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Novel Human Breast Cancer Cell Lines IBH-4, IBH-6, and IBH-7 Growing in Nude Mice

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Breast cancer is the most frequent cancer in women. However, in vivo hormone receptor positive and metastatic models are scarce. The aim of the present manuscript was to assess if the novel steroid receptor positive human cell lines IBH-4, IBH-6, and IBH-7 developed in our laboratory from primary infiltrant ductal carcinomas are good models to study in vivo human breast cancer. Cell lines or tumors were inoculated to nude mice in the presence or absence of hormone supplementation. Growth was analyzed by ANOVA followed by Tukey–Kramer's test. Steroid hormone expression was assessed by immunohistochemistry and Western blotting. The histology of the tumors was analyzed. IBH-4 and IBH-6 cells were inoculated to nude mice and 100% of the injected mice developed tumors in the presence or absence of hormone treatment, although tamoxifen inhibited growth. IBH-4 and IBH-6 cell lines in vivo gave rise to poorly differentiated carcinomas with areas of solid growth and sarcomatoid areas showing no morphological signs of epithelial differentiation. Distinct features of malignancy were observed. IBH-7 tumors in animals receiving estradiol were semi-differentiated adenocarcinomas. IBH-7 cells grew only in the presence of estradiol, but even with hormone addition, the tumor take was 20%. These tumors metastasized to the uterus and lung and vascular tumor emboli were evident. IBH-7 tumors were invasive and able to break through the peritoneum. As a conclusion, IBH-4 and IBH-6 are good models for studying tumor progression, whereas IBH-7 is a good model for tumor take, being metastatic and strictly estrogen-dependent.

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Breast cancer is by far the most frequent cancer in women (23% of all cancers), with an estimated 1.15 million new cases in 2002, ranking second overall when both sexes are considered together (Parkin et al., 2005). Despite the large variations in incidence, mortality and survival between different countries, regions, and within specific regions (Hortobagyi et al., 2005), breast cancer is the most common form of cancer among women in North and South America (Pecorelli et al., 2003; Parkin, 2004). With more than 410,000 deaths each year, the disease accounts for more than 1.6% of all female deaths worldwide (Anderson et al., 2006). With the increase in the age of the female population, prevention and treatment of breast cancer will continue to represent a major challenge.

Breast cancer is not a single disease. It is instead a collection of breast diseases that have diverse histopathologies, genetic and genomic variations, and clinical outcomes (Vargo-Gogola and Rosen, 2007). A major challenge in advancing our understanding of the biology of breast cancer is the availability of experimental model systems that recapitulate the many forms of this disease (Vargo-Gogola and Rosen, 2007). The differences between the cell lines and primary tumors probably reflect that many of the cell lines were obtained from advanced-stage tumors and pleural effusions, and, therefore, may represent the most malignant variants that could be adapted to culture (Vargo-Gogola and Rosen, 2007). It should be possible to establish new cell lines from primary tumors and different breast cell lineages. These new cell lines may be more representative, and thus may expand the utility of cell culture models (Vargo-Gogola and Rosen, 2007). We have developed three cell lines which arose from primary tumors from three different patients at diverse stages of disease (Vazquez et al., 2004). They express estrogen receptors (ER) α and β, progesterone (PR) and EGF receptors, and respond to all the ligands for these receptors with increased proliferation (Vazquez et al., 2004).

The aim of the present manuscript was to assess if these cell lines are good models in vivo, growing in nude mice for studying human breast cancer.

Materials and Methods

Animals

The animals used were congenitally athymic nude mice on a Swiss background (N:NIH(S)-nu) bred at *Bioterio de la Universidad de La Plata*, Argentina. The mice were sexually mature females, 8 weeks old. They were housed in ventilated racks, pathogen-free conditions under a 12 h light–dark photoperiodicity and with controlled humidity and temperature ($20 \pm 2^{\circ}$C). Boxes, bedding, food and water were sterilized. Sterility was maintained during the surgical procedures used for the inoculation of the cells to give rise to solid tumors and for subsequent removal and transplantation of tumors. Animal care and manipulation was in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission of Life National Research, 1996).

Breast cancer cell lines and culture conditions

IBH-4, IBH-6, and IBH-7 human breast cancer cell lines were originally isolated from primary human breast carcinomas (Vazquez et al., 2004). Cells were maintained in Dulbecco's Minimun Essential/Ham F12 (1:1) medium supplemented with

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heat-inactivated 10% fetal bovine serum (FBS, Invitrogen Life Technology, Carlsbad, CA), 2 mM glutamine (Invitrogen Life Technology), 2 mg/ml bovine insulin (Invitrogen Life Technology), 100 μg/ml penicillin (Invitrogen Life Technology), 100 μg/ml streptomycin (Invitrogen Life Technology) and 15 mM HEPES (Sigma–Aldrich, St. Louis, MO). Cells were grown on plastic, incubated at 37°C in 5% CO_2 and subcultured at ~80% confluence using 0.25% trypsin–0.025% EDTA (both from Invitrogen Life Technology). Except when stated in the text, the subcultures used for inoculation were from in vitro passages 30–40.

Preparation of cell suspension for injections

Tumor cells were dispersed with 0.25% trypsin–0.025% EDTA, washed twice with medium and adjusted to $1–2 \times 10^7$ viable cells, suspended in 200 μl. The cells were s.c. inoculated on the flank through a 21-gauge needle tunneled 1–2 cm to prevent leakage of the cell *inoculum*.

When stated, mice were supplemented with 17β-estradiol (E_2, 0.5 mg/pellet) (Sigma–Aldrich) in the form of slow-release s.c. pellets placed in the interscapular region, with medroxyprogesterone acetate depot (MPA, 20 mg) (Gador Laboratories, Buenos Aires, Argentina) or with both. Tamoxifen was s.c. injected daily at a 5 mg/kg doses (Lamb et al., 2003).

Tumors

Tumor growth was monitored every second day. The two major diameters were measured with a caliper and the volume was calculated with the formula: volume $= (4/3)\pi \times$ minor radius$^2 \times$ major radius (Bruzzone et al., 2008). Fragments of each tumor were transplanted s.c. by trocar. At the end-point animals were sacrificed and autopsies performed. Organs (including lungs, lymph nodes, liver, spleen, pancreas, uterus, ovaries and any other tissues of abnormal appearance) were examined superficially for evidence of macroscopic metastasis. Primary tumors and organs harvested at autopsy were formalin-fixed, paraffin-embedded and thin-sectioned. Standard hematoxylin and eosin (H&E) staining procedures were used to observe histopathologic characteristics and to determine metastatic involvement. Mitotic index was calculated as the mean of the mitotic images observed in 10 fields at a magnification of 200× in the H&E stained slides.

