of BCCs, MSCs, and hybrid BCCs that have engulfed MSCs in human samples, we performed RNA sequencing of MDA-MB-231 BCCs and MSCs isolated from patient’s metastasis previously characterized in our lab (13). We identified fibroblast activation protein-alpha (FAP), as one of the most significantly upregulated proteins in MSCs with undetectable expression in MDA-MB-231 cells, which was further validated by WB (Supplementary Figure 1A-B). Next, we sought to determine the presence of BCCs expressing a hybrid phenotype in 38 clinical tissue samples including 20 primary invasive carcinomas with adjacent normal breast and 18 distant breast cancer metastasis that have failed chemotherapy. We employed quantitative multiplexed fluorescence immunohistochemistry with pan-cytokeratin (panCK), FAP antibodies and an artificial intelligence (AI) pipeline which allows quantification of specific cell populations (Figure 1A). In normal breast lobules, we observed a clear separation between panCK+ breast epithelial cells and FAP+ stromal cells. In contrast, primary invasive carcinomas and distant metastases contain a unique population of cells that co-express panCK and FAP, indicating a hybrid phenotype (Figure 1B, Supplementary Figure 1C, Supplementary Tables 1 and 2). Quantification of the percentage of panCK+/FAP+ cells in whole tissue sections showed that distant metastases have significantly higher percentage of hybrid cells compared to primary tumors (Figure 1C).
To detect the formation of hybrid cells in vivo, we injected GFP labeled MDA-MB-231 cells (GFP-231) and DsRed labeled-MSCs (DsRed-MSCs) in the mammary fat pads of NOD/SCID mice. Using in vivo two photon fluorescence microscopy of orthotopic tumors and ex vivo imaging of lungs, we identified GFP+/DsRed+ hybrid cells in orthotopic tumors and in spontaneous distant lung metastasis (Supplementary Figure 2A). In addition, to investigate the metastatic potential of the hybrid cells, we sorted by flow cytometry the GFP+/DsRed+ cells from the hybrid cell-enriched co-culture of GFP-231 with DsRed-MSCs and intracardially injected the GFP+/DsRed+ in NOD/SCID mice. We observed that hybrid cells can form metastasis that grow over time using bioluminescence imaging (Supplementary Figure 2B). Together, these data provide direct evidence that hybrid cells form in situ in live mammary tumors, have the ability to disseminate, and demonstrate that hybrid cells can be detected in tissue samples of primary breast cancer and metastasis using a combination of panCK and FAP markers.

**Generation and characterization of hybrid cells in co-cultures of breast cancer cells and mesenchymal stem cells**

We co-cultured a panel of GFP-labeled BCCs, including MDA-MB-231 (GFP-231), MDA-MB-436 (GFP-436), and patient-derived T4 BCCs (14) with DsRed-labeled MSCs (DsRed-MSCs) for 72 h (Figure 2A). In addition, we generated GFP-CCN6 knockout (KO) cells from MMTV-Cre;Ccn6<sup>fl/fl</sup> mammary breast carcinomas, which recapitulate the histopathology and genomic features of human spindle metaplastic TNBC (15, 16) and cultured GFP-CCN6KO cells with DsRed-MSCs for 7 days.

Flow cytometry live imaging stream single-cell analysis showed the presence of hybrid cells in co-cultures of GFP-231 and DsRed-MSCs (Figure 2B) and three-dimensional renderings of confocal Z stacks (Video 1). In concordance with our in vivo observations (Supplementary Figure 2A), hybrid cells characterized by GFP and DsRed signals were found in the co-cultures
of GFP-436, GFP-T4, GFP-CCN6KO with DsRed-MSCs analyzed by immunofluorescence microscopy (Supplementary Figure 2C).

Co-culture of GFP-231 with MSCs resulted in 89% GFP+/DsRed+ hybrid cells (Figure 2C) and co-culture of GFP-CCN6KO cells with DsRed-MSC cells for 7 days, led to 95% hybrid cells (Supplementary Figure 2D), representing well-suited experimental cell models to investigate the role of hybrid cells in breast cancer progression.

Hybrid cells exhibit a senescent phenotype

In order to characterize the hybrid cell population that we identified in vivo and in vitro, we first investigated the presence of DNA copy number changes in hybrid cells analyzing their DNA content by flow cytometry. We found that in the co-culture of GFP-231 with DsRed-MSCs, the GFP+/DsRed+ hybrid cells are polyploid with increased DNA content (>4N+) compared to GFP+ cells (Figure 2C). Since polyploidization is often a feature of senescence, we hypothesized that hybrid cells may display a senescent phenotype (17-19). We used cytokine arrays and ELISA to compare the secretome profiles of hybrid-enriched co-cultures, BCCs, and MSCs. Compared to the supernatant of BCCs and MSCs mixed in the ratio 1:1, hybrid-enriched co-cultures exhibit increased levels of several senescence-associated secretory phenotype (SASP) factors, including interleukin (IL) 6, C-C Motif Chemokine Ligand (CCL) 2, Osteopontin (OPN), Thrombospondin (THBS) 1, Urokinase-type plasminogen activator receptor (uPAR) (Figure 2D and Supplementary Figure 3A) (12, 20-22). Similar results were observed using GFP-436 and -T4 patient-derived BCCs (Supplementary Figure 3B and C). To investigate whether the direct contact between BCCs and MSCs is required for secretion of SASP factors, DsRed-MSCs were cultured physically divided from GFP-231 using a transwell system followed by analysis of the supernatants by cytokine array (Figure Supplementary 3D). We found a reduced secretion of the SASP factors in the supernatants of GFP-231 and DsRed-MSCs cultured in the transwell
system compared to the direct contact co-culture, demonstrating that the direct contact between BCCs and MSCs is necessary for secretion of SASP.

We next measured the senescence-associated (SA) beta-galactosidase activity, a well-characterized marker of cellular senescence (23), in co-cultures of DsRed labeled MDA-MB-231 (DsRed-231) with unlabeled MSCs. Flow cytometry analysis (Figure 2E) and SA-beta-galactosidase staining (Supplementary Figure 4A and B) revealed an increased percentage of DsRed+ cells expressing SA-beta-galactosidase in the hybrid cell-enriched co-culture compared to single culture. Moreover, we observed higher SA-beta-galactosidase staining in the metastases from mice injected with flow-sorted hybrid cells compared to Luc-GFP-231 (Figure 2F). Together, these data indicate that hybrid cells are enriched in polyploid cells with a senescent phenotype.

