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Heterogeneity of Phenotype in Breast Cancer Cell Lines

Bruce C. Baguley and Euphemia Leung
*Auckland Cancer Society Research Centre, University of Auckland,
New Zealand*

1. Introduction

Breast cancer is a major challenge to current medicine; it is the disease with highest death rate in the female population and is even of significance to the male population. Although breast cancer is effectively treated by surgery at early stages, patients who present with breast cancer metastases at diagnosis or who subsequently develop metastatic disease have a much poorer prognosis. A feature of the normal breast endothelium is its regulation by the endocrine system; steroid hormones such as oestrogen are released predominantly by the ovary and control both proliferation and differentiation of epithelial cells. The proliferation of corresponding carcinoma cells that arise in early breast cancer has a similar dependence on endocrine hormones. Thus, one of the main methods for treating early breast cancer, apart from surgery, is to block the growth promoting action of oestrogen, either by blocking the downstream action with antioestrogens such as tamoxifen or by reducing the concentration of circulating oestrogen through oophorectomy or treatment with aromatase inhibitors. While such treatment is generally effective, the consequent emergence of aggressive tumours is common and poses a major barrier to successful disease management. Heterogeneity has been postulated to be a key property of both breast cancer and epithelial subtypes of normal breast tissue (Visvader, 2009). MCF-7, a commonly used breast cancer cell line, has been propagated for many years by multiple groups and it might be expected that such propagation would select a single phenotype that had the highest growth rate. However, the finding of extensive heterogeneity among MCF-7 lines used by different groups (Nugoli et al., 2003) suggests that mechanisms may be operating within proliferating MCF-7 populations to generate phenotypic diversity continuously. The aim of this chapter is to discuss the evidence of the way that the MCF-7 breast cancer cell line is heterogeneous with respect to both the expression of hormone receptors and to the utilization of the signalling pathways linked to these receptors. Such heterogeneity may be reflected by the presence of multiple phenotypes within a tumour population that differ markedly in their relative expression of receptors such as progesterone receptor (PR), oestrogen receptor (ER), epidermal growth factor receptor (EGFR) and epidermal growth factor receptor-2 (HER2; also known as ErbB2).

2. Detection of cellular heterogeneity in the MCF-7 breast cancer cell line

Several approaches have been employed to investigate phenotypic heterogeneity in MCF-7 cell lines. Cassanelli et al. (1995) isolated a series of individual MCF-7 clones and measured

their expression of PR, finding that two thirds were PR-negative and that the remainder showed various degrees of PR expression. They also showed that PR expression was related to proliferation rate in culture. Coser et al. (2009) isolated multiple MCF-7 clones using two different culture conditions. Firstly, they picked individual colonies from low density MCF-7 cultures to establish antioestrogen-sensitive MCF-7 sub-lines. Secondly, they picked individual antioestrogen-resistant colonies from MCF-7 cultures that had previously been grown to high density and exposed to either 4-hydroxytamoxifen (1 μ M) or to fulvestrant (10–100 nM) for 21 days. The surviving cells (less than 0.001% of the population) were allowed to recover in drug-free growth medium for 7 days before isolation of the colonies, and all sub-lines subsequently characterized within a 20 population doubling period of culture. The isolated sub-lines could be distinguished by morphology, gene expression profile, gene copy number variations and the presence of individual genetic changes. The results indicated that all of the antioestrogen-resistant MCF-7 sub-lines were derived from a common ancestor.

In a somewhat different approach, Leung et al. (2010) set out to mimic, *in vitro*, the conditions that lead to the development clinical resistance to antioestrogens (Leung et al., 2010). Three different conditions were used to generate sub-lines. Firstly, MCF-7 cells were grown continuously in standard growth medium in the presence of increasing amounts of tamoxifen to produce the TamR7 sub-line. Oestrogen (which is present in foetal bovine serum) was not specifically excluded, thus mimicking clinical antioestrogen therapy. Secondly, cells were grown continuously in culture medium in the absence of both oestrogen and phenol red (which has oestrogenic properties) to produce the TamC3 and TamC6 sub-lines. The foetal bovine serum, used as a source of growth factors, had been previously absorbed with charcoal to remove oestrogen, thus mimicking the clinical effects of either oophorectomy or treatment with aromatase inhibitors such as letrozole. Thirdly, cells were grown continuously as above in the absence of oestrogen but with the addition of tamoxifen to produce the TamR3 and TamR6 sub-lines, thus mimicking the effect of combined therapy with antioestrogens plus aromatase inhibitors. Independent studies were undertaken using different batches of foetal calf serum, one batch giving the “3-series” (TamC3 and TamR3) and another giving the “6-series” (TamC6 and TamR6). Each condition resulted initially in the death of a high proportion of the cell population, followed over a period of at least 6 months by the emergence of resistant cells; however, the sub-lines were not clonally derived. Some of the properties of these sub-lines are shown in Table 1; all were shown by microsatellite analysis to be related to the parental MCF-7 cell line (Leung et al., 2010).

