THE ROLE OF ESTROGEN IN THE INITATION OF BREST CANCER

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Abstract

Estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. Their role as breast carcinogens has long been suspected and recently confirmed by epidemiological studies. Three major mechanisms are postulated to be involved in their carcinogenic effects: stimulation of cellular proliferation through their receptor-mediated hormonal activity, direct genotoxic effects by increasing mutation rates through a cytochrome P450-mediated metabolic activation, and induction of aneuploidy. Recently it has been fully demonstrated that estrogens are carcinogenic in the human breast by testing in an experimental system the natural estrogen 17β-estradiol (E_2) by itself or its metabolites 2-hydroxy, 4-hydroxy, and 16-a-hydroxy-estradiol (2-OH-E_2, 4-OH-E_2, and 16-α-OH E_2) respectively, by inducing neoplastic transformation of human breast epithelial cells (HBEC) MCF10F *in vitro* to a degree at least similar to that induced by the chemical carcinogen benz(a)pyrene (BP). Neither TAMOXYFEN (TAM) nor ICI-182,780 abrogated the transforming efficiency of estrogen or its metabolites. The E_2 induced expression of anchorage independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, is associated with the loss of 9p11-13 and only invasive cells that exhibited a 4p15.3-16 deletion were tumorigenic. Tumors were poorly differentiated ER-α and progesterone receptor negative adenocarcinomas that expressed keratins, EMA and E-cadherin. The E_2 induced tumors and tumor-derived cell lines exhibited loss of chromosome 4, deletions in chromosomes 3p12.3-13, 8p11.1-21, 9p21-qter, and 18q, and gains in 1p, and 5q15-qter. The induction of complete transformation of the human breast epithelial cell MCF-10F *in vitro* confirms the carcinogenicity of E_2, supporting the concept that this hormone could act as an initiator of breast cancer in women. This model provides a unique system for understanding the genomic changes that intervene for leading normal cells to tumorigenesis and for testing the functional role of specific genomic events taking place during neoplastic transformation.

Keywords

estrogen; invasiveness; CGH; breast cancer

1.Introduction

Breast cancer is a malignancy whose dependence on ovarian function was first recognized through the regression of both advanced cancer (1) and metastatic disease (2) induced by oophorectomy in premenopausal women. Ulterior correlation of ovarian function with estrogen

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production (3), and the isolation of the estrogen receptor protein (4,5), combined with the observed greater incidence of estrogen receptor positive tumors in postmenopausal women (6-16), led to the identification of a strong association of estrogen dose and length of exposure with increased breast cancer risk (6,10,13,14). The importance of ovarian steroidogenesis in normal breast development and in the genesis of breast cancer is highlighted by the facts that early menarche and late menopause are associated with greater breast cancer risk, whereas late menarche and early menopause, that occurring before 40 years of age, result in a significant reduction of the same (17-20). Breast development at puberty and during sexual maturity is stimulated by 17β-estradiol (E_2), which is the predominant circulating ovarian steroid and the most biologically active hormone in breast tissue (21,22). At menopause E_2 plasma levels decrease by 90% (17-19). In spite of the markedly different circulating levels of estrogens in pre- and postmenopausal women, the concentrations of E_2 in breast cancer tissues do not differ between these two groups of women, an indication that its uptake from the circulation might not contribute significantly to the total content of this hormone in breast tumors, but rather that *de novo* biosynthesis, i.e., peripheral aromatization of ovarian and adrenal androgens, plays a more significant role (23,24).