**Hybrid cells drive breast cancer metastatic progression and chemoresistance**

The main reason for chemotherapy failure in TNBC is the emergence of chemoresistance, which ultimately leads to patient death. Thus, we investigated whether hybrid cells might contribute to breast cancer chemoresistance. Towards this, we treated hybrid cell-enriched co-cultures or single cultures of GFP-BCCs with doxorubicin (DOXO) or paclitaxel (PTX), two first-line chemotherapeutic agents used in patients with breast cancer (24, 25). Treatment with DOXO or PTX significantly reduced the GFP signal in GFP-231, -436 and CCN6KO cells in single culture. However, neither drug reduced the GFP signal in the hybrid-enriched co-cultures (Figure 3A, Supplementary Figure 5A and B). To investigate in greater detail the effect of chemotherapy specifically on hybrid cells, we treated the co-cultures with DOXO, PTX, or vehicle and sorted the GFP+ and GFP+/DsRed+ hybrid cells by flow cytometry. As shown in Figure 3B, DOXO or PTX treatment effectively reduced the percentage of GFP+ 231 cells compared to vehicle treated samples. However, neither drug reduced the percentage of GFP+/DsRed+ cells, with DOXO increasing the percentage of hybrid cells. We next investigated the impact of chemotherapy on each single hybrid cell using our microfluidic single-cell paring device, which allows to follow the
formation of hybrid cells and the fate of each cell over time (11). For this, we loaded GFP-231 alone or with DsRed-MSC (1:1) in each microfluidic chamber and after 72 h incubation, we treated the cells with PTX for 48 h. We observed that PTX treatment significantly reduced cell viability of GFP+ 231 cells without affecting cell viability of hybrid cells. Moreover, hybrid cells had higher cell viability compared to single cells upon PTX treatment. These data demonstrate that hybrid cells show chemoresistant properties at single cell resolution level (Figure 3C).

Based on these results, we set out to test the ability of hybrid cells to progress during chemotherapy in the presence of primary mammary tumors, modeling the clinical setting of neoadjuvant chemotherapy. Hybrid-enriched co-cultures of GFP-231 with DsRed-MSCs containing 89% hybrid cells were orthotopically inoculated into the mammary fat pads of NOD/SCID mice. When tumors became palpable, mice received PTX every 3 days. As shown in Figure 4A, PTX reduced the size of primary tumors compared to vehicle, whereas it increased the percentage of PanCK+/FAP+ compared to vehicle treated controls (Figure 4B), analyzed using multiplex immunofluorescence staining. Notably, in mice bearing hybrid enriched tumors, PTX had no effect on the number of spontaneous pulmonary metastases compared to controls (Figure 4C).

To directly test the ability of hybrid cells to acquire chemoresistance in vivo, GFP-231 labeled with luciferase (Luc-GFP-231) in single culture and in co-cultures with DsRed-MSCs were injected intracardially in NOD/SCID mice. We began treatment with DOXO 20 days after intracardiac injection and euthanized mice at day 27, when they developed clinical signs of metastasis. Quantification of the number of distant metastases by GFP immunofluorescence of fresh tissues at necropsy (Figure 4D) and by histopathological studies (not shown) demonstrated that, as expected, DOXO reduced the number of distant metastases in mice injected with Luc-GFP-231 cells compared to vehicle. In contrast, DOXO treatment had no significant effect in the number of metastases in mice injected with hybrid-enriched co-culture compared to vehicle (Figure 4D).
Cell senescence has been linked to drug resistance and metastasis (26-29). Thus, we next investigated the chemoresistant properties of hybrid cells and the relevance of the senescent cells within the hybrid population to metastasis. For these experiments we employed CCN6KO BCCs in syngeneic FVB immune competent mice. We cultured GFP-CCN6KO with DsRed-MSCs for 5 days which resulted in 95% hybrid GFP+/DsRed+ cells (Supplementary Figure 2D) and treated the cells with Navitoclax, a senolytic drug which selectively eliminates non proliferative cells (30). Navitoclax significantly reduced the CCN6KO-GFP signal of hybrid cells compared to control but had no significant effect in CCN6KO-GFP cells in the single culture (Supplementary Figure 5C). Next, we intracardially injected Luc-GFP-CCN6KO and hybrid cells pretreated with Navitoclax or vehicle. Additionally, we treated the control mice with PTX or vehicle for 10 days (Figure 4E and F). Validating the data with DOXO treatment, PTX significantly reduced distant metastases of Luc-GFP-CCN6KO but had no significant effect on the distant metastasis of hybrid cells compared to the vehicle control group. Notably, pretreatment of hybrid cells, but not BCC single cultures, with Navitoclax significantly reduced metastatic burden compared to controls (Figure 4F). Collectively, these data show that hybrid cells enhance breast cancer metastatic progression and chemoresistance and that the senescent cells within the hybrid population are important for metastasis.

WNT5A downregulation rescues MSC engulfment and hybrid cell chemoresistance

We have reported that MSC engulfment induces persistent gene expression changes in MDA-MB-231 cells with significant upregulation of WNT5A by hybrid cancer cells compared to non-engulfing counterparts (11). To directly investigate the role of WNT5A in hybrid cell formation, we knocked-down the endogenous expression of WNT5A (shWNT5A) in GFP-231 BCCs using lentiviral delivered short harpin RNA (Figure 5A). We also established shWNT5A in GFP-T4 patient-derived BCCs (Supplementary Figure 6A). shWNT5A effectively reduced WNT5A protein levels in both cell lines (Figure 5A and Supplementary Figure 6A) and reduced
phosphorylation of WNT5A downstream targets RAC (RAC 1/2/3), Protein kinase C-alpha (PKC-\(\alpha\)) and Phospholipase C 1-gamma-1 (PLC-1-\(\gamma\)-1) (31) compared to GFP-231-shC cells (Figure 5A, Supplementary Figure 6B). WNT5A shRNA downregulation or WNT5A blocking antibody significantly reduced the percentage of hybrid cells by flow cytometry (Figure 5B), immunofluorescence (Supplementary Figure 6C-D) and reduced the rate of MSC engulfment measured using a microfluidics cell pairing device (Figure 5C). Demonstrating the relevance to chemoresistance, WNT5A blocking antibody reduced the viability of hybrid cells treated with PTX in the microfluidic device compared to vehicle (Figure 5D). Altogether, these data show that the downregulation of WNT5A reduces MSC engulfment and generation of hybrid cells. These results also demonstrate that WNT5A knockdown leads to phenotypic changes in the hybrid cells reducing their chemoresistant properties.

