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Breast cancer stem cells are involved in Trastuzumab resistance through the HER2 modulation in 3D culture

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ABSTRACT

Breast cancer human cells culture as spheroids develop autophagy and apoptosis, which promotes Trastuzumab resistance in HER2 overexpressing cells. Our aim was to study the association of the hostile environment developed in 3D with the breast cancer stem cells population and the HER2 modulation.

Human mammary adenocarcinoma cell lines were cultured as spheroids using the hanging drop method. We generated hypoxia conditions by using a hypoxic chamber and $CoCl_2$ treatment. Breast cancer stem cells were measured with mammosphere assays, the analysis of $CD44+CD24^{low}$ population by flow cytometry and the pluripotent gene expression by RT-qPCR. HER2 expression was evaluated by flow cytometry and Western blot. MTS assays were conducted to study cell viability.

Hostil environment developed in spheroids, defined by hypoxia and autophagy, modulated the response to Trastuzumab. In HER2+ cells with acquired resistance, we observed an increase in the breast cancer stem cell population. In BT474 spheroids, Trastuzumab induced the acquisition of resistance, along with an increase in breast cancer stem cells. Also, in 3D culture conditions we determined a modulation in the HER2 expression. Moreover, breast cancer stem cells showed enhanced HER2 expression. Finally, cells without HER2 gene amplification cultured as spheroids were sensitive to Trastuzumab, diminishing HER2 expression and cancer stem cells.

Our findings show that 3D architecture is able to modulate breast cancer stem cell population and HER2 distribution, modifying the cell response to Trastuzumab.

ERBB2/HER2 is a member of the human epidermal growth factor receptor (HER/ErbB) family of tyrosine kinases which also includes EGFR, HER3 and HER4. Human breast cancers with overexpression of HER2, occur in about 20 % of patients and are associated with poor prognosis (Press et al., 1997). Trastuzumab (Herceptin) is a humanized monoclonal antibody that binds the extracellular region of HER2 and inhibits its functions (Ghosh et al., 2011). Even though Trastuzumab is the alternative choice in the clinical treatment of HER2-positive breast cancer treatment (Vogel et al., 2002), only a fraction of metastatic patients respond to Trastuzumab as single agent and approximately 60% develop resistance after initial response (Fiszman & Jasnis, 2011).

Tumor microenvironment plays an important role as pro-survival factor for remaining living cells after initial chemotherapy and it is also involved in mechanisms that facilitate drug-resistance (Correia & Bissell, 2012). Several drugs show strong anti-cancer efficacy in conventional in vitro assays; however, these results can only be partially translated to experimental outcomes *in vivo* (Mikhail, Eetezadi, & Allen, 2013). Spheroids are a three-dimensional cell culture that represents small avascular tumors, by developing a gradient of oxygen and nutrient supply towards the central core, subjected to hypoxic conditions (Muñoz et al., 2010).

Cancer stem cells are a subset of cells within tumors that possess the ability for self-renewal and differentiation (Liu, Dontu, & Wicha, 2005), widely associated with resistance to radio and chemotherapy (Abdullah & Chow, 2013; Knezevic et al., 2015). Evidence suggests that HER2 is able to modulate breast cancer stem cells (BCSC) population in HER2+ breast tumors (Hasan Korkaya & Wicha, 2013) and HER2 blockade with Trastuzumab decreases the BCSC population (H Korkaya, Paulson, Iovino, & Wicha, 2008).

We have previously showed that basal autophagy and apoptosis are developed in spheroids, conferring HER2 overexpressing cells with survival advantage against Trastuzumab (Rodríguez, Reidel, Bal de Kier Joffé, Jasnis, & Fiszman, 2015). Since it has been reported that autophagy plays a critical role in cancer stem cells maintenance and tumorigenicity (Gong et al., 2013), we hypothesize that the resistance acquired in 3D culture conditions is related to the BCSC population. Here we provide evidence that the hostile environment developed in spheroids has a key role in the acquisition of resistance to Trastuzumab and is associated with an increase in the number of breast cancer stem cells as well as a modulation in HER2 expression.

MATERIALS AND METHODS

Cell cultures and generation of tumor spheroids

Trastuzumab (Herceptin) was used at 50 µg/ml; an unrelated human IgG (UNC Hemoderivados, Argentina) was used as isotype control. Human mammary adenocarcinoma BT474 and MCF7 cell lines, obtained from American Tissue Culture Collection (ATCC), were grown in RPMI 1640 and DMEM-F12 medium respectively (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco) and 80 ug/ml gentamicine. Serial passages were carried out by treatment with 0.25% trypsin and 0.075% EDTA (Sigma).

Trastuzumab- resistant BT474 cells (BT474-MR) were obtained by continuous treatment of BT474 monolayers with Trastuzumab (10 µg/ml) during up to 6 months. Resistance of these cells was periodically tested by using the colorimetric MTS assay (Cell Titer 96 non-radioactive cell proliferation assay kit, Promega) to evaluate cell viability in the presence of increasing concentrations of Trastuzumab (0,001-100 µg/ml).

