

# Cancer cells enter dormancy after cannibalizing mesenchymal stem/stromal cells (MSCs)

Thomas J. Bartosh<sup>a,b,1</sup>, Mujib Ullah<sup>a</sup>, Suzanne Zeitouni<sup>a</sup>, Joshua Beaver<sup>a,b</sup>, and Darwin J. Prockop<sup>a,1</sup>

<sup>a</sup>Institute for Regenerative Medicine, College of Medicine, Texas A&M University System Health Science Center, Temple, TX 76502; and <sup>b</sup>Medical Physiology, College of Medicine, Texas A&M University System Health Science Center, Temple, TX 76504

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Patients with breast cancer often develop malignant regrowth of residual drug-resistant dormant tumor cells years after primary treatment, a process defined as cancer relapse. Deciphering the causal basis of tumor dormancy therefore has obvious therapeutic significance. Because cancer cell behavior is strongly influenced by stromal cells, particularly the mesenchymal stem/stromal cells (MSCs) that are actively recruited into tumor-associated stroma, we assessed the impact of MSCs on breast cancer cell (BCC) dormancy. Using 3D cocultures to mimic the cellular interactions of an emerging tumor niche, we observed that MSCs sequentially surrounded the BCCs, promoted formation of cancer spheroids, and then were internalized/degraded through a process resembling the well-documented yet ill-defined clinical phenomenon of cancer cell cannibalism. This suspected feeding behavior was less appreciable in the presence of a rho kinase inhibitor and in 2D monolayer cocultures. Notably, cannibalism of MSCs enhanced survival of BCCs deprived of nutrients but suppressed their tumorigenicity, together suggesting the cancer cells entered dormancy. Transcriptome profiles revealed that the resulting BCCs acquired a unique molecular signature enriched in prosurvival factors and tumor suppressors, as well as inflammatory mediators that demarcate the secretome of senescent cells, also referred to as the senescence-associated secretory phenotype. Overall, our results provide intriguing evidence that cancer cells under duress enter dormancy after cannibalizing MSCs. Importantly, our practical 3D coculture model could provide a valuable tool to understand the anti-tumor activity of MSCs and cell cannibalism further, and therefore open new therapeutic avenues for the prevention of cancer recurrence.

cancer | dormancy | MSC | cannibalism | inflammation

Cancer dormancy, a divergent stage in tumor progression in which residual disease becomes indolent and resistant to conventional therapies, creates obvious clinical challenges (1–5). The phenomenon is poorly understood but conceptually explains the gap or “latency period” between successful primary tumor eradication and subsequent life-threatening resurgence, locally or systemically, in patients otherwise considered free of disease. Dormant tumor cells are commonly found in patients with ductal carcinoma of the breast, often present at early stages of the disease (6, 7) and often suspected of driving incurable recurrent disease many years, or even decades, after mastectomy (8). Precisely how cancer cells enter dormancy is currently unclear. Data from preclinical models indicate that tumor dormancy broadly manifests itself as either solitary cells deficient in appropriate proliferation signals or small clusters of cancer cells (i.e., tumor mass dormancy) that maintain balanced expansion and death due to inadequate neovascularization and/or immune surveillance (2, 9). Interestingly, many molecular programs that promote dormancy are thought to resemble those molecular programs regulating self-renewal of adult stem cells, or those molecular programs that promote growth arrest and autophagy of normal tissues in response to various hostile conditions (4, 10). Disruption of dormancy-permissive signals can, in effect, encourage cancer regrowth (11, 12). Ultimately, understanding the driving force of tumor dormancy has important therapeutic implications for preventing relapse in patients with a history of cancer (1, 2).

Recent investigations describe an essential role for the tumor microenvironment, or niche, in regulating cancer dormancy (2, 4, 9, 10).

The cells found in this microenvironment include fibroblasts, immune and inflammatory cells, neural cells, endothelium, and a variety of tissue-specific parenchymal cells (13). To varying degrees, it also includes the heterogeneous class of multipotent progenitor cells referred to as mesenchymal stem/stromal cells (MSCs) that are found in most tissues and that participate in tissue homeostasis and injury repair (14, 15). Through means that emulate their migration into classical wounds, MSCs can infiltrate developing tumors, interact with cancer cells, and shape tumor phenotype (16). In addition, cross-talk between cancer cells and MSCs occurs when disseminated tumor cells enter skeletal tissue (17, 18), ironically one of the most prevalent sites of breast cancer relapse (19). In fact, it has been suggested that bone marrow might serve as a reservoir for dormant tumor cells that recirculate, when conditions become favorable, and invade other organs (20). Nonetheless, the effect of MSCs on tumor progression has been quite contradictory, with studies demonstrating both tumor-promoting and tumor-suppressive responses (21). Collectively, the need to unravel mechanisms underlying MSC-mediated modulation of tumor cell behavior is apparent not only for identifying new targeted treatments but for using MSCs in the clinic safely.

There has been a growing interest in using 3D nonadherent culture platforms to understand MSC biology better and enhance MSC-based therapies (22–24). By mirroring natural conditions *in vivo*, 3D cultures are superior to 2D cultures for understanding complex cell-to-cell and cell-to-matrix interactions, and therefore

## Significance

In many patients with cancer, some tumor cells tolerate conventional treatments and persist for years in an undetectable/dormant state, after which these same cells can mysteriously resume their growth and seed, almost invariably fatal, recurrent cancerous lesions. The therapeutic challenges of tumor dormancy and need to decode the underlying mechanisms involved are apparent. Here, we revealed that mesenchymal stem/stromal cells (MSCs), recognized determinants of breast cancer cell (BCC) behavior, were readily cannibalized by the BCCs they mingled with in 3D cocultures, a process that distinctly altered cancer cell phenotype, suppressed tumor formation, and supported tumor dormancy. Our discoveries provide original insight into the interactions between MSCs and cancer cells, with the potential to identify novel molecular targets for cancer therapy.

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<sup>1</sup>To whom correspondence may be addressed. Email: bartosh@medicine.tamhsc.edu or prockop@medicine.tamhsc.edu.

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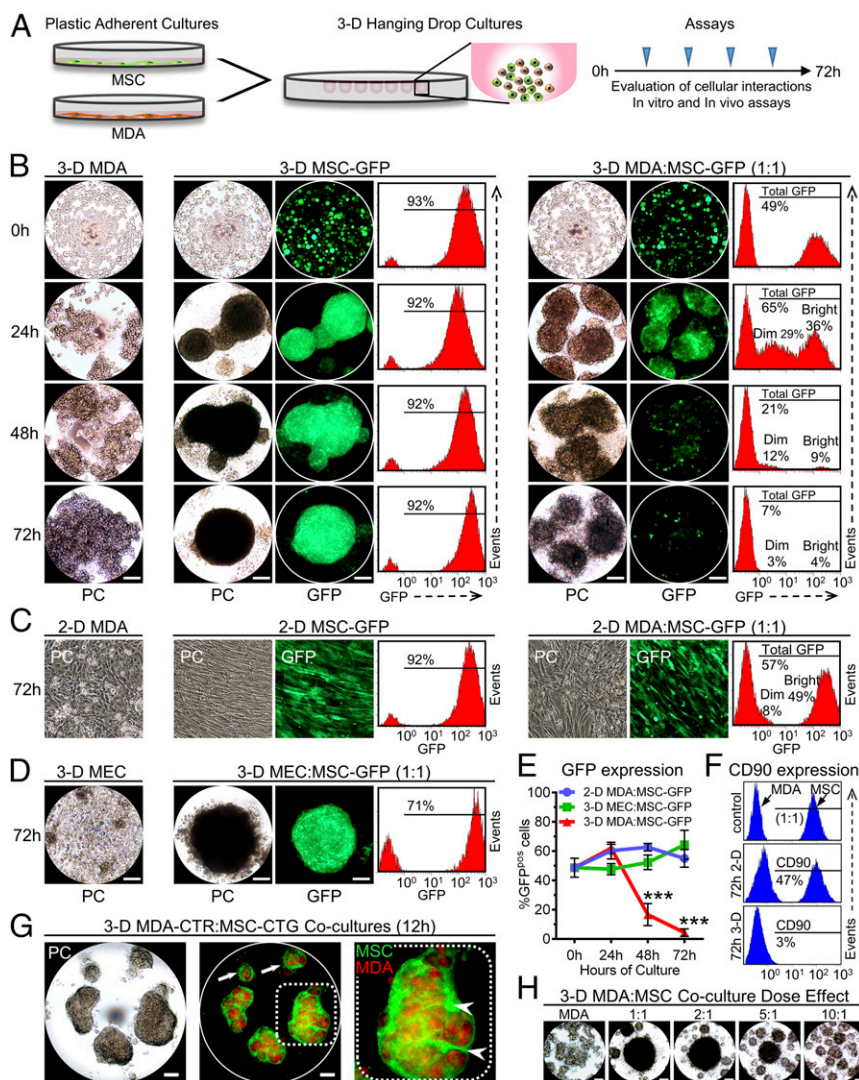
have been regularly used as tumor models and in drug discovery (25). We previously observed that within 3D hanging drop cultures of MSCs, sphere formation was accompanied by a dramatic reduction in the physical size of viable MSCs, transient cellular quiescence, and heightened cellular stress responses (26–28). Moreover, we (26, 29) and others (30) have observed enhanced anticancer properties of MSCs prepared in 3D cultures. Of particular importance, nonadherent cultures have been used effectively to study various cell engulfment programs, including the cell-eat-cell phenomenon referred to as cell cannibalism (31). In general, cell cannibalism, also called xenocannibalism, describes a process by which a cell encloses and ultimately eliminates one or more neighboring target cell(s) of either similar (homotypic) or different (heterotypic) type (31, 32). Cell cannibalism is evolutionarily conserved and has been observed in mammalian systems for more than 100 y (31, 33), particularly in cancer tissue specimens (34–37), although few reports have evaluated its biological significance.