**Hybrid cells are generated through a phagocytosis-like mechanism mediated by a WNT5A-IL6-CCL2 cytokine network**

Studies have shown that WNT5A regulates IL6 expression in melanoma and modulates the expression of SASP factors in tendon stem cells, and that IL6 exhibits a reciprocal regulation with CCL2 in non-small cell lung cancer (32, 33). These data, together with our results that hybrid cell-enriched co-cultures secrete SASP factors (Figure 2D), suggest the novel hypothesis that WNT5A may maintain the generation of hybrid cells through a cytokine network involving SASP proteins. To further examine the detailed mechanisms involved in the generation of hybrid cells, we first investigated the cell type responsible for specific SASP protein secretion. Using cytokine arrays and ELISA of the conditioned media of GFP-BCCs and DsRed-MSCs, we found that BCCs are the main source of IL6, OPN, THBS1 and uPAR and that MSCs secrete CCL2 (Supplementary Figure 7A-C). WNT5A downregulation in GFP-231 significantly reduced secretion of IL6 in the conditioned media of hybrid enriched populations (Figure 5E) and IL6 recombinant protein was sufficient to increase CCL2 secretion by MSCs (Figure 5F). Collectively,
these data reveal a cytokine network between BCC and MSC in which WNT5A released by BCCs
stimulates IL6 secretion that in turn, increases the release of CCL2 by MSCs.

Next, we investigated whether WNT5A might mediate hybrid cell formation through a
phagocytosis-like mechanism. GFP-231-shC and -shWNT5A cells were cultured with MSCs
labeled with a pHrodo dye that becomes fluorescent when MSCs enter in the acid phagosome,
allowing to follow the phagocytosis flux. After 24 h, we found that the pHrodo emission increased
over time and was maximal at 72 h (Supplementary Figure 7D). WNT5A downregulation
significantly reduced phagocytosis compared to controls (Figure 5G and Supplementary Figure
7E), which was rescued by addition of recombinant IL6 and WNT5A proteins. Further, inhibition
of the CCL2 receptor, CCR2, using a specific antagonist (CCR2i) or IL6 receptor blockade using
Tocilizumab, a monoclonal antibody against IL6R, effectively reduced phagocytosis in co-cultures
of GFP-231 with MSCs labeled with pHrodo (Figure 5G). Taken together, these data show that
hybrid cell formation occurs through a phagocytosis-like mechanism mediated by a WNT5A-IL6-
CCL2 network.

The WNT5A-IL6-CCL2 protein-protein interaction network is associated with
chemoresistance and metastasis in human TNBC

To ascertain the significance of our studies to human disease, we analyzed the WNT5A-
IL6-CCL2 network in a proteomics dataset that contains expression levels of over 5,000 proteins
deregulated in human TNBC that we have recently reported (16). Differential expression analyses
show that chemoresistant metastatic TNBCs show upregulation of 113 proteins compared to non-
metastatic tumors (n=4 independent patient samples with replicates, per group), with significant
upregulation of proteins in the metabolic and nuclear processes, and with downregulation of
proteins in secretory functions (Figure 6A, Supplementary Figure 8A, Supplementary Table
3). We identified a significant WNT5A-IL6-CCL2 predicted protein-protein interaction (PPI)
network in metastatic TNBC tumors (Figure 6B). GO analyses of the predicted interactors in the
WNT5A-IL6-CCL2 network show significant representation of endoplasmic reticulum lumen, extracellular space, and extracellular region processes (Figure 6C). Supporting these data, independent analysis of the GSE183947 publicly available human breast cancer dataset revealed a significant positive correlation between IL6 and CCL2 transcript levels in breast cancer metastasis compared to primary tumors (primary tumors: r=0.24, p=0.383 vs. metastasis: r=0.64, p=0.0009, Supplementary Figure 8B).

To expand on this analysis in the specific setting of breast cancer chemoresistance, we analyzed the TCGA dataset using the KM plotter bioinformatics platform (https://kmplot.com/analysis/). This dataset contains 5-year relapse free survival and gene expression data from patients with breast cancer treated with anthracyclines (e.g., DOXO, n=383) or taxanes (e.g., PTX, n=237). These analyses revealed that IL6R and CCR2 expression is associated with response to chemotherapy. Specifically, tumors with high IL6R or CCR2 expression had a shorter 5-year recurrence-free survival compared to tumors with low IL6R or CCR2 (Figure 6D). Collectively, these results show that WNT5A-IL6-CCL2 are enriched in TNBC tumors that develop metastasis and that high expression of IL6R or CCR2 is associated with breast cancer chemoresistance and recurrence.
Discussion

Although initially responsive to chemotherapy, TNBC patients frequently develop drug resistant metastatic progression (3). In recent years, studies have shown that TNBC tumors are highly heterogeneous and that this property fuels chemoresistance through selection of resistant subclones (34, 35). In this study, we investigated whether the existence of breast cancer hybrid cells generated by MSC engulfment influences TNBC progression. Using clinical samples of breast cancer metastases and in vitro and in vivo models, we discovered a hybrid cell population enriched in senescent and polyploid cells that enhance TNBC chemoresistance, suggesting a novel mechanism of TNBC progression.

Using a new high throughput microfluidic cell pairing chip to dynamically study the interaction between two single cells, we have demonstrated that BCCs can engulf MSCs resulting in the generation of hybrid cells with markers of BCCs and MSCs. In this study, we observed a unique hybrid population expressing panCK and the mesenchymal cell marker FAP in clinical tissue samples of primary invasive carcinomas and distant breast cancer metastasis that have failed chemotherapy. We show that FAP is selectively expressed by MSCs and can be used as a potential marker for the identification of BCC-engulfing MSCs in breast cancer metastasis.

To evaluate the biological relevance of the hybrid cells in breast cancer drug resistance and dissemination, we generated hybrid cells in vitro culturing a panel of BCCs with MSCs and we employed primary orthotopic and intracardiac injection models for further evaluation in vivo. We found that PTX significantly reduced primary tumor size and increased the percentage of panCK+/FAP+ hybrid cells. Moreover, PTX failed to reduce the number of distant metastases. In two independent models of breast cancer metastasis, neither PTX nor DOXO treatments reduced distant metastasis formed by hybrid cells compared to BCCs alone. Thus, we identified a hybrid cell population that enhances breast cancer chemoresistance and facilitates metastatic
progression. Based on our studies, targeting this specific population could hold promise in triggering a more robust tumor response to chemotherapy and combating drug resistance.

An important finding in our study is that the hybrid cell population generated through MSC engulfment is enriched in polyploid cells with senescent properties. Although cellular senescence has been previously considered as a tumor suppressive mechanism (36), studies have shown that senescent cells maintain a metabolically active state (37) supporting tumorigenesis. Indeed, senescent cells release several mediators, including pro-inflammatory molecules, growth factors and proteases that create a pro-tumorigenic milieu. In agreement with a previous study (12), we observed that hybrid cells show increased secretion of SASP factors including IL6, CCL2, THBS1, uPAR and OPN. We found that approximately 50% of hybrid cells express senescence-associated beta-galactosidase (SA-beta-Gal) a well-characterized marker of senescence (23) compared to 3% of BCCs, and that lung metastasis derived from hybrid cells have higher expression of SA-β-Gal than those derived from BCCs. Further supporting the senescent phenotype of hybrid cells and their relevance to metastasis, we found that the senolytic drug Navitoclax reduced hybrid cell viability and resulted in hybrid cells with reduced metastatic ability.

