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14. ABSTRACT

The goal of this proposal was to determine whether cell fusion between tumor cells and hematopoietic cells is the precipitating event for breast cancer metastasis and whether viral fusion proteins enable or catalyze this event. Our results suggest therapeutic strategies to disrupt or inhibit cell fusion could reduce tumor heterogeneity and limit the formation of metastases. In support of this statement we demonstrated the following in the course of the funded award. First, we found mesenchymal stem cells are a potent fusion partner for breast cancer cells of all phenotypes. Fusion occurred spontaneously at rates of up to 1 in 250 cells. Rates were increased with decreased availability of oxygen. This finding is especially relevant given the hypoxic nature of the tumor microenvironment. Fusion products were able to migrate faster and invade collagen gels more rapidly and at greater distances than unfused counterparts. In addition, the transcriptome of fusion products was highly diverse, capable of upregulating critical oncogenes and downregulating tumor suppressors. Further, we found apoptotic cells can promote cell fusion and we are now extending this line of study to identify molecular targets on cancer cells that are responsive to apoptotic cell engagement, especially members the receptor family for lipid engagement. Such targets could prove quite useful for the development of a new class of pharmaceutical agent capable of inhibiting or limiting cell-cell fusion.

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INTRODUCTION

Ninety percent of breast cancer-related deaths are due to secondary tumors, or metastases, that form at sites far removed from the primary tumor. Exactly how metastases form is yet unclear. The goal of this proposal is to determine whether cell fusion between tumor cells and hematopoietic cells is the precipitating event for breast cancer metastasis and whether viral fusion proteins enable or catalyze this event. If successful, this discovery would dramatically change our approach to breast cancer therapy in the following specific ways. First, the studies proposed here will help identify fusion partner(s) able to fuse with breast cancer cells to promote a metastatic phenotype. With this information, it may be possible to design strategies to limit interaction with breast cancer cells, including removal of the cell type. In addition, future studies could identify the specific receptor-ligand interactions necessary for cell fusion, to produce a target for drug therapy. Post-fusion events might also be investigated, including the molecular steps governing the integration or rearrangement of genomic DNA to form a single hybrid genome or those steps necessary for activation of genes that regulate the migratory or invasive phenotype. Second, the studies proposed here will investigate the possibility that exogenous, virus-associated proteins might facilitate breast cancer cell fusion. If viral fusogens are found to promote tumor cell fusion, viral vaccination regimes may be appropriate as a prevention strategy. Vaccines might be developed to target viral fusion genes (i.e., fusogens) exclusively, so that the immune system would recognize the protein, even in the context of a eukaryotic cell membrane. Third, the studies proposed here will establish new tools for the study of the complex processes of cell fusion. The inducible bipartite nature of these strategies assures the accurate identification of fusion products, and allows for longitudinal assays both *in vitro* and *in vivo.*

BODY

Specific Aim 1. To determine the specificity and functional capacity of hematopoietic cells that spontaneously fuse with breast cancer cells in vitro.

Aim 1.A To identify the type(s) of cell capable of spontaneously fusing with breast cancer cells

Task 1. Purify hematopoietic subpopulations

To accomplish Task 1 a VarioMACS separation unit (Part No. 130-043-102) was purchased from Miltenyi Biotec, Inc along with CD33 Microbeads (130-045-501) for the positive selection of blood cells of myeloid lineage and a Pan Monocyte Isolation Kit (130-096-537) for negative selection of classical, non-classical, and intermediate monocytes. Human buffy coat was purchased from Innovative Research (IPLA-WB5) to optimize MACS separation before donor blood samples are obtained. Separation was assessed by 2D flow cytometry analysis using fluorescently conjugated antibodies specific for cell surface markers CD14, CD16 (monocyte markers), and CD33, a myeloid lineage marker. (AbD Serotec). Optimization of the magnetic sorting procedure led to a maximum of 4.8-fold enrichment in myeloid lineage cells and a 3.3-fold enrichment in monocytes when compared to unsorted leukocytes (**Table 1**). Representative flow cytometry charts are included in **Figure 1**. This level of enrichment was determined to be sufficient for comparison to unsorted leukocytes in future blood cell-cancer cell cocultures. In a similar way, lymphoid lineage cells and hematopoietic stem cells were enriched. In addition, human mesenchymal stem cells were obtained from bone marrow.

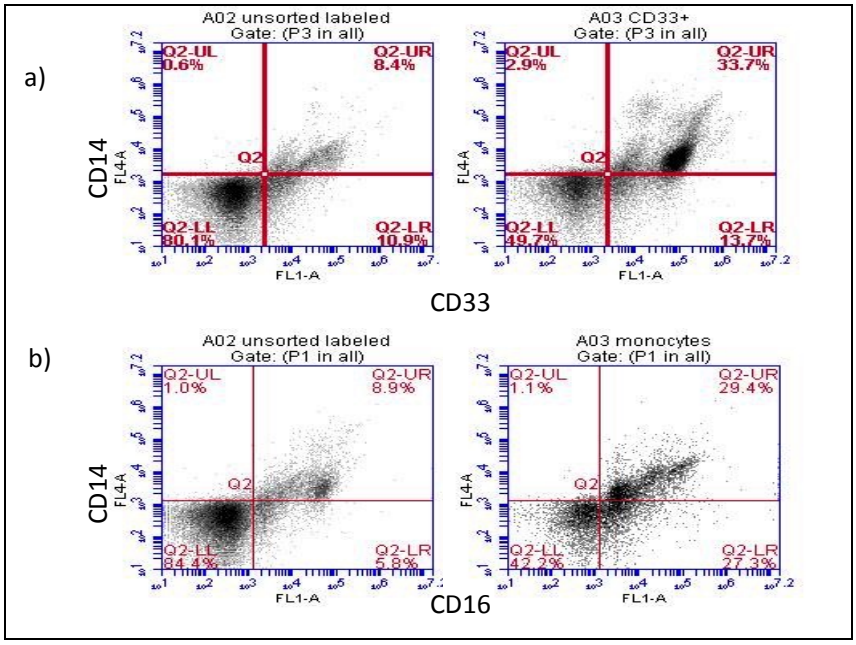


Figure 1. Representative flow cytometry plots for MACS blood cell sorting. a) CD33 (myeloid) sorting. Left, unsorted leukocytes. Right, retentate after positive CD33 selection. b) Pan monocyte sorting. Left, unsorted leukocytes. Right, flowthrough after negative selection.

Table 1. Quantification Sorted Hematopoietic Cells using MACS.

	CD33	CD33 +2 rinses	Mon	Mon +2 rinses
Total Sorted	25000000	16500000	30000000	40000000
Post-sorted	1000000	3300000	25243	6332
Theoretical Max	3170000	3192750	1830000	3668000
Actual # Isolated	606300	1561890	2791	3330
% Efficiency	**19.13%**	**48.92%**	**0.15%**	**0.09%**
% Purity	**60.63%**	**47.33%**	**11.06%**	**30.65%**
Fold enrichment	**4.78**	**2.45**	**1.81**	**3.34**

Task 2. Optimize lentiviral BiFC transduction parameters for hematopoietic subpopulations.

To accommodate transduction of sensitive cell types to be used in blood cell-cancer cell cocultures such as inactivated monocytes and nontumorigenic mammary epithelial cells, we sought to subclone our BiFC DNA constructs into a lentiviral vector with an RFP reporter. In addition to being gentler on sensitive cells than electroporation, lentiviral transduction can reach high efficiencies (up to 95%) in both adherent and non-adherant cells. In addition, we deleted Histone H3.1 coupling from one half of the BiFC probe set. In this way, BiFC still couples in the nucleus, but irreversible binding does not involve linking of histones and instead just the fluorescent moieties. With this approach proliferation was possible and cell viability long term was improved (**Figure 2**).

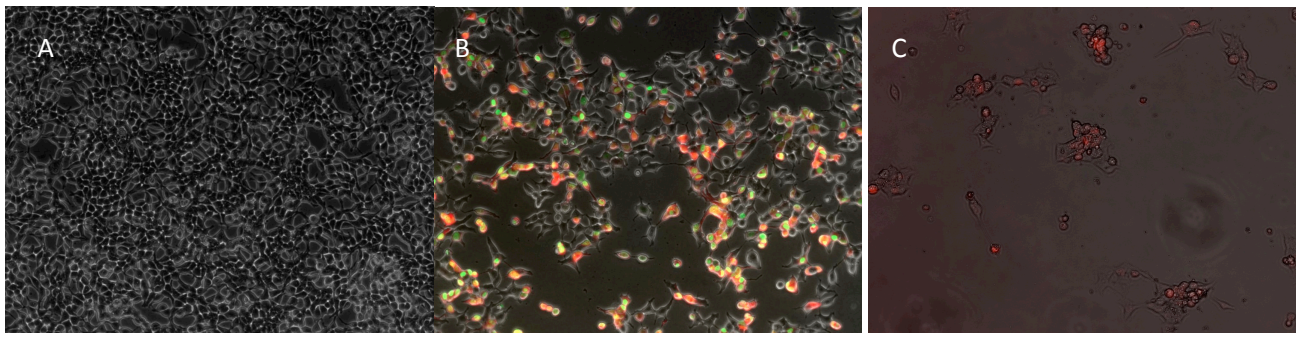


Figure 2. Viability of MDA-MB-231 cells following transfection with BiFC plasmids. A, Untransfected MDA-MB-231 cells. B, MDA-MB-231 cells transfected with modified VN-YC BiFC pairing. Red indicates successful transfection with one BiFC half. Green indicated successful transfection of both BiFC halves (simulating a fusion product) C, MDA-MB-231 cells transfected with original VN-YC BiFC pairing. Red indicates successful transfection with one BiFC half. Green indicated successful transfection of both BiFC halves (simulating a fusion product). Note limited cell survival and no evidence of survival of cells containing both BiFC halves.

Task 3. Established controlled conditions for inducing hypoxic and hypoglycemic microenvironments.

Hypoglycemia was induced in tissue culture by removing glucose in the media. Hypoxic treatment was by removing from standard tissue culture conditions (21% O_2, 5% CO_2, 74% N_2) and put in conditions of tissue culture oxygen deprivation (2% O_2, 5% CO_2, and 93% N_2) for 3hrs. We recognize that standard tissue culture conditions are actually hyperoxic relative to normal tissue, but for the purposes of this study we term the condition of 2% O_2 "hypoxic".

Task 4. Prepare breast cancer cell-hematopoietic subpopulation co-cultures

The design of co-culture experiments is illustrated in **Figure 3**. In our study, we transduced blood cell types, including MSCs with lentiviral particles expressing the BiFC complex, VNH3.1 (GFP, histone H3.1, nuclear localization) and the breast epithelial cells (MCF10A) or breast cancer cells (T47D, MDA-MB-231, MCF7) with corresponding BiFC complex, YCH3.1 (GFP, histone H3.1, nuclear localization). The histone H3.1 tag brings the BiFC halves in close association in the nucleus. We made several attempts to modify co-culture conditions of epithelial cell types with blood cell types without success. The primary challenge was the

nonadherent nature of the blood cell types and adherent nature of the epithelial cell types. In a standard 2D format it is challenging to attain an high enough cell density (as it would be in the tissue) to appropriately test the possibility of spontaneous cell-cell fusion. In the absence of an appropriate 3D model system, we focused our energy on spontaneous fusion observed with two adherent cell types – mesenchymal stem cells and epithelial cell types. Thus, MSCs were mixed with each epithelial cell type (i.e. MSCs with MDA-MB-231, MSCs with T47D, MSC with MCF7, and MSCs with MCF10a) in 4 well chamber slides such that the stromal population was mixed with each epithelial cell type. The ratio of cells for co-cultures was 80,000 MSC cells for 35,000, 112,000, 100,000, and 90,000 MDA-MB-231, T47D, MCF7, and MCF10a cells respectively. Cell densities were selected such that surface area coverage for each cell type was approximately 50% at 24 hours after initiating co-cultures (**Figure 4**). Forty-eight hours after initiating co-cultures, the cells were fixed and stained with DAPI. Fusion products were identified using fluorescence microscopy. A signal (green fluorescence) was considered positive if it was stronger than the background in the FITC channel for unaltered co-cultures, was nuclear (overlapped with DAPI) and, did not crossover to the Rhodamine and CyV channels (**Figure 5**). The frequency of fusion products was reported relative to the total number of nuclei in at least 200 fields of view per experiment with about 110 cells per field. We performed each experiment at least three times.

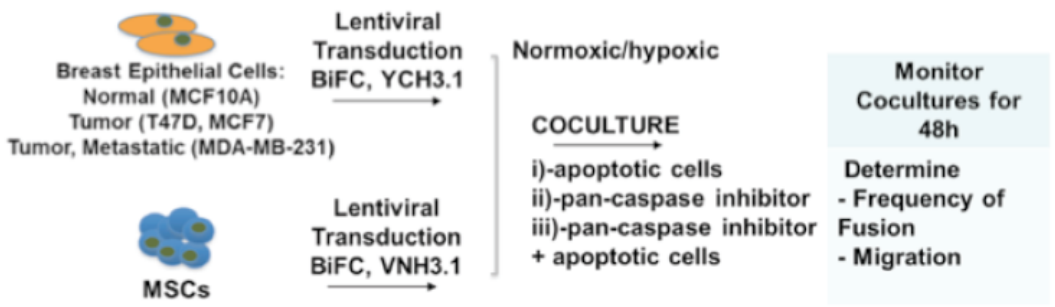


Figure 3. Summary of co-culture experiments

Task 5. Analyze time course data to determine fusion partners (BiFC positive) most apt to fuse

Task 6. Isolate cell types of the subpopulation found most apt to fuse in Task 5

Task 7. Prepare breast cancer cell-hematopoietic cell type co-cultures

Co-cultures for time lapse microscopy were initiated as described in the co-culture protocol above. In brief, MSCs were transduced with lentiviral particles from the BiFC component VNH3.1 and T47D or MCF7 were transduced with lentiviral particles from the BiFC component YCH3.1. 48 hours after initiation of co-cultures, the fusion products were identified using fluorescence microscopy (Nikon TE200). The cells were maintained at 5% CO_2, 37°C, in a humidified atmosphere. Phase contrast and fluorescent images of hybrids were taken every 5min for 3hrs using a 20x objective (0.5 NA, plan fluor 20X phase 1). Images of double transduced parental cells were taken as well as control. The paths of migrating cells were analyzed using the manual tracking and chemotaxis and migration tool from NIH FIJI software(27). In brief, the center of a given nucleus was selected for each frame. Based on the resultant path created, the software was used to calculate velocity, total distance and directionality.

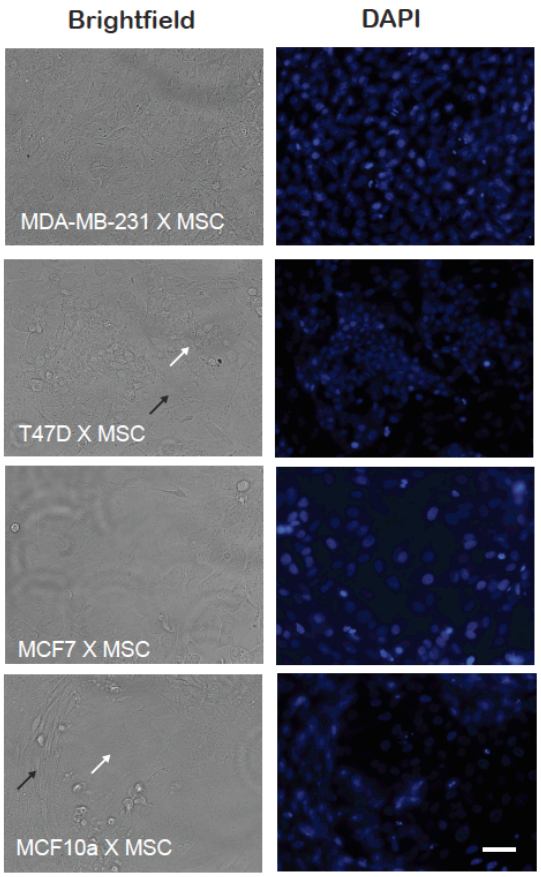


Figure 4. Co-culture experimental protocls and visualization of morphology and distribution. Representative views of the organization of breast epithelial cells in co-cultures with MSCs. Note in many cases, elongated MSCs (black arrow) form circles or boundaries around small populations of breast epithelium (white arrow). Scale bar, 50 μm.

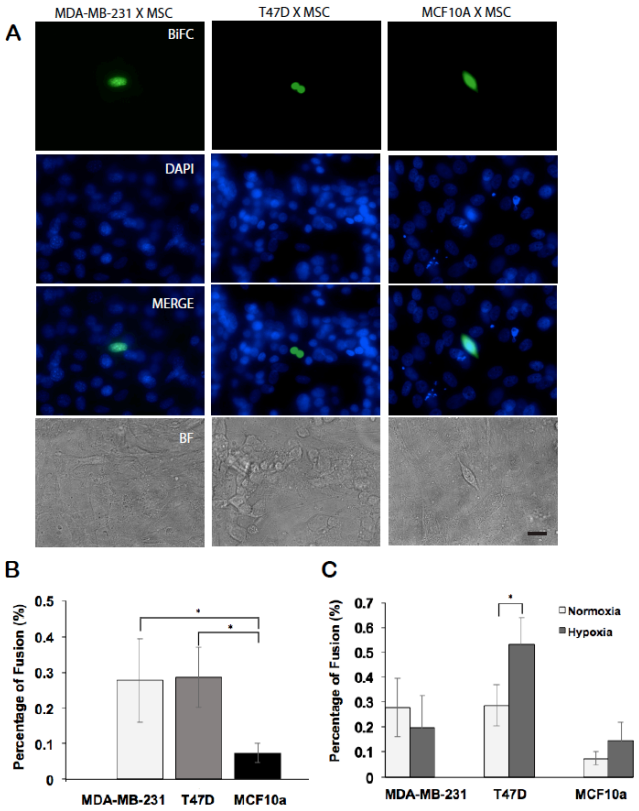


Figure 5. MSCs fuse spontaneously with breast epithelial cells. A. Representative fusion products from MSC x breast epithelial cell co-cultures. Scale bar, 25 μm. **B**. Frequency of fusion of MSCs with breast epithelial cells. Average of 3 independent experiments done in triplicate. ANOVA Tukey's HSD *post hoc* test (*P<0.05, mean ± SD). **C.** Percentage of cell fusion between MSCs and MDA-BM-231, T47D, or MCF10a under hypoxic and normoxic conditions. Each value represents the average of three independent experiments done in triplicate. ANOVA Tukey's HSD *post hoc* test (*P<0.05, mean ± SD).

For long term tracking (72 hrs) MSC and T47D were transiently transfected with mCherry and GFP expressing plasmids respectively. Sixteen hours post transfection, co-cultures were initiated and fusion products (expressing both red and green fluorescence) were identified 24hrs later using a fluorescence microscope. Phase contrast and green fluorescent images of hybrids were taken every 10 min for 72 hrs using 10x objective in the Citation 3 imaging reader (Biotek company Inc.). The cells were maintained at 5% CO_2 and 37°C and the medium was replaced every 24 hrs. At 72 hours a static fluorescence image (green and red) of the tracked hybrid was obtained to confirm hybrid identity.

We found that MSCs fuse spontaneously with all breast epithelial cell types (**Figure 5**) and that the rate of fusion is approximately 1/1000 for cancer cell types and 1/2000 for the non-tumorigenic cell line, MCF10a ($P< 0.05$, **Figure 5**). We also found hybrids acquire the enhanced motility of the MSC parent, but are non-directional, like their T47D breast cancer cell parent (**Figure 6**). The same trend in motility and directionality was also observed in hybrids between MCF7 cells and MSCs.

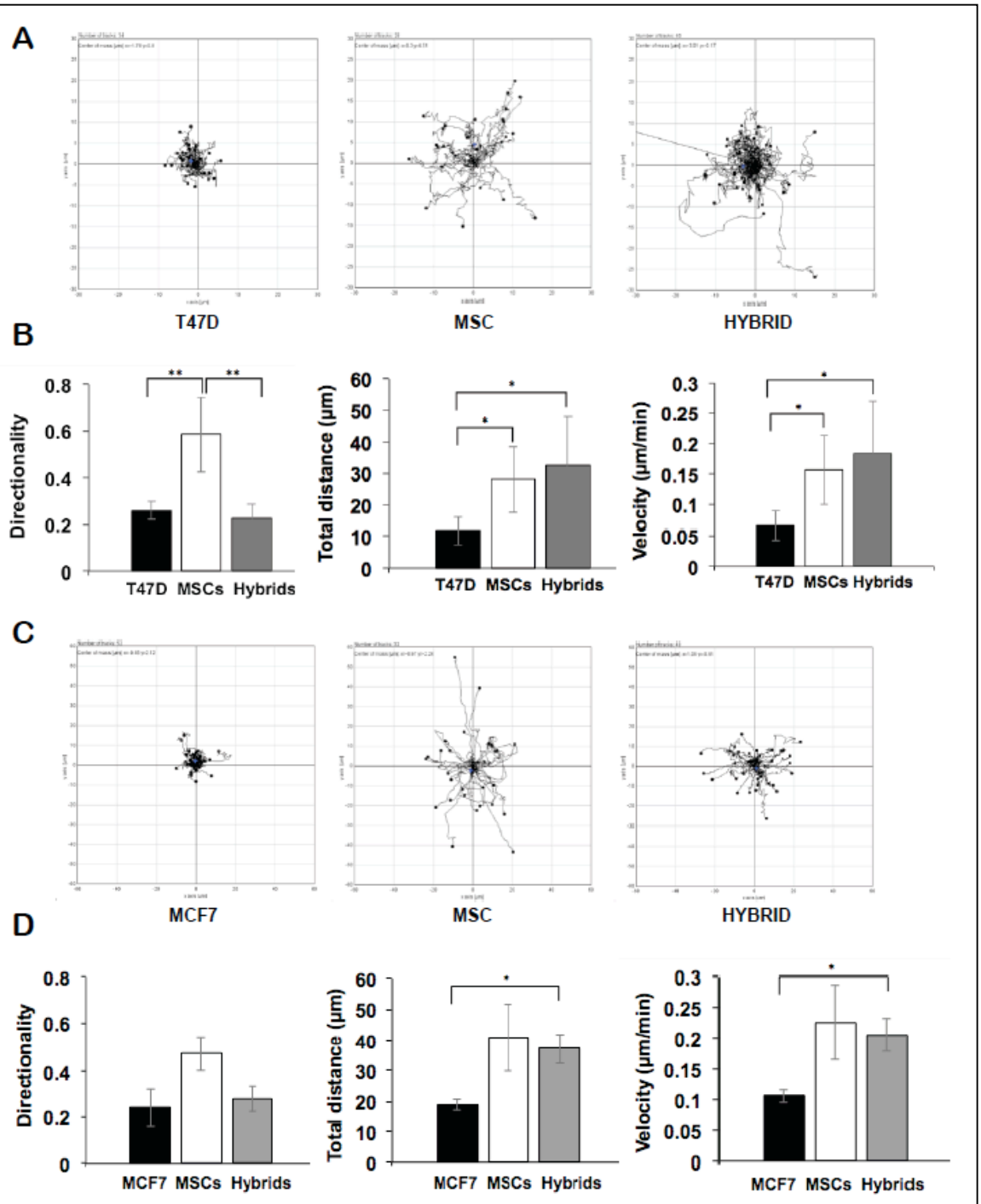


Figure 6. Hybrids between MSCs and T47D or MSCs and MCF7 show higher migratory capability **A**. Representation of motility pattern of T47Ds, MSCs, and hybrids**. B**. Directionality of motion of cells, Total accumulated distance, and Velocity. Average of four independent experiments with the analysis of at least 40 cells per group and per experiment. ANOVA Tukey's HSD *post hoc* test (*P<0.05, **P<0.01, mean ± SD). **C.** Representation of motility pattern of MCF7, MSCs, and hybrids. **D.** Migration of hybrid cells compared to that of MSCs alone or MCF7 alone. Average of 3 experiments with analysis of at least 40 cells per group and per experiment. ANOVA Tukey's HSD *post hoc* test (*P<0.05, mean ± SD).

Task 8. If cell fusion is enhanced with cell stress (hypoxia/hypoglycemia), add 3-Methyladenine

to test possibility of an autophagic response

Given its potential relevance for breast cancer metastasis, it is critical to understand the mechanisms governing cell fusion. In the studies above we note that hypoxia had little impact on the ability of metastatic cancer cells MDA-MB-231 to spontaneously fuse with MSCs. This suggests that hypoxia might promote fusion of nonmetastatic cancer cells with MSCs and may therefore enable metastasis, an event not necessary or advantageous for the already metastatic MDA-MB-231 cells. A recent study identified signaling *via* the phosphatidylserine (PtdSer) receptor BAI1 as a cue to promote fusion of healthy myoblasts, involving the ELMO/Dock180/Rac1 pathway. Blocking apoptosis potently impaired myoblast fusion, and adding back apoptotic myoblasts restored fusion. The added apoptotic myoblasts exposed PtdSer but did not attach to the tissue culture plate, or fuse on their own. They were necessary to promote myoblast fusion, requiring PtdSer-dependent cell-cell contact between apoptotic and viable myoblasts. Since apoptosis is increased for certain cell types in hypoxic conditions (we tested whether the same was true of breast epithelial cells used in this study. In fact, apoptosis was only enhanced with hypoxia (2% O_2, 3 hours) for nonmetastatic cancer cells, T47D and MCF7 (**Figure 7**). Since we found that hypoxia considerably increases fusion between MSCs and the breast tumor cells T47D and MCF7 but not between MSCs and MDA-MB-231 or MCF10a (**Figure 5**), we investigated first whether apoptosis was involved in fusion of cancer cells. Co-cultures between MSCs and T47D cells were initiated as described in Materials and Methods. Sixteen hours following initiation of co-cultures, they were supplemented with T47D apoptotic cells and fusion products were recorded. We found that addition of apoptotic cells to co-cultures significantly increases cell fusion between MSCs and T47D cells under normoxic and hypoxic conditions ($p<0.05$) (**Figure 8**). To continue the investigation of the role of apoptosis in cancer cell fusion, we assessed whether inhibition of apoptosis could impair cell fusion. Apoptosis was inhibited in the fusion partners prior to co-culture initiation using the pan-caspase inhibitor z-VAD-FMK (1μM). The pan-caspase inhibitor remained in the co-culture medium during the entire co-culture experiments and did not seem to affect the morphology or viability of the cells. We analyzed the ability of MSCs and T47D to fuse under normoxic and hypoxic conditions. We found that

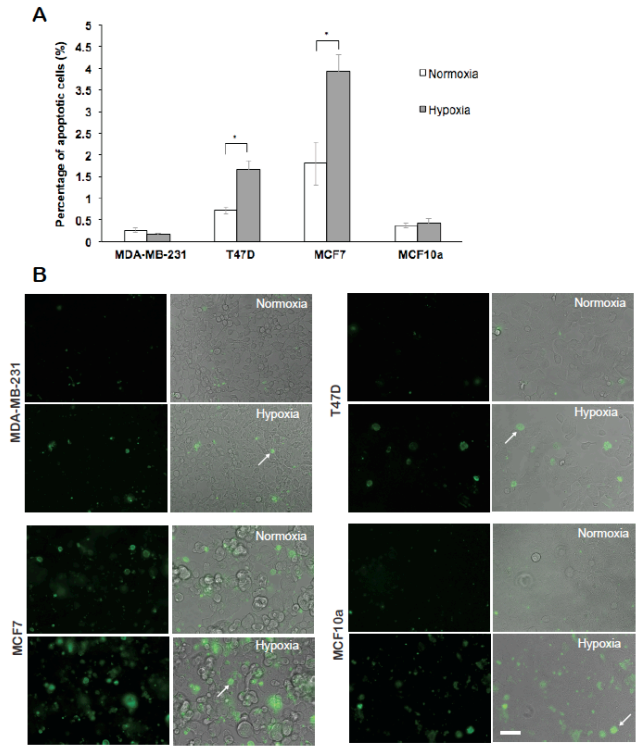


Figure 7. Hypoxia-induced apoptosis of epithelial cells Epithelial cells were exposed to low oxygen for 3 hrs and subsequently stained with Annexin V staining. Apoptotic cells were detected using fluorescence microscopy. **A.** Percentage of breast epithelial cells undergoing apoptosis following exposure to normoxic and hypoxic conditions. Student's *t* test (*P<0.05, mean ± SD). **B.** Representation of hypoxia-induced apoptotic cells as detected using fluorescence microscopy for MDA-BM-231, T47D, MCF7, and MCF10a. White arrows indicate representative apoptotic cells. Scale bar, 50 μM

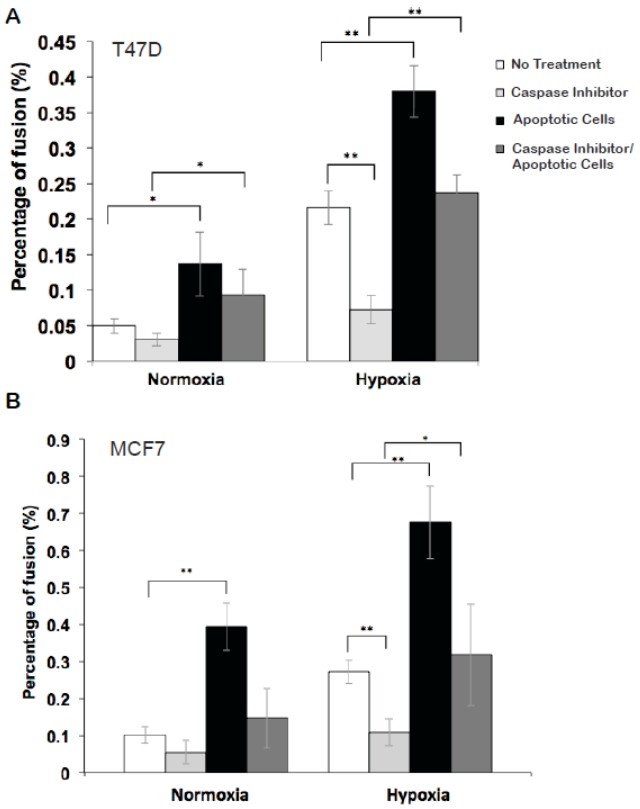


Figure 8. Apoptosis enhances fusion between MSCs and T47Ds, or MSCs and MCF7s
A. Fusion between MSCs and T47Ds is impaired by the pan-caspase inhibitor (z-VAD-FMK) treatment of the cells. However, addition of apoptotic cells enhances fusion between the two cell types and could even rescue the fusion inhibited by the caspase inhibitor. **B.** Fusion between MSCs and MCF7s is impaired by the pan-caspase inhibitor (z-VAD-FMK) treatment of the cells as well. However, addition of apoptotic cells enhances fusion between the two cell types and could also rescue the fusion inhibited by the caspase inhibitor. ANOVA Tukey's HSD post hoc test (*P<0.05, **P<0.01, mean ± SD).

inhibition of apoptosis reduces cell fusion under normoxic conditions and this reduction was significant in hypoxic conditions (P<0.05), whereas addition of apoptotic cells could rescue the fusion diminished by the pan-caspase inhibitor (**Figure 8**). Apoptosis appears also to regulate fusion of nonmetastatic MCF7 cells and MSCs (**Figure 8**). This suggests that the mechanism by which cancer cells fuse with MSCs can involve apoptosis.

Aim 1.B Determine the migratory and invasive capacity of blood cell-tumor cell fusion products

Task 1. Prepare breast cancer cell-hematopoietic cell type co-cultures according to the results of Aim 1.A, Task 7. **See figures 4, 5.**

Task 2. Purify fusion products using FACS and the YFP signal induced by cell fusion.
We found sorting of fusion products detrimental to viability not only as a function of the sorting process, but also as a function of the limited cell density after sorting this relatively rare cell population (1 in 500 cells). We were able to avoid this procedure by simply using cell tracking via fluorescence microscopy.

Task 3. Determine migratory capacity of fusion products using long-term imaging and tracking analysis software. **See figure 6.**

Task 4. Determine invasive capacity of fusion products using long-term imaging and a modified Boyden migration assay chamber

We have devloped an inverted vertical invasion assay capable of assessing the invasion capabilities of fusion products in a three dimensional environment. Our results show that mesenchymal stem cells, which are one of the main fusion partners of cancer cells, invade vertically into the assay we are developing when under the influence of growth factors chemoattractants. This assay will aid in analyzing the metastatic capability of both cancerous cells and associated fusion hybrids and help understand better the mechanisms of metastasis

with the hope of developing new therapeutic targets to inhibit cancer cell fusion and metastatic spread.

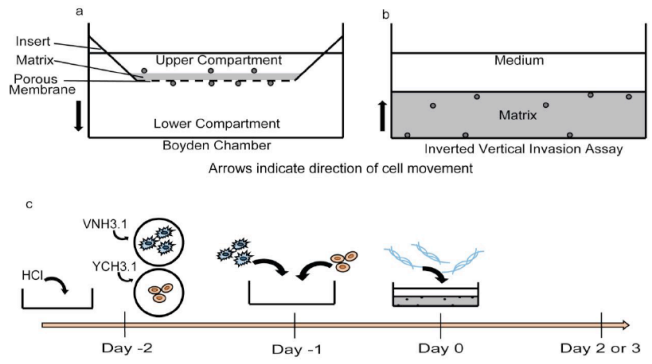


Figure 9. Invasion assay designs. a) Traditional Boyden Chamber or transwell invasion assay. In this assay, cells are collected from their original culture environment and plated onto the transwell insert. The arrow indicates the direction of cell movement during the assay. **b)** Inverted vertical invasion assay currently proposed. In this design, cells remain in their original microenvironment and the matrix protein is laid over the cell layer. The arrow indicates the direction of cell movement during the assay. **c)** Schematic detailing timeline of the inverted vertical invasion assay procedure. Day -2: MatTek dishes are treated with HCl and the two cell lines to be cocultured are transfected with the BiFC constructs as previously described[18]. If fused cells are not the focus of the invasion assay, the BiFC step is eliminated. Day -1: Transfected cells are cocultured on the treated MatTek dish. Day 0: Collagen gel mixture is made and laid over the cells. Day 2 or 3: cells are fixed, stained and subsequently imaged.

Current design allows cells to remain in original microenvironment. We attempted to perform assays in several different plate designs, but ultimately concluded that a 35 mm MatTek dish with a 10 mm glass bottom well is the most effective plate for the inverted vertical invasion assay (**Figure 9**). Other attempts were made using 24-well plate wells, and glass bottom chamber slides. The advantage of the MatTek dishes comes from several properties. First, the glass bottom of the wells allow for clear and distinct images when compared with those of the standard plastic bottom. Second, the 10 mm well allows for a thin layer of collagen gel to be overlaid atop the cells. Other plate designs required a thicker gel layer that eventually killed the cells beneath due to the lack of oxygen diffusion through the gel. Most importantly, the current design allows the cells to remain within the original microenvironment, unlike the more commonly used Boyden chamber (**Figure 9**).

Assessment of chemoattractant to promote invasion. Originally, we attempted the assay using 20% FBS in the α-MEM used to formulate the collagen gels. Thus, after diluting the growth medium into the collagen gel, the gel layer consisted of approximately 3.8% FBS. Images of the assay three days after plating the collagen gel showed that very few MSCs invaded into the gel matrix (**Figure 10**). Days one and two presented with even fewer invading cells (not shown). The use of IGF-1 and PDGF-BB as chemoattractants within the gel showed increased invasion on the part of MSCs. We performed the assay using both insulin-like growth factor-I (IGF-I) and platelet-derived growth factor-BB (PDGF-BB). We compared the percentage of cells invading to that when performing the assay using only FBS. The concentrations used were based off a previous study that determined the optimum growth factor concentrations for optimum MSC chemotaxis. Results showed that the 18.4 ng/mL concentration of IGF-I produced significantly improved invasion when compared to the assay using solely 20% FBS. Platelet-derived growth factor produced invasion similar to that of solely 20% FBS.

