

The analysis of estrogen receptor- α positive breast cancer stem-like cells unveils a high expression of the serpin proteinase inhibitor PI-9: Possible regulatory mechanisms

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Abstract. Breast cancer stem cells seem to play important roles in breast tumor recurrence and endocrine therapy resistance, although the underlying mechanisms have not been well established. Moreover, in some tumor systems the immunosurveillance failure against cancer cells has been related to the presence of the granzyme B inhibitor PI-9. This study explored the status of PI-9 in tumorspheres isolated from estrogen receptor- α positive (ER α ⁺) breast cancer MCF7 cells. Studies were performed in tertiary tumorspheres which possess high levels of stemness markers (Nanog, Oct3/4 and Sox2) and self-renewal ability. The exposure to estrogens (17- β estradiol and genistein) increased the number and sizes of tumorspheres, promoting cell proliferation as demonstrated by the increase in the proliferating cell nuclear antigen (PCNA). The study of the three isoforms (66, 46 and 36 kDa) of ER α disclosed that tertiary tumorspheres exhibit a marked increase in ER α 36, while the level of ER α 66, which is highly expressed in MCF7 cells, declines. Although it is known that PI-9 is a transcriptional target of ER α 66, surprisingly in tertiary tumorspheres, despite the reduced level of ER α 66, the protein and mRNA content of PI-9 is higher than in MCF7 cells. Treatment with estrogens further increased PI-9 level while decreased that of ER α 66 isoform thus excluding the involvement of this receptor isoform in the event. Moreover, our studies also provided evidence that tertiary tumorspheres express elevated levels

of CXCR4 and phospho-p38, suggesting that the high PI-9 content might be ascribed to the activation of the proliferative CXCR4/phospho-p38 axis. Taken together, these events could supply a selective advantage to breast cancer stem cells by interfering with immunosurveillance systems and open up the avenue to new possible targets for breast cancer treatment.

Introduction

Breast cancer (BC) is the most common endocrine tumor and the second leading cause of cancer-related death in women (1). Approximately 75% of BC cases is characterized by the presence of hormone receptors (ERs) whose expression is considered an important prognostic and therapeutic indicator (2-4). ERs are nuclear receptors that regulate the transcription of estrogen-responsive genes in target cells after ligand binding and dimerization (5,6). Moreover, it became evident that other pathways can be activated by ERs that involve cytoplasmic signaling proteins, growth factor receptors or other membrane signaling circuits (7). There are two ER types, ER α and ER β which are responsible for both the canonical genomic and the non-genomic rapid pathways (8). In 2005 Wang *et al* identified and cloned a novel isoform of full-length ER α 66 with a molecular weight of 36 kDa (ER α 36) which is characterized by the lack of transcriptional activation domains and retains a partial ligand-binding domain (9). Although the prevalent subcellular localization of ER α 36 (cytosol and cell membrane) and the lack of intrinsic transcriptional activity associate ER α 36 to a non-genomic rapid estrogen signaling (10), the interplay between the diverse variants of ERs has not been fully established so far. It has been demonstrated that ER α 36 can act as a dominant-negative regulator of signaling mediated by ER α 66 but, on the other hand, the ER α 36 expression seems to be subjected to an ER α 66-dependent negative regulation (11). The presence of ERs in breast cancer cells allows the employment of ER antagonists, such as 4-hydroxy-tamoxifen, which are able to block their proliferative rate (12). However, although great strides have been made in BC therapy in recent years, 30-50% of cases of ER-positive BC still displays *de novo* or intrinsic resistance to ER antagonists and frequently tumor recurrence is observed (13,14).

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Abbreviations: BC, breast cancer; ER, estrogen receptor; CTL, cytotoxic T lymphocytes; NK, natural killer; PCNA, proliferating cell nuclear antigen; PI-9, proteinase inhibitor 9

Key words: serpin proteinase inhibitor 9, breast cancer stem-like cells, breast cancer, estrogen receptors

There is substantial evidence that the main cause of cancer relapse and chemoresistance is the presence of cancer stem cells (CSCs) in tumor tissues (15). CSCs represent a small population within the tumor mass that not only drives tumor initiation and growth, but also mediates tumor metastasis and therapy resistance. They are very hard to eradicate due to their robust survival mechanisms. Although many proliferative pathways were identified in BC stem-like cells obtained from cancer tissues or cell lines, the role of estrogen signaling has not been fully established and the expression of ER α 66 still remains controversial. Recently, Deng *et al* found that stem cells derived from ER-positive BC cells express high levels of ER α 36 that play important roles in mediating anti-estrogen resistance (16).