Immunohistochemistry

An immunohistochemical approach was developed to confirm the epithelial origin of the tumors. Tissue sections were deparaffinized with xylene and rehydrated in graded ethanol. Sections were treated with trypsin 0.25% during 30 min. Endogenous peroxidase was blocked by treatment with 10% hydrogen peroxide for 10 min at room temperature, after which, non-specific binding was blocked by incubation with 10% FBS (Sigma). The rabbit anti-bovine cytokeratin Z0622 polyclonal (Dako Corporation, Carpinteria, CA) was used (at 1:500). After that, cells were treated for 60 min. with anti-rabbit-peroxidase conjugate (LSAB+ System, Dako Corporation).

Steroid receptors were first assessed by immunohistochemistry as described (Montero et al., 2007). Briefly, paraffin-embedded sections were reacted with various antibodies using the avidin biotin peroxidase complex technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Briefly, after dewaxing and hydrating, endogenous peroxidase activity was inhibited using 10% H_2O_2 in distilled water. Blocking solution (10% FCS) was used before specific antibody. Polyclonal antibodies to ER-α (MC-20; Santa Cruz Biotechnology, Santa Cruz, CA), PR (C-19; Santa Cruz Biotechnology) were used at 1:100 dilution and incubated overnight at 4°C. After incubation with the appropriate secondary antibodies (Vector Laboratories) the reactions were developed with the KIT DAKO Cytomation Liquid DAB+ substrate Chromogen System. Specimens were counterstained with hematoxylin, dehydrated, and mounted.

Western blotting for steroid hormone receptors

Steroid receptor expression was tested as previously described (Lamb et al., 2005). The samples (100 μg total protein/lane) were separated on 8% SDS–PAGE using Laemmli's buffer system. The proteins were dissolved in sample buffer (6 mM Tris, 2% SDS, 0.002% bromophenolblue, 20% glycerol, 5% mercaptoethanol, pH 6.8) and boiled for 4 min. After electrophoresis they were blotted onto a nitrocellulose membrane and blocked overnight in 5% dry skimmed milk dissolved in phosphate-buffered saline (PBS)/Tween 0.1% (0.8% NaCl, 0.02% KCl, 0.144% Na_2PO_4, 0.024% H_2PO_4, pH 7.4, 0.1% Tween 20). Following several washes with PBS/Tween, the membranes were incubated with the primary antibody against PR (C-19; Santa Cruz Biotechnology) and ER-α (MC-20; Santa Cruz Biotechnology), at 4°C overnight. Primary antibodies were used at 1:500 concentrations. Blots were probed with anti-mouse or anti-rabbit IgG, horseradish peroxidase-conjugated whole antibody (Amersham Life Science, GE Healthcare, Buckinghamshire, UK). The luminescent signal was generated with ECL Western blotting detection reagent kit (Amersham Pharmacia Biotech, Uppsala, Sweden), and the blots were exposed to a medical X-ray film (Curix RP1; Agfa, Buenos Aires, Argentina) from 10 sec to 5 min. To control the efficiency of transference, membranes were stained with Ponceau S.

Statistical analysis

Statistical analysis of tumor volume was performed by ANOVA followed by Tukey–Kramer test (Dowdy and Wearden, 1983). Differences were considered statistically significant when $P < 0.05$.

Results

Growth of the different cell lines in nude mice

The first experiment was designed to test cell number inoculation to nude mice. The cell number tested ranged from 2.5 to 10×10^6 for IBH-4 and IBH-6, and from 5 to 20×10^6 for IBH-7. All cell numbers tested were able to generate IBH-4 and IBH-6 tumors although the optimal cell number was 10×10^6 (data not shown).

Early in vitro passages (number 10) and later in vitro passages (number 60) were inoculated to nude mice in order to assess if the time spent in culture changed tumor growth. In IBH-4 cells, early passages achieved a tumor volume of 600 mm^3 at 31 days whereas passage 60 achieved that volume in approximately 72 days. Similarly, for IBH-6, the time elapsed to attain 850 mm^3 was 22 days for early passages and 45 days for later passages. These results suggest that the time spent in culture selected a population of cells which were more adapted to the in vitro conditions.

IBH-4 and IBH-6 cells were inoculated to nude mice in three separate experiments for each cell line. In every case 100% of the injected mice developed tumors. The primary tumors were palpable 1 week post-injection and tumor take was 100%. Both cell lines were able to grow without hormone treatment. The tumors which arose from these cell lines were then transplanted into other nude mice and grown until animals were sacrificed and the tumor passed. After the first passage in nude mice the tumors grew significantly faster. Figure 1 shows tumor growth of cells and tumors from successive in vivo passages for IBH-4 (part a) and IBH-6 (part b) tumors. For IBH-7 cell lines, mice had small palpable non-growing nodules which persisted for weeks at the injection site.

As the three cell lines expressed steroid receptors and were stimulated by both E_2 and MPA in vitro, the tumors were assessed for their response in vivo to these hormones by supplementing them with E_2 (0.5 mg/pellet) and/or MPA (20 mg). As shown in Figure 2, neither IBH-4 (part a) nor IBH-6 (part c) showed a significant response to these hormones with