Thus we demonstrate a viable tool in the measurement of invasion of cancer cell fusion product invasion. The design has proven that viable cell cultures can be maintained for up to a minimum of three days under the collagen gel matrix. Likewise, the gel integrity is maintained for just as long, except in the case of excess motion or other disturbances to the culture. We have also shown that the inverted invasion assay in the glass bottom MatTek dishes allow for clear z-stack

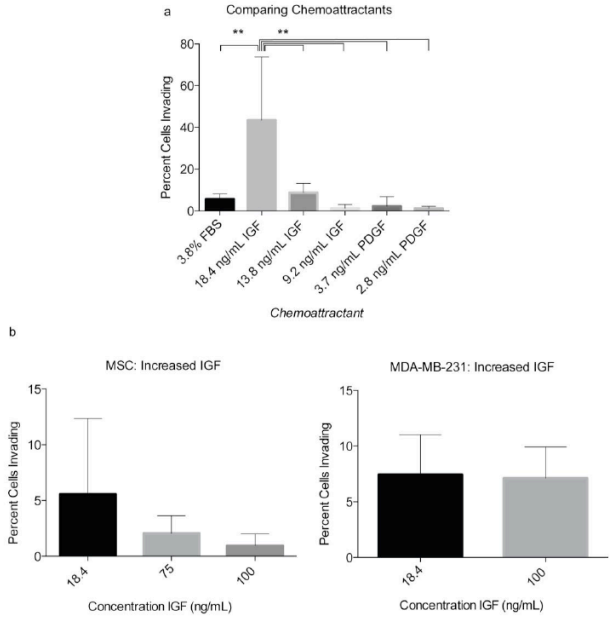


Figure 10. Optimization of chemoattractants using MSCs and MDA-MB-231. a) Various concentrations of PDGF-BB and IGF-1 were tested initially with MSCs. The percentage of invading MSCs was assessed 3 days following the layering of the collagen gel over the cells. **b)** After identifying IGF-1 as the more effective chemoattratant, its concentration was optimized using both MSCs and MDA-MB-231. Each number represents the average of the percentages of invading cells in eight unique locations within a plate. Experiments were performed in triplicate. A two-tailed unpaired ANOVA with Tukey's HSD post hoc test. **P < 0.01.

fusion between MSCs and the nonmetastatic breast cancer cells MCF7. For this assay, MSCs were transfected with the BiFC halve construct VNH3.1 and MCF7 with the BiFC halve counterpart, YCH3.1. Cocultures were initiated and covered with collagen gel as described in materials and methods. Two days following the collagen overlay, the cells were fixed, stained with DAPI, and imaged. Z-stack image analysis of the cells showed that cancer cell fusion products were able to invade into the collagen gel to an average distance of 10.7 +/- 12.4 µm. The MCF7 cells showed an average invasion distance of 0.04 +/- 1.8 µm, and MSCs an average invasion distance of 41.3 +/- 76.0 µm (**Figure 11**). The difference in the invasion levels between MCF7 and MSCs was shown to be significantly different (P<0.01), while the fusion product invasion was shown to be insignificant when images to be taken of the collagen gel, and thus cell invasion can be noted. We did show that the assay requires a stronger a chemoattractant than fetal bovine serum, most likely due to the preferable environment on the glass-bottom of the well. When we performed the assay using insulin-like growth factor-1, a known strong chemoattractant of MSCs, we saw that a significantly higher percentage of the MSCs invaded into the gel. While not shown above, it was seen that in the assays using growth factors that many MSCs would invade in excess of 300 micrometers into the collagen gel. Thus, the growth factors not only increased the number of invading cells, but also the distance of which they invaded.

We then investigated the *in vitro* invasive capacity of cancer cell fusion products. We looked specifically at hybrid cells arising from spontaneous

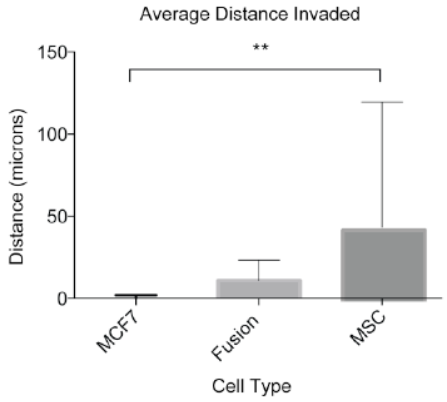


Figure 11. Invasion capability of cancer cell fusion products. We validated our assay by quantifying the invasion capacity of cancer cell fusion products. These numbers represent the average distance covered by four independent fusion products from three separate cocultures and their parental cell lines analyzed from eight different locations within each assay. This experiment was performed in triplicate. ANOVA with Tukey's HSD post hoc test. **P < 0.01.

compared to both the MCF7 (P=0.85) and MSCs (P=0.21). These results indicate that our inverted vertical invasion assay not only is efficient and suitable to quantify *in vitro* invasive capacity of cancer cell fusion products, but also suggests that the fusion products have a higher migratory and invasive capability than the parental cancer cells, MCF7, as suggested by our previous study using 2-D time lapse microscopy.

Specific Aim 2. To determine whether tumor cell fusion products give rise to metastatic tumors.

Aim 2.A.1 Track the course of fusion products with tumor metastasis/sensitivity of detection

Task 1. Inject luciferase expressing cells into mice and image; Xenogen system

Task 2. Inject YFP expressing cells into mice and image; multiphoton microscopy

Task 3. Collect data and define detection sensitivity for each labeling regime

We injected luciferase and YFP expressing cells into the fat pad, bone marrow and tail vein to test the sensitivity of cell detection in the primary (fat pad) and metastatic (bone marrow and lung) sites. We were never able to robustly detect YFP cells in any location using the IVIS system or multiphoton microscopy with up to 10 million cells. We were not surprised that the IVIS system did not work as it has not been optimized for deep tissues; similarly it appears the depth needed was also too great for mulitphoton microscopy (3 mm). We were able to visualize with multiphoton microscopy after dissection, but this would negate the possibility of tracking a single mouse over time. For this reason we abandoned using fluorescence and felt it unnecessary for successful completion of the aims. Luciferase expression was quite robust and we were able to reliably detect 100 cells in both the bone marrow and fat pad (**Figure 12**). Detection limit in the lung (following transit through the veinous system) was much more challenging to detect. We believe this was likely a function not only of the limitations of the imaging system for deep tissues, but also a function of cell transit. We predicted (and other literature reports suggest), the majority of cells will be trapped in lung tissue when injected into a primary vein. However, we did not detect many cells with this approach. Thus, the number of cells needed to detect fusion products in lung metastasis is unclear and therefore histologic analyses will be needed to confirm or refute fusion products in the lung.

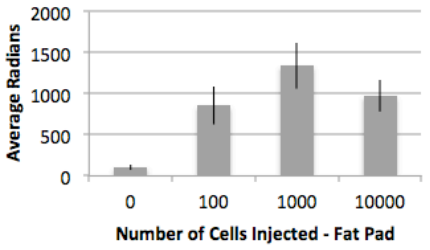
Aim 2.A.2 Track the course of fusion products with tumor metastasis/point of metastasis

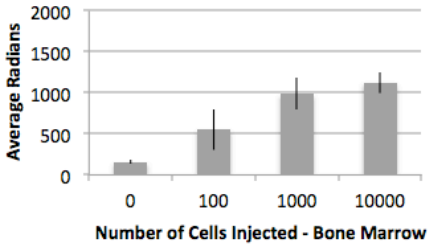
Task 1. Breeding of autochthonous mice and *Cre* mice

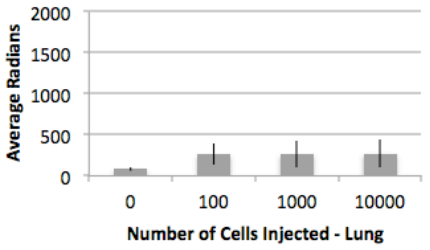
Task 2. Isolation and culture of breast cancer cells from autochthonous mice

Following breeding and confirmation *Cre* positivity, breast cancer cells were isolated from MMTV mice and expanded in culture. Cells group rapidly with an average doubling time of 34 hours. A representative image is shown in **Figure 13**.

Task 3. Optimization of Loxp luciferase/YFP lentiviral transduction efficiency for tumor cells
Transduction efficiency of MMTV tumor cells with Loxp luciferase was on average 65%. We did not add YFP to the cassette as we were unsuccessful in imaging YFP in mice with either the IVIS imaging system or multiphoton microscopy.







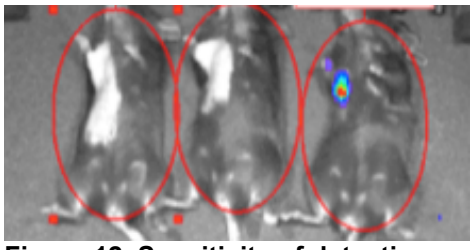


Figure 12. Sensitivity of detection using *in vivo* imaging. Intensity of the bioluminescent signal is reported in average radians as a function of number of cells injected. Cells of the fat pad were most easily detected (100 Cells), while cells of the bone marrow required at least 1000 cells and cells of the lung can not be reliably detected and will require a secondary detection modality. Bottom panel shows mouse with 1000 cells in the fat pad relative to controls (with cells + no substrate; without cells).

Task 4. Transplant modified tumor cells into isogenic (FVB) mice

Modified tumor cells were injected initially at a dose of 10 million cells according to previously published reports utilizing these cells with fat pad injection. However, the primary tumor grew large very quickly; too quickly for metastases to form before the animal needed to be sacrificed according to our protocol guidelines (**Figure 14**). We reduced the number from 10 million to 5 million and then 1 million and ultimately 1000 cells. Finally at 1000 cells we were are able to sustain the primary tumor for sufficient time to allow for the formation of metastases (**Figure 14**).

Task 5. Tracking of transplanted cells in live mice using Xenogen system

We have imaged all animals (39 to date) at 1, 3, 5, 8 and 15 days and then at sacrifice. We have yet to detect luciferase luminescence, even in organs extracted at the time of necropsy. However, we have only just begun to detect metastases and it is in the metastases that we anticipate detecting fusion. We are in the process of analyzing histologically the primary tumors and metastases for evidence of luciferase positive staining via immunofluorescence.

Task 6. Optimization of VSV-G lentiviral transduction efficiency by tumor cells

We have not yet optimized VSV-G lentiviral transduction of tumor cells. This was an alternative approach in the original application and will only be necessary if we do not observe fusion in the metastases. We have alternately injected 100 tumor cell – MSC hybrid cells predicting that injecting hybrids directly may prime the system for fusion-driven metastases. We have just begun this approach and have yet to complete the *in vivo* imaging and associated histology. It should be noted that these ongoing studies are funded using start up funds of the PI as the original grant funds were not sufficient to support the unanticipated transfer of institutions of the PI and delays and additional expenses associated with such a move.

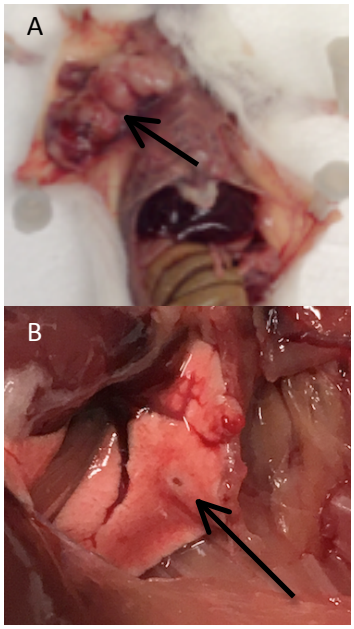


Figure 13. Representative image of MMTV tumor in Cre mice. A. MMTV modified tumor cells were injected into the fat pad of *Cre* mice. Primary tumors for 10 million, 5 million and 1 million cells were too large to allow time for metastases to form. **B.** More recently we have been injected 1000 modified cells and 1000 cells enriched for fusion products. These cells have given rise to metastases in the lung.

Task 7. Collect data and confirm origin of tumor cell fusion products

As noted above, these studies are ongoing. We have completed the *in vivo* studies as planned, but have yet to locate fusion products associated with metastases. We have not given up however, as we believe conditions need to be tweaked in order to best promote and detect fusion in this experimental scenario. As mentioned above, because the PI and associated personnel find merit and high potential impact in the work it has continued beyond the funding granted via this award mechanism.

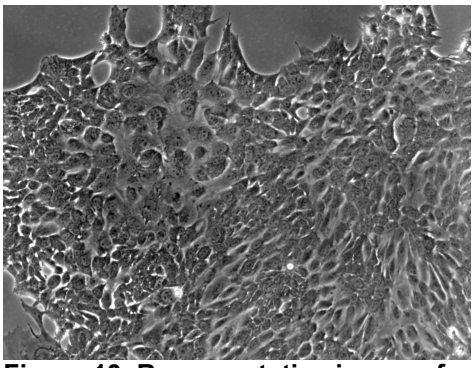


Figure 13. Representative image of MMTV tumor cells. MMTV mice generate spontaneous tumors and such cells were isolated and expanded in culture. Cells for *in vivo* experiments were used between passage 8 and 10.

KEY RESEARCH ACCOMPLISHMENTS

- Optimization of MACS magnetic bead separation of myeloid and monocyte populations from human buffy coat
- Optimization of electroporation transduction conditions for T47D cells
- Optimization of lentiviral constructs for BiFC transfection
- Engineering of new BiFC pairs to promote viability and proliferative capacity of all cell types of interest for this proposal
- Indication of spontaneous fusion between MCF10A, MDA-MD-231 and hMSCs and between T47D and hMSCs in *in vitro* cocultures
- Fate of putative T47DxhMSC fusion products tracked over time; some fusion products were found to undergo proliferation
- The course of fusion products after transplantation of MSCs expressing the floxed luciferase to cre mice was tracked.
- Developed a 3D invasion assay to determine metastatic potential *in vitro*
- Development of an *in vivo* model to detect fusion in mice over time.

The primary outcomes of this work are the 1) discovery tumor cells can fuse *spontaneously* with stromal cells at a frequency higher than originally believed (~1 in 300 cells) and 2) that the frequency is increased with hypoxia which can be a characteristic of the tumor microenvironment. We have yet to confirm or refute whether fusion of this type drives metastases, but these challenging studies are ongoing.

REPORTABLE OUTCOMES

Manuscript:

Harkness, TE; Weaver, BA; Alexander, CM; Ogle, BM. Cell Fusion in Tumor Development: Accelerated Genetic Evolution. *Critical Reviews in Oncogenesis*. 18(1-2):19-42. 2013.

Noubissi, FK; Harkness T; Alexander CM; Ogle BM. Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis. *FASEB Journal.* 2015 Jun 17. pii: fj.15-271098. [Epub ahead of print]

Noubissi, FK; Ogle BM. Cancer Cell Fusion: Mechanisms Slowly Unravel. *International Journal of Molecular Science.* 2016 Sept 21;17(9). Pii.E1587.

McArdle, TJ; Ogle BM; Noubissi FK. An in vitro inverted vertical invasion assay to avoid manipulation of rare or sensitive cell types. *Journal of Cancer.* 7(15):2333-2340. 2016

Degrees awarded:
Ty Harkness, M.S., University of Wisconsin-Madison.

Employment received: Ty Harkness, Research Scientist, Pfizer, San Francisco, CA; Felicite Noubissi, Assistant Professor, University of Mississippi-Jackson.

Research tools developed: Bimolecular fluorescence complementation lentiviral vectors for study of cell fusion *in vitro*; specifically, VN-H3.1, YC-H3.1 and VN-null. Floxed luciferase lentiviral vectors for study of cell fusion *in vivo*. 3D cell invasion assay, with special applicability to sensitive cultures such as those associated with cell fusion.

CONCLUSIONS AND FUTURE STUDIES

In the upcoming months we expect to complete and publish two studies. One related to the invasive quality of breast cancer cell hybrids *in vitro* and associated mechanism of fusion related to apoptosis and signaling associated with phosphatidyl serine receptors. The second manuscript will share results associated with *in vivo* studies designed to test whether breast cancer cells can fuse at the primary tumor site and whether fusion of this type can enable metastasis. If the hypothesis holds, we expect this to garner significant attention in the field.

SUPPORTING DATA

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Cell Fusion in Tumor Development: Accelerated Genetic Evolution

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ABSTRACT: The majority of human tumor cells have highly aberrant karyotypes, typically ascribed to errors during tumor cell division, potentially linked to a failure of DNA repair, or telomeric insufficiency. Here we discuss another option, that of cell fusion which can lead to the re-assortment of chromosomes during post-fusion mitosis. The observation of hyperdiploid cells has a long history in cancer genetics, but the concept of cell fusion has been difficult to test in practice. Here, we examine the role of cell fusion during normal development, and relate that to potential cellular fusion partners for primary tumor cells. In particular, we describe the potential for stromal partner fusion during metastatic mobilization. The evidence for genetic and cytoplasmic diversity in heterotypic fusion partners is described, together with the new tools available to help the evaluation of this process as a tumor driver.

KEY WORDS: metastasis, chromosomal instability, multipolar mitosis, polyploidy, reprogramming

ABBREVIATIONS

BMDCs: bone marrow-derived cells; **CENP-E:** Centromere-associated Protein-E; **CIN:** chromosomal instability; **FISH:** fluorescence *in situ* hybridization; **GFP:** green fluorescent protein; **TAMs:** tumor-associated macrophages

I. INTRODUCTION

Cell fusion is an incompletely understood process that occurs spontaneously during normal development as well as in response to viral infection, including infections known to promote transformation, such as HPV.[1] By increasing DNA content as well as centrosome number, cell fusion rapidly alters cellular genotypes and phenotypes and catalyzes genetic diversity. Through stimulating genetic diversity, cell fusion may contribute to the formation, propagation, and metastasis of tumor cells. For instance, fusion of a healthy epithelial cell with an activated fibroblast of the local stroma can allow it to acquire the ability to proliferate inappropriately. Fusion of a tumor cell with a mesenchymal stem cell can allow it to degrade extracellular matrix proteins and migrate beyond the basement membrane. Fusion of an epithelial tumor cell with a macrophage could lead to the temporary acquisition of macrophage-associated properties of transit through the vasculature and invasion of distant tissue sites.

The current view of cancer is evolving beyond the accumulation of genomic and epigenetic aberrations in epithelial cells to one that appreciates the impact of the "soil,"[2] or microenvironment, on the formation, propagation, and metastasis of tumor cells. Focus of this effort centers on the impact of close contact with cellular members of the stroma and immune system, which generate insoluble extracellular matrix proteins and/or soluble paracrine and autocrine factors. Activated stromal fibroblasts secrete a myriad of soluble factors including SDF-1,[3] MMPs,[4] VEGF-A,[5] HGF,[6] Sdc1[7] and TGF-β[8] that have been shown

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to enhance tumor growth and angiogenesis. As one example, cells of the human breast cell line MCF-7 are weakly tumorigenic in SCID mice, but are strongly tumorigenic when inoculated with stromal fibroblasts.[9-10] In addition to secreted factors, the impact of cell to cell communication is also coming to the fore as a means by which the microenvironment facilitates oncogenesis. Recent work by Apostolopoulou, et al., shows that MCF-7 breast cancer epithelial cells form cadherin-23-dependent, heterotypic adhesions with normal breast fibroblasts when grown in co-culture, suggesting that cadherin-23 upregulation may play a role in the early stages of metastasis.[11] A logical extension of cell adhesion or close cell contact is the possibility that tumor cells or their precursors fuse with cells of the local microenvironment. Known examples of cell fusion along with their demonstrated and potential roles in oncogenesis and metastasis are reviewed here.

II. CONTEXT AND OUTCOMES OF CELL FUSION

A. Spontaneous Heterotypic Fusion Occurs in Nature

Fusion occurs spontaneously between cells of the same type (homotypic) and cells of different types (heterotypic). Homotypic cell fusion was first observed more than eighty years ago in the formation of foreign body giant cells[12] and was soon followed by reports of fusion between trophoblasts of the placenta, myoblasts of muscle fibers, and osteoclasts of bone.[13] However, it was not appreciated until recently that fusion products may form between heterogeneous cell types *in vivo* and that nuclei themselves often fuse to give rise to synkaryons.[14] Spontaneous heterotypic cell fusion *in vivo* was first observed in transplantation studies in animal models and later in humans, both in healthy and diseased states.[15-18] Mesenchymal stem cells and other bone marrow-derived cells (BMDCs) have often been implicated in these studies, especially in synkaryon formation.[19-20]

B. Cell Fusion Generates Diversity

The immediate consequence of both homotypic and heterotypic cell fusion is tetraploidy, a doubling of the genetic material available to the cell and the first opportunity to augment clonal diversity. Given the correlation between copy number and gene expression, tetraploidy alone has the potential to radically alter cell state.[21-22] Tetraploidy has also been reported to increase the sensitivity of both yeast and human cells to DNA-damaging agents.[23-24] Fusion between cells in different stages of the cell cycle can also result in DNA damage—particularly double-strand breaks mediated by premature chromosome condensation.[25-26] In addition, tetraploidy has been suggested to "overwhelm" the mitotic machinery, making chromosome missegregation more likely.[25] A second immediate consequence of cell fusion is an increase in centrosome number. Supernumerary centrosomes can result in multipolar spindles or the missegregation of individual chromosomes after multipolar spindles are focused into bipolar spindles.[27-28] In any scenario, fusion would be expected to increase genetic diversity of the fusion product compared to individual fusion partners.

Of course genetic diversity is likely to result in phenotypic diversity, dependent on the degree of change and the localization of change. Perhaps the most familiar example of how a fusion product attains diversity of phenotype is the hybridoma cell, a Sendai virus-induced fusion product of a murine myeloma cell with a B cell from an immunized mouse.[29] The entire hybridoma system is based on the assumption that the fusion product has a competitive advantage over the parental cells, as neither parental cell line can survive in selective media. The fusion products of these experiments were known to have highly variable phenotypes. In practice, this made the process of hybridoma selection frustrating, since only a percentage of fusions resulted in nuclear hybrids, a fraction of those produced antibodies, and a small percentage of antibody-producing hybrids were able to be expanded into functional hybridomas.[30] The ability of hybridomas to "evolve" upon continuous culture was even used to map genes to specific chromosomes.[31] In a study that mapped an

"IL-6 dependency gene" to human chromosome 21, it was found that newly-fused hybridomas displayed IL-6 dependence; however, this dependency was lost at frequencies up to 50% upon continuous culture, suggesting that genetic material was lost after fusion.[31] Karyotyping of stable clones showed a great variety of seemingly randomized chromosome content. Thus, early hybridomas show a high degree of chromosomal instability (CIN), defined here as an increased and continuous rate of large chromosomal aberrations, including deletions, duplications, or translocations as well as loss or gain of whole chromosomes. Despite the great degree of genetic rearrangement after fusion, hybridoma genotypes eventually stabilize in culture, as evidenced by the extent of their use in industry to provide an extremely pure monoclonal antibody product in a highly reproducible fashion.[32-33]

Although the progression from tetraploidy to aneuploidy or diploidy in hybridomas may be dismissed as a product of extreme culture conditions, there is considerable evidence that this process also occurs in normal culture conditions and even *in vivo*. Observations that tetraploid yeast cells could progress to aneuploidy[34] and that cells with more than four centrioles could be found in mouse models,[35] along with the fact that multipolar mitoses are often seen in human cancers,[36] led to the hypothesis that supernumerary centrosomes lead to multipolar cell division in tetraploid cells and diverse aneuploid progeny.[36-37] However, multipolar spindles are frequently focused into bipolar spindles before anaphase onset and chromosome segregation.[28] Ganem et al. recently showed that even when cells ultimately proceed through a bipolar division, extra centrosomes promote missegregation of individual chromosomes in a variety of human cell lines by merotelic attachment, in which a single kinetochore attaches to microtubules originating from multiple centrosomes.[27] If these centrosomes are focused into different spindle poles, this phenomenon can lead to chromosome lagging during anaphase and the production of diverse aneuploid progeny even in the absence of multipolar division.

Diversification of the genome and the phenome could be advantageous in many scenarios beyond the generation of hybridomas. As one example, Grompe and colleagues have reported detailed studies of the consequences of hepatocyte fusion with BMDCs in a regenerative context *in vivo*. It was first shown that hepatocyte-BMDC fusion products had a competitive advantage in the diseased liver microenvironment and were able to completely repopulate the host liver.[38-39] After diploid fusion products (as determined by a Cre-lox reporter system) were observed,[19] genetic studies were performed to elucidate the mechanism of chromosome loss after cell fusion. Using a three-marker system in which fusion-derived hepatocytes alone expressed β-gal, FAH, and bore a Y chromosome, ploidy reduction was demonstrated, frequently resulting in aneuploid progeny of diverse karyotypes.[40] Subsequent single-cell PCR genotyping revealed that parent-specific markers segregated independently via ploidy reduction after cell fusion, with the incidence of loss ranging from 33–70% of cells analyzed. Only 13% of fusion products tested retained all parental markers, suggesting that ploidy reduction is a very common phenomenon after cell fusion *in vivo*. It is also interesting that the incidence of single-marker loss did not cluster around 50%, as would be expected for random chromosome loss, suggesting that environmental pressure may enrich for cells with favorable phenotypes. Later studies also implicated merotely and chromosome lagging in aneuploidy generation in this process.[41] In addition, it was shown that hepatocytes can regularly increase and reduce their ploidy while maintaining viability and producing high levels of genetic heterogeneity—a process termed the "ploidy conveyor".[41] It is interesting to note that regrowth of the liver after hepatectomy is associated with an increase in polyploid cells.[42] Populations of tetraploid fibroblasts have also been observed in a variety of wound-healing contexts in humans and mice and the frequency of tetraploidy has been shown to increase during the proliferative phase of wound healing.[43] Importantly, these tetraploid cells often appeared in clusters suggesting a clonal evolution, possibly from an initial cell–cell fusion event. It is easy to envision an invading inflammatory BMDC being involved in this context as well. These examples suggest that polyploidy may in fact be an adaptive response to the need to repopulate a damaged tissue.[44]

While genetic recombination is certainly an important event in heterotypic fusion, the mixing of epigenetic factors from diverse cell types may be just as crucial. In a process termed "nuclear reprogramming,"[45-46] cell fusion can result in activation or silencing of genes of one parent via outside epigenetic factors. The most well-known example of nuclear reprogramming by the cytoplasm is probably somatic cell nuclear transfer, which has been utilized to reset a mature nucleus to a pluripotent or totipotent state and resulted in the cloning of Dolly the sheep.[47] Decades ago, it was shown that trans-acting epigenetic factors from one nucleus of a heterokaryon can silence or activate genes in a partner nucleus.[48-49] Through work in stem cell biology, it has been shown that fusion of pluripotent murine germ cells with thymocytes from adult mice alters the methylation status of previously imprinted somatic genes.[50] However, when embryonic stem cells were used as fusion partners instead of germ cells, imprinted genes were not demethylated but hyperacetylation of histones H3 and H4 led to a pluripotent state.[51-52] The result of these epigenetic changes can be expression of genes already active in one (or both) fusion partners[48] or expression of genes not expressed in either parent, an outcome seen more recently in the context of cancer.[53]

An altered phenotype may also be produced solely by the physical changes of a fusion product, specifically an increase in cytoplasmic and/or nuclear volume. Effects of increased volume could include less efficient transfer or decreased concentration of transcription factors and other proteins, less efficient interactions between organelles, and lowered efficiency of membrane-dependent activities due to an increased ratio of cell volume to organelle surface area.[44]

The process of combining genetic or phenotypic information to create diversity and selective competitive advantages is also an important concept in evolutionary biology. There is considerable evidence of polyploidization in the evolutionary history of many organisms including mammals,[54] which, although not well understood, is thought to increase evolutionary innovation through genome recombination.[55] Furthermore, a recent study on a newly-formed (~80 yr) naturally polyploid plant species revealed massive chromosomal instability, with 69% of individuals studied displaying aneuploidy for one or more chromosomes and 76% showing intergenomic translocations.[56] The high degree of genomic diversity still being generated after ~40 generations of this biennial species suggests that prolonged periods of chromosomal instability may commonly precede genomic stabilization in naturally-forming polyploid species. The evolutionary theory of symbiogenesis takes this idea a step further.[57] It holds that mergers of species, rather than Darwinian evolution via accumulation of mutations, are the drivers of diversity and speciation. The most convincing evidence to support the symbiogenic theory is the finding that mitochondria and chloroplasts are bacterial in origin.[58] What fraction of evolutionary adaptations can be ascribed to symbiogenesis is unknown, but it is not difficult to imagine a corollary to symbiogenesis, which states that one means by which species or structures of species are merged is via fusion.

III. MECHANISMS OF CELL FUSION

A. Spontaneous Cell Fusion

Cell membranes allow the cell to function independently and preserve self-regulation from outside influence. In normal development, this compartmentalization is overcome by cell–cell fusion only in a tightly regulated manner. The process of fusion is thermodynamically unfavorable owing to the need to expunge water between the cells, the negative and opposing charges of the outer phospholipid bilayer, and the need to disrupt the membrane. One way that the formation of a syncytium is enabled is via specific integral membrane proteins, termed fusogens, which minimize the incredible energy cost required to overcome the merger of the two hydrophobic membranes.[59] Establishing true fusogens has proven difficult since removal of facilitating proteins from the system reduces overall fusion rates. As technologies advance, many "fusogens" have been contested and shown to be merely adhesion proteins that bring cell membranes in close apposition without actually facilitating fusion. Although the mechanisms of spontaneous cell fusion

are still not well understood, studies of fusion in normal development have given us some clues.

B. Homotypic Fusion: Macrophages

While some cells, such as myoblasts and placental trophoblasts, are required to fuse for normal development, macrophages are normally present as mononuclear cells in the body, and undergo rare (and inducible) fusion events to form multinucleated osteoclasts and giant cells.[60] As such, macrophages may prove to be the most useful model to study inducible fusion. When the recent evidence of BMDC fusion with somatic and cancer cells is taken into account, the mechanisms of macrophage fusion become even more interesting.

Osteoclast and giant cell development are thought to progress similarly and include a series of functional steps including induction of a fusion-competent status, chemotaxis, cell–cell attachment, cytoskeletal rearrangements, and fusion.[61-62] IL-4 and IL-13 are secreted by a number of immune cells and can induce giant cell formation *in vitro* and *in vivo*,[61] while RANKL and M-CSF can induce osteoclast formation *in vitro*.[63] These soluble factors are thought to lead to a fusion competent status by upregulating fusion mediator expression on macrophage cell membranes. For example, IL-4 stimulates DC-STAMP and E-cadherin expression, both of which are implicated in macrophage fusion.[64-66] Internal signaling via DAP12 also seems to be important in this process by mediating transcription of other fusion mediators.[67] Little is known about chemotactic and adhesive factors in macrophage fusion. CCL2 (MCP1) is thought to be important in chemotaxis, as deficiency in mice has been shown to impair giant cell formation while leaving macrophage recruitment unaffected,[68] and E-cadherin has been implicated in macrophage adhesion.[61] Final temporal regulation of macrophage fusion may be determined by the macrophage fusion receptor (MFR) and its ligand CD47. CD47 is expressed ubiquitously, while MFR is expressed only in myeloid cells and neurons. Additionally, MFR is expressed strongly and transiently in macrophages at the onset of fusion.[69] Notably, CD47[70-71] and CCL2[72-73] are both expressed in diverse cancers[74] and IL-4 and IL-13 are secreted by $CD4^+$ T cells in the breast tumor microenvironment.[75]

It has recently been shown that a podosome-like structure is created around actin foci in fusion-competent myoblasts. These podosomes invade adjacent founder myoblasts and mediate fusion pore formation.[76] Interestingly, lamellipodia form in IL-4-stimulated macrophages before giant cell formation, and these structures have been suggested to be critical in the fusion process.[77]

C. Heterotypic Fusion: Gametes

Despite its essential role in the generation of life, the most well known example of developmental heterotypic cell fusion is similarly shrouded in mystery. Mammalian reproduction occurs through heterotypic gamete fusion. As a fusion product, the fertilized embryo can proliferate and differentiate into all the tissues of the adult body as well as the extra-embryonic tissues. CD9 is an egg-associated putative fusogen. Knockout mice for CD9 have shown severely hampered fertilization,[78] which is restored with polyadenylated CD9 mRNA.[79] Additionally, CD9 has been shown to generate the strongest observed interactions with the sperm.[80] In contrast, IZUMO is a putative fusogen found only on the sperm membrane following acrosomal reaction.[81] Supporting the role of IZUMO as a fusogen is the finding that IZUMO-deficient mice have normal sperm migration into the oviduct, motility, zona pellucida penetration, and acrosomal reaction, but are completely sterile.[82] Furthermore, successful fertilization occurs after artificial injection of IZUMO-deficient sperm into oocytes, indicating that IZUMO-null mice lacked other developmental defects. Although the expression of each protein is essential for fusion and fertilization, the molecular mechanism of IZUMO/CD9 induced fusion is not well understood. For example, it is not known whether other facilitating proteins are required.[59, 82]

D. Viral-Mediated Fusion

In contrast to spontaneous cell cell fusion, mechanisms of many virus cell fusion events have been

well characterized.[83] The first step of viral fusion is attachment; capsid proteins bind to specific receptor proteins on the cell membrane. Next, depending upon the type of virus, one of two pathways occurs: plasma membrane fusion or endocytosis followed by endosomal membrane fusion. Typically, a hidden fusogenic protein in the envelope is activated either by the induction of a conformational change upon receptor binding, or exposure to low pH within the endosome. Class I fusion proteins, such as the human immunodeficiency virus type-1 (HIV-1) envelope protein (env), use a hydrophobic fusion peptide that is only exposed after specific receptor binding or under low pH conditions. The fusion protein then undergoes a conformational change that brings the viral and cell membranes into close apposition to mediate fusion.[84]

After viral infection, cells may express fusogenic proteins such as env on their cell membranes, facilitating fusion with adjacent healthy cells. This characteristic has been used to induce cell fusion *in vitro* for years,[85] and viruses that are able to fuse cells are nearly ubiquitous in humans.[26] This property is especially interesting in the context of oncogenesis in light of recent findings that certain viruses, such as HPV, are indeed causative of cancer and capable of inducing cell fusion.[1, 86] It is also worth noting that tumors often create an acidic microenvironment that could activate pH-sensitive viral fusogens.[87]

IV. CELL FUSION AND ONCOGENESIS

CIN and aneuploidy are classic hallmarks of cancer[88-89] and are documented consequences of cell fusion.[25] However, it is unclear, exactly, how CIN is initiated in cancer progression. Supernumerary centrosomes, with or without tetraploidy, can lead to CIN due to the formation of merotelic attachments.[27, 36] It has also been suggested that CIN could arise as a direct result of extra chromosomes through an increased rate of DNA damage[34, 90] or that increased chromosome content could "overwhelm" the mitotic machinery resulting in missegregation of chromosomes.[25, 37] As was discussed earlier, cell fusion-induced tetraploidy can lead to aneuploid progeny through a transient period of CIN. Fittingly, the first clue that fusion may be involved in oncogenesis is that tetraploidy is common in premalignant lesions and often gives way to aneuploidy in later stages.[25, 91-92] The best-studied example of this phenomenon is probably Barrett's esophagus, a premalignant condition in which tetraploid cells are predictive of progression into both aneuploidy and cancer.[93-94] Tetraploidy has also been detected in premalignant lesions in cervical cancer,[95] head and neck squamous cell carcinoma,[96] and Kaposi sarcoma;[97] however, it is unknown whether tetraploid cells in these lesions progress to malignancy. Direct evidence for carcinogenesis as a result of tetraploidy comes from a 2005 study in which $p53^{-/-}$ tetraploid cells were derived through chemically inhibiting cytokinesis.[98] These $p53^{-/-}$ tetraploid cells formed tumors when implanted subcutaneously in nude mice while isogenic $p53^{-/-}$ diploid cells did not. Karyotyping of resultant tumors showed they were near-tetraploid with numerous structural rearrangements. The observations that a majority of cancer cell lines in the NCI-60 drug screening panel[99] and elsewhere[100-102] are hyperdiploid and that karyotypes are generally preserved between cell lines and the primary tumors they were derived from[100, 103] suggest that the progression from tetraploidy to aneuploidy may be common in many cancers *in vivo*.