	Parental	TamR7	TamC3	TamR3	TamC6	TamR6
DNA content (ploidy)	1.5	1.9	1.4	1.4	2.0	2.1
Modal cell volume (pL)	2.4	2.6	1.6	1.7	2.2	2.0
Cell cycle time (hours)	31	31	27	27	36	37

Table 1. Characteristics of the MCF-7 line and of its sub-lines. From previously published data (Leung et al., 2010).

The above sub-lines were each found to be resistant to tamoxifen and were initially characterized by DNA content, cell cycle time and cell size. Flow cytometry was utilized to measure DNA content following staining of DNA with propidium bromide; cell size was

determined by forward scatter in a flow cytometer; cell cycle time was measured by a stathmokinetic method involving the arrest of cell division by the mitotic poison paclitaxel and subsequent measurement of the reduction in the incorporation of ^3H -thymidine by the S-phase (Baguley et al., 1995). Surprisingly, DNA content alone distinguished four of the five sub-lines (Fig. 1). The parental MCF-7 line was aneuploid with a DNA content of 1.5x diploid, while the ploidy of derived sub-lines ranged from 1.4x diploid to 2.1x diploid. Changes in ploidy arise as a consequence of chromosomal instability, which in turn is related to the presence of extra centrosomes (Ganem et al., 2009). Control of centrosome number is in turn influenced by the expression of oncogenic and tumour suppressor proteins (Fukasawa, 2007). Changes in ploidy of a tumour population appear to occur gradually as a function of cell division, providing an effective measure of divergence of individual cells in a population.

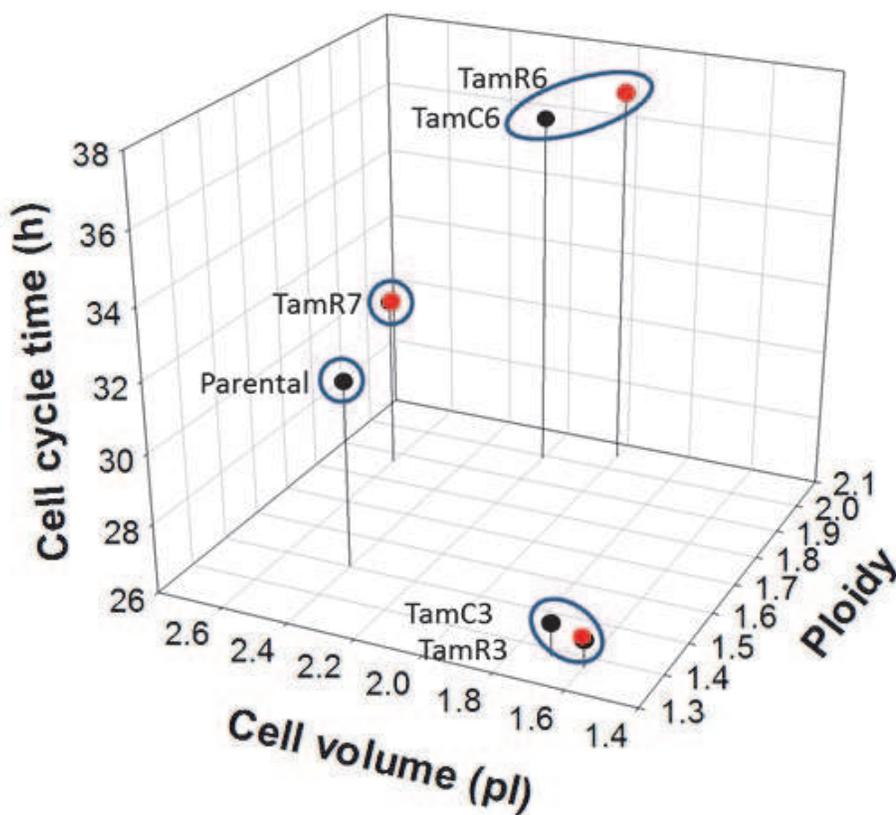


Fig. 1. Relationship between DNA ploidy, modal cell volume and cell cycle time of MCF-7 sub-lines. Figure reproduced from previously published data (Leung et al., 2010).

Mean cell cycle time and modal cell volume were also used to distinguish the sub-lines. Surprisingly, cell volume did not appear to be related to DNA content (Fig. 1). It was of interest that two lines, TamC6 and TamR6, which were derived under very different growth conditions (one in the presence of tamoxifen and one in its absence) showed very similar DNA content, cell cycle time and modal cell volume; the same situation applied to TamC3 and TamR3, which are very similar to each other although differing substantially from TamC6 and TamR6 (Fig. 1). Although it is likely that the constituent cell lines in each of these pairs are not identical, their similar properties suggest they are closely related. Such

hierarchies might be expected on the basis of molecular signatures derived from gene expression arrays (Coser et al., 2009).