In this study, we used hanging drop cultures to focus on interactions between bone marrow-derived MSCs and MDA-MB-231 (MDA) breast cancer cells (BCCs). Our results provide evidence that BCCs under duress in these 3D cocultures can eat or “cannibalize” MSCs, a process that mirrored, from a morphological perspective, the infrequent but well-documented clinical phenomenon of cancer cell cannibalism (34, 35) and that enhanced

inflammatory response and cell resiliency while impeding tumor formation. Taken together, the results indicated that cannibalism of MSCs under demanding conditions naturally encountered during tumor development *in vivo* supports tumor dormancy.

## Results

**MSCs Stimulated Compaction of MDA Cancer Spheroids in 3D Hanging Drops and then Rapidly Disappeared from the Cultures.** Despite expanding awareness that MSCs strongly influence tumor evolution, our understanding of their direct impact on cancer cell behavior is not complete and has been limited by complexities of the tumor microenvironment *in vivo*. Because 3D cultures exhibit features that mimic natural cellular interactions better than conventional plastic-adherent conditions (25), we used a 3D hanging drop culture system here to study BCC-MSC cross-talk (Fig. 1A). When suspended alone in hanging drops (3D MDA), MDA BCCs formed loose aggregates over 72 h, whereas GFP-expressing bone marrow MSCs (3D MSC-GFP) formed compact spheroids near the lower surface of the drop (Fig. 1B), as we reported previously (26, 38). In 3D cocultures initiated by mixing equal amounts of GFP MSCs (94% GFP-positive; Fig. S1A) and cancer cells, compact spheroids formed by 24 h that contained both cell types (Fig. 1B and Fig. S1B). Interestingly, we observed a simultaneous decrease in the number of bright GFP MSCs and emergence of a



**Fig. 1.** MSCs disappeared from hanging drop cocultures with MDA cells after first surrounding the cancer cells and promoting formation of spheroids. (A) Schematic representation of hanging drop cultures and the plan to evaluate cellular interaction. (B) Phase-contrast (PC) microscopy and flow cytometry showing time-dependent changes in formation of spheroids and expression of GFP in 3D hanging drop cultures of MDA cells (3-D MDA) or GFP-expressing MSCs (3-D MSC-GFP), and in cocultures comprising MDA cells and GFP MSCs [3-D MDA/MS (MDA:MSC-GFP)]. For flow cytometry, only live cells (annexin V-negative) were analyzed. (C) Microscopy and flow cytometry of high-density 2D cultures of MDA cells or GFP MSCs, and cocultures of MDA cells and GFP MSCs at 72 h. (D) Microscopy and flow cytometry of 3D cultures of normal human MECs or 3D cocultures of MECs and GFP MSCs. (E) Graph showing percentage of GFP cells in groups from A–C. GFP percentage was determined by counting GFP-positive cells in single-cell suspensions. Data, expressed as means  $\pm$  SD, were analyzed by ANOVA and compared using Tukey's posttest ( $n = 3$  per group;  $***P < 0.001$  compared with corresponding time point of other groups). (F) Level of CD90 was measured by flow cytometry at 0 h and 72 h in 2D and 3D cocultures of MDA cancer cells and MSCs. At baseline MDA cells are CD90-negative, whereas MSCs are strongly positive for CD90 (arrows). (G) Immunofluorescence imaging of hanging drop cocultures (1:1) of CTR MDA cells (MDA-CTR) and CTG MSCs (MSC-CTG). White arrows depict MDA encapsulation by MSCs in small aggregates. Coalescence of small aggregates promotes internal tracks of MSCs (white arrows). (H) Micrographs of MDA cells cocultured with different amounts of MSCs in hanging drops for 48 h. (Scale bars, 100  $\mu$ m).

dim GFP population at 24 h (Fig. 1B). It is important to note that cells with reduced GFP signal were not dead, because we only analyzed the viable (annexin V-negative) cell population. At 48 h, total GFP signal (bright and dim populations) of viable cells within 3D cocultures decreased appreciably, and by 72 h, most of the GFP signal had dissipated (Fig. 1B). In contrast, MSCs cultured alone in spheroids maintained high expression of GFP (Fig. 1B and Fig. S1B). Levels of GFP MSCs were also preserved, for up to 72 h, in high-density 2D adherent cocultures of MDA cells and GFP MSCs (Fig. 1C) and in hanging drop cocultures of GFP MSCs and normal mammary epithelial cells (MECs) (Fig. 1D). Disappearance of GFP-positive cells from hanging drop cocultures at 48 and 72 h was further quantified by counting numbers of fluorescent cells in the single-cell suspensions (Fig. 1E). In the same fashion, the level of CD90, which is expressed by MSCs but not by MDA cells, decreased from ~50% in 1:1 cocultures at 0 h to ~3% in 3D MDA/MSc cocultures after 72 h but did not radically decrease in high-density 2D cocultures (Fig. 1F). Of interest, MSCs also enhanced formation of compact cancer spheroids when cocultured with A549, PANC-1, and PC3 cancer cell lines (Fig. S1C), and the percentage of GFP MSCs was also reduced after 48 h in cocultures with these various cancer cells, but not in coculture with normal dermal fibroblasts (DFs) (Fig. S1D). This report shows that MSCs can rapidly disappear in 3D cultures as a result of their interactions with some cancer cells.

To evaluate potential mechanism(s) involved in sphere formation, we labeled MSCs with cell tracker green (CTG) and MDA cells with cell tracker red (CTR), and then monitored their interactions in developing spheroids. We observed that MSCs first surrounded the cancer cells in small clusters that eventually coalesced into larger spheroids leaving temporary tracks of MSCs through the interior of the cell aggregates (Fig. 1G). The data suggested that the MSCs essentially pulled the cancer cells together and perhaps changed their phenotype. We recently reported a similar mechanism of cellular attraction that promoted spheroid formation of MSCs cultured in hanging drops alone (26). Formation of MEC spheroids was also strongly influenced by the presence of MSCs (Fig. S1B) and appeared to progress in a manner comparable to the manner observed with MDA cells (Fig. 1G and Fig. S1E). Formation of large cancer spheres diminished when the number of MSCs added to the cocultures was reduced (Fig. 1H), further indicating that MSCs were the driving force behind sphere compaction and coalescence.