It has been reported that there exists a p53-dependent "tetraploidy checkpoint" that must be overcome for proliferation of these cells.[104-105] However, subsequent experiments using the same cell type have failed to replicate this finding.[24, 106] Even though cell cycle arrest is not directly related to DNA content, suppression of proliferation is common in fusion products, as evidenced by the low survival of hybridomas[30] and lack of proliferation of developmental fusion products such as osteoclasts and muscle fibers.[13] Additional proof was provided by Duelli et al. in the context of viral-induced cell fusion. It was observed that cell fusion induced by viral infection of normal human fibroblasts, but not the viral infection itself, caused cell cycle arrest.[107] However, if one parental cell expressed the adenoviral oncogene E1A or a mutated form of the tumor suppressor p53, the fusion products proliferated, producing diverse aneuploid progeny, some

of which were capable of producing tumors in nude mice.[108] Furthermore, Duelli et al. have suggested that fusion-specific mechanisms of combining DNA content from cells in different epigenetic states, possibly including premature chromosome condensation, may result in instability, double strand breaks, and consequent translocations often seen in aneuploid progeny.[26]

An alternative explanation of the appearance of diverse aneuploid progeny after a period of CIN in cancer progression is progression through "telomere crisis." This theory holds that while a great majority of cells will trigger senescence or apoptosis pathways upon sufficient erosion of telomeres (i.e., reaching the Hayflick limit), oncogene expression may allow continued proliferation to the point of crisis. Telomere crisis is characterized by genetic aberrations including chromosomal end-to-end fusions, translocations, losses, and duplications. Out of this period of genetic instability, rare cells emerge that are able to maintain telomere length by reactivating telomerase. These dysregulated aneuploid cells may then go on to form malignant tumors.[109-110]

In a landmark study of *in situ* genome instability in breast cancer, Chin et al. demonstrated a transient period of genomic instability coinciding with telomerase activation and with transition from ductal hyperplasia to ductal carcinoma *in situ*.[110] This period of instability was attributed to telomere crisis, although telomere length was also shown to decrease at a steady rate throughout disease progression. However, two observations made by Chin and colleagues are reminiscent of CIN following cell fusion. First, it was noted that the increase in genome instability was contemporaneous to an increase in DNA content. Second, the frequency of anaphase bridges were low in hyperplasia, highest during the period of instability, and reduced thereafter. The occurrence of anaphase bridges is often used as an indicator of telomere crisis,[111-112] but can also occur as a consequence of chromosome lagging due to supernumerary centrosomes and merotely, as discussed earlier.[27, 113] Thus, the same temporal pattern of anaphase bridge frequency, peaking alongside increased DNA content and genomic instability, would be expected to occur after cell fusion.

Further confusing the debate around CIN and aneuploidy in the context of cancer is that aneuploidy can act as a tumor promoter or suppressor depending on the cellular context.[114] Aneuploidy caused by depletion of the mitotic checkpoint component BubR1 promotes tumor formation in the colon of $APC^{Min/+}$ mice, which are predisposed to intestinal tumors due to heterozygous expression of a truncation mutant of the Adenomatous Polyposis Coli (APC) tumor suppressor, but inhibits tumor formation in the small intestine.[115] Similarly, monosomy of 33 genes on mouse chromosome 16 enhances intestinal tumor formation in $APC^{Min/+}$ mice, but trisomy of these same 33 genes suppresses tumor formation.[116] Aneuploidy caused by partial reduction of the mitotic checkpoint component Bub1 drives liver tumor formation, but further reducing the level of Bub1 causes an increased rate of chromosome missegregation and suppresses liver tumors.[117] Both loss and overexpression of securin, a protein that prevents premature sister chromatid separation, result in aneuploidy.[118-119] However, loss of securin reduces pituitary tumor formation in $Rb^{+/-}$ animals,[120] while overexpression of securin facilitates pituitary tumor development.[121] Finally, epidemiological studies have shown that Down's syndrome/trisomy 21 patients have much higher rates of hematopoietic malignancies, but lower incidence of solid tumors.[122-124]

In another study, Weaver *et al.* utilized a low-expression centromere-associated protein E (CENP-$E^{+/-}$) model to induce aneuploidy and CIN due to whole chromosome gain and loss *in vitro* and *in vivo*.[114] When CENP-$E^{+/-}$ mice were inspected for spontaneous tumor formation, it was found that heterozygous animals developed higher rates of lymphomas and lung tumors than their littermates, but had significantly decreased rates of tumorigenesis in tissues normally prone to tumor formation, such as the liver. Additionally, CENP-$E^{+/-}$ animals exhibited decreased tumorigenesis compared to wild type animals when exposed to the well characterized carcinogen DMBA and had a highly significant increase in tumor-free survival in the absence of the ARF tumor suppressor when compared to $ARF^{-/-}$, CENP-$E^{+/+}$ littermates. Another important observation of the study was that aneuploidy in nontransformed cells *in*

vivo was characterized by a disproportionate tendency toward whole-chromosomal loss relative to gain.

Taken together, these results argue that aneuploidy promotes tumorigenesis in otherwise genetically stable tissues and cells but inhibits tumor formation in tissues with a preexisting rate of CIN. This mechanism may explain why cell fusion is tumor suppressive in certain contexts,[74, 125-126] which led to the early belief that tumor cell fusion always suppressed malignancy and even aided in the discovery of tumor suppressor genes.[127]

If high or sustained levels of CIN could result in the extinction of a neoplasia, Storchova et al. raise the possibility that cell fusion via the introduction of additional genetic material gives a sort of "cushion" to the deleterious effects of CIN, as redundant genes could compensate for the loss of single alleles or whole chromosomes.[88] It is also possible that a polyploid genotype after fusion has a larger selection of dormant genes that can be activated in response to environmental challenge and, thus, display the remarkable adaptive ability found in tumor cells.[48,51] In any case, in order for a clinically significant tumor to arise, at least one stable genome capable of continuous proliferation must be generated from the period of CIN (Fig. 1A). This seems to be the case in many cancers by the time of clinical appearance,[44, 60, 128] and is further supported by the observation that karyotypes from relapsed tumors many years after treatment can be identical to the original tumor.[129-130]

V. CELL FUSION AND METASTASIS

Although much progress has been made in recent years in understanding the pathways of metastasis and the role of the microenvironment in this process,[131] very little is known about the generation of metastatic cells within a previously non-metastatic primary tumor. The question of why only a tiny fraction of transformed cells are able to free themselves from the cell and ECM adhesions of the tumor site, migrate through the surrounding tissues and basement membranes, intravasate into the bloodstream or lymphatic system, and extravasate, invade, and proliferate at a distant site is a critical one for the development of effective therapeutics. The importance of understanding this process is underscored by the fact that a majority of cancer deaths are attributed not to primary tumors, but to complications arising from distal metastases. A recent publication[132] further emphasizes the importance of understanding metastasis by pointing out that of the six famed "hallmarks of cancer,"[133] only one – tissue invasion and metastasis – can be used to distinguish a life-threatening malignant tumor from an essentially harmless benign tumor.

The dogmatic view of metastasis is that it evolves gradually during tumor evolution, as part of the selective adaptation of the tumor genome.[134] In this linear progression model, primary tumor cells accumulate genetic changes randomly, those mutations that support survival and proliferation in the tumor microenvironment are selected for, and clonal expansion occurs within the tumor. Eventually, a cell arises with a combination of chance genetic alterations that enable it to depart the primary tumor and take up residence elsewhere in the body. This model has become popular for good reason. First, it follows logically from the discovery of oncogenes in malignant transformation and second, it does an excellent job of explaining the positive clinical correlation between tumor size and frequency of metastasis.[134-135] A large tumor has presumably been in existence longer than a smaller tumor, giving it both more time for accumulation of mutations and a larger cell population in which to select for cells capable of metastasis. However, several lines of evidence argue against the linear progression model of metastasis.

First, advances in parallel-sequencing technology now allow for whole-genome analysis of primary and metastatic tumor cells. One recent study compared the whole genome of a brain metastasis to that of the primary breast tumor and found only two *de novo* mutations in the metastatic tumor.[136] One was a silent mutation while the other was determined to be non-essential to metastasis, suggesting that a mechanism other than genetic mutation initiated the metastatic process. It is important to note that this methodology would not detect polyploidy of the tumor cells.

Another argument involves the growth kinetics of primary and metastatic tumors. The linear progression hypothesis predicts that significant time and cell divisions will be required for a tumor cell to gain and "fix" the chance mutations needed for metastasis. Therefore, according to this hypothesis primary tumors should be of sufficient size to sustain the establishment of clonogens that form with low frequency. In fact, metastases are often seen in early disease stages, and 5–10% of patients diagnosed with cancer in Europe and the United States present with unknown primary tumors.[137-138] For the linear progression hypothesis to hold in these cases, growth rates of metastases would need to be far greater than those of the primary tumor. Primary and metastatic breast cancer growth rates have been well studied[139, 140] and argue against this possibility. Several methods of investigation have shown that growth rates of primary and metastatic tumors are comparable at the time of diagnosis, typically within a factor of two.[134]

A third, primarily hypothetical argument against linear progression has been put forward by George Parris[141] and expands on the discussion of CIN and consequent genomic stabilization above. If a tumor is conceptualized as a new, "parasitic" species within the host,[142] it will be subject to the ecological pressures of any other species. By the widely known ecological concept of Muller's Ratchet,[143] an asexual population with only genetic mutations available to generate diversity may gain a momentary genetic advantage via a desirable mutation. However, when a deleterious mutation occurs in this asexual population it must (with the exception of back mutations) be passed on to all progeny. As a given mutation is more likely to be deleterious than advantageous, undesirable mutations build up in an asexual population until it finally becomes extinct. In the context of cancer progression, this theory would allow for the formation of neoplasia due to somatic mutations, but tumors would be expected to become extinct before clinical relevance or metastasis. We and others[13, 74, 141] argue that a "sexual" method of reproduction – such as cell fusion – that allows for genetic recombination offers a more likely explanation for tumor progression into metastasis than the linear progression model (Fig. 1B). This hypothesis is initially supported by the correlation between the most immediate consequence of genetic recombination – aneuploidy – and poor prognosis in a variety of cancers[92, 144-147] as well as the finding that polyploidy is strongly correlated with growth rate in many species.[148] In more concentrated studies, it was found that polyploidy in the liver was increased in faster growing mouse pups[149] and in rat hepatocytes after growth hormone stimulation.[150] Thus, fusion-induced polyploidy may be an adaptive response to the increased rate of proliferation throughout disease progression.

A. Tumor Cell Fusion with Other Tumor Cells

If fusion is a viable means by which metastasis is initiated, the next question is "which two (or more) cells fuse?" The definitive answer to this question remains elusive, but several studies are suggestive of certain pairings. One pairing is fusion between tumor cells. If tumor cells fuse with each other, detection of such an event within a single tumor is challenging, as most detection techniques rely on overlapping lineage-specific markers to identify fusion partners. Accordingly, a fusion event between phenotypically identical tumor cells would be undetectable in most experimental systems. However, spontaneous fusion between cancer cells has been observed *in vitro*. As early as 1984, it was observed that multi-nucleated giant cells arose in suspension cultures of B16 melanoma cells.[151] More recently, co-culture of two variants of the MDA-MB-231 breast cancer line, one of which metastasizes almost exclusively to bone and the other primarily to the lung, resulted in fusion hybrids that had gene expression signatures of both parental strains and retained strong metastatic ability to both lung and bone *in vivo*.[152] Interestingly, fusion products were phenotypically and genomically stable at a nearly doubled genome size after passage *in vivo* up to the time of publication (almost 1 year).

B. Tumor Cell Fusion with Local Non-malignant Cells

Evidence of fusion between xeno- or allogeneic transplanted malignant cells and host cells *in vivo* was

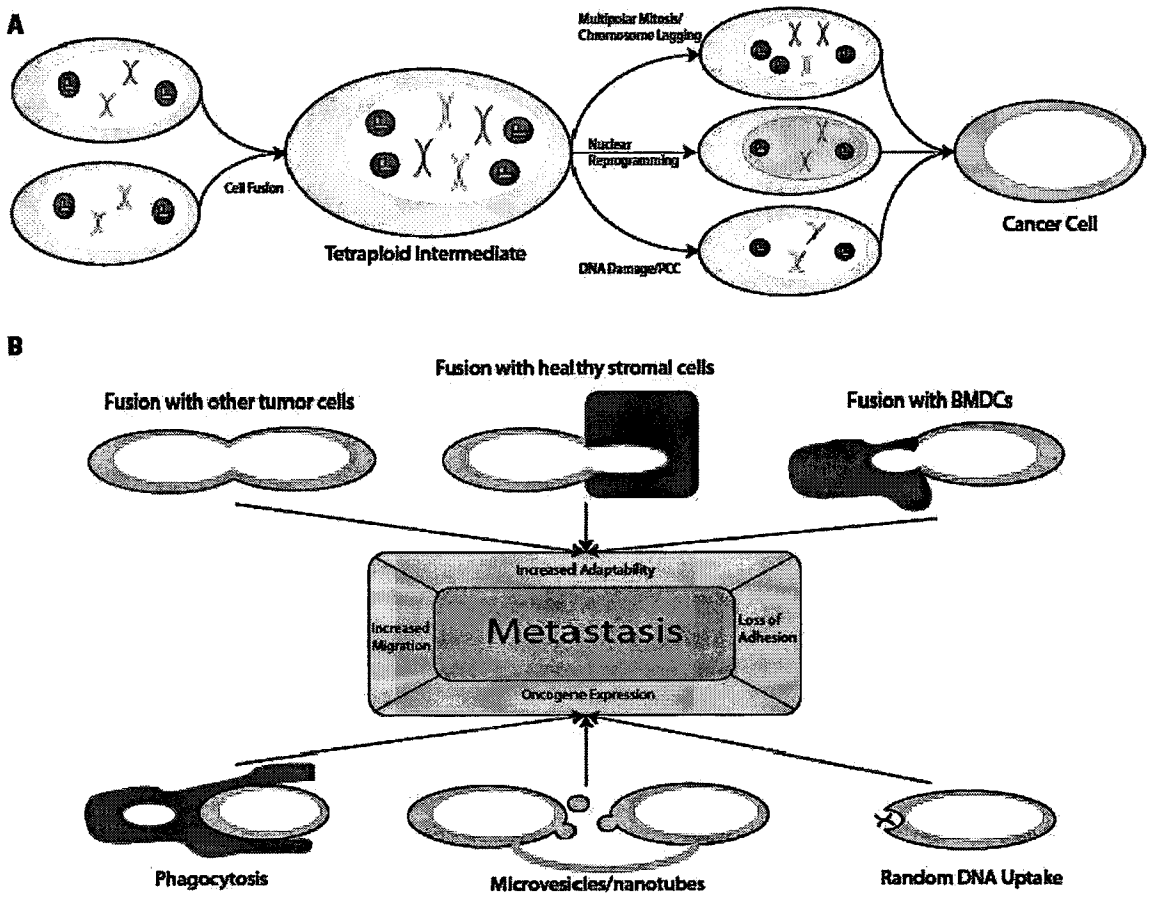

FIGURE 1. Schematic of cancer progression via cell fusion. A) Fusion can lead to oncogenesis. An initial fusion event leads to an intermediate tetraploid fusion product with doubled centrosome content. This intermediate is unstable and is capable of progression into malignancy through several mechanisms. **B)** Fusion can lead to metastasis. Cell fusion or alternative methods of genetic transfer impart new phenotypic capabilities to the tumor cell, which enable the cell to metastasize

reported repeatedly in the 1970s and early 1980s,[153-154] often resulting in increased metastatic ability. However, it was not until recently that advances in genetic profiling techniques such as fluorescence *in situ* hybridization (FISH) could provide conclusive evidence of synkaryon formation.

In the last decade, fusion of epithelial tumor cells with local endothelial and other stromal cells *in vivo* has been reported. In one study, human breast cancer cells were injected into the tail vein of nude mice. Subsequent analysis of lung sections revealed that 0.5–2.0% of tumor cell nuclei contained both human and murine DNA.[155] A portion of these hybrid cells also stained positively for the endothelial marker von Willebrand factor, suggesting that at least some of the murine fusion partners were of endothelial origin. FISH analysis of the hybrid cells revealed consistent spatial separation of human and murine DNA into distinct subcompartments of hybrid nuclei, a phenotype that was reproduced *in vitro*. Although the relevance of this spatial separation is unknown, it is interesting to speculate on the epigenetic and mitotic

consequences of this arrangement. It is possible that chromosomes of one fusion partner are epigenetically favored over the other, resulting in transcriptional profiles very similar to parent cells. Alternatively, this compartmentalization could allow for lineage-specific DNA content to be easily separated in subsequent asymmetric cell divisions,[37] resulting in progeny with little or no genomic alterations. Because of the great similarities between the progeny of fusion products and unfused cells, either of these mechanisms could lead to underestimation of the frequency of cell fusion by common detection techniques.

Further evidence for tumor cell-stromal cell fusion comes from implantation of primary human breast cancer cells from a pleural effusion into the mammary glands of nude mice.[156] Resultant tumor nuclei stained positive for both human and murine DNA. In addition, a cell line was created from the tumor that had a spindle-shaped stromal morphology and stained positive for several stromal markers. After immortalization, FISH analysis indicated that ~64% of interphase nuclei were mouse-human hybrids, while a significant portion of mitotic cells showed mouse/human chromosomal translocations. The DNA content of the analyzed synkaryons was ~4N in early passages, but gradual reduction of chromosomes was observed upon serial passaging. These data suggest that increased chromosomal content was beneficial for tumor cells adapting to a new environment *in vitro*. Gradual loss of chromosomes with passage number could represent the stabilization of a new karyotype after cell fusion-induced CIN or simply the reduction of genetic load of cultured cells by loss of DNA content unnecessary for survival. Similar behavior is often observed in antibiotic resistant bacterial and mammalian cell lines upon removal of the selective pressure of antibiotics in the culture media.[157]

A more recent study investigated long-term gene expression of hybrid cells formed after transplantation of a primary human glioblastoma into a hamster cheek pouch.[158] After a year of passage *in vivo*, metastases were found to contain primarily hamster DNA, but still retained genes from at least six different human chromosomes as detected by PCR. Additionally, histochemical examination showed protein expression of at least three human genes after one year of passage. All three of the human proteins detected (CD74, CXCR4, and PLAGL2) have been implicated in cancer progression,[158] again suggesting selective retention and/or expression of genes beneficial for tumor cell survival and proliferation after cell fusion.

C. Tumor Cell Fusion with BMDCs

Perhaps the longest[159] and most extensively investigated tumor cell fusion partners have been bone-marrow derived cells, especially immune cells such as macrophages. This is in part because the consequences of tumor cell fusion with immune cells agree well with numerous observations of cancer progression.

Tumors have been described as "wounds that do not heal"[160] and as such, recruit immune cells through the activation of the body's natural inflammation response. A plethora of BMDCs are known to be present in the tumor microenvironment (reviewed in[161]) and many, including $CD4^+$ T cells,[162] mast cells,[163] B cells,[164] and macrophages[165] have been implicated in cancer progression or poor prognosis. Interestingly, chronic inflammation or other tissue injury has been shown to induce BMDC fusion in a wide variety of tissues, both in a cancer context[53,166] and in normal regenerative processes.[38-39, 167-168] Especially interesting is the observation that BMDC fusion with diseased hepatocytes results in fusion products that repopulate the liver due to a selective advantage in the microenvironment[19]—a model that could be recapitulated in the tumor microenvironment.

Given these observations, it is not surprising that all three clinical case studies implicating cancer cell fusion have suggested BMDCs as the fusion partner. In two studies reported by Pawelek and colleagues, patients with a prior bone marrow transplant developed renal cell carcinomas.[20,169] In both cases, marrow–donor DNA was detected in mononuclear tumor cells. In the most striking example, the donor Y-chromosome was identified via FISH analysis in mononuclear tumor nuclei also containing three copies of the female host's 17th chromosome, a signature of the tumor. Importantly, the Y chromo-

some was detected in a small area at the border of the tumor accounting for ~10% of the tumor area. This observation suggests a possible clonal expansion from the initial fusion event. In a third case, FISH analysis indicated that up to 48% of nuclei in multinucleated osteoclasts of a multiple myeloma patient were of myeloma origin, with all nuclei maintaining activation of transcription.[170]

It has also been suggested that carcinoma cells can undergo an epithelial-to-mesenchymal conversion during the transition from benign to malignant tumors. During this transition, cells lose tight cell–cell contacts and E-cadherin expression, and gain migratory and invasive capacities of mesenchymal cells (reviewed in[171]). This change in phenotype can be easily imagined as a result of fusion of a tumor cell with an immune cell that depends on efficient migration to function effectively. Indeed, melanoma cell/macrophage fusion products have shown increased migratory capacity *in vitro*[172] and greater metastatic potential *in vivo*[173] compared to the parent melanoma line.

Macrophages are an especially promising fusion partner candidate, as they routinely fuse *in vivo* to form osteoclasts and giant cells.[69] It is worth noting that macrophages also share many characteristic properties of tumors, such as angiogenesis, protease secretion, and growth factor and other cytokine production.[174] Pollard and colleagues have pioneered the study of tumor-associated macrophages (TAMs) in mice models and have suggested that TAMs are essential for metastasis.[175] In human patients, macrophage density around the primary tumor has been correlated to poor prognosis in several studies.[165,176-177]

The characteristics of macrophage/melanoma fusion products *in vitro* have been studied extensively by Pawelek and colleagues and have been shown to exhibit multiple metastatic characteristics absent in the parent melanoma line, including increased motility,[172] increased proto-oncogene expression,[178] and macrophage-associated expression of β1,6 oligosaccharides.[179] Notably, many of these characteristics were reproduced after culturing cells from spontaneous metastases developed *in vivo*.[173] In this important study, a nude mouse with a homozygous tyrosinase mutation (c/c) developed a tumor after subcutaneous implantation of wild type (C/C) melanoma cells. Genetic analysis of metastases revealed a C/c genotype and a 30–40% increase in DNA content, suggesting the tumor cells had fused with host cells, possibly with metastasis as a direct consequence. Subsequent *in vitro* culture of cells isolated from the metastases revealed characteristics similar to artificially derived melanoma-macrophage hybrids, such as increased motility and β1, 6-branched oligosaccharide expression.

Additional evidence of *in vivo* tumor cell fusion with macrophages has been supplied recently in a study by Powell et al., in which green fluorescent protein (GFP)-labeled macrophages were introduced via parabiosis into a host harboring β-galactosidase-expressing intestinal tumors.[53] Double-labeled cells were observed in up to 20% of the tumor epithelia after parabiosis, however, as the intestinal epithelium completely renews every 3–5 days[180] it is unclear whether labeled macrophages fused with tumor epithelia or rather with a progenitor cell that later differentiated into epithelium, as has been shown previously.[166] In any case, transcriptome analysis of fusion products revealed transcription profiles with similarities to both native epithelial and macrophage transcriptomes. Significantly, over 3% of differentially regulated transcripts in the fusion products were unique in the fusion product compared to either parental cell line, providing evidence that nuclear reprogramming following cell fusion can result in gene expression completely distinct from either parent cell.

VI. ALTERNATIVE MECHANISMS OF GENE TRANSFER

Although cell fusion offers a promising route for genetic recombination and diversity generation within tumors, it is not the only possible mechanism. Macrophages also routinely perform phagocytosis and digestion of apoptotic cells, a process that has been shown to result in horizontal transfer of oncogenes into the phagocytic host.[181-182] Macrophages are known to associate extensively with necrotic areas in advanced tumors[177] and it is reasonable to speculate

that clearance of necrotic debris by macrophages may also result in genetic transfer of tumor cell DNA into the macrophage genome.

Tumor cell secretion of microvesicles via membrane blebbing and other mechanisms has been a recent focus of study.[183] These microvesicles have not been shown to contain DNA, but commonly harbor RNA and proteins, including activated oncogenic proteins.[184] Microvesicles could therefore transfer information between tumor cells or from tumor cells to the stroma through RNA or protein, bypassing the need for genetic recombination. Similarly, membrane nanotubes allow for transfer of vesicles and signaling molecules between cells, although evidence of these structures *in vivo* is lacking.[185]

It is also possible that information flows in the opposite direction, from the stroma to the tumor. A recent model of tumor progression returns to the idea of the tumor as a parasite. The model posits that tumor cells induce autophagy in the stromal microenvironment through oxidative stress, resulting in a release of nutrients that are used by the tumor for continued growth.[186] Although autophagy is normally thought to preserve genomic DNA content, mitochondrial DNA could be transferred to the tumor in this manner. Alternatively, information could again be transferred via RNA or proteins.

Finally, naked DNA is known to be taken up and expressed by a variety of cell types *in vivo*.[187] It is possible that DNA is released into the tumor microenvironment through apoptosis or necrosis and is simply randomly incorporated into the genome of tumor or stromal cells.

VII. DETECTION OF CELL FUSION

Given the potential impact, it is surprising that fundamental aspects of tumor cell fusion are unknown, including the soluble or insoluble signals that trigger fusion, the cell surface proteins responsible for mediating fusion, the kinetics of fusion within the metastatic cascade, or the mechanism of reprogramming and the functional capacity of hybrid cells after fusion. Hindering discovery in this area is lack of appropriate technology to 1) identify fusion partners poised to fuse and to 2) track fusion products over time. To address this problem we have recently developed imaging and characterization technologies to accurately identify fusion products immediately after cell fusion and to track fusion products over time both *in vitro* and *in vivo*.

A. Historical Methods

The origin of a cell as the product of a fusion event can be difficult to deduce since in many cases hybrid cells are morphologically identical to unfused cells (Fig. 2B). Three methods have been used traditionally to overcome this difficulty. First, cell fusion can be detected using fluorescent cytoplasmic dyes, which diffuse freely through the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes react with intracellular components to produce cells that are fluorescent for at least 24 hours after labeling. Different dyes are used to label each fusion partner and fusion products are discerned by detecting overlapping fluorescence emission via flow cytometry or fluorescence microscopy. This method is ideal for short-term *in vitro* studies; however *in vivo* studies are not possible and the fluorescence signal is undetectable at 72 hours in proliferating cells. In addition, dead cells can be endocytosed and the cell dye transferred as a consequence leading to false positives. Second, cell fusion can be detected by the complementary action of genes; most commonly, the Cre-lox system.[18] This method is robust, detecting only true fusion events and can be used *in vivo*. However, available reporter systems (i.e., beta-galactosidase, fluorescent proteins) limit *in vivo* analysis to excised tissues precluding the tracking of fusion events in the same organism over time. Third, fusion events can be detected by probing the DNA of different species or genders (X, Y chromosomes). When a cell of one species/gender fuses with a cell of another species/gender, *in situ* hybridization probes can be generated to species/gender-specific sites in the genome. More recently, genetic techniques such as single nucleotide polymorphism (SNP) and short-tandem repeat (STR) analysis have been utilized to identify DNA from multiple parents in fusion products.[188] However, these methods of detection

of fusion events are also limited to excised tissue. Thus, there is a distinct lack of means to detect and track fusion products long term *in vitro* and *in vivo*.

B. New Approaches

We have developed two new approaches for the detection of fusion products. The first utilizes bimolecular fluorescence complementation (BiFC). BiFC is a method of viewing the association of proteins inside living cells. Intact GFP (and its variants YFP, BFP, RFP, etc.) is fluorescent, and this property can be reconstituted when these proteins are broken into two halves by making each into an interactive fusion protein (Fig. 2A).[189-191] Fluorescence is detected and recorded via traditional, time lapse, fluorescence microscopy. We believe this technique to be a powerful tool for detecting fusion *in vitro* and will be especially useful to determine the mechanism by which the fate of tumor cell hybrids is accomplished (i.e., nuclear fusion, rearrangement of genetic material or other). Due to the inducible nature of the signal (i.e., fluorescent signal is only detected after a fusion event, instead of detection of overlapping fluorescent signals), the incidence of false-positives is essentially zero. In addition, the hybrids synthesize accumulating amounts of BiFC genes over time and so signal intensity increases over time (Fig. 2B), instead of

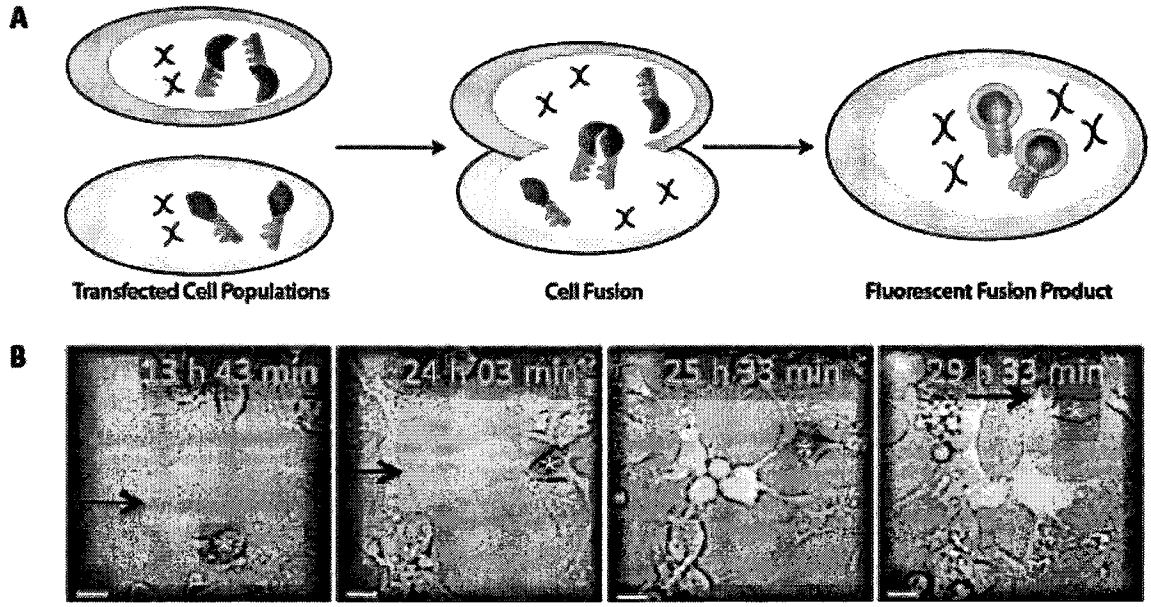

FIGURE 2. Detection of cell fusion *in vitro* via BiFC. A) Schematic of BiFC mechanism. Each BiFC construct contains half a YFP gene fused to genes encoding histone H3.1, which naturally dimerizes in the cell. The two BiFC constructs are transduced into separate cells. When the cells fuse, the YFP protein is reconstituted and a fluorescent signal is generated. **B)** Morphologic comparison of fusion products with unfused cells.[191] Plasmids corresponding to complementary BiFC constructs (YN-CBX5 and YC-CBX5) were transfected into separate populations of COS-1 cells. After transfection, populations were mixed and fusion was induced with poly(ethylene glycol). Fusion signals (green) were detected using fluorescence microscopy. The morphology of fused cells was monitored via time-lapse imaging for 30 hours. The initial fusion product is a clearly larger cell (black arrow, first two frames). When this cell divides, the four daughters remain green (and would be difficult to distinguish from other non-fused cells (white asterisk) were it not for the BiFC label).

degrading over time as is the case for cytoplasmic dyes. However, this approach is limited in that it cannot easily be adapted to *in vivo* systems.

To detect fusion products *in vivo* we have developed an approach to trigger bioluminescence upon fusion. We have developed a construct encoding the firefly (*Photinus pyralis*) luciferase gene placed downstream of a stop codon flanked by loxP sequences (Fig. 3A). When cells expressing this gene fuse with cells expressing the Cre protein, the LoxP sites are recombined resulting in excision of the stop signal and expression of luciferase (Fig. 3B).[192] Akin to the BiFC method, this is an inducible method thereby limiting the incidence of false-positive signals. Unlike existing methods, which utilize the Cre-lox system, we have incorporated a "living" detection signal and thereby afford for the first time the opportunity to track the kinetics of cell fusion *in vivo*.

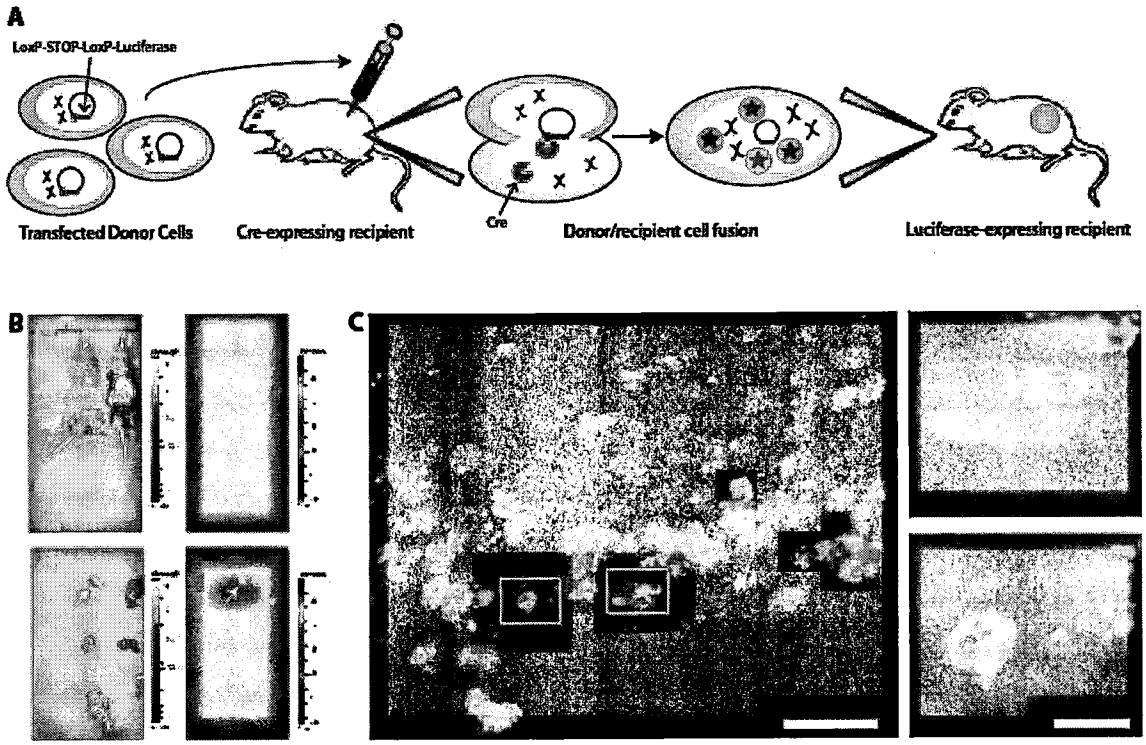

FIGURE 3. Detection of cell fusion *in vivo* via Cre-LoxP bioluminescence. A) Schematic of Cre-LoxP bioluminescence mechanism. Upon donor/recipient cell fusion, the Cre protein excises the floxed stop codon and luciferase is expressed in the fusion product. **B)** Detection of cell fusion *in vivo* via bioluminescent imaging. Human MSCs were transfected with two plasmids, one containing luciferace downstream of a floxed stop codon and the other containing the viral fusogen VSVG. Transfected MSCs were then transplanted into Cre-expressing mice. Luminescent signal was present in experimental mice (top, right mouse) but not in sham controls (top, left mouse). Excision of organs (bottom) revealed luminescent signal, indicative of fusion, in multiple tissues including the heart (arrow). **C)** FISH analysis of bioluminescent heart issue.[193] Insets display fusion products with nuclei staining positive for both human centromeres (red) and mouse centromeres (green). One fusion product (bottom right) is undergoing mitosis. Scale bar = 25μm.