3. Steroid hormone receptor expression

The results in the previous section demonstrate the presence of heterogeneity in the MCF-7 cell lines and imply that small sub-populations existing in the parental line can be expanded under appropriate selective conditions. The time scale of the *in vitro* selection process (6 months or more) is consistent with the long period of time that occurs clinically in the development of resistance to antioestrogens or aromatase inhibitors in breast cancer patients. However, a critical question with regard to therapy is whether the emerging sub-lines express altered receptors and associated signalling pathways. This question was addressed using the sub-lines in Table 1. Expression of ER is shown in Fig. 2 and that of PR is shown in Fig. 3. MCF-7 is an ER⁺ tumour but as can be seen from Fig. 2, ER expression was weak when compared to that of the tamoxifen-resistant sub-lines with the exception of TamC6, which also expressed ER weakly. On the other hand, expression of PR was strong in the parental line, weak in TamC6 and virtually absent in the remaining sub-lines.

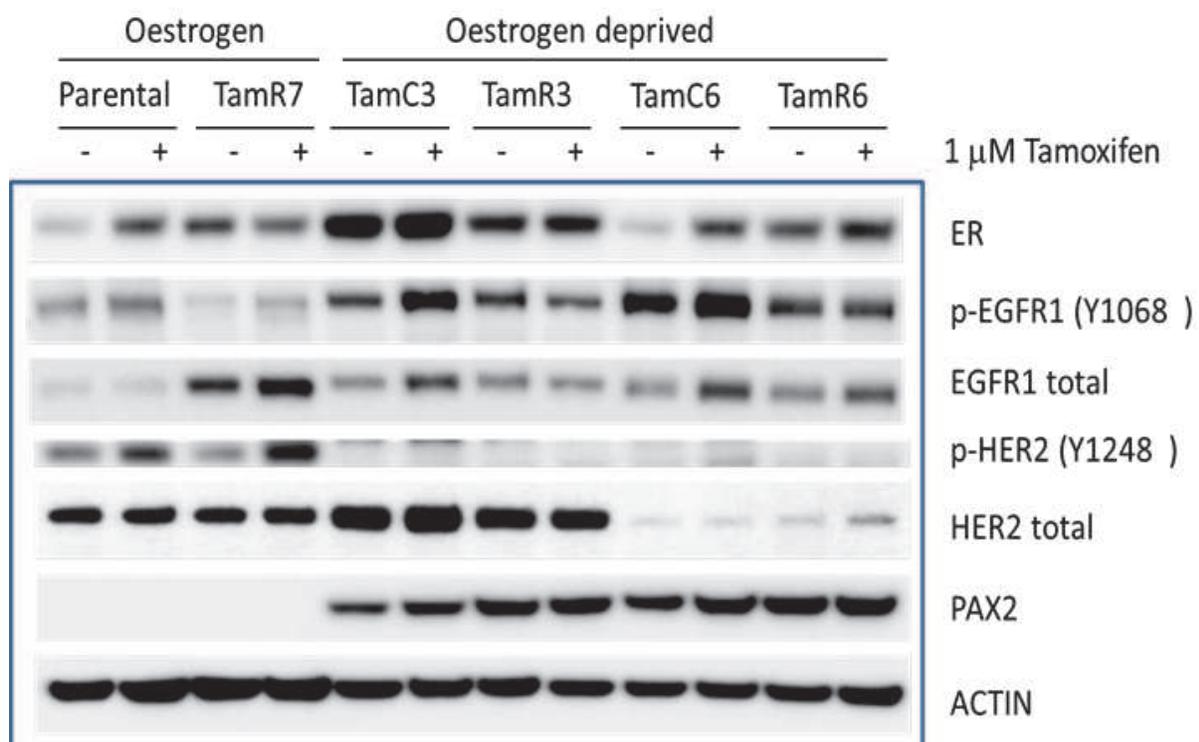


Fig. 2. Relative expression of ER, EGFR and HER2 by MCF-7 and its sub-lines in the absence (-) and presence (+) of tamoxifen (1 μ M). Reproduced from previously published data (Leung et al., 2010).

None of the tamoxifen-resistant lines showed complete absence of ER expression but this may have been a result of the selection method. Tamoxifen is a partial ER agonist, probably acting on ER associated with the plasma membrane and selection in the presence of tamoxifen may favour cells expressing ER. An alternative approach was carried out by Liu et al. (2006), who used fulvestrant as a “pure” antioestrogen for cell line selection. This

resulted in the generation of a MCF-7 sub-line (MCF-7/F) that did not express ER and that grew independently of either oestrogen or antioestrogens (Liu et al., 2006).

