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Cancer Cell Plasticity: Rapid Reversal of Chemosensitivity and Expression of Stemness Markers in Lung and Breast Cancer Tumorspheres

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Abstract

In cancer cells, the reversible nature of the stemness status in terms of chemoresistance has been poorly characterized. In this study, we have simulated on cycle of environmental conditions to study such reversibility by first generating floating tumorspheres (FTs) from lung and breast cancer cells by culturing them in serum-free media without the addition of any external mitogenic stimulation, and subsequently (after two weeks) re-incubating them back in serum-containing media to simulate routine culture conditions (RCCs). We found that cancer cells are extremely plastic: cells grown under RCCs become multidrug-resistant when grown as FTs, but upon re-incubation under RCCs quickly re-attach and lose the acquired resistance. These phenotypic changes are accompanied by concomitant changes in the expression of key proteins associated with multiple pathways important for chemoresistance, survival and stemness maintenance. Therefore, our strategy provides an excellent experimental model to study environmental factors that modulate the plasticity of cancer cells.

Keywords

cancer; plasticity; stemness; chemoresistance; tumorspheres

Introduction

The presence of a subpopulation of cancer stem cells (CSCs) or cancer stem-like cells (CS-LCs) has been associated with chemoresistance and tumor relapse in multiple cancers (Mohammed et al., 2016; Zhao, 2016). CSCs/CS-LCs were initially described as a rare subpopulation of cancer cells with unlimited self-renewal capacity and ability to differentiate and repopulate the entire tumor; according to this model, only CSCs could generate non-CSCs. CSCs/CS-LCs are highly resistant to anticancer drugs and often exhibit multidrug resistance (An and Ongkeko, 2009; Weiswald et al., 2015). Therefore, eliminating the CSCs/CS-LCs fraction present in a given tumor (provided that non-CSCs/CS-LCs can be easily eliminated with conventional anticancer drugs) should theoretically eradicate all

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cancer-producing cells from the body and thus prevent tumor relapse. However, there is increasing evidence to suggest that such a model may not accurately represent most cancers.

We had previously proposed an alternative model (The Stemness Phenotype Model, SPM) in which, depending on the microenvironment, cancer cells can adopt stemness properties that can range from a pure non-CSC phenotype to a CSC phenotype by a poorly understood process called interconversion (Cruz et al., 2010; Cruz et al., 2012). Other models of CSCs such as the “complex system model”, the “reprogramming model” and the “plasticity model” that have implications similar to that of SPM have also been proposed (reviewed in (Cruz et al., 2012)). A central theme of the SPM and other such models is that that cancer cells are extremely plastic in term of stemness, and non-CSCs have the ability to generate CSCs as well. Indeed, experimental evidence of interconversion between non-CSCs and CSCs has been described for breast, lung, colon cancers and melanoma (Cruz et al., 2012; Luo et al., 2014b; Safa et al., 2015). The SPM predicts that in order to effectively cure cancer and prevent tumor relapse, not only the CSCs/CS-LCs fraction but all cancer cells must be eliminated at once, which is supported by a mathematical model that found that the chemotherapeutic elimination of *in vitro* cultures of heterogeneous cancer cells will be effective only by targeting and inducing significant cell death of all cancer cell type subpopulations (Dilão, 2015).

Heterogeneity in stemness has been recently found *in vivo* in gliomas: 1) where gliomas cells from the periphery showed a stem cell phenotype when compared to gliomas cells localized in the core (Munthe et al., 2016), and 2) multiple subpopulations of brain tumor-initiating cells (BTICs) characterized by distinct surface marker expression and single-cell molecular profiles coexist in human glioblastoma (Piccirillo et al., 2009; Rennert et al., 2016). Generation of floating tumorspheres is a widely used method to isolate and characterize putative CSCs/SC-LCs, and it is thought that the ability to form clonal spheres is a unique characteristic of CSCs (Pastrana et al., 2011; Tirino et al., 2013). However, tumorspheres may be generated only in a cell line-dependent manner, and tumorspheres-forming cells sometimes display lower tumorigenic potential than adherent cells (Calvet et al., 2014). CSCs/CS-LCs have been isolated from multiple cancer types including lung (Levina et al., 2008; Shi et al., 2012; Zhang et al., 2015a) and breast cancer (Cioce et al., 2010), and typically display chemoresistance to anticancer drugs such as Cisplatin, Etoposide, Doxorubicin and Paclitaxel (Barr et al., 2013; Gomez-Casal et al., 2013; Larzabal et al., 2013; Levina et al., 2008). Although the generation of floating tumorspheres with an enriched proportion of CSC from adherent cultures has been established as a model to study cancer stemness, the plasticity of cancer cells that can allow for dynamic interconversion between CSCs/CS-LCs and non-CSCs, and its implications for chemosensitivity and changes in microenvironmental cues has not been extensively characterized.