To generate spheroids, we adapted the hanging drop method (Del Duca, Werbowetski, & Del Maestro, 2004). Briefly, $1x10^4$ cells were seeded on the cover of 48-well plates in 20 µl drops. Covers were inverted and incubated for 72 h, after which spheroids were transferred into individual wells coated with 1.5% agarose. To evaluate spheroids growth, microphotographs were taken periodically and then analyzed using Graph pad software. Treatment was initiated when spheroids reached 550 µm diameters.

Mammosphere assay

To obtain mammospheres, a suspension containing 10^4 cells was plated in low attachment 35 mm culture dishes in serum-free RPMI medium supplemented with B27 (1:50) (Life Technologies) and 20 ng/ml epidermal growth factor (BD Biosciences). To propagate the mammospheres in vitro, primary mammospheres were enzymatically dissociated with 0.05% trypsin for 15 min at 37°C to obtain a single-cell suspension that was further cultured in suspension as described above, to produce the next generation of mammospheres (secondary mammospheres). In both cases the number and size of mammospheres were recorded 7 days after seeding.

Western blot

Protein extracts were prepared by homogenizing monolayers or spheroids in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% de SDS, 1% NP40, 0.5% deoxycholate and 1 mM EDTA) containing protease and phosphatase inhibitor cocktails (Calbiochem). Primary antibodies used were anti-Akt, anti-phospho Akt (Ser 475), anti-β-actin (Santa Cruz Biotechnology), and anti-LC3B (Cell Signaling). Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

Cytometry Analysis

BT474 and MCF7 spheroids chronically treated with Trastuzumab or control IgG were disaggregated with Trypsin-EDTA for 15 minutes at 37ºC, cells were washed and counted to obtain a concentration of 10^6 cells/ml. Cells were incubated with specific antibodies: CD44-FITC, CD24-PE and HER2-APC (Biolegend), and analyzed by flow cytometry. Experiments were performed by triplicate. Data was analyzed with FloJo software and statistics were done with GraphPad Prism software.

Cell viability

Cell viability was determined using the colorimetric MTS assay (Cell Titer 96 non-radioactive cell proliferation assay kit, Promega). Briefly, after 5 days treatment, medium was replaced with MTS/PMS solution (1 ml MTS: 20 µl PMS). Absorbance was measured at 490 nm using the Multiscan Ascent microplate reader. To determine the effect of autophagy inhibition, cells were treated simultaneously with 1 µg/ml Trastuzumab and 1 mM 3-methyl adenine (Santa Cruz Biotechnology) and left unchanged for 3 days. Viability was quantified with the Trypan Blue exclusion method.

Quantitative real-time PCR analysis

RNA was extracted from cells cultured as monolayers or spheroids using TRIzol reagent (MRC Inc., USA) and was reverse transcribed using the Gentra Pure script RNA isolation kit from Qiagen. (Valencia, CA). cDNA was prepared with the iScript cDNA synthesis kit from Bio-Rad. qPCR was performed in a CFX96 real-time PCR detection system (Bio-Rad) using the SYBR green method based on the manufacturer´s instructions. The amplification of unique products in each reaction was verified by melting curve. Expression level of each gene was normalized to GAPDH expression level using delta Ct method and specific primers. Means and standard deviation (SD) from at least three experiments were calculated and shown as fold induction with respect to the control. PCR products were

obtained using Nanog, Sox2 and OCT4 primers previously described elsewhere (Berardi et al., 2015).

Statistical analysis

Statistical analyses were performed by corresponding t student tests or ANOVA tests and differences between groups were compared by Bonferroni posttest analysis, utilizing the GraphPad Prism V software. At least three independent experiments were performed and data were considered statistically significant when $p<0.05$.

RESULTS

Hostile environment induces resistance to Trastuzumab

Since we have previously reported that HER2+ spheroids chronically treated with Trastuzumab acquired resistance to the antibody (Rodríguez et al., 2015), we aim to investigate if this resistance was due to the hostile environment developed in 3D architecture. To this end, we cultured HER2+ breast cancer human cells BT474 as spheroids and treated them with 50 µg/ml Trastuzumab or control IgG during 15 days, until the spheroids growth was inhibited by 66%, compared to control IgG [(4,5 ± 0,6 vs 13,1 ± 0,7) $x10^7$ mm^3 respectively] (Fig 1a). To evaluate the influence of the 3D enhanced autophagy in the development of resistance to Trastuzumab, we disaggregated the spheroids and cultured the remaining cells as monolayers in the presence of the autophagy inhibitor 3-MA. We found that resistant cells derived from Trastuzumab treated spheroids (BT474-Tz) became susceptible to Trastuzumab after autophagy inhibition, decreasing viability by 33%, while cells derived from IgG control spheroids (BT474-IgG) behaved as parental cells (Fig 1b). In order to recreate the hostile microenvironment developed in 3D, we treated BT474 cells with CoCl2 to induce a pseudo hypoxia state, similar to that found

in the inner core of spheroids. In these conditions, cells also developed resistance to Trastuzumab treatment, which was also reversible after autophagy inhibition (Fig 1c). We corroborated these results by using a hypoxic chamber, instead of CoCl2, to induce hypoxia in the cells and obtained the same outcome (data not shown). These results suggest that the hostile environment developed in the spheroids can modulate the response to Trastuzumab in HER2+ breast cancer cells.