It has been largely demonstrated that the malignancy of tumor cells is also sustained by the ability to escape immunosurveillance controlled by immune system cells such as cytotoxic T lymphocytes (CTL) and natural killer (NK) cells that recognize and kill cancer cells (17). Immune system cells eradicate tumor cells through two different death pathways: the perforin/granzyme B and Fas/Fas ligand pathway (18). In perforin/granzyme B-mediated apoptosis, the recognition of a target cell triggers the release of granules containing perforin and the granzyme protease. Perforin promotes granzyme delivery to the cytosol of target cell and, on entry, this protease triggers rapid and efficient apoptosis by the direct cleavage of caspase and non-caspase substrates (19). The activity of granzyme B, the main effector molecule of CTL and NK cells, is regulated by PI-9 (proteinase inhibitor 9), a member of the serine proteinase inhibitor family collectively called serpins (20). PI-9 is a pseudo-substrate for granzyme B leading to the irreversible inhibition of protease upon interaction (20).

We demonstrated that tumorspheres derived from ER α -positive BC MCF7 cells express a high level of PI-9, supporting a role for the granzyme B inhibitor in immunosurveillance escape of BC stem cells.

Materials and methods

Cell culture and reagents. MCF7 cells were purchased from Istituto Scientifico Tumori (Genoa, Italy) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. For estrogen challenge, 4-5 days before the experiments medium was replaced with phenol red-free DMEM containing 5% charcoal-stripped FBS (Life Technologies, Monza, Italy). All reagents, except where differently reported, were purchased from Sigma-Aldrich (Milan, Italy).

Tumorsphere production. For tumorsphere preparation, 10⁴ MCF7 cells were seeded in ultra low-attachment 6-well plates in a conditioned medium bereft of serum and containing phenol red-free DMEM/F12, supplemented with 1X B27, 20 ng/ml EGF, 20 ng/ml bFGF (Life Technologies). Growth factors were added every two days (without removing the old media) and in these conditions cells grew as non-adherent spherical clusters named tumorspheres. Formed spheres were visualized under a bright-field Leica DMR inverted microscope (Wetzlar, Germany) and their sizes and number were

measured and counted using IM50 Leica software (Wetzlar, Germany).

Tumorsphere passage. For sphere passage experiments, the tumorspheres were collected by centrifugation and dissociated by means of enzymatic (0.25% trypsin-EDTA) and mechanical treatment. After centrifugation to remove the enzyme, pellets were resuspended in serum starved conditioned medium to allow tumorsphere re-growth. The dissociated single sphere-forming cells were diluted to a density of 1,000 cells/ml and culture passaged every 7 days according to Dontu *et al* (21). Sphere formation efficiency (SFE) was calculated dividing the number of tumorspheres by the number of seeded cells.

Cell viability. To determine cell viability after estrogen treatments, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed as previously reported (22). Briefly, 20 μ l of MTT (11 mg/ml) was added to the cells and incubation was protracted for 2 h at 37°C. Then, medium was replaced with 100 μ l lysis buffer (20% SDS, 10% dimethylformamide and 20% acetic acid) to lyse the cells. After solubilisation, the absorbance was read at 570 and 690 nm. Each condition was tested six times and results were analyzed for statistical significance.

RNA extraction and qPCR analysis. Total RNA was prepared using Direct-zolTM RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Then, after removal of residual genomic DNA with DNase I (Zymo Research), oligo(dT)-primed reverse transcription was performed on 1 μ g of total cellular RNA using the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories Srl, Milan, Italy), following the manufacturer's instructions. For real-time PCR analyses, each cDNA sample was amplified by iQTM SYBR Green Supermix (Bio-Rad), using the following QuantiTect primers (Qiagen, Milan, Italy): Oct3/4 (POU5F1: QT00210840), Sox2 (QT00237601), Nanog (QT01025850), Klf4 (QT00061033), CXCR4 (QT00223188). As for PI-9 amplification, the following primers were used: Fw 5'-CGCTGGACTAGGTGGCAG-3' and Rv 5'-CAGAACACGTTGTGCGAAGG-3' (Life Technologies).

All PCR reactions were performed in triplicate in the iQ5 thermal cycler instrument (Bio-Rad, Laboratories Srl, Milan, Italy), using a PCR cycling as previously reported (23). The relative quantification of analyzed genes was calculated using the 2^{- $\Delta\Delta C_t$} method and data were normalized by using GAPDH (QT01192646; Qiagen) as endogenous control. Data processing and statistical analysis were performed by iQ5TM Optical System software (Bio-Rad).

Western blot analyses. The procedure of western blot analysis was performed as previously described (24). Briefly, proteins (30 μ g) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad) for detection with primary antibodies. All antibodies were from St. Cruz Biotechnology (St. Cruz, CA, USA) except for p38 and phospho-p38 (Cell Signaling Technology, Beverly, MA, USA). Then, filters were incubated with horseradish-peroxidase-conjugated secondary antibodies and the detections were developed by ECL PlusTM western blotting reagents (Amersham, GE Healthcare Life Science, Milan, Italy) according to the manu-