We are the first group to have developed tumorspheres from the human H460 lung cancer cell line in the absence of any external mitogenic stimulation (Yakisich et al., 2016b), an approach that may offer significant advantages to study the role of exogenous factors on stemness formation and maintenance. We have extended the application of our approach to the generation of mammospheres without the addition of external mitogenic stimulation as well. In this study, we use tumorspheres from lung and breast cancer cell lines to study the

plasticity of cancer cells when subjected to at least one cycle of microenvironmental change in terms of both chemosensitivity intracellular pathways that drive cellular plasticity. In our experimental model, parental cells growing under routine culture conditions (RCCs, in serum-containing media) were cultured as floating tumorspheres (FTs) in serum-free media and after two weeks, re-incubated back in serum-containing media. By characterizing the chemosensitivity to anticancer drugs as well as the expression of important proteins associated with stemness maintenance and multidrug resistance, this strategy allowed us to evaluate the plasticity of cancer cells. In addition, we evaluated the impact of exogenously added epidermal growth factor (EGF) on sphere formation and differences in drug sensitivity of tumorspheres to EGFR inhibitors both in the absence and presence of EGF. Our results demonstrate that cancer cells are extremely plastic in terms of chemosensitivity and expression of key proteins, and that addition of exogenous EGF promotes viability of parental cells and FTs in H460 but not in MCF-7 cells and improves the sensitivity of tumorspheres to the tyrosine kinase inhibitors Gefitinib and Erlotinib.

Materials & Methods

Chemicals and Reagents

Drugs—Hydroxyurea (HU), Paclitaxel (PX) and Colchicine (CX), Digitoxin (DIG), Poly-HEMA (Poly(2-hydroxyethyl methacrylate) and MTT (Thiazolyl Blue Tetrazolium Bromide) were purchased from Sigma-Aldrich (St. Louis, MT). Obatoclax (OBT), Nigericin (NIG), Salinomycin (SAL), Erlotinib (ERL) and Gefitinib (GEF) were purchased from VWR (Radnor, PA). HU and CX were prepared as stock solutions (500 mM, 10 mM respectively) in distilled sterile water and stored in aliquots at -20°C . PX, OBT, ERL and GEF were prepared as stock solution (1 mM, 10 mM, 10 mM and 10 mM, respectively) in DMSO and stored in aliquots at -20°C .

Epidermal growth factor (BD Biosciences) was resuspended in PBS (1 mg/ml) and stored in aliquots at -20°C .

Detailed protocols for routine cell culture, generation of Floating Tumorspheres (MSs, and LTs), short-term viability assays for adherent cells (MTT assay) and FTs (CCK assay) and Western blotting are provided as supplemental material,

Statistical analysis

All Pairwise Multiple Comparison Procedures (ANOVA, Student-Newman-Keuls Method) have been performed using Sigmaplot (V. 11.0) software.

Results & Discussion

MCF-7 and H460 cancer cells have the ability to grow as floating tumorspheres without exogenous mitogenic stimulation