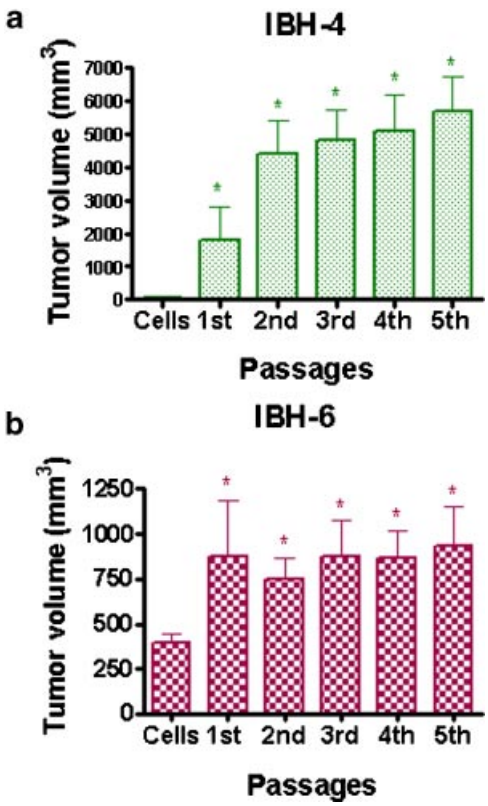

Fig. 1. Tumor growth of IBH-4 (part a) and IBH-6 (part b) after 20 days in nude mice. Cells or tumors were inoculated and measured as detailed in Materials and Methods Section. Bars represent the mean ± SD of three animals. The significantly different bars (as analyzed by ANOVA followed by Tukey–Kramer multiple test) are marked in the graph. The experiment was repeated with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

respect to tumor growth. However, when the effect of tamoxifen treatment was assessed, both tumors inhibited their growth (part b for IBH-4 and d for IBH-6).

On the other hand, the IBH-7 (part e) tumors showed a highly estrogen-dependent behavior, with virtually no growth in the absence of E_2. However, even with hormone addition, the tumor take for IBH-7 tumor was 20%. Even the transplantation of tumors which were able to grow presented a tumor take of 30%. When linearizing the curves, the slope obtained for the growth of tumors in the presence of E_2 was significantly higher than the control ones ($P < 0.001$ by ANOVA followed by Tukey's Multiple Comparison Test). The addition of MPA to estrogen or controls had no noticeable effect on tumor growth.

In order to assess if the tumors still expressed hormone receptors, immunohistochemistry was performed for PR A and B isoforms and ER α. As can be seen in Figure 3, part a, IBH-4 tumors showed both nuclear and cytoplasmic staining for ERα, even in giant multinucleated cells. IBH-6 cells showed strong staining only in some nuclei (Fig. 3, part c) and IBH-7 tumors, a constant nuclear staining in the majority of the tumor nests (Fig. 3, part e). The expression of PR was less evident, with a lower proportion of stained nuclei and cells showing both nuclear and cytoplasmic expression (part b for IBH-4, part d for IBH-6 and part f for IBH-7 tumors). The expression of these hormone receptors was confirmed by Western blotting. As shown in Figure 3, part g, the expression of PRB was evident for IBH-4, IBH-6, and IBH-7. PRA was expressed in lower proportion in all three tumors. ERα was expressed in all three cell lines. Surprisingly, the highly estrogen-dependent IBH-7 tumors showed two different bands, the superior band of 67 kDa should be native receptor (Leclercq, 2002). These tumors do not grow in the absence of estrogen, and were therefore the only extracts from estrogen-bearing mice, and therefore could display a different phosphorylation pattern. These results confirmed that steroid hormone receptors were still expressed in the tumors.

Histology of tumors

Figure 4 depicts the histology of the different tumors with the treatments previously shown in Figure 2. The IBH-4 cells in vivo (parts a–f) gave rise to poorly differentiated carcinomas with areas of solid growth. All these tumors presented a biphasic growth pattern, with sarcomatoid areas showing no morphological signs of epithelial differentiation, and other areas with a definite carcinomatous growth pattern with solid groups of polygonal cells. In the control mice (part a), tumors showed a solid pattern of growth with the presence of sarcomatoid areas. These tumors experienced an extensive necrosis and slight pleomorphism. The animals receiving E_2 pellets (part b) showed a sarcomatoid diffuse pattern with little necrosis and a very marked pleomorphism. The animals which received both treatments (part c) showed groups of polygonal cells. In every case, the tumors are highly undifferentiated and very aggressive: high polymorphism, bizarre, big nuclei with irregularly distributed chromatin, and one or more nucleoli. The mitotic index was very high. The presence of giant multinucleated cells was observed (part d) IBH-4 cells stained for cytokeratins (part e) but not for vimentin (data not shown). Only animals treated with E_2 showed spontaneous lung metastases. Table 1 shows the proportion of mice with metastasis and Figure 4, part f, an image of a lung metastasis.

IBH-6 cell line in vivo gave rise to poorly differentiated carcinomas with absent signs of glandular differentiation. In the case of tumors in control animals, spindle shaped, fibroblastic-like cells are predominant (part g), whereas the E_2 alone animals showed an anaplastic pattern (part h). The animals receiving both hormones (part i) had a mixed cell pattern. In every case, the extracellular matrix is slack and amorphous (part j). Numerous mitotic and apoptotic images were observed. Malignant characteristics similar to IBH-4 tumors were evident. The mitotic index was also very high. The lesion infiltrated extensively the surrounding tissues. Table 1 shows the proportion of mice showing invasion with the different treatments. The epithelial origin of the tumor was assessed by positive staining with cytokeratins (part k) and negative staining with vimentin (data not shown).

IBH-7 tumors in animals receiving E_2 (part l) disclosed a semi-differentiated adenocarcinoma with extensive necrosis and abundant apoptosis and mitosis (both typical and atypical). Tumors in animals receiving both hormones (part m) showed differentiated adenocarcinomas. The neoplastic cells were polygonal, disclosing big nuclei with irregularly distributed chromatin and scanty cytoplasm. Several duct formations were visible. The cytokeratin staining is depicted in part n, with no staining for vimentin (data not shown). The IBH-7 tumor metastasized to uterus, lung (part o) and vascular tumor emboli were observed (part p) and exclusively in the presence of E_2, the tumor was invasive and was able to break through the peritoneum. Table 1 shows the proportion of mice with metastasis at different locations.

Table 2 summarizes the predominant cell type and the biological behavior of the tumors in the presence and absence of E_2 and/or MPA.