VIII. CANCER RELEVANCE

Despite mounting evidence that cell fusion or transfer of genetic material by various mechanisms could contribute to carcinogenesis and metastasis, critics contend that spontaneous fusion occurs too rarely to contribute in a relevant way to the initiation and propagation of diseases like cancer. In response, we offer a few points for consideration. First, we point to the lack of technology sensitive enough to determine the relative frequency of spontaneous fusion between tumor cells or their precursors with surrounding cell types. As noted above, the task is complicated by the uncertainty of rearrangement of cytoplasmic and nuclear components and the relative speed with which rearrangement takes place.

The choice of model system will be important to the assessment of the role of fusion in the future. Since it is unlikely that fusion assays will ever be done in humans, rodent models are an obvious choice. However, many mouse models are not spontaneously metastatic. The human tumor cell lines or human grafts that are in common use have already progressed past the point of genetic evolution likely to be catalyzed by cell fusion, and results from these may not reveal the full potential of this mechanism. Even if early stage lesions were transplanted, the mouse hosts will be immune-compromised, and this microenvironment may therefore be deficient in key cell fusion partners. It will be important to choose the correct assay to test this hypothesis.

IX. CONCLUSION

We have discussed the processes that regulate cell fusion, as it occurs naturally and spontaneously to govern normal tissue development and functionality, and the evidence for novel cell fusion events (and/or genetic transfer) that occur during pathogenic processes. These include viral infections, which include fusion events at various points of their life cycles, and the immune reactions that characterize inflammation. The details of these processes are known, including their molecular mediators. Given the emerging importance of inflammatory mediators during metastasis, it seems a logical extension to propose that cell fusion will be associated with this process, and that it could indeed be a functional effector of neoplastic changes. Cell fusion offers an opportunity for rapid genomic evolution. It may offer practical solutions to explain the (arguably) paradoxical timelines for tumors, and to generate mechanisms for the evolution of the massively rearranged genomes that are so characteristic of these tumors. Cell fusion can account for re-assortment of combinations of mutant and normal genomes in various copy numbers. It also offers the potential to acquire cytoplasmic determinants of function (including mitochondria, trafficking components and cell signaling-related functions) that have been shown historically to be powerful mediators of the cancer cell phenotype. These functionalities may be acquired only transiently, to enable for example the enhanced motility or altered metabolism required for metastasis, and may largely revert after the fusion products resolve to stable daughter cells in metastatic outgrowths. The potential of this novel hypothesis stands on the brink of a full evaluation, with the development of new tools and methods. If these studies generate evidence in support of this hypothesis, this avenue opens the way to a whole new class of therapeutics that are likely to be highly selective for pathogenic processes.

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REFERENCES

1. Gao P, Zheng J. High-risk HPV E5-induced cell fusion: a critical initiating event in the early stage of HPV-associated cervical cancer. J Virol. 2010;7:238.
2. Paget S. The distribution of secondary growths in cancer of breast. Lancet. 1889;1:571–3.
3. Huang M, Li Y, Zhang H, Nan F. Research Breast cancer stromal fibroblasts promote the generation of CD44+CD24-cells through SDF-1/CXCR4 interaction. J Exp Clin Cancer Res. 2010;29:80.
4. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010;141:52–67.
5. Pinto MP, Badtke MM, Dudevoir ML, Harrell JC, Jacobsen BM, Horwitz KB. Vascular Endothelial

Growth Factor Secreted by Activated Stroma Enhances Angiogenesis and Hormone-Independent Growth of Estrogen Receptor–Positive Breast Cancer. Cancer Research. 2010;70:2655.
6. Tyan SW, Kuo WH, Huang CK, Pan CC, Shew JY, Chang KJ, Lee, EYHP, Lee WH. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PloS One. 2011;6:e15313.
7. Su G, Blaine SA, Qiao D, Friedl A. Shedding of syndecan-1 by stromal fibroblasts stimulates human breast cancer cell proliferation via FGF2 activation. World J Biol Chem. 2007;282:14906–15.
8. Wu M, Jung L, Cooper AB, Fleet C, Chen L, Breault L, Clark K, Cai Z, Vincent S, Bottega S. Dissecting genetic requirements of human breast tumorigenesis in a tissue transgenic model of human breast cancer in mice. Proceedings of the National Academy of Sciences. 2009;106:7022.
9. Trimis G, Chatzistamou I, Politi K, Kiaris H, Papavassiliou AG. Expression of p21waf1/Cip1 in stromal fibroblasts of primary breast tumors. Hum Mol Genet. 2008;17:3,596–600.
10. Kiaris H, Chatzistamou I, Trimis G, Frangou-Plemmenou M, Pafiti-Kondi A, Kalofoutis A. Evidence for nonautonomous effect of p53 tumor suppressor in carcinogenesis. Cancer Research. 2005;65:1,627–30.
11. Apostolopoulou M, Ligon L. Cadherin-23 Mediates Heterotypic Cell-Cell Adhesion between Breast Cancer Epithelial Cells and Fibroblasts. PloS One. 2012;7:e33289.
12. Forkner CE. The Origin and Fate of Two Types of Multi-nucleated Giant Cells in the Circulating Blood. J Exp Med. 1930;52:279.
13. Duelli D, Lazebnik Y. Cell fusion: a hidden enemy? Cancer Cell. 2003;3:445–8.
14. Ogle BM, Cascalho M, Platt JL. Biological implications of cell fusion. Nat Rev Mol Cell Biol. 2005;6:567–75.
15. Gussoni E, Bennett RR, Muskiewicz KR, Meyerrose T, Nolta JA, Gilgoff I, Stein J, Chan Y, Lidov HG, Bonnemann CG. Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. J Clin Invest. 2002;110:807–14.
16. Gibson AJ, Karasinski J, Relvas J, Moss J, Sherratt TG, Strong PN, Watt DJ. Dermal fibroblasts convert to a myogenic lineage in mdx mouse muscle. J Cell Sci. 1995;108(Pt 1):207–14.
17. Weimann JM, Johansson CB, Trejo A, Blau HM. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. Nat Cell Biol. 2003;5:959–66.
18. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature. 2003;425:968–73.
19. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature. 2003;422:897–901.
20. Yilmaz Y, Lazova R, Qumsiyeh M, Cooper D, Pawelek J. Donor Y chromosome in renal carcinoma cells of a female BMT recipient: visualization of putative BMT-tumor hybrids by FISH. Bone Marrow Transplant. 2005;35:1021–4.
21. Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringnér M, Sauter G, Monni O, Elkahloun A. Impact of DNA amplification on gene expression patterns in breast cancer. Cancer Research. 2002;62:6240.
22. Pollack JR, Sørlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Børresen-Dale AL, Brown PO. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. Proceedings of the National Academy of Sciences. 2002;99:12963.
23. Hau PM, Siu WY, Wong N, Lai PB, Poon RY. Polyploidization increases the sensitivity to DNA-damaging agents in mammalian cells. FEBS Lett. 2006;580:4727–36.
24. Wong C, Stearns T. Mammalian cells lack checkpoints for tetraploidy, aberrant centrosome number, and cytokinesis failure. BMC Cell Biol. 2005;6:6.
25. Gao P, Zheng J. Oncogenic virus-mediated cell fusion: new insights into initiation and progression of oncogenic viruses--related cancers. Cancer Lett. 2011;303:1–8.
26. Duelli D, Lazebnik Y. Cell-to-cell fusion as a link between viruses and cancer. Nat Rev Cancer. 2007;7:968–76.
27. Ganem NJ, Godinho SA, Pellman D. A mechanism linking extra centrosomes to chromosomal instability. Nature. 2009;460:278–82.
28. Yang Z, Lončarek J, Khodjakov A, Rieder CL. Extra centrosomes and/or chromosomes prolong mitosis in human cells. Nat Cell Biol. 2008;10:748–51.

29. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256:495–7.
30. Olsson L, Kronstrom H, Cambon-De Mouzon A, Honzik C, Brodin T, Jakobsen B. Antibody producing human-human hybridomas. I. Technical aspects. J Immunoll Methods. 1983;61:17–32.
31. Ebeling SB, Bos HMK, Slater R, Overkamp WJI, Cuthbert AP, Newbold RF, Zdzienicka MZ, Aarden LA. Human chromosome 21 determines growth factor dependence in human/mouse B-cell hybridomas. Cancer Research. 1998;58:2863–8.
32. Dalm MCF, Jansen M, Keijzer TMP, van Grunsven WMJ, Oudshoorn A, Tramper J, Martens DE. Stable hybridoma cultivation in a pilot-scale acoustic perfusion system: Long-term process performance and effect of recirculation rate. Biotechnology and Bioengineering. 2005;91:894–900.
33. Dewar V, Voet P, Denamur F, Smal J. Industrial implementation of *in vitro* production of monoclonal antibodies. ILAR J. 2005;46:307.
34. Mable BK, Otto SP. Masking and purging mutations following EMS treatment in haploid, diploid and tetraploid yeast (Saccharomyces cerevisiae). Genetical Research. 2001;77:9–26.
35. Levine DS, Sanchez CA, Rabinovitch PS, Reid BJ. Formation of the tetraploid intermediate is associated with the development of cells with more than four centrioles in the elastase-simian virus 40 tumor antigen transgenic mouse model of pancreatic cancer. Proceedings of the National Academy of Sciences. 1991;88:6427.
36. Nigg EA. Centrosome aberrations: cause or consequence of cancer progression? Nat Rev Cancer. 2002;2:815–25.
37. Vitale I, Senovilla L, Jemaà M, Michaud M, Galluzzi L, Kepp O, Nanty L, Criollo A, Rello-Varona S, Manic G. Multipolar mitosis of tetraploid cells: inhibition by p. 53 and dependency on Mos. EMBO J. 2010;29:1272–84.
38. Grompe M. The role of bone marrow stem cells in liver regeneration. Semin Liver Dis. 2003;23:363–72.
39. Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature. 2003;422:901–4.
40. Duncan AW, Hickey RD, Paulk NK, Culberson AJ, Olson SB, Finegold MJ, Grompe M. Ploidy reductions in murine fusion-derived hepatocytes. PLoS Genet. 2009;5:e1000385.
41. Duncan AW, Taylor MH, Hickey RD, Newell AEH, Lenzi ML, Olson SB, Finegold MJ, Grompe M. The ploidy conveyor of mature hepatocytes as a source of genetic variation. Nature. 2010;467:707–10.
42. Gupta S. Hepatic polyploidy and liver growth control. Semin Cancer Biol. 2000;10:161–71.
43. Oberringer M, Lothschütz D, Jennewein M, Koschnick M, Mutschler W, Hanselmann RG. Centrosome multiplication accompanies a transient clustering of polyploid cells during tissue repair. Mol Cell Biol Res Comm. 1999;2:190–6.
44. Storchova Z, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol. 2004;5:45–54.
45. Gurdon J, Melton D. Nuclear reprogramming in cells. Science. 2008;322:1811.
46. Lin HP, Ogle BM. Nuclear Reprogramming. Encyclopedia for Stem Cell Research. CA: Sage Publications; 2008. 369–99.
47. Wilmut I, Schnieke A, McWhir J, Kind A, Campbell K. Viable offspring derived from fetal and adult mammalian cells. Clones and Clones: Facts and Fantasies about Human Cloning. 1999:21.
48. Blau HM, Chiu CP, Webster C. Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell. 1983;32:1171–80.
49. Davidson RL, Ephrussi B, Yamamoto K. Regulation of pigment synthesis in mammalian cells, as studied by somatic hybridization. Proceedings of the National Academy of Sciences of the United States of America. 1966;56:1437.
50. Tada M, Tada T, Lefebvre L, Barton SC, Surani MA. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J. 1997;16:6510–20.
51. Yamanaka S, Blau HM. Nuclear reprogramming to a pluripotent state by three approaches. Nature. 2010;465:704–12.
52. Kimura H, Tada M, Nakatsuji N, Tada T. Histone code modifications on pluripotential nuclei of reprogrammed somatic cells. Mol Cell Biol. 2004;24:5710.
53. Powell AE, Anderson EC, Davies PS, Silk AD, Pelz C, Impey S, Wong MH. Fusion between intestinal epithelial cells and macrophages in a cancer context results in nuclear reprogramming. Cancer Research. 2011;71:1497.
54. Wolfe KH. Yesterday's polyploids and the mystery of diploidization. Nature Reviews Genetics. 2001;2:333–41.

55. Wendel JF. Genome evolution in polyploids. Plant Mol Biol. 2000;42:225–49.
56. Chester M, Gallagher JP, Symonds VV, da Silva AVC, Mavrodiev EV, Leitch AR, Soltis PS, Soltis DE. Extensive chromosomal variation in a recently formed natural allopolyploid species, Tragopogon miscellus (Asteraceae). Proceedings of the National Academy of Sciences. 2012;109:1176–81.
57. Eldredge N, Gould SJ. Punctuated equilibria: an alternative to phyletic gradualism. Models in Paleobiology. 1972;82:115.
58. Sagan L. On the origin of mitosing cells. J Theor Biol. 1967;14:225-74, IN1–IN6.
59. Oren-Suissa M, Podbilewicz B. Cell fusion during development. Trends Cell Biol. 2007;17:537–46.
60. Chen EH, Grote E, Mohler W, Vignery A. Cell–cell fusion. FEBS Lett. 2007;581:2181–93.
61. Helming L, Gordon S. Molecular mediators of macrophage fusion. Trends Cell Biol. 2009;19:514–22.
62. Shinn-Thomas JH, Mohler WA. Chapter five - New Insights into the Mechanisms and Roles of Cell–Cell Fusion. In: Kwang WJ, editor. Int Rev Cell Mol Biol: Academic Press; 2011. p.149–209.
63. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337–42.
64. Moreno JL, Mikhailenko I, Tondravi MM, Keegan AD. IL-4 promotes the formation of multinucleated giant cells from macrophage precursors by a STAT6-dependent, homotypic mechanism: contribution of E-cadherin. J Leukoc Biol. 2007;82:1542–53.
65. Yagi M, Ninomiya K, Fujita N, Suzuki T, Iwasaki R, Morita K, Hosogane N, Matsuo K, Toyama Y, Suda T. Induction of DC-STAMP by Alternative Activation and Downstream Signaling Mechanisms. J Bone Miner Res. 2007;22:992–1,001.
66. Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, Morita K, Ninomiya K, Suzuki T, Miyamoto K. DC-STAMP is essential for cell–cell fusion in osteoclasts and foreign body giant cells. Journal Exp Med. 2005;202:345.
67. Helming L, Tomasello E, Kyriakides TR, Martinez FO, Takai T, Gordon S, Vivier E. Essential role of DAP12 signaling in macrophage programming into a fusion-competent state. Sci Signal. 2008;1:ra11.
68. Kyriakides TR, Foster MJ, Keeney GE, Tsai A, Giachelli CM, Clark-Lewis I, Rollins BJ, Bornstein P. The CC chemokine ligand, CCL2/MCP1, participates in macrophage fusion and foreign body giant cell formation. The American Journal of Pathology. 2004;165:2157.
69. Vignery A. Macrophage fusion: the making of osteoclasts and giant cells. J Exp Med. 2005;202:337–40.
70. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, van Rooijen N, Weissman IL. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell. 2009;138:286–99.
71. Liu AY. Differential expression of cell surface molecules in prostate cancer cells. Cancer Research. 2000;60:3429.
72. Baier P, Eggstein S, Wolff-Vorbeck G, Baumgartner U, Hopt U. Chemokines in human colorectal carcinoma. Anticancer Research. 2005;25:3581.
73. Zijlmans HJ, Fleuren G, Baelde H, Eilers PHC, Kenter G, Gorter A. The absence of CCL2 expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11. 2. Journal Pathol. 2006;208:507–17.
74. Pawelek JM, Chakraborty AK. The cancer cell-leukocyte fusion theory of metastasis. Adv Cancer Res. 2008;101:397–444.
75. Aspord C, Pedroza-Gonzalez A, Gallegos M, Tindle S, Burton EC, Su D, Marches F, Banchereau J, Palucka AK. Breast cancer instructs dendritic cells to prime interleukin 13–secreting CD4+ T cells that facilitate tumor development. J Exp Med. 2007;204:1037.
76. Sens KL, Zhang S, Jin P, Duan R, Zhang G, Luo F, Parachini L, Chen EH. An invasive podosome-like structure promotes fusion pore formation during myoblast fusion. J Cell Biol. 2010;191:1013.
77. Jay SM, Skokos E, Laiwalla F, Krady MM, Kyriakides TR. Foreign body giant cell formation is preceded by lamellipodia formation and can be attenuated by inhibition of Rac1 activation. Am J Pathol. 2007;171:632.
78. Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A. Requirement of CD9 on the egg plasma membrane for fertilization. Science. 2000;287:321.
79. Kaji K, Oda S, Miyazaki S, Kudo A. Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion. Developmental Biology. 2002;247:327–34.
80. Jégou A, Ziyyat A, Barraud-Lange V, Perez E, Wolf JP, Pincet F, Gourier C. CD9 tetraspanin generates fusion competent sites on the egg membrane for mammalian fertilization. Proceedings of the National Academy of Sciences. 2011;108:10,946.

81. Okabe M, Adachi T, Takada K, Oda H, Yagasaki M, Kohama Y, Mimura T. Capacitation-related changes in antigen distribution on mouse sperm heads and its relation to fertilization rate *in vitro*. J Reprod Immunol. 1987;11:91–100.
82. Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature. 2005;434:234–8.
83. Larsson LI, Bjerregaard B, Talts JF. Cell fusions in mammals. Histochem Cell Biol. 2008;129:551–61.
84. Chen EH, Olson EN. Unveiling the mechanisms of cell-cell fusion. Science. 2005;308:369.
85. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256:495–7.
86. Hu L, Plafker K, Vorozhko V, Zuna RE, Hanigan MH, Gorbsky GJ, Plafker SM, Angeletti PC, Ceresa BP. Human papillomavirus 16 E5 induces bi-nucleated cell formation by cell-cell fusion. Virology. 2009;384:125–34.
87. Iessi E, Marino ML, Lozupone F, Fais S, De Milito A. Tumor acidity and malignancy: novel aspects in the design of anti-tumor therapy. Cancer Therapy. 2008;6:55–66.
88. Storchova Z, Kuffer C. The consequences of tetraploidy and aneuploidy. J Cell Sci. 2008;121:3,859.
89. Hansemann D. Ueber asymmetrische Zelltheilung in Epithelkrebsen und deren biologische Bedeutung. Virchows Archiv. 1890;119:299–326.
90. Storchová Z, Breneman A, Cande J, Dunn J, Burbank K, O'Toole E, Pellman D. Genome-wide genetic analysis of polyploidy in yeast. Nature. 2006;443:541–7.
91. Lu X, Kang Y. Cell fusion as a hidden force in tumor progression. Cancer Research. 2009;69:8,536.
92. Rajagopalan H, Lengauer C. Aneuploidy and cancer. Nature. 2004;432:338–41.
93. Barrett MT, Pritchard D, Palanca-Wessels C, Anderson J, Reid BJ, Rabinovitch PS. Molecular phenotype of spontaneously arising 4N (G2-tetraploid) intermediates of neoplastic progression in Barrett's esophagus. Cancer Research. 2003;63:4,211.
94. Rabinovitch PS, Longton G, Blount PL, Levine DS, Reid BJ. Predictors of progression in Barrett's esophagus III: baseline flow cytometric variables. Am J Gastroenterol. 2001;96:3071–83.
95. Olaharski AJ, Sotelo R, Solorza-Luna G, Gonsebatt ME, Guzman P, Mohar A, Eastmond DA. Tetraploidy and chromosomal instability are early events during cervical carcinogenesis. Carcinogenesis. 2006;27:337–43.
96. Abou-Elhamd KEA, Habib TN. The flow cytometric analysis of premalignant and malignant lesions in head and neck squamous cell carcinoma. Oral Oncol. 2007;43:366–72.
97. Dictor M, Fernö M, Baldetorp B. Flow cytometric DNA content in Kaposi's sarcoma by histologic stage. Comparison with Angiosarcoma. Analytical and Quantitative Cytology and Histology/the International Academy of Cytology [and] American Society of Cytology. 1991;13:201.
98. Fujiwara T, Bandi M, Nitta M, Ivanova EV, Bronson RT, Pellman D. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature. 2005;437:1043–7.
99. Roschke AV, Tonon G, Gehlhaus KS, McTyre N, Bussey KJ, Lababidi S, Scudiero DA, Weinstein JN, Kirsch IR. Karyotypic complexity of the NCI-60 drug-screening panel. Cancer Research. 2003;63:8,634.
100. Abdel-Rahman WM, Katsura K, Rens W, Gorman PA, Sheer D, Bicknell D, Bodmer WF, Arends MJ, Wyllie AH, Edwards PAW. Spectral karyotyping suggests additional subsets of colorectal cancers characterized by pattern of chromosome rearrangement. Proceedings of the National Academy of Sciences. 2001;98:2,538.
101. Sirivatanauksorn V, Sirivatanauksorn Y, Gorman PA, Davidson JM, Sheer D, Moore PS, Scarpa A, Edwards PAW, Lemoine NR. Non-random chromosomal rearrangements in pancreatic cancer cell lines identified by spectral karyotyping. Int J Cancer. 2001;91:350–8.
102. Davidson J, Gorringe K, Chin S, Orsetti B, Besret C, Courtay-Cahen C, Roberts I, Theillet C, Caldas C, Edwards P. Molecular cytogenetic analysis of breast cancer cell lines. Br J Cancer. 2000;83:1,309.
103. Wistuba II, Bryant D, Behrens C, Milchgrub S, Virmani AK, Ashfaq R, Minna JD, Gazdar AF. Comparison of features of human lung cancer cell lines and their corresponding tumors. ClinCancer Res. 1999;5:991–1,000.
104. Andreassen PR, Lohez OD, Lacroix FB, Margolis RL. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. Mol Biol Cell. 2001;12:1,315–28.
105. Margolis RL, Lohez OD, Andreassen PR. G1 tetraploidy checkpoint and the suppression of tumorigenesis. J Cell Biochem. 2003;88:673–83.
106. Uetake Y, Sluder G. Cell cycle progression after cleavage failure. J Cell Biol. 2004;165:609.

107. Duelli DM, Hearn S, Myers MP, Lazebnik Y. A primate virus generates transformed human cells by fusion. J Cell Biol. 2005;171:493–503.
108. Duelli DM, Padilla-Nash HM, Berman D, Murphy KM, Ried T, Lazebnik Y. A virus causes cancer by inducing massive chromosomal instability through cell fusion. Curr Biol: CB. 2007;17:431–7.
109. Greenberg R. Telomeres, crisis and cancer. CurrMol Med. 2005;5:213-8.
110. Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K. *In situ* analyses of genome instability in breast cancer. Nat Genet. 2004;36:984–8.
111. Lin TT, Letsolo BT, Jones RE, Rowson J, Pratt G, Hewamana S, Fegan C, Pepper C, Baird DM. Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. Blood. 2010;116:1,899–907.
112. Stewénius Y, Gorunova L, Jonson T, Larsson N, Höglund M, Mandahl N, Mertens F, Mitelman F, Gisselsson D. Structural and numerical chromosome changes in colon cancer develop through telomere-mediated anaphase bridges, not through mitotic multipolarity. Proceedings of the National Academy of Sciences of the United States of America. 2005;102:5,541.
113. Chan JY. A Clinical Overview of Centrosome Amplification in Human Cancers. Int J Biol Sci. 2011;7:1,122.
114. Weaver BAA, Silk AD, Montagna C, Verdier-Pinard P, Cleveland DW. Aneuploidy acts both oncogenically and as a tumor suppressor. Cancer Cell. 2007;11:25–36.
115. Rao CV, Yang YM, Swamy MV, Liu T, Fang Y, Mahmood R, Jhanwar-Uniyal M, Dai W. Colonic tumorigenesis in BubR1+/–ApcMin/+ compound mutant mice is linked to premature separation of sister chromatids and enhanced genomic instability. Proceedings of the National Academy of Sciences of the United States of America. 2005;102:4,365.
116. Sussan TE, Annan Yang FL, Ostrowski MC, Reeves RH. Trisomy represses ApcMin-mediated tumours in mouse models of Down's syndrome. Nature. 2008;451:73–5.
117. Jeganathan K, Malureanu L, Baker DJ, Abraham SC, Van Deursen JM. Bub1 mediates cell death in response to chromosome missegregation and acts to suppress spontaneous tumorigenesis. J Cell Biol. 2007;179:255–67.
118. Wang Z, Yu R, Melmed S. Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. Mol Endocrinol. 2001;15:1,870–9.
119. Yu R, Lu W, Chen J, McCabe CJ, Melmed S. Overexpressed pituitary tumor-transforming gene causes aneuploidy in live human cells. Endocrinology. 2003;144:4,991–8.
120. Chesnokova V, Kovacs K, Castro AV, Zonis S, Melmed S. Pituitary hypoplasia in Pttg–/– mice is protective for Rb+/– pituitary tumorigenesis. Mol Endocrinol. 2005;19:2,371–9.
121. Donangelo I, Gutman S, Horvath E, Kovacs K, Wawrowsky K, Mount M, Melmed S. Pituitary tumor transforming gene overexpression facilitates pituitary tumor development. Endocrinology. 2006;147:4,781–91.
122. Yang Q, Rasmussen SA, Friedman J. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet. 2002;359:1,019–25.
123. Satge D, Sommelet D, Geneix A, Nishi M, Malet P, Vekemans M. A tumor profile in Down syndrome. Am J Med Genet. 1998;78:207–16.
124. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000;355:165–9.
125. Stanbridge EJ. Suppression of malignancy in human cells. Nature. 1976;260:17.
126. Harris H, Miller O, Klein G, Worst P, Tachibana T. Suppression of malignancy by cell fusion. Nature. 1969;223:363.
127. Anderson M, Stanbridge E. Tumor suppressor genes studied by cell hybridization and chromosome transfer. FASEB J. 1993;7:826.
128. Mitelman F, Johansson B, Mertens F. Mitelman database of chromosome aberrations and gene fusions in cancer. 2011.
129. Kuukasjärvi T, Karhu R, Tanner M, Kähkönen M, Schäffer A, Nupponen N, Pennanen S, Kallioniemi A, Kallioniemi OP, Isola J. Genetic heterogeneity and clonal evolution underlying development of asynchronous metastasis in human breast cancer. Cancer Research. 1997;57:1,597–604.
130. Waldman FM, DeVries S, Chew KL, Moore II DH, Kerlikowske K, Ljung BM. Chromosomal alterations in ductal carcinomas *in situ* and their *in situ* recurrences. J Nat Cancer Inst. 2000;92:313–20.

131. Hunter KW, Crawford N, Alsarraj J. Mechanisms of metastasis. Breast Cancer Res. 2008;10:S2.
132. Lazebnik Y. What are the hallmarks of cancer? Nature Reviews Cancer. 2010;10:232–3.
133. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
134. Klein CA. Parallel progression of primary tumours and metastases. Nat Rev Cancer. 2009;9:302–12.
135. Katoh M, Neumaier M, Nezam R, Izbicki J, Schumacher U. Correlation of circulating tumor cells with tumor size and metastatic load in a spontaneous lung metastasis model. Anticancer Research. 2004;24:1,421–6.
136. Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, Harris CC, McLellan MD, Fulton RS, Fulton LL. Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature. 2010;464:999–1,005.
137. Abbruzzese JL, Abbruzzese MC, Hess KR, Raber MN, Lenzi R, Frost P. Unknown primary carcinoma: natural history and prognostic factors in 657 consecutive patients. J ClinOncol. 1994;12:1,272–80.
138. Van de Wouw A, Janssen-Heijnen M, Coebergh J, Hillen H. Epidemiology of unknown primary tumours; incidence and population-based survival of 1285 patients in Southeast Netherlands, 1984–1992. Eur J Cancer. 2002;38:409–13.
139. Friberg S, Mattson S. On the growth rates of human malignant tumors: implications for medical decision making. J Surg Oncol. 1997;65:284–97.
140. Peer PGM, Van Dijck JAAM, Verbeek ALM, Hendriks JHCL, Holland R. Age-dependent growth rate of primary breast cancer. Cancer. 1993;71:3,547–51.
141. Parris GE. Clinically significant cancer evolves from transient mutated and/or aneuploid neoplasia by cell fusion to form unstable syncytia that give rise to ecologically viable parasite species. Med Hypotheses. 2005;65:846–50.
142. Duesberg P, Fabarius A, Hehlmann R. Aneuploidy, the primary cause of the multilateral genomic instability of neoplastic and preneoplastic cells. IUBMB Life. 2004;56(2):65–81.
143. Muller H. The relation of recombination to mutational advance. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 1964;1:2–9.
144. Risques RA, Moreno V, Ribas M, Marcuello E, Capellà G, Peinado MA. Genetic pathways and genome-wide determinants of clinical outcome in colorectal cancer. Cancer Research. 2003;63:7,206.
145. Staibano S, Franco R, Mezza E, Chieffi P, Sinisi A, Pasquali D, Errico M, Nappi C, Tremolaterra F, Somma P. Loss of oestrogen receptor ☒, high PCNA and p53 expression and aneuploidy as markers of worse prognosis in ovarian granulosa cell tumours. Histopathology. 2003;43:254–62.
146. Choma D, Daures J, Quantin X, Pujol J. Aneuploidy and prognosis of non-small-cell lung cancer: a meta-analysis of published data. Br J Cancer. 2001;85:14.
147. outhern JF, Warshaw AL, Lewandrowski KB. DNA ploidy analysis of mucinous cystic tumors of the pancreas: correlation of aneuploidy with malignancy and poor prognosis. Cancer. 1996;77:58–62.
148. Vinogradov A, Anatskaya O, Kudryavtsev B. Relationship of hepatocyte ploidy levels with body size and growth rate in mammals. Genome. 2001;44:350–60.
149. Brodsky VY, Delone G. Functional control of hepatocyte proliferation. Comparison with the temporal control of cardiomyocyte proliferation. Biomed Sci. 1990;1:467.
150. Torres S, Díaz BP, Cabrera JJ, Díaz-Chico JC, Díaz-Chico BN, López-Guerra A. Thyroid hormone regulation of rat hepatocyte proliferation and polyploidization. AmJ Physiol Gastrointest Liver Physiol. 1999;276:G155–G63.
151. Klein PA, Xiang J, Kimura AK. Melanoma cells growing in aggregates on a non-adhesive poly (HEMA) substrate exhibit polykaryocytosis but do not develop an increased metastatic capability. Clin Exp Metastasis. 1984;2:287–95.
152. Lu X, Kang Y. Efficient acquisition of dual metastasis organotropism to bone and lung through stable spontaneous fusion between MDA-MB-231 variants. Proceedings of the National Academy of Sciences. 2009;106:9,385.
153. Goldenberg DM, Pavia RA, Tsao MC. *In vivo* hybridisation of human tumour and normal hamster cells. Nature. 1974;250:649–51.
154. Kerbel RS, Lagarde A, Dennis J, Donaghue T. Spontaneous fusion *in vivo* between normal host and tumor cells: possible contribution to tumor progression and metastasis studied with a lectin-resistant mutant tumor. Mol Cell Biol. 1983;3:523.
155. Mortensen K, Lichtenberg J, Thomsen P, Larsson LI. Spontaneous fusion between cancer cells and endothelial cells. Cell Mol Life Sci. 2004;61:2,125–31.
156. Jacobsen BM, Harrell JC, Jedlicka P, Borges VF, Varella-Garcia M, Horwitz KB. Spontaneous fusion with, and transformation of mouse stroma by,

malignant human breast cancer epithelium. Cancer Research. 2006;66:8,274.
157. Smith MA, Bidochka MJ. Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. CanJ Microbiol. 1998;44:351–5.
158. Goldenberg DM, Zagzag D, Heselmeyer-Haddad KM, Berroa Garcia LY, Ried T, Loo M, Chang CH, Gold DV. Horizontal transmission and retention of malignancy, as well as functional human genes, after spontaneous fusion of human glioblastoma and hamster host cells *in vivo*. Int J Cancer. 2012;131:49–58.
159. Aichel O. Ueber Zellverschmelzung mit qualitativ abnormer Chromosomenverteilung als Ursache der Geschwulstbildung, von Prof. Dr. Med. Et phil. Otto Aichel: W. Engelmann; 1911.
160. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315:1,650.
161. Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol. 2009;9:259–70.
162. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell. 2009;16:91–102.
163. Ribatti D, Crivellato E, Roccaro A, Ria R, Vacca A. Mast cell contribution to angiogenesis related to tumour progression. ClinExp Allergy. 2004;34:1,660–4.
164. De Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell. 2005;7:411–23.
165. Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC, Fisher RI, Braziel RM, Rimsza LM, Grogan TM. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N EnglJ Med. 2004;351:2,159–69.
166. Rizvi AZ, Swain JR, Davies PS, Bailey AS, Decker AD, Willenbring H, Grompe M, Fleming WH, Wong MH. Bone marrow-derived cells fuse with normal and transformed intestinal stem cells. PNAS. 2006;103:6,321–5.
167. Johansson CB, Youssef S, Koleckar K, Holbrook C, Doyonnas R, Corbel SY, Steinman L, Rossi FMV, Blau HM. Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation. Nat Cell Biol. 2008;10:575–83.
168. Nygren JM, Liuba K, Breitbach M, Stott S, Thorén L, Roell W, Geisen C, Sasse P, Kirik D, Björklund A. Myeloid and lymphoid contribution to non-haematopoietic lineages through irradiation-induced heterotypic cell fusion. Nat Cell Biol. 2008;10:584–92.
169. Chakraborty A, Lazova R, Davies S, Bäckvall H, Ponten F, Brash D, Pawelek J. Donor DNA in a renal cell carcinoma metastasis from a bone marrow transplant recipient. Bone Marrow Transplant. 2004;34:183–6.
170. Andersen TL, Boissy P, Sondergaard T, Kupisiewicz K, Plesner T, Rasmussen T, Haaber J, Kølvraa S, Delaissé J. Osteoclast nuclei of myeloma patients show chromosome translocations specific for the myeloma cell clone: a new type of cancer–host partnership? J Pathol. 2007;211:10–7.
171. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139:871–90.
172. Rachkovsky M, Pawelek J. Acquired melanocyte stimulating hormone-inducible chemotaxis following macrophage fusion with Cloudman S91 melanoma cells. Cell Growth & Differentiation: The Molecular Biology Journal of the American Association for Cancer Research. 1999;10:517.
173. Sodi S, Chakraborty A, Platt J, Kolesnikova N, Rosemblat S, Keh-Yen A, Bolognia J, Rachkovsky M, Orlow S, Pawelek J. Melanoma× Macrophage Fusion Hybrids Acquire Increased Melanogenesis and Metastatic Potential: Altered N-Glycosylation as an Underlying Mechanism. Pigment Cell Research. 1998;11:299–309.
174. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer. 2004;4:71–8.
175. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006;124:263–6.
176. Bingle L, Brown N, Lewis C. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol. 2002;196(3):254–65.
177. Pollard JW. Macrophages define the invasive microenvironment in breast cancer. J Leukoc Biol. 2008;84:623–30.
178. Chakraborty AK, Kolesnikova N, JdF S, Espreafico EM, Peronni KC, Pawelek J. Expression of c-Met Proto-oncogene in Metastatic Macrophage Melanoma Fusion Hybrids: Implication of Its Possible Role in MSH-Induced Motility. Oncology Research

Featuring Preclinical and Clinical Cancer Therapeutics. 2003;14:163–74.
179. Chakraborty AK, Pawelek J, Ikeda Y, Miyoshi E, Kolesnikova N, Funasaka Y, Ichihashi M, Taniguchi N. Fusion hybrids with macrophage and melanoma cells up-regulate N-acetylglucosaminyltransferase V, β1-6 branching, and metastasis. Cell Growth and Differentiation. 2001;12:623–30.
180. Wright NA. Epithelial stem cell repertoire in the gut: clues to the origin of cell lineages, proliferative units and cancer. IntJ Exp Pathol. 2000;81:117–43.
181. Holmgren L, Szeles A, Rajnavölgyi E, Folkman J, Klein G, Ernberg I, Falk KI. Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood. 1999;93:3,956.
182. Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, Holmgren L. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proceedings of the National Academy of Sciences. 2001;98:6,407.
183. Al-Nedawi K, Meehan B, Rak J. Messengers and mediators of tumor progression. Cell Cycle. 2009;8:2,014–8.
184. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol. 2008;10:619–24.
185. Davis DM, Sowinski S. Membrane nanotubes: dynamic long-distance connections between animal cells. Nat Rev Mol Cell Biol. 2008;9:431–6.
186. Martinez-Outschoorn UE, Whitaker-Menezes D, Pavlides S, Chiavarina B, Bonuccelli G, Trimmer C, Tsirigos A, Migneco G, Witkiewicz AK, Balliet R. The autophagic tumor stroma model of cancer or "battery-operated tumor growth": A simple solution to the autophagy paradox. Cell Cycle. 2010;9:4,297.
187. Wolff JA, Budker V. The mechanism of naked DNA uptake and expression. Adv Genet. 2005;54:1–20.
188. Nagler C, Hardt C, Zänker KS, Dittmar T. Cocultivation of murine BMDCs with 67NR mouse mammary carcinoma cells give rise to highly drug resistant cells. Cancer Cell Int. 2011;11:21.
189. Kerppola TK. Visualization of molecular interactions by fluorescence complementation. Nat Rev Mol Cell Biol. 2006;7:449–56.
190. Kerppola TK. Bimolecular fluorescence complementation: visualization of molecular interactions in living cells. Methods Cell Biol. 2008;85:431–70.
191. Lin HP, Vincenz C, Eliceiri KW, Kerppola TK, Ogle BM. Bimolecular fluorescence complementation analysis of eukaryotic fusion products. Biol Cell. 2010;102(Pt 9):525.
192. Sprangers A, Freeman B, Kouris N, Ogle B. A Cre-Lox P Recombination Approach for the Detection of Cell Fusion *In Vivo*. J VisExp: JoVE. 2012;(59):e3581.
193. Kouris NA, Schaefer JA, Hatta M, Kawaoka Y, Kamp TJ, Ogle BM. Directed fusion of mesenchymal stem cells with cardiomyocytes via VSV-G facilitates stem cell programming. Stem Cells Int. 2012;2012:414038.