The ability of cancer cells to sustain proliferative signaling and divide in the absence of exogenous mitogenic stimulation, leading to unregulated proliferation is one of the important hallmarks of cancer (Hanahan and Weinberg, 2011). In order to study the modulation of stemness by cues from the immediate microenvironment, it is important that

cells be grown in the complete absence of any external stimulation. Traditionally, the isolation of CS-LCs as tumorspheres are done in serum-free media containing exogenous mitogens such as EGF and basic fibroblast growth factor (bFGF) (Chung et al., 2015; Pastrana et al., 2011) with the addition of supplements such as insulin, insulin-transferrin-selenium (combination of insulin, transferrin, and selenium), B27 and hydrocortisone (Gomez-Casal et al., 2013; Luo et al., 2014a; Weiswald et al., 2015; Zhang et al., 2015a; Zhang et al., 2015b). For instance, bFGF that is routinely included in culture media to isolate putative CSCs/CS-LSCs, can *per se* increase the stemness due to its pleiotropic effects as seen with stem cells from the apical papilla (Wu et al., 2012). In MCF-7 cells, bFGF downregulated Bcl-2, promoted apoptosis and sensitized cells to Etoposide and 5-Fluorouracil (Wang et al., 1998), clearly indicating that external mitogenic stimulation may have a profound effect on chemosensitivity. Since growth factors can affect multiple signaling pathways and cellular functions, the generation of FTs in the absence of external mitogens could be useful to investigate the role of one or more specific factors related to stemness and chemoresistance properties of cancer cells.

At present, the ability of cancer cells to grow as spheres in the absence of any mitogenic stimulation has been demonstrated only for glioma CSCs/CS-LCs (Kelly et al., 2009; Rafiee et al., 2015) and lung cancer cells (Yakisich et al., 2016b) but not for breast cancer. In this study, we were successful in obtaining FTs from the breast cancer cell line MCF-7 in the absence of any external mitogenic stimulation (Figure 1S). Under these conditions, MCF-7 cells spontaneously formed mammospheres (MSs) within 72 h that could be propagated and maintained for several months. The preparation and characterization of lung tumorspheres (LTs) in a similar manner from the H460 lung cancer cell line was previously reported (Yakisich et al., 2016b).

This result validates our approach of generating a simple experimental system that can be used in a reproducible manner to investigate the role of specific factors on the basal signaling network responsible for maintenance of the stemness status and the regulation of their key components, and further supports the independence of cancer cells from requiring external signals.

Chemoresistance of lung and breast tumorspheres are quickly reversed by reincubation in CM

Both LTs and MSs were able to reattach and proliferate when were re-incubated back in poly-HEMA uncoated petri dishes (See examples of floating MSs and re-attachment of MSs in Figure 1S). These revertant cells were morphologically indistinguishable from the original parental cells. After obtaining FTs in the absence of any external mitogenic stimulation from two major human cancers (lung and breast), we characterized their chemosensitivity profile when cells were subjected to one cycle of microenvironmental changes. In order to study the chemosensitivity of the revertant cells, we carried out the experiment outlined in Figure 1 to compare the sensitivity of parental cells, tumorspheres and revertant cells to five different anticancer drugs: Colchicine (CX, 1 μ M), Hydroxyurea (1 mM), Obatoclax (OBT, 1 μ M), Nigericin (NIG, 1 μ M) and Salinomycin (SAL, 1 μ M). At day 14, FTs were incubated back in CM and allowed to re-attach – these cells were called revertants. The chemosensitivity of

parental cells, FTs and revertant cells to five different anticancer drugs (CX, HU, OBT, NIG and SAL) were evaluated using viability assays. Our results demonstrated that while parental cells (P1 and P2) were highly sensitive to the drugs tested, they become highly resistant when cultured as FTs (MSs or LTs). However, when tumorspheres were re-incubated in CM, the sensitivity was quickly (within 2–5 days) and completely reversed, indicating that both H460 and MCF-7 cancer cells are highly plastic in terms of sensitivity to anticancer drugs and they can quickly reverse from a high to low resistant phenotype (Figure 2). This experiment demonstrates that the multidrug-resistant property of cancer cells is highly dependent on the culture conditions and that it is a reversible property at least *in vitro* and possibly *in vivo*.