Trastuzumab-refractary cells are enriched in BCSCs

Since it has been described that autophagy is necessary for cancer stem cells survival (Gong et al., 2013), we hypothesize that the Trastuzumab resistance derived from an hypoxic and autophagic environment, might be related to an enhance in the BCSC population. To address this hypothesis, we first compared the BCSCs population between normoxic and hypoxic cell cultures by using a mammosphere assay, where mammary BCSCs/progenitor cells can be enriched and propagated in culture as floating spherical colonies. First, we cultured BT474 cells in a hypoxic chamber while control cells were cultured as usual in a CO_2 incubator. After 48 hs, we harvested the cells and a single-cell suspension from both conditions was cultured under mammosphere conditions and after 7-10 days culture, colonies were analyzed. We did not detect significant differences between the size of treated and control mammospheres, but when we quantified the colonies, we observed that hypoxic cells developed a higher proportion of BCSCs than normoxic cells (Fig 2a). Next, we compared the BCSC population in sensitive BT474 cells versus Trastuzumab-refractory BT474-MR cells developed in our laboratory employing a mammosphere assay. We observed that BT474-MR cells were able to generate a higher number of mammospheres than parental BT474 cells (Fig 2b). Moreover, we compared pluripotent genes expression between sensitive and resistant cells and observed that BT474-

MR cells had enhanced expression of Nanog, SOX2 and Oct4 mRNA (Fig 2c). In order to corroborate these findings, we analyzed the CD44+CD24^{low} phenotype, widely associated with breast cancer stem cells. As expected, BT474-MR cells presented a larger CD44+CD24^{low} subpopulation than parental BT474 cells (Fig 2d). Altogether, these results show that acquired resistance to Trastuzumab is associated with an increase in the cancer stem cell population.

BCSCs are increased in spheroids after Trastuzumab chronic treatment

Next, we analyzed if the 3D hostile environment developed in spheroids could lead to the acquisition of resistance through, at least in part, an increase in the BCSCs population. To explore this hypothesis, we treated BT474 spheroids with 50 µg/ml Trastuzumab or control IgG during 15 days as described before. At the end of the experiment, we harvested the spheroids and analyzed their CD44+CD24^{low} phenotype. Results showed that this population was increased by 2.3 fold in the treated spheroids (Fig 3a). The analysis of pluripotent genes demonstrate that Trastuzumab treated spheroids presented higher Nanog, SOX2 and Oct4 mRNA expression than controls (2.08, 2.05 and 2.11 fold, respectively, $p<0.05$) (Fig 3b). It is important to emphasize that in no more than 15 days of treatment; cells cultured in a 3D hypoxic environment with induced autophagy not only developed resistance but also increased the BCSC subpopulation.

HER2 expression is modulated by 3D architecture

Taking these results in consideration, we sought to assess whether the expression of HER2 could be playing a role in the BCSC modulation. In order to investigate this possibility, we first evaluated total HER2 expression and observed that 3D culture conditions presented higher amounts of the receptor than 2D cultures, while it was not modulated by Trastuzumab treatment. Interestingly, when we analyzed HER2 in

mammosphere cultures from BT474 and BT474-MR cells, we observed an even higher expression of HER2. As control, we evaluated MCF7 breast cancer cell line, without HER2 genomic amplification, and not expressing HER2 receptor at protein level (Fig 4a). Considering that spheroids present different cell subpopulations, we then studied whether they also exhibited different levels of HER2 expression by flow cytometry. As expected, 2D cultures of BT474 and BT474-MR cells presented a homogeneous distribution of HER2 in similar amounts (data not shown). However, 3D cultures showed that 88% of alive cells were HER2+, divided in two subpopulations, $HER2^{high}$ and $HER2^{low}$ (Fig 4b). Moreover, after Trastuzumab chronic treatment, the ratio of these subpopulation changed, increasing $HER2^{high}$ in resistant spheroids (38.2% vs 50.1%, IgG and Trastuzumab treated spheroids respectively, $p<0.05$). Since by Western Blot we did not detect differences in HER2 expression between mammospheres derived from sensitive or from Trastuzumab-resistant BT474 cells (Fig 4a), next we studied HER2 expression in the $CD44^{+}CD24^{low}$ subpopulation of BT474 and BT474-MR cell lines. As expected, we found that they both were nearly 100% HER2+ in the $CD44^{+}CD24^{low}$subpopulation (data not shown). Interestingly, a higher percentage of BCSCs from spheroids treated with Trastuzumab were HER2+ compared with untreated controls (93% vs 86%, respectively, $p<0.05$) (Fig. 4c).

BCSC population in MCF7 spheroids are targeted by Trastuzumab

To further study the relation between HER2 expression and the BCSC population developed in 3D architecture in response to Trastuzumab treatment, we used MCF7 cells, which do not respond to Trastuzumab either in 2D or in 3D culture conditions. However, when we analyzed the BCSC population in 15 day treated spheroids, we observed a 30% reduction in the $CD44^{+}CD24^{low}$ subpopulation respect to control IgG-treated spheroids (Fig. 5a). Even though we could not detect HER2 expression in MCF7 monolayers (Fig. 4a),

when we analyzed the presence of the receptor in spheroids by flow cytometry, we observed a low expression of HER2 in 82 % of the cells, which was surprisingly reduced to 61 % after Trastuzumab treatment (Fig 5b). Furthermore, only 28% of BCSCs expressed HER2 in their surface and this population was also reduced to 10% after Trastuzumab treatment (Fig. 5c). Therefore, our results suggest that Trastuzumab could be able to target BCSCs developed in 3D through their HER2 expression, no matter how low this could be.