Considerable epidemiological and clinical evidence link cumulative and sustained exposure to estrogens with increased risk of developing breast cancer. However, there is no clear understanding of the mechanisms through which estrogens cause cancer. In experimental animal models it has been demonstrated that E_2, 11β-methoxyethinylestradiol (Moxestrol), and diethylstilbestrol (DES), as well as their 4-hydroxycatechols, induce kidney cancer in castrated male Syrian golden hamsters (25-27). In rats, continuous administration of supraphysiological doses of estrogens induces a high percentage of mammary adenocarcinomas, whereas low doses given over long periods induce fibroadenomas (28). In both models, however, the tumorigenic effects of estrogens are associated with marked hyperprolactinemia and pituitary hyperplasia resulting from an increase in number of hyperplastic prolactin secreting cells. The dependence on a functional pituitary gland has been further confirmed in hypophysectomized rats in which estrogens are ineffective as carcinogens (29). Nevertheless, the most widely acknowledged mechanism of estrogen carcinogenicity is its binding to its specific nuclear receptor alpha (ER-α) for exerting a potent stimulus on breast cell proliferation through its direct and/or indirect actions on the enhanced production of growth factors (21,22). However, the fact that ER-α knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) develop mammary tumors provides direct evidence that estrogens may cause breast cancer through a genotoxic, non-ER-α-mediated mechanism (30,31). This postulate is further supported by the observations that when ovariectomized mice are supplemented with E_2 they develop a higher tumor incidence with shorter latency time than controls, even in the presence of the pure antiestrogen ICI-182,780. Experimental studies on estrogen metabolism (32,33), formation of DNA adducts (34), carcinogenicity (35-37), mutagenicity (38), and cell transformation (39-42) have supported the hypothesis that reaction of specific estrogen metabolites, namely, catechol estrogen-3,4-quinones (CE-3,4-Q) and to a much lesser extent, CE-2,3-Q, can generate critical DNA mutations that initiate breast, prostate and other cancers (43).

2. Rationale for an *in vitro-in vivo* model of cell transformation

In order to definitively outline the pathways through which estrogens act as carcinogens in the human breast and for assessing whether one or more of the mechanisms described above are responsible of carcinogenic initiation, it is needed an experimental system in which E_2 by itself or its metabolites induce transformation of human breast epithelial cells (HBEC) in a well controlled environment, preferentially *in vitro*. Recently it has been reported an *in vitro-in vivo* system of cell transformation that fulfills these requirements (44). Using this model it has been demonstrated that E_2 and its metabolite 4-hydroxyestradiol (4-OH-E_2) induce

transformation of MCF-10F, an ER-αnegative human breast epithelial cell line (39-42,45). In response to estrogen treatment the cells form colonies in agar methocel, lose the capacity to differentiate by forming three-dimensional structures when grown in a collagen matrix, or their ductulogenic capacity, forming instead spherical and solid masses, and exhibit an increase in cell proliferation and in their invasive capabilities in Matrigel (39-42,45). More importantly, the expression of these phenotypes indicative of neoplastic transformation was not abrogated by their simultaneous treatment with the anti estrogen ICI-182,780 (ICI), suggesting that the transformation of MCF-10F cells by these compounds did not require the presence of the ER-α(40,41). E_2-induced transformation of HBEC*in vitro* increased the invasive potential of the cells. In addition, the selection of the most highly invasive cells in the Matrigel chambers identified transformed cells that express phenotypic and genotypic variations that correlate with their tumorigenic potential in a heterologous host, but still maintained their cell lineage characteristics. It also has been reported that the induced tumors exhibit genomic alterations that are similar to those reported in primary breast cancer, as determined by comparative genomic hybridization (CGH) (44).

3. The experimental model of transformation of MCF-10F cells by 17-β estradiol treatment

Treatment of the spontaneously immortalized ER-α and progesterone receptor (PgR) negative human breast epithelial cell line MCF-10F (Figure 1) with 70nM E_2 twice a week for two weeks formed colonies in agar methocel (Figure 2) and the colony efficiency increased from 0 in controls to 12.0±1 in the treated cells. The positive control cells BP1-Tras and MDA-MB231 cells had a moderately (p<0.02) and significantly (p<0.001) higher colony efficiency than E_2-transformed cells, respectively (44). This treatment also affected the ductulogenic pattern of cells grown in collagen gel (Figure 3), which was quantitatively evaluated by counting the total number of ductules and spherical masses formed by 10,000 cells plated in collagen. Control MCF-10F cells formed an average of 110 ductular structures, but did not form solid masses. After treatment with E_2, MCF-10F cells almost completely lost their ductulogenic capacity, while acquiring the ability to form spherical solid masses (Figure 3). BP1-Tras and MDA-MB 231 exhibited a complete absence of ductule formation, forming instead solid masses in collagen gel whose values were not significantly different from those formed by E_2-treated cells. The differences were highly significant (p<0.0001) (44).

