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Partial acquisition of stemness properties in tumorspheres obtained from interleukin-8-treated MCF-7 cells

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Abstract

The interleukin-8 is an important regulator of the tumor microenvironment, promoting the epithelial–mesenchymal transition and the acquisition of stem-like cell properties in cancer cells. The tumorsphere-formation assay has been used for the identification of cancer stem cell. Interleukin-8 induces the formation of larger tumorspheres in Michigan Cancer Foundation-7 (MCF-7) cells, suggesting cancer stem cell enrichment. In this work, we aimed to study the phenotypic and functional characteristics of the cells present within the tumorspheres of MCF-7 cells previously treated with interleukin-8. MCF-7 cells treated for 5 days or not with this cytokine were further cultivated in ultralow attachment plates for another 5 days to allow tumorspheres formation. We showed that the enhanced sphere formation by MCF-7 cells was not a consequence of higher cell proliferation by interleukin-8 stimulation. Despite maintaining an epithelial–mesenchymal transition phenotype with the presence of epithelial and mesenchymal markers, basic stemness properties were impaired in tumorspheres and in those treated with interleukin-8, while others were increased. Self-renewal capacity was increased in interleukin-8-treated cells only in the first generation of tumorspheres but was not sustained in consecutive assays. Accordingly, self-renewal and reprogramming gene expression, differentiation capacity to adipocytes, and clonogenicity were also impaired. We showed also that tumorspheres were enriched in differentiated luminal cells (EpCAM+/CD49f−). Nevertheless, cells were more quiescent and maintain a partial epithelial–mesenchymal transition, consistent with their increased resistance to Paclitaxel and Doxorubicin. They also presented higher migration and interleukin-8-directed invasion. Therefore, the breast cancer cell line MCF-7, having a low stemness index, might partially acquire some stem-like cell attributes after interleukin-8 stimulation, increasing its aggressiveness.

Keywords

Cancer stem cells, interleukin-8, self-renewal, drug resistance, migration/invasion

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Introduction

The cancer stem cell (CSC) hypothesis suggests that tumors are driven and maintained by a minor cell subpopulation that displays stem-like cell properties (self-renewal and multilineage differentiation ability).[1–3] Mani et al.[4] and Morel et al.[5] showed a close relationship between CSC and the epithelial–mesenchymal transition (EMT), a biological program consisting of some biochemical/cellular modifications allowing cells to shift from an epithelial stationary state to a mesenchymal migratory phenotype.[6,7] The EMT/CSC condition represents an advantage for cancer progression and

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metastasis.[8,9] Recently, it has been shown that the EMT and CSC programs are temporary and reversible processes characterized by many intermediate states, the hybrid phenotypes in the EMT, and the transient states of enhanced plasticity in CSC, endowing cells with tumor-initiating, invasion, and metastatic capabilities.[10–13]

The tumor microenvironment (TME) significantly influences the properties of CSC. In particular, abundantly infiltrated cells secrete interleukin-8 (IL-8) having a considerable pro-tumorigenic effect.[14] This is achieved by its binding to the CXCR1/CXCR2 receptors and modulation of several signaling pathways,[15–17] affecting the expression of the EMT-associated transcription factors (TFs) (SNAI1, ZEB1, and TWIST).[18] Accordingly, high levels of IL-8 were responsible for augmenting the invasiveness of a panel of breast cancer cell lines.[19,20] We have shown that exogenous addition of IL-8 promotes both an EMT and the acquisition of stem-like cell properties in the non-secreting IL-8 luminal breast cancer cell line Michigan Cancer Foundation-7 (MCF-7).[21] Also, IL-8 accurately simulates the effect of senescence in CSC properties and tumor-initiating capabilities.[21] This is striking in several ways; first, because senescence is another key cellular response to drug resistance;[22] second, since cells that escape from senescence gain increased aggressiveness;[23] and third, because the elimination of CSC or the blocking of specific signals from TME are the focus of new cancer therapies.[24–27]

In fact, CSC properties (i.e. heterogeneity, plasticity, and self-renewal) are connected to disease recurrence and drug resistance.[28–30] For this reason, it would be useful to determine what specific stem properties the CSC have, in other words, how much stemness do they acquire in particular circumstances. This issue was recently addressed using bioinformatics approaches and a strong correlation of stemness with dedifferentiation and pluripotency was found.[31,32] In breast cancer, a higher stemness index (mRNAsi) is associated with dedifferentiation processes, basal cancer subtypes, p53 mutations, and spread of tumor cells.[33] In another study in breast cancer, the mRNAsi was associated to cell fate decisions, that is, cell cycle, cellular senescence, and mitotic division.[34] Of note, the cancer breast luminal A subtype was the least associated with stemness.[33–35] Nevertheless, in these studies, genomic data were obtained from primary tumors that are already in pre-configured stem states. Interestingly, the majority of breast cancer cell lines contain CSC, although in a very variable range, as evaluated by the Aldefluor(+) cell population.[36] In particular, the MCF-7 cell line possesses less than 1% of CSC,[36] in total agreement with its low stemness index.[33–35] Since it would be important to distinguish between the inherent stemness of cancer cells and that induced by cues from TME, an experimental system in which cells are able to acquire features of stemness would reveal better CSC function dynamics. In this sense, the luminal breast cancer cell line MCF-7 seems to be ideal.

A tumorsphere-forming assay, in low-binding conditions, was developed to identify, obtain, and propagate these CSC.[37] Also, breast tumor cells and cell lines are able to form tumorspheres, in a similar assay used to isolate and propagate CSC.[37] Interestingly, previous incubation of MCF-7 cells with IL-8 induced a faster and larger (>500 μm) tumorsphere formation than untreated cells.[21] In this work, we aimed to study if this higher sphere-forming ability may indicate the acquisition of particular stem-like cell functions by the MCF-7 cells. We hypothesized that the generation of an EMT by IL-8 stimulation, followed by induction of tumorspheres formation in MCF-7 cells, would allow to determine the acquisition of some particular stemness properties. In addition, the characterization of the cells present within these spheres would allow the understanding of CSC dynamics, their relationship to progenitors, and more differentiated cells.[38]

Materials and methods

Culture of MCF-7 cells

The MCF-7 (HTB-22) cell line was obtained from the ATCC. Cells were cultured in complete RPMI 1640 medium (Gibco) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco) and incubated in standard conditions. Fresh MCF-7 cells were periodically thawed for the different experiments.

IL-8 treatment

MCF-7 cells were cultured at a density of 1×10^5 cells/mL until they reached 40% confluence, washed with phosphate-buffered saline (PBS) 1×, and then serum-starved overnight. Then, 50 ng/mL of hrIL-8 (PHC0084, Invitrogen) were added in Roswell Park Memorial Institute (RPMI) 1640 medium with 0.5% FBS, and cells were further cultured for 5 days; fresh medium was added every 2 days. MCF-7 cells cultured in RPMI 1640 with 0.5% FBS were used as control.