DISCUSSION

The tumor microenvironment has important implications in the response to therapies, as cells can be shaped by their surrounds to better resist anti cancer treatments. Spheroids represent a 3D *in vitro* tumor model where heterogeneous cell architecture is developed due to limited availability of oxygen and nutrients. We have previously reported that HER2+ spheroids chronically treated with Trastuzumab are increased in cell autophagy, which correlated with the acquisition of resistance (Rodríguez et al., 2015). In this work we show that cells grown in hypoxic and autophagic conditions acquire resistance to Trastuzumab through an increase in the BCSC subpopulation and this behavior is associated to the expression of the HER2 receptor.

We first observed that cells growing in hypoxic and autophagic conditions are resistant to Trastuzumab treatment. It is known that hostile environment might cause chemotherapy resistance (velaei, Samadi, Barazvan, & Soleimani Rad, 2016) and some authors suggested autophagy is involved in breast cancer resistance to HER2-targeted therapies (Lozy, Vazquez, & Karantza, 2014; Vazquez-Martin, Oliveras-Ferraros, & Menendez, 2009), but little is known on the effect of hypoxia over Trastuzumab treatment. In a prospective clinicopathological study, Koukourakis et al demonstrated a link between hypoxia,

glycolytic metabolism and active autophagic pathways in HER2 breast cancer (Koukourakis et al., 2014). In that paper, the author observed that the overexpression of hypoxia inducible factor 1 (HIF1) was strongly correlated with the resistance of breast tumors to Trastuzumab. We here observed that hypoxic cells are resistant to Trastuzumab but can be re-sensitized by autophagy inhibition. Our results are important as cells inside the spheroids are exposed to hypoxia and autophagy, which seems to collaborate in the acquisition of resistance to Trastuzumab in 3D cultures.

Recent studies have linked HIF1 and autophagy in the development of cancer stem cell (CSC) phenotype (Zhu et al., 2013). Moreover, increasing evidence suggests that environmental signals from autophagic and hypoxic niches are necessary for stem cell maintenance (Carnero & Lleonart, 2016). By using a hypoxic chamber, we determined that hypoxic cultures had more BCSCs than normoxic cultures. To further understand the relationship between the acquisition of resistance to Trastuzumab and the BCSC population we studied this subpopulation through several parameters in BT474-MR resistant cells and observed higher levels of stemness in the resistant cells compared to sensitive parental BT474 cells.

Taking these results in consideration, we hypothesize that the hostile environment of spheroids might modulate the cancer stem cells subpopulation. Interestingly, we observed that 15 day-Trastuzumab treated spheroids presented a higher percentage of BCSCs than control IgG-treated spheroids, even though they both share the same nutritional restrictions due to the 3D architecture. In fact, 3D cultures showed higher levels of pluripotent genes than 2D cultures. This could suggest that even if the hostile environment is capable of modulating the BCSC population, the presence of Trastuzumab is key to induce a resistant phenotype that is stable after the environmental conditions have changed. It would be

interesting to define if the hostile environment is just maintaining the self renewal of BCSCs or can also control the plasticity of these cells, modulating the switch from non-stem cancer cells population into BCSCs. In that sense, a recent study on glioblastoma showed that chemotherapy-induced HIF drove glioma cells to reprogram from a differentiated state to an undifferentiated CSC-like state, leading to chemoresistance acquisition (Deheeger, Lesniak, & Ahmed, 2014).

In the past years, a key role of HER2 in the regulation of breast cancer stem cells was demonstrated by several authors (H Korkaya et al., 2008; Trastuzumab et al., 2013). Moreover, HER2 expression and signaling pathways can be modulated by culture conditions (Breslin & O'Driscoll, 2014; Pickl & Ries, 2009). According with these reports, in spheroids we detected more HER2 expression than in 2D cultures and in mammospheres, this receptor was even more upregulated. Interestingly, when we further analyzed HER2 in BT474 spheroids we observed two populations with different HER2 expression, which were not present in monolayers. Considering that in spheroids there are different cell subpopulations due to 3D architecture, as we and other have described (McMahon et al., 2012; Rodríguez et al., 2015), it should not be surprising that these different subpopulations express different amounts of HER2. This result holds great clinical importance, since each population might not show the same response to anti HER2 therapy; in fact, after Trastuzumab treatment, we detected a change in the spheroid subpopulations, increasing the cells with higher HER2 expression, which correlated with cell resistance to Trastuzumab. When we analyzed the BCSC population in spheroids, we observed that after treatment, BCSCs held higher amounts of HER2 than controls. In line with these results, upcoming reports point that the intrinsic plasticity of HER2+ tumors to switch between epithelial and mesenchymal BCSC states might differ among the heterogeneous phenotypes

that coexist in solid tumors, thus modifying their response to Trastuzumab (Oliveras-Ferraros et al., 2012).