The ability of cells to invade a Matrigel membrane *in vitro* is a widely accepted criterion of cell transformation. Control MCF-10F cells exhibited a low invasive capacity (Figure 4A), whereas the invasive capacity of E_2-transformed cells at their 9th passage was significantly higher (Figure 4B) (44). BP1-Tras and MDA-MB231 cells had an invasive index that was significantly higher than that of MCF-10F control and E_2 transformed cells (44)

MCF-10F cells between passages 130-132 and E_2-treated cells between their passages 7 and 9 were injected to 10 SCID mice each for testing their tumorigenic capabilities. Neither control nor E_2-treated cells formed tumors after a six-month follow up period. Instead, BP1-Tras and MDA-MB231 cells were highly tumorigenic with a short latency period (44). Because the tumorigenic response of these two cell lines was associated with a highly invasive phenotype, it was tested whether selection of more invasive cells among E_2-transformed MCF-10F cells would allow them to express the tumorigenic phenotype, and further to determine whether this phenotype was exclusively induced by estrogen, and not the result of the selection of more invasive control cells. For this purpose, MCF-10F cells in their 133rd passage and E_2-treated MCF-10F cells in their 10th passage were trypsinized and seeded in the upper chamber of seven and four matrigel invasion chambers, respectively (44). Those cells that at 22 hours post-seeding had crossed the Matrigel membrane were cultured, giving origin to seven MCF-10F cell lines that were labeled A1 to A-7. From the E_2-treated cells four lines were obtained, and

were designated B2, C3, C4, and C5 (44). Injection of A1 to A7 cells to SCID mice did not induce a tumorigenic response even after six months of follow up. After injection of the E_2-transformed cells B2, C3, C4, and C5 to SCID mice, only C3 and C5 were tumorigenic in 2/12 and 9/10 animals injected, respectively. The clone C5 produced tumors that were larger than the ones produced by C3 (Figure 5). From the 9 tumors obtained from C5 cells, four tumoral cell lines, designated C5-A1-T1, C5-A4-T4, C5-A6-T6 and C5-A8-T8 were derived. These cells were subsequently injected to another set of five SCID mice per cell line for testing their tumorigenic capabilities. All these cell lines formed palpable tumors, being C5-A8-T8 the fastest growing tumor. Cell lines B2 and C4 did not induce tumors even after a nine-month follow up (44).

Histopathological analysis revealed that all the E_2 70nM-C5 cells formed tumors and those tumors formed by their derived cells were poorly differentiated adenocarcinomas. They invaded the mammary fat pad and the skeletal muscle of the abdominal wall (Figure 6). The immunocytochemical reactivity of the E_2-induced tumors in SCID mice were positive for AE1 and AE3, human low and high molecular weight cytokeratins were expressed in the cytoplasm of the neoplastic cells in all E_2 induced tumors in a pattern similar to those observed in normal breast tissues, in primary invasive ductal carcinomas of the breast and in MCF-10Fcells. The cytokeratin peptide 7 and 8 (CAM5.2) diffusely stained the cytoplasm of neoplastic cells with greater variations in the degree of intensity than in the invasive ductal carcinoma of the breast used as positive control. E-cadherin was positive in all E_2-induced carcinomas, exhibiting a diffuse and moderate reactivity, which was less intense than that observed in the invasive ductal carcinoma used as a positive control. Epithelial membrane antigen (EMA) had similar level of reactivity in E_2-induced tumors than in primary breast cancer, in normal breast tissues and in MCF-10F cells. Estrogen receptor alpha (ER-α), which was positive in normal breast tissues and in primary breast cancer, was negative in MCF-10F cells and in all E_2-induced tumors in SCID mice. The same pattern of reactivity was observed for progesterone receptor (44).