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Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis

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ABSTRACT **Although cancer cell fusion has been suggested as a mechanism of cancer metastasis, the underlying mechanisms defining this process are poorly understood. In a recent study, apoptotic cells were newly identified as a type of cue that induces signaling *via* phosphatidylserine receptors to promote fusion of myoblasts. The microenvironment of breast tumors is often hypoxic, and because apoptosis is greatly increased in hypoxic conditions, we decided to investigate whether the mechanism of breast cancer cell fusion with mesenchymal stem/multipotent stromal cells (MSCs) involves apoptosis. We used a powerful tool for identification and tracking of hybrids based on bimolecular fluorescence complementation (BiFC) and found that breast cancer cells fused spontaneously with MSCs. This fusion was significantly enhanced with hypoxia and signaling associated with apoptotic cells, especially between nonmetastatic breast cancer cells and MSCs. In addition, the hybrids showed a significantly higher migratory capacity than did the parent cells. Taken together, these findings describe a mechanism by which hypoxia-induced apoptosis stimulates fusion between MSCs and breast tumor cells resulting in hybrids with an enhanced migratory capacity that may enable their dissemination to distant sites or metastases. In the long run, this study may provide new strategies for developing novel drugs for preventing cancer metastasis.—Noubissi, F. K., Harkness, T., Alexander, C. M., Ogle, B. M. Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis. *FASEB J.* 29, 000–000 (2015). www.fasebj.org**

Key Words: hybrid · hypoxia · migration · mesenchymal stem cell · bimolecular fluorescence complementation

FUSION OF BREAST CANCER CELLS with cells of the tumor microenvironment has been proposed as a means of generating widespread genetic and epigenetic diversity (1–4). Diversity created in this way could rapidly enhance the formation, propagation, and metastasis of tumor cells or could quickly alter drug sensitivity. Yet to be reconciled are the precise mechanisms governing hybrid reprogramming after cell fusion and the means by which cancer cells undergo spontaneous fusion in the first place. The latter was the focus of this work.

One way that spontaneous cell fusion is enabled is *via* specific integral membrane proteins, termed fusogens, which provide cell anchorage and disrupt cell membranes, to minimize the energy necessary to overcome the merger of the 2 hydrophobic membranes (5). The structure and function of many viral fusogens are well characterized, but eukaryotic fusogens are more difficult to establish because removal of facilitating proteins from the system reduces overall fusion rates. As technologies advance, many putative fusogens have been contested and shown to be merely adhesion proteins that bring cell membranes in close apposition but do not actually facilitate fusion. Take, for example, heterotypic gamete fusion. As a fusion product, the fertilized embryo can proliferate and differentiate into all the tissues of the adult body as well as the extraembryonic tissues. CD9 is an egg-associated putative fusogen. Cluster of differentiation (CD)9-knockout mice have shown severely hampered fertilization (6) that is restored with polyadenylated CD9 mRNA (7). In addition, CD9 has been shown to generate the strongest observed interactions with the sperm (8). However, it is not known whether it specifically facilitates the disruption and merging of membranes or whether other facilitating proteins or conditions are necessary (5).

Bona fide fusogens may not be necessary for cell fusion if cell membranes can be disrupted by other means. Cell stress, including nutrient deprivation, electroporation, and hypoxia, can render cell membranes leaky or unstable (9–11). For example, changes in the ratio of cholesterol to phospholipid and in the double-bond index of the associated fatty acids have been shown to alter plasma membrane structure during hypoxic injury (12). In addition, alterations in lipid structure with hypoxia can lead to changes in membrane protein structure and function, resulting in dysregulated enzyme activity and transport properties that could also lead to leakiness of the plasma membrane barrier (13). Unstable cell membranes are biophysically

Abbreviations: αMEM, α-minimum essential medium; BAI, brain-specific angiogenesis inhibitor; BiFC, bimolecular fluorescence complementation; CCL, chemokine ligand; CD, cluster of differentiation; CMV, cytomegalovirus; COI, cytochrome *c* oxidase I; Dock, dedicator of cytokines; EBV, Epstein-Barr virus; ELMO, engulfment cell and mobility protein; GFP,

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susceptible to membrane fusion (14, 15). It may be for this reason that cell fusion appears to occur more readily in the context of hypoxia than during normoxia (16, 17). In contrast, cell fusion may be enhanced with hypoxia *via* signaling events triggered by hypoxic stress. For example, human hematopoietic progenitor cells upregulate VCAM-1 with hypoxia, a protein found to enhance adhesion and fusion with cardiomyocytes. Similarly, fusion of skeletal myocytes is increased during hypoxia, in part because of signaling events associated with apoptotic cells. Apoptotic cells exhibit exposed phosphatidyl serine and choline, which binds associated phosphatidyl serine and choline receptors expressed by skeletal muscle. Binding of these receptors in skeletal muscle can engage the engulfment cell and mobility protein (ELMO)/dedicator of cytokines (Dock)180/ras-related C3 botulinum toxin substrate (Rac)-1 module to enhance myoblast fusion (18).

To accurately test whether apoptotic cells enable cell fusion, we used a unique tool developed in our lab to detect fusion products that was based on bimolecular fluorescence complementation (BiFC). With this approach, a fluorescent signal is induced by hybrid formation and can be detected in living cells. In addition, the signal intensity in hybrids increases over time as BiFC products are continuously synthesized. Using this robust tool, we showed that spontaneous fusion of multipotent stem/stromal cells (MSCs) with breast cancer cells occurs more readily in hypoxia than in normoxia, a significant finding, given that the microenvironment of breast tumors is often hypoxic. In addition, we found that, like skeletal muscle, cell fusion with hypoxia is enhanced as a consequence of the signaling associated with apoptotic cells. These findings are among the first to address how cancer cells spontaneously fuse in the first place and, as a consequence, reveal novel untapped therapeutic targets.

MATERIALS AND METHODS

Cell lines and culture

MSCs were a generous gift from Dr. Peiman Hematti (University of Wisconsin, Madison, WI, USA). They were derived from human embryonic stem cells in accordance with guidelines of the University of Wisconsin Institutional Review Board (19) and maintained in α-minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). We reconfirmed the identity of the MSCs in our lab by flow cytometry for specific MSC markers. Human mammary epithelial cells (MCF10a), human ductal breast epithelial tumor cells (T47D), human breast adenocarcinoma cells (MDA-MB-231), and human breast cancer cells (MCF7) (MCF indicates the Michigan Cancer Foundation where the MCF10a and MCF7 lines were established) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in accordance with the recommendations of ATCC; the cells were not passaged for more than 6 mo from the time of receipt. MCF10as were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by short tandem repeat (STR) analysis and cytochrome *c* oxidase I (COI) assay, 3) sterility by BacT/ALERT 3D (bioMérieux, St. Louis, MO, USA), and 4) the human pathogens HIV, hepatitis B and C (HepB, HepC), human papilloma virus (HPV), Epstein Barr virus (EBV), and cytomegalovirus (CMV). T47Ds were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR and COI assays, and 3) sterility by BacT/ALERT 3D. MDA-MB-231s were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR and COI assay, 3) sterility by BacT/ALERT 3D, and 4) the human pathogens listed above. MCF7s were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR, 3) sterility by BacT/ALERT 3D, and 4) the human pathogens listed above.

BiFC approach

BiFC is a method of viewing living hybrid cells (20, 21) (Supplemental Fig. S1*A*). Thus, the fluorescence of green fluorescent protein (GFP) is reconstituted when 2 nonfluorescent halves, expressed in fusion partners, are coexpressed and are in proximity in fusion products (22–26). To encourage proximity, each nonfluorescent half is coupled to histone 3.1 (H3.1; plasmids, a generous gift of Dr. Thomas Kerppola, University of Michigan, Ann Arbor, MI, USA). Fluorescence was detected and recorded *via* traditional fluorescence microscopy (excitation 485, emission 530; Olympus IX81; Olympus America, Center Valley, PA, USA). Because of the conditional nature of the signal (*i.e.,* fluorescent signal is detected only after a fusion event), the incidence of false positives was significantly reduced. A fluorescence signal was deemed positive if it was localized to the nucleus (as a consequence of the H3.1 fusion protein), fluoresced above the background fluorescence of unaltered cocultures, and did not fluoresce with excitation/emission at alternate wavelengths. Under these criteria, transduction of the cells with each BiFC half alone did not yield a detectable false-positive signal (Supplemental Fig. S1*B*).

Coculture protocol

The design of coculture experiments is illustrated in Supplemental Fig. S2*A*. In our study, we transduced MSCs with lentiviral particles expressing the BiFC complex amino terminus GFP, histone H3.1 (VNH3.1), and the breast epithelial cells (MCF10a) or breast cancer cells (T47D, MDA-MB-231, and MCF7), with the corresponding BiFC complex carboxyl terminus GFP, histone H3.1 (YCH3.1) (Supplemental Fig. S2*A*). The histone H3.1 tagged to each construct brought the BiFC halves in close association in the nucleus. MSCs were mixed with each epithelial cell type (*i.e.,* MSC with MDA-MB-231, MSC with T47D, MSC with MCF7, and MSC with MCF10a) in 4-well chamber slides, such that the stromal population was mixed with each epithelial cell type. The ratio of cells for cocultures was 80,000 MSCs to 35,000, 112,000, 100,000, and 90,000 MDA-MB-231, T47D, MCF7, and MCF10a cells, respectively. Cell densities were selected so that surface area coverage for each cell type was approximately 50% at 24 h after the cocultures were initiated (Supplemental Fig. S2B). Forty-eight hours after coculturing began, the cells were fixed and stained with DAPI. Fusion products were identified by fluorescence microscopy. A signal (green fluorescence) was considered positive if it was stronger than the background in the FITC channel for unaltered cocultures, was nuclear (overlapped with DAPI), and did not

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green fluorescent protein; HepB, hepatitis B; HepC, hepatitis C; HPV, human papillomavirus; HSD, honest significant difference; MCF7, human breast cancer cells; MCF10a, mammary epithelial cells; MDA-MB-231, human breast adenocarcinoma cells; MSC, multipotent stromal/mesenchymal stem cell; NOD, nonobese diabetic; PtSer, phosphatidylserine; Rac, ras-related C3 botulinum toxin substrate; STR, short tandem repeat; T47D, human ductal breast epithelial tumor cell; VNH3.1, amino terminus GFP, histone H3.1; YCH3.1, carboxyl terminus GFP, histone H3.1; Z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone

crossover to the rhodamine and cyanine (Cy)5 channels. The frequency of fusion products was reported relative to the total number of nuclei in at least 200 fields of view per experiment, with about 110 cells per field. We performed each experiment at least 3 times.

Hypoxic treatment

Hypoxic treatment was performed 16 h after the initiation of cocultures. The cocultures were removed from standard tissue culture conditions (21% O_2, 5% CO_2, and 74% N_2) and put in conditions of oxygen deprivation (2% O_2, 5% CO_2, and 93% N_2) for 3 h. At the end of coculturing (48 h after initiation) the cells were fixed and stained with DAPI, and the number of fusion products was recorded. We recognize that standard tissue culture conditions are actually hyperoxic relative to normal tissue, but for the purposes of this study, we considered the condition of 2% O_2 to be hypoxic.

Apoptotic cell treatment

Apoptosis was induced by treatment of T47Ds with 1 μM staurosporine (Sigma-Aldrich) for 12 h. Annexin V staining determined that 50–75% of the cells were apoptotic. Floating and attached cells were collected and used in the experiments. To investigate the involvement of apoptotic cells in cancer cell fusion, MSCs and T47Ds were transduced, and cocultures were initiated as described above. Sixteen hours after initiation of the cocultures, apoptotic cells were added at a ratio of 1 apoptotic cell for 2 coculture cells. The cocultures were analyzed under normoxic and hypoxic conditions, as described above.

Annexin V apoptosis assay

Breast epithelial cells exposed to low oxygen for 3 h were stained with the ApoAlertAnnexin V-FITC Apoptosis Kit (Clontech, Mountain View, CA, USA), in accordance with the recommendations of the manufacturer. Apoptotic cells were detected with a fluorescence microscope.

Caspase inhibitor treatment

Caspase activity was inhibited in cocultures of fusion partners by using the small molecule, Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone; Promega, Madison, WI, USA) and the ability of cells to fuse under normoxic and hypoxic conditions was analyzed. Z-VAD-FMK is a cell-permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases and is widely used to inhibit apoptosis. In our study, each cell population (MSCs, T47D, and MCF7) was transduced with the lentiviral particles expressing the BiFC complex VNH3.1 for MSCs, and the corresponding BiFC complex YCH3.1 for T47Ds or MCF7s, as described above. Twelve hours after transduction, each cell population was treated with Z-VAD-FMK (1 μM) for 1 h, and the cocultures were initiated. The coculture medium was supplemented with Z-VAD-FMK during the entire experiment (36 h), and the cocultures were assessed under normoxic and hypoxic (2% O_2, 5%CO_2, and 93% N_2) conditions, with and without the addition of apoptotic cells. The cells were fixed and stained with DAPI, and the fusion products were recorded.

Time-lapse microscopy

Cocultures for time-lapse microscopy were initiated as described in the protocol above. In brief, MSCs were transduced with lentiviral particles from the BiFC component VNH3.1, and T47D or MCF7s were transduced with lentiviral particles from the BiFC component YCH3.1. At 48 h after initiation of the cocultures, the fusion products were identified by fluorescence microscopy (TE200; Nikon, Inc., Melville, NY, USA). The cells were maintained at 5% CO_2, 37°C, in a humidified atmosphere. Phase-contrast and fluorescent images of hybrids were taken every 5 min for 3 h with a ×20 objective (0.5 NA, plan fluor ×20 phase 1). Images of double-transduced parental cells and of control cells were taken. The paths of migrating cells were analyzed by using the manual tracking and chemotaxis and migration tool in NIH Fiji software (ImageJ; National Institutes of Health, Bethesda, MD, USA) (27). In brief, the center of a given nucleus was selected for each frame. Based on the resultant path created, the software was used to calculate velocity, total distance, and directionality.

For long-term tracking (72 h), MSCs and T47Ds were transiently transfected with mCherry- and GFP-expressing plasmids, respectively. Sixteen hours after transfection, the cocultures were initiated, and fusion products (expressing both red and green fluorescence) were identified 24 h later with a fluorescence microscope. Phase-contrast and green fluorescent images of hybrids were taken every 10 min for 72 h with a ×10 objective in the Citation 3 imaging reader (Bio-Tek, Winooski, VT, USA). The cells were maintained at 5% CO_2 and 37°C, and the medium was replaced every 24 h. At 72 h, a static fluorescence image (green and red) of the tracked hybrid was obtained to confirm the hybrid's identity.

Statistical analysis

All data are presented as means ± SD. When 2 groups were compared, a 2-tailed Student's *t* test was used. When more than 2 groups were compared, ANOVA with Tukey's honest significant difference (HSD) *post hoc* test was used. $P < 0.05$ denoted statistically significant differences.

RESULTS

Breast tumor cells fuse with MSCs spontaneously

Fusion between tumor cells and host cells has been shown by several studies, many of which implicate bone marrow–derived cells of myeloid lineage as host fusion partners [reviewed in (17, 28)]. We propose that fusion of tumor cells with cells of the stroma gives rise to hybrid cells capable of dissemination and new tumor growth. In the initial step of investigating the role of cell fusion in breast cancer metastasis, we examined the ability of MSCs to fuse spontaneously with breast cancer cells with different degrees of aggressiveness (T47D cells, which are nonmetastatic, and MDA-MB-231 cells, which are metastatic) and nontumorigenic MCF10a breast epithelial cells . We have developed a powerful tool for the detection of fusion products *in vitro* that uses BiFC (see Materials and Methods and Supplemental Fig. S1*A*). Because of the conditional nature of the signal, the incidence of false positives is significantly reduced. Moreover, transduction of the cells with each BiFC half did not yield any detectable false positives (Supplemental Fig. S1*B*). Lentiviral particles expressing each half of the BiFC complex were delivered to the cells, and cocultures were initiated as described in Materials and Methods (Supplemental Fig. S2*A*). Fusion products were identified with an epifluorescence microscope. The MSCs fused spontaneously with all breast epithelial cell types (**Fig. 1*A*** and Supplemental Fig. S3*A*) at a fusion rate of ~1:1000 for cancer cell types and ~1:2000 for the

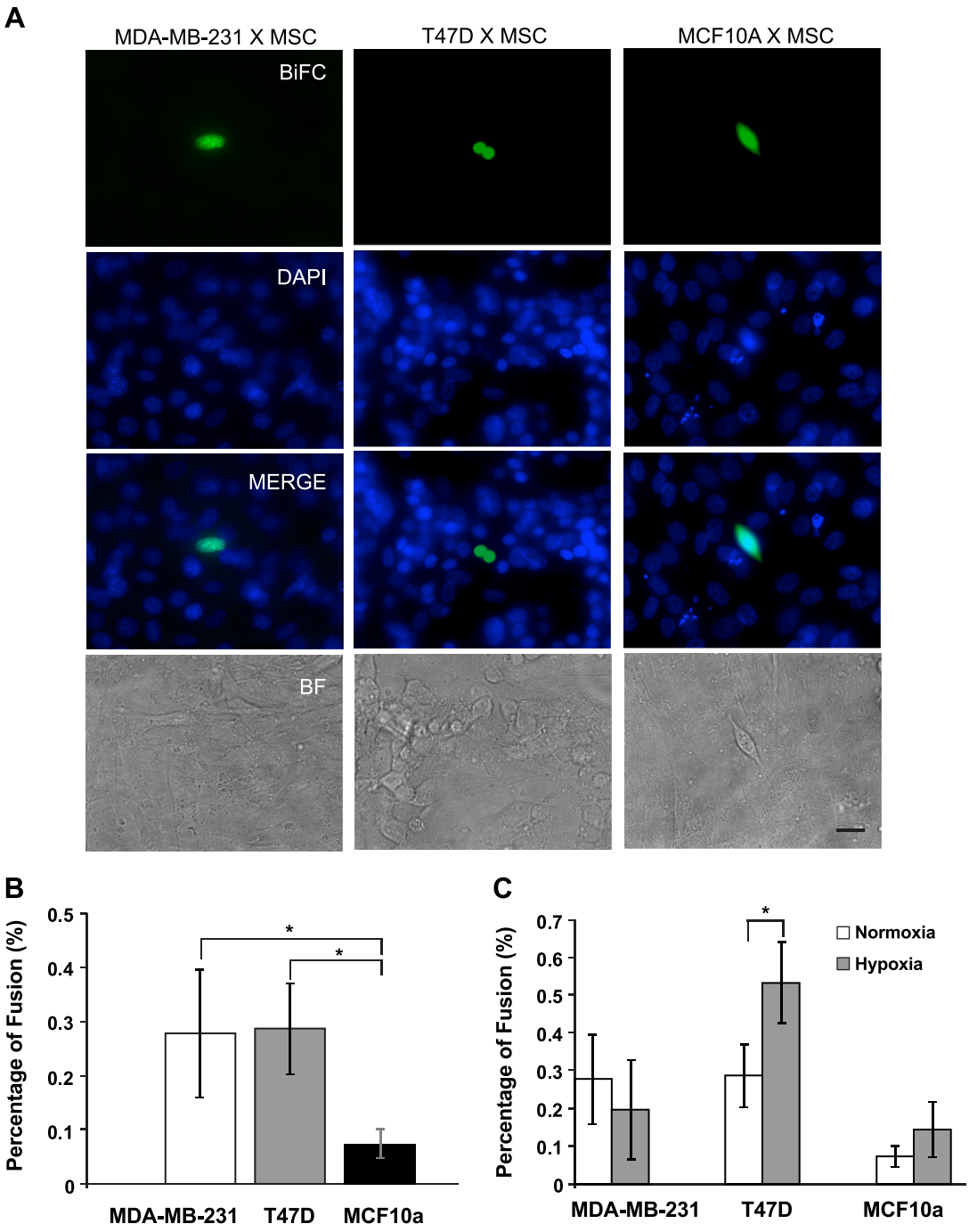

Figure 1. MSCs fuse spontaneously with breast epithelial cells. *A*) Representative fusion products from MSC-breast epithelial cell cocultures. Scale bar, 25 μm. *B*) Frequency of fusion of MSCs with breast epithelial cells. *C*) Percentage of cell fusion between MSCs and MDA-BM-231s, T47Ds, or MCF10as under hypoxic and normoxic conditions. *B*, *C*) Each value (mean ± SD) represents the average of 3 independent experiments performed in triplicate. ANOVA with Tukey's HSD *post hoc* test. $^*P < 0.05$.

nontumorigenic cell line, MCF10a ($P < 0.05$) (Fig. 1*B* and Supplemental Fig. S3*B*).

Fusion is enhanced with hypoxia

To test whether the rate of fusion could be regulated by factors associated with tumor microenvironments, we evaluated the effect of hypoxia (29). This hypoxic microenvironment puts cancer cells under stress and may promote their fusion with cells of the stroma recruited to the tumor microenvironment, as a means of survival. Cells were transduced with lentiviral particles expressing each half of the BiFC complex, and cocultures were initiated between MSCs and breast epithelial cells. The cocultures were subsequently exposed to low oxygen for 3 h, and fusion products were recorded, as described in Materials and Methods. We found that hypoxia stimulates a significant increase in fusion between MSCs and the T47D nonmetastatic breast cancer cells ($P < 0.05$; Fig. 1*C*). This

increase in fusion was not restricted to T47Ds, but was also observed with the nonmetastatic MCF7 cancer cells ($P < 0.05$; Supplemental Fig. S3*B*).

Fused breast tumor cells show increased migratory capability

To study the contribution of spontaneous cell fusion to metastasis, we used time-lapse microscopy to analyze the migratory capability of hybrids derived from the fusion between T47Ds and MSCs. Cocultures were initiated between the MSCs and the T47Ds. Forty-eight hours after the initiation of the cocultures, the hybrids were identified and tracked every 5 min for 3 h with an inverted fluorescence microscope. The MSCs were highly motile and showed directed travel patterns. The T47Ds were 3 times less motile and showed little directional travel. The hybrids acquired the enhanced motility of the MSC parent, but were nondirectional, similar to their T47D breast cancer cell parent (**Fig. 2*A, B***). The same trend in motility and directionality was observed in hybrids between MCF7s and MSCs (Fig. 2*C, D*).

The mechanism of fusion of breast cancer cells involves apoptosis

Given its potential relevance in breast cancer metastasis, it is critical to understand the mechanisms governing cell fusion. In the experiments described above, we noted that hypoxia had little impact on the ability of MDA-MB-231 metastatic cancer cells to fuse spontaneously with MSCs. This observation suggests that hypoxia promotes fusion of nonmetastatic cancer cells with MSCs and therefore enables metastasis, an event that is not necessary or advantageous for the already metastatic MDA-MB-231 cells. A recent study identified signaling *via* the phosphatidylserine (PtdSer) receptor brain-specific angiogenesis inhibitor (BAI)-1 as a cue to promote fusion of healthy myoblasts, involving the ELMO/Dock180/Rac1 pathway (18). Blocking apoptosis potently impaired myoblast fusion, and adding back apoptotic myoblasts restored fusion. The added apoptotic myoblasts exposed PtdSer, but did not attach to the tissue culture plate or fuse on their own. They were necessary to promote myoblast fusion, requiring PtdSer-dependent cell-cell contact between apoptotic and viable myoblasts (18). Because apoptosis is increased in certain cell types in hypoxic conditions [reviewed in (30)], we tested whether the same would be true of the breast epithelial cells used in this study. In fact, apoptosis was enhanced by hypoxia (2% O_2, 3 h) only in the T47D and MCF7 nonmetastatic cancer cells (**Fig. 3**). Because we found that hypoxia considerably increases fusion between MSCs and the T47D and MCF7 breast tumor cells but not between MSCs and MDA-MB-231s or MCF10as (Fig. 1*C* and Supplemental Fig. S3*B*), we investigated first whether apoptosis is involved in the fusion of cancer cells. Cocultures between MSCs and T47Ds were initiated as described in Materials and Methods. Sixteen hours after initiation of cocultures, they were supplemented with T47D apoptotic cells, and fusion products were recorded. We found that addition of apoptotic cells to cocultures significantly increased cell fusion between MSCs and T47Ds in normoxic and hypoxic conditions ($P < 0.05$; **Fig. 4*A***). To continue the investigation of the role of apoptosis in cancer cell fusion, we assessed whether inhibition of apoptosis impairs cell fusion. Apoptosis was inhibited in the fusion partners before coculture initiation with the pan-caspase inhibitor Z-VAD-FMK (1 μM). The inhibitor remained in the coculture medium during the entire coculture experiments and did not seem to affect the morphology or viability of the cells. We analyzed the ability of MSCs and T47D to fuse in normoxic and hypoxic conditions. We found that inhibition of apoptosis reduces cell fusion in normoxic conditions, and this reduction was significant in hypoxic conditions ($P < 0.05$), whereas addition of apoptotic cells rescued the fusion diminished by the pan-caspase inhibitor (Fig. 4*A*). Apoptosis appeared also to regulate the fusion of nonmetastatic MCF7s and MSCs (Fig. 4*B*), suggesting that the mechanism by which cancer cells fuse with MSCs involves apoptosis.

DISCUSSION

Our findings describe a novel mechanism by which cancer cells fuse with MSCs, an event that could underlie the development of metastases. The breast cancer cells fused spontaneously with the MSCs. This fusion was significantly enhanced by hypoxia, especially between the nonmetastatic breast cancer cells and MSCs, and was considerably increased by apoptosis. In addition, the hybrids formed showed a significantly higher migratory capability, lending support to the prediction that fusion contributes to cancer metastasis.

The possibility that cell fusion gives rise to the metastatic phenotype was first put forward nearly a century ago by Aichel (1) and later by Mekler (31) and Goldenberg (32). In recent years, this hypothesis has gained further support. Spontaneous fusion has been reported between normal breast epithelium and breast cancer cells (33–36), among breast tumor cells themselves (37–39), between breast cancer epithelium and endothelial cells (40), and between breast cancer epithelium and tumor stromal cells (12, 13). *In vitro* studies of hybrids formed between normal breast epithelium (M13SV1-EGFP-Neo) and breast cancer cells (HS578T-Hyg) showed increased locomotory activity, compared with the normal parental lines. This fusion-enhanced migration was associated with altered chemokine ligand (CCL)21/chemokine receptor (CCR)-7 signaling, which has been linked to the metastatic spread of breast cancer to lymph nodes (34). Increased metastatic potential of hybrids was also observed *in vivo* when breast cancer cell variants (MDA-MB-231) with tropism for either lung or bone injected in nude mice gave rise to hybrids capable of metastases to both organs (37). Drug susceptibility was also found to be altered in hybrids. Hybrids formed between parental breast cancer cells (MCF-7), with and without resistance to doxorubicin, were heterogeneous, with some exhibiting resistance and others not (39). Likewise, hybrids derived from breast epithelial cells (M13SV1-EGFP-Neo) and breast cancer cells (MDA-MB-435-Hyg) showed altered sensitivity to the PI3K inhibitor, Ly294002, as a consequence of differential RAF-AKT crosstalk among hybrids (36). Close probing of the heterogeneity generated by the formation of stromal

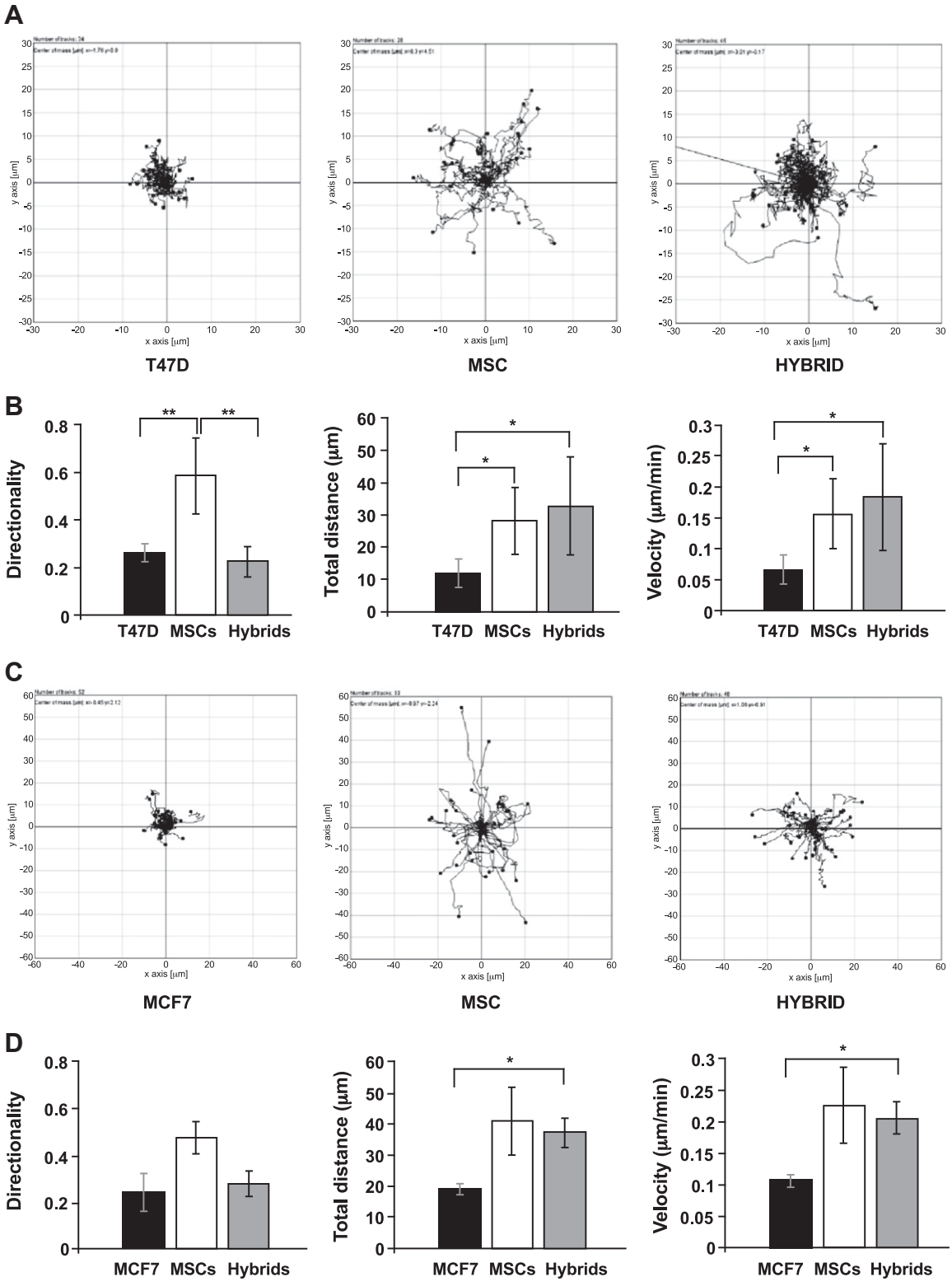

Figure 2. Hybrid cells from MSC-T47D or MSC-MCF7 cocultures show higher migratory capability. Migration of hybrid cells compared with that of MSCs alone or T47Ds alone (time-lapse microscopy). *A*) Representation of motility pattern of T47Ds, MSCs, and hybrids. *B*) Directionality of motion of cells. Total accumulated distance, and velocity. Each value (mean ± SD) represents the average of 4 independent experiments with the analysis of at least 40 cells per group and per experiment. ANOVA with Tukey's HSD *post hoc* test. $*P < 0.05$, $**P < 0.01$. *C*) Representation of the motility pattern of MCF7s, MSCs, and hybrids. Cells of cocultures of MCF7s and MSCs were identified and tracked by time-lapse microscopy. *D*) Hybrids between MCF7s and MSCs showed increased migratory capability. Migration of hybrid cells compared to that of MSCs alone or MCF7s alone (time-lapse microscopy) including directionality of motion of cells, total accumulated distance, and velocity. Each value (mean ± SD) represents the average of 3 independent experiments with analysis of at least 40 cells per group and per experiment. ANOVA with Tukey's HSD *post hoc* test. $*P < 0.05$.