A remaining question is whether the proliferative or the senescent cells within the hybrid cell population is responsible for their chemoresistant properties. Our data support that senescent hybrid cells remain metabolically active, secreting cytokines that allow the communication with various components of the microenvironment and potentiate the aggressiveness of breast cancer. These findings support the idea that targeting hybrid cells could serve as a promising therapeutic strategy to counteract breast cancer metastasis and overcome chemoresistance.

To elucidate the mechanism of hybrid cell formation, we investigated the involvement of WNT5A, a non-canonical signaling member of the WNT family, which we previously found to be highly expressed by BCC-engulfing MSCs (11). The role of WNT5A in tumorigenesis is not completely understood. A recent study showed that WNT5A initially supports the dissemination and seeding of melanoma cells in the lungs, activating dormancy (38). Another study
demonstrated that WNT5A also induced cellular senescence in stem/progenitor cells (39). Here, we demonstrate that WNT5A downregulation reduces MSC engulfment, hybrid cell formation and chemoresistance, supporting an important role in maintaining the aggressive properties of hybrid cells. Although further studies are needed to explore the role of WNT5A in hybrid cell formation in vivo, our mechanistic data demonstrate that WNT5A is a crucial player of hybrid cell formation through a phagocytosis-like mechanism. Data reported in the literature have shown that WNT5A can regulate the secretion of IL6 and CCL2 in other types of cancers, including melanoma and non-small cell lung cancer (32, 33). Our study defines a mechanism by which WNT5A increases the production of IL6 by BCCs, which, in turn, interacts with MSCs, inducing the secretion of CCL2. Using a pharmacological approach, we found that blocking IL6 receptor or CCR2 is sufficient to reduce hybrid cell formation. Thus, these results suggest that these proteins and their receptors may be potential targets to improve sensitivity to chemotherapy and/or biomarkers of therapy response. Significantly, our analysis of 620 breast cancer patients treated with anthracyclines or taxanes showed that patients with primary tumors expressing high levels of IL6 receptor or CCR2 are significantly less likely to respond to either chemotherapy compared to low receptor expression.

In conclusion, we provide direct evidence of a hybrid cell population in human breast cancer that can be identified by co-expression of cytokeratin and FAP proteins. Our work defines that hybrid cells form through a phagocytosis-like mechanism mediated by a WNT5A-IL6-CCL2 network. Our data show that the hybrid cell population is enriched in senescent cells that contribute to TNBC chemoresistance and metastatic progression. We identify new predictive biomarkers and potential targets to overcome breast cancer drug resistance.
**Materials and Methods**

**Tissue samples and multiplexed fluorescence immunohistochemistry.** Tissue samples from primary breast cancers and breast cancer distant metastasis from 38 patients were retrieved from the Surgical Pathology files at the University of Michigan with IRB approval and reviewed by two pathologists. Paraffin embedded 5 micron-thick sections of each block were subjected to multiplexed fluorescence immunohistochemistry using immunofluorescence. Briefly, Heat induced antigen retrieval was performed using Leica Bond Epitope Retrieval Buffer 1 (Citrate solution, pH6.0) for 20 minutes. Non-specific antibody binding was blocked using Novolink Protein Block (Leica, cat#RE7280-CE) for 30 minutes. A cocktail of both primary antibodies was applied for overnight incubation at 4°C. Goat anti-mouse IgG Alexa Fluor 546 (ThermoFisher, Cat#A11030, Lot#2026145) and goat anti-rabbit IgG Alexa Fluor 647 (ThermoFisher, Cat#A32733, Lot#VA294744) were applied for 60 minutes. A DAPI nuclear counterstain (blue) was applied. Once stained, whole slide images were generated using the Akoya Phenoimager HT and an integrated AI-powered image quality control tools that automatically assess focus, tissue and slide artifacts, and image quality at scale (Reveal Biosciences). Positive cells in whole slide images were identified and quantitated within stained tissue sections as the number of positive cells within the total image analysis area for each sample. The number of positive cells were counted and measured as the total amount of positive cells, total positive cell area and percentage of positive cells within the total image analysis area for each sample. The number of pan-cytokeratin and FAP positive cell was recorded and served to calculate the percentage of pan-cytokeratin+/FAP+ per sample.

**Animal studies.** In vivo experiments were performed following the instructions of the NIH Guide for the Care and Use of Laboratory Animals and were allowed by the Institutional Animal Care and Use Committee at the University of Michigan. To evaluate the formation of hybrid cells in vivo, we injected GFP-MDA-MB-231 (GFP-231) single culture (1.5 x10^6) and GFP-MDA-MB-231 and DsRed-MSC co-cultures (1:2) in the inguinal mammary fat pads of female NOD/SCID mice (n=6).
Two weeks after cell injections, primary orthotopic tumors were imaged in vivo and lungs were imaged ex vivo immediately after euthanizing the mice using confocal two photon microscopy as described previously (40).

To investigate the effect hybrid cells on tumor growth, GFP-231 and DsRed-MSC co-cultures (1:1) were orthotopically injected into the right inguinal mammary fat pad of anesthetized mice at a concentration of 1x10^6 cells in matrigel (3-4 mice per group). When tumors were palpable, mice were treated with Paclitaxel (NDC 63323-763-50), 10 mg/kg i.p. every 3 days or PBS for 15 days. In a separate experiment, GFP- 231 single culture or in co-cultures with equal amounts of DsRed-MSC cells were injected in the mammary fat pad, as above at a concentration of 1.5x10^6 cells (10 mice per group). Mice were treated with PTX 10 mg/kg i.p, every three days. The first day of PTX injection was assigned as day zero of the experiment and mice were treated every three days for 3 weeks. Primary tumor size was measured twice a week using a calliper and mice were euthanized (tumor volume = (length x width^2)/2). Metastases were quantified by GFP pixels in the lungs using ImageJ and confirmed by histology.