Tumorspheres-forming assay

MCF-7 cells were seeded at a density of 5×10^4 cells/mL in ultralow attachment 6-well plates (Nalgene-Nunc International). Cells were grown for 5 days in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium supplemented with 20 ng/mL basic fibroblast growth factor (PHG0266, Gibco), 20 ng/mL epidermal growth factor (PHG0315, Gibco), 10 μg/mL insulin transferrin selenium (Sigma), 2% B27 supplement (Gibco), 0.5 μg/mL hydrocortisone (Sigma),

and 1.5% methylcellulose (Sigma). Every 2 days, 500 μL of fresh medium was added to each well. Tumorspheres with a size greater than 100 μm were counted. Tumorspheres from adherent cells without previous stimulation with IL-8 were used as a control.

Cell proliferation assay and Ki67 expression

MCF-7 cells were seeded at a density of 1×10^4 cells/mL in duplicates in 24-well plates and cultured as mentioned above. On day 1, 2, 3, and 5, cells were counted, and viability was evaluated using the Trypan blue exclusion method. Control and IL-8-treated adherent cells were labeled with Ki67 according to the manufacturer's guidelines (Biolegend). The expression of Ki67 was evaluated on day 5 by flow cytometry and data analysis was performed using FlowJo software (FlowJo, LLC). At least 50,000 cells were analyzed in each experiment.

RNA extraction and qRT-PCR

Total RNA was extracted from adherent cells and from tumorspheres in the different conditions. Adherent cells were harvested by trypsinization on day 5. Tumorspheres were collected on day 5 and passed through a cell strainer with a pore size of 100 μm (Corning) and they were placed in a 50-mL Falcon tube and washed twice with PBS 1× before RNA extraction (Supplementary information).

Western blot assay of EMT markers

Total protein lysates were prepared from adherent cells and from tumorspheres in the different conditions using 2× Assay/Lysis Buffer (Cell Biolabs, Inc., 240102) and a protease inhibitor cocktail (Sigma, P8340). Protein quantification was done using the Pierce BCA Protein Assay Kit (Thermo scientific). Details of Western blot (WB) and antibodies used are provided in Supplementary information.

Consecutive tumorsphere-forming assay

MCF-7 cells from untreated controls and previously IL-8-treated cells were seeded at a cell density of 5×10^4 cell/mL and stained with CellTrace™ CFSE according to the manufacturer's guidelines (Invitrogen). Sphere formation was induced as described above (first generation), and after 5 days of cultures, the tumorspheres were trypsinized for 10 min at 37°C, mechanically dissociated by repeated pipetting and seeded in the same conditions as above (second generation). The procedure was repeated once more (third generation). Tumorspheres were observed by fluorescence microscopy, counted, and photographed.

Wound-healing, directed-cell migration, and invasion assays

After 5 days of culture in adherent conditions or as tumorspheres, cells were harvested by trypsinization, counted, and seeded in complete medium in 24-well plates. Wound-healing, Transwell (TW) migration, and invasion assays were performed as described in Supplementary information.

Differentiation assays to adipo-, chondro-, and osteogenic lineages

After 5 days of culture in adherent condition or as tumorspheres, cells were harvested by trypsinization, counted, and seeded (2×10^4cells/mL) in complete media for 24 h in 24-well plates for the differentiation assay (Supplementary information).

Gene expression analysis of multipotent differentiation

Nine days after the differentiation induction, cells were harvested by trypsinization and total RNA was extracted, and processed as described above. PPAR-γ, Aggrecan, and ALP expression was evaluated for adipo-, chondro-, and osteogenic differentiation, respectively. Controls and data analysis were done as described above. The specific sets of primers used are listed in Supplementary Table 1.

Chemoresistance and MTT cell viability assay

After 1 day of culture as tumorspheres, the treatment with Paclitaxel (PLX, 0.5 and 1 μM) and Doxorubicin (DOX, 0.5, 1, and 2 μM) was initiated. After 72 h, cell viability was determined by the MTT assay (details are provided in Supplementary information).

FACS analysis

After trypsinization, 1×10^6 MCF-7 cells from adherent conditions and from tumorspheres were resuspended in 500 μL of PBS 1× with 0.2% bovine serum albumin. Details of the procedure and antibodies used are provided in Supplementary information.

Limiting dilution assay

The tumorspheres-forming assay was performed at different low cell densities (1.000, 500, and 100 cells/mL) for 5 days. The frequency of CSC and the statistical analysis was performed using the extreme limiting dilution analysis (ELDA) software.[39]

Clonogenic assay

A density of 500 cells/mL from adherent condition and cells obtained from tumorspheres were seeded in triplicates into plates in six-well plates and cultured in complete medium for 12 days (fresh medium was added every 3 days). The medium was removed and washed twice with PBS 1 ×, and then colonies were stained with Crystal violet and counted with Image J software.[40]

Statistical analysis

Statistical analysis was performed with GraphPad Prism v5 software (GraphPad Software, Inc.). All data were expressed as the mean and standard error of the mean (SEM). Student's t-test was used for tumorsphere-forming assay, Gene expression analysis, wound-healing, directed-cell migration, and invasion assays. One-way analysis of variance (ANOVA) was used for clonogenic assay and two-way ANOVA was used for consecutive tumorsphere-forming assay, and chemoresistance (MTT cell viability assay). Significant p values were considered as follows: *$p < 0.05$; **$p < 0.01$; or ***$p < 0.001$.

Results

The formation of larger tumorspheres by IL-8 stimulation is not due to a higher rate of cell proliferation

IL-8 stimulation of MCF-7 induced more (Figure 1(a)) and larger (Figure 1(b)) tumorspheres than control cells. Even if the number of spheres is the same, the size increases with time (Figure 1(c) and (d)). We first sought to investigate if these differences in size were due to a higher proliferation rate induced in IL-8-stimulated cells. As can be seen in Supplementary Figure 1(a), there were no differences in the proliferation rate between IL8-stimulated and untreated MCF-7 cells. In addition, the cell proliferation marker Ki67 was equally expressed in both conditions (Supplementary Figure 1(b)). MCF-7 cells stimulated or not with IL-8 were labeled with CFSE and then tumorspheres were induced to form. As can be seen in Supplementary Figure 2(a), dividing cells (low green fluorescence) were more abundant in control tumorspheres. At day 5, cells with CFSE fluorescence (more quiescent) were more evident in IL-8-treated cell cultures (Supplementary Figure 2(b)). Because the tumorspheres are first selected in a cell strainer (with a mesh of 100 μM) and then counted, it is then likely that smaller (< 100 μm) spheres are formed in the control dishes. We performed the same experiment, but at the end of the incubations period, tumorspheres were dissociated and total number of cells were counted. As can be seen in Figure 1(e), the number of cells was very similar in MCF-7 treated or not with IL-8. These experiments suggest that the larger size of the tumorspheres observed after the treatment with IL-8 is not due to a greater proliferation of adherent MCF-7 cells treated with the chemokine or to a late increase in cell proliferation when the IL-8-treated MCF-7 cells are induced to form tumorspheres. IL-8 stimulation and tumorsphere-formation induction must change the properties of the cells in some unknown way to induce larger spheres

An EMT program, induced by IL-8 in adherent cells, is partially maintained during tumorspheres formation