4. Growth factor receptor expression

The growth of breast cancer cells is controlled not only by ER and PR but also by plasma membrane-associated growth factor receptors. Two particularly important members of this large family are EGFR, which is activated by epidermal growth factor (EGF), and HER2. The expression of the two receptors was compared in MCF-7 cells and in their tamoxifen-resistant sub-lines. EGFR was expressed very weakly in parental MCF-7 cells but was upregulated in the sub-lines with strongest expression in the TamR7 sub-line (Leung et al., 2010). It was also upregulated in an MCF-7 sub-line (MCF-7/F) that does not express ER (Liu et al., 2006). Since EGFR is activated by phosphorylation, expression of the phosphorylated form of EGFR is more indicative of activity. As shown in Fig. 2, the parental and TamR7 cell lines show weak phosphorylation and TamC6 strong phosphorylation, while the other lines show intermediate phosphorylation (Leung et al., 2010). For most of the sub-lines, addition of tamoxifen increased EGFR phosphorylation, suggesting a relationship between ER and EGFR pathway utilization, in keeping with other studies implicating cross-talk between these pathways in breast cancer (Johnston, 2010).

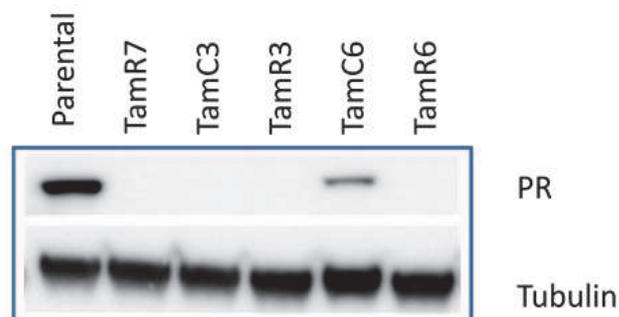


Fig. 3. Relative expression of PR by MCF-7 and its sub-lines.

Expression of the HER2 also varied across the cell lines. It was moderate in the parental MCF-7 line and TamR7, increased in TamC3 and TamR3, and low or absent in the TamC6 and Tam R6 sub-lines (Fig. 2). Previously, it was reported that PAX2 played an important role in the HER2 expression (Hurtado et al., 2008) and that of two MCF-7 phenotypes identified in this study, one expressed HER2 but not PAX2, while the other expressing PAX2 but not HER2. PAX2 is a paired box protein involved in lineage determination that is expressed during development and is commonly expressed in breast cancers (Muratovska et al., 2003). Hurtado et al. (2008) suggested that this inverse relationship arises because PAX2 competes with ER and its co-activator SRC-3 for binding sites on the *HER2* promoter, so that expression of PAX2 leads to repression of HER2 expression. While these two phenotypes are each evident in Fig. 2, a third phenotype expressing both HER2 and PAX2 proteins is also apparent as represented by TamC3 and TamR3. This suggests that the regulation of HER2 expression is more complex and that HER2 can sometimes be co-expressed with PAX2.

As discussed in the previous two sections, MCF-7 sub-lines demonstrate a wide divergence in the relative expression of ER, PR and HER2. It would be interesting to determine whether

a sub-line of MCF-7 exhibiting “triple negative” properties could be isolated using appropriate selection procedures; this might form a useful model for understanding triple negative breast cancers that are encountered in clinical practice. Thus, the generation of variants of a single cancer cell line might be able to recapitulate the development of multiple phenotypes in clinical cancer.