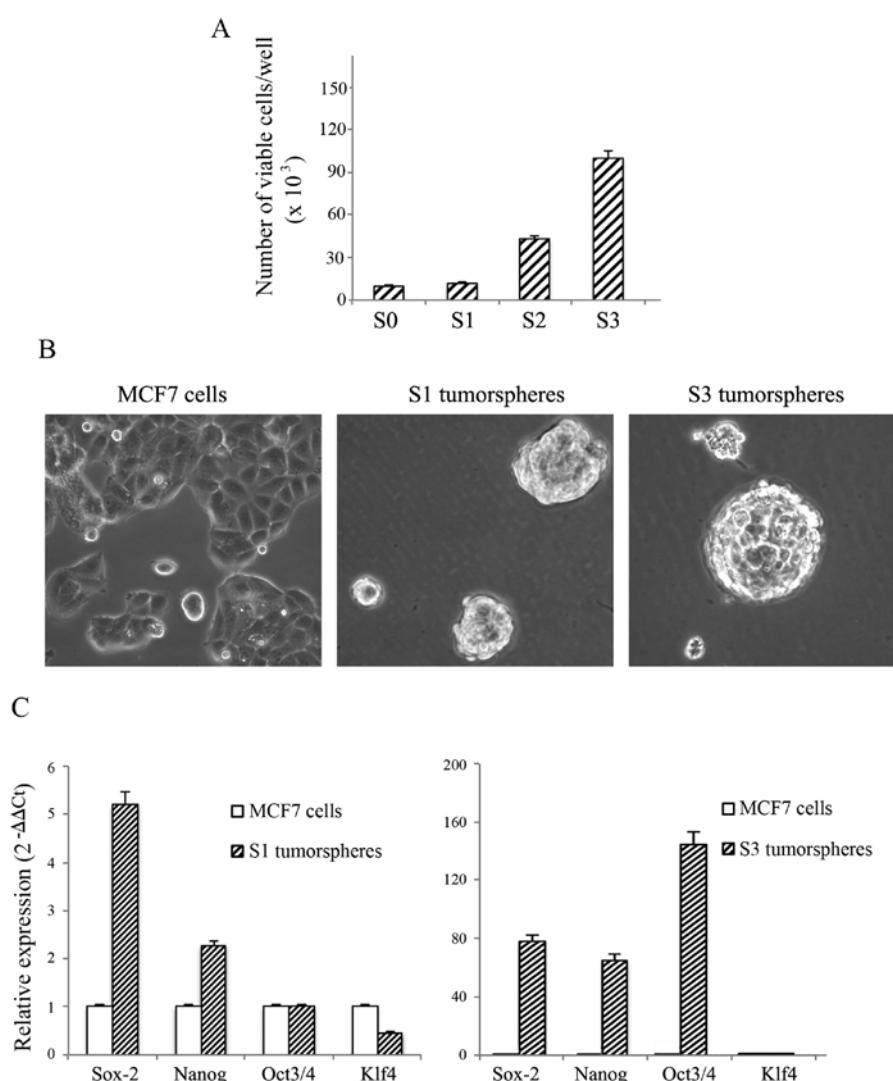


Figure 1. Morphological features and biochemical characterization of breast cancer derived tumorspheres. (A) Analysis of self-renewal ability. Sphere formation efficiency of serially passaged cells cultured as tumorspheres up to three generations was analyzed. After each serial passage, the number of viable dissociated cells was calculated at each generation and plotted against passage number. (B) Representative phase contrast images of adherent MCF7 cells, primary (S1) and tertiary (S3) tumorspheres obtained, respectively, after 7 and 21 days of culturing in a conditioned medium in ultra-low adherent plates. Micrographs were taken by a Leica microscope equipped with a computer-imaging system. The original magnification was at x100. (C) Real-time PCR analysis of stemness markers in parental MCF7 cells and breast cancer tumorspheres. Total RNA from MCF7 cells, S1 and S3 tumorspheres was reverse transcribed and amplified with appropriate primers in iQ5 cycler instrument as described in Materials and methods. Histograms report relative amounts of Sox2, Nanog, Oct3/4 and Klf4 to the amount of GAPDH calculated from data generated by iQ5 software package. The results are the means of three independent experiments.

facturer's instructions. Bands were visualized and acquired by ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

Densitometric analysis of the bands was performed using the NIH Image J 1.40 analysis software distributed by National Institutes of Health (Bethesda, MD, USA). The correct protein loading was ascertained by both red Ponceau staining and immunoblotting for β -actin. All the blots shown are representative of at least three separate experiments.

Statistical analysis. Data, reported as means \pm SD from at least three independent experiments, were analyzed using the Student's t-test. Differences were considered significant at $P < 0.05$.