**MSCs Were Interiorized and Consumed by MDA Cells in Hanging Drop Cultures.** To understand better why MSCs disappeared from 3D cocultures with BCCs, we carefully monitored interactions between MSCs, labeled green with CTG, and MDA cells, labeled red with CTR. In cytospin preparations of single cells obtained by enzymatic dissociation of spheroids (at 24–48 h), some CTG MSCs appeared as small cells attached to the membrane of MDA cells, whereas others had condensed nuclei and were inside the cytoplasm of the MDA cells (Fig. 2A and B). Taken together, the data suggested that the MSCs were being internalized, similar to the internalization previously observed to occur at lower efficiency within 2D cocultures of umbilical cord-derived MSCs and MDA cells (39). In some cases, nuclei of the CTR MDA cells were exceedingly large, and in other cases, the nucleus was pushed to the cell periphery, presumably to allow space for MSC degradation within the cytoplasm. The latter phenomenon has been observed to occur with cell cannibalism (32) and during the rho kinase (ROCK)-dependent, cell-in-cell invasion process referred to as entosis (37). MSC destruction was nearly absent in the presence of a ROCK inhibitor (Fig. 2C), suggesting that cannibalism of MSCs occurred through a process resembling entosis. Interestingly, inhibition of ROCK appeared to enhance cell fusion (Fig. 2C), consistent with a recent report (40).

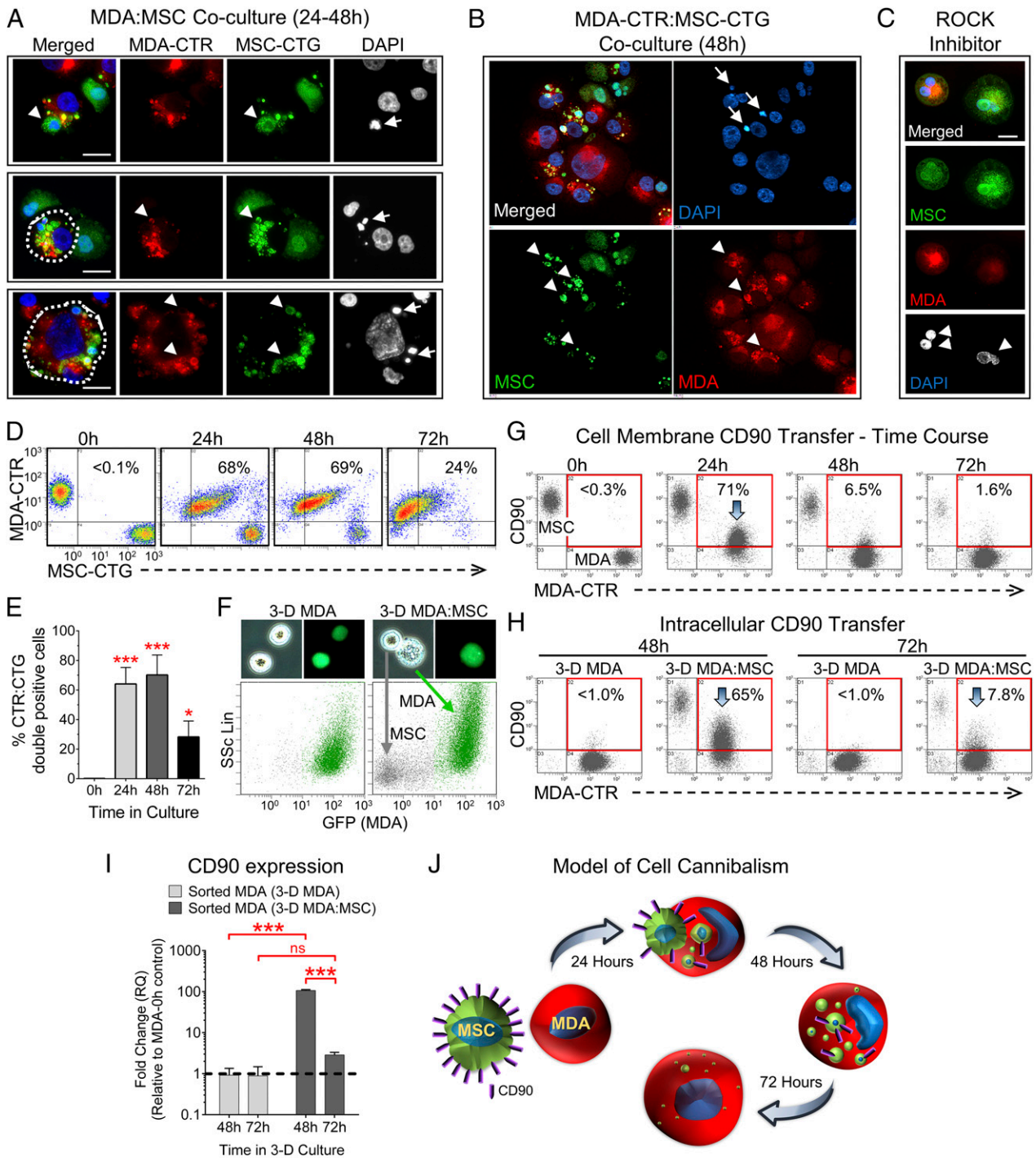
To quantify the extent of interactions, we examined changes in the number of double-positive cells (CTG and CTR), as a function

of time, by flow cytometry (Fig. 2D and E). At 24 and 48 h, nearly 70% of cells within 3D cocultures were double-positive, and by 72 h, most CTG MSCs had disappeared as expected based on our prior results using GFP MSCs (Fig. 1). At 72 h, the number of double-positive cells also decreased to an average of just over 20%, further supporting the notion that MSCs were degraded. Also, cell granularity and/or surface topography of some MDA cells was considerably altered by coculture with MSCs in hanging drops as demonstrated by microscopy and flow cytometry (Fig. 2F). To validate that MSCs were internalized, we monitored time-dependent changes in levels of cell membrane-associated CD90 (Fig. 2G) and intracellular CD90 (Fig. 2H) by flow cytometry. At 0 h, when cocultures were initiated, all MSCs expressed CD90, whereas MDA cells were CD90-negative as anticipated. At 24 h, more than 70% of the CTR MDA cells within 3D cocultures also appeared to be labeled positive for CD90. However, surface expression of CD90 on MDA cells diminished to 6.5% at 48 h and less than 2% by 72 h (Fig. 2G). Interestingly, levels of intracellular CD90 were elevated to 65% in MDA cells at 48 h, a signal that decreased 24 h later (Fig. 2H), suggesting the MSCs were indeed internalized and then rapidly destroyed. Temporary appearance of CD90 signal within the cytoplasm of MDA cells was verified using GFP-expressing MDA cells, which also formed compact spheroids in the presence of MSCs (Fig. S2A–D). These data were further corroborated by results from real-time RT-PCR experiments that showed higher gene expression levels of CD90 in MDA cells purified by FACS from 48-h cocultures compared with 72-h cocultures (Fig. 2I). We also observed that DNA content transiently increased in MDA cells cocultured in hanging drops with MSCs, after the MSCs were internalized (Fig. S3A), further supporting the concept that the MSCs were indeed cannibalized. The data suggested that within 3D hanging drop cocultures, MSCs first encapsulated and/or invaded clusters of MDA cells and then were cannibalized by the cancer cells (Fig. 2J). Overall, the results indicate that hanging drop cultures represent a practical model to study tumor cell cannibalism.