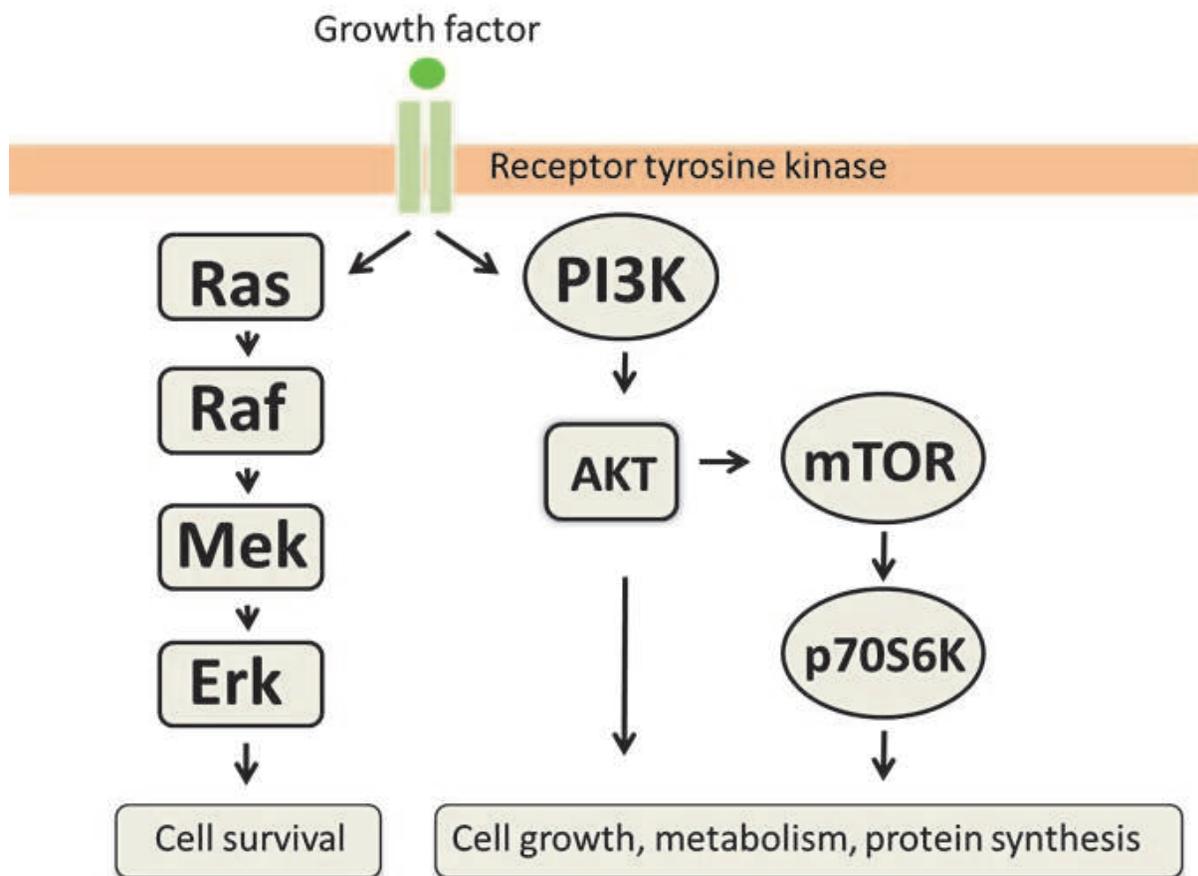


Fig. 4. Simplified diagram of the main pathways involving ERK, AKT (PKB) and mTOR.

5. Growth factor receptor pathways

Growth factor receptors such as EGFR and HER2 largely signal through a common pathway; self-association of adjacent receptors leads through tyrosine kinase phosphorylation to the activation of the receptor complex and recruitment of a series of associated signalling proteins that include RAS, phosphoinositide-3-kinase (PI3K) and RAF, ultimately contributing to the control of proliferation and survival; a simplified summary of these pathways is shown in Fig. 4. Members of the GTPase RAS family of proteins activate both the PI3K and RAF proteins, which in turn activate three key pathways: AKT (PKB; protein kinase B), ERK (extracellular related kinase) and mTOR (mammalian target of rapamycin). RAF activates ERK through the intermediate kinase MEK (mitogen-activated protein kinase kinase), while mTOR activates p70S6K, which is involved in the regulation of protein synthesis. Some measure of the crucial significance of these pathways to cancer growth is indicated by the incidence of mutations of the genes which control these signalling proteins; in particular, mutations of PIK3CA, the gene

specifying PI3K, are found in 15-40% of patients with breast cancer (Isakoff et al., 2005) and a *PIK3CA* mutation is also found in MCF-7 cells. The impact of these mutations is to provide a decreased dependence on external stimulation of the pathways by growth factors such as EGF. Measurement of the utilization of the above pathways demonstrated considerable variation among the different MCF-7 sub-lines (Leung et al., 2010) and the results are summarized in Fig. 5. Utilization of the PI3K pathway can be assessed by phosphorylation of AKT; since the *PIK3CA* mutation and the gain of *PIK3CA* copy number in the MCF-7 cell line (Wu et al., 2005) lead to constitutive activation of PI3K, one might expect a high degree of AKT phosphorylation.