Results

Isolation and characterization of ER α -positive breast cancer tumorspheres. In our study we first isolated and character-

ized tumorspheres derived from adherent ER α ⁺ MCF7 breast cancer cells to individuate new selective targets in BC stem cells. To this purpose MCF7 cells were seeded in ultra-low attachment plates and maintained in serum-starved medium to form non-adherent spherical clusters named tumorspheres. These spheres were already visible after 4-5 days and, when they reached a diameter $>50 \mu\text{m}$ (approximately after 7 days), were serially passaged for different generations. Single cells obtained from sphere dissociation were propagated forming again new tumorspheres with a sphere formation efficiency (SFE) $\sim 2\%$. The morphological and molecular features of tumorspheres were analyzed after 7 (primary spheres, S1), 14 (secondary spheres, S2) and 21 (tertiary spheres, S3) days, respectively. As shown in Fig. 1A, the number of viable cells, calculated and plotted against the equivalent passage number, progressively increased at each generation. In Fig. 1B the characteristic morphological aspects of S1 and S3 tumorspheres

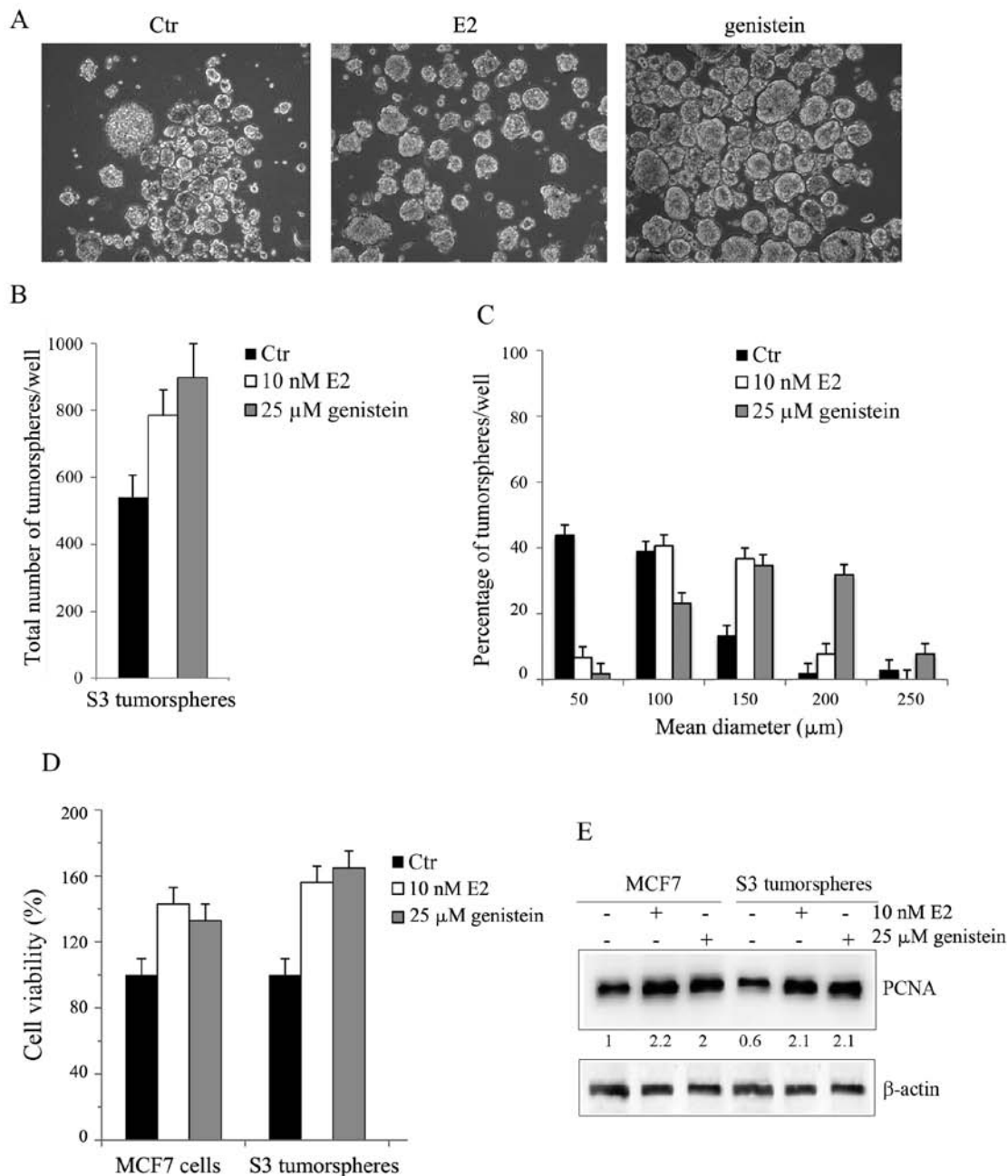


Figure 2. Analysis of proliferating effects of 17-β estradiol and genistein on S3 tumorspheres. (A) Micrographs of S3 tumorspheres cultured in low-attachment plates and treated with 10 nM 17-β estradiol (E2) or 25 μM genistein for 48 h. Micrographs at original x100 magnification are reported. (B and C) Effect of E2 and genistein on number (B) and size (C) of S3 tumorspheres. The total number of tumorspheres and the corresponding diameters were calculated as reported in Materials and methods. (D) Effects of E2 and genistein on S3 tumorsphere cell viability in comparison to MCF7 cells. Cells were treated with 10 nM E2 or 25 μM genistein for 48 h; then MTT assay was performed as reported in Materials and methods. (E) Western blot analysis of PCNA in parental MCF7 cells and S3 tumorspheres. In (B-D) values are the means of three independent experiments \pm SD.

grown in anchorage-independent conditions are reported. As shown, both S1 and S3 tumorspheres grew in spheroidal clusters floating in the medium and were deprived of the flattened cell morphology, typical of adherent parental MCF7 cells.