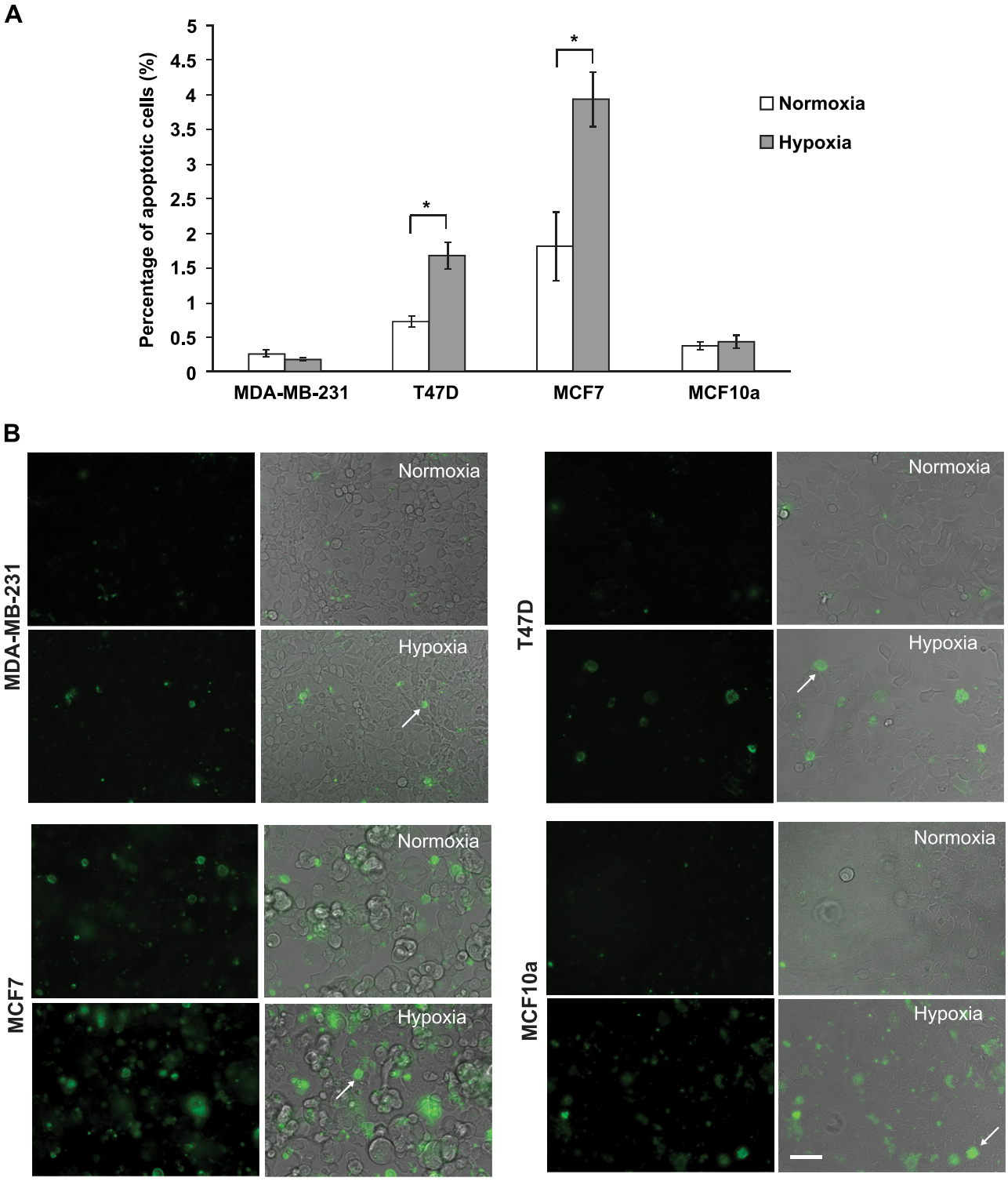

Figure 3. Hypoxia-induced apoptosis of epithelial cells. Epithelial cells were exposed to low oxygen for 3 h and subsequently stained with annexin V staining. Apoptotic cells were detected by fluorescence microscopy. *A*) Percentage of breast epithelial cells undergoing apoptosis after exposure to normoxic and hypoxic conditions. Student's *t* test. $*P < 0.05$. *B*) Representative micrographs of hypoxia-induced apoptotic cells as detected by fluorescence microscopy for MDA-BM-231, T47D, MCF7, and MCF10a cells. Arrows: apoptotic cells. Mean ± SD. Scale bar, 50 μm.

cell-breast cancer cell hybrids indicated that hybrids can undergo DNA ploidy reduction and morphologic switching from mesenchymal-like to breast carcinoma–like. In addition, analysis of coding single-nucleotide polymorphisms by RNA sequencing showed genetic contributions from both fusion partners in the primary tumor and metastases (41).

Spontaneous fusion was also reported with other cancer cell types. A study showed that mesenchymal stem cells

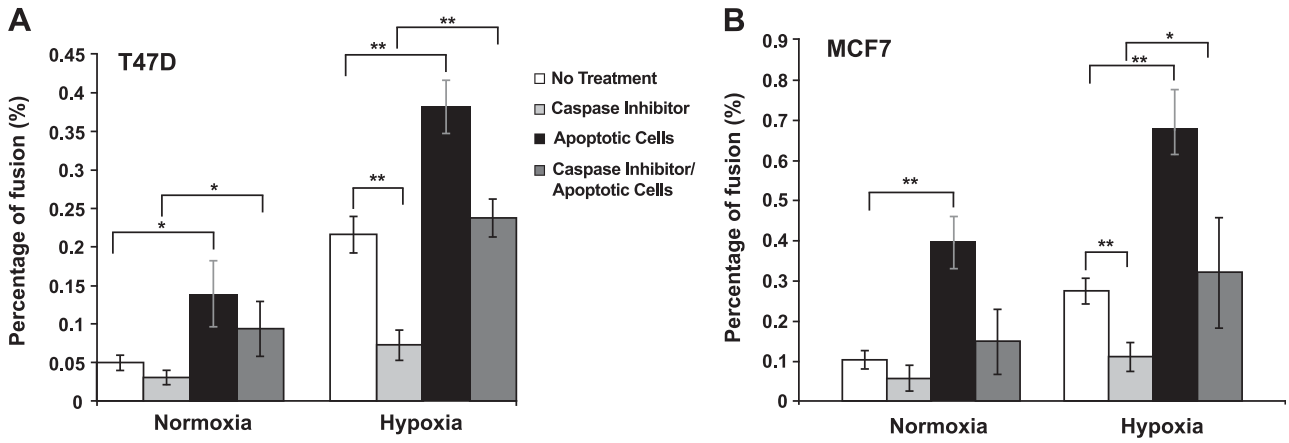

Figure 4. Apoptosis enhances fusion between MSCs and T47Ds or MSCs and MCF7s. *A*) Fusion between MSCs and T47Ds was impaired by treatment with the pan-caspase inhibitor Z-VAD-FMK. However, addition of apoptotic cells enhanced fusion between the 2 cell types and even rescued the fusion inhibited by the caspase inhibitor. *B*) Fusion between MSCs and MCF7s was impaired by the pan-caspase inhibitor z-VAD-FMK, as well. However, addition of apoptotic cells enhanced fusion between the 2 cell types and also rescued the fusion inhibited by the caspase inhibitor (mean ± SD). ANOVA with Tukey's HSD *post hoc* test. $*P < 0.05$; $**P < 0.01$.

could spontaneously fuse with lung cancer cells *in vitro*. Fusion was also detected *in vivo* when freshly mixed lung cancer cells and MSCs were xenografted by subcutaneous injection into nonobese diabetic (NOD)/SCID immunodeficient mice (42). The hybrids formed acquired increased motility and invasiveness and were found to contribute to highly malignant subpopulations with both epithelial-to-mesenchymal and stem cell–like properties (42). In addition, cells of a melanoma clone (wild-type for tyrosinase, *C/C*) implanted into BALB/c nu/nu mice (homozygous mutation for albino tyrosinase, *c/c*) developed massive pulmonary metastases a few weeks later. Analysis of chromosomes of cells from the metastatic tumors showed that most clones had acquired the *c* allele (same as that of the BALB/c recipient) while maintaining the *C* allele. Thus, lung metastases primarily comprised host-tumor hybrids and it is interesting that these hybrids expressed the same traits of enhanced motility and MSH/BMX responsiveness as *in vitro*–derived melanoma-macrophage hybrids (4).

Clinical studies have confirmed the presence of cell fusion in tumors as well, as found in patients who had undergone hematopoietic stem cell transplantation, with subsequent development of tumors showing evidence of donor genes in their cells (22, 43). In addition, a new report used STR polymorphism and forensic genetic techniques to suggest that a metastatic melanoma lesion in a patient arose from the fusion between a bone marrow–derived cell the patient received as a transplant and a tumor cell (44).

Our findings are in line with those in previous studies supporting the notion that cell fusion contributes to the ability of cancer cells to disseminate and perhaps metastasize. Moreover, a previous study showed that the motility-associated integrin subunits α2, α3, α5, α6, αv, β1, and β3, which are involved in the migration of leukocytes and cancer cells, were significantly upregulated in metastatic macrophage-melanoma hybrids compared with parental melanoma cells (45). A more recent study also indicated that fusion between cancer cells (ovarian and lung) and hematopoietic cells of the myeloid lineage gave rise to hybrids expressing significantly higher levels of the promigratory marker CXC chemokine receptor (CXCR)-4, which was conferred by the parental myeloid cells (46). Collectively, the increase in motility-associated integrin subunits and CXCR4 levels in hybrids may equip them with superior migratory potential and help their dissemination to various secondary organs, therefore explaining how fusion could provide a means by which adherent cancer cells acquire new qualities necessary to form metastases (*i.e.,* enhanced motility and matrix degradation) under conditions conducive to hematopoietic survival and later resume tumorlike activities (*i.e.,* rapid proliferation) under conditions conducive to epithelial survival. Cancer cell fusion may also provide a nonmutational mechanism that could contribute to aberrant gene expression patterns and give rise to highly malignant subpopulations with properties of cancer stem cells.

In contrast, a study showed that fusion between hematopoietic stem cells and human esophageal cancer cells slowed the growth speed of esophageal cancer cells and decreased the clone formation ability and tumorigenicity in NOD/SCID mice (47). So far, only the tumorigenicity of some hybrids that were formed *in vitro* between normal epithelial cells or fibroblasts and tumorigenic cancer cells has been shown to be generally suppressed compared to the parental cancer cells (48–50), probably because of activation of some tumor-suppressor genes, although some exceptions to this observation have been shown (20). The hybrids obtained in this way may deserve in depth analyses to understand the involvement of fusion in their tumor suppressor phenotype.

MSCs are known to be present in both healthy and cancerous breast tissue (51, 52) and have even been observed trafficking directly from bone marrow to sites of primary breast tumors (53). These studies have implicated MSCs in encouraging the migration and metastasis of primary tumors, primarily through MSC secretion of chemotactic cytokines such as CCL5. Our results suggest that cell fusion may be an additional mechanism by which MSCs encourage breast cancer metastasis.

Although cell fusion occurs naturally in many fundamental biologic processes [homotypic fusion of myoblasts,

placental trophoblasts (21), and macrophages (21, 54) and heterotypic gamete fusion (21)] and some pathologic conditions [viral-mediated fusion (55)], the mechanisms by which this phenomenon occurs are poorly understood, especially in the context of cancer. Studies have shown that cancer cell fusion can be induced by viruses (2, 56) or cell-cell invasion mechanisms of cellocytosis (cell-cell internalization) (54, 57) or entosis (a nonapoptosis cell death mechanism) (58). A recent work, however, identified apoptotic cells as a new type of cue that induces signaling *via* the PtdSer receptor BAI1 to promote fusion of healthy myoblasts (18). The 7-transmembrane protein BAI1 (a member of the adhesion type GPCR family) mediates recognition of PtdSer on apoptotic cells and signals through the ELMO/Dock180/Rac1 pathway to enhance mammalian myoblast fusion. In that study, blocking apoptosis potently impaired myoblast fusion, and adding back apoptotic myoblasts restored it (18). Our findings are consistent with those in that study, as addition of apoptotic cells significantly increased cell fusion between MSCs and T47D breast cancer cells. We also observed reduction of cell fusion when apoptosis was inhibited, but this reduction was rescued by supplementation of Z-VAD-FMK-treated cocultures with apoptotic cells. It has been shown that myoblasts and macrophages use some of the same molecular components in fusion, including the Dock180 protein (59), suggesting that PtdSer-BAI1 signaling is used by other cell types for fusion in addition to myoblasts. Moreover, BAI1 is well expressed in other cell types, including breast cancer cells and bone marrow–derived cells. These findings, together with our data, indicate that the mechanism by which cancer cells fuse with MSCs can involve apoptosis and may operate *via* PtdSer-BAI1 signaling.

We therefore propose that hypoxia-induced apoptosis in primary tumors stimulates cell fusion between MSCs and tumor cells, resulting in hybrids that exhibit both parental properties (mobility and proliferation) that enable their dissemination and new tumor growth at distant sites or metastases. *In vivo* studies addressing whether metastasis can in fact be initiated by fusion of cancer cells will complement this study. Investigating the involvement of BAI1 in cancer cell fusion will help delineate the molecular mechanisms of fusion of cancer cells and potentially the mechanisms of metastasis development. In the long run, this study may provide new strategies for developing alternate drugs for cancer treatment and preventing metastatic spread. FJ

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REFERENCES

1. Aichel, O. (1911) Über Zellverschmelzung mit qualitativ abnormer Chromosomenverteilung als Ursache der Geschwulstbildung *[*About *c*ell merging with high abnormal chromosome distribution as a cause of tumor formation*]*, Engelmann, Leipzig, Germany
2. Duelli, D. M., Padilla-Nash, H. M., Berman, D., Murphy, K. M., Ried, T., and Lazebnik, Y. (2007) A virus causes cancer by inducing massive chromosomal instability through cell fusion. *Curr. Biol.* **17**, 431–437
3. Pawelek, J. M., and Chakraborty, A. K. (2008) The cancer cell: leukocyte fusion theory of metastasis. *Adv. Cancer Res.* **101**, 397–444
4. Chakraborty, A. K., Sodi, S., Rachkovsky, M., Kolesnikova, N., Platt, J. T., Bolognia, J. L., and Pawelek, J. M. (2000) A spontaneous murine melanoma lung metastasis comprised of host x tumor hybrids. *Cancer Res.* **60**, 2512–2519
5. Oren-Suissa, M., and Podbilewicz, B. (2007) Cell fusion during development. *Trends Cell Biol.* **17**, 537–546
6. Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M., and Mekada, E. (2000) Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321–324
7. Kaji, K., Oda, S., Miyazaki, S., and Kudo, A. (2002) Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion. *Dev. Biol.* **247**, 327–334
8. Jégou, A., Ziyyat, A., Barraud-Lange, V., Perez, E., Wolf, J. P., Pincet, F., and Gourier, C. (2011) CD9 tetraspanin generates fusion competent sites on the egg membrane for mammalian fertilization. *Proc. Natl. Acad. Sci. USA* **108**, 10946–10951
9. Hashizume, H., Baluk, P., Morikawa, S., McLean, J. W., Thurston, G., Roberge, S., Jain, R. K., and McDonald, D. M. (2000) Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol.* **156**, 1363–1380
10. McDonald, D. M., and Foss, A. J. (2000) Endothelial cells of tumor vessels: abnormal but not absent. *Cancer Metastasis Rev.* **19**, 109–120
11. Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K., and McDonald, D. M. (2002) Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* **160**, 985–1000
12. Jezková, J., Nováková, O., Kolár, F., Tvrzická, E., Neckár, J., and Novák, F. (2002) Chronic hypoxia alters fatty acid composition of phospholipids in right and left ventricular myocardium. *Mol. Cell. Biochem.* **232**, 49–56
13. Van Blitterswijk, W. J., Hilkmann, H., and Storme, G. A. (1987) Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* **22**, 820–823
14. Mondal Roy, S., and Sarkar, M. (2011) Membrane fusion induced by small molecules and ions. *J. Lipids* **2011**, 528784
15. Hafez, I. M., Ansell, S., and Cullis, P. R. (2000) Tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipids. *Biophys. J.* **79**, 1438–1446
16. Paltsyn, A. A., Manukhina, E. B., Goryacheva, A. V., Downey, H. F., Dubrovin, I. P., Komissarova, S. V., and Kubatiev, A. A. (2014) Intermittent hypoxia stimulates formation of binuclear neurons in brain cortex: a role of cell fusion in neuroprotection? *Exp. Biol. Med. (Maywood)* **239**, 595–600
17. Lazova, R., Chakraborty, A., and Pawelek, J. M. (2011) Leukocyte-cancer cell fusion: initiator of the Warburg effect in malignancy? *Adv. Exp. Med. Biol.* **714**, 151–172
18. Hochreiter-Hufford, A. E., Lee, C. S., Kinchen, J. M., Sokolowski, J. D., Arandjelovic, S., Call, J. A., Klibanov, A. L., Yan, Z., Mandell, J. W., and Ravichandran, K. S. (2013) Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. *Nature* **497**, 263–267
19. Trivedi, P., and Hematti, P. (2007) Simultaneous generation of CD34 + primitive hematopoietic cells and CD73+ mesenchymal stem cells from human embryonic stem cells cocultured with murine OP9 stromal cells. *Exp. Hematol.* **35**, 146–154
20. Defendi, V., Ephrussi, B., Koprowski, H., and Yoshida, M. C. (1967) Properties of hybrids between polyoma-transformed and normal mouse cells. *Proc. Natl. Acad. Sci. USA* **57**, 299–305

21. Aguilar, P. S., Baylies, M. K., Fleissner, A., Helming, L., Inoue, N., Podbilewicz, B., Wang, H., and Wong, M. (2013) Genetic basis of cell-cell fusion mechanisms. *Trends Genet.* **29**, 427–437
22. Yilmaz, Y., Lazova, R., Qumsiyeh, M., Cooper, D., and Pawelek, J. (2005) Donor Y chromosome in renal carcinoma cells of a female BMT recipient: visualization of putative BMT-tumor hybrids by FISH. *Bone Marrow Transplant.* **35**, 1021–1024
23. Kerppola, T. K. (2006) Visualization of molecular interactions by fluorescence complementation. *Nat. Rev. Mol. Cell Biol.* **7**, 449–456
24. Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J. M., Fike, J. R., Lee, H. O., Pfeffer, K., Lois, C., Morrison, S. J., and Alvarez-Buylla, A. (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* **425**, 968–973
25. Croce, C. M., Shander, M., Martinis, J., Cicurel, L., D'Ancona, G. G., Dolby, T. W., and Koprowski, H. (1979) Chromosomal location of the genes for human immunoglobulin heavy chains. *Proc. Natl. Acad. Sci. USA* **76**, 3416–3419
26. Erikson, J., Martinis, J., and Croce, C. M. (1981) Assignment of the genes for human lambda immunoglobulin chains to chromosome 22. *Nature* **294**, 173–175
27. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682
28. Kemp, K., Wilkins, A., and Scolding, N. (2014) Cell fusion in the brain: two cells forward, one cell back. *Acta Neuropathol.* **128**, 629–638
29. Vaupel, P., and Harrison, L. (2004) Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *Oncologist* **9**(Suppl 5), 4–9
30. Greijer, A. E., and van der Wall, E. (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J. Clin. Pathol.* **57**, 1009–1014
31. Mekler, L. B. (1971) [Hybridization of transformed cells with lymphocytes as 1 of the probable causes of the progression leading to the development of metastatic malignant cells]. *Vestn. Akad. Med. Nauk SSSR* **26**, 80–89
32. Goldenberg, D. M. (1968) [On the progression of malignity: a hypothesis]. *Klin. Wochenschr.* **46**, 898–899
33. Berndt, B., Zänker, K. S., and Dittmar, T. (2013) Cell fusion is a potent inducer of aneuploidy and drug resistance in tumor cell/normal cell hybrids. *Crit. Rev. Oncog.* **18**, 97–113
34. Berndt, B., Haverkampf, S., Reith, G., Keil, S., Niggemann, B., Zänker, K. S., and Dittmar, T. (2013) Fusion of CCL21 non-migratory active breast epithelial and breast cancer cells give rise to CCL21 migratory active tumor hybrid cell lines. *PLoS ONE* **8**, e63711
35. Dittmar, T., Schwitalla, S., Seidel, J., Haverkampf, S., Reith, G., Meyer-Staeckling, S., Brandt, B. H., Niggemann, B., and Zänker, K. S. (2011) Characterization of hybrid cells derived from spontaneous fusion events between breast epithelial cells exhibiting stem-like characteristics and breast cancer cells. *Clin. Exp. Metastasis* **28**, 75–90
36. Ozel, C., Seidel, J., Meyer-Staeckling, S., Brandt, B. H., Niggemann, B., Zänker, K. S., and Dittmar, T. (2012) Hybrid cells derived from breast epithelial cell/breast cancer cell fusion events show a differential RAF-AKT crosstalk. *Cell Commun. Signal.* **10**, 10
37. Lu, X., and Kang, Y. (2009) Efficient acquisition of dual metastasis organotropism to bone and lung through stable spontaneous fusion between MDA-MB-231 variants. *Proc. Natl. Acad. Sci. USA* **106**, 9385–9390
38. Yang, J. J., Milton, M. N., Yu, S., Liao, M., Liu, N., Wu, J. T., Gan, L., Balani, S. K., Lee, F. W., Prakash, S., and Xia, C. Q. (2010) P-glycoprotein and breast cancer resistance protein affect disposition of tandutinib, a tyrosine kinase inhibitor. *Drug Metab. Lett.* **4**, 201–212
39. Yang, J. Y., Ha, S. A., Yang, Y. S., and Kim, J. W. (2010) p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance. *BMC Cancer* **10**, 388
40. Mortensen, K., Lichtenberg, J., Thomsen, P. D., and Larsson, L. I. (2004) Spontaneous fusion between cancer cells and endothelial cells. *Cell. Mol. Life Sci.* **61**, 2125–2131
41. Rappa, G., Mercapide, J., and Lorico, A. (2012) Spontaneous formation of tumorigenic hybrids between breast cancer and multipotent stromal cells is a source of tumor heterogeneity. *Am. J. Pathol.* **180**, 2504–2515
42. Xu, M. H., Gao, X., Luo, D., Zhou, X. D., Xiong, W., and Liu, G. X. (2014) EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. *PLoS ONE* **9**, e87893
43. Chakraborty, A., Lazova, R., Davies, S., Bäckvall, H., Ponten, F., Brash, D., and Pawelek, J. (2004) Donor DNA in a renal cell carcinoma metastasis from a bone marrow transplant recipient. *Bone Marrow Transplant.* **34**, 183–186
44. Lazova, R., Laberge, G. S., Duvall, E., Spoelstra, N., Klump, V., Sznol, M., Cooper, D., Spritz, R. A., Chang, J. T., and Pawelek, J. M. (2013) A melanoma brain metastasis with a donor-patient hybrid genome following bone marrow transplantation: first evidence for fusion in human cancer. *PLoS ONE* **8**, e66731
45. Chakraborty, A. K., Pawelek, J., Ikeda, Y., Miyoshi, E., Kolesnikova, N., Funasaka, Y., Ichihashi, M., and Taniguchi, N. (2001) Fusion hybrids with macrophage and melanoma cells up-regulate N-acetylglucosaminyltransferase V, beta1-6 branching, and metastasis. *Cell Growth Differ.* **12**, 623–630
46. Ramakrishnan, M., Mathur, S. R., and Mukhopadhyay, A. (2013) Fusion-derived epithelial cancer cells express hematopoietic markers and contribute to stem cell and migratory phenotype in ovarian carcinoma. *Cancer Res.* **73**, 5360–5370
47. Fan, H., and Lu, S. (2014) Fusion of human bone hemopoietic stem cell with esophageal carcinoma cells didn't generate esophageal cancer stem cell. *Neoplasma* **61**, 540–545
48. Stanbridge, E. J. (1976) Suppression of malignancy in human cells. *Nature* **260**, 17–20
49. Harris, H. (1988) The analysis of malignancy by cell fusion: the position in 1988. *Cancer Res.* **48**, 3302–3306
50. Weinberg, R. A. (1991) Tumor suppressor genes. *Science* **254**, 1138–1146
51. Zhao, M., Sachs, P. C., Wang, X., Dumur, C. I., Idowu, M. O., Robila, V., Francis, M. P., Ware, J., Beckman, M., Rizki, A., Holt, S. E., and Elmore, L. W. (2012) Mesenchymal stem cells in mammary adipose tissue stimulate progression of breast cancer resembling the basal-type. *Cancer Biol. Ther.* **13**, 782–792
52. Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., Richardson, A. L., Polyak, K., Tubo, R., and Weinberg, R. A. (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**, 557–563
53. Liu, S., Ginestier, C., Ou, S. J., Clouthier, S. G., Patel, S. H., Monville, F., Korkaya, H., Heath, A., Dutcher, J., Kleer, C. G., Jung, Y., Dontu, G., Taichman, R., and Wicha, M. S. (2011) Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res.* **71**, 614–624
54. Vignery, A. (2005) Macrophage fusion: are somatic and cancer cells possible partners? *Trends Cell Biol.* **15**, 188–193
55. Podbilewicz, B. (2014) Virus and cell fusion mechanisms. *Annu. Rev. Cell Dev. Biol.* **30**, 111–139
56. Pawelek, J. M., and Chakraborty, A. K. (2008) Fusion of tumour cells with bone marrow-derived cells: a unifying explanation for metastasis. *Nat. Rev. Cancer* **8**, 377–386
57. Vignery, A. (2005) Macrophage fusion: the making of osteoclasts and giant cells. *J. Exp. Med.* **202**, 337–340
58. Overholtzer, M., Mailleux, A. A., Mouneimne, G., Normand, G., Schnitt, S. J., King, R. W., Cibas, E. S., and Brugge, J. S. (2007) A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. *Cell* **131**, 966–979
59. Pajcini, K. V., Pomerantz, J. H., Alkan, O., Doyonnas, R., and Blau, H. M. (2008) Myoblasts and macrophages share molecular components that contribute to cell-cell fusion. *J. Cell Biol.* **180**, 1005–1019

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Research Paper

An *In Vitro* Inverted Vertical Invasion Assay to Avoid Manipulation of Rare or Sensitive Cell Types

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Abstract

The ability to quantify cell migration and invasion is critical in the study of cancer metastasis. Current invasion assays, such as the Boyden Chamber, present difficulties in the measurement of the invasion of cells that are few in number and are intrinsically tied to the cell microenvironment. There exists a need for a three-dimensional invasion assay that is easily reproduced, accessible for most laboratories, and requires no displacement of cells from their original microenvironment. Here we present a simple design for an inverted vertical invasion assay able to assess the invasion capabilities of cells in a three dimensional, extracellular matrix-based environment without displacement from the original culture location. We used the assay to determine the migratory capacity of hybrids between mesenchymal/multipotent stem/stroma cells (MSCs) and breast cancer cells MCF7. These hybrids are formed reliably but rarely (1 in 1,000 cells) and for this reason require an invasion assay that does not involve extensive cell manipulation. Using this assay, we found that MSCs, breast cancer cells, and corresponding fusion products are able to migrate and invade through the extracellular matrix and that hybrids invade in a manner more similar to stromal cells than cancer cells. Thus, this assay can aid the study of the invasive capacity of both cancerous cells and associated fusion hybrids and could augment testing of therapeutic strategies to inhibit metastatic spread.

Key words: invasion, hybrids, metastasis, 3D assay, collagen.

Introduction

Approximately 90% of cancer-related deaths are caused by local invasion and distant metastasis of tumor cells. To successfully relocate in the body cancer cells must invade local tissues by degrading extracellular matrix (ECM) components and ultimately, transverse the basement membrane. Once in circulation, these cells can form metastatic colonies at secondary locations. Thus, increased invasion and migration capabilities of cancer cells often indicate increased metastatic potential [1]. Different assays have been designed to quantify the metastatic capability of tumor cells. These assays include wound healing or scratch assay, time-lapse microscopy, and the transwell or Boyden Chamber assay. In the body, cells exist in a 3D ECM environment rich in type I collagen. The frequently used wound healing assay and time lapse microscopy are two dimensional systems and do not closely mimic the *in vivo* environment especially with respect to cell adhesion and associated cell motility [2-7]. The more physiologically relevant transwell or Boyden Chamber assay, which is a 3D system, requires the cells to be removed from their original environment and seeded on a layer of ECM in the upper chamber of the transwell. The cells then invade through the gel into a lower chamber containing a chemoattractant [6]. This technique, although valuable, appears challenging and unsuitable for cells susceptible to the

microenvironment and/or significantly limited in number. As one example cell hybrids, formed as result of fusion between cancer cells and cells of the tumor microenvironment, are rare and are significantly influenced by the local microenvironment.

In previous studies including ours, hybrids arising from fusion between cancer cells and cells of the tumor microenvironment (mesenchymal stem/stroma cells, macrophages) have been proposed to contribute to tumor metastasis [8-18]. In particular, hybrids might acquire the migratory capability of the stroma cell parent and the proliferative property of the cancer cell parent leading to dissemination and new tumor growth at a distant site. However, traditional cell-based assays are not suitable to quantify the migration and invasion capability of hybrids since hybrids are vulnerable to the microenvironment in culture and the pool of hybrid cells is very small occurring at a frequency of 1 in 1000 cells [18] or less. These features of hybrids have hindered the study of their role in the development of metastases. Therefore, designing of a customized assay to quantify migration and invasion capability of hybrids is imperative. This assay should function at a per cell scale and should limit disruption to the cell microenvironment. In order to fulfill these criteria, we have developed an inverted vertical invasion assay. Using this newly developed assay, we efficiently analyzed the migration and invasion capacity of fusion products and parental lines. This assay could be used in different laboratories to study other complex cell types or to screen for pharmacological agents affecting cell migration and invasion.

Materials and Methods

Cell lines and culture

To optimize and validate our inverted vertical invasion assay design, we used MSCs and the breast cancer cells MDA-MB-231 and MCF7.

MSCs were a generous gift from Dr. Peiman Hematti (University of Wisconsin, Madison, WI, USA). They were derived from human embryonic stem cells in accordance with guidelines of the University of Wisconsin Institutional Review Board (Trivedi and Hematti, 2007) and maintained in α-minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). We reconfirmed the identity of the MSCs in our lab by flow cytometry for specific MSC markers, CD73, CD90 and CD105.

The human breast cancer cells MDA-MB-231 and MCF7 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in accordance with the recommendations of ATCC; the cells were not passaged for more than 6 months from the time of receipt. MDA-MB-231 cells were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR and COI assay, 3) sterility by BacT/ALERT 3D, and 4) the human pathogens. MCF7s were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR, 3) sterility by BacT/ALERT 3D, and 4) the human pathogens.

Transfection and Coculture Protocol. We used the BiFC approach to identify fusion products [18]. In brief, MSCs were transfected with the BiFC halve construct VNH3.1 and the breast cancer cells MCF7 were transfected with the corresponding BiFC halve construct YCH3.1. Transfected MSCs and MCF7 cells were cocultured in a MatTek dish at the ratio of 35,000 MSCs for 75,000 MCF7 cells. At this cell density, the surface area coverage for each cell type was approximately 50% at 24 h after the cocultures were initiated. The cocultures were initiated and maintained in α-MEM (Sigma-Aldrich, St. Louis, MO, USA) media supplemented with 10% heat inactivated defined FBS (Hyclone, Logan, UT, USA), 1% NEAA (Sigma-Aldrich, St. Louis, MO, USA), and 1% L-Glutamine (Sigma-Aldrich, St. Louis, MO, USA). At the end of the experiment, the cells were fixed, stained with DAPI and the fusion products were identified by fluorescence microscopy as previously described [18]. Figure 1C displays the timeline followed for this protocol.

Preparation of culture plates. The inverted vertical invasion assay was performed in MatTek 35 mm plates with 10 mm glass-bottom wells (MatTek P35G-1.5-10-C). Before initiating the cocultures, each plate was washed with 1 M hydrochloric acid to lower the hydrophobicity of the glass-bottom and to allow the collagen gel to adhere evenly to the surface. The plates were subsequently washed twice with phosphate buffered saline solution to remove any trace of acid, twice with 70% ethanol, and once with cell culture medium (Dulbecco's modified eagle's medium or α minimum essential medium).

Preparation of collagen gel. Collagen used for the inverted vertical invasion assay was prepared by combining on ice, rat tail collagen I (Corning, Corning, NY, USA), 10x DMEM (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1x cell culture medium containing heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific Inc, Waltham, MA, USA) and the appropriate chemoattractant to a final concentration of collagen of 2.4 mg/ml. Sodium bicarbonate solution, 4.5%, was used to neutralize the collagen gel for its proper

polymerization. Three chemoattractants were tested; FBS, platelet-derived growth factor BB (PDGF-BB), and insulin-like growth factor 1 (IGF-1). Eighty microliters of the prepared solution was laid over the cells within the well of MatTek dishes. The gel was allowed to polymerize at 37°C in a CO_2 incubator with humidifier for thirty minutes, and then 2 mL of cell culture medium was added to the plate to cover the gel. The added medium used for experiments involving the chemoattractant IGF-1 or PDGF-BB contained 2% FBS, whereas it contained 10% FBS for experiments using FBS as the chemoattractant.

Imaging and assay analysis. The ability of cells to invade vertically was investigated at various time points during our study. At the end of each experiment, the cells were fixed with 4% paraformaldehyde for fifteen minutes and subsequently stained with a DAPI solution (100 ng/ml) at room temperature for twenty minutes. The fixed cells were imaged using a confocal fluorescent microscope (Olympus IX81; Olympus America, Center Valley, PA, USA). The total cell populations were imaged in the DAPI channel, while the GFP^+ fusion products were imaged in the FITC channel. A fluorescence signal was considered positive if it was localized to the nucleus, fluoresced above background fluorescence of unaltered cocultures and did not fluoresce with excitation/emission at alternate wavelengths (Rhodamine, Cy5) [18]. Each plate was imaged in eight unique locations, taking 400 µm z-stack images of each location. Images were then processed using ImageJ software. The 3D image depicted in Figure 2C was captured after staining an assay of MSCs with DAPI and phalloidin. Images were taken using a mode-locked Ti:Sapphire multi-photon laser scanning microscope (MPLSM) (Prarie Technologies, Sioux Falls, SD, USA), with a 40x (NA 0.8) objective. 3D reconstructions were generated using Imaris 7.5.2 software (Bitplane Inc., Zurich, CH).