In summary treatment of the immortalized estrogen receptor alpha (ER-α) negative human breast epithelial cell line MCF-10F with E_2 and its metabolites 2- and 4-hydroxyestradiol induce anchorage independent growth, loss of ductulogenic pattern, and invasiveness in a Matrigel basement membrane (2,44,46). The transforming capabilities of estrogens have been confirmed in MCF-10A, another ER-α negative immortalized human breast epithelial cell line in which E_2 and estrogenic substances, such as Zeranol (Ralgro), a nonsteroidal agent with estrogenic activity that is used as a growth promoter in the U.S. beef and veal industry (47) and 4-hydroxyequilenin (48) induce anchorage independent growth. These observations support the concept that estrogens induce neoplastic transformation through non-receptor alpha mediated mechanisms, exerting direct genotoxic effects, as previously suggested (32-38). Our findings of specific mutations in p53, and loss of heterozygosity (LOH) in chromosomes 11 and 13 further support this concept (40,49).

4. Genomic pathway of 17 beta estradiol induced neoplastic transformation

Using Comparative Genomic Hybridization (CGH) that is a molecular cytogenetic method for screening gains and losses at chromosomal and subchromosomal levels, it has been detected that MCF-10F cells transformed by E_2 had lost 9p11-13, a loss that persisted in the invasive cell line E_2-70 nM-C5 (Figure 7). This locus contains the serine protease family member PRSS3 (trypsinogen-IV), a putative tumor suppressor gene (50) in which an allelic imbalance has been reported in hepatocellular carcinoma (51), carcinoma *in situ* of the bladder (52) and renal cell carcinoma (53). The loss of 9p11-13 was not detected by CGH technique in the tumors and tumor-derived cell lines, probably because the change did not reach the threshold for detection or because the cell population was heterogeneous. However, losses in 9p21-pter were clearly evident in the tumor and tumor cell lines. Losses of chromosome 9 regions are frequently

reported in bladder carcinoma, especially in premalignant lesions such as hyperplasia and carcinoma in situ (CIS). Simultaneous losses in 9p11-q12 and in 9p21 have been reported in CIS of the bladder (54). Losses in this locus have also been reported in peripheral T cell lymphoma (55), melanoma cell lines (56), malignant fibrous histiocytoma (57) and parathyroid adenomas (58). The 9p21-pter, region includes both the p16 and p15 genes. These observations indicate that loss of these tumor suppressor genes on 9p contribute to the progression of the invasive to the tumorigenic phenotypes in the natural progression of the disease.

5. Genomic changes during the tumorigenic stage of malignant transformation

E_2 induces, in addition to the expression of early phenotypes of neoplastic transformation, tumorigenesis in a heterologous host (Figure 8). This phenomenon became possible only after the selection of invasive cells that exhibit specific changes, such as the deletion of chromosome 4p15.3-16, which was the first one, detected (Figures 7 and 8). Interestingly enough, injection of these cells to SCID mice resulted in the formation of tumors in which the entire chromosome 4 was deleted, a change that became a permanent feature of all tumors and tumor-derived cell lines. Allelic losses at one or both arms of chromosome 4 have been frequently reported in several tumor types, including breast cancers, either sporadic or occurring in BRCA1 and BRCA2 germline mutation carriers (59,60). Regions that have been frequently reported to be deleted are 4p16.3 (50 %), 4p15.1-15.3 (57%), 4q25-26 (63%), and 4q33-34 (76%) (61). The tumors induced by E_2-transformed cells in SCID mice are fast growing and ER negative, being similar in these aspects to the tumors exhibiting similar deletions and that are diagnosed in young women, in whom tumors are large at the time of diagnosis, having a high percentage of cells in S-phase and being negative for estrogen receptors (59,60). Chromosome 4 contains numerous genes of potential interest in cancer development, among them is *Slit2*, a gene located at 4p15.2 that encodes a protein that inhibits leukocyte chemotaxis and is a putative ligand for the ROBO receptors gene (62). *SLIT2* is primarily a secreted protein that in conditioned medium suppresses the growth of several breast cancer lines (62). Therefore the loss of the 4p15.3-16 region in E_2-70 nM C5 cells could be the event that triggers a cascade that select tumorigenic cell population.