We then sought to investigate if the expression of EMT cell markers—which were induced in adherent MCF-7 cells after IL-8 stimulation (Supplementary Figure 2(c))—were maintained after the sphere-formation assay. The epithelial markers, TJP1 and E-cadherin, were down-regulated in tumorspheres formed after IL-8 stimulation, while the expression of the EMT-TFs, SNAI2, and ZEB1 remained high in IL-8-stimulated MCF-7-derived tumorspheres (Figure 2(a)), as evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Nevertheless, E-cadherin expression in tumorspheres—whether previously treated or not with IL-8—was higher than in adherent MCF-7 cells when analyzed by WB (Figure 2(b)). Densitometric analysis showed that E-cadherin expression was increased by more than 50% in tumorspheres compared to adherent cells (Supplementary Figure 2(d)). SNAI2 expression was found increased in tumorspheres compared to adherent cells, but again this effect was independent of IL-8 treatment (Figure 2(b); Supplementary Figure 2(d)). ZEB1 expression was very similar in all conditions. Therefore, the tumorsphere microenvironment partially maintains an EMT-like program in MCF-7 cells with the presence of cells with the simultaneous expression of epithelial and mesenchymal markers. Nevertheless, the modulation of these changes by IL-8 was reduced.

CSC frequency is not increased, and self-renewal is not sustained in tumorspheres obtained from IL-8-treated MCF-7 cells

The permanence of CFSE signals for a longer period of time in cells from tumorspheres obtained from MCF-7 cultures in the presence of IL-8 (Supplementary Figure 2(a) and (b)), suggested that they might be enriched in quiescent cells, a feature of some CSC. We then proceeded to determine the frequency of CSC using the limiting dilution assay.[39] CSC were not significantly enriched in cells obtained from IL-8-derived tumorspheres (Supplementary Table 2). In theory, serial

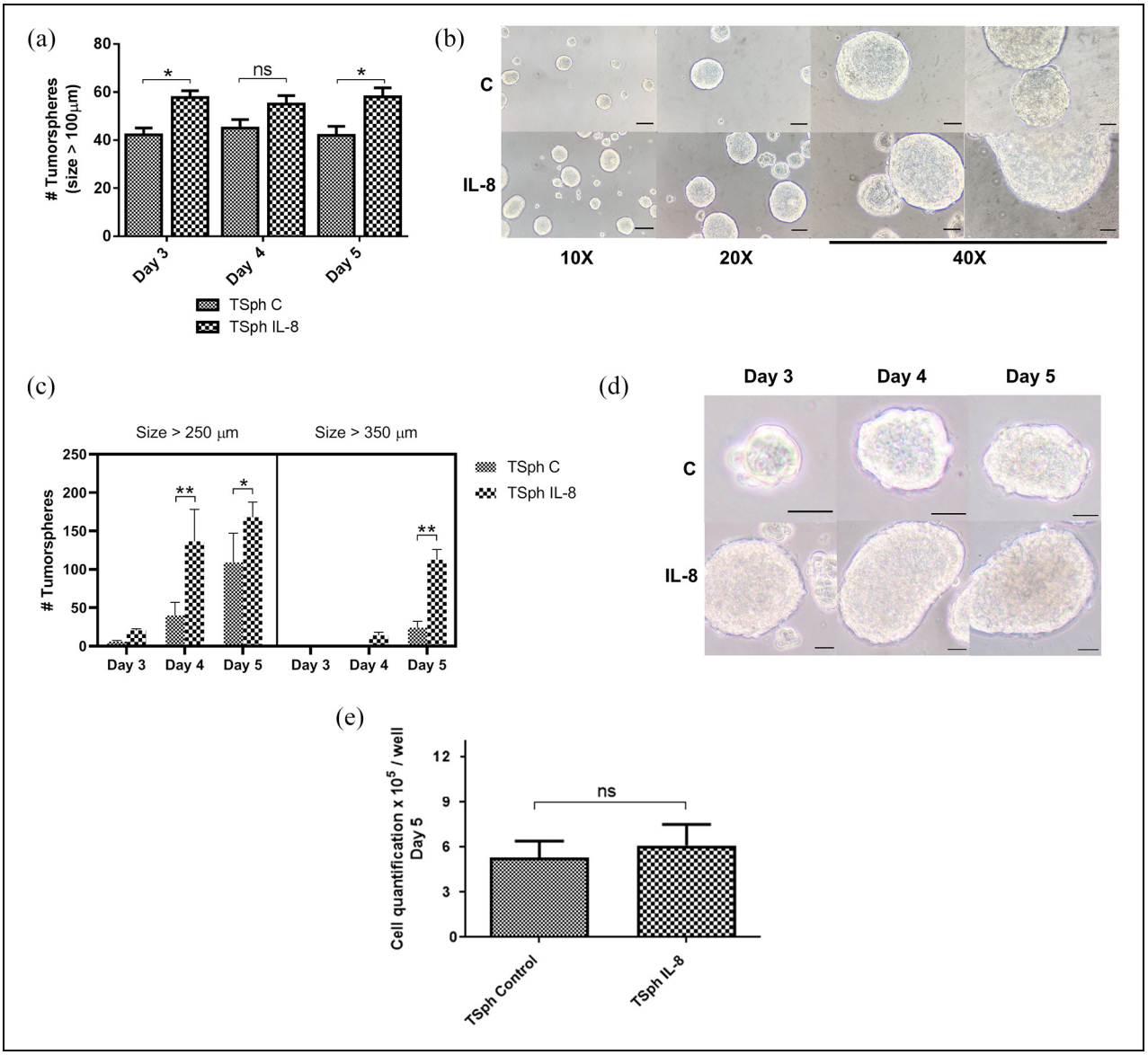

Figure 1. Tumorsphere-formation capacity of MCF-7 cells treated or not with IL-8. (a) Quantification of the number of tumorspheres (TSph) with size >100 μm in days 3–5 in the presence (TSph IL-8) or absence (TSph C) of IL-8. (b) Morphology and size of TSph after 5 days of culture at different magnifications in the presence (IL-8) or absence (control, C) of IL-8. A representative image of the experiments is shown. The scale bar represents 100 μm. (c) Number of tumorspheres of different sizes (>250 and >350 μm) on day 3, 4, and 5 in the presence (TSph IL-8) or absence (TSph C) of IL-8. (d) Representative images of the different tumorspheres on day 3, 4, and 5. The scale bar represents 100 μm. (e) Quantification of cells isolated from tumorspheres after trypsinization on day 5. Cell count was done using the Trypan blue exclusion method. Bars represent the standard error of the mean SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns: not significant).

tumorsphere-forming assays should evidence progenitor or stem cells ability of self-renewal, with progenitor cells decreasing this ability in few (two to three) generations, while CSC maintaining it indefinitely.[41] This evaluation[38] showed more tumorspheres in the IL-8-treated cells (166 ± 17) than in the controls (85 ± 5) ($p < 0.001$) (Figure 2(c)). In the second and third generations, we found a drastic reduction in the number of tumorspheres ($p < 0.001$) in both conditions. These results showed that although IL-8-treated cells were very efficient in producing tumorspheres, they lose its self-renewal capacity after the first generation.