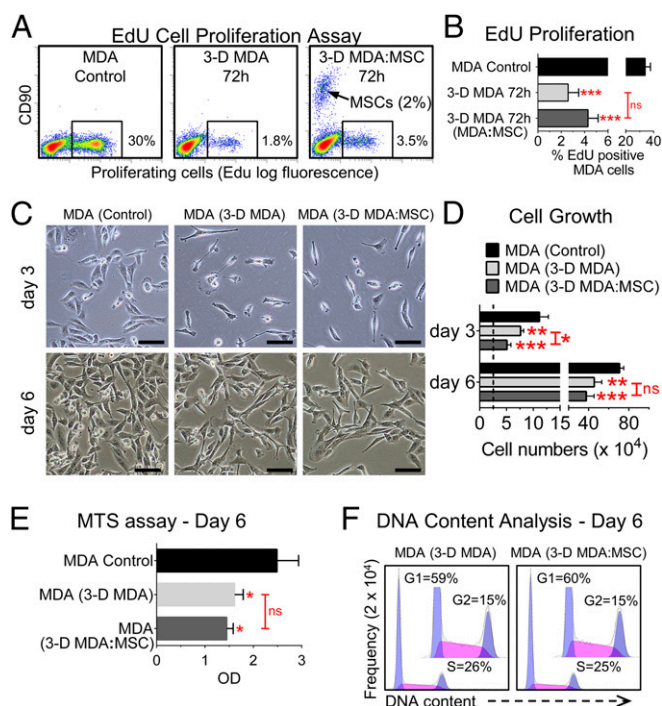
**Evaluation of BCC Growth and Tumorigenicity Following Cannibalization of MSCs.** Here, we first assessed the effects of cannibalism on the proliferation of cells in hanging drop cultures. As expected, the number of MDA cells synthesizing DNA [5-ethynyl-2'-deoxyuridine (EdU)-positive] decreased significantly from more than 30% in standard monolayer cultures to less than 5% in 3D cultures (Fig. 3A and B), probably because of limitations in access to nutrients and oxygen. The results were verified by an experiment where MDA cells were colabeled with EdU and the cell cycle dye FxCycle (Fig. S3). As expected, the number of EdU/S-phase double-positive MDA cells decreased from ~37% in monolayer cultures to 2.7% and 4.2% in 3D MDA cultures and 3D MDA/MSc cocultures, respectively.

Subsequently, we evaluated effects of cannibalism on growth of MDA cells under conventional plastic-adherent conditions. MDA cells were sorted by FACS from 3D cultures based on the absence of CD90 expression (Fig. S4A and B), or high expression of GFP when GFP MDA cells were used (Fig. S5A and B), and then seeded in growth medium containing 10% FBS. As expected, a modest delay in growth of MDA cells (Fig. 3C–E) and GFP MDA cells (Fig. S5C–E) from 3D cultures was observed. This growth delay was slightly greater when the MDA cells were obtained from 3D cocultures with MSCs. It is important to note that the number of population doublings among groups was similar between days 3 and 6, and after 5–6 d in culture, the percentage of cells in S-phase returned to levels comparable to the levels of control cells (Fig. 3F and Fig. S5F). The data suggested that after a brief pause, the original growth rate of MDA cells observed in monolayer cultures was restored. Previously, we detected a similar transient delay in growth of MSCs derived from spheroids (26).

To explore effects of cannibalism on tumor formation and growth, a xenograft model of human breast cancer was induced



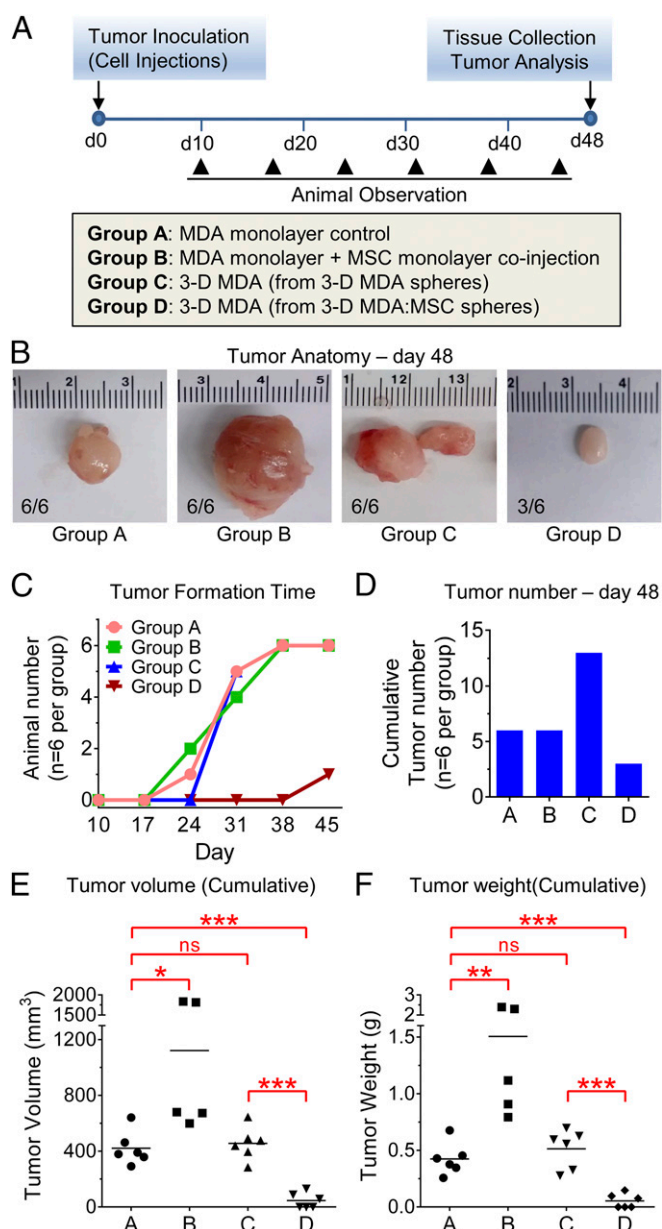
**Fig. 2.** MSCs are readily cannibalized by cancer cells in 3D cultures. (A) Immunofluorescence images of CTG MSCs (MSC-CTG) and CTR MDA cells (MDA-CTR) fixed as cytopsin preparations after dissociating spheroids formed in hanging drop cocultures for 24–48 h. Nuclei were counterstained with DAPI. Most MSCs appeared as small round cells associated with the plasma membrane or cytoplasm of MDA cells (arrowheads). Many internalized MSCs had condensed nuclei (arrows). (Scale bar, 50  $\mu$ m.) (B) Representative confocal image of a cytopsin preparation from A. (C) Representative cytopsin preparation of MDA cells and MSCs cultured in hanging drops for 48 h in the presence of the ROCK inhibitor Y-27632. (Scale bar, 20  $\mu$ m.) (D) Flow cytometric quantification of MDA–MSC interactions in hanging drops over time. Percentage of double-positive cells is shown in the upper right quadrant of each dot plot. (E) Graph displaying replicate values from D ( $n = 3$ ).  $^*P < 0.05$  and  $^{***}P < 0.001$  compared with 0-h time point. (F) Cell granularity was determined by microscopy and flow cytometry [side scatter (SSC)] after 48 h in 3D cultures. (G and H) MDA internalization of MSCs was verified by monitoring time-dependent changes in levels of MDA cell membrane CD90 and intracellular CD90. The percentage of MDA cells expressing CD90 is presented (red box) on the dot plots. Peak expression is highlighted by arrows. (I) Real-time RT-PCR measuring relative changes in expression of CD90 in MDA cells sorted from 3D cocultures at 48 and 72 h, relative to MDA cells cultured as monolayers [relative quantity (RQ) of 1, dotted line].  $^{***}P < 0.001$ ; ns, not significant. (J) Model depicting cannibalism of CD90-positive MSCs by cancer cells in 3D cultures. As shown, CD90-negative MDA cells can appear to express CD90 (purple bars), whereas the membrane of the CD90-positive MSCs is temporarily exposed to the extracellular milieu during engulfment (24 h). Once the internalization process is complete (48–72 h), the CD90-positive MSC membrane is no longer exposed to the cell exterior, resulting in loss of cell surface CD90 (48 h). The MSCs are subsequently degraded within the cytoplasm of the MDA cells, resulting in complete loss of CD90 expression in the MDA cells by 72 h.