Additional losses that were initially detected in the tumors and that were maintained in the tumor-derived cell lines were in chromosomes, 3 p12.3-13, 8 p11-21 and 18q. The region lost in chromosome 3 (p12.3-13) has been reported to exhibit imbalances in MCF-7 cells developing resistance to tamoxifen (63); the region 8p11-21 encodes the frizzled-related gene FRP1/FRZB, that is turned off in 78% of breast carcinomas (64), and associated with androgen in prostate cancer (65); the loss of chromosome arm 18q is a common event in primary breast cancers (60-70), ductal hyperplasia (71), and in breast cancer cell lines (72), and it is often interpreted as representing loss of one or more tumor-suppressor genes. The relevance of these losses in estrogen-induced cell transformation is that among the genes located in the q arm of chromosome 18 are two independent tumor-suppressor loci in segment 18q21.1, one at SMAD4 and the other potentially at an enhancer of DCC or an unrelated novel gene (66,70).

Treatment of MCF-10F cells with E_2 induced genomic gains in 1p and 5q15-qter, both of which became evident in tumors and remained at the same level of expression in all tumor-derived cells. Amplification of 1p has already been reported in primary breast cancer (73-78) and in established breast cancer cell lines (79). Gain in 5q15-qter has not been frequently found in breast cancer (80), but it has been reported in previously immortalized human ovarian surface epithelial (HOSE) cells using HPV16E6E7 ORFs (81) and in the cell lines SW480 and SW620, derived from different stages of colon carcinoma in the same patient (82). Although at the present time the role played by these gains in 1p and 5q15-qter in the process of estrogen-induced tumorigenesis is not known, a likely explanation is that the gains resulted from

amplifications of smaller chromosomal segments that probably arose through real DNA amplification processes, suggesting that many genes present in these chromosomal loci are potential targets for the carcinogenic effect of 17-β-estradiol (83).

6. Conclusions

17-β-estradiol is able to induce complete neoplastic transformation of human breast epithelial cells, as proven by the formation of tumors in SCID mice. This model demonstrates a sequence of chromosomal changes that correlates with specific stages of neoplastic progression. The data also support the concept that 17-β-estradiol can act as a carcinogenic agent without the need of the ERα, although we cannot rule out thus far the possibility that other receptors such as ERβ, or other mechanisms could play a role in the transformation of human breast epithelial cells. These are areas of active research in our laboratory. The knowledge that breast cancer in women is associated with prolonged exposure to high levels of estrogens gives relevance to this model of estrogen induced carcinogenesis (6,8-10,15,16). For this reason this model is extremely valuable for furthering our understanding of estrogen induced carcinogenicity.