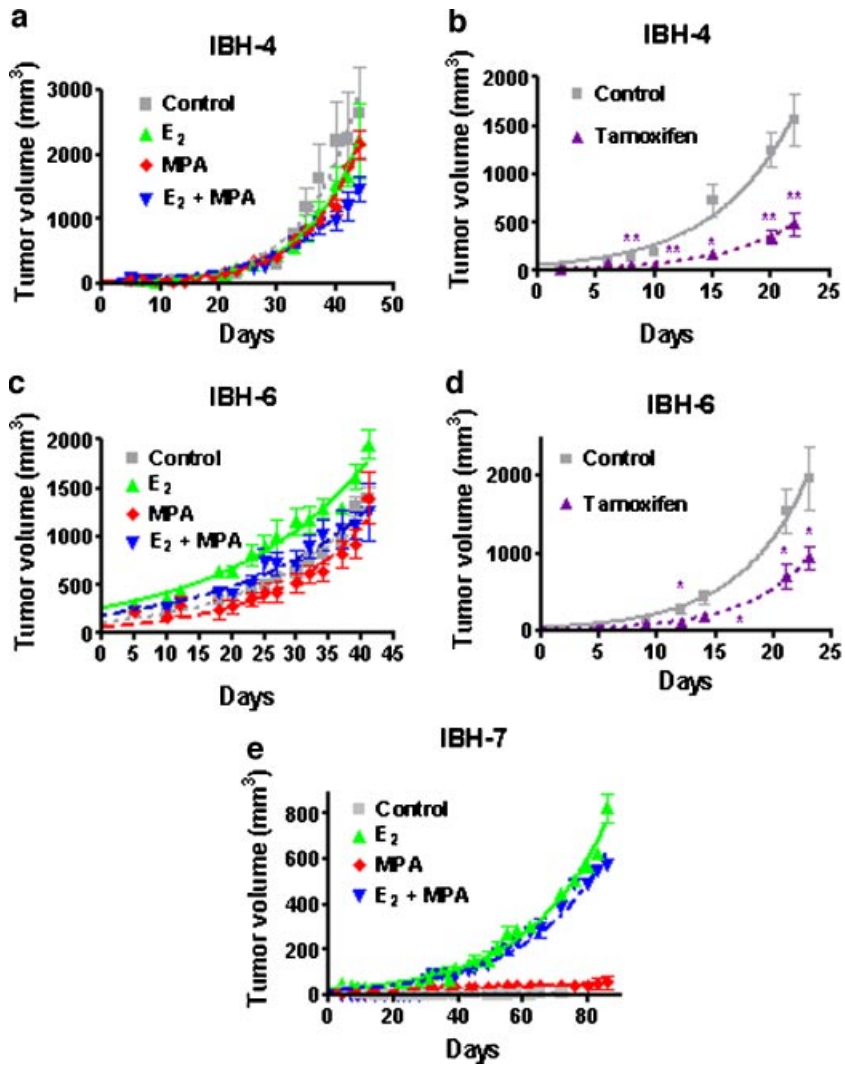

Fig. 2. Effect of the administration of steroid hormones to the nude mice on tumor growth. The tumors IBH-4 (parts a,b), IBH-6 (parts c,d) and IBH-7 (part e) were inoculated and measured as detailed in Materials and Methods Section. The tumors were passed and the animals in parts a, c, and e received simultaneously a pellet with no hormone, (control, ■), 17β-estradiol (E_2, 0.5 mg/pellet, ▲), with medroxiprogesterone acetate depot (MPA, 20 mg, ◆) or with both (E_2 + MPA, ▼). The experiment was repeated with similar results. Parts b and d show the effect of daily administration of tamoxifen (▲) (5 mg/kg) on tumor growth, as compared to control animals (■) $^{*}P < 0.05$; ** <0.01. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Discussion

Growth of the different cell lines in nude mice

The characteristics of growth pattern, hormonal requirements and metastatic capabilities of human breast cells lines growing in athymic nude mice are reported. IBH-4, IBH-6, and IBH-7 cells lines derived from primary human breast carcinoma. IBH-4 cells came from a post-menopausal, 69-year-old Caucasian woman, who underwent tumorectomy and axillary dissection for an invasive ductal carcinoma in her right breast, with positive axillary nodes. IBH-6 arose from a pre-menopausal 34-year-old Caucasian woman with locally advanced ductal carcinoma, who underwent neoadjuvant chemotherapy followed by mastectomy, with positive lymph nodes. IBH-7 was cultured from a tumor of a pre-menopausal 40-year-old Caucasian woman with invasive ductal carcinoma who underwent tumorectomy with axillary dissection, remaining asymptomatic after 7 years (Vazquez et al., 2004).

When setting up the growth conditions for the different cell lines in nude mice, IBH-4 and IBH-6 cells showed that the time spent in culture impaired tumor growth, as if the cells adapted by selection to growing in plastic. Once the cells began to grow in nude mice, they grew fast in animals, in the case of IBH-4 progressively and in the case of IBH-6, from the first animal passage. This reversibility suggests a selection of cells which are better fitted for plastic growth. Other cell types may be present however, and are probably selected again in the animal passages.

All three cell lines in culture expressed both ER-α and ER-β and were able to bind to a tritiated progestin, and respond to the incubation with the correspondent agonists by enhancing cell proliferation (Vazquez et al., 2004). It has been reported that intact adult athymic mice have low circulating E_2 concentration, slightly higher than that of post-menopausal women (Shafie and Liotta, 1980; Shafie and Grantham, 1981; Seibert et al., 1983). Since nude mice are unable to optimally support the growth of MCF-7 tumors, some animals were supplemented with E_2 pellets with or without progestin