The notion that Trastuzumab is able to attack BCSC through their HER2 receptor became more evident when we experimented with MCF7 cells, a breast cancer cell line devoid of HER2 genomic amplification. We observed that treated MCF7 spheroids showed a reduction in their BCSC population, which correlated with a decrease in HER2 expressing cells and especially with the HER2 expressing BCSCs. In accordance with these results, Ithimakin et al have demonstrated that Trastuzumab targets the cancer stem cell population of MCF7 tumors, in a process that does not require HER2 gene amplification, as it can be modulated by the environment (Trastuzumab et al., 2013). Moreover, other studies show that Heregulin (HRG), a ligand for HER3, plays a key role in mammosphere formation in cells derived from human breast cancer tissues expressing HER2 at moderate levels (Hinohara et al., 2012). Since Trastuzumab can sensitize HRG-overexpressing breast cancer cells to chemotherapy (Menendez, Mehmi, & Lupu, 2006), this could suggest that Trastuzumab might target BCSCs in the breast cancer tissues in which HRG is overexpressed even if HER2 is expressed at moderate levels. It is important to highlight that in HER2 overexpressing spheroids, Trastuzumab chronic treatment lead to the development of resistance, associated with an increase in BCSCs. These results might seem opposite to our finding in MCF7 spheroids, where after Trastuzumab treatment the BCSC population is diminished. However, it must be taken in consideration that in MCF7 cells, Trastuzumab chronic treatment does not induce resistance, since these cells are not responsive per se. It is more likely that 3D architecture modulates HER2 and BCSCs in both BT474 and MCF7 spheroids, resulting in two different outcomes based on the cells initial response to Trastuzumab. In this scenario, when 3D architecture in MCF7 spheroids

induces an increase in the BCSC population with higher HER2 expression, they become accessible and responsive to Trastuzumab.

In conclusion, our results show that the 3D environment not only modulates BCSCs population and HER2 distribution, but also generates the environmental conditions to allow Trastuzumab exert its effect over cells that are clinically defined as HER2 negative. This potential impact in the BCSC population rather than in the bulk tumor must be taken in consideration at the time of determining the treatment of patients without HER2 genomic amplification.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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FIGURE LEGENDS

Figure 1: 3D hostile environment induces Trastuzumab resistance. (a) Percentage size change of spheroids treated with 50 µg/ml Tz or control IgG during 15 days respect to initial size (n=6). Inserts correspond to photographs of representative spheroids (x40 magnification, scale bar: 50 µm); (b) Viability assay of parental BT474 and spheroid-derived BT474-IgG and BT474-Tz (respectively, derived from spheroids chronically

treated with 50 µg/ml control IgG or Tz), cultured as monolayers and treated for 3 days with 1 µg/ml Tz, IgG or 1mM 3MA; (c) Viability assay of BT474 cell monolayers treated for 3 days with 50µg/ml Tz, IgG or 1mM 3MA; hypoxic condition was induced by constant presence of $CoCl_2$ in the cell culture. *$p<0.05$. Tz: Trastuzumab. 3MA: 3 methyl-adenine.

Figure 2: BCSCs are enhanced in Trastuzumab refractory cells (a) Mammosphere formation rates of BT474 cells cultured in normoxic and hypoxic conditions for 48 hs (n=6). Inserts correspond to representative photographs of a mammosphere (x40 magnification, scale bar: 50 µm); (b) Mammosphere formation rates of BT474 and BT474-MR cell lines (n=3). Inserts correspond to representative photographs of mammosphere cultures (x40 magnification, scale bar: 50 µm); (c) RNA from BT474 and BT474-MR cells was isolated and expression of different pluripotent genes was analyzed by qPCR. Each data point represents the mean ±S.D. of triplicate determinations, $p<0.05$ versus control (2 ways ANOVA); (d) Left, representative flow cytometry dot plots of CD24 and CD44 expression of BT474 and BT474-MR cells. The square indicates the distribution of $CD44^{+}CD24^{low}$ cell subpopulation. Right, quantification of the $CD44^{+}CD24^{low}$ cells determined by flow cytometry relative to control BT474 cells. $p<0.05$ versus control (t student).

Figure 3: BCSCs are enhanced in Trastuzumab refractory spheroids (a) Left, representative flow cytometry dot plots of CD24 and the CD44 expression in cells derived from spheroids treated with control IgG (upper insert) or 50 µg/ml Trastuzumab (down insert). The square indicates the distribution of $CD44^{+}CD24^{low}$ cell subpopulation. Right, quantification of the $CD44^{+}CD24^{low}$ cells determined by flow cytometry relative to control

spheroids. $p<0.05$ versus control (t student); (b) RNA from spheroids treated with 50 µg/ml Tz or control IgG during 15 days was isolated and expression of different pluripotent genes was analyzed by qPCR. Each data point represents the mean ±S.D. of triplícate determinations, $p<0.05$ versus control (2 ways ANOVA).