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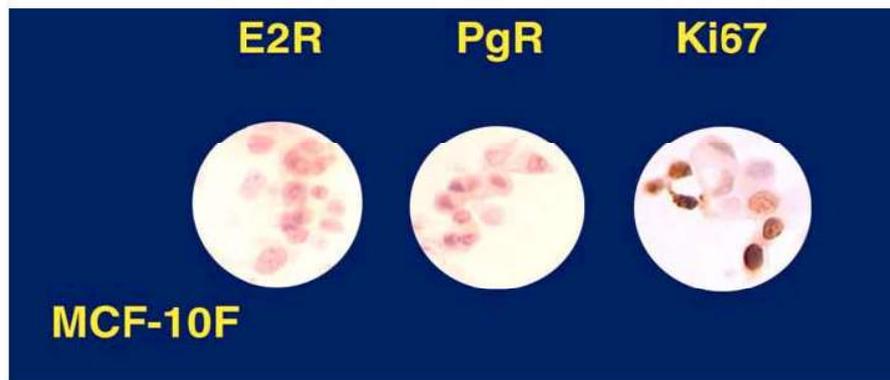
Figure 1.
MCF-10F cells are proliferating cells (Ki67 positive), E_2R (estrogen receptor) and PgR (progesterone receptor) negative.
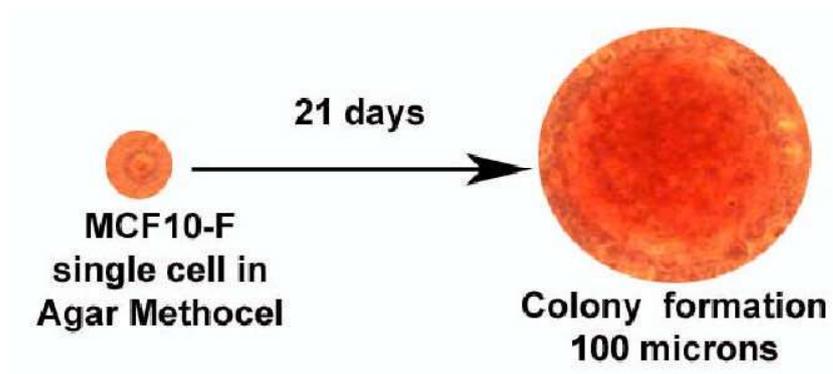
Figure 2.
Anchorage independent growth is only observed in MCF-10F cells transformed with estradiol forming colonies of 100 micros in diameter.
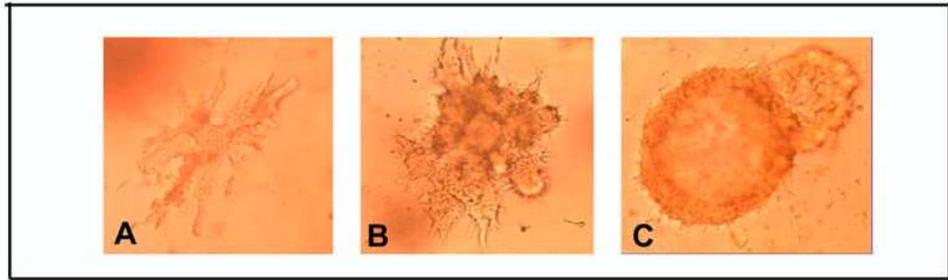
Figure 3.
A) MCF-10F cells in collagen matrix form ductules, B) MCF-10F cells transformed with 70nM of E_2 have lost the ability to form ductules and C) form solid masses. Phase contrast micrographs, X20)
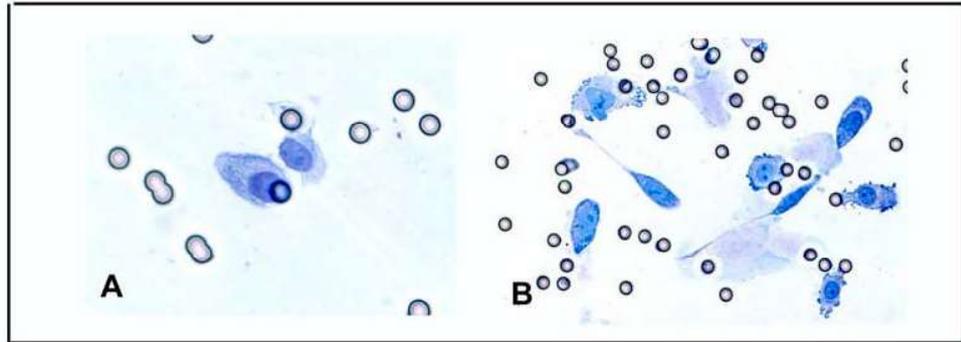
Figure 4.
A)Invasion of f MCF-10F Control cells and B) $E_2$70nM transformed cells.
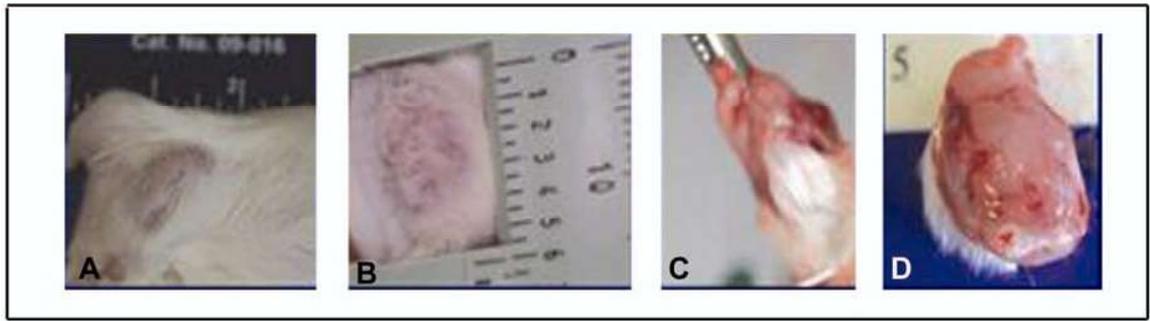
Figure 5.
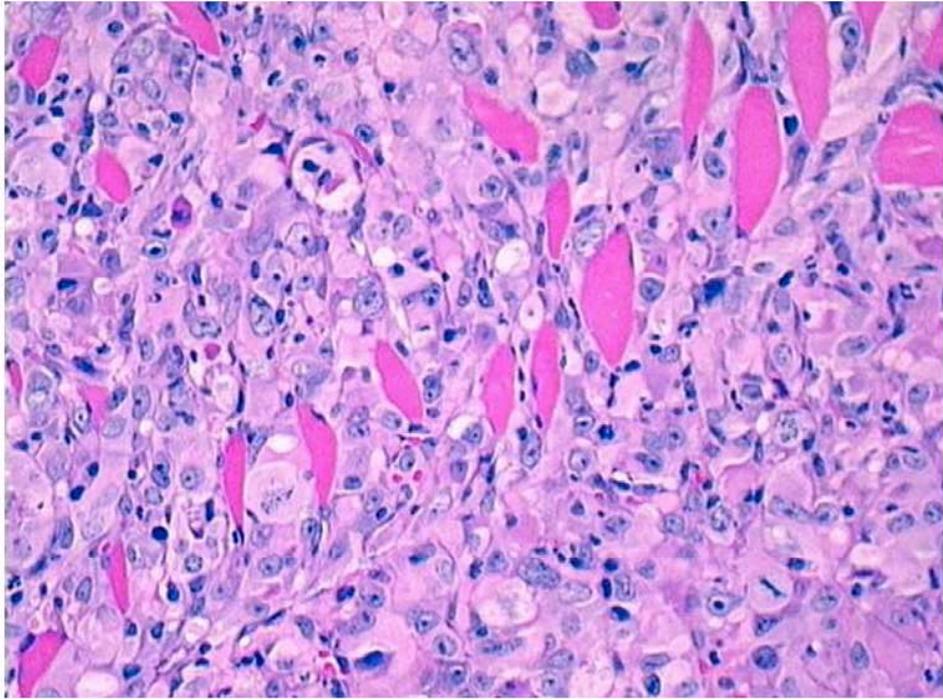A-D) Palpable tumor formed by E$_2$ 70nM-C5 cells.