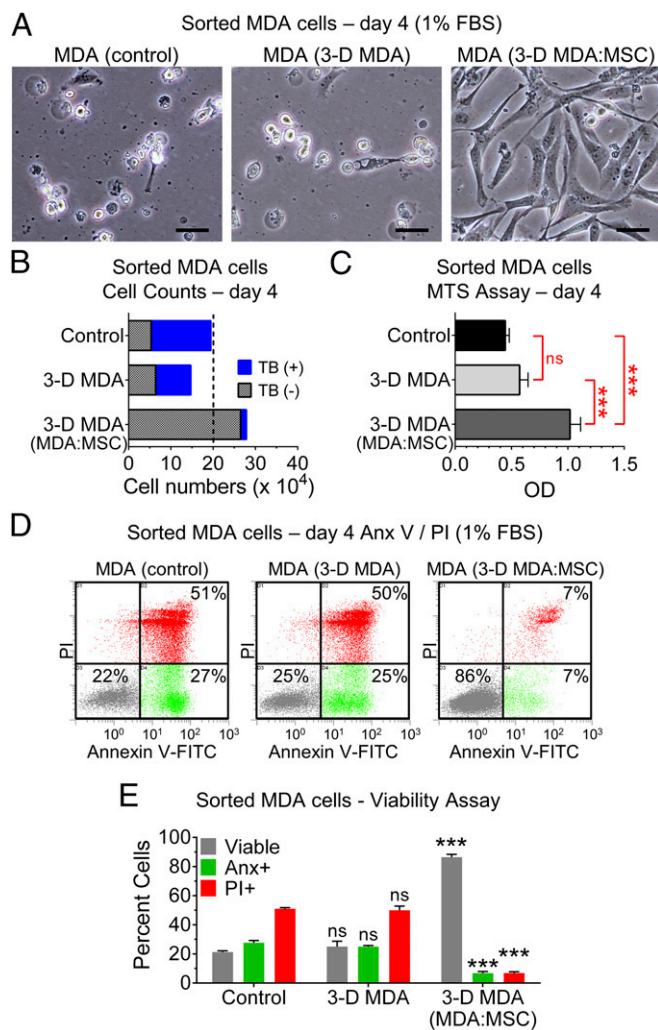
**Fig. 3.** Effects of cannibalization on cancer cell proliferation in vitro. (A) Level of proliferating cells in hanging drop cultures was determined by measuring EdU incorporation into DNA of dividing cells. MDA cells were cultured as monolayers (control), alone in hanging drops for 72 h (3-D MDA 72h), or in hanging drops with MSCs for 72 h (3-D MDA:MSC 72 h). Samples were counterstained with CD90 to exclude MSCs from analysis. Dual-parameter dot plots obtained by flow cytometry are shown. The percentage of EdU-positive cells is presented on the image (box). (B) Graph of EdU-positive cells is presented on the image (box). (C) Images of MDA cells from monolayer cultures (MDA control), 72-h 3D cultures (3-D MDA), and 72-h 3D hanging drop cocultures (3-D MDA:MSC) after purification and seeding of the cells into six-well plates for 3 d and 6 d. (Scale bars, 100  $\mu$ m). (D) Quantification of cell growth by counting cells. The dotted line indicates a seeding density of 25,000 cells per well. Values are presented as means  $\pm$  SD and were analyzed by ANOVA ( $n = 3$ ). (E) MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) proliferation assay after 6 d of growth ( $n = 3$ –4). Values are presented as in D. OD, optical density. (F) Cell cycle analysis of MDA cells by flow cytometry, 6 d after dissociating spheroids from 3D MDA and 3D MDA:MSC hanging drop cultures, and culture of the cells as monolayers. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, not significant.

by injecting MDA cells ( $0.5 \times 10^6$ ) into the left inguinal mammary fat pad of female immune-deficient mice. Tumor formation was observed weekly beginning at day 10, and tissues were collected for analysis on day 48 (Fig. 4A and B). Remarkably, time to tumor formation was delayed by more than 2 wk when mice were inoculated with MDA cells obtained from MDA/MSCs spheroids following cannibalization of the MSCs (group D), and only three of the six mice in this group developed tumors by day 48 (Fig. 4B and C). Moreover, the tumors formed (in group D) were significantly smaller in volume (Fig. 4E) and weight (Fig. 4F) relative to those tumors formed by injecting mice with MDA monolayer control cells (group A) or MDA cells cultured in hanging drops alone (group C). As expected, mice inoculated by coinjections of MDA cells and MSCs formed larger tumors (Fig. 4B–F). Interestingly, cannibalistic cells were not readily discernible in vivo 24 h or 72 h following cell coinjections. Also of interest, multiple tumors were observed in each animal injected with MDA cells from 3D cultures (group C; Fig. 4D). Overall, the data suggested that cannibalization of MSCs by BCCs in a 3D microenvironment exerts tumor-suppressive effects that could be a result of the cells acquiring a dormant phenotype.

**MDA Cells from 3D Cocultures Exhibited a Robust Survival Advantage Under Stressful Culture Conditions.** Dormant cancer cells possess a profound survival advantage when under duress (4). Moreover, cell cannibalism has been considered a mechanism for cells to maintain metabolic fitness when deprived of essential factors (32). Here, we evaluated the effects of nutrient deprivation on viability of cultured MDA cells (Fig. 5A–E) and GFP MDA cells (Fig. S5C–G) obtained from standard adherent monolayers (controls) or sorted from aggregates/spheroids (Figs. S4A and B and S5A and B) produced in hanging drops. The MDA cells were seeded in six-well or 96-well plates and cultured for 4–5 d in medium containing



**Fig. 4.** Cannibalization of MSCs by BCCs suppressed tumorigenicity. (A) Schematic representation of the human breast cancer xenograft mouse model and groups of cells used for injections. (B) Representative images of tumors collected 48 d after cell injections. (C) Graph depicting tumor formation time in each group. (D) Graph showing total number of tumors in each group ( $n = 6$  per group). (E) Measurement of cumulative tumor volume. (F) Measurement of tumor weight. Data, expressed as means  $\pm$  SD, were analyzed by ANOVA and compared using Tukey's posttest ( $n = 5$  or 6 per group). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, not significant.



**Fig. 5.** Cell cannibalism enhanced survival of MDA cells. MDA cells from monolayer cultures (control), 3D cultures (3-D MDA), and hanging drop cocultures (3-D MDA:MSC) were sorted by FACS and plated in nutrient-poor medium (1% FBS) for 4–5 d. (A) Micrographs of MDA cells cultured for 4 d in 1% FBS. (Scale bars, 50  $\mu$ m.) (B) Cell counts and trypan blue (TB) exclusion (dotted line indicates seeded density of 200,000 cells). (C) MTS viability assay. Values are presented as means  $\pm$  SD ( $n = 3$ ). \*\*\* $P < 0.001$ ; ns, not significant. (D) Measurement of cell viability by flow cytometry. Representative dot plots with percentage of dead cells (labeled with annexin V and PI) are displayed. (E) Graph of replicates in D. Data are expressed as means  $\pm$  SD ( $n = 3$  per group). Groups were analyzed by one-way ANOVA and Tukey's posttest. \*\*\* $P < 0.001$  compared with the respective label of both control and 3D MDA groups.