To investigate distant dissemination in the absence of primary tumor, GFP-231 labeled with Firefly-luciferase (Luc-GFP-231), were cultured alone or with DsRed-MSCs for 72 h and injected intracardially in eight-week-old female severe combined immunodeficiency mice (The Jackson Laboratories) at a concentration of 1x10^5 cells (GFP-231 alone) or 1.5x10^5 (GFP-231/DsRed-MSCs) resuspended in 50 μl of PBS (n = 10 mice per group). At day 20, mice were divided into 2 groups (n=3-4 mice per group) and treated every three days i.p with DOXO dissolved in saline at doses of 4 mg/kg or vehicle. Metastases were monitored using bioluminescence imaging as previously described (11). Bioluminescence images were acquired using the IVIS imaging system (Xenogen) within approximately 2–5 minutes after injection. Analysis was performed as described (13). Mice were sacrificed and necropsied at day 27. Metastases were identified by GFP fluorescence microscopy right after collecting the tissues at necropsy. The number of metastases per mice per group was quantified using ImageJ. To study
distant dissemination in a immunocompetent mouse model, GFP-CCN6KO cells labeled with
Firefly-luciferase (Luc-GFP-CCN6KO), were cultured alone or with DsRed-MSCs for 5 days and
treated with vehicle or Navitoclax 5 µM for 48h prior being injected intracardially in 8 week old
FVB mice at the concentration of 1×10^5 cells (Luc-GFP-CCN6KO) (n=6-14 mice per group). After
2 days, mice injected with untreated Luc-GFP-CCN6KO in single culture or co-culture were
treated with vehicle or PTX 10 mg/kg for 10 days. Mice were sacrificed and necropsied at day 13.

**Reagents and Antibodies.** FAP antibody was purchased from Cell Signaling (#66562).
Doxorubicin (#D1515), Paclitaxel (#T7402) and C-C Motif Chemokine Receptor 2 (CCR2)
antagonist (#227016) were purchased from Sigma Aldrich (St. Louis, MO, USA). Navitoclax (ABT-
263) (#S1001) was purchased from Selleckchem (Houston, Texas, USA). Recombinant
Interleukin (IL) 6 protein (#7270-IL), recombinant Wnt Family Member 5A (WNT5A) protein were
acquired from R&D System (#645-WN, Minneapolis, USA). Tocilizumab was acquired from Novus
Biologicals (#NBP2-75193, Colorado, USA). Puromycin was acquired from Gibco (#A11138). Cell
Signaling Technology antibodies (Denver, MA, USA): anti-PKC-α (#59754S), anti-p-PKC- α/β II
(#9375S), anti-p-PLC-γ1 (#8713S), anti-PLC-γ1 XP (#5690S), anti-p-RAC1/cdc42 (Ser71)
(#2461), anti-RAC1/2/3 (#2465), anti-WNT5A/B (#MA5-15502), anti-cleaved caspase-3
(#9661S). Mouse monoclonal β-Actin-HRP (#47778, Santa Cruz Biotechnology, Dallas, TX, USA)
was used as loading control. Anti-Ki67 was acquired by BD Horizon (#565929, San Jose, CA,
USA).

**Cell culture.** Breast cancer cell lines MDA-MB-231, MDA-MB-436, MCF-7, MDA-MB-468 were
purchased from the American Type Culture Collection and grown under recommended conditions.
Patient-derived cancer (T4) as well as mesenchymal stem/stromal cells (MSCs) isolated from
fresh human breast cancer metastasis to a supraclavicular lymph node (LN-MSCs) and to the
liver (Lv-MSCs) were isolated and characterized in our lab (13). CCN6KO cells derived from
MMTV-Cre;Ccn6fl/fl mammary breast carcinomas developed and validated in our lab (15) were
cultured with DsRed-MSCs for 7 days. Cell lines were authenticated using STR profiling and were
tested for mycoplasma infection using Sigma LookOut Mycoplasma PCR Detection Kit (Cat MP0035). BCCs and MSCs were labeled with GFP and DsRed, respectively. In another set of experiments, BCCs were labeled with DsRed. To generate hybrid cells, $2 \times 10^5$ DsRed-MSCs and $1 \times 10^5$ GFP-BCCs were cultured in completed medium (50% of BCC medium and 50% of MSC medium) for 72 hours or 7 days as indicated.

Human WNT5A knockdown was achieved using stable short-harping interfering RNA (MISSION shRNA, Sigma Aldrich) (TRCN0000288987, TRCN0000296083 for WNT5A) as previously reported (41). Background control was Lenti-PuroEMPTY-VSVG. Cells were selected with 1 μg/mL puromycin overtime to eliminate un-infected cells. WNT5A protein expression in stable clones were evaluated by immunoblotting.

**Fluorescent imaging.** DsRed-MSCs and GFP-MDA-MB-231, -MDA-MB-436, -T4 or -CCN6KO BCCs were seeded in 6-multiwell plate and cultured in complete medium for 72 h. In another set of experiments, BCCs were cultured alone or with DsRed-MSCs in 24-multiwell plate for 48 h and then treated with doxorubicin or paclitaxel as indicated. GFP and DsRed pixels were quantified by viewing five separate fields at 10x magnification (OLYMPUS IX-71), using ImageJ software. GFP and DsRed signals from DsRed-MSCs and $1 \times 10^5$ GFP-MDA-MB-231 co-culture were also captured using an inverted spinning disk confocal system head from Yokogawa (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan), a cooled digital CCD camera (AxioCamMRm; Zeiss), and two laser lines (488 nm and 561 nm, Zeiss, Jena, Germany) attached to the spinning disc confocal scan head. The system is enclosed in a custom environmental chamber for temperature (37° C) and CO$_2$ (5%) control. Images were recorded using a Plan-Apochromat ×63/1.44 DIC oil objective operated via the Zen 2012 (Blue edition) software platform.

**Enzyme-Linked Immunosorbent Assay.** Levels of IL6, C-C Motif Chemokine Ligand 2 (CCL2), Osteopontin (OPN), Thrombospondin-1 (THBS-1), Urokinase-type plasminogen activator receptor (uPAR) were measured in supernatants derived from BCCs (MDA-MB-231, MDA-MB-436, T4).
and MSCs cultured alone or in co-culture for 72 h. Supernatants from MSCs and BCCs plated in single culture were diluted 1:1 and used as control. Levels of CCL2 were measured in supernatants derived from MSCs after treatment with IL6 rh 30 ng/ml for 24 h. All ELISAs were performed using human ELISA kits according to manufacturer's instructions (R&D Systems).

**Cell Senescence Studies.** MDA-MB-231 TNBC cells and patient-derived MSCs cells isolated and validated, were used to evaluate cellular senescence. Single and co-culture 1:1 ratio of MDA-MB-231 and MSCs were seeded in 6-well plates and cultured for 1 week, then subjected to a beta-galactosidase Staining Kit (Cell Signaling, Cat.no #9860). Following manufacturer's instructions, cells were fixed for 15 min in the fixative solution at room temperature. Cells were washed twice with PBS and incubated with beta-galactosidase staining solution at 37°C overnight in a dry incubator (no CO2). For each sample of the single cultures and co-cultures, 10 randomly chosen fields were photographed using a camera-equipped bright field microscope (Olympus) at 10x of magnification. Data was plotted as the percentage of cells with beta-galactosidase activity.