Next, tumorspheres were collected by centrifugation and characterized by real-time RT-PCR to evaluate changes in the gene expression of stemness markers such as Sox2, Nanog, Oct3/4, and Klf4. The analysis reported in Fig. 1C shows an increase in the levels of Sox2 and Nanog stemness markers in S1 tumorspheres with respect to parental MCF7 cells. The gene expression of these markers consistently increased in S3

tumorspheres where a remarkable upregulation of Oct3/4 was also observed (Fig. 1C). Our data also provided evidence that no significant change was present in the content of Klf4. In light of these results, since S3 tumorspheres exhibited the highest content of the examined stemness markers, we performed the next experiments in these tumorspheres comparing data to parental MCF7 cells.

Effects of 17-β estradiol and genistein in adherent MCF7 cells and S3 tumorspheres. Since a possible involvement of BC stem cells has been postulated in anti-estrogen resistance

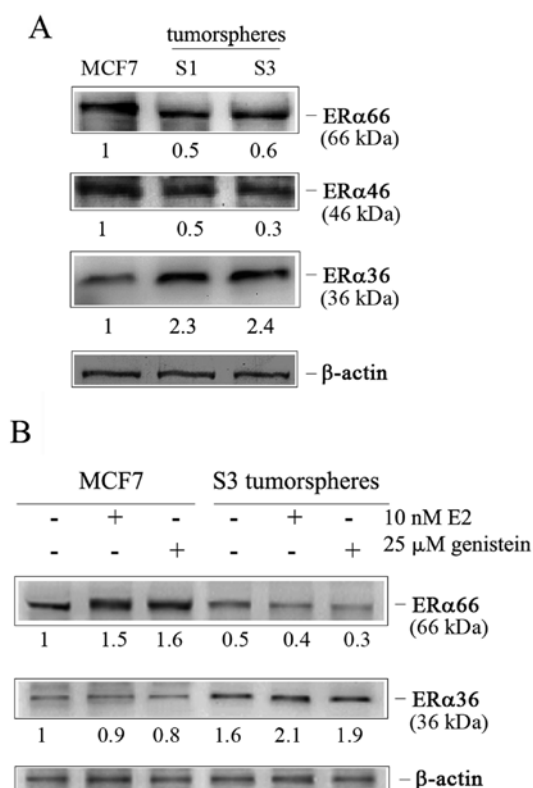


Figure 3. Comparative analysis of ERα isoforms in adherent MCF7 cells and tumorspheres. (A) Immunoblotting of ERα receptor isoforms. Cells and tumorspheres (S1 and S3) were lysed and protein extracts were submitted to western blot analysis as reported in Materials and methods. (B) Effect of estrogen treatment on ERα variants. MCF7 cells and tumorspheres were exposed to hormone treatment (10 nM E2 or 25 μM genistein) for 48 h. Then cells were collected, lysed and subjected to western blot analysis. The correct amount of proteins onto the gel was checked by immunoblotting for β-actin. The images are representative of three independent experiments with similar results.

of ER-positive BC (25), we aimed to clarify the estrogen responsiveness of BC tumorspheres. At first, we treated S3 tumorspheres with 17-β estradiol (E2) or genistein, a natural isoflavonoid phytoestrogen that possesses BC preventive properties (26). The doses (10 nM E2 or 25 μM genistein) employed for these studies were chosen on the basis of preliminary experiments demonstrating that the incubation of tumorspheres or parental MCF7 cells with different concentrations (100 pM-100 μM) of hormones for 48 h increased cell number at low doses while reduced cell viability at higher concentrations (50-100 μM) (not shown). In light of these data we evaluated the effect of the hormones in S3 tumorspheres seeded in low attachment conditions as reported in Materials and methods. As shown in Fig. 2A and B, the treatment increased S3 tumorsphere population by 45% with E2 and by 66% with genistein at 48 h. Moreover, a remarkable increase in tumorsphere sizes (Fig. 2C) was also observed. Approximately 86% of E2-treated tumorspheres showed a diameter >50 μm, most of which with a mean diameter of 100-150 μm. This percentage amounted to 98% in genistein-treated tumorspheres, showing an enrichment in the population with a mean diameter of 150-200 μm.

The effect of estrogens on cell viability of S3 tumorspheres was also analyzed by MTT assay. As Fig. 2D reports, the

incubation with low doses of hormones stimulated the growth of S3 tumorspheres by about 50% with E2 and by 63% with genistein, values which were higher than those obtained in MCF7 cells.

In accordance with these results, western blot analyses provided evidence that, albeit S3 tumorspheres expressed a lower level of the proliferating marker PCNA in comparison to parental MCF7 cells, it significantly increased after E2 or genistein stimulation (Fig. 2E).