Figure 6.
Histological section of an invasive adenocarcinoma growing in the fat pad of a SCID mouse
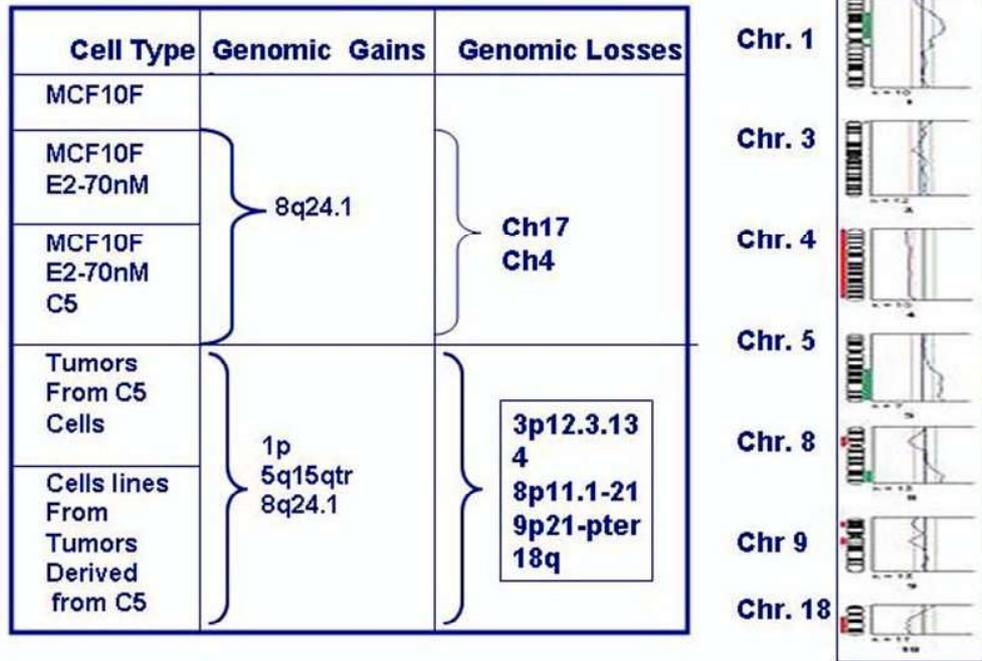(H&E, 10X).