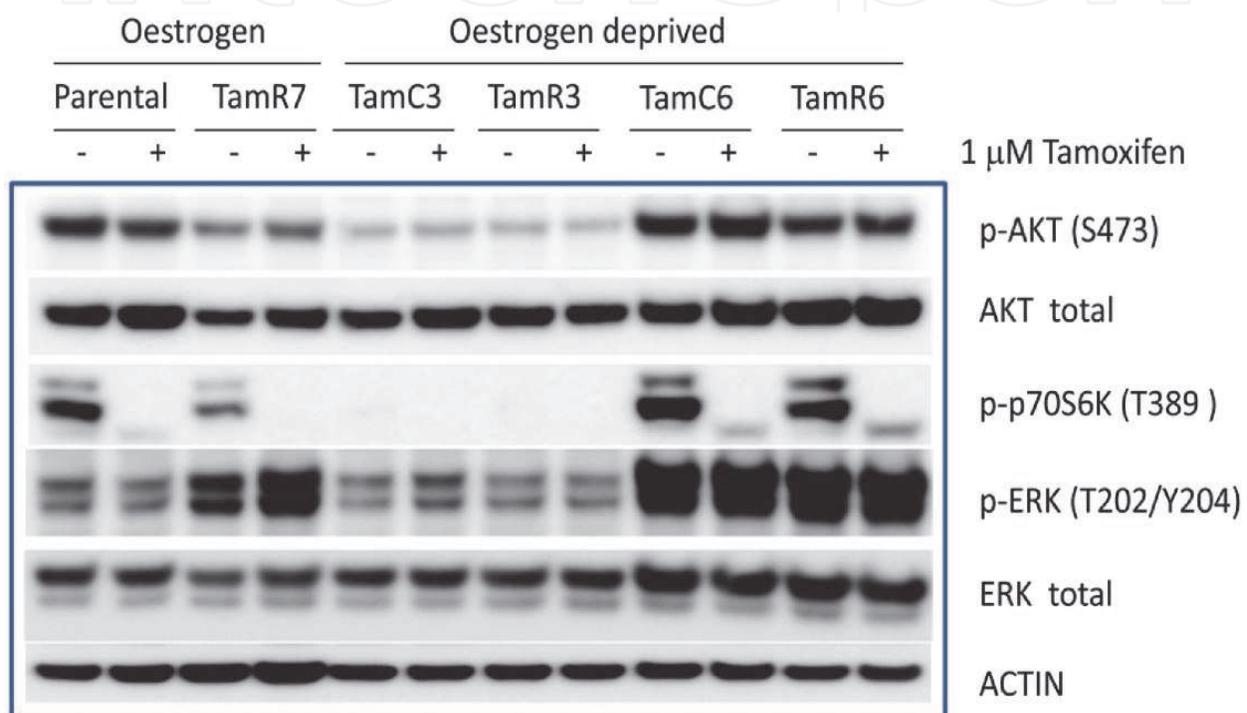


Fig. 5. Relative expression of AKT and its phosphorylated form p-AKT, phosphorylated p-p70S6K, ERK and phosphorylated form p-ERK. Data are shown for MCF-7 and its sub-lines in the absence (-) and presence (+) of tamoxifen (1 μM). Reproduced from previously published data (Leung et al., 2010).

Surprisingly, two of resistant MCF-7 sub-lines, TamC3 and TamR3, show low levels of phosphorylation of AKT, suggesting a comparatively low level of utilization. Utilization of the RAF pathway was assessed by measurement of ERK phosphorylation and here the parental line, as well as the TamC3 and TamR3 sub-lines, exhibited low phosphorylation while TamC6 and TamR6 exhibited high utilization and TamR7 showed intermediate phosphorylation. Utilization of the mTOR pathway was assessed by measurement of phosphorylation of the downstream signalling molecules p70S6K. Here the parental line, as well as the TamC3, TamR3 and TamR7 sub-lines, exhibited low phosphorylation levels while the TamC6 and TamR6 show higher utilization. The wide divergence in pathway utilization among the different sub-lines, as well as a lack of correlation between pathway utilization and expression of ER, PR, EGFR and HER2, was a surprising aspect of this study.

6. Response of MCF-7 sub-lines to therapeutic agents

It is clear from the previous section that the MCF-7 sub-lines vary considerably in their utilization of the AKT (PKB), ERK and mTOR signalling pathways. An important question arising from this observation is whether a high level of utilization of a particular pathway is related to sensitivity to inhibitors of this pathway. For instance, based on the data in Fig. 4, are the parental and TamC6 cell lines, which show higher phosphorylation of AKT than the other sub-lines, differentially susceptible to inhibitors of PI3K? Furthermore, do PI3K inhibitors differentially inhibit AKT phosphorylation in cell lines showing increased phosphorylation? It should be noted that MCF-7 cells have a PIK3CA mutation and the consequently activated PI3K activity makes them generally more sensitive to inhibitors of PI3K than other cell lines containing the wild type enzyme (Serra et al., 2008).