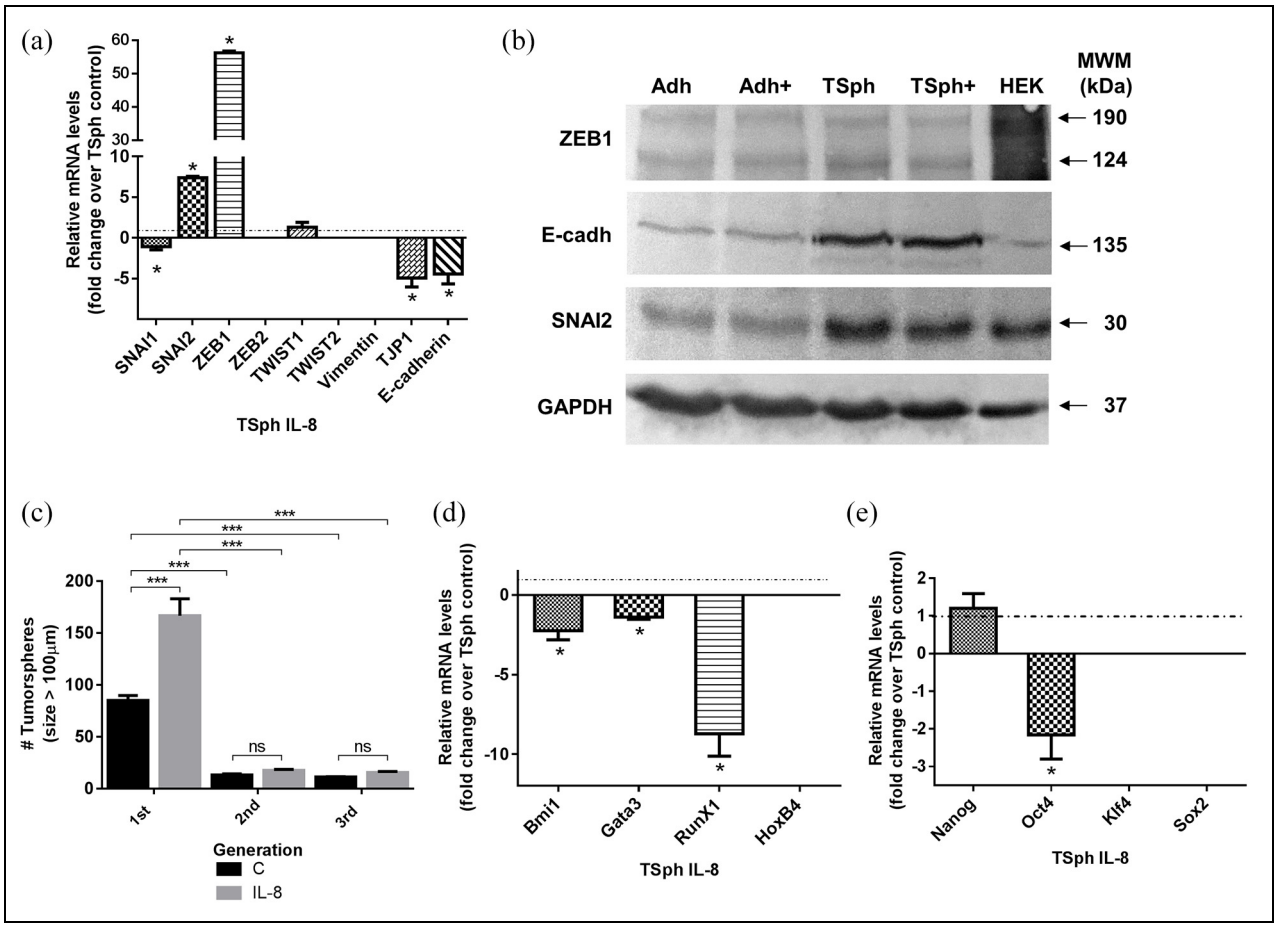

Figure 2. EMT markers, self-renewal, and cell reprogramming gene expression. (a) Gene expression levels of the EMT-TFs SNAI1, SNAI2, ZEB1, ZEB2, TWIST1, TWIST2, Vimentin, TJP1, and E-cadherin (E-cadh) in TSph cells previously treated with IL8, as determined by qRT-PCR. The values were normalized to GAPDH and fold changes were calculated with respect to control cells. (b) Western blot analysis for ZEB1, E-cadh, SNAI2, and GAPDH. Adh: IL-8 untreated adherent cells (control); Adh+: IL-8-treated adherent cells; TSph: IL-8 untreated tumorsphere cells; TSph+: IL-8-treated tumorsphere cells; HEK: HEK293 cell line (positive control for ZEB1 and SNAI2); MWM: molecular weight markers. (c) Quantification of the number of tumorspheres (size $>$ 100 μm) in three consecutive tumorspheres assays in the presence (IL-8) or absence (C) of IL-8. (d) Gene expression levels of the self-renewal TFs Bmi1, GATA 3, RunX1, and HoxB4 in TSph previously treated with IL-8, as determined by qRT-PCR. The values were normalized to GAPDH and the fold change was calculated with respect to control cells. (e) Gene expression levels of the cell reprogramming TFs Nanog, Oct4, Klf4, and Sox2 in TSph previously treated with IL-8, as determined by qRT-PCR. The values were normalized to GAPDH and the fold change was calculated with respect to control cells. Bars represent the standard error of the mean SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ns: not significant).

Genes involved in self-renewal and cell reprogramming present lower expression in IL8-derived tumorspheres

All self-renewal genes tested were down-regulated in IL-8-derived MCF-7 tumorspheres (Figure 2(d)), although no expression of HoxB4 was found. In relation to the reprogramming TFs, we found no expression of Sox2 and Klf4, important down-regulation of Oct4 in IL-8-derived MCF-7 tumorspheres, and similar expression of Nanog (Figure 2(e)). These results agree with the reduction of self-renewal capacity through generations.

Directed-cell migration and invasion were increased in cell obtained from IL-8-derived tumorspheres

We showed above that a partial EMT was maintained in IL-8-treated MCF-7-derived tumorspheres. This program allows cancer cells to acquire migration capacities that are in general associated with more aggressiveness, disease progression, and metastasis.[7] The wound-healing assay showed that migration was increased in IL-8-treated MCF-7-derived tumorspheres (Figure 3(a) and (b)). Migration toward FBS or IL-8 in the TW system showed no differences between these cell populations (Figure 3(c) and (d)); instead, IL-8 directed-cell