Reversal of sensitivity is accompanied by global expression changes of key stemness-associated markers which are differentially expressed based on tissue origin

In order to evaluate whether the plasticity associated with chemoresistance was accompanied by changes in the expression of proteins important for key cellular processes, we collected protein lysates from both parental H460 and MCF-7 cell lines, LTs and MSs as well as revertant cells (see Figure 1 for details). Proteins were separated by SDS-PAGE and subjected to immunodetection by Western Blots. We evaluated the expression of: i) Stemness associated markers proteins Sox2, Oct4 and Nanog, ii) the multidrug resistance proteins MDR-1 and ABCG2, iii) key proteins of the Wnt signaling pathway: p-LRP6, LRP6, Wnt5a/b, β -catenin and Axin, iv) EGFR and p-EGFR, v) the prosurvival protein Bcl-2 and vi) Vimentin. GAPDH was used as loading control. Figure 3 shows that i) All proteins showed changes in their expression when cells were growing as tumorspheres compared to their parental cells, and ii) protein expression levels were restored to levels similar to the parental cells when tumorspheres were incubated back in CM, and iii) both H460 and MCF-7 cells have a distinct pattern of regulation for cancer-associated proteins: when comparing the two different cancer cells, both cells showed a similar chemosensitivity pattern – both parental and revertant cells were sensitive to CX, HU, OBT, NIG and SAL, and the FTs isolated from both cell lines showed increased resistance to these drugs. At the molecular level, although both cell lines showed a similar trend in the context of reversibility, the individual signatures of each cell line was different – for instance, while Sox2 levels were markedly increased in H460 FTs, they were slightly decreased in MCF-7 FTs. Sox2 is a stem cell transcription factor involved in maintenance of stemness-related processes such as clonogenicity, pluripotency, and self-renewal (Basu-Roy et al., 2015; Santini et al., 2014; Yang et al., 2013). The Sox2 gene is frequently amplified in small-cell lung cancer cells obtained from primary cells and cell lines (Rudin et al., 2012). The opposite pattern was observed for Nanog, also a well-established protein associated with stemness. Bcl-2, a prosurvival protein, is associated with chemoresistance (Nishioka et al., 2014), showed similar trends in both cell lines. Another protein that showed markedly difference between cell lines was pEGFR (Figure 3).

Overall, these results demonstrate that the plasticity associated with chemoresistance is indeed accompanied by significant plastic changes in the expression of proteins, and that distinct biomarkers may drive chemoresistance based on the tissue or origin for the cancer. This also suggests that increased sensitivity in LTs and MSs is the result of not one but

multiple pathways and that, in order to be effective, anticancer agents may need to simultaneously target several key pathways. In agreement with this hypothesis, we have recently found that cells growing under prolonged periods of serum starvation become multidrug-resistant likely by rewiring multiple chemoresistance and survival pathways (MDR1, ABCG2 and Bcl-2), but are still sensitive to the cardiac glycoside Digitoxin, a drug that downregulates key signaling proteins such as MDR1, ABCG2, Bcl-2, phospho-AKT, β -catenin (Yakisich et al., 2016).

EGF downregulates the expression of EGFR in FTs

Since our tumorspheres grow in SFM in the absence of any external mitogenic stimulation, they provide an excellent system to evaluate the effect of individual growth factors on stemness status. FTs (14 days old) were incubated in media with the addition of external EGF (20 ng/ml) for 24 h. Western blot analysis revealed i) that both MCF-7 and H460 cell lines have a functional EGFR signaling axis since as expected addition of external EGF downregulated the expression of the EGFR (Figure 4), ii) that while EGF was able to downregulate the expression of EGFR, no significant effect on other proteins such as Nanog, Oct4, Sox2, AKT and p-AKT was observed (Figure 4).

EGF promotes viability of H460 LTs but not MCF-7 MSs

We first evaluated the effect of EGF on parental cells. H460 or MCF-7 cells were incubated in SFM in the absence or presence of EGF (20, 50 or 100 ng/ml) for 48 h. Figure 5A shows that EGF was able to promote the viability of parental H460 cells but not parental MCF-7 cells. FTs grown without the addition of any external mitogenic stimulation were used later to study the role of epidermal growth factor (EGF) on tumor formation and its potential effect on chemotherapy. We generated FTs that were grown from day 1 (see figure 1) to day 21 in the presence or absence of EGF (20 ng/ml). We tested the effect of EGF at several points during sphere formation (at day, 3, 7, 10, 14, and 21). While parental MCF-7 cells and their corresponding FTs (MSs) were largely insensitive to EGF (Figure 5A–B, top panels) in H460 cells, the addition of 20–100 ng/ml EGF significantly ($P < 0.05$) increased the viability of parental cells and “early” FTs (day 14) by approximately 28% at day 3, and by approximately 15% at days 7, 10 and 14 (Figure 5A–B, bottom panels). However, this effect was lost in “late” FTs at day 21 (Figure 5 and Table I). The effect of additional EGF supplementation was the same in FTs which were grown in the presence or absence of EGF at day 1. In FTs grown in the absence of EGF, addition of supplemental EGF (20 ng/ml) at day 14 produced a significant decrease in the expression of EGFR (Figure 4) indicating the presence of a functional feedback mechanism that leads to downregulation of EGFR expression. Overall, our results showed that addition of exogenous EGF promotes the viability of parental H460 cells and LTs early during development under SFM. However, LTs demonstrate a gradual resistance to exogenous EGF at later stages, and a significant proportion of LTs at day 21 is mainly composed of EGF-insensitive cells. In contrast, parental MCF-7 cells are independent of external EGF, likely because these cells produce sufficient levels of their own EGF, and breast FTs remain independent of exogenous EGF at all times (from day 3 to day 21).