Cells were also subjected to a Quantitative Cellular Senescence Assay Kit (SA-beta-gal, Fluorometric) (Cell biolabs, Inc Cat.no # CBA-232), flow cytometry and epifluorescence were used for beta-galactosidase activity detection. GFP or DsRed-MDA-MB-231 cells and MSCs were plated as above and pretreated for 2 hrs at 37°C with the 1X solution. SA-β-gal substrate solution was added to the cells and incubated overnight at 37°C. For flow cytometry cells were collected and washed in cold PBS, spin-down at low speedy for 5 minutes and resuspended the pellets in PBS+DAPI with 2% of FBS. For epifluorescence analysis the cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed three time with PBS and counterstained with DAPI (60x of magnification). All experiments were performed at least three times with similar results. Data were analyzed by Student’s t test using GraphPad Prism 7. *p<0.05.

Immunohistochemistry for β-gal staining was performed using citrate retrieval at pH 6.0, and a pressure cooker. Anti-GLB1/Beta-galactosidase antibody (ab203749) was used at 1:250 for 1 hour. We used HRP Polymer for 30 minutes, as well as hematoxylin. Quantification of
immunostaining was evaluated by histopathology and quantified in five different fields per condition using ImageJ.

**Human Cytokine Array.** Human XL Cytokine Array Kits (R&D Systems) were used to analyze the secreted proteins in the supernatants derived from MSCs and MDA-MB-231 in single culture, direct or transwell co-culture as described in the manufacturer’s protocol. Transwell co-cultures were established using trans-well inserts (0.4 μm pore polycarbonate membrane insert Corning, catalogue #3412), loading MSCs in the upper inserts and MDA-MB-231 into the lower compartment of the culture well. The intensity of selected spots was quantified using ImageJ software.

**Immunoblot Analysis.** Immunoblot analyses were carried out with 100 μg of whole-cell extract derived as previously reported (41) Membranes were blocked and incubated with primary antibodies in 4% milk (Sigma-Aldrich, #A3059) in TBS-T (Bio-Rad, #161–0372, with 0.05% Tween20) at 4°C overnight.

**Flow cytometry analysis.** The % of GFP+ and GFP+/DsRed+ population in Ds-Red-MSCs cultured with BCCs for 72 h was determined by FACS analyses. In another set of experiments, MSCs cultured with BCCs for 48 h were treated with Doxorubicin or Paclitaxel as described. Single cell pictures were taken from the Ds-Red+/GFP+ population using Life Imaging Stream Flow Cytometry. All flow cytometry analyses were completed using the UM Flow Cytometry Core, in triplicate.

**Cell cycle analysis using flow cytometer.** Cells were collected by trypsinization, re-suspended in ice cold PBS and fixed by adding ice cold ethanol. After 20 min of incubation, cells were centrifuged, re-suspended in 0.5 ml PBS/RNase solution containing 50 μg/ml DAPI for 20 min in the dark and FACs analyzed. To determine the % of Ki67low cells in G0-G1 phase of the cell cycle, cells were fixed in ethanol and stained in 100 μl of Brilliant Stain Buffer (BD Horizon) with anti-Ki67 for 30 min in the dark. After two washes in the Brilliant Stain Buffer, cells were resuspended in regular medium, stained with Vybrant DyeCycle Ruby (Invitrogen, California, USA) and FACs
analyzed by Bio-Rad ZE5 #2 Cell Analyzer (Bio-rad, Laboratories, California, USA) at the UM Flow Cytometry Core.

**Microfluidic chip design and fabrication.** The high-throughput cellular engulfing device was built by a PDMS (polydimethylsiloxane) piece with microfluidic patterns bonded to a glass slide. The PDMS was patterned by standard soft lithography. The SU-8 mold used for soft-lithography was created by a 3-layer photolithography process with 10 μm, 40 μm, and 100 μm thick SU-8 (Microchem) following the manufacturer’s protocol. The pattern was designed using computer aided design software (AutoCAD 2015, Autodesk®), and the masks were made by a mask-making instrument (μPG 101, Heidelberg instruments). The SU-8 mold was treated by vaporized trichloro(3,3,3-trifluoropropyl)silane (452807 ALDRICH) under vacuum overnight to promote the release of cured PDMS. PDMS (Sylgard 184, Dow Corning) was prepared by mixing with 10 (elastomer): 1(curing agent) (w/w) ratio, poured on SU-8 molds, and cured at 80°C for 4 hours before peeling. Inlet and outlet holes were created by biopsy punch cutting. The PDMS piece with microfluidic channel structures and glass slide were treated using oxygen plasma (100W for 60 s) and bonded. The devices were heated at 80°C overnight to ensure bonding quality.

**Cell engulfment on chip.** We employed the microfluidic cell pairing device as reported in our previous study (11). Briefly, the microfluidic chip was sanitized by UV radiation prior to use to ensure aseptic conditions. Before cell loading, collagen solution (1.45mL Collagen (Collagen Type 1, 354236, BD Biosciences), 0.1mL acetic acid in 50mL DI Water was flowed through the device for one hour to coat collagen on the substrate to enhance cell adhesion. Devices were then rinsed with PBS for one hour to remove the residual collagen solution. Cancer cells and MSCs were harvested from culture plates and re-suspended in culture media to a concentration of $2 \times 10^4$cells/mL. Cancer cell suspension and MSC suspension were mixed 1:1 by volume. 100 μL of the cell suspension solution was then pipetted into the chip inlet, and the cells were driven into the chip by gravity flow. After the cell loading for 5 minutes, the cell suspension solution was replaced by 100 μL of cell culture media to stop cell loading, and the chip was imaged to readout.
cell pairing scenarios initially. The entire chip was placed into a cell culture incubator. Cells were treated with control IgG, anti-WNT5A/B, or untreated. After 72 h cells were treated with vehicle or PTX 10 µM for 48 h. 10 cells for each condition were counted, in biological triplicate. Engulfment rate was measured based on the final status of each device.

**Phagocytosis assay.** Phagocytosis assay was performed using Incucyte pHrodo Red Cell Labeling Kit (Essen BioScience (#4649, Michigan, USA), according to the manufacturer’s instruction. Briefly, 1×10^5 GFP-MDA-MB-231 or -T4 were seeded in 24-well plate for 24 h and incubated with 2×10^5 MSCs labeled with pHrodo dye according to the manufacturer’s instruction. In a set of experiments, GFP-MDA-MB-231 were treated, as described for 30 min before MSCs were plated on top of BCCs. Fluorescence was photographed with OLYMPUS IX-71 microscope, 20x magnification. Integrated red intensity above threshold standardized of pHrodo MSCs was obtained using ImageJ software and normalized to number of cells.