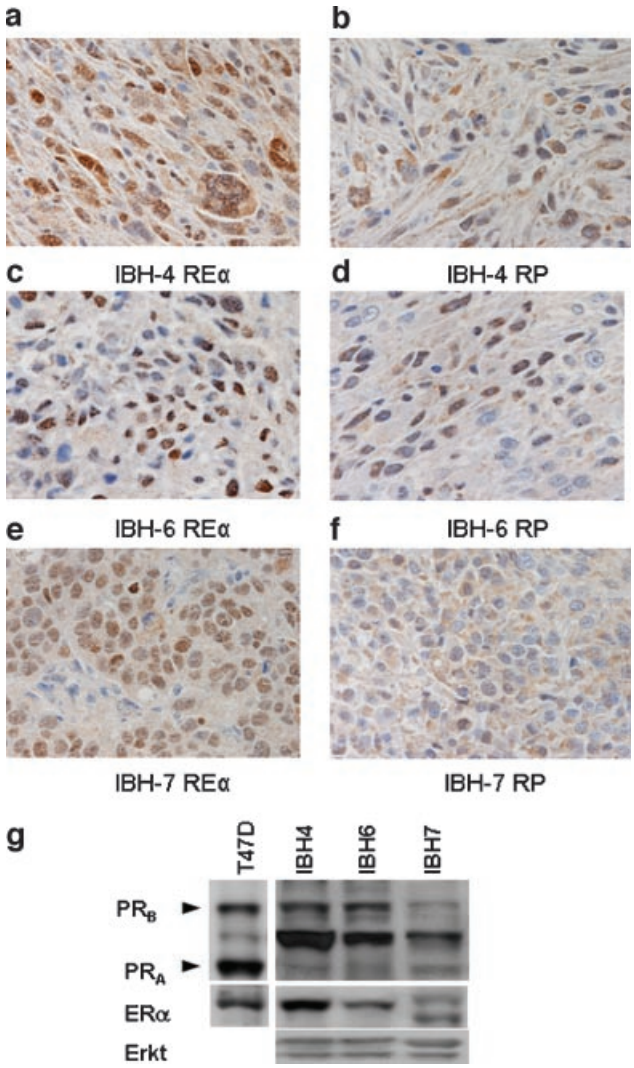

Fig. 3. Immunohistochemistry and Western blotting for steroid receptors. Immunohistochemistry and Western blotting were performed as described in Material and Methods Section. Immunohistochemistry for IBH-4, part a: estrogen receptor-α (ERα), part b: progesterone receptors (PR); for IBH-6, part c: ERα, part d: PR and for IBH-7, part e: ERα, part f: PR. Part g: Western blotting for PR and ERα. As a positive control, human breast cancer T47D cells were used. The experiment was repeated with similar results.

administration. The results varied with the cell line. Whereas IBH-4 and IBH-6 did not show any response to these hormones with respect to tumor growth, the IBH-7 grew only in the presence of E_2. The effect of this hormone could be either direct through their ER or systemic, by enhancing angiogenesis and stromal cell recruitment (Gupta et al., 2007). However, both IBH-4 and IBH-6 were able to respond to tamoxifen with a significant inhibition of tumor growth. Although the usual behavior of tumors is to respond to both estrogen and tamoxifen, this and other SERMs may even inhibit growth of ER α-negative cells and tumors (Medina et al., 2005; Stuart and Rosengren, 2008).

An interesting feature of these cell lines is the differential in vivo/in vitro hormone responsiveness. In vitro, IBH-4 and IBH-6 cell lines showed a very sharp response to E_2, with EC_{50} values of 39.5 and 53.2 pM, respectively (Vazquez et al., 2004) whereas the in vivo response was not evident. On the other hand, IBH-7 cells with a more discrete in vitro response with an EC_{50} value of 1.51 nM (Vazquez et al., 2004) was highly dependent on estrogen for in vivo growth. A similar feature has been described (Lanari et al., 2001) in an experimental model of ER and PR positive transplanted mammary tumors originally induced by medroxyprogesterone acetate (MPA) in female BALB/c mice. Curiously, similar differences between in vitro/in vivo hormone sensibility were described (Lanari et al., 2001). The different hormone sensitivity in vitro and in vivo could be due to the well-established influence of the stromal microenvironment in the tumors (Orimo et al., 2005). The accumulated evidence indicates that tumor cells actively recruit stromal cells, such as inflammatory cells, vascular cells, and fibroblasts into the tumor, and that this recruitment is essential for the generation of a microenvironment that actively fosters tumor growth (Orimo et al., 2005).

Histology of tumors

The IBH-4 tumors in nude mice are poorly differentiated. These tumors presented a biphasic growth pattern, with sarcomatoid areas showing no morphological signs of epithelial differentiation, and other areas showing a definite carcinomatous growth pattern with solid groups of polygonal cells. Even if the growth of these cells is not influenced by the administration of E_2, the tumors are even more malignant and metastatic in its presence. The administration of this hormone selects the more sarcomatoid cell type as predominant, whereas in the presence of MPA, the polygonal-type cells are selected. IBH-6 displays a similar behavior as the IBH-4 cells, with an influence of E_2 in malignancy although no response in tumor growth is observed. The IBH-4 and IBH-6 cells grow very fast, so the animals have to be sacrificed before the appearance of massive metastases, especially in the case of the latter cell line. With respect to IBH-7 cells growing in nude mice, the pattern of semi-differentiated tumors in E_2-bearing animals changed to differentiated tumors in the presence of the progestin, suggesting a differentiating influence of this hormone. As a general feature, it can be concluded that although estrogen regulates only IBH-7 cell growth, this hormone is able to render all three tumors more malignant. Epithelial to mesenchymal transition (EMT) is an important change in cell phenotype which allows the escape of epithelial cells from the structural constraints imposed by tissue architecture (Hugo et al., 2007). Over the last decade, evidence has mounted for EMT as the means through which solid tissue epithelial cancers invade and metastasize (Hugo et al., 2007). EMT involves dedifferentiation of polarized epithelial cells to a migratory fibroblastoid phenotype, a phenomenon that is increasingly considered to be an important event during cancer progression and metastasis (Huang et al., 2007). Breast cancer cell lines with increased invasiveness in vitro, and metastatic potential in vivo, exhibited expression of the mesenchymal intermediate filament protein vimentin (Hugo et al., 2007). In all the cell lines described here in nude mice, no expression of vimentin was found, suggesting that even if there may be some degree of EMT induced by E_2, mainly in the metastatic capacity of the cells, and the shape they adopt, the transition is not complete.

Metastasis suppressor genes have been defined as genes in which loss of function specifically enhances metastasis without affecting primary tumor growth (Nguyen and Massague, 2007). One such gene (Mitchell et al., 2006), *KiSS-1* or *KiSS1*, originally identified as a metastasis suppressor in melanoma lines, has been found to have lost or reduced expression in a variety of tumor metastasis, including breast cancer (Mitchell et al., 2006). The binding of the KiSS-1 peptide to the membrane-bound G-protein-coupled receptor GPCR54 initiates a series of cellular changes as well as morphological changes, such as up-regulating focal adhesion and stress fiber formation, inhibiting chemotaxis