EGF increases the sensitivity of FTs to the EGFR inhibitors Erlotinib and Gefitinib

The potential translational impact of EGF on chemotherapy was tested by evaluating the effect of Erlotinib (ERL) and Gefitinib (GEF) in FTs growing in the presence or absence of supplemental EGF (20 ng/ml). ERL and GEF are EGFR inhibitors (Rothenberg et al., 2015). Figure 6 shows that “early” LTs (14-day culture) grown in the presence of EGF were more sensitive to both GEF and ERL compared to LTs grown without EGF. However, “late” LTs (21-day culture) grown in the presence or absence of EGF showed similar sensitivity to both EGFR inhibitors. This effect was observed only in “early” FTs, recapitulating the “age” effect previously seen with EGF on cell viability. Similar results were observed with MSs except that “early” MSs were sensitive to ERL but insensitive to GEF. Once again, “late” MSs were insensitive to both EGFR inhibitors.

Our data reveals that the adaptation of bulk cancer cells to environmental changes (e.g. from CM to SFM) may have at least two linked components; i) a rapid change in their phenotype perhaps as a quick adaptation mechanism to survive extreme changes in the microenvironment, which is reflected in their profile of chemoresistance and expression of protein markers associated with chemoresistance and stemness, and ii) a slow adaptation to the absence of prolonged periods of external mitogenic signals. This issue is reflected in changes in their sensitivity to EGF as well as to tyrosine kinase inhibitors when compared “early” versus “late” FTs. The mechanism behind the slow adaptation may involve the consolidation of rewired signaling network that better fits the new environment. Metabolic re-wiring due to chronic exposure to the Src family kinases (SFK) inhibitor Dasatinib has been observed in the triple negative breast cancer cell line (BT20) (Pinedo-Carpio et al., 2016). In general, rewiring has been reported in a variety of cancer including melanoma (Ratnikov et al., 2016), chronic lymphocytic leukemia (Porakishvili et al., 2015) and lung cancer (Yenerall and Kittler, 2015), and has been associated with cancer resistance (Komurov et al., 2012; Locasale, 2012; Yakisich et al., 2016). However, rewiring of signaling pathways can be exploited to more effectively kill cancer cells as it has been demonstrated in breast cancers (Lee et al., 2012).

Significance and future applications

We are the first to report that MCF-7 breast cancer cells (this study) and lung cancer cells (Yakisich et al., 2016b) can grow as tumorspheres in the absence of any external mitogenic factors as has been demonstrated for gliomas (Kelly et al., 2009). Therefore this system provides an excellent model to study the role of exogenous factors on stemness formation and maintenance. Moreover, due to the ability of FTs to re-attach, and grow as adherent monolayers when incubated back under routine culture conditions (the same as parental cells) our experimental model depicted in Figure 1 serves as an effective tool to study several aspects of cancer biology (such as chemosensitivity, gene expression profiles) in response to environmental changes. In the context of the “age” (early vs late) of the tumorsphere, at present there is no literature addressing this issue. This is likely because most experiments are performed as soon as the tumorspheres have been obtained from a cell line or tissue sample, a process that takes between 3–12 days as has been described for MSs (Rota et al., 2012; Shaw et al., 2012) and metastatic lung cancer (Nolte et al., 2013). The “age” of the tumorspheres and its impact on chemotherapy may have potential clinical

implications due to dynamic microenvironmental changes in factors such as hypoxia and pH that exist *in vivo*, and that affect the stemness and chemosensitivity of cancer cells. In such case, the duration of the microenvironmental change will ultimately dictate the sensitivity to a particular anticancer drug. From a technical standpoint, the screening of anticancer drugs using only ‘early’ FTs may give artifactual results since as it has been demonstrated in the present study drugs that are effective in “early” FTs may become ineffective in “late” FTs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental design