Figure 4: HER2 is modulated by 3D culture conditions (a) Western blot analysis of HER2 levels in 2D cultures of BT474, BT474-MR and MCF7, in spheroids treated or not with 50 µg/ml Tz during 15 days (BT474 3D+Tz and BT474 3D+IgG respectively) and in mammospheres from sensitive and resistant cells (BT474 m and BT474-MR m respectively). β-actin was used as internal loading control; (b) HER2 expression of cells derived from BT474 spheroids treated or not with 50 µg/ml Tz during 15 days. Left, representative cytometry histograms; right, quantification of the HER2+ cells, distinguishing between $HER2^{low}$ and $HER2^{high}$ subpopulations. At least three independent experiments were performed, $p<0.05$ (ANOVA); (c) HER2 expression in $CD44^{+}CD24^{low}$ subpopulation corresponding to 3D cultures of BT474 treated or not with 50 µg/ml Tz during 15 days. Left, representative cytometry histograms; right, quantification of HER2+ cells. $p<0.05$ (ANOVA).

Figure 5: HER2 in MCF7 (a) Left, representative flow cytometry dot plots of CD24 and CD44 expression in cells derived from spheroids treated with control IgG (upper insert) or 50 µg/ml Trastuzumab (down insert). The square indicates the distribution of the $CD44^{+}CD24^{low}$ cell subpopulation. Right, quantification of $CD44^{+}CD24^{low}$ cells determined by flow cytometry relative to control spheroids. $p<0.05$ versus control (t Student); (b) HER2 expression in 3D cultures of MCF7 cells treated or not with 50 µg/ml

Tz during 15 days. Upper insert corresponds to representative cytometry histograms; down, quantification of the HER2+ cells. $p<0.05$ (ANOVA); (c) HER2 expression in the $CD44^{+}CD24^{low}$ subpopulation corresponding to MCF7 spheroids treated or not with 50 µg/ml Tz during 15 days. Up, representative cytometry histograms; down, quantification of the HER2+ cells. $p<0.05$ (ANOVA).