Since there are currently no PI3K inhibitors in routine clinical use, the question of differential sensitivity to PI3K inhibition was addressed using NVP-BEZ235 and GSK2126458 (Leung et al., 2011). NVP-BEZ235 is currently being tested in phase I/II clinical trials in breast cancer patients with advanced disease while GSK2126458 is being evaluated in a phase I trial in patients with solid tumours or lymphoma¹. Examination of drug inhibitory properties showed that at drug concentrations of 1 μ M and 50 nM, respectively, the parental and TamR7 lines were the most sensitive while TamC6 and TamR6 were the most resistant (Fig. 6). Thus, drug sensitivity was not related to pathway utilization. The question of differential inhibition of AKT phosphorylation was also examined; the parental and TamR7 were the most sensitive while TamC6 and TamR6 were least sensitive (Leung et al., 2011). This correlated well with inhibition of cell proliferation. A subsequent commentary on this work emphasises the importance of understanding the principles underlying sensitivity and resistance to inhibitors of this pathway (Butt, 2011).

A second example is provided by the mTOR pathway. Rapamycin is the classical inhibitor of this pathway and mTOR inhibitors such as everolimus and temsorolimus are in clinical trial. Moreover, treatment of mice with rapamycin sensitizes MCF-7 tumour xenografts to inhibition by tamoxifen (deGraffenried et al., 2004). Are MCF-7 sub-lines that highly phosphorylate the mTOR substrate p70S6K differentially sensitive to rapamycin? As measured by growth inhibition assays, the parental line and TamR7 were the most sensitive (Leung et al., 2010) while TamC6 and TamR6, where p70S6K are also highly phosphorylated, are resistant (Fig. 5). A third example is provided by the ERK pathway. No inhibitors of MEK, the enzyme that phosphorylates ERK, are yet in clinical use but CI-1040 has undergone clinical trial. Are MCF-7 sub-lines that show high phosphorylation of ERK, the MEK substrate, differentially sensitive to CI-1040? This does not appear to be the case because TamC3 was the most sensitive to CI-1040 despite showing only moderate phosphorylation of the ERK protein (Fig. 5).

Taken together, these results indicate a lack of a clear relationship between the degree of utilization of a particular pathway and the degree of dependence of cell growth on that pathway. While this may at first seem counter-intuitive, it should be kept in mind that phosphorylation pathways are utilized by the cell not only to promote proliferation but also to promote survival. Growth in culture in the presence of the multiple growth factors and antioxidants provided by foetal bovine serum is very different from growth *in vivo*, where

¹ <http://clinicaltrials.gov/ct2/show/NCT00620594> and <http://clinicaltrials.gov/ct2/show/NCT00972686>.