Intrigued by these results we focused on ERα receptor isoforms which mediate estrogen effects (27). Considering the different sensitivity of MCF7 cells and S3 tumorspheres to both E2 and genistein, we wondered whether the observed effects were linked to a different expression of ER receptors. Thus, we evaluated the presence of the isoforms of ERα (ERα66, its splice variant ERα46, and the recently identified variant ERα36) in S1 and S3 tumorspheres in comparison to MCF7 cells. As Fig. 3A shows, tumorspheres expressed a very low amount of ERα66 and ERα46 isoforms respect to MCF7 cells, while a remarkable expression of ERα36 isoform was visible. Moreover, the exposure of S3 tumorspheres to estrogen treatment (E2 and genistein) further increased ERα36 content reducing that of ERα66. Such an effect was dissimilar to that observed in parental MCF7 cells (Fig. 3B), where hormone treatment increased the ERα66 level.

Breast cancer tumorspheres express high levels of serpin proteinase inhibitor 9. In light of the results obtained on ERα receptor isoforms, our attention was next focused on PI-9 (proteinase inhibitor 9), a member of serpin family which is subjected to the hormonal induction mediated by ERα66-dependent transcriptional regulation (28). Since S3 tumorspheres showed a higher expression of ERα36 receptor which lacks transcriptional activity, we expected to find a lower level of PI-9 in tumorspheres than parental MCF7 cells. Surprisingly, western blot analysis revealed that the level of PI-9 protein was 1.6 and 3.5-fold higher in S1 and S3 tumorspheres, respectively (Fig. 4A). Based on these data, we wondered whether this upregulated level could be ascribed to an increase in mRNA level. To verify this hypothesis, we performed comparative analyses by real-time RT-PCR. Data reported in Fig. 4B revealed that S3 tumorspheres also expressed a pronounced level of PI-9 mRNA which was ~110-fold higher than that found in adherent MCF7 cells, while a very modest increase of this factor was observed in S1 tumorspheres.

Interestingly, 17-β estradiol or genistein treatment further increased the level of PI-9, thus indicating a possible relationship between PI-9 expression and estrogen-mediated signaling (Fig. 4C).

S3 tumorspheres show an active CXCR4 /phospho-p38 proliferative axis. Next, we explored possible molecular scenarios responsible for PI-9 upregulation in S3 tumorspheres. In this regard, we focused particular attention on CXCR4, the chemokine receptor specific for the stromal-derived-factor-1 (29). This receptor represents a prognostic factor in different types of cancer and plays a role in chemotaxis, stemness and drug resistance (30,31). Moreover, recent data have also described this factor as a key regulator of breast cancer invasion, directing

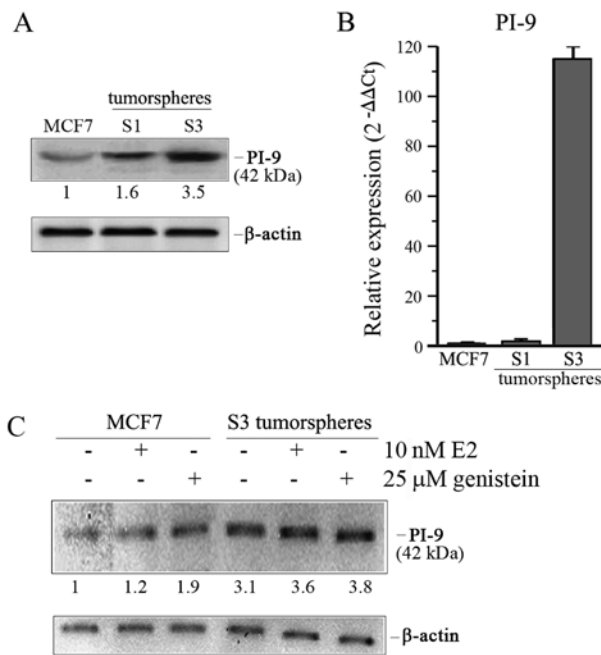


Figure 4. Analysis of proteinase inhibitor PI-9 in adherent MCF7 cells and tumorspheres. (A) Immunoblotting of PI-9 level. Cells and tumorspheres were lysed and protein extracts were submitted to western blot analysis as reported in Materials and methods. (B) Quantitative RT-PCR of PI-9 in adherent MCF7 and tumorspheres. mRNA from MCF7 cells and tumorspheres was reverse-transcribed as reported in Materials and methods and gene expression was analyzed by real-time PCR. (C) Effect of E2 or genistein treatment on PI-9 protein content. After exposure of MCF7 cells and tumorspheres to hormone treatment for 48 h, cells were collected, lysed and subjected to western blot analysis. In (A) and (C) the correct amount of proteins onto the gel was checked by immunoblotting for β -actin. In (B) error bars depict standard deviations. All experiments were performed in triplicates.