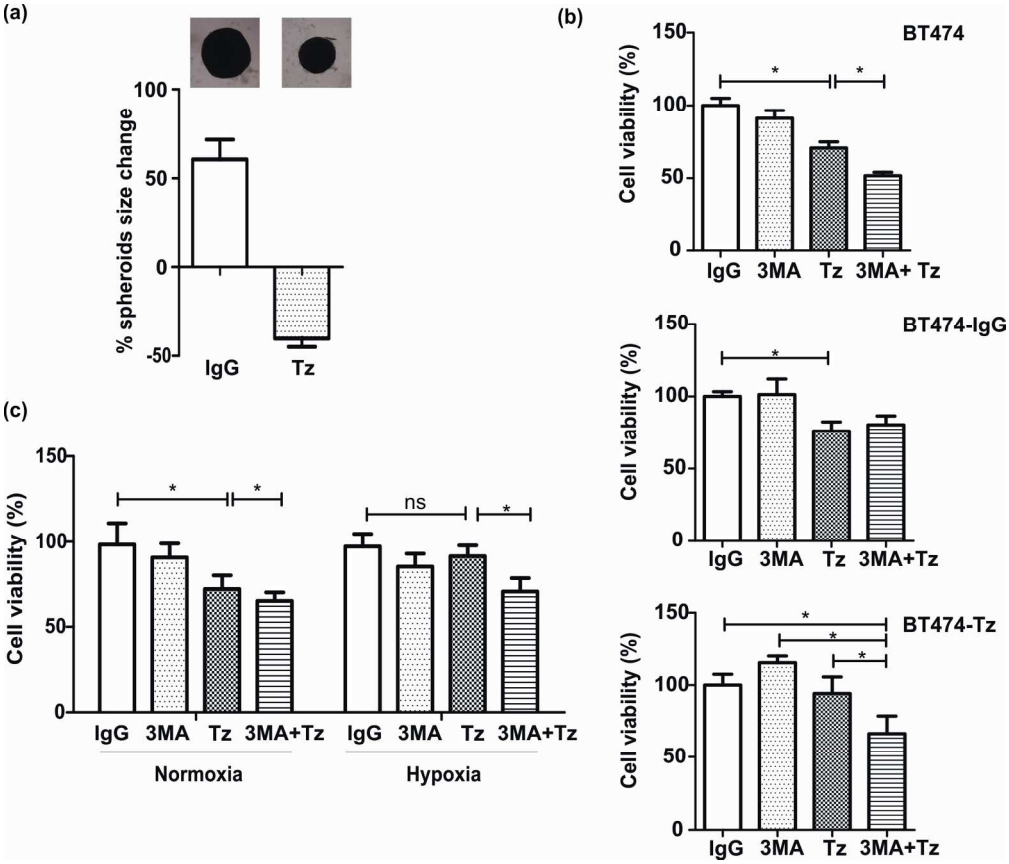

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Fig. 1

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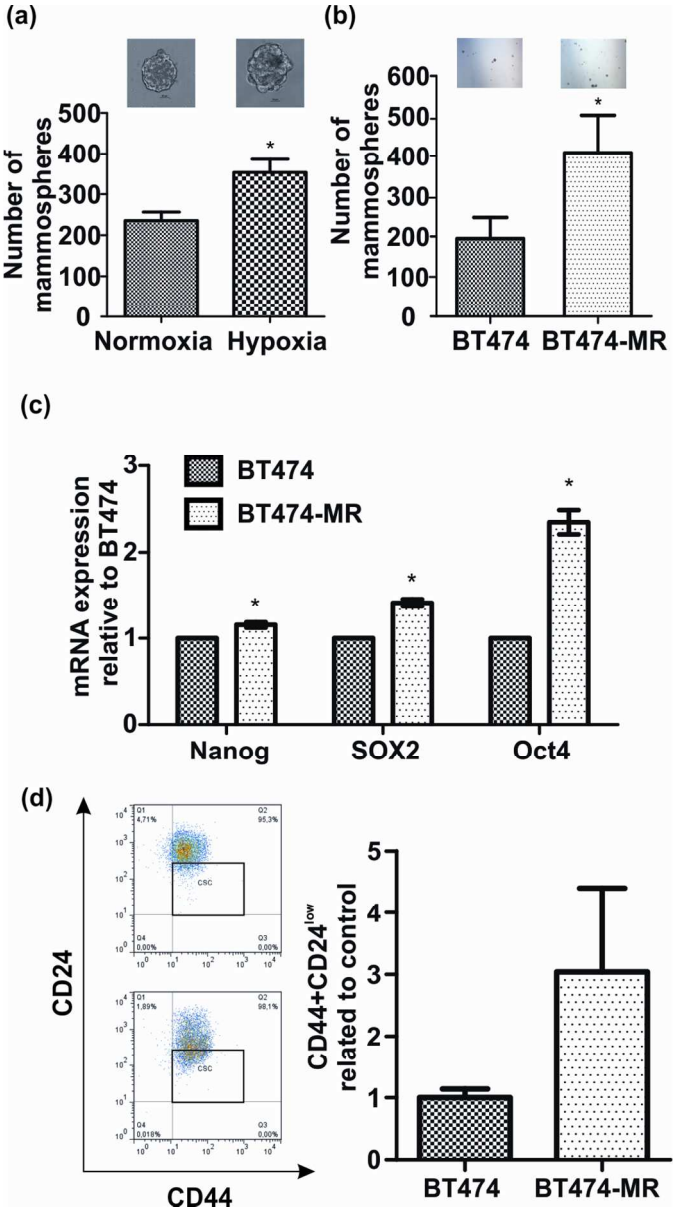

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Fig. 2

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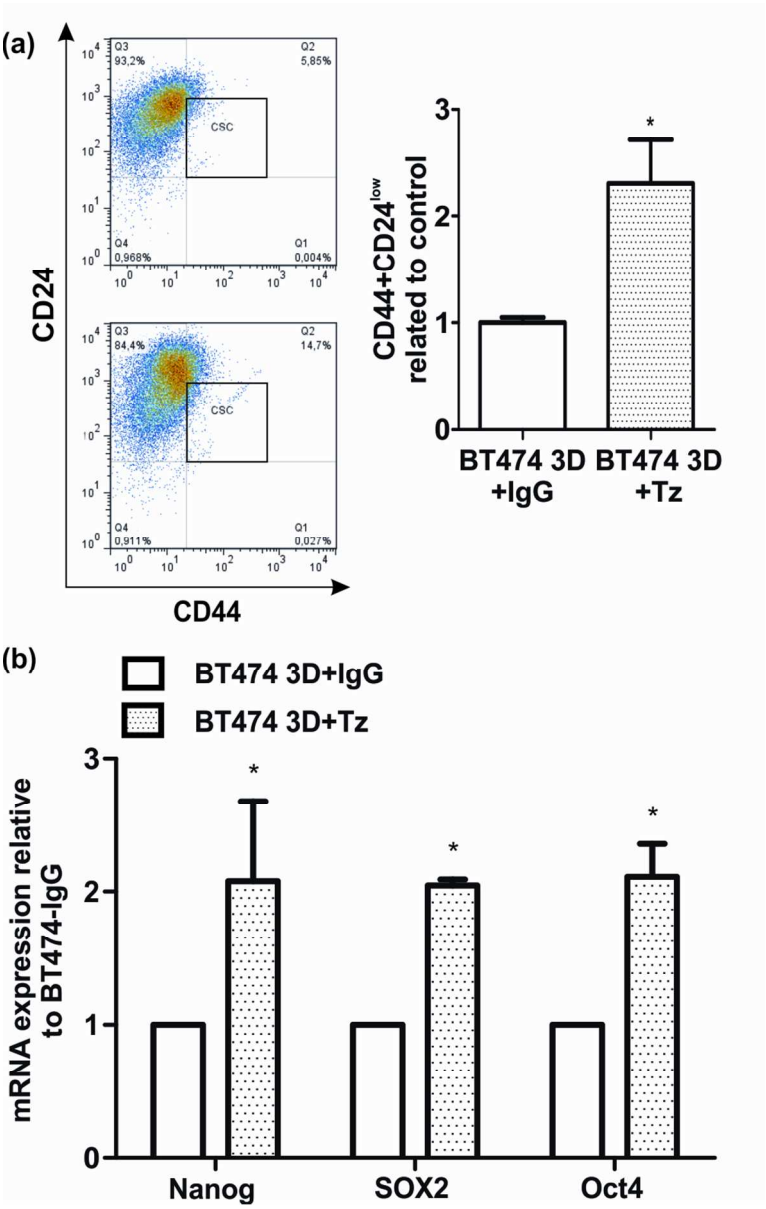