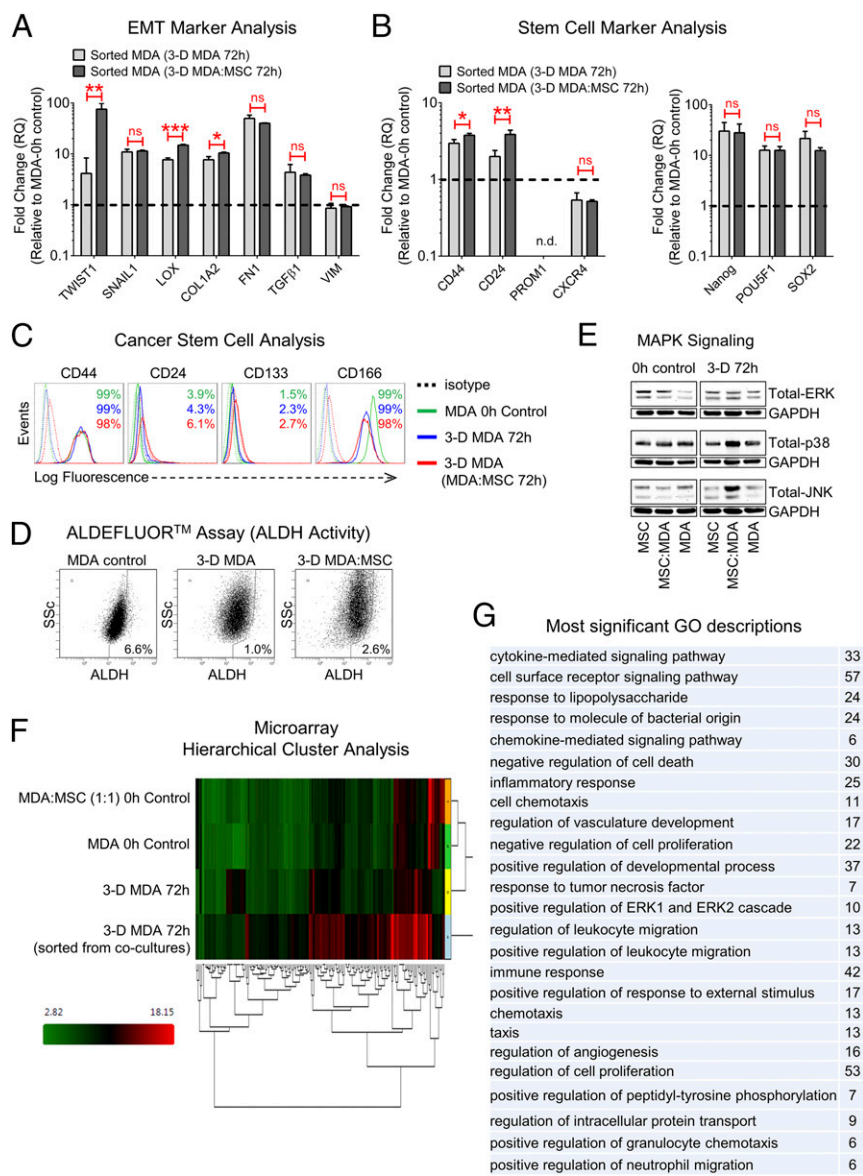
1% FBS. Upon examination of cell morphology, most control MDA cells and MDA cells from 3D cultures were floating in the medium with large amounts of debris/apoptotic bodies/dead cells (Fig. 5A and Fig. S5C). In contrast, MDA cells (or GFP MDA cells) sorted from 3D cocultures, after cannibalizing MSCs, were attached to the dish and displayed a fibroblast-like morphology with numerous long extensions (Fig. 5A and Fig. S5C). As a result, the number of MDA cells obtained after 4–5 d was markedly greater from the 3D MDA/MSc group relative to the control group and/or 3D MDA group (Fig. 5B and C and Fig. S5D and E). Assays for cell viability confirmed that the MDA cells (or GFP MDA cells) from 3D cocultures, after cannibalizing MSCs, were highly resistant to stresses imposed by nutrient deprivation (Fig. 5B–E and Fig. S5G). Specifically, less than 7% of these stress-resistant MDA cells stained positive for trypan blue, whereas more than 50% in control/3D MDA groups stained blue (Fig. 5C).

Consistent with these findings, notably lower levels of apoptotic [annexin V-positive/propidium iodide (PI)-negative] and necrotic [PI- or 7-aminoactinomycin D-positive (7AAD)] MDA cells were observed after cannibalizing MSCs in 3D cocultures (Fig. 5D and E and Fig. S5G). The data suggested that cannibalism of MSCs by cancer cells fuels survival under hostile conditions, a recognizable attribute of dormant cancer cells (4).

**Analysis of MDA Phenotype Following Cannibalism of MSCs.** Next, we aimed to elucidate putative mechanisms underlying the functional effects of cell cannibalism by evaluating changes in MDA cell phenotype. Initially, we measured levels of epithelial-mesenchymal transition (EMT; Fig. 6A) and stem cell markers (Fig. 6B–D) because these phenotypes are considered fundamental for tumor cell dissemination, metastasis, and relapse (9, 10, 41). With the exception of transcription factor *TWIST1* and lysyl oxidase (*LOX*) expression, both of which increased significantly in BCCs following cannibalization of MSCs (Fig. 6A), changes in markers of EMT and cancer stem cells (CSCs) were unremarkable. Moreover, the CD44<sup>hi</sup>/CD166<sup>hi</sup>/CD24<sup>low</sup>/CD133<sup>low</sup>/aldehyde dehydrogenase<sup>low</sup> (ALDH)<sup>low</sup> surface phenotype of the MDA cells was not radically altered by cell cannibalism (Fig. 6C and D). Interestingly, simply transferring MDA cells from plastic-adherent to hanging drop cultures enhanced expression of many EMT markers (Fig. 6A), as well as the pluripotency transcription factors *NANOG*, *POU5F1* (Oct4), and *SOX2* (Fig. 6B). We then evaluated effects of cell cannibalism on stress-activated mitogen-activated protein kinase (MAPK) signaling pathways, which are commonly implicated in cytoprotection and cellular quiescence/dormancy (4). Although changes in the level of total ERK and p38 MAPK proteins were minimal, we did observe a marked increase in expression of jun N-terminal kinase (JNK) in 3D cocultures of MDA cells and MSCs after 72 h (Fig. 6E). Due to time/technical requirements for spheroid processing, measurements of phosphorylated proteins are unreliable; therefore, only levels of total proteins are shown.

To understand mechanisms that might link cell cannibalism to tumor dormancy better, we assessed genome-wide transcriptional changes by microarrays. Expression of numerous genes was increased in MDA cells following their interactions with MSCs in 3D cultures that were not highly expressed by MDA cells or MSCs in monolayer cultures, or by MDA cells cultured in hanging drops alone for 3 d (Fig. 6F). Specifically, our analysis revealed that 204 genes were up-regulated and 43 genes were down-regulated by at least fourfold in MDA cells following cell cannibalism relative to MDA cells cultured alone in hanging drops. In addition, over 30 genes were up-regulated by approximately 10-fold or more (Table S1). As we anticipated, expression of genes involved in cell cycle progression was decreased in both 3D cultures of MDA cells and MDA/MSc cocultures (Table S2). On the other hand, cell cannibalism significantly increased genes with ontologies associated with cytokine and chemokine signaling, inflammatory and immune response, negative regulation of cell death and proliferation, and vascular development, among numerous other terms (Fig. 6G). Gene ontologies related to autophagy were, however, not significantly altered following cannibalism of MSCs. Real-time RT-PCR assays were used to validate findings from microarrays (Fig. 7A) and showed robust increases in expression of factors associated with inflammatory/immune response, including *CSF3* [granulocyte colony-stimulating factor (G-CSF)], *PTGS2* (COX2), *TNFA*, *IL1A*, *IL1B*, *IL6*, *IL8*, *CXCL1*, *CXCL2*, *CXCL10* (IP10), and *CCL20*, as well as the antiapoptotic factor *IF16* and the tumor suppressor *EGRI*. With subsequent ELISAs (Fig. 7B), we found that for most of the cytokines/chemokines tested, the highest levels were secreted by 3D MDA/MSc cocultures relative to both 3D MDA cells and 3D MSCs cultured independently in hanging drops.

Finally, we determined if the enhanced inflammatory secretome of cannibalistic MDA cells was maintained after injection