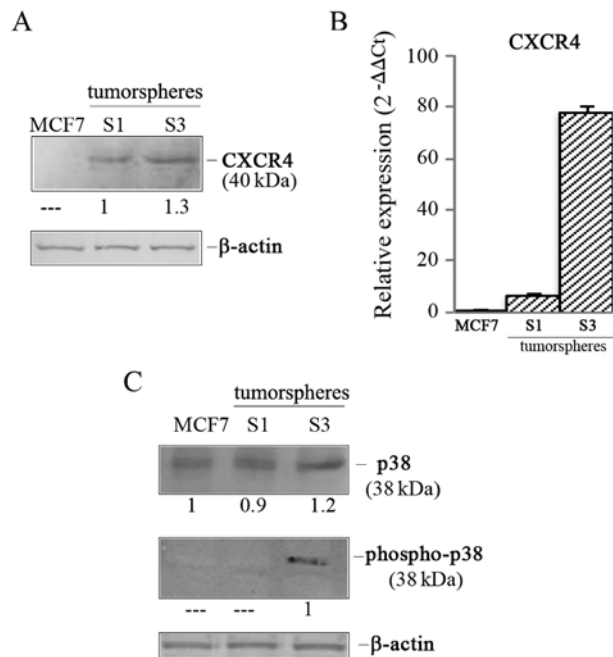


Figure 5. Analysis of CXCR4, p38 and phospho-p38 in MCF7 cells and BC tumorspheres. Western blot analysis (A) and real-time PCR (B) of CXCR4 expression in MCF7 cells and S1 and S3 tumorspheres. (C) Immunoblotting of p38 and phospho-p38 by western blotting as reported in Materials and methods. In (A) and (C) the correct amount of proteins onto the gel was checked by immunoblotting for β -actin. In (B) error bars depict standard deviations. All experiments were performed in triplicates.

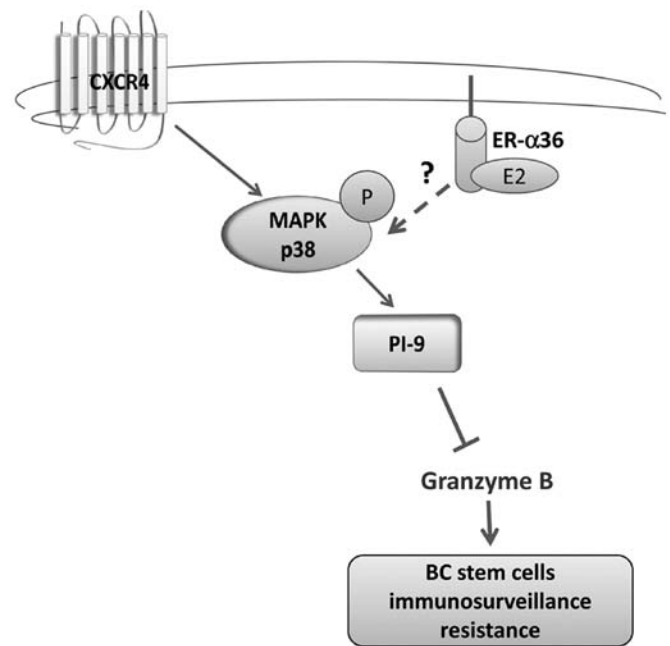


Figure 6. Schematic representation of the possible mechanism of action controlling the high PI-9 levels in BC tumorspheres.

homing of BC cells to particular sites of metastases (32). In our experiments we observed that CXCR4 was undetectable in parental MCF7 cells, while it appeared in tumorspheres increasing from S1 to S3 generation (Fig. 5A). Accordingly, mRNA analysis by real-time PCR showed a 77.7-fold higher content in S3 tumorspheres than MCF7 cells (Fig. 5B).

Since Bots *et al* (33) have shown that PI-9 is induced through p38 MAP kinase activation in dendritic cells and the activation of this kinase can be linked to CXCR4, we analyzed the level of p38 kinase in S3 tumorspheres. As shown in Fig. 5C, we demonstrated that p38 was present in both parental MCF7 cells and tumorspheres, but its content was only 1.2-fold higher in S3 tumorspheres than parental cells. Interestingly, the phosphorylated and active form of the protein, detected by using an antibody directed against the dual phosphorylated sites Thr180 and Tyr182, was found only in S3 tumorspheres (Fig. 5C), indicating the presence of an active p38 MAP kinase pathway in this stem-like population.

Discussion

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells can specifically identify and eliminate tumor cells in a process termed cancer immunosurveillance that exerts a crucial role in inhibiting carcinogenesis and maintaining cellular homeostasis (34). Unfortunately, tumor cells develop multiple strategies to evade host immune responses, including reduced expression of major histocompatibility complex molecules and loss of tumor antigens (35). Recently, it has been suggested that the inhibition of granzyme B activity in neoplastic cells may constitute one of the mechanisms that tumors use to escape immunosurveillance (36,37). Granzyme B belongs to a group of serine proteases commonly found in the cytotoxic granules of CTL and NK cells and it mediates apoptosis in target cells together with the pore forming protein perforin

(38). The proteinase inhibitor 9 (PI-9), a member of family of serine proteinase inhibitors, is a direct inhibitor of granzyme B in human (39). Its overexpression has been observed in several types of cancer, such as breast (28), prostate (40) and lung cancer (41) and has been correlated to the protection of tumor cells from granzyme B-mediated cytotoxicity. However, no data concerning the status of PI-9 in cancer stem cells are available so far.