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Fig. 3

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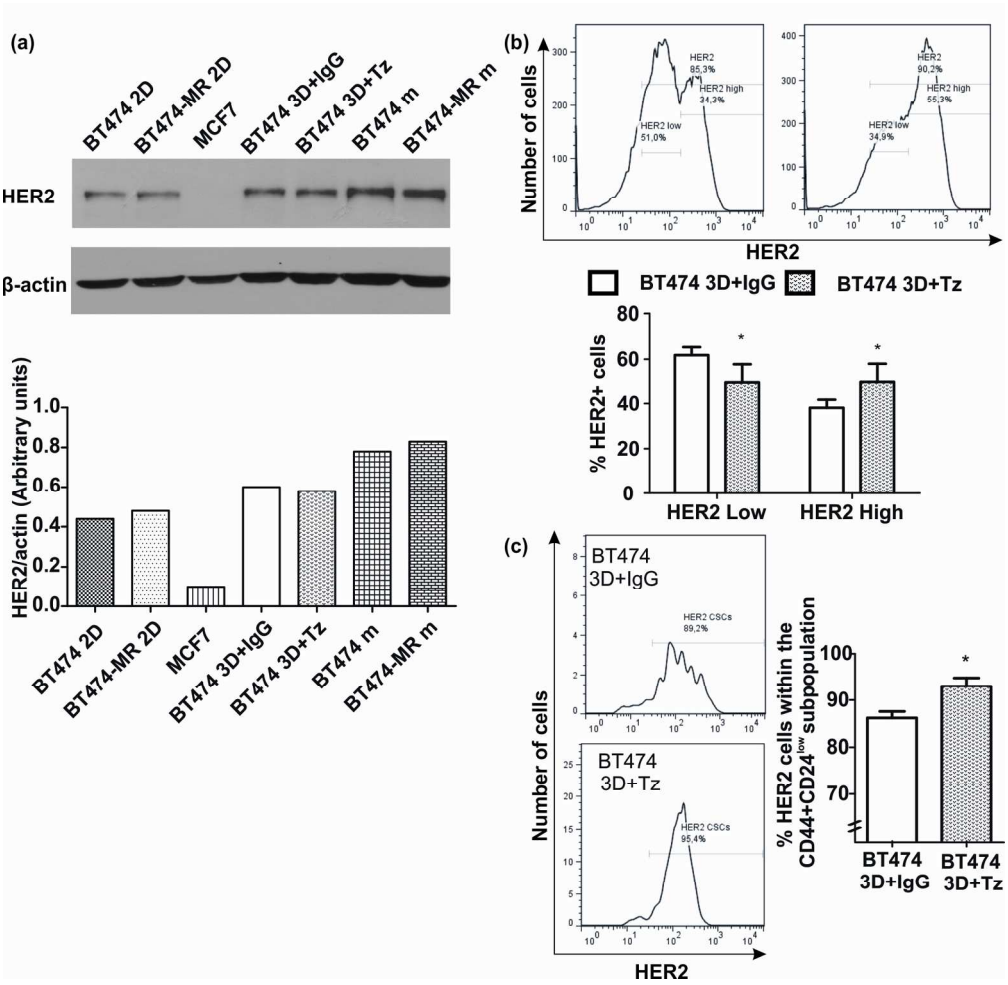

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Fig. 4

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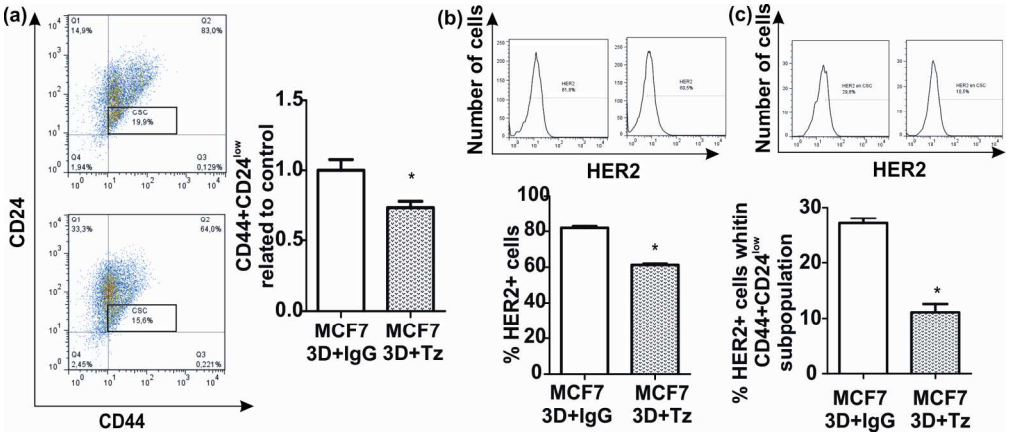

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Fig. 5

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