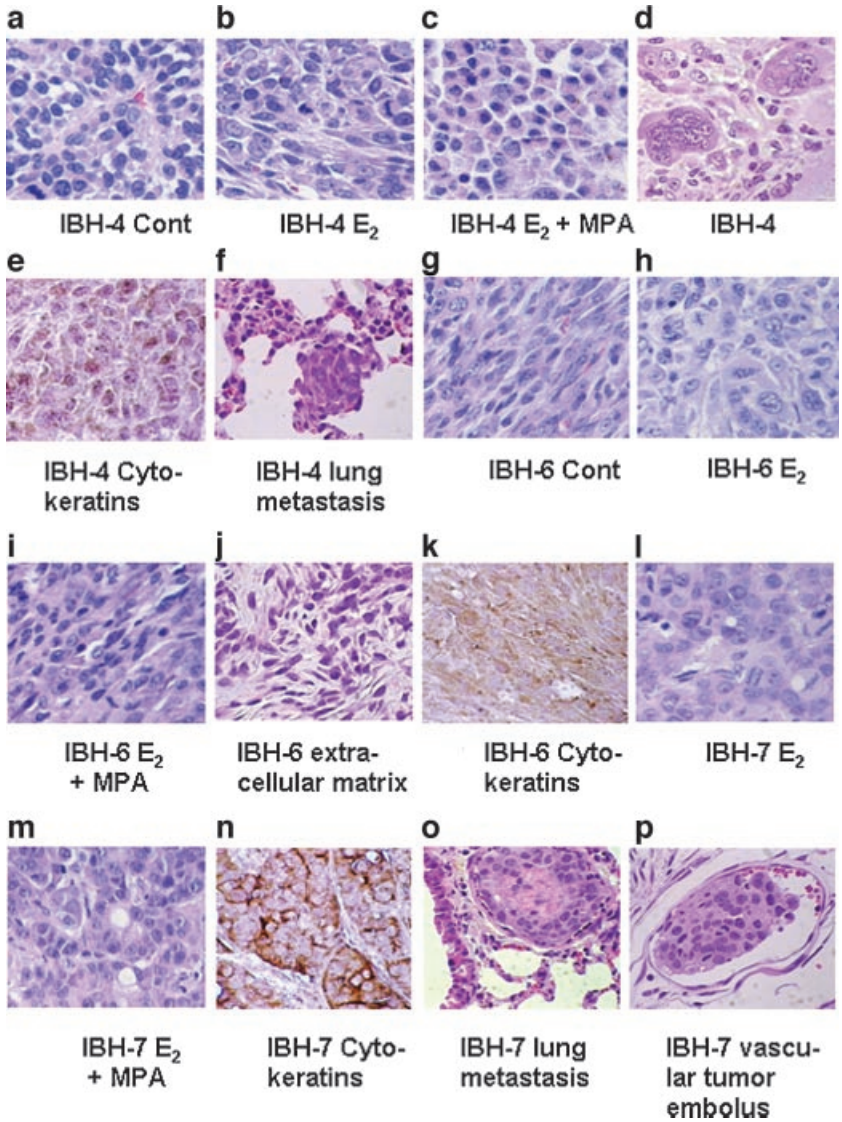

Fig. 4. Histology of the different tumors. Hematoxylin and eosin (H&E) staining was used in order to analyze the histopathologic features of the tumors, except in parts e, k, and n, where the cytokeratin staining is shown. IBH-4 tumors: a: control, b: estradiol (E_2)-treated animals, c: E_2 + medroxyprogesterone acetate (MPA) treated mice, d: detail of a control tumor, showing giant multinucleated cells, e: cytokeratin staining, f: lung metastasis. IBH-6: g: control animal, h: E_2-treated, i: E_2 + MPA, j: detail of extracellular matrix from control tumor, k: cytokeratin staining. IBH-7: l: E_2-treated, m: E_2 + MPA, n: cytokeratin staining, o: lung metastasis, p: vascular tumor embolus. Augmentation: 400×.

and invasion and reducing the incidence of tumor metastasis (Mitchell et al., 2006). It has recently been demonstrated (Li et al., 2007) that the interaction of ERα with Sp1 transactivates KiSS1 promoter activity, whereas its interaction with Sp3 functions as a transcriptional repressor in ER-α positive hypothalamic GT1–7 cells. Although this item has not been yet analyzed, we speculate that the regulation of this metastasis suppressor gene by the interaction of E_2 and Sp3 on Sp-1 target DNA elements or the interaction with another metastasis suppressor gene could be an eventual explanation for the metastatic effect of this hormone without affecting tumor growth in IBH-4 tumors.

IBH-4, IBH-6, and IBH-7 xenografts as models for human breast cancer

The growth of breast cancer cell lines as xenografts allows investigation in an in vivo environment, which includes the complex tumor–stroma interactions that facilitate tumor formation and progression. Many facets of breast cancer biology have been investigated using xenografts, including the genetic alterations that contribute to tumor initiation and growth (Gonzalez et al., 2006; Iliopoulos et al., 2007; Scaltriti et al., 2007), signal transduction in tumor progression (Kasper et al., 2007), the role of the microenvironment (tumor cell–extracellular matrix interactions (Kasper et al., 2007), inflammation and angiogenesis), and the multistage process of metastasis (Bandyopadhyay et al., 2007; Vargo-Gogola and Rosen, 2007). Therapeutic inhibition of breast cancer xenograft tumor growth, with (Arpino et al., 2007) or without (Yao et al., 2000; Paulus et al., 2006; Halpern et al., 2007) gene modification has also been studied successfully. As considering the models in vitro and in vivo, huge differences have been reported in the expression of estrogen-regulated genes in the same T47D human breast cancer cells growing in vivo and in vitro, with only 11% overlap (Harvell et al., 2006). Moreover, occasionally, the

TABLE 1. Invasion and/or metastasis with different hormone treatments

	Control	Estradiol	Estradiol + MPA
IBH-4	0/4 metastasis	2/4 lung metastasis	0/4 metastasis
IBH-6	4/4 peritoneum invasion	1/4 peritoneum invasion	1/4 peritoneum invasion
IBH-7	No tumor	2/4 peritoneum invasion	0/4 metastasis
		1/4 uterine metastasis	
		2/4 lung metastasis	

Summary of the invasive and/or metastatic behavior of the different tumors and treatments.

TABLE 2. Predominant cell type and biological behavior

	Predominant cell type	Biological behavior
IBH-4 tumor		
Control	Spindle and polygonal cells	No metastases
E_2	Spindle cells	Lung metastases
E_2 + MPA	Polygonal cells	No metastases
IBH-6 tumor		
Control	Spindle cells	All able to invade the peritoneum and seed secondary tumors
E_2	Anaplastic cells	
E_2 + MPA	Spindle and anaplastic cells	
IBH-7 tumor		
Control	—	—
E_2	Polygonal cells	Vascular tumor emboli. Uterine and lung metastases. Occasionally able to invade the peritoneum and seed secondary tumors
E_2 + MPA	Polygonal cells with duct formation	No metastases

same gene is regulated in opposite directions in vitro compared with in vivo (Harvell et al., 2006).