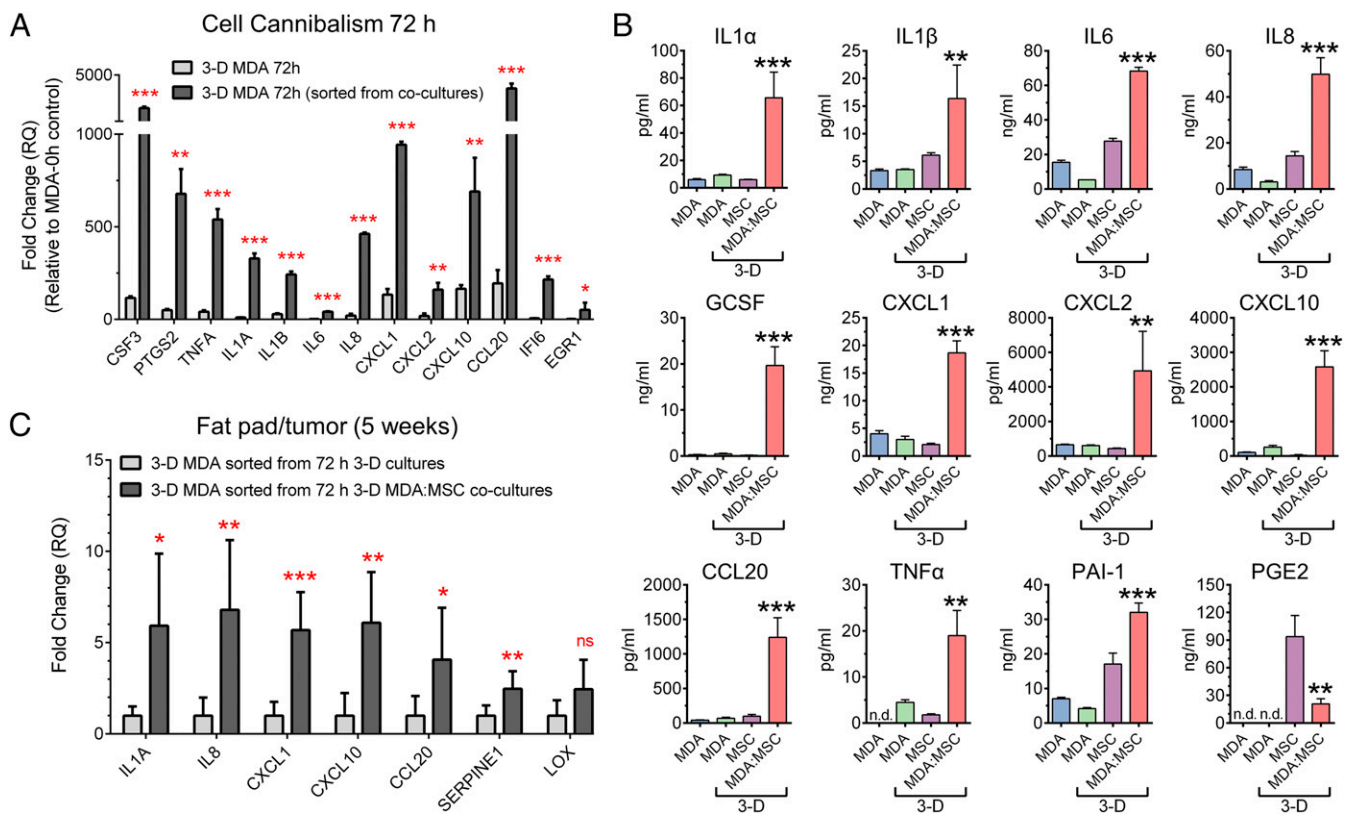
**Fig. 6.** Analysis of BCC phenotype after internalizing and degrading MSCs. (A) Real-time RT-PCR of EMT-related genes. MDA cells were purified from 72-h hanging drop cultures by FACS. Fold changes relative to MDA monolayer cultures (RQ of 1, dashed line) are shown. Values are presented as means  $\pm$  SD and were analyzed by Student's *t* test ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . n.d., not detectable; ns, not significant. (B) Expression of the indicated stem cell markers was assessed by real-time RT-PCR as in A. (C) Flow cytometry assays of select stem cell markers. MSCs (CD90-positive) were removed by selective gating. (D) Measurements of aldehyde ALDH1 activity by flow cytometry. Gates were established from cells coincubated with the ALDH1 inhibitor N,N-diethylaminobenzaldehyde (DEAB). (E) Immunoblots of MAPK signaling factors ERK, p38, and JNK in monolayer cultures of MDA cells and MSCs at 0 h and in 3D hanging drop cultures at 72 h. GAPDH antibody was used as a loading control. (F) Microarray heat map generated by hierarchical clustering of differentially expressed genes (fourfold up-regulated and fourfold down-regulated) in MDA cells sorted from 72-h cocultures after cannibalism of MSCs, relative to the other groups shown. High signal intensities are red, and low signal intensities are green. (G) Most significant gene ontology (GO) terms ( $P < 10^{-4}$ ) for genes up-regulated in MDA cells following cannibalization of MSCs are presented, along with the number of genes that matched each GO term.

of the cells into the mammary fat pad. For these experiments, only  $5 \times 10^4$  MDA cells sorted from 3D MDA cultures or 3D MDA/MSCC cocultures were administered to provide a longer pretumor period for evaluation. Before formation of large palpable tumors, the fat pad tissue/small developing tumors ( $\sim 5$  wk) at the site of injection were collected and the MDA cells were analyzed for expression of select inflammatory markers using human-specific primer/probes. Expression of several inflammatory factors was significantly greater in the injected MDA cells derived from 3D cocultures relative to the MDA cells cultured alone in hanging drops (Fig. 7C), suggesting that the senescence-associated inflammatory secretome of cannibalistic MDA cells was maintained in vivo to some extent. In this experiment, we observed small developing tumors in five of six animals injected with MDA cells from 3D cultures, whereas only one of six mice had a developing tumor in the group injected with MDA cells purified from the 3D MDA/MSCC cocultures, corroborating our prior findings when  $0.5 \times 10^6$  MDA cells were injected (Fig. 4). Taken together, interactions between MDA cells and MSCs that result in cannibalization of the MSCs promote a unique cancer cell gene signature, highlighted by factors involved in inflammation, stress response, and dormancy.

## Discussion

A myriad of signals originating in the milieu surrounding cancer cells strongly influence tumor progression. Here, we used a 3D culture platform to understand further the biological outcome of interactions between BCCs and bone marrow MSCs that potentially transpires following breast cancer dissemination to bone (17) or after the MSCs are actively recruited into primary tumors (42). Specifically, we demonstrated that when MDA BCCs and bone marrow MSCs were permitted to interact directly in 3D cultures, the MSCs quickly encapsulated the MDA cells and promoted formation of compact cancer spheres, and then were engulfed/cannibalized by the BCCs.

From a mechanistic perspective, cell cannibalism, a live-cell feeding behavior, is thought to be distinct from conventional phagocytosis used by macrophages to eliminate apoptotic cells (32, 33, 43). It is also considered distinct from the live-cell engulfment programs entosis (44) and emperipolesis (45) that involve active invasion/penetration of one cell into the cytoplasm of another, although one outcome of entosis, similar to cell cannibalism, is demise of the internalized cell (44). Another difference is that entosis is regarded as a homotypic cell-in-cell



**Fig. 7.** Cytokine production is augmented in 3D hanging drop cocultures of MSCs and MDA cells. (A) Real-time RT-PCR assays of inflammatory markers/survival factors expressed by MDA cells after culture for 72 h in 3D hanging drops or sorted from 72-h 3D MDA:MSC cocultures. Fold changes were determined from MDA monolayer cultures (RQ of 1). (B) ELISAs of select cytokines/chemokines secreted by MDA cells that were cultured for 72 h as in A. Data are displayed as means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and not significant (ns) compared with all other groups (one-way ANOVA with Tukey's posttest). n.d., not detectable. (C) Real-time RT-PCR assays of select inflammatory/mesenchymal markers expressed by MDA cells 5 wk following injection of  $5 \times 10^4$  sorted cells into the mammary fat pad. Data are shown as means  $\pm$  SD and were analyzed by Student's  $t$  test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , and not significant (ns) compared with the respective label of the 3D MDA control group.

interaction, whereas cannibalism and emperipolesis can occur between cells of different types (31, 32). Interestingly, we observed that the percentage of cannibalistic cells in 3D cocultures was reduced in the presence of a ROCK inhibitor, suggesting that the molecular machinery normally implicated during entosis (37) was also engaged here. Future studies are needed to define the precise mechanisms driving cancer cell cannibalism of MSCs.

Here, we focused on the consequences of cell cannibalism because few pathophysiological roles for the processes have been revealed. Nonetheless, observations of the phenomenon are abundant, particularly with regard to breast cancer and other carcinomas (34–37). In fact, cell cannibalism has been considered an indicator of tumor aggressiveness and perhaps a method to distinguish benign from malignant lesions (34, 35, 46, 47). Despite these observations, the impact of cannibalistic events on tumor progression has remained, in part, a mystery. Initially, cell cannibalism was considered a feeding behavior that would allow cancer cells to obtain resources for survival similar to that observed in unicellular microorganisms (33), or in the case of lymphocyte engulfment, a potential mechanism to escape immune recognition (47). Additional studies reported that cell cannibalism or related processes might facilitate oncogenesis by obstructing cytokinesis and triggering formation of aneuploid cells (36, 48), or by permitting transfer of genes that harbor beneficial traits (49, 50). In some contexts, however, cancer cell internalization of neighboring cancer cells (51) or of umbilical cord-derived MSCs (39) was also shown to result in termination of the host cell, an event that would ultimately oppose tumor growth and metastasis.