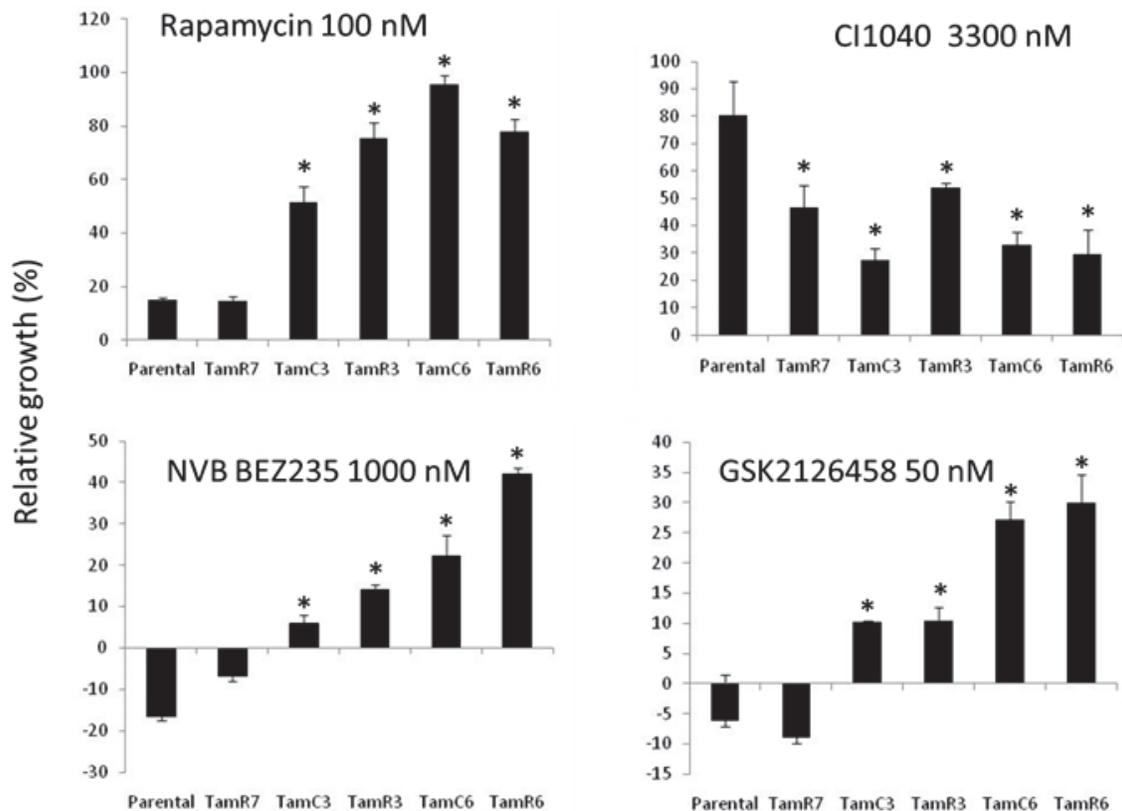


Fig. 6. Relative effects of inhibitors of mTOR (rapamycin), MEK (CI-1040) and PI3K (NVP BEZ235 and GSK2126458). Asterisks represent significant increases ($p < 0.05$) and negative values indicate evidence of cell killing. The two lower graphs are reproduced from previously published data (Leung et al., 2011).

the microenvironment is much more hostile. It is of interest in the case of the inhibitors of PI3K that the induction of apoptosis, if observed at all, was seen only at the highest drug concentrations tested, and that the main basis for inhibition of culture growth, particularly in the determination of IC_{50} values, was the induction of cell cycle arrest (Leung et al., 2011). It can thus be hypothesized that there are many alternative pathways that can be accentuated to promote cell survival and that different sub-lines have developed individual combinations of survival mechanisms. Only a small proportion of the overall signalling may be required to maintain cells in a proliferating state.

7. Conclusion

The hypothesis that emerges from these studies is that the human breast MCF-7 line, although often treated as a single entity, comprises a large number of individual phenotypes, most of which constitute only small proportions of the total population. These phenotypes differ in gene expression profile, receptor expression and signalling pathway usage. Despite differences in proliferation rate of individual phenotypes, a balance of multiple phenotypes is somehow maintained during progressive culturing of the line, perhaps by some type of signalling co-operation. The proportion of the dominant phenotype may be maintained by the growth conditions; in the case of MCF-7 the predominance of the ER+ phenotype could be maintained by the presence of small amounts of oestrogen in the

foetal bovine serum. However, extended growth in the absence of oestrogen would select for variants that rely on EGFR, HER2 and other stimulators of signalling pathways.

If the above hypothesis is correct, it raises three important questions. The first is whether such heterogeneity is a feature of all human breast cancer cell lines. Based on current literature, this is likely to be the case. Multiple antigenic phenotypes have been identified in several breast cancer cell lines (Edwards et al., 1985). Studies with T47D breast cancer cells using multidimensional flow cytometry have shown the presence of different phenotypes that differ not only in the expression of PR but also in DNA content (Graham et al., 1992). Further research is needed to explore the generality of these observations.

The second question is whether human breast cancers growing *in vivo* show similar levels of heterogeneity to those of the derived cell lines. This question is difficult to answer unless individual cells can be identified, but evidence of heterogeneity of *EGFR* copy number has been detected in fine needle biopsies from 29 breast cancer patients, as well as in samples of the MCF-7, SKBR3, and T47D cell lines (Sauer et al., 2005). An answer to this question is critical because it could imply a common ancestry not only for breast cancer cells that differ in receptor status but also in histological status and growth rate.

The third question is whether the mechanisms responsible for generating the heterogeneity of breast cancer cell types also apply to normal mammary tissue. There is increasing evidence for the existence of a differentiation hierarchy in the adult mammary epithelium, where precursor cells at various levels of the hierarchy are able to switch expression of proteins involved in differentiation; such switching includes the epidermal-mesenchymal transition (Visvader, 2009). The mechanism of such phenotype switching is not yet understood but is thought to involve epigenetic changes mediated by changes in DNA methylation, histone modification and concentrations of non-coding RNA (Huang & Esteller, 2010). These studies imply that the mechanism for generation of multiple phenotypes in breast cancer cells could be based on existing mechanisms that occur in the normal breast epithelium, with additional tumour-specific mechanisms arising from genetic alteration, chromosomal instability and possibly the presence of a mutated PI3K enzyme (Meyer et al. 2011).

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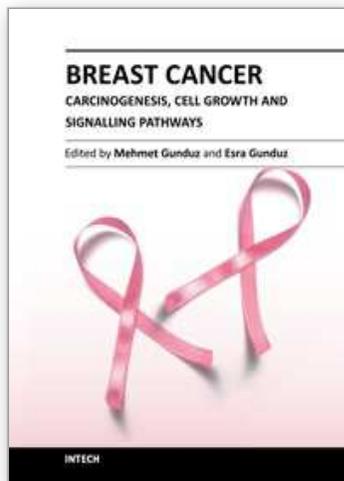
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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