Results

Optimization of culture substrate for the inverted vertical invasion assay design

The inverted vertical invasion assay was optimized using a variety of cell culture dishes including 24-well plates, glass bottom chamber slides, and MatTek dishes. The 35 mm MatTek dish with a 10 mm glass bottom well in its center was found to be the most effective culture dish for the inverted vertical invasion assay (Fig. 1B). The MatTek dish has been used in a previous collagen based assay, the design of which was used as a basis for the inverted vertical invasion assay proposed in this study [19]. The advantages of this MatTek dish are threefold: 1) the thin glass bottom of the MatTek dish allows effective imaging and accurate assessment of cell position within the gel, 2) the 10 mm well within the MatTek dish restricts the gel to a thickness appropriate for oxygen diffusion and cell survival, and 3) most importantly the cultured cells can be analyzed within their microenvironment during the experiment (Fig 1B) and are not displaced as is the case in the Boyden Chamber assay (Fig 1A). The glass bottom chamber slides and the 24-well plates yielded uneven gel layers as a function of the meniscus formed at the edge of the well or chamber. The thickened gel at the edge of the wells limited gas and

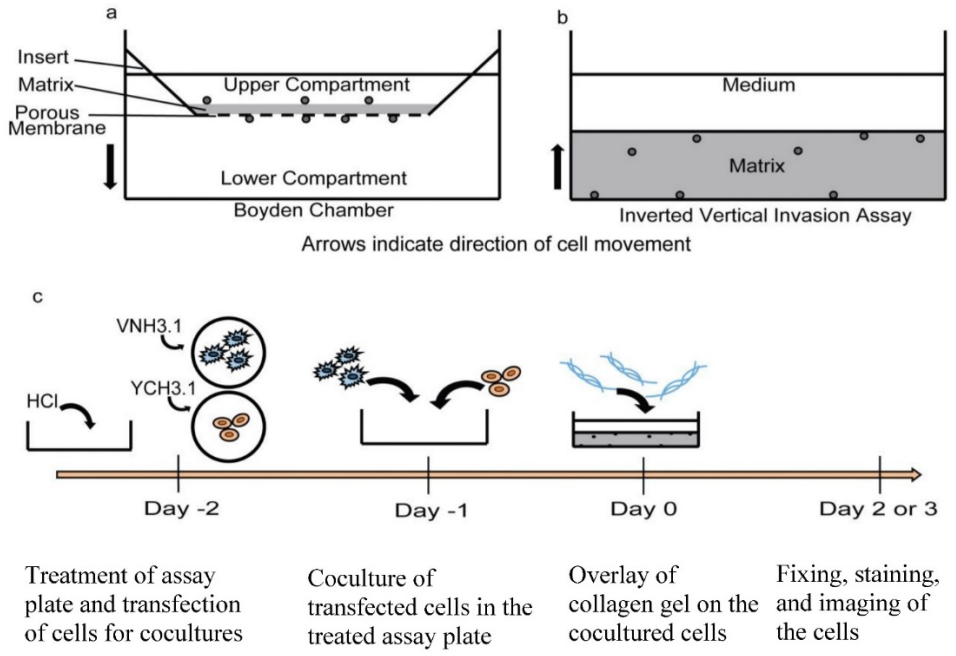
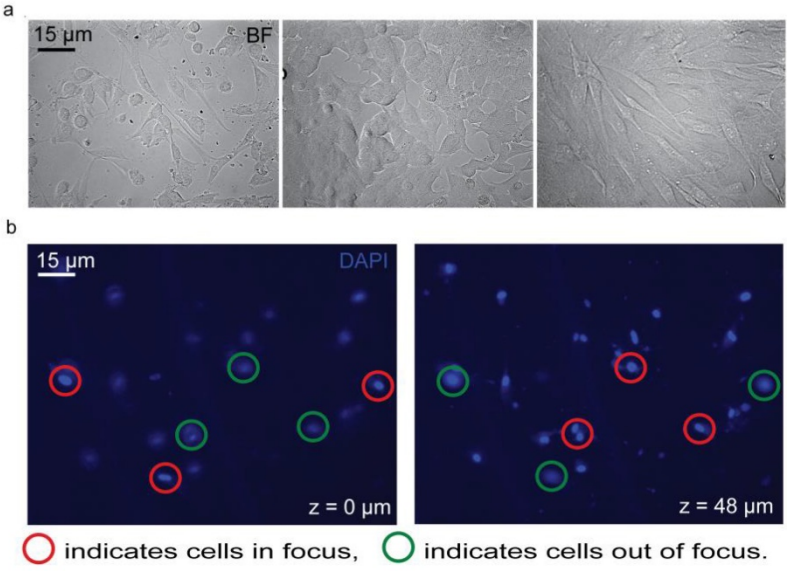


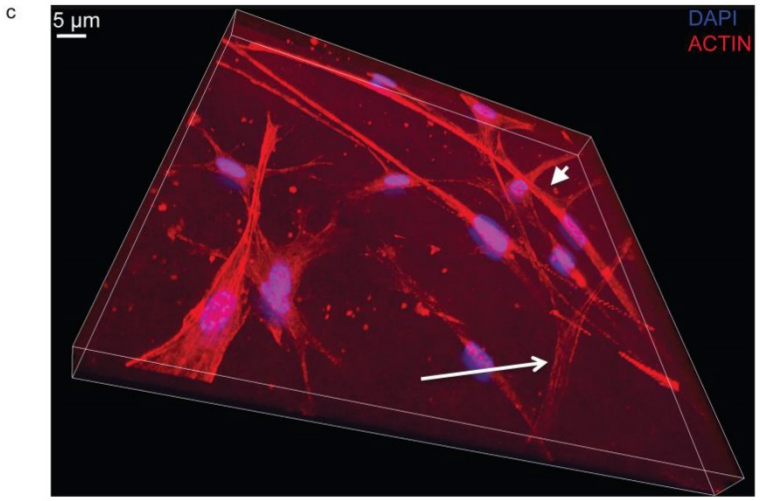
Figure 1: Invasion assay designs. a) Traditional Boyden Chamber or transwell invasion assay. In this assay, cells are collected from their original culture environment and plated onto the transwell insert. The arrow indicates the direction of cell movement during the assay. **b)** Inverted vertical invasion assay currently proposed. In this design, cells remain in their original microenvironment and the matrix protein is laid over the cell layer. The arrow indicates the direction of cell movement during the assay. **c)** Schematic detailing timeline of the inverted vertical invasion assay procedure. Day -2: MatTek dishes are treated with HCl and the two cell lines to be cocultured are transfected with the BiFC constructs as previously described [18]. If fused cells are not the focus of the invasion assay, the BiFC step is eliminated. Day -1: Transfected cells are cocultured on the treated MatTek dish. Day 0: Collagen gel mixture is made and laid over the cells. Day 2 or 3: cells are fixed, stained and subsequently imaged.

nutrient diffusion to the cells causing substantial cell death. In addition, the 24-well plates were not suitable for imaging due to the thickness of the plate and associated long working distance.

Cell viability and imaging potential within the MatTek dish

As a result of the MatTek design, the collagen gel layer of the assay was thin with even distribution. The concentration of collagen was 2.4 mg/mL and thus was thick enough to remain stable within the assay chamber during the course of the assay. The gels were thicker than the 200 µm nutrient diffusion limit in static conditions (350 +/- 75 µm). However, the wall height is substantially shorter than standard well plates and we predicted nutrient and gas exchange might occur readily from beneath and to the media-exposed sides of the gel. To test cell viability, especially in the gel center, we assessed cell morphology and numbers three days following gel polymerization. We found cells were able to survive and proliferate throughout the gel matrix. In particular, MCF7, MSCs, and the resulting cocultures maintained healthy morphologies to at least three days following initial gel formation (Fig 2A). In addition, the inverted design allowed for clear distinction of cells at distinct z planes within the collagen gel (Fig 2B, 2C). In this way, precise quantification of migratory distance into the gel could be assessed. We associate this migratory Z distance with *in vitro* invasive capacity.





Long arrow: Appendage of an MSC invading into the gel

Arrowhead: MSC aligned along the bottom of the gel

Figure 2: Cell viability and Z series of the inverted vertical invasion assay. a) 20x bright field images of MCF7 and MSC coculture, MCF7, and MSC (left to right) showing healthy cells under the collagen gel after two days, indicating that the assay environment maintained adequate conditions for cell survival. **b)** Representation of z series showing one location within a dish from the bottom (Z=0 µm) to the top (Z=48 µm). 3.8% FBS was used as a chemoattractant. Red circles indicate cells in focus. Green circles indicate cells out of focus. **c)** Representation of z series showing one location within a dish of MSCs from the bottom (Z=0 µm) to the Z=20 µm. 3.8% FBS was used as a chemoattractant. The long arrow indicates the appendage of an MSC invading into the gel. The arrowhead indicates an MSC, which is aligned along the bottom of the gel, and not invading. The blue shows the nuclear DAPI stain while the red shows the phalloidin stained actin filaments.

Reproducibility of the inverted vertical invasion assay

The reproducibility of the inverted vertical invasion assay was assessed by measuring the *in vitro* invasive capability of MSCs into the collagen gel in two separate experiments using 3.8% FBS as a chemoattractant. MSCs were previously shown to be highly migratory and invasive [20-23] and are therefore appropriate for testing of the reproducibility of our assay design. Each set of experiments consisted of three cultures of MSCs, fixed at days one, two, and three after collagen gel plating and the cells were imaged at eight different locations in each plate. No invasion was observed at day 1, little invasion was seen at day 2 (0.4 +/- 1.1% of the cells), and at day 3 substantial invasion was observed (2.2 +/- 3.8% of the cells) (Fig 3). However, due to the relatively low fraction invading cells, the variability appears large. To increase the percentage of cells able to

invade the collagen gel, we tested stronger chemoattractants including PDGF- BB and IGF-1.

Optimization of chemoattractants in the inverted vertical invasion assay

In order to optimize the level of observable invasion, the chemoattractants PDGF-BB and IGF-1 were tested at various concentrations within the collagen gel (Fig 4). The initial concentrations of IGF-1 and PDGF-BB were selected based on previously optimized concentrations used to achieve maximum MSC migration [24]. We found that IGF-1 at 18.6 ng/mL could induce up to 43.5% of MSCs to invade into the collagen gel. IGF-1 tested at 9.2, 75, and 100 ng/mL had a less substantial effect on MSC invasion into the collagen (Fig 4A-B). PDGF-BB produced levels of invasion similar to that of FBS and significantly less than that of IGF-1. At 2.8 ng/ml, PDGF-BB induced 1.21% of MSCs to invade into the collagen gel and at 3.1 ng/mL induced 6% MSC invasion (Fig 4A). Therefore, of the chemoattractants and concentrations used in the assay, IGF-1 at 18.6 ng/mL showed the most profound effect on MSC migration and invasion (Fig 4A). In order to confirm the chemoattractant effects of IGF-1 and further optimize the assay, the breast cancer cells MDA-MB-231 were tested in the assay. This breast cancer cell line is highly metastatic and characterized by elevated migratory and invasive ability [25, 26]. We observed that 8.4% of the MDA-MB-231 cells could invade into the gel in two days when IGF-1 was used at a concentration of 18.6 ng/mL (Fig 4B). A higher concentration of IGF-1 (100 ng/ml) did not increase the percentage of invading MDA-MB-231 cells (Fig 4B).

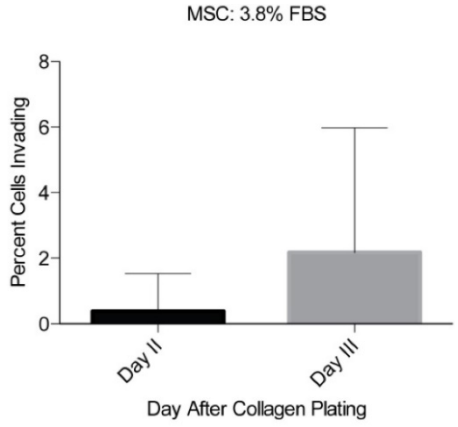


Figure 3: Reproducibility of the inverted vertical invasion assay. The percentage of invading MSCs was assessed in two separate experiments, in eight fields of view on days two and three following the collagen gel placement. While little invasion was seen on days one and two, day three showed substantial levels of invasion throughout the gel and in both experiments.

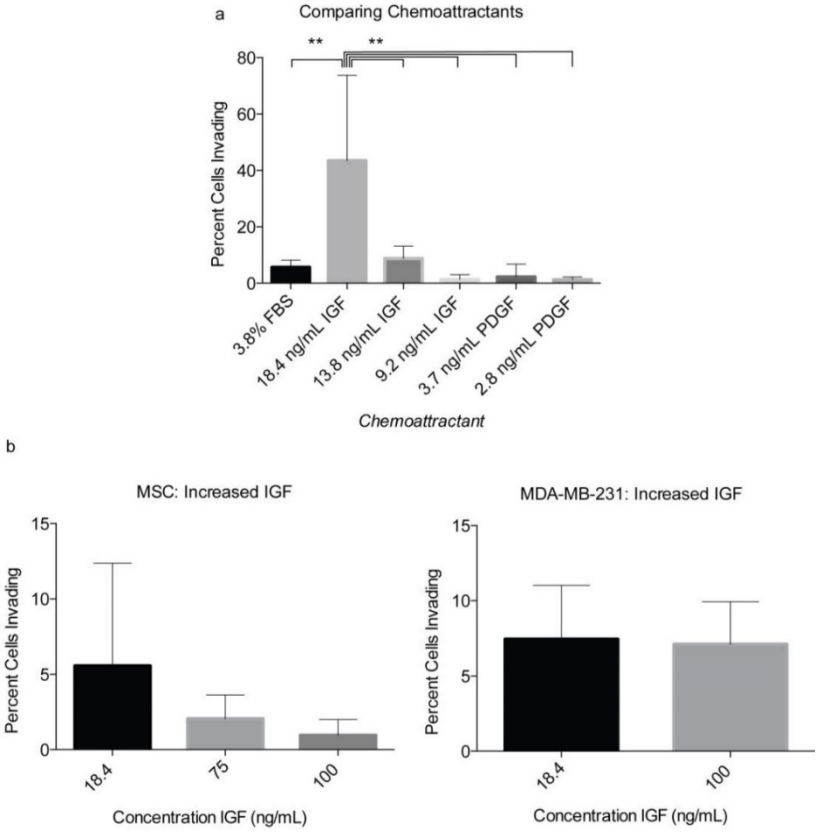


Figure 4: Optimization of chemoattractants using MSCs and MDA-MB-231. a) Various concentrations of PDGF-BB and IGF-1 were tested initially with MSCs. The percentage of invading MSCs was assessed 3 days following the layering of the collagen gel over the cells. **b)** After identifying IGF-1 as the more effective chemoattratant, its concentration was optimized using both MSCs and MDA-MB-231. Each number represents the average of the percentages of invading cells in eight unique locations within a plate. Experiments were performed in triplicate. A two-tailed unpaired ANOVA with Tukey's HSD post hoc test. **P < 0.01.

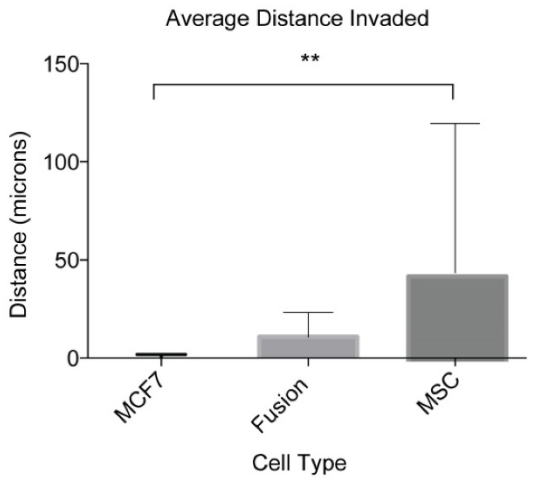


Figure 5: Invasion capability of cancer cell fusion products. We validated our assay by quantifying the invasion capacity of cancer cell fusion products. These numbers represent the average distance covered by four independent fusion products from three separate cocultures and their parental cell lines analyzed from eight different locations within each assay. This experiment was performed in triplicate. ANOVA with Tukey's HSD post hoc test. **P < 0.01.

Validation of the inverted vertical invasion assay using fusion products

The ultimate goal of this study was to develop an assay suitable for quantifying the migratory and invasive capabilities of rare and sensitive cell types including cancer cell fusion products. As a result of the optimizing experiments with MSCs and MDA-MB-231s, IGF-1 at 18.6 ng/mL was selected as the appropriate chemoattractant and the optimum concentration to investigate the *in vitro* invasive capacity of cancer cell fusion products. We looked specifically at hybrid cells arising from spontaneous fusion between MSCs and the nonmetastatic breast cancer cells MCF7. For this assay, MSCs were transfected with the BiFC halve construct VNH3.1 and MCF7 with the BiFC halve counterpart, YCH3.1. Cocultures were initiated and covered with collagen gel as described in materials and methods. Two days following the collagen overlay, the cells were fixed, stained with DAPI, and imaged. Z-stack image analysis of the cells showed that cancer cell fusion products were able to invade into the collagen gel to an average distance of 10.7 +/- 12.4 µm (Fig 5). The MCF7 cells showed an average invasion distance of 0.04 +/- 1.8 µm, and MSCs an average invasion distance of 41.3 +/- 76.0 µm (Fig 5). The difference in the invasion levels between MCF7 and MSCs was shown to be significantly different ($P<0.01$), while the fusion product invasion was shown to be insignificant when compared to both the MCF7 ($P=0.85$) and MSCs ($P=0.21$). These results indicate that our inverted vertical invasion assay not only is efficient and suitable to quantify *in vitro* invasive capacity of cancer cell fusion products, but also suggests that the fusion products have a higher migratory and invasive capability than the parental cancer cells, MCF7, as suggested by our previous study using 2-D time lapse microscopy [18].

Discussion

Although cell fusion has been proposed for many decades as a mechanism of cancer metastasis, relevant cell-based assays to investigate the metastatic potential of hybrids are still limited. Traditional cell-based invasion assays like the transwell or Boyden Chamber assay require excess manipulation of the cells that affect cell recovery and survival. While acceptable for many cell lines, such features of traditional assays make impossible the quantification of invasive qualities of sensitive and low frequency cell types including cancer cell hybrids. Therefore, there is a need to develop a cell-based assay, which avoids manipulation of cells and is suitable for cells, which are sensitive and/or limited in number. In this study, we developed and validated a collagen gel-based, inverted vertical invasion assay as a viable tool that can be used to quantify *in vitro* invasive capacity of cancer cell fusion products and other such scarce and sensitive cell types. The assay was shown to be cell-compatible and enabled easy identification and tracking of rare cell types.

We have shown here that while the inverted assay design results in easy and reliable cell invasion measurements, it does require a stronger chemoattractant than FBS. We observed that IGF-1 at a concentration of 18.6 ng/mL constitutes a sufficiently strong chemoattractant for the use of the assay with MSCs and cancer cells lines [27]. However, the use of other chemoattractants appropriate for the cell type being tested could be used in other studies. In the current experiment, for example, cytokines CSCL12/SDF-1α or CCL21/6Ckine could have been used, as both have shown to promote metastasis of breast cancer cell lines [28]. Furthermore, PDGF-BB may have been a sufficiently strong chemoattractant should we have further optimized the concentration. However, as IGF-1 was already found to promote sufficient invasion of the MSCs and MDA-MB-231s, this was not done. Chemoattractants could also be chosen in order to mimic a specific bodily environment. For example, osteonectin has been shown to promote the invasiveness of bone-metastasizing prostate carcinoma [29]. The inverted vertical invasion assay could be used to develop a model system of such organ-specific cancer metastasis. The need for a strong chemoattractant like IGF-1 in this assay may result from the preference of

the cells for their environment on the glass-bottom of the well [1], and/or from the effect of gravity.

While producible and efficient itself, the major advantage of the inverted vertical assay is the circumvention of issues presented by the Boyden chamber assay and better control of the culture substrate. Cells are not displaced from the original cell culture environment, thus ensuring better control of cells that are intrinsically linked to the microenvironment. This is especially important for the case of cancer/stromal cell co-cultures, which are frequently used in order to mimic the tumor microenvironment. Studies that aim to quantify the invasiveness of the co-cultured cells in a 3D environment typically use a 3D spheroid assay, or displace the cells into Boyden chamber assay [30,31]. It has also been shown that the fate of stem cells is in part determined by previous culture substrate [21]. The inverted vertical invasion assay design that we propose here avoids the complications of a spheroid assay, can maintain the co-culture microenvironment, and better controls the culture substrate, all within a 3D environment.

While the inverted design does offer many benefits, there exist some drawbacks when compared to other 3D invasion assays. First, the MatTek dish design requires a relatively large amount of collagen to produce only a single invasion assay. For our purposes this was sufficient. However, in order to expand the versatility of the inverted assay design, it could be optimized for smaller culture wells, allowing for multiple simultaneous assays in a multi-well plate. Second, we found that there exists a need to balance the collagen gel strength while keeping the gel sufficiently thin for cell survival. This results in a collagen gel layer that is sensitive to large movements; thus, the assay must be handled delicately in order to prevent lifting of the gel. In addition to the small technical difficulties presented by the inverted assay design, it also provides less mimicry of the *in vivo* conditions compared to the dynamic system of microfluidic invasion assays. These assays provide a similar 3D environment as the inverted invasion assay, yet can maintain cells in an environment with flow similar to that of a bodily microenvironment [32]. While the static nature of the inverted invasion assay limits the degree to which it can mimic bodily microenvironments, it offers far greater simplicity and cost-effectiveness when compared to a microfluidic design.

The purpose of this study was to develop and standardize an assay that could be readily used to quantify the invasion capabilities of sensitive and rare cell types such as cancer cell fusion products. The need to develop this assay was highlighted by the lack of 3D systems that enable the quantification of cell invasion without affecting the integrity of the cell microenvironment [33,34]. And, although we used the inverted invasion assay to study the invasive capabilities of stem/cancer cell hybrids, invasion is a biological phenomenon that is not unique to cancer metastasis. Our proposed assay could also assist in the study of embryonic development, wound healing, and/or inflammatory responses, all of which require some form of cell migration/invasion [2]. The assay also could be customized to address many other questions including the effect of ECM engagement on cell invasion, the role of different chemoattractants on cell invasion, as well as the contribution of cancer stem cells or tumor circulating cells in cancer progression and metastasis. This assay could also be a useful tool for screening of pharmacological agents effective in inhibiting metastasis. Investigating these questions would not only increase understanding of cell interaction with the 3D environment, but could help in the design of therapeutic strategies to prevent metastatic spread.

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med. 2006; 231(1): 39-49.
2. Kramer N, Walzl A, Unger C, et al. In vitro cell migration and invasion assays. Mutat Res. 2013; 752(1): 10-24.
3. Birgersdotter A, Sandberg R, Ernberg I. Gene expression perturbation in vitro--a growing case for three-dimensional (3D) culture systems. Semin Cancer Biol. 2005; 15(5): 405-412.

4. Weaver VM, Petersen OW, Wang F, et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cancer Biol. 1997; 137(1): 231-245.
5. Bhadriraju K, Chen CS. Engineering cellular microenvironments to improve cell-based drug testing. Drug Discov Today. 2002; 7(11): 612-620.
6. Valster A, Tran NL, Nakada M, et al. Cell migration and invasion assays. Methods. 2005; 37(2): 208-215.
7. Baharvand H, Hashemi SM, Kazemi Ashtiani S, Farrokhi A. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. Int J Dev Biol. 2006; 50(7): 645-652.
8. Aichel O. Vortrage und Aufsatze uber Entvickelungsmechanik Der Organismen Leipzig. Wilhelm Engelmann. 1911.
9. Pawelek JM, Chakraborty AK. The cancer cell--leukocyte fusion theory of metastasis. Adv Cancer Res. 2008; 101: 397-444.
10. Chakraborty AK, Sodi S, Rachkovsky M, et al. A spontaneous murine melanoma lung metastasis comprised of host x tumor hybrids. Cancer Res. 2000; 60(9): 2512-2519.
11. Yilmaz Y, Lazova R, Qumsiyeh M, Cooper D, Pawelek J. Donor Y chromosome in renal carcinoma cells of a female BMT recipient: visualization of putative BMT-tumor hybrids by FISH. Bone Marrow Transpl. 2005; 35(10): 1021-1024.
12. Mekler LB. Hybridization of transformed cells with lymphocytes as 1 of the probable causes of the progression leading to the development of metastatic malignant cells. Vestn Akad Med Nauk+. 1971; 26(8): 80-89.
13. Goldenberg DM. On the progression of malignity: a hypothesis. Klin Wochenschr. 1968; 46(16): 898-899.
14. Rappa G, Mercapide J, Lorico A. Spontaneous formation of tumorigenic hybrids between breast cancer and multipotent stromal cells is a source of tumor heterogeneity. Am J Pathol. 2012; 180(6): 2504-2515.
15. Xu MH, Gao X, Luo D, et al. EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. PloS One. 2014; 9(2): e87893.
16. Chakraborty A, Lazova R, Davies S, et al. Donor DNA in a renal cell carcinoma metastasis from a bone marrow transplant recipient. Bone Marrow Transplant. 2004; 34(2): 183-186.
17. Lazova R, Laberge GS, Duvall E, et al. A Melanoma Brain Metastasis with a Donor-Patient Hybrid Genome following Bone Marrow Transplantation: First Evidence for Fusion in Human Cancer. PloS One. 2013; 8(6): e66731.
18. Noubissi FK, Harkness T, Alexander CM, Ogle BM. Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis. Faseb J. 2015.
19. Hooper S, Marshall JF, Sahai E. Tumor cell migration in three dimensions. Method Enzymol. 2006; 406: 625-643.
20. Steingen C, Brenig F, Baumgartner L, et al. Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells. J Mol Cell Cardiol. 2008; 44(6): 1072-1084.
21. Yang C, Tibbitt MW, Basta L, Anseth KS. Mechanical memory and dosing influence stem cell fate. Nat Mater. 2014; 13(6): 645-652.
22. Ponte AL, Marais E, Gallay N, et al. The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem Cells. 2007; 25(7): 1737-1745.
23. Ries C, Egea V, Karow M, et al. MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. Blood. 2007; 109(9): 4055-4063.
24. Ozaki Y, Nishimura M, Sekiya K, et al. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells Dev. 2007; 16(1): 119-129.
25. Farina AR, Tacconelli A, Cappabianca L, Gulino A, Mackay AR. Inhibition of human MDA-MB-231 breast cancer cell invasion by matrix metalloproteinase 3 involves degradation of plasminogen. Eur J Biochem. 2002; 269(18): 4476-4483.
26. Munoz R, Man S, Shaked Y, et al. Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. Cancer Res. 2006; 66(7): 3386-3391.
27. Zahorec P, Koller J, Danisovic L, Bohac M. Mesenchymal stem cells for chronic wounds therapy. Cell Tissue Bank. 2015; 16(1): 19-26.
28. Müller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001; 410: 50-56.
29. Jacob K, Webber M, Benayahu D, Kleinman HK. Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. Cancer Res. 1999; 59: 4453-4457.
30. Knuchel S, Anderle P, Werfelli P, Diamantis E, Rüegg C. Fibroblast surface-associated FGF-2 promotes contact-dependent colorectal cancer cell migration and invasion through FGFR-SRC signalling and integrin $\alpha_V\beta_5$-mediated adhesion. Oncotarget. 2015; 6(16): 14300-14317.
31. Chen D, Liu S, Ma H, et al. Paracrine factors from adipose-mesenchymal stem cells enhance metastatic capactiy through Wnt signalling pathway in colon cancer cell co-culture model. Cancer Cell Int. 2015; 15: 42-55.
32. Sakolish CM, Esch MB, Hickman JJ, et al. Modeling barrier tissues in vitro: methods, achievements, and challenges. EBioMedicine. 2016; in press.
33. Li F, Tiede B, Massague J, Kang Y. Beyond tumorigenesis: cancer stem cells in metastasis. Cell Res. 2007; 17(1): 3-14.
34. Li H, Feng Z, Tsang TC, et al. Fusion of HepG2 cells with mesenchymal stem cells increases cancerassociated and malignant properties: an in vivo metastasis model. Oncol Rep. 2014; 32(2): 539-547.

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Review

Cancer Cell Fusion: Mechanisms Slowly Unravel

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Abstract: Although molecular mechanisms and signaling pathways driving invasion and metastasis have been studied for many years, the origin of the population of metastatic cells within the primary tumor is still not well understood. About a century ago, Aichel proposed that cancer cell fusion was a mechanism of cancer metastasis. This hypothesis gained some support over the years, and recently became the focus of many studies that revealed increasing evidence pointing to the possibility that cancer cell fusion probably gives rise to the metastatic phenotype by generating widespread genetic and epigenetic diversity, leading to the emergence of critical populations needed to evolve resistance to the treatment and development of metastasis. In this review, we will discuss the clinical relevance of cancer cell fusion, describe emerging mechanisms of cancer cell fusion, address why inhibiting cancer cell fusion could represent a critical line of attack to limit drug resistance and to prevent metastasis, and suggest one new modality for doing so.

Keywords: cell fusion; genomic instability; phosphatidyl serine receptor; metastasis; genetic diversity

1. Introduction

Approximately 90% of cancer-related deaths are caused by the local invasion and distant metastasis of tumor cells. Metastasis is arguably the most poorly understood aspect in cancer. To successfully relocate in the body, a tumor cell must acquire transient properties that enable dissemination, followed by the reestablishment of the original primary phenotype at a distant site. Exactly how this is accomplished is still unclear, and reliable treatments are therefore lacking. One hypothesis suggests that a variety of genetic and epigenetic changes lead to the development of breast cancer. These changes involve somatic gene mutations, copy number aberrations, exon sequencing changes, alterations in miRNA and protein expression levels, and changes in methylation [1–4]. Hence, the unstable cancer genome combined with host selective pressures generates metastatic cells in the otherwise non-metastatic primary tumor [5]. This view continues to provide some framework for envisioning tumor progression. However, it is difficult to imagine how this might occur through successive, stepwise mutations, as the generation of a metastatic phenotype would require the activation and silencing of large numbers of genes in the primary tumor cell. Moreover, a recent report compared the entire genome of a primary tumor cell with a corresponding metastatic tumor cell, and found only two de novo mutations in the metastatic tumor with neither mutation essential to the metastatic process [6]. A second widely accepted paradigm for cancer progression is that epithelial cells undergo a mesenchymal transition, during which they lose apical-basal polarity and intercellular adhesions, and express mesenchymal genes such as *N*-cadherin

and vimentin. Then, single mesenchymal cells escape from the epithelial tumor mass and enter the lymphatic system or bloodstream, through which they disseminate. At ectopic sites in the body, the tumor cells extravasate, revert to an epithelial phenotype, and colonize surrounding tissue to form metastases [7,8]. However, epithelial-to-mesenchymal transition (EMT) is not essential for tumor invasion, as epithelial cells can collectively invade [9,10]. Furthermore, circulating tumor cells isolated from cancer patients show the expression of markers for both mesenchymal and epithelial cells [11,12]. A third more recent hypothesis suggests that the tumor bulk contains a heterogeneous tumor cell population that is derived from a subset of cells that show the characteristics of stem cells, termed tumor-initiating cells or cancer stem cells (CSCs) [13,14]. They are capable of dividing asymmetrically to produce one stem cell, which enables self-renewal, and one progenitor cell, which allows the production of phenotypically-diverse cancer cells that constitute tumors. The CSCs might result from the deregulation of normal stem cell self-renewal and differentiation pathways [14–16], or may develop from EMTs [17,18]. This current idea has yet to be universally adopted, as the origin of CSCs is still controversial. A fourth possibility (which is the topic of this review) stipulates that the fusion of tumor cells with cells of hematopoietic lineage or stromal lineage gives rise to hybrid cells capable of dissemination and new tumor growth. The possibility that cell fusion gives rise to the metastatic phenotype was first put forward nearly a century ago by Aichel [19], and later on by Mekler [20] and Goldenberg [21]. Since then, the hybrid theory has been proposed as an explanation for tumor metastasis [22–24]. In this review, we will present various studies pointing to the contribution of cancer cell fusion to metastasis, the possible role of cancer cell fusion in chemoresistance, and some potential mechanisms governing cancer cell fusion.

2. Cell Fusion and Metastasis

Several in vitro and in vivo studies have shown that metastatic cells result from the fusion of primary tumor cells and cells of hematopoietic lineage [24–27] or other cell types of the tumor microenvironment [28–31]. These fusion events were shown to occur spontaneously in many cases. For instance, spontaneous fusion was observed in vitro between normal breast epithelium and breast cancer cells [28–31], among breast tumor cells themselves [32], between breast cancer epithelium and endothelial cells [33], between breast cancer epithelium and stroma cells [22,34,35], and between lung cancer cells and stroma cells [36]. Further analysis of hybrids resulting from these spontaneous fusion events showed that they harbored metastatic properties. For example, hybrids formed between normal breast epithelium (M13SV1-EGFP-Neo) and breast cancer cells (HS578T-Hyg) showed increased locomotory activity compared to the normal parental line. This fusion-enhanced migration was associated with altered CCL21/CCR7 signaling, which was previously linked to the metastatic spreading of breast cancer to lymph nodes [28]. We found in our studies that breast tumor cells could spontaneously fuse with mesenchymal stem cells (MSCs) to form hybrids presenting increased invasion and migratory capacity [34,37].

The increased metastatic potential of hybrids was also observed in vivo when breast cancer cell variants (MDA-MB-231) with tropism for either lung or bone injected in nude mice gave rise to hybrids capable of metastases to both organs [32]. Moreover, fusion was detected when freshly-mixed lung cancer cells and MSCs were xenografted by subcutaneous injection into nonobese diabetic severe combined immunodeficient (NOD/SCID) mice [36]. The hybrids formed acquired epithelial-to-mesenchymal (EMT) properties and increased motility and invasiveness. They also displayed stem cell markers and were found to contribute to highly-malignant subpopulations enriched for lung cancer-initiating cells [36]. Additionally, cells of a melanoma clone (wild type for tyrosinase, C/C) implanted into BALB/c nu/nu mice (homozygous mutation for albino tyrosinase, c/c) developed massive pulmonary metastases a few weeks later. Analysis of chromosomes of cells from the metastatic tumors showed that most clones had acquired the c allele (the same as that of the BALB/c recipient), while maintaining the C allele. Thus, lung metastases were comprised primarily of host-tumor hybrids; interestingly, these hybrids expressed the same traits of enhanced

motility and MSH/BMX responsiveness as in vitro-derived melanoma-macrophage hybrids [38]. The motility-associated integrin subunits α2, α3, α5, α6, αv, β1, and β3—which are involved with the migration of leucocytes and cancer cells—were significantly upregulated in metastatic macrophage-melanoma hybrids compared with parental melanoma cells. They also produced high levels of β1,6-branched oligosaccharides—predictors of poor survival in patients with melanoma or carcinomas of the breast, lung, and colon [39,40]. A more recent study also indicated that fusion between cancer cells (ovarian and lung) and hematopoietic cells of the myeloid lineage gave rise to hybrids expressing significantly higher levels of the promigratory marker C-X-C chemokine receptor type 4 (CXCR4) that was conferred by the parental myeloid cells [23].

Collectively, the increase in motility-associated integrin subunits and CXCR4 levels in hybrids might equip them with superior migratory potential and help their dissemination to various secondary organs, and therefore explain how fusion could provide a means by which adherent cancer cells acquire new qualities necessary to form metastases (i.e., enhanced motility and matrix degradation) under conditions conducive to hematopoietic survival, and later resume tumor-like activities (i.e., rapid proliferation) under conditions conducive to epithelial survival. Another potent example of cancer cell fusion-driven metastasis is the study by Li et al. [41] that showed that human hepatocellular carcinoma cells with low metastatic potential exhibited significantly increased metastatic potential following fusion with MSCs, as proven by the gross examination of tumors. The parental hepatocellular carcinoma cells induced well-differentiated noninvasive tumors, whereas fused cells induced poorly-differentiated and invasive tumors when implanted in mouse liver.