Parental (P) MCF-7 or H460 cells were split and incubated in either complete media (CM) or serum-free media (SFM) in uncoated plates (UPs). After overnight incubation (day 0), serum-starved cells were trypsinized and plated in Poly-HEMA coated plates (CPs) and maintained in SFM to form floating tumorspheres (FTs). At day 14, FTs were plated back in CM in UPs to obtain revertant cells (R). After 48 h, revertant cells were trypsinized and plated in 96-wells plates for MTT assay (performed at day 17, R1) and in 100 mm Petri dishes to collect protein lysates for western blots (at day 18, π). Revertant cells were also propagated in CM until day 25, when another MTT assay (R2) was performed and protein lysate was obtained at day 26 (at days 26, π). In parallel, age-matched parental cells were maintained in CM to perform MTT assays (P1 and P2) and to collect protein lysates for comparison (days 18 and 26, π). Viability of FTs was assayed at day 17 using the CCK assay and protein lysates from FTs were collected at day 18. π indicates the time point when protein lysates were collected for Western blots.

Figure 2. Chemosensitivity profile of parentals (P), revertants (R) and floating tumorspheres (MSs and LTs) derived from MCF-7 and H460 cells

Quantification of the effects of different anticancer agents on MSs (A) or LTs (B) as compared to their parental (P) and revertant (R) cells. In all cases P, R and MSs or LTs cells were incubated with the indicated concentrations of drugs for 72 h. Cell viability was assessed by the MTT assay for parental and revertant cells and by the CCK assay for MSs and LTs. Data (mean $\pm$ SD) are representative of 2 independent experiments performed in sextuplicates. P1, R1 are cells at day 18, and P2, R2 are cells at day 26 (see Figure 1).

Figure 3. Global protein expression profile of parentals (P), revertants (R) and floating tumorspheres (MSs and LTs) derived from MCF-7 and H460 cells
Expression of key proteins of the Wnt signaling pathway (Wnt), stemness-associated markers (SAM), multidrug resistance and survival (MDR) and epidermal growth factor pathway (EGF) in H460 and MCF-7 parental cells (P1 and P2), revertant cells (R1 and R2) and FTSS (LTs and MSs) grown in SFM without external mitogens.

Figure 4. EGF downregulates the expression of EGFR but has little effect on other proteins
H460 and MCF-7 Parental (P) cells and their respective tumorspheres (LTs and MSs) were incubated with EGF (20 ng/ml for 24 h. Expression of various proteins indicated were determined by Western Blots. GAPDH was used as loading control.

A. Parental Cells **B. Floating tumorspheres (14 days old)** **(21 days old)**

Figure 5. LTs but not MSs grown in absence of external mitogenic stimulation are sensitive to EGF

A) Parental H460 and MCF-7 cells grown under routine culture conditions were incubated overnight in SFM prior to the addition of EGF (20–100 ng/ml) and incubated for 48 h. Cell viability was determined by the MTT assay. B) Floating tumorspheres grown in the absence (black columns) or in the presence of EGF (20 ng/ml) were incubated washed with culture media, plated in 96-well plates with EGF (20–100 ng/ml) for 48 h. Cell viability was measured by the CCK assay. * indicates $P < 0.05$ compared to control value. ** indicates $P < 0.01$ compared between parental and spheres.

Floating tumorspheres (14 days old)

(21 days old)

Figure 6. Cell type and time dictates effects of EGFR inhibitors on FTs

FTs grown in the absence (black columns) or presence (white columns) of EGF (20 ng/ml) for 14 days ("Early" FTs) or 21 days ("Late" FTs) were incubated with the indicated concentrations of ERL or GEF for 72 h. Cell viability was measured by the CCK assay. ** indicates $P < 0.01$ compared between parental and spheres.