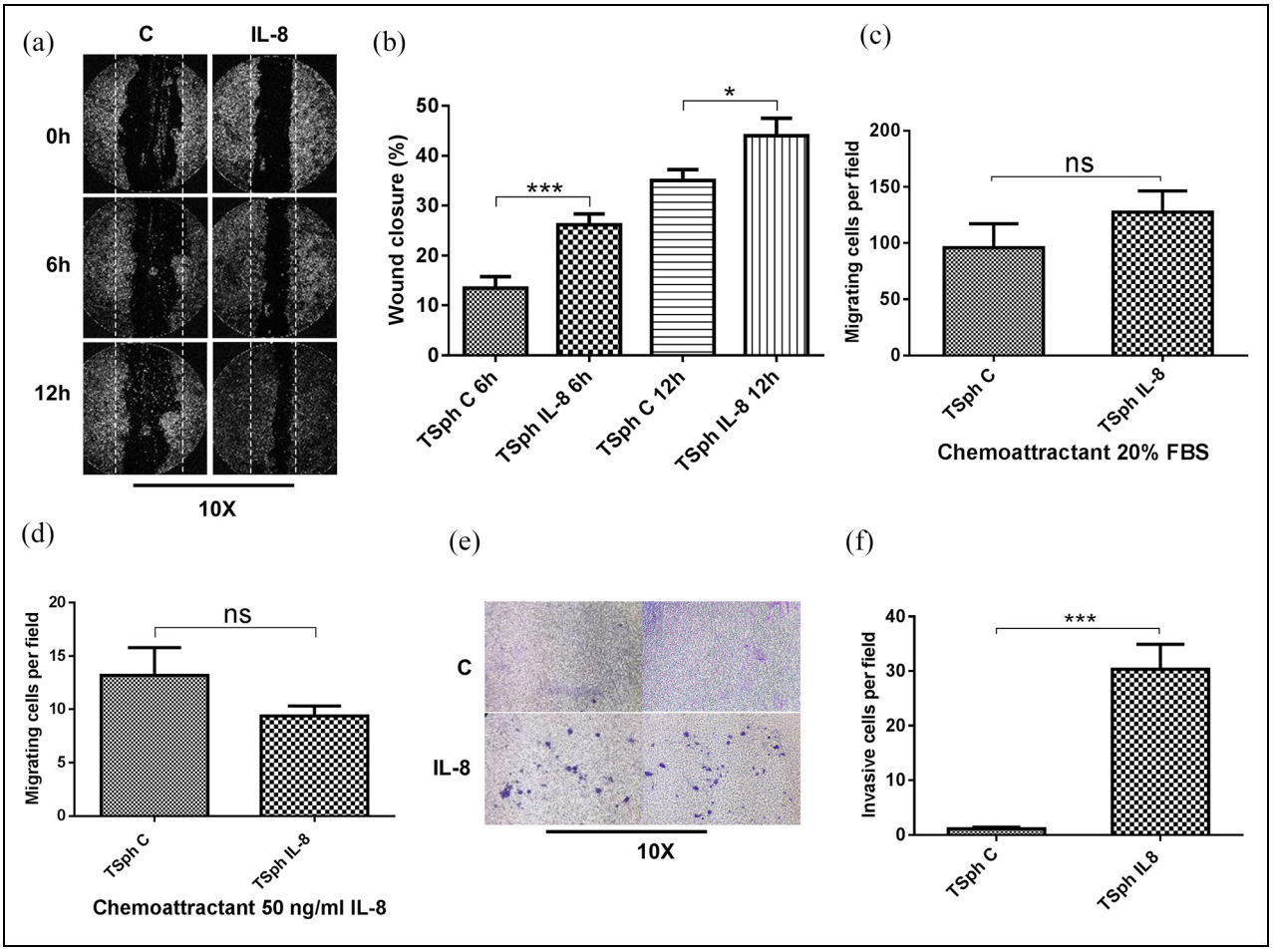

Figure 3. Migration and invasion capacities of MCF-7 cells obtained from tumorspheres previously treated or not with IL-8. (a) Cell migration analysis of cells from tumorspheres by the wound-healing assay. Images were taken at 0, 6, and 12 h. The area of the wound is shown between the two white dashed lines. A representative image of the experiments is shown. C: control TSph cells; IL-8: TSph cells previously treated with IL-8. Magnification: 10×. (b) Quantification of the percentage of wound closure. (c) Quantification of migrating cells per field; 20% FBS was used as a chemoattractant in the TW lower compartment. (d) Quantification of migrating TSph cells per field; 50 ng/mL of IL-8 was used as a chemoattractant in the TW lower compartment. (e) Cell invasion assay with Matrigel used to cover the insert in the upper compartment; 50 ng/mL of IL-8 was used as a chemoattractant in the lower compartment. The bottom surface of the filter in the TW system was stained with Crystal violet. A representative image of the experiments is shown. Magnification: 10×. (f) Quantification of invasive TSph cells per field. Bars represent the standard error of the mean SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ns: not significant).

invasion using Matrigel in the TW system was importantly increased in cells obtained from IL-8-treated cells (Figure 3(e) and (f)). Interestingly, this effect of IL-8 on cell migration was not observed in MCF-7 adherent cells in either the wound-healing assays (Supplementary Figure 3(a) and (b)) or the TW assays (Supplementary Figure 3(c) and (d)).

Multilineage differentiation and clonogenic capacities were impaired in cells isolated from IL-8-derived tumorspheres

In MCF-7 cells obtained by dissociation of tumorspheres, we observed a slight and anomalous decrease in adipogenic differentiation while osteoblastic and chondrogenic differentiation was normal in cells from IL-8-treated MCF-7-derived tumorspheres (Figure 4(a)). This was confirmed by the expression of specific genes involved in each of the differentiation; PPARγ was consistently reduced in IL-8-derived tumorspheres (Figure 4(b)). On the contrary, as judged by the intensity of the different staining reactions, a slight increase in differentiation (adipo- and chondrogenesis) was observed in adherent cells treated with IL-8 (Supplementary Figure 3(e)), as previously reported.[21] The evaluation of the expression of specific genes involved in each of the differentiation showed no significant differences on adherent cells (Supplementary Figure 3(f)).