Clinical studies have also confirmed the presence of cell fusion in tumors. This was first demonstrated in patients who had received hematopoietic stem cell transplantation and later on developed tumors showing evidence of donor genes in their cells [42,43]. Other discoveries in cancer patients of circulating tumor cells expressing both carcinoma and leucocyte cell markers also points to fusion events between bone marrow-derived cells and tumor cells [25,44,45]. More recently, a study found macrophage-melanoma hybrids in the peripheral blood of patients with cutaneous melanomas. The study further demonstrated that those hybrids, when transplanted subcutaneously in nude mice, produced metastatic lesions at distant sites [46]. Those hybrids in patients' peripheral blood might have been on their way to distant sites to develop metastases. Another recent report used short tandem repeat length-polymorphism and forensic genetic techniques to show that a metastatic melanoma lesion in a patient arose from the fusion between a tumor cell and a bone marrow-derived cell that the patient received as a transplant [47]. Xu et al. [36] also proposed that, after fusion between cancer cells and bone marrow-derived cells, the hybrids undergo EMT that facilitates their migration and invasion and also the acquisition of stem cell-like properties that enhance tumorigenicity and the ability to metastasize. As time goes by, however, those hybrids reacquire epithelia-like morphology by a process termed mesenchymal-epithelial transition, or MET. Thus, cell fusion could encompass or account for both the first transition from cell–cell or cell–matrix bound to unattached, and the second transition from blood transit to proliferation at the point of metastasis. However, although cancer cell fusion is seen in cancer patients and some metastases, and though it represents an attractive mechanism to explain the process of metastasis, it has yet to be clearly demonstrated in vivo that cancer cell fusion in the primary tumor is at the origin of metastasis.

3. Cancer Cell Fusion and Tumor Heterogeneity

Cancer is a clinically and genomically heterogeneous disease (reviewed in [48]). This heterogeneity is observed between tumors as well as within individual tumors [49]. This diversity in the populations of tumor cells is thought to be responsible for the emergence of subpopulations resistant to treatment and the development of metastasis. Although the origin of heterogeneity in a given patient is likely multifaceted, fusion between tumor cells and cells of the tumor microenvironment might represent a key mechanism generating the critical population diversity needed to evolve resistance to therapy and metastasis. Studies have suggested that the fusion of cells in general (and fusion between

cancer cells and cells of the tumor microenvironment especially) is a means to generate widespread genetic and epigenetic diversity [19,27,38,40,50–52]. Diversity created in this way could rapidly enhance the formation, propagation, and metastasis of tumor cells, or quickly alter drug sensitivity. Close examination of the heterogeneity generated by the heterotypic formation of stromal cell–breast cancer cell hybrids indicated that hybrids exhibited mixed gene expression profiles and could undergo DNA ploidy reduction and morphologic switching from mesenchymal-like to breast carcinoma-like. In addition, analysis of coding single-nucleotide polymorphisms by RNA sequencing revealed genetic contributions from both fusion partners to primary tumors and metastasis [22].

A recent study proposed a potent explanation of cell fusion-driven heterogeneity and metastasis mechanism. Zhou et al. [53], used normal intestinal crypt epithelial cells from rats to demonstrate that cell fusion generates populations of cells in which about 1 of 200 could form tumors. In addition, fusion engenders aneuploidy, DNA damage, phenotypic heterogeneity, transformation, and the capacity to form tumors, and these properties were established immediately or within a few cell divisions after the fusion event. They found that tumors formed after the implantation of fusion-derived clones obtained from the same parental line exhibited distinct rates of growth and histology. Some of the clones generated rapidly-growing high-grade undifferentiated tumors, whereas other clones formed slowly-growing moderately differentiated tumors exhibiting a high degree of invasiveness. Overall, these studies support the premise that cell fusion events induce increases in genetically diversified cell populations.

4. Cancer Cell Fusion and Chemoresistance

The diversity of tumor cell populations could account for the drug susceptibility found to be altered in hybrids. Hybrids formed between parental breast cancer cells (MCF-7) with and without resistance to doxyrubicin were heterogeneous in nature; some exhibiting resistance, and others not [54]. Similarly, hybrids derived from breast epithelial cells (M13SV1-EGFP-Neo) and breast cancer cells (MDA-MB-435-Hyg) showed altered sensitivity to the phosphoinositide 3-kinase (PI3K) inhibitor Ly294002 as a consequence of differential RAF-AKT (Rapidly Accelerated Fibrosarcoma-Akt) crosstalk among hybrids [31]. Moreover, studies by Wang [55] showed that the fusion of stem cells and liver cancer cells generated hybrids which were highly tumorigenic and chemoresistant compared with the parental hepatocellular carcinoma cells. Co-cultivation of mouse bone marrow-derived cells and murine 67NR mammary carcinoma cells resulted in the origin of cells exhibiting markedly increased expression levels of the ABC multidrug resistance transporters Abcb1a and Abcb1b, associated with an enhanced resistance towards chemotherapeutic drugs [35]. Thus, the fusion theory offers a compelling explanation for the tumor heterogeneity-driven emergence of cells resistant to chemotherapeutics.

Early detection of the sub-population of treatment resistant and/or metastasis-prone cancer cells and the characterization of residual metastatic cancers would significantly improve cancer management, as it would direct the course of treatment appropriate for the patient. Precision medicine and personalized therapy based on the identification of the molecular drivers of cancer by genome sequencing has been proposed as a strategy to overcome the effects of tumor heterogeneity on metastasis [48,49,56]. This approach has been proven beneficial in various cancers (chronic myeloid leukemia, breast, melanoma, colorectal, and others; reviewed in [57]), however targeted cancer cells can quickly develop resistance to the drug. In addition, not all types of cancer have personalized treatment, and are still under exploration as cutting-edge tools and technologies are developed to identify critical targets implicated in their progression. Another limitation of precision medicine is that not all identified targets that modulate cancer progression are druggable. Moreover, there are a large number of genomic and epigenomic aberrations that have been discovered in cancer. It would be extremely expensive to try to develop targeted therapy for each of those aberrations. Targeted therapy attacks tumor heterogeneity after it is established. However, tackling tumor heterogeneity before inception could represent a more advantageous approach. This could be achieved by preventing cancer cell fusion. Molecular mechanics of hybrid formation could therefore present prime targets, and might

include (1) members of pathways that facilitate close apposition of cell membranes of fusing partners; (2) specific cell surface receptors involved in cell–cell contact-induced fusion; (3) members of pathways that govern the integration of parental fusion partner genes into hybrid genomes; (4) epigenetic modifications enabling transcript expression of hybrid genomes; and even (5) immune modulation to disable or enhance macrophage fusion [58].

5. Potential Molecular Mechanisms and Signaling Pathways Driving Cancer Cell Fusion

The mechanisms governing cancer cell fusion are still underexplored, and this review represents a call for increased study in this area. Here we consider elements that have been unraveled and consider their possible interplay to facilitate tumor cell fusion. Our studies showed that fusion between MSCs and breast tumor cells was significantly increased in hypoxic condition, and was regulated by a mechanism involving apoptosis. We found that the inhibition of apoptosis reduced cell fusion, whereas the addition of apoptotic cells to co-cultures could significantly enhance fusion [34]. A previous work also identified apoptotic cells as a new type of cue that promotes fusion of myoblasts by inducing signaling via the phosphatidylserine receptor BAI1 (brain specific angiogenesis inhibitor 1) pathway [59]. Myoblasts and macrophages have been shown to use some of the same molecular components in fusion, and in these studies, activation of BAI1 triggers ELMO/Dock180/Rac1-associated pathways [60].

ELMO/Dock180/Rac proteins are a conserved signaling module for the promotion of the internalization of apoptotic cell corpses. ELMO and Dock180 function together as a guanine nucleotide exchange factor (GEF) for the small GTPase Rac, and thereby regulate the phagocyte actin cytoskeleton during engulfment [61]. BAI1 was identified as a receptor upstream of ELMO, and as a receptor that can bind PtdSer on apoptotic cells. BAI1 forms a trimeric complex with ELMO and Dock180, and functional studies suggest that BAI1 cooperates with ELMO/Dock180/Rac to promote the maximal engulfment of apoptotic cells. Decreased BAI1 expression or interference with BAI1 function was shown to inhibit the engulfment of apoptotic targets ex vivo and in vivo. Thus, BAI1 is a PtdSer recognition receptor that can directly recruit a Rac-GEF complex to mediate the uptake of apoptotic cells [62]. ELMO and Dock180 are overexpressed in breast cancer cell lines [63]. Activation of the ELMO-Dock signaling pathway has also been shown to be involved in breast cancer metastasis [63,64]. Moreover, ELMO1 has been identified as a modifier of breast cancer risk for BRCA mutation carriers [65]. Rac1 was shown to be overexpressed in proliferative breast disease, pre-invasive and invasive breast carcinoma, as well as lymph node metastases [66,67]. It was also shown to be implicated in the molecular mechanism of cancer metastasis driven by episodes of hypoxia and re-oxygenation [68], and its overexpression was found to be associated with the aggressive form of breast cancer [67,69]. Rac1 is a member of the Ras superfamily of small guanosine triphosphatases (GTPases) that acts as molecular switches to control cytoskeletal rearrangements and cell growth. Rac1 activity, as a modulator of the cytoskeleton, is critical for a number of normal cellular activities, including phagocytosis, mesenchymal-like migration, axonal growth, adhesion and differentiation of multiple cell types, as well as reactive oxygen species (ROS)-mediated cell killing (reviewed in [70]). Rac1 also plays a major role in the moderation of other signaling pathways involved in cellular growth and cell cycle regulation [71], the formation of cell–cell adhesions [72], and the process of contact inhibition [73]. These Rac1-mediated activities appear central to the processes that underlie malignant transformation, including tumorigenesis, angiogenesis, invasion, and metastasis. Building on the role of the cytoskeleton in cell fusion, a recent study in drosophila showed that cell fusion was driven by the mechanical tension of cell membranes [74]. They demonstrated that, during cell–cell fusion, the receiving fusion partner mounts a non-muscle Myosin II (MyoII)-mediated mechanosensory response to the invasive force from the attacking cell. MyoII is recruited to the fusogenic synapse because of its intrinsic ability to sense mechanical strains in the actin network, whereas chemical signaling from membrane-associated molecules Rho and Rok increased the amount of activated MyoII to amplify the response. The accumulated MyoII generated cortical tension required to resist podosome-like structure invasion, thereby promoting membrane

juxtaposition and fusion [74]. The possible mechanistic link between apoptosis and cytoskeletal activation to encourage fusion is delineated in Figure 1. Taken together, one could identify therapeutic targets that may be less promiscuous than directly tapping the cytoskeleton, but still potentially successful in limiting cell–cell fusion in the tumor bed.

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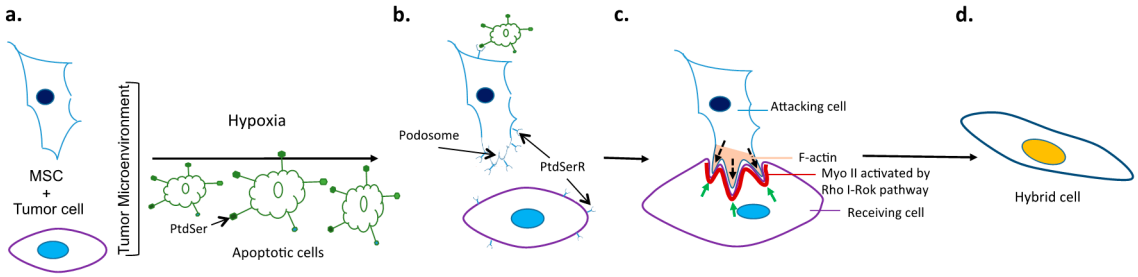

Figure 1. Unraveling and targeting mechanisms of cancer cell fusion. (**a**) Tumor cells and mesenchymal stem cells (MSCs) are capable of spontaneous fusion, which is augmented with hypoxia; (**b**) Fusion in hypoxic conditions can be facilitated by the engagement of the exposed phosphatidyl serine (PtdSer) of apoptotic cells with PtdSer receptors (PtdSerR) on tumor cells or MSCs. Engagement of this type facilitates podosome formation that ultimately leads to robust activation of the F actin of the attacking fusion partner and MyoII of the receiving cell; (**c**) Green arrows indicate resisting forces from the actomyosin network, and black arrows indicate pushing forces from invasive protrusions of the attacking cell; (**d**) Hybrids formed in this way represent an accelerated evolution of sorts, sometimes giving rise to cells with enhanced metastatic potential or the ability to resist drug treatment. Inhibiting the engagement of apoptotic cells via PtdSer represents one potential therapeutic approach to the prevention of tumor cell fusion.

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While cell fusion is observed in various pathological conditions, it is also a fundamental requirement in numerous developmental and physiological processes in eukaryotes. These processes include the homotypic fusion of myoblasts, trophoblasts [75], and macrophages [75,76], as well as the heterotypic fusion of gametes [75]. Inhibiting cancer cell fusion could be harmful for these naturally occurring processes, and should be considered while devising treatment to inhibit cancer cell fusion and prevent metastasis. Inhibitors of fusion should be cell-specific to target only the tumor bulk, be delivered and sequestered locally, or used at a dose that is not toxic for the normal biological events.

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As we learn more of the processes involved in cell fusion in cancer, better strategies should emerge for targeting critical steps in fusion and hybrid formation for the prevention of metastasis and better cancer management. Given the growing body of observational studies linking spontaneous fusion and cancer progression, there is valid evidence to justify pushing the field forward by way of mechanistic studies. As a result, the effective inhibition of fusion might be formally tested as a means to avoid tumor metastasis and drug resistance.

As we learn more of the processes involved in cell fusion in cancer, better strategies should emerge for targeting critical steps in fusion and hybrid formation for the prevention of metastasis and better cancer management. Given the growing body of observational studies linking spontaneous fusion and cancer progression, there is valid evidence to justify pushing the field forward by way of mechanistic studies. As a result, the effective inhibition of fusion might be formally tested as a means to avoid tumor metastasis and drug resistance.

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References

1. Stephens, P.J.; Tarpey, P.S.; Davies, H.; van Loo, P.; Greenman, C.; Wedge, D.C.; Nik-Zainal, S.; Martin, S.; Varela, I.; Bignell, G.R.; et al. The landscape of cancer genes and mutational processes in breast cancer. *Nature* **2012**, *486*, 400–404. [CrossRef] [PubMed]
2. Curtis, C.; Shah, S.P.; Chin, S.F.; Turashvili, G.; Rueda, O.M.; Dunning, M.J.; Speed, D.; Lynch, A.G.; Samarajiwa, S.; Yuan, Y.; et al. The genomic and transcriptomic architecture of 2000 breast tumours reveals novel subgroups. *Nature* **2012**, *486*, 346–352. [PubMed]
3. Elsheikh, S.E.; Green, A.R.; Rakha, E.A.; Powe, D.G.; Ahmed, R.A.; Collins, H.M.; Soria, D.; Garibaldi, J.M.; Paish, C.E.; Ammar, A.A.; et al. Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. *Cancer Res.* **2009**, *69*, 3802–3809. [CrossRef] [PubMed]
4. Byler, S.; Goldgar, S.; Heerboth, S.; Leary, M.; Housman, G.; Moulton, K.; Sarkar, S. Genetic and epigenetic aspects of breast cancer progression and therapy. *Anticancer Res.* **2014**, *34*, 1071–1077.

4. Byler, S.; Goldgar, S.; Heerboth, S.; Leary, M.; Housman, G.; Moulton, K.; Sarkar, S. Genetic and epigenetic aspects of breast cancer progression and therapy. *Anticancer Res.* **2014**, *34*, 1071–1077. [PubMed]
5. Rodenhiser, D.I.; Andrews, J.; Kennette, W.; Sadikovic, B.; Mendlowitz, A.; Tuck, A.B.; Chambers, A.F. Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. *Breast Cancer Res.* **2008**, *10*, R62. [CrossRef] [PubMed]
6. Ding, L.; Ellis, M.J.; Li, S.; Larson, D.E.; Chen, K.; Wallis, J.W.; Harris, C.C.; McLellan, M.D.; Fulton, R.S.; Fulton, L.L.; et al. Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature* **2010**, *464*, 999–1005. [CrossRef] [PubMed]
7. Thiery, J.P.; Acloque, H.; Huang, R.Y.; Nieto, M.A. Epithelial-mesenchymal transitions in development and disease. *Cell* **2009**, *139*, 871–890. [CrossRef] [PubMed]
8. Wang, Y.; Zhou, B.P. Epithelial-mesenchymal transition—A hallmark of breast cancer metastasis. *Cancer Hallm.* **2013**, *1*, 38–49. [CrossRef] [PubMed]
9. Friedl, P.; Zallen, J.A. Dynamics of cell-cell and cell-matrix interactions in morphogenesis, regeneration and cancer. *Curr. Opin. Cell Biol.* **2010**, *22*, 557–559. [CrossRef] [PubMed]
10. Nguyen-Ngoc, K.V.; Cheung, K.J.; Brenot, A.; Shamir, E.R.; Gray, R.S.; Hines, W.C.; Yaswen, P.; Werb, Z.; Ewald, A.J. ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E2595–E2604. [CrossRef] [PubMed]
11. Yu, M.; Bardia, A.; Wittner, B.S.; Stott, S.L.; Smas, M.E.; Ting, D.T.; Isakoff, S.J.; Ciciliano, J.C.; Wells, M.N.; Shah, A.M. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* **2013**, *339*, 580–584. [CrossRef]
12. Chatterjee, S.; Damle, S.G.; Sharma, A.K. Mechanisms of resistance against cancer therapeutic drugs. *Curr. Pharm. Biotechnol.* **2014**, *15*, 1105–1112. [CrossRef]
13. Velasco-Velazquez, M.A.; Homsi, N.; de La Fuente, M.; Pestell, R.G. Breast cancer stem cells. *Int. J. Biochem. Cell Biol.* **2012**, *44*, 573–577. [CrossRef]
14. Al-Hajj, M.; Wicha, M.S.; Benito-Hernandez, A.; Morrison, S.J.; Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3983–3988. [CrossRef] [PubMed]
15. Ponti, D.; Costa, A.; Zaffaroni, N.; Pratesi, G.; Petrangolini, G.; Coradini, D.; Pilotti, S.; Pierotti, M.A.; Daidone, M.G. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res.* **2005**, *65*, 5506–5511. [CrossRef] [PubMed]
16. Ginestier, C.; Korkaya, H.; Dontu, G.; Birnbaum, D.; Wicha, M.S.; Charafe-Jauffret, E. The cancer stem cell: The breast cancer driver. *Med. Sci.* **2007**, *23*, 1133–1139.
17. Morel, A.P.; Lievre, M.; Thomas, C.; Hinkal, G.; Ansieau, S.; Puisieux, A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS ONE* **2008**, *3*, e2888. [CrossRef] [PubMed]
18. Mani, S.A.; Guo, W.; Liao, M.J.; Eaton, E.N.; Ayyanan, A.; Zhou, A.Y.; Brooks, M.; Reinhard, F.; Zhang, C.C.; Shipitsin, M.; et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **2008**, *133*, 704–715. [CrossRef] [PubMed]
19. Aichel, O. Über Zellverschmelzung mit Qualitativ Abnormer Chromosomenverteilung als Ursache der Geschwulstbildung. In *Vorträge und Aufsätze über Entwicklungsmechanik der Organismen*; Engelmann: Leipzig, Germany, 1911; p. 115S.
20. Mekler, L.B. Creation of antineoplastic preparations on the basis of the theory of organ-tissue mosaicism of malignant cells. *Farmakol. Toksikol.* **1971**, *34*, 713–718. [PubMed]
21. Goldenberg, D.M.; Pavia, R.A.; Tsao, M.C. In vivo hybridisation of human tumour and normal hamster cells. *Nature* **1974**, *250*, 649–651. [CrossRef] [PubMed]
22. Rappa, G.; Mercapide, J.; Lorico, A. Spontaneous formation of tumorigenic hybrids between breast cancer and multipotent stromal cells is a source of tumor heterogeneity. *Am. J. Pathol.* **2012**, *180*, 2504–2515. [CrossRef] [PubMed]
23. Ramakrishnan, M.; Mathur, S.R.; Mukhopadhyay, A. Fusion-derived epithelial cancer cells express hematopoietic markers and contribute to stem cell and migratory phenotype in ovarian carcinoma. *Cancer Res.* **2013**, *73*, 5360–5370. [CrossRef] [PubMed]
24. Pawelek, J.M. Fusion of bone marrow-derived cells with cancer cells: Metastasis as a secondary disease in cancer. *Chin. J. Cancer* **2014**, *33*, 133–139. [CrossRef] [PubMed]

25. Chakraborty, A.; Lazova, R.; Davies, S.; Backvall, H.; Ponten, F.; Brash, D.; Pawelek, J. Donor DNA in a renal cell carcinoma metastasis from a bone marrow transplant recipient. *Bone Marrow Transpl.* **2004**, *34*, 183–186. [CrossRef] [PubMed]
26. Sodi, S.A.; Chakraborty, A.; Platt, J.; Kolesnikova, N.; Rosemblat, S.; Keh-Yen, A.; Bolognia, J.; Rachkovsky, M.; Orlow, S.; Pawelek, J. Melanoma x macrophage fusion hybrids acquire increased melanogenesis and metastatic potential: Altered *N*-glycosylation as an underlying mechanism. *Pigment Cell Res.* **1998**, *11*, 299–309. [CrossRef] [PubMed]
27. Pawelek, J.M.; Chakraborty, A.K. The cancer cell—Leukocyte fusion theory of metastasis. *Adv. Cancer Res.* **2008**, *101*, 397–444. [PubMed]
28. Berndt, B.; Haverkampf, S.; Reith, G.; Keil, S.; Niggemann, B.; Zanker, K.S.; Dittmar, T. Fusion of CCL21 non-migratory active breast epithelial and breast cancer cells give rise to CCL21 migratory active tumor hybrid cell lines. *PLoS ONE* **2013**, *8*, e63711. [CrossRef] [PubMed]
29. Berndt, B.; Zanker, K.S.; Dittmar, T. Cell fusion is a potent inducer of aneuploidy and drug resistance in tumor cell/normal cell hybrids. *Crit. Rev. Oncog.* **2013**, *18*, 97–113. [CrossRef] [PubMed]
30. Dittmar, T.; Schwitalla, S.; Seidel, J.; Haverkampf, S.; Reith, G.; Meyer-Staeckling, S.; Brandt, B.H.; Niggemann, B.; Zanker, K.S. Characterization of hybrid cells derived from spontaneous fusion events between breast epithelial cells exhibiting stem-like characteristics and breast cancer cells. *Clin. Exp. Metastasis* **2011**, *28*, 75–90. [CrossRef] [PubMed]
31. Ozel, C.; Seidel, J.; Meyer-Staeckling, S.; Brandt, B.H.; Niggemann, B.; Zanker, K.S.; Dittmar, T. Hybrid cells derived from breast epithelial cell/breast cancer cell fusion events show a differential RAF-AKT crosstalk. *Cell Commun. Signal.* **2012**, *10*, 10. [CrossRef] [PubMed]
32. Lu, X.; Kang, Y. Efficient acquisition of dual metastasis organotropism to bone and lung through stable spontaneous fusion between MDA-MB-231 variants. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9385–9390. [CrossRef] [PubMed]
33. Mortensen, K.; Lichtenberg, J.; Thomsen, P.; Larsson, L.I. Spontaneous fusion between cancer cells and endothelial cells. *Cell. Mol. Life Sci.* **2004**, *61*, 2125–2131. [CrossRef] [PubMed]
34. Noubissi, F.K.; Harkness, T.; Alexander, C.M.; Ogle, B.M. Apoptosis-induced cancer cell fusion: A mechanism of breast cancer metastasis. *FASEB J.* **2015**, *29*, 4036–4045. [CrossRef] [PubMed]
35. Nagler, C.; Zanker, K.S.; Dittmar, T. Cell fusion, drug resistance and recurrence CSCs. *Adv. Exp. Med. Biol.* **2011**, *714*, 173–182. [PubMed]
36. Xu, M.H.; Gao, X.; Luo, D.; Zhou, X.D.; Xiong, W.; Liu, G.X. EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. *PLoS ONE* **2014**, *9*, e87893. [CrossRef] [PubMed]
37. McArdle, T.J.O.B.; Noubissi, F.K. An in vitro inverted vertical invasion assay to avoid manipulation of rare or sensitive cell types. *J. Cancer* **2016**. [CrossRef]
38. Chakraborty, A.K.; Sodi, S.; Rachkovsky, M.; Kolesnikova, N.; Platt, J.T.; Bolognia, J.L.; Pawelek, J.M. A spontaneous murine melanoma lung metastasis comprised of host X tumor hybrids. *Cancer Res.* **2000**, *60*, 2512–2519. [PubMed]
39. Chakraborty, A.K.; Pawelek, J.; Ikeda, Y.; Miyoshi, E.; Kolesnikova, N.; Funasaka, Y.; Ichihashi, M.; Taniguchi, N. Fusion hybrids with macrophage and melanoma cells up-regulate *N*-acetylglucosaminyltransferase V, β1-6 branching, and metastasis. *Cell Growth Differ.* **2001**, *12*, 623–630. [PubMed]
40. Pawelek, J.M. Cancer-cell fusion with migratory bone-marrow-derived cells as an explanation for metastasis: New therapeutic paradigms. *Future Oncol.* **2008**, *4*, 449–452. [CrossRef] [PubMed]
41. Li, H.; Feng, Z.; Tsang, T.C.; Tang, T.; Jia, X.; He, X.; Pennington, M.E.; Badowski, M.S.; Liu, A.K.; Chen, D.; et al. Fusion of HepG2 cells with mesenchymal stem cells increases cancerassociated and malignant properties: An in vivo metastasis model. *Oncol. Rep.* **2014**, *32*, 539–547. [PubMed]
42. Varley, K.E.; Mutch, D.G.; Edmonston, T.B.; Goodfellow, P.J.; Mitra, R.D. Intra-tumor heterogeneity of MLH1 promoter methylation revealed by deep single molecule bisulfite sequencing. *Nucleic Acids Res.* **2009**, *37*, 4603–4612. [CrossRef] [PubMed]
43. Mekler, L.B. Hybridization of transformed cells with lymphocytes as 1 of the probable causes of the progression leading to the development of metastatic malignant cells. *Vestnik Akad. Med. Nauk SSSR* **1971**, *26*, 80–89.

44. Andersen, T.L.; Boissy, P.; Sondergaard, T.E.; Kupisiewicz, K.; Plesner, T.; Rasmussen, T.; Haaber, J.; Kolvraa, S.; Delaisse, J.M. Osteoclast nuclei of myeloma patients show chromosome translocations specific for the myeloma cell clone: A new type of cancer-host partnership? *J. Pathol.* **2007**, *211*, 10–17. [CrossRef] [PubMed]
45. Yilmaz, Y.; Lazova, R.; Qumsiyeh, M.; Cooper, D.; Pawelek, J. Donor Y chromosome in renal carcinoma cells of a female BMT recipient: Visualization of putative BMT-tumor hybrids by FISH. *Bone Marrow Transpl.* **2005**, *35*, 1021–1024. [CrossRef] [PubMed]
46. Clawson, G.A.; Matters, G.L.; Xin, P.; Imamura-Kawasawa, Y.; Du, Z.; Thiboutot, D.M.; Helm, K.F.; Neves, R.I.; Abraham, T. Macrophage-tumor cell fusions from peripheral blood of melanoma patients. *PLoS ONE* **2015**, *10*, e0134320. [CrossRef] [PubMed]
47. Lazova, R.; Laberge, G.S.; Duvall, E.; Spoelstra, N.; Klump, V.; Sznol, M.; Cooper, D.; Spritz, R.A.; Chang, J.T.; Pawelek, J.M. A melanoma brain metastasis with a donor-patient hybrid genome following bone marrow transplantation: First evidence for fusion in human cancer. *PLoS ONE* **2013**, *8*, e66731. [CrossRef]
48. Collisson, E.A.; Cho, R.J.; Gray, J.W. What are we learning from the cancer genome? *Nat. Rev. Clin. Oncol.* **2012**, *9*, 621–630. [CrossRef] [PubMed]
49. Russnes, H.G.; Navin, N.; Hicks, J.; Borresen-Dale, A.L. Insight into the heterogeneity of breast cancer through next-generation sequencing. *J. Clin. Investig.* **2011**, *121*, 3810–3818. [CrossRef] [PubMed]
50. Duelli, D.M.; Padilla-Nash, H.M.; Berman, D.; Murphy, K.M.; Ried, T.; Lazebnik, Y. A virus causes cancer by inducing massive chromosomal instability through cell fusion. *Curr. Biol.* **2007**, *17*, 431–437. [CrossRef] [PubMed]
51. Mohr, M.; Zaenker, K.S.; Dittmar, T. Fusion in cancer: An explanatory model for aneuploidy, metastasis formation, and drug resistance. *Methods Mol. Biol.* **2015**, *1313*, 21–40.
52. Freeman, B.T.; Jung, J.P.; Ogle, B.M. Single-cell RNA-seq reveals activation of unique gene groups as a consequence of stem cell-parenchymal cell fusion. *Sci. Rep.* **2016**, *6*, 23270. [CrossRef] [PubMed]
53. Zhou, X.; Merchak, K.; Lee, W.; Grande, J.P.; Cascalho, M.; Platt, J.L. Cell fusion connects oncogenesis with tumor evolution. *Am. J. Pathol.* **2015**, *185*, 2049–2060. [CrossRef] [PubMed]
54. Yang, J.Y.; Ha, S.A.; Yang, Y.S.; Kim, J.W. p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: Correlations with cell fusion and doxorubicin resistance. *BMC Cancer* **2010**, *10*, 388. [CrossRef] [PubMed]
55. Wang, R.; Chen, S.; Li, C.; Ng, K.T.; Kong, C.W.; Cheng, J.; Cheng, S.H.; Li, R.A.; Lo, C.M.; Man, K.; et al. Fusion with stem cell makes the hepatocellular carcinoma cells similar to liver tumor-initiating cells. *BMC Cancer* **2015**, *16*, 56. [CrossRef] [PubMed]
56. Butler, T.M.; Johnson-Camacho, K.; Peto, M.; Wang, N.J.; Macey, T.A.; Korkola, J.E.; Koppie, T.M.; Corless, C.L.; Gray, J.W.; Spellman, P.T. Exome Sequencing of cell-free DNA from metastatic cancer patients identifies clinically actionable mutations distinct from primary disease. *PLoS ONE* **2015**, *10*, e0136407. [CrossRef] [PubMed]
57. Garay, J.P.; Gray, J.W. Omics and therapy—A basis for precision medicine. *Mol. Oncol.* **2012**, *6*, 128–139. [CrossRef] [PubMed]
58. Maisel, D.; Birzele, F.; Voss, E.; Nopora, A.; Bader, S.; Friess, T.; Goller, B.; Laifenfeld, D.; Weigand, S.; Runza, V. Targeting tumor cells with anti-CD44 antibody triggers macrophage-mediated immune modulatory effects in a cancer xenograft model. *PLoS ONE* **2016**, *11*, e0159716. [CrossRef] [PubMed]
59. Hochreiter-Hufford, A.E.; Lee, C.S.; Kinchen, J.M.; Sokolowski, J.D.; Arandjelovic, S.; Call, J.A.; Klibanov, A.L.; Yan, Z.; Mandell, J.W.; Ravichandran, K.S. Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. *Nature* **2013**, *497*, 263–267. [CrossRef] [PubMed]
60. Pajcini, K.V.; Pomerantz, J.H.; Alkan, O.; Doyonnas, R.; Blau, H.M. Myoblasts and macrophages share molecular components that contribute to cell–cell fusion. *J. Cell Biol.* **2008**, *180*, 1005–1019. [CrossRef] [PubMed]
61. Lu, M.; Ravichandran, K.S. Dock180-ELMO cooperation in Rac activation. *Methods Enzymol.* **2006**, *406*, 388–402. [PubMed]
62. Park, D.; Tosello-Trampont, A.C.; Elliott, M.R.; Lu, M.; Haney, L.B.; Ma, Z.; Klibanov, A.L.; Mandell, J.W.; Ravichandran, K.S. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature* **2007**, *450*, 430–434. [CrossRef] [PubMed]

63. Li, H.; Yang, L.; Fu, H.; Yan, J.; Wang, Y.; Guo, H.; Hao, X.; Xu, X.; Jin, T.; Zhang, N. Association between Galphai2 and ELMO1/Dock180 connects chemokine signalling with Rac activation and metastasis. *Nat. Commun.* **2013**, *4*, 1706. [CrossRef] [PubMed]
64. Abu-Thuraia, A.; Gauthier, R.; Chidiac, R.; Fukui, Y.; Screaton, R.A.; Gratton, J.P.; Cote, J.F. Axl phosphorylates Elmo scaffold proteins to promote Rac activation and cell invasion. *Mol. Cell. Biol.* **2015**, *35*, 76–87. [CrossRef] [PubMed]
65. Walker, L.C.; Spurdle, A.B. Prioritizing candidate genetic modifiers of BRCA1 and BRCA2 using a combinatorial analysis of global expression and polymorphism association studies of breast cancer. *Methods Mol. Biol.* **2010**, *653*, 23–34. [PubMed]
66. Feng, H.; Hu, B.; Vuori, K.; Sarkaria, J.N.; Furnari, F.B.; Cavenee, W.K.; Cheng, S.Y. EGFRvIII stimulates glioma growth and invasion through PKA-dependent serine phosphorylation of Dock180. *Oncogene* **2014**, *33*, 2504–2512. [CrossRef] [PubMed]
67. Bid, H.K.; Roberts, R.D.; Manchanda, P.K.; Houghton, P.J. RAC1: An emerging therapeutic option for targeting cancer angiogenesis and metastasis. *Mol. Cancer Ther.* **2013**, *12*, 1925–1934. [CrossRef] [PubMed]
68. Lee, J.W.; Ryu, Y.K.; Ji, Y.H.; Kang, J.H.; Moon, E.Y. Hypoxia/reoxygenation-experienced cancer cell migration and metastasis are regulated by Rap1- and Rac1-GTPase activation via the expression of thymosin β-4. *Oncotarget* **2015**, *6*, 9820–9833. [CrossRef] [PubMed]
69. Feng, M.; Bao, Y.; Li, Z.; Li, J.; Gong, M.; Lam, S.; Wang, J.; Marzese, D.M.; Donovan, N.; Tan, E.Y.; et al. RASAL2 activates RAC1 to promote triple-negative breast cancer progression. *J. Clin. Investig.* **2014**, *124*, 5291–5304. [CrossRef] [PubMed]
70. Heasman, S.J.; Ridley, A.J. Mammalian Rho GTPases: New insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 690–701. [CrossRef] [PubMed]
71. Saci, A.; Cantley, L.C.; Carpenter, C.L. Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size. *Mol. Cell* **2011**, *42*, 50–61. [CrossRef] [PubMed]
72. Ehrlich, J.S.; Hansen, M.D.; Nelson, W.J. Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell-cell adhesion. *Dev. Cell* **2002**, *3*, 259–270. [CrossRef]
73. Bosco, E.E.; Nakai, Y.; Hennigan, R.F.; Ratner, N.; Zheng, Y. NF2-deficient cells depend on the Rac1-canonical Wnt signaling pathway to promote the loss of contact inhibition of proliferation. *Oncogene* **2010**, *29*, 2540–2549. [CrossRef] [PubMed]
74. Kim, J.H.; Ren, Y.; Ng, W.P.; Li, S.; Son, S.; Kee, Y.S.; Zhang, S.; Zhang, G.; Fletcher, D.A.; Robinson, D.N.; et al. Mechanical tension drives cell membrane fusion. *Dev. Cell* **2015**, *32*, 561–573. [CrossRef] [PubMed]
75. Aguilar, P.S.; Baylies, M.K.; Fleissner, A.; Helming, L.; Inoue, N.; Podbilewicz, B.; Wang, H.; Wong, M. Genetic basis of cell–cell fusion mechanisms. *Trends Genet.* **2013**, *29*, 427–437. [CrossRef] [PubMed]
76. Vignery, A. Macrophage fusion: Are somatic and cancer cells possible partners? *Trends Cell Biol.* **2005**, *15*, 188–193. [CrossRef] [PubMed]

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