**Whole transcriptome sequencing.** We retrieved 100,000 – 200,000 MDA-MB-231 andMSCs for next-generation whole transcriptome sequencing to identify differentially expressed genes. The RNA was extracted as previously described (11). We identified genes with significant differences in expression between MSCs and BCCs as defined by p value < 0.01 and logarithmic fold change > 0.5. For visualization of gene expression signature, R-based Seurat (42) was used in analysis. For pathway analysis, top-ranked significantly differential genes were applied to Enrichr (http://amp.pharm.mssm.edu/Enrichr/), and the pathway dataset of NCI-Nature 2016 was used. The data have been deposited on GEO (accession number GSE92485)

**Statistics.** Data are represented as mean ± SEM, and experiments were performed at least in triplicate. Number of replicates is indicated in the dot plots in the figures and corresponds to biological replicates. Differences between two groups were examined by unpaired Student’s t test. One-way ANOVA or two-way ANOVA with Tukey’s or Dunn’s multiple comparison test were used to compare more than 2 groups, as indicated. P value of less than 0.05 was considered significant.
Study approval. This study was conducted with approval from the University of Michigan UCUCA protocol# PRO00010731 and IRB protocol# HUM00050330.

Data availability. All data are available in the main text or the supplementary materials.

Author contributions. MEG, GA and CGK formulated the hypothesis and study design. MEG, GA, AP, BB, AE, and CGK performed experiments, analyzed, and interpreted data. GL, JB, and MEG designed and performed the in vivo confocal microscopy studies, YCC and EY developed and performed the microfluidic engulfing assay, SD and AN performed and analyzed the TMT proteomics, LM, DB and SA contributed to aspects of the study design. GA, MEG and CGK wrote the paper.

Acknowledgments. We are grateful to members of the Kleer laboratory for helpful discussion during this project. This work was supported by National institutes of Health (NIH) grants R01CA125577 and R01CA107469 (C.G.K.), Department of Defense Breast Cancer Research Program grant W81XWH-19-1-0093 (C.G.K) and University of Michigan Rogel Cancer Center support grant P30CA046592.

Footnotes

"The authors have declared that no conflict of interest exists."
References


Figure 1. Analysis of clinical samples using quantitative multiplex fluorescence immunostaining.

A. Workflow for multiplex image analysis. We included clinical samples from primary breast cancers (n = 20) and breast cancer distant metastasis (n = 18) using pan-cytokeratin (panCK), the mesenchymal cell marker fibroblast activating protein-α (FAP), and DAPI. For data processing and quantification, we used an automated artificial intelligence (AI) system. B. Representative images of human breast samples showing the presence of hybrid cancer cells co-expressing panCK and FAP. Normal breast tissue, panCK is expressed by epithelial cells in the acini (arrows) and FAP is expressed in the stromal cells (arrowhead). In contrast, primary invasive carcinomas and metastasis contain a population of cancer cells with a hybrid panCK+/FAP+ phenotype. Scale Bar: 30 μm.

C. Quantification of the percentage of panCK+/FAP+ cells as well as total cells analyzed per sample. Data are expressed as individual values with mean ± SEM analyzed with 2-tailed unpaired Student’s t test.
Polyploidy (>4N+):

- GFP+ cells
- DAPI x10
- >4N+ Count

- DsRed+/GFP+ Hybrid cells
- DAPI x10
- Polyploidy (>4N+)

- IL6
- CCL2
- OPN
- THBS1
- uPAR

- SA-beta-Gal

- Lung metastasis

- H&E
- BF
- GFP
- DsRed
- Overlay
Figure 2. MSC engulfment by breast cancer cells (BCCs) generates a hybrid polyploid population characterized by a senescent phenotype.

A. Schematic representation of BCCs cultured with MSCs to obtain hybrid-enriched population. B. Representative flow cytometry live imaging stream pictures showing a Ki67<sub>low</sub> hybrid cell. GFP-labeled MDA-MB-231 (GFP-231) BCCs were cultured with DsRed-labeled MSCs (DsRed-MSCs) for 72 h and stained with Ki67 antibody. Ki67<sub>low</sub> cells were sorted, fixed and nuclei were stained with DAPI. An overlay of all fluorescence channels shows a multinucleated GFP+/DsRed+ hybrid cell. Cell phase image (BF) is included to display cell morphology. C. Cell-cycle analysis by flow cytometry in the co-culture of GFP-231 BCCs with DsRed-MSCs after 72 h showing the emergence of a polyploid population in GFP+/DsRed+ (hybrid) compared to GFP+ cells. Bar graph shows the quantification of the flow cytometry analyses. D. Enzyme linked immunosorbent assay (ELISA) analyses of Interleukin (IL) 6, C-C Motif Chemokine Ligand (CCL) 2, Osteopontin (OPN), Thrombospondin (THBS) 1, Urokinase-type plasminogen activator receptor (uPAR) in supernatants of GFP-231 BCCs and MSCs diluted 1:1 (GFP-231) or in co-culture (GFP-231+DsRed-MSCs). E. Senescence-associated beta-galactosidase (SA-beta-Gal) activity analyzed by flow cytometry using DsRed labeled MDA-MB-231 cells (DsRed-231) and MSC (unlabeled) in single cultures and co-cultures for 1 week. Bar graph shows the percentage of SA-beta-Gal/DsRed+cells. F. Lung metastases from mice injected intracardially with GFP-231 or FACS-sorted GFP+/FAP+ hybrid cells stained with H&E and SA-beta-Gal by immunohistochemistry (n = 3 per group). Bar graph shows the quantification of the SA-beta-Gal in five different fields per condition (n = 4 per group) using ImageJ. Scale bars: 20 μm. For all panels data are shown as individual values with mean ± SEM. In C, D and F, 2-tailed unpaired Student’s t test was employed; in E one-way ANOVA with Tukey’s multiple comparison test was employed *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.
**Figure 3.** Hybrid cells exhibit chemoresistance properties  

**A.** GFP signal of GFP-231 cells alone or in co-culture with DsRed-MSCs, treated with doxorubicin (DOXO) 1 µM, paclitaxel (PTX) 10 µM, or vehicle for 24 h. GFP was quantified in five different fields per condition using ImageJ. Representative pictures of GFP signal upon drug treatment are shown. Scale bar: 200 µm.  

**B.** Effect of chemotherapy on the specific GFP+ and GFP+/DsRed+ populations of co-cultures of GFP-231 with DsRed-MSCs treated with DOXO 1 µM, PTX 10 µM, or vehicle. Bar graphs show the quantification of flow cytometry analyses expressed in fold changes of each population of cells with respect to vehicle treated samples, in biological triplicates.  