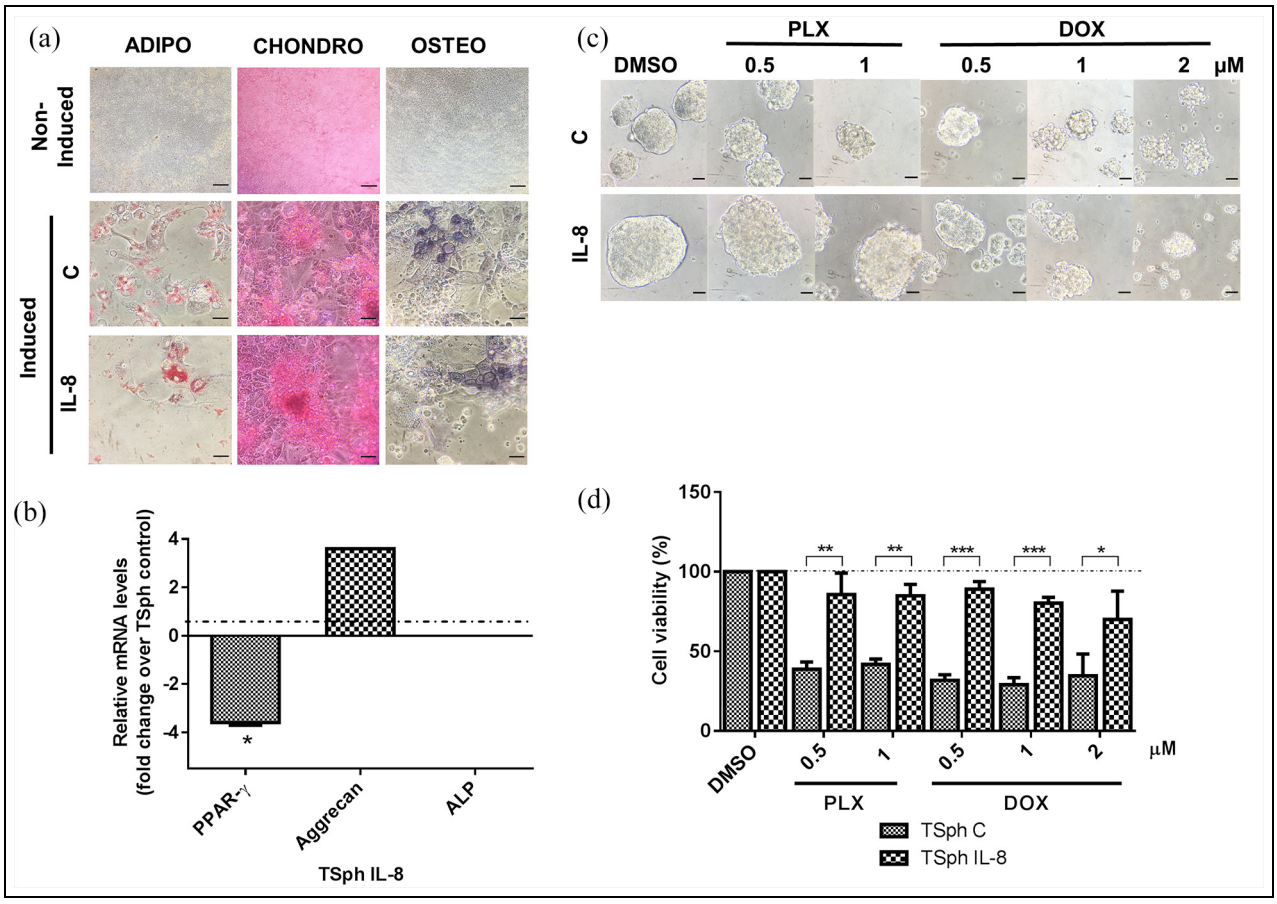

Figure 4. Multilineage differentiation capacity and chemoresistance of cells obtained from tumorspheres previously treated or not with IL-8. (a) Differentiation capacity of tumorsphere cells into mesenchymal cell lineages. Specific stains for Adipogenic (Oil red-O), Chondrogenic (Safranin O), and Osteogenic (Alkaline phosphatase, ALP) were used. C: control tumorsphere cells; IL-8: tumorsphere cells previously treated with IL-8. A representative image of the experiments is shown. (b) Gene expression levels of specific genes involved in adipo- (*PPAR-γ*), chondro- (Aggrecan), and osteogenic differentiation (ALP) in TSph previously treated or not with IL-8, as determined by qRT-PCR. Bars represent the standard error of the mean SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ns: not significant). (c) The cytotoxic effect of Paclitaxel (PLX) and Doxorubicin (DOX) was evaluated on tumorspheres previously treated (IL-8) or not (C) with IL-8. Bright-field microscopy of the tumorspheres on day 4 of treatment is shown and (d) percentage of cell viability after the treatment of tumorspheres with different concentrations of PLX and DOX.

In addition, clonogenic capacity of cells obtained from tumorspheres was consistently reduced compared to adherent cells ($p < 0.05$), but this was unrelated to IL-8 treatment (Supplementary Figure 3(g) and (h)). These findings suggest that cells obtained from the tumorspheres, although able to avoid anoikis, are somehow impaired in some stemness functions.

Chemoresistance to PLX and DOX was increased in cell isolated from IL-8-derived tumorspheres

Since cells isolated from tumorspheres presented characteristics compatible with drug resistance (partial EMT, expression of SNAI2, quiescence, and low proliferation rate), we tested the cytotoxic effect of PLX and DOX using the MTT assay (Figure 4(c) and (d)). Control tumorspheres were severely affected by these treatments with loss of the defined sphere contour and shedding of cell clusters of different sizes. This was much less evident in tumorspheres derived from IL-8-treated MCF-7 cells (Figure 4(c)). Remarkably, cytotoxicity was totally inhibited in cells from IL-8-derived tumorspheres (Figure 4(d)).

Differential expression of cell surface markers was revealed in cell subpopulations obtained from tumorspheres

CSC have also been identified by the expression of cell surface markers. In breast cancer, high CD44 and low CD24 expression ($CD44^{+}/CD24^{-/low}$) was associated with CSC.[42] Nevertheless, MCF-7 does not express