Seventy to 80% of primary breast cancers are ER and/or PR positive and considered hormone responsive. Importantly, if the primary tumors are ER+, >80% of lymph node metastases and 65–70% of distant metastases retain their receptors (Harrell et al., 2006). Even if the knowledge of breast cancer was greatly enhanced lately, there is a general concordance about a certain lack in experimental models. The ER+ human breast cancer cell lines most widely used for xenograft studies are MCF-7, T47D, ZR75 and BT-474 cells (Harrell et al., 2006; Liang et al., 2007). They develop tumors in mammary glands of nude mice in response to E_2 supplementation. However, such tumors are poorly invasive and rarely, if ever, metastasize (Harrell et al., 2006). These cell lines can be genetically modified in order to enhance their metastatic capacity (Zhang et al., 2007a,b). Most metastasis models do not use these cells, relying instead on ER negative cell lines like MDA-MB-231 (Wang et al., 2006), MDA-MB-435 (Martinelli et al., 2007) and MDA-MB-468 (Allan et al., 2006) that express putative aggressiveness markers and do metastasize in nude mice (Harrell et al., 2006). Even a xenograft model based on a spontaneously immortalized human breast epithelial cell line, (MCF-10A) rendered tumorigenic, but not metastatic by genetic manipulation was successfully used in in vivo studies (Dawson et al., 1996; Tang et al., 2007).

Alternative approaches for studying tumor aggressiveness with ER+ breast cancer cells involve their modification to overexpress oncogenes or growth factors or their receptors in an effort to enhance tumor progression while suppressing estrogen-dependent behavior (as recently reviewed in Harrell et al., 2006). From the work referenced, a certain lack of steroid receptor positive and metastatic behavior is evident.

Conclusions

From the results shown in the present manuscript it may be concluded that the IBH-4 and IBH-6 are good models for in vivo studying several features of advanced human breast cancers, including pharmacological studies. On the other hand, IBH-7 cells with their difficult and highly estrogen-dependent growth are a good model for analyzing the effects on tumor take, as well as estrogen dependent metastasis. All these tumors were invasive and/or metastatic, at least in the presence of E_2 pellets.

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Literature Cited

Allan AL, George R, Vantyghem SA, Lee MW, Hodgson NC, Engel CJ, Holliday RL, Girvan DP, Scott LA, Postenka CO, Al Katib W, Stitt LW, Uede T, Chambers AF, Tuck AB. 2006. Role of the integrin-binding protein osteopontin in lymphatic metastasis of breast cancer. Am J Pathol 169:233–246.

Anderson BO, Shyyan R, Eniu A, Smith RA, Yip CH, Bese NS, Chow LW, Masood S, Ramsey SD, Carlson RW. 2006. Breast cancer in limited-resource countries: An overview of the Breast Health Global Initiative 2005 guidelines. Breast J 12:S3–S15.

Arpino G, Gutierrez C, Weiss H, Rimawi M, Massarweh S, Bharwani L, De Placido S, Osborne CK, Schiff R. 2007. Treatment of human epidermal growth factor receptor 2-overexpressing breast cancer xenografts with multiagent HER-targeted therapy. J Natl Cancer Inst 99:694–705.

Bandyopadhyay A, Wang L, Chin SH, Sun LZ. 2007. Inhibition of skeletal metastasis by ectopic ERalpha expression in ERalpha-negative human breast cancer cell lines. Neoplasia 9:113–118.

Bruzzone A, Pinero CP, Castillo LF, Sarappa MG, Rojas P, Lanari C, Luthy IA. 2008. alpha(2)-Adrenoceptor action on cell proliferation and mammary tumour growth in mice. Br J Pharmacol 155:494–504.

Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. 1996. MCF10AT: A model for the evolution of cancer from proliferative breast disease. Am J Pathol 148:313–319.

Dowdy S, Wearden S. 1983. Statistics for research. New York: John Wiley & Sons, Inc.

Gonzalez L, Agullo-Ortuno MT, Garcia-Martinez JM, Calcabrini A, Gamallo C, Palacios J, Aranda A, Martin-Perez J. 2006. Role of c-Src in human MCF7 breast cancer cell tumorigenesis. J Biol Chem 281:20851–20864.

Gupta PB, Proia D, Cingoz O, Weremowicz J, Naber SP, Weinberg RA, Kuperwasser C. 2007. Systemic stromal effects of estrogen promote the growth of estrogen receptor-negative cancers. Cancer Res 67:2062–2071.

Halpern M, Zahalka MA, Traub L, Moroz C. 2007. Antibodies to placental immunoregulatory ferritin with transfer of polyclonal lymphocytes arrest MCF-7 human breast cancer growth in a nude mouse model. Neoplasia 9:487–494.

Harrell JC, Dye WW, Allred DC, Jedlicka P, Spoelstra NS, Sartorius CA, Horwitz KB. 2006. Estrogen receptor positive breast cancer metastasis: Altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. Cancer Res 66:9308–9315.

Harvell DM, Richer JK, Allred DC, Sartorius CA, Horwitz KB. 2006. Estradiol regulates different genes in human breast tumor xenografts compared with the identical cells in culture. Endocrinology 147:700–713.