**C.** A single GFP-231 alone or with a DsRed-MSC was loaded in the microfluidic device. After 72 h, cells were treated with PTX 10 µM for 48 hours (days 4 and 5). Representative images of hybrid cells and GFP-231 breast cancer cells are shown (scale bar: 20 µm). Note that PTX treatment induced reduced cell viability only in GFP-231 cells. Bar graph shows the percent of cell viability ± SEM of hybrid cells and GFP-231 after 48 h of PTX treatment (number of cells= 10, in biological triplicates). For all panels data are expressed as individual values with mean ± SEM analyzed. In A and C, two-way ANOVA with Tukey’s multiple comparison test was applied; in B one-way ANOVA with Tukey’s multiple comparison test was applied. *p<0.05; **p<0.005; ns: not significant.
Figure 4

**GFP-231 + DsRed-MSC**

### A. Primary tumor

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<tr>
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<tr>
<td>Tumor volume (mm$^2$)</td>
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<td>PanCK+FAP+ cells (%)</td>
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### B. Primary tumor

- **Vehicle**
- **PTX**

### C. Lung metastasis

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### D. DOXO

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### E. CCN6KO

- **Coculture**
- **Hybrids**

### F. Navitoclax

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<td>Metastasis photon flux (p/s)$\times 10^7$</td>
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**GFP-231**

- **GFP-231**
- **GFP-231 + DsRed-MSC**

**DOXO**

- **DOXO**
- **DOXO +**

**CCN6KO**

- **2 days**
- **1x10^5**
- **+/ PTX**

**Navitoclax**

- **+/ PTX**

**BLI**
Figure 4. In vivo, hybrid cells drive resistance to chemotherapy and senescent hybrid cells specifically enhance metastasis

A. Quantification of primary tumor volume of co-cultures injected in the mammary fat pads of NOD/SCID mice treated with vehicle (-) or with PTX 10 mg/kg every 3 days i.p. for 20 days (n = 3-4 per group). B. Primary mammary tumors in A were subjected to quantitative fluorescence multiplex immnostaining for panCK and FAP to quantify the panCK+/FAP+ hybrid cells. Shown are representative images of vehicle or PTX treated tumors. Scale bar: 50 µm. C. Number of spontaneous lung metastasis of per mice per group of mice in (A) assessed by histopathology. D. Luc-GFP-231 (1.0x10^5) alone or co-cultured with DsRed-MSCs for 72 h (1.5x10^5 cells) were injected intracardially in NOD/SCID mice. At day 20, mice were treated with DOXO (4 mg/kg every three days) or vehicle for one week (n = 3-4 per group). Shown are representative images of GFP+ lung metastases and bioluminescence images of distant metastases at necropsy in mice treated with DOXO or vehicle. Quantification of the number of metastasis in mice using GFP pixels. Scale bar: 20 µm. E. Schematic illustrating that Luc-GFP-CCN6KO BCCs (1.0x10^5) alone or with DsRed-MSC were cultured for 5 days and treated with Navitoclax (5 µM for 48 hours) or vehicle (Veh). Cells (1x10^5) were intracardially injected in FVB mice. After 2 days control mice were treated with vehicle or PTX 10 mg/kg for 10 days and followed using bioluminescence imaging (BLI). F. Representative bioluminescence images of distant metastases at indicated conditions (n= 6-14 per group). In A–C, the 2-tailed unpaired Student’s t test was employed; in D one-way ANOVA with post hoc Tukey HSD/Tukey-Kramer; in F two-way ANOVA with Tukey’s multiple comparison test. Data are expressed as individual values with mean ± SEM. *p<0.05; **p<0.005; ***p<0.0005; ns: not significant.
Figure 5

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Cell pairing of GFP-231 + DsRed-MSC

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Figure 5. WNT5A, IL6 and CCL2 mediate hybrid cell formation through a phagocytosis-like mechanism.

A. Immunoblots of GFP-231 transduced with lentivirus control (-shC) or with two independent shWNT5A (1 and 2). Beta-actin was used as loading control. Numbers below the blots represent the average fold change over GFP-231-shC. B. Representative flow cytometry of indicated cells show that WNT5A shRNA reduces the percent of GFP+/DsRed+ hybrid cells. Quantification is shown as fold change ± SEM. C. A single GFP-231 and a single DsRed-MSC were loaded in the microfluidic cell-pairing device and treated with vehicle or anti-WNT5A over time. Bar graph shows percent of engulfment ± SEM (number of cells= 10, in biological triplicate). Representative images illustrating that anti-WNT5A inhibited hybrid cell formation. Scale bar: 20 μm. D. GFP-231 and MSC loaded in the microchip and after 3 days were treated with PTX 1μM for 48 h. Bar graph shows percent of viability ± SEM (n=10 cells/condition, in biological triplicate). Scale bar: 20 μm. E. ELISA for IL6 protein secretion in supernatants of co-cultures of DsRed-MSCs and GFP-231-shC or –shWNT5A after 72 h. F. ELISA for CCL2 protein secretion in the supernatants of DsRed-MSCs treated with vehicle (-) or with IL6 recombinant protein (rh), 30 ng/ml. G. Representative pHrodo and merge fluorescence images of GFP-231-shC treated with vehicle, Tocilizumab 30 μg/ml or CCR2 inhibitor (CCR2i) 40 μM and GFP-231-shWNT5A treated with vehicle, IL6 rh 30 ng/ml and WNT5A rh 1 μg/ml before incubation with pHrodo labeled MSCs for 48h. Scale ba: 50 μm. Bars quantify total red fluorescence normalized to cell number, pHrodo signal was quantified in 3 fields/condition using ImageJ. Data are expressed as individual values with mean ± SEM. In B, one-way ANOVA with Tukey’s multiple comparison test was employed; in C-F, 2-tailed unpaired Student’s t test was employed; in G two-way ANOVA with Tukey’s multiple comparison test was employed*p<0.05, **p<0.005, ***p< 0.0005; ****p<0.0001.
Figure 6. WNT5A, IL6 and CCL2 proteins are enriched in human chemoresistant metastatic breast carcinomas.

A. Differential expression analysis on the proteomics profile of metastatic and non-metastatic human TNBC tumors (n = 4 per group). Volcano plots show differential up and down regulated proteins. We considered all protein in the region p<0.05 and FC>1 and outliers within p<0.001 and between -1<FC<1. B. Protein network graph (STRING) depicts predicted protein-protein interaction networks of WNT5A, IL6, and CCL2 with up and down regulated proteins in A. Note formation of two nodes (red and green). C. GO analysis showing the significant biological processes of the WNT5A, IL6, and CCL2 significantly predicted protein-protein interaction network in metastatic vs. non-metastatic TNBC. D. Analyses of publicly available datasets containing treatment response information on 1,329 breast cancer patients treated with Antracyclines (e.g., doxorubicin) or Taxanes (e.g., paclitaxel) in relationship to relapse-free survival at 5 years after treatment. Shown are box plots (top) and corresponding ROC curves. We used KM Plotter (Kmplot.com).