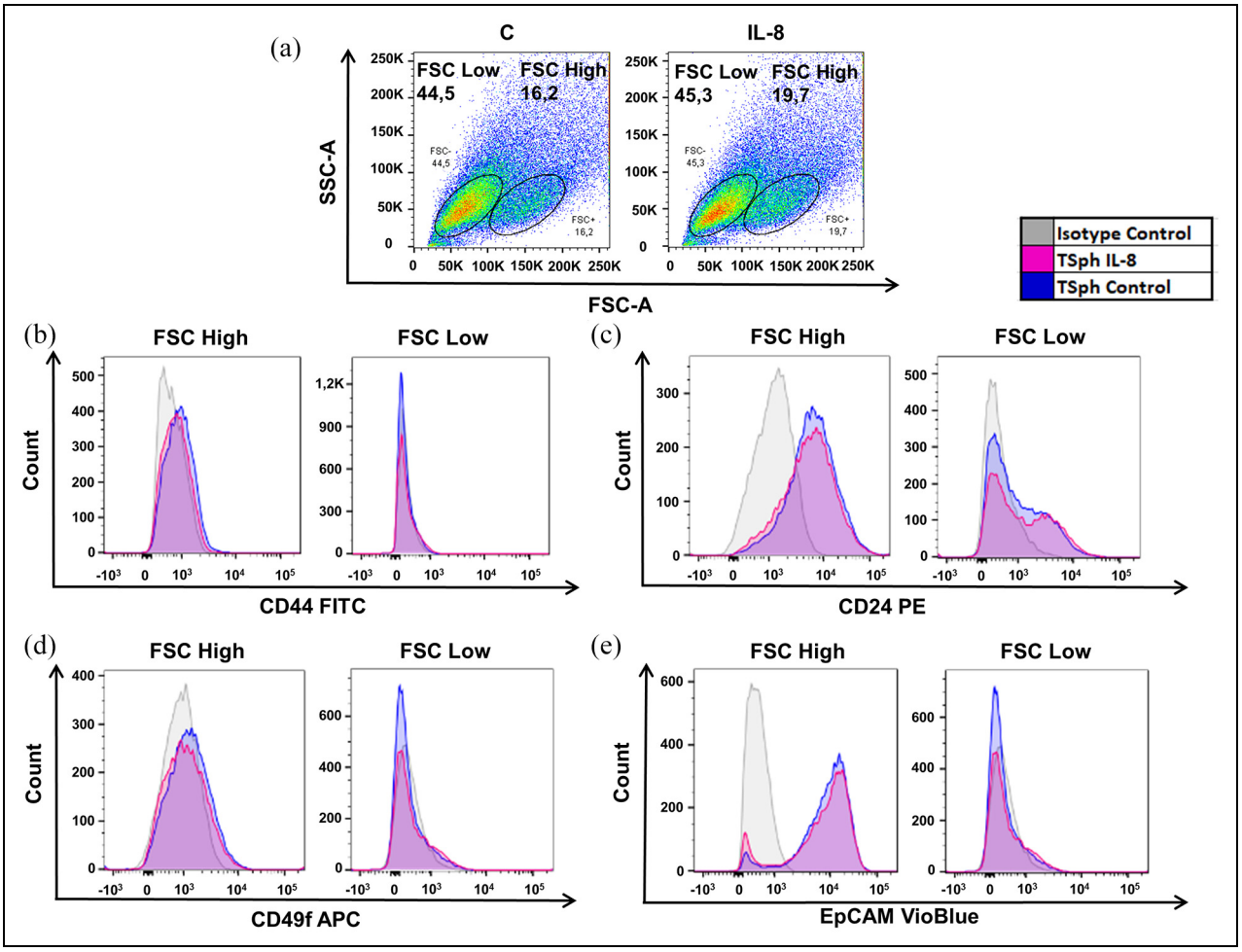

Figure 5. Phenotypic characterization of cells within tumorspheres treated or not with IL-8. (a) Representative flow cytometry of forward scatter (FSC-A) versus side scatter (SSC-A) of cells isolated from IL-8-derived tumorspheres (IL-8) or untreated controls (C). Representative flow cytometry histograms of the expression of (b) CD44, (c) CD24, (d) CD49f, and (e) EpCAM, in the FSC-low and FSC-high cell subpopulations treated (TSph IL-8) or not (TSph C) with IL-8.

CD44, but there is a slight increase after pro-inflammatory cytokine stimulation.[21] Since tumorspheres showed two well-defined cell populations when analyzed by forward scatter (FSC) and side scatter (SSC) (Figure 5(a)), we evaluated CD44 (Figure 5(b)) and CD24 (Figure 5(c)) expression in these two cell subpopulations. The FSC-high cell population had more CD24 expression than the FSC-low one (Figure 5(c)); no differences were observed in CD44 expression when comparing both cell subpopulations or the IL-8-treatment and control (Figure 5(b)). We proceed then to evaluate the expression of additional cell surface markers, that is, CD49f and EpCAM. CD49f was not or very low expressed in both cell subpopulations (Figure 5(d)). On the contrary, EpCAM was highly expressed in the FSC-high cell population, while no expression was observed in the FSC-low cell population (Figure 5(e)). Yet, all these results were independent of IL-8 treatment

Interestingly, the same two cell subpopulations were observed in adherent cells, but the proportion of FSC-low and FSC-high was inverted, with FSC-low cell population being three times more abundant in cells obtained from tumorspheres than in adherent cells (Supplementary Figure 4(a)), please compare to Figure 5(a). Surface marker evaluation in adherent cells showed that FSC-high expressed more CD24 (MFI = 9.891) and EpCAM (MFI = 11.873) than cells obtained from the tumorspheres (MFI = 5.149 and MFI = 6.579, respectively) (please compare Supplementary Figure 4(c) and (e) with Figure 5(c) and (e)).

The simultaneous evaluation of CD49f and EpCAM expression has been used to identify CSC and progenitors cells in breast cancer.[43,44] We showed a decreased expression of EpCAM+/CD49f+ (luminal progenitors) in tumorspheres FSC-high (54%) compared to adherent FSC-high cells (88%) (right panels of Figure 6(a) and (b)). This is consistent with the inhibitory effect of

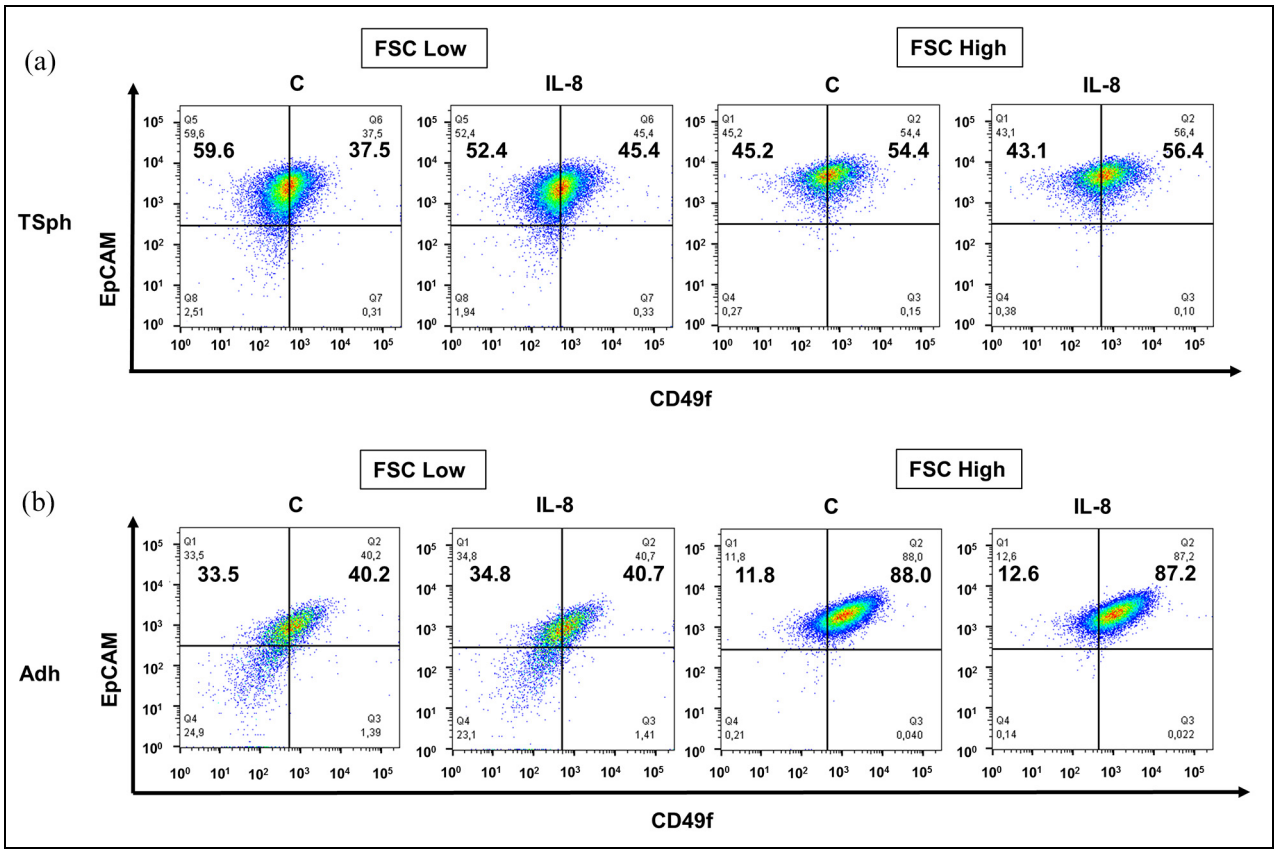

Figure 6. CD49f and EpCAM expression in adherent cells and in cells obtained from tumorspheres treated or not with IL-8. Representative plot of fluorescence intensity for CD49f and EpCAM in FSC-low and FSC-high cell subpopulation. (a) Cells from tumorspheres previously treated (IL-8) or not (C) with IL-8 for 5 days and (b) adherent cells previously treated (IL-8) or not (C) with IL-8 for 5 days.