Here, we report a different overall result of cannibalism and alternate hypothesis of its effect. Specifically, we showed that after cannibalizing bone marrow-derived MSCs, the BCCs displayed a robust ability to survive in conditions with diminished access to nutrients as anticipated, but their potential to form tumors in mice was markedly suppressed. Together, our results indicated the BCCs acquired a phenotype that is characteristic of dormant cancer cells or that, at a minimum, encouraged tumor dormancy after inoculation. The 3D tumor niche model we used here was essential to augment cell feeding behaviors effectively, and therefore determine consequences of cell cannibalism, because we did not obtain a high degree of cell cannibalism in 2D adherent cultures or in vivo after coinjections of MDA cells and MSCs, consistent with a prior report showing that cell cannibalism is a relatively infrequent event in tumors of patients with breast cancer (34). Our findings were also consistent with the paradigm that processes resulting in formation of cell-in-cell structures are driven by loss of cell adhesion (44) and competition between cells for available nutrients (52). Importantly, our findings corroborate prior studies that showed MSCs can provoke BCC quiescence/dormancy through transfer of cell cycle inhibitory microRNA via gap junctions and/or exosomes (53, 54). Given the rapid cannibalism of MSCs in 3D cultures, the contribution of microRNA transfer to tumor dormancy in our study was likely minimal. However, we did note that TWIST1 was significantly up-regulated in MDA cells derived from 3D cocultures. These results were intriguing because a recent report showed a relationship between TWIST1 expression and preservation of growth-inhibitory signals such as p38, as well as a correlation between TWIST1



expression in disseminated BCCs and recurrent disease in patients, suggesting a potential role for TWIST1 in micrometastatic dormancy (55). In addition, we observed up-regulated expression of LOX, a TWIST1 transcriptional activator that is implicated in MSC-mediated breast cancer malignancy (56), and of JNK, a stress-activated MAPK signaling factor that was previously reported to phosphorylate and stabilize TWIST1 protein (57). Taken together, these data suggest that TWIST1 could, in part, influence BCC dormancy following cannibalization of MSCs through cooperative regulation by LOX, JNK, and p38.

With further evaluation of MDA phenotype by microarray assays, we observed that cannibalism of MSCs resulted in a robust up-regulation of numerous cytokines/chemokines. Although the role of inflammation in tumor progression has been controversial, the results were intriguing because inflammatory mediators such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, CXCL1, CXCL2, CCL20, GCSF, and PAI-1 (SERPINE1), all of which were up-regulated following MSC cannibalism, are products of senescence cells and are key factors of the senescence-associated secretory phenotype (SASP) (58–60). Although senescent cells are generally considered to have permanent growth restrictions, they are also recognized to be highly active and contribute to a variety of physiological and pathological processes (58). The SASP, in particular, provides senescent cells with diverse functionality. In opposition to tumor growth, factors associated with the SASP can alert the immune system (61), reinforce the phenotype, and propagate growth arrest signals to surrounding cells (62, 63), implying that senescence programs exert bystander effects (64). In fact, senescent MSCs were recently demonstrated to secrete factors with antitumor activity (65). Paradoxically, senescent cells can also contribute to tumor progression, perhaps through the inflammatory cytokines they produce and/or as a consequence of aging (66). Further studies are warranted to evaluate the role of the SASP in regulating tumor dormancy and relapse.

It is important to note that cell senescence can be triggered not only by telomere attrition (replicative senescence) but also by various cellular stresses (acute senescence) and autophagy (67), a process that, by definition, has been considered an exacerbation or form of cell cannibalism (i.e., self-cannibalism) (68), although autophagy and xeno-cannibalism appear to engage, at least in part, distinct mechanisms (69). It is also important to note that cell senescence is governed largely by tumor suppressors such as p53, and that p53 inactivation can reverse growth restrictions (70). Subsequently, dormant cancer cells with p53 mutations might have evolved an ability to escape some consequences of senescence, such as permanent growth arrest, but perhaps exploit the benefit of other functions, including the SASP. Taken together, these factors could explain why MDA cells obtained from 3D MDA/ MSC cocultures in our study propagated *in vitro*, after a slight delay, but showed limited tumorigenicity.

Overall, this study has greatly expanded our knowledge of the biological outcome of interactions between cancer cells and bone marrow MSCs. Our results indicate that cannibalism of MSCs within the tumor niche represents a unique mechanism supporting cancer dormancy, which is a logical cause-and-effect relationship because both phenomena (i.e., cell cannibalism, dormancy) implicate cell survival strategies, are associated with growth arrest, are observed most often in highly aggressive cancers, and are represented by a minor/residual population of cells. However, because the resulting MDA cells were not uniquely stem cell-like, the data suggested that cannibalistic/dormant cells might represent a population distinct from conventional CSCs, which also exist as a minor drug-resistant cell population (41). It is important to note

that tumor dormancy is governed by a variety of contextual cues, many of which remain a mystery. In fact, there is evidence that primary tumor dormancy and metastatic dormancy are distinct processes (10, 71), and that reactivation requires cancer cells to overcome organ-specific signals of growth suppression (11). Moreover, evidence is emerging that MSC source and status might contribute to cancer cell feeding behaviors (72). Thus, regulation of tumor dormancy initiated by cannibalizing MSCs after their recruitment to the primary tumor could be discernible from dormancy provoked by cancer cells that metastasize to bone marrow or other tissues and, afterward, eat the native MSCs. It would be interesting in future studies to evaluate these potential differences. Nonetheless, the therapeutic implications from our results are notable. On one hand, we have identified an outcome and unique cancer phenotype associated with BCC-MSC cross-talk that could open new avenues for therapeutic intervention. Additionally, the study suggests that our practical 3D coculture model could be a useful tool to understand and exploit antitumor properties of MSCs and cell cannibalism further.

## Materials and Methods

Detailed information is provided in *SI Materials and Methods*.

**Cell Culture and 3D Modeling of Cancer Cell–MSC Interactions.** Human bone marrow-derived MSCs were obtained from the Center for Preparation and Distribution of Adult Stem Cells at Texas A&M University, Health Science Center. The MSCs were cultured as previously reported (26). For the experiments described herein, MSCs in passage 3 or 4 were used. Human adult DFs and the human cancer cell lines MDA-MB-231, A549, PANC-1, and PC3 were purchased from the American Type Culture Collection. MDA cells expressing GFP (CopGFP) were kindly provided by Fei Liu, Institute for Regenerative Medicine, Texas A&M University, College of Medicine. All cultures were performed under sterile conditions in a humidified atmosphere at 37 °C in 5% (vol/vol) CO<sub>2</sub>.

Hanging drop cultures were used to generate 3D aggregates/spheroids as we previously described (26–29, 38), but with some modifications. Briefly, cells were suspended in 30- $\mu$ L droplets of growth medium, at 10,000–15,000 cells per drop, on the underside of culture dish lids (Corning) for up to 3 d. Cocultures were prepared by mixing MSCs and cancer cells at a 1:1 ratio, unless otherwise indicated. In some experiments, cells were prelabeled with fluorescent tags. For most downstream assays, the aggregates/spheroids that formed in hanging drops were dissociated by chemical (trypsin/EDTA) and mechanical (pipetting) means to obtain a single-cell suspension. For Western blots, intact aggregates were collected, washed in PBS, and lysed. Levels of secreted inflammatory cytokines were determined by ELISA using conditioned medium collected from hanging drops after 72 h.

**Breast Cancer Xenograft Model.** Animal studies were approved by the Institutional Animal Care and Use Committee of Texas A&M Health Science Center and Scott & White Healthcare. MDA BCCs ( $0.5 \times 10^6$  in 100  $\mu$ L of HBSS) obtained from monolayer cultures and 3D hanging drop cultures were injected into the left inguinal mammary fat pad of female NOD/SCID mice (NOD.CB17-Prkdcscid/J; Jackson Laboratory) at 2–3 mo of age. In one group, the MDA cells were coinjected with MSCs. Tumor formation was monitored at regular intervals for 48 d, after which tumors were excised and evaluated. In a separate experiment,  $5 \times 10^4$  MDA BCCs were injected. After ~5 wk, fat pads/small tumors were collected and analyzed for expression of select inflammatory markers by real-time RT-PCR using human-specific primer/probes (Life Technologies).

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