Figure 7.
Comparative Genomic Hybridization (CGH) that is a molecular cytogenetic method for
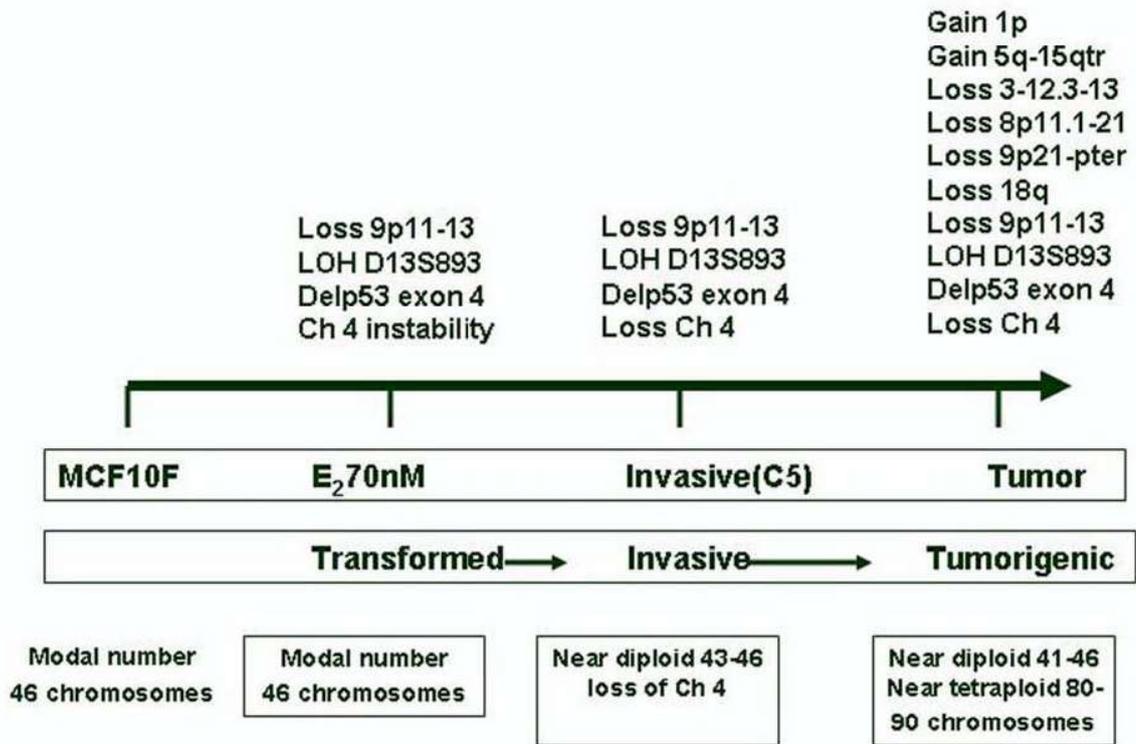screening gains and losses at chromosomal and sub chromosomal levels.

Figure 8.
Schematic representation of the cumulative genomic changes observed in the MCF10F cells
transformed with 17 beta estradiol.