Hortobagyi GN, de la Garza SJ, Pritchard K, Amadori D, Haidinger R, Hudis CA, Khaled H, Liu MC, Martin M, Namer M, O'Shaughnessy JA, Shen ZZ, Albain KS. 2005. The global breast cancer burden: Variations in epidemiology and survival. Clin Breast Cancer 6:391–401.
Huang Y, Fernandez SV, Goodwin S, Russo PA, Russo IH, Sutter TR, Russo J. 2007. Epithelial to mesenchymal transition in human breast epithelial cells transformed by 17beta-estradiol. Cancer Res 67:11147–11157.
Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW. 2007. Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. J Cell Physiol 213:374–383.
Iliopoulos D, Fabbri M, Druck T, Qin HR, Han SY, Huebner K. 2007. Inhibition of breast cancer cell growth in vitro and in vivo: Effect of restoration of Wwox expression. Clin Cancer Res 13:268–274.
Institute of Laboratory Animal Resources Commission on Life National Research. 1996. Guide for the care and use of laboratory animals. Washington, DC: National Academy Press.
Kasper G, Reule M, Tschirschmann M, Dankert N, Stout-Weider K, Lauster R, Schrock E, Mennerich D, Duda GN, Lehmann KE. 2007. Stromelysin-3 over-expression enhances tumourigenesis in MCF-7 and MDA-MB-231 breast cancer cell lines: Involvement of the IGF-1 signalling pathway. BMC Cancer 7:12.
Lamb CA, Helguero LA, Fabris V, Lucas C, Molinolo AA, Lanari C. 2003. Differential effects of raloxifene, tamoxifen and fulvestrant on a murine mammary carcinoma. Breast Cancer Res Treat 79:25–35.
Lamb CA, Helguero LA, Giulianelli S, Soldati R, Vanzulli SI, Molinolo A, Lanari C. 2005. Antisense oligonucleotides targeting the progesterone receptor inhibit hormone-independent breast cancer growth in mice. Breast Cancer Res 7:R1111–R1121.
Lanari C, Luthy I, Lamb CA, Fabris V, Pagano E, Helguero LA, Sanjuan N, Merani S, Molinolo AA. 2001. Five novel hormone-responsive cell lines derived from murine mammary ductal carcinomas: In vivo and in vitro effects of estrogens and progestins. Cancer Res 61:293–302.
Leclercq G. 2002. Molecular forms of the estrogen receptor in breast cancer. J Steroid Biochem Mol Biol 80:259–272.
Li D, Mitchell D, Luo J, Yi Z, Cho SG, Guo J, Li X, Ning G, Wu X, Liu M. 2007. Estrogen regulates KiSS1 gene expression through estrogen receptor alpha and SP protein complexes. Endocrinology 148:4821–4828.
Liang Y, Besch-Williford C, Brekken RA, Hyder SM. 2007. Progestin-dependent progression of human breast tumor xenografts: A novel model for evaluating antitumor therapeutics. Cancer Res 67:9929–9936.
Martinelli M, Bonezzi K, Riccardi E, Kuhn E, Frapolli R, Zucchetti M, Ryan AJ, Taraboletti G, Giavazzi R. 2007. Sequence dependent antitumour efficacy of the vascular disrupting agent ZD6126 in combination with paclitaxel. Br J Cancer 97:888–894.
Medina D, Kittrell FS, Hill J, Shepard A, Thordarson G, Brown P. 2005. Tamoxifen inhibition of estrogen receptor-alpha-negative mouse mammary tumorigenesis. Cancer Res 65:3493–3496.
Mitchell DC, Abdelrahim M, Weng J, Stafford LJ, Safe S, Bar-Eli M, Liu M. 2006. Regulation of KiSS-1 metastasis suppressor gene expression in breast cancer cells by direct interaction of transcription factors activator protein-2alpha and specificity protein-1. J Biol Chem 281:51–58.
Montero GG, Vanzulli SI, Cerliani JP, Bottino MC, Bolado J, Vela J, Becu-Villalobos D, Benavides F, Gutkind S, Patel V, Molinolo AA, Lanari C. 2007. Association of estrogen receptor alpha and progesterone receptor A expression with hormonal mammary carcinogenesis: Role of the host microenvironment. Breast Cancer Res 9:R22.
Nguyen DX, Massague J. 2007. Genetic determinants of cancer metastasis. Nat Rev Genet 8:341–352.
Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121:335–348.
Parkin DM. 2004. International variation. Oncogene 23:6329–6340.
Parkin DM, Bray F, Ferlay J, Pisani P. 2005. Global cancer statistics, 2002. CA Cancer J Clin 55:74–108.
Paulus P, Stanley ER, Schafer R, Abraham D, Aharinejad S. 2006. Colony-stimulating factor-1 antibody reverses chemoresistance in human MCF-7 breast cancer xenografts. Cancer Res 66:4349–4356.
Pecorelli S, Favalli G, Zigliani L, Odicino F. 2003. Cancer in women. Int J Gynaecol Obstet 82:369–379.
Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J, Di Cosimo S, Matias-Guiu X, Cajal S, Arribas J, Baselga J. 2007. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst 99:628–638.
Seibert K, Shafie SM, Triche TJ, Whang-Peng JJ, O'Brien SJ, Toney JH, Huff KK, Lippman ME. 1983. Clonal variation of MCF-7 breast cancer cells in vitro and in athymic nude mice. Cancer Res 43:2223–2239.
Shafie SM, Grantham FH. 1981. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 67:51–56.
Shafie SM, Liotta LA. 1980. Formation of metastasis by human breast carcinoma cells (MCF-7) in nude mice. Cancer Lett 11:81–87.
Stuart EC, Rosengren RJ. 2008. The combination of raloxifene and epigallocatechin gallate suppresses growth and induces apoptosis in MDA-MB-231 cells. Life Sci 82:943–948.
Tang B, Yoo N, Vu M, Mamura M, Nam JS, Ooshima A, Du Z, Desprez PY, Anver MR, Michalowska AM, Shih J, Parks WT, Wakefield LM. 2007. Transforming growth factor-beta can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. Cancer Res 67:8643–8652.
Vargo-Gogola T, Rosen JM. 2007. Modelling breast cancer: One size does not fit all. Nat Rev Cancer 7:659–672.
Vazquez SM, Mladovan A, Garbovesky C, Baldi A, Luthy IA. 2004. Three novel hormone-responsive cell lines derived from primary human breast carcinomas: Functional characterization. J Cell Physiol 199:460–469.
Wang Y, Lam JB, Lam KS, Liu J, Lam MC, Hoo RL, Wu D, Cooper GJ, Xu A. 2006. Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. Cancer Res 66:11462–11470.
Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC. 2000. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036.
Zhang Y, Pu X, Shi M, Chen L, Qian L, Song Y, Yuan G, Zhang H, Yu M, Hu M, Shen B, Guo N. 2007a. c-Jun, a crucial molecule in metastasis of breast cancer and potential target for biotherapy. Oncol Rep 18:1207–1212.
Zhang Y, Pu X, Shi M, Chen L, Song Y, Qian L, Yuan G, Zhang H, Yu M, Hu M, Shen B, Guo N. 2007b. Critical role of c-Jun overexpression in liver metastasis of human breast cancer xenograft model. BMC Cancer 7:145.