In this study we employed tumorspheres obtained by us from MCF7 cells serially passaged up to the third generation (S3 tumorspheres) and expressing high levels of stem cell markers. Interestingly, we show for the first time that ER α ⁺ breast cancer tumorspheres express higher levels of PI-9 protein than parental MCF7 cells and the content of this serpin proteinase inhibitor progressively increases with the passaging, resulting more pronounced in S3 tumorspheres. Real-time RT-PCR also provided evidence that the increased expression of PI-9 is related to gene upregulation.

It has been shown that estrogens increase PI-9 level through an ER α 66-dependent transcriptional regulation in breast cancer cells (28). In particular, in PI-9 promoter it has been identified a sequence located 200 bp downstream of the transcription start site and binding the estrogen/ER α complex (27). These observations prompted us to evaluate a possible involvement of estrogens in PI-9 induction in MCF7-derived tumorspheres. After proving that estrogens (17- β estradiol and genistein) increase both the number and sizes of tertiary tumorspheres as well as the level of proliferation marker PCNA, we demonstrated that both the hormones markedly increase PI-9 level in tumorspheres, suggesting their involvement in PI-9 upregulation. Estrogens are known to regulate different cellular processes stimulating both genomic and non-genomic pathways (3-8). The classical mechanism of estrogen action involves the binding of the hormones to specific nuclear receptors (ER α 66, its splice variant ER α 46, and the closely related estrogen receptor ER β) (7,8) which bind to estrogen responsive elements (EREs) located in the promoter of target genes (8). The experiments described here demonstrated that the levels of both ER α 66 and ER α 46 are lower in tertiary tumorspheres than in parental MCF7 cells. Moreover, both 17- β estradiol and genistein further decreased the level of ER α 66 isoform. The divergent trends between the increase in PI-9 and the decrease in ER α 66 observed in tertiary tumorspheres seem to indicate that ER α 66 isoform is not involved in PI-9 upregulation in ER α -positive BC-derived spheres.

Estrogens were also reported to bind cell surface receptors to transduce membrane-initiated estrogen signaling (7). These effects seem to be related to the expression of ER α 36, a variant of ER α 66 (9) which lacks both the transcriptional activation domains (AF-1 and AF-2). Being mainly located near the plasma membrane and in the cytoplasm, it mediates the activation of MAPK/ERK and PI3K/AKT signaling pathways (42,43). Our results showed that ER α 36 is expressed at a higher level in tertiary tumorspheres than MCF7 cells and its content is further increased by 17- β estradiol or genistein treatment. These results are in accordance with the observation of Deng *et al* (44) showing that ER α 36 expression is markedly increased in stem cells derived from ER-positive breast cancer cells where it plays an important role in expanding breast cancer stem-like cell population. Thus, we postulated that

the activation of non-genomic signaling involving estrogen/ER α 36 could be responsible for PI-9 increased expression in tertiary tumorspheres. This hypothesis is also corroborated by the observation that ER α 36 stimulation is involved in the activation of MAPK/ERK signaling pathway (45) and that the expression of PI-9 is regulated via the p38/MAPK pathway in human dendritic cells (33). Interestingly, we demonstrate that both parental MCF7 cells and tertiary tumorspheres express p38, while the phosphorylated and active form of this protein only occurs in tumorspheres of third generation, thus suggesting that this event could be related to the increase in PI-9 content found in tertiary tumorspheres.

Recently, several lines of evidence suggested that the biological effects of CXCR4, the chemokine receptor specific for the stromal-derived-factor-1 (SDF-1), are mediated by the activation of MAPK family members (46). The SDF-1-CXCR4 signaling axis is a known mediator of metastasis in breast cancer (32) as demonstrated by CXCR4 expression observed in human breast cancer specimens (47). A recent study has also shown that CXCR4 maintains a cancer progenitor population in tamoxifen-resistant breast cancer cells (48). Data reported herein showing that tertiary tumorspheres express high levels of both CXCR4 mRNA and protein suggest that the activation of this receptor could be also involved in p38 MAPK activation.

Taken together, our results demonstrate for the first time that ER α ⁺ breast cancer stem-like cells express a high content of granzyme B inhibitor PI-9 and that this event could be ascribed to the activation of a CXCR4-p38 axis. Considering that ER α 36 has been reported to play a role in MAPK signaling activation (43), it is plausible to hypothesize an involvement of ER α 36 isoform which is overexpressed in tertiary tumorspheres in this signaling circuit. A possible molecular mechanism controlling this pathway in BC tumorspheres is illustrated in Fig. 6.

In conclusion, we consider that PI-9 overexpression found in mammary tumorspheres could cause a short-circuit in immunosurveillance's signaling preserving these cells from cytotoxic T lymphocytes and NK cell-mediated apoptosis. Therefore, this experimental system might represent a suitable *in vitro* model to study breast cancer-initiating cells and to explore the response of these tumorspheres to granzyme B and agents molecularly interfering with immunosurveillance system.

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