Slug (SNAI2) and ZEB1 in luminal differentiation in breast cancer,[45] two EMT-TFs that were increased in IL-8-derived tumorspheres. No differences were observed in the FSC-low subpopulations or between IL-8-treated and control cells. It seems then that tumorspheres are enriched in differentiated luminal cells (EpCAM+/CD49f−). This may explain in part some of the reduced stemness properties described above. Notwithstanding, an undetermined number of cells with specific stemness features (drug-resistant and directed migration/invasion) developed within tumorspheres as a consequence of previous IL-8 exposure.

Discussion

CSC, a minor cell subpopulation in tumors, is believed to be responsible for tumor growth, resistance to treatment, relapse, and metastasis.[1,46,47] The gaining of stem-like cell features and de-differentiated phenotypes may enhance tumor progression.[31,32,35] Particularly, the role of chronic stimulation of pro-inflammatory cytokines or senescent conditions in this process have been well documented.[21,48–51] The in vitro mammo- or tumorsphere-forming assay allows the identification and propagation of cells with stem properties and is a practical tool for the enrichment of the CSC population enabling cell functional studies.[37,38] Here, we sought to study the characteristics and functional properties of the cells present in tumorspheres that have been previously incubated with IL-8. We have chosen this cytokine since it has been used as a prognostic marker in different subtypes of breast cancer, and its expression level correlates with the in vitro anchorage independent growth and importantly, metastatic breast cancer and poor prognosis.[52,53] Under these stimulation conditions, MCF-7 cells, a non-aggressive breast cancer cell line, acquire characteristics compatible with an EMT program and stemness.[21] IL-8 confers a more aggressive phenotype to a Luminal A breast cancer cell line supposedly devoid or diminished of a cancer stemness signature.[21,54] As with other breast cancer cell lines Aldefluor(+),[36] IL-8-treated MCF-7 cells formed faster and larger tumorspheres than untreated cells. We have shown here that larger sphere formation is not due to increased proliferation of adherent cells after IL-8 treatment or after sphere-formation induction. This

ability is probably the consequence of the small differences found in the high FSC cell population increased after IL-8 treatment, and/or the small differences in surface marker expression, or even the differences in stemness functions found in MCF-7 cells isolated from tumorspheres previously treated with IL-8. But this issue deserves more experimentation.

Since the cell fraction that survives to anoikis has more stem-like cell properties,[21,37,55,56] we assumed that pre-treatment with IL-8 and further induction of tumorspheres, would favors the acquisition of robust or particular stemness features. A clear effect on the gaining of particular stemness features was observed in cells obtained from tumorspheres derived from MCF-7 cells treated with IL-8. Nevertheless, in some cases, stemness features were severely affected, suggesting a differential modulation of these properties by IL-8. A partial EMT program[11,12] was evident based on morphology and expression of some epithelial and mesenchymal markers. This partial EMT program was maintained in tumorspheres and was only moderately affected by IL-8. Intriguingly, although E-cadherin mRNA was reduced in tumorspheres, the protein was upregulated suggesting particular considerations in relation to the regulation of its expression. Of note, more cellular plasticity and tumorigenicity has been found in hybrid cells that tend to be more epithelial.[57,58] Also, E-cadherin expression has been shown to be important to support sphere formation.[59,60] Recent evidence shows that E-cadherin expression is important for the activation of survival signaling pathways in non-adherent conditions.[59] The up-regulation of SNAI2 in tumorspheres was noteworthy, since it has been identified as a prognostic marker in aggressive cancers and is correlated with a worse clinical outcome in breast cancer.[61–63]

Although self-renewal, as evaluate by the tumorspheres-forming assay, was increased by IL-8 treatment in the first generation of tumorspheres, it was not sustained in consecutive assays. This is in complete agreement with the reduction found in the expression of genes involved in self-renewal (Bmi1, GATA 3, and RunX1) and in the expression of the reprogramming TF Oct4 detected in IL-8-derived tumorspheres cells. Multilineage differentiation potential was also partially affected by IL-8. Accordingly, it was found that epithelial cells from reductive mammoplasties subjected to the mammosphere-formation assay had a reduced clonogenic potential and adipogenic differentiation.[64] In addition, cells obtained from the tumorspheres have less capacity for clonogenic growth compared to adherent cells, although this effect was independent of IL-8 treatment. Therefore, an important impairment of stemness functions is produced by IL-8 pre-treatment of MCF-7 cells or sphere-formation induction.

IL-8 had a heterogeneous effect on cells depending on their intrinsic characteristics. Interestingly, we found two cell subpopulations based on the FSC cytometry parameter in both cells isolated from tumorspheres and in adherent cells. Nevertheless, their proportion was inverted, with the FSC-high cells considerably reduced in tumorspheres and being more heterogeneous and much more complex, as evaluated by SSC-A in flow cytometry. The tumorspheres were composed by a reduced number of stem cells and luminal progenitors EpCAM+/CD49f+ and enriched in differentiated cells EpCAM+/CD49f−.[65,66] We showed that FSC-high cells were enriched in more differentiated luminal cells, in accordance with our above-described results showing reduced self-renewal capacity, clonogenicity, and primitive cells within tumorspheres.

Nonetheless, cells obtained within tumorspheres previously treated with IL-8 are slightly more quiescent and accordingly they were resistant to Paclitaxel and Doxorubicin treatments and they also exhibit higher migration and directed invasion. These results are in agreement with previous work showing that IL-8 induces enhanced migration, invasion, and chemoresistance to Oxaliplatin in colon and gastric cancer cells.[67,68] This situation fairly resembles what probably happens in vivo, a TME able to drive the differentiation states contributing to cell heterogeneity, while at the same time inducing the appearance of a cell subpopulation that develops stemness features capable of sustaining CSC growth, drug resistance, and propagation. Interestingly, blockade of the IL-8 receptor CXCR1 decreased CSC population in vitro and in xenografts models and induced apoptosis in the remaining bulk of cancer cells,[69] showing the relevance of this signaling pathway for novel therapeutic strategies. We have shown here that the non-aggressive and non-secreting IL-8 MCF-7 breast cancer cell line, having low stemness index[33,35] and few (<1%) Aldefluor(+) cells,[42] could partially acquire specific and relevant stem cell features for cancer progression under inflammatory conditions. This also suggests that a global evaluation of stemness gene expression (e.g., stemness index), although useful, do not really show the functional capabilities that cells may acquire under particular stimulation, as it may happen in the TME.

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Author contributions

J.P.V. was involved in conceptualization, funding acquisition, and project administration and supervision; N.O.M. and

J.P.V. were involved in formal analysis, validation, and paper writing; and N.O.M. was involved in experiments. Both the authors have read and agreed to the published version of the article.

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Ethical approval

This work was approved by the Ethical Committee (Evaluation Report No. 012-157-16) of the Facultad de Medicina, Universidad Nacional de Colombia.

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Supplemental material

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