CHAPTER TWO

LITERATURE REVIEW

As a prelude to this chapter, it should be emphasised that, at the outset, the discovery of ERα prompted intensive research with regards to breast cancer. Subsequently, when a second ER, ERβ, was discovered, this stimulated new interest in the two ERs. However in recent years, this focus has dwindled to a certain extent and this is reflected in the paucity of current research in this field, with the regulatory effects of epigenetics and microRNA coming to the fore. Nevertheless, oestrogen receptors and their splice variants remain an important area of study and this is reflected in the current literature review presented below, that encompasses the field at large.

2.1

Hormones and Breast Cancer

2.1.1

Historical Perspective

A relationship between ovarian secretions and the growth of some human breast cancers was suggested as early as 1836 (Cooper, 1836) when cyclical fluctuations in tumour growth during different phases of the menstrual cycle was observed. The use of oophorectomy for the treatment of inoperable breast cancer was

described by Beatson in 1896 (Beatson, 1896). This introduced the concept that ovarian secretions were somehow necessary for the maintenance and growth of breast tissue. In the 1940's orchiectomy was introduced for the treatment of prostatic cancer in males (Huggins and Hodges, 1941) thereby reviving interest in Beatson's observations. Ablative endocrine therapy, removing sources of oestrogenic hormones, either by oophorectomy or by ablation of extra ovarian sites of oestrogenic hormone production, became an accepted therapy for patients with advanced breast cancer. Additive hormonal treatment became available in the 1950's with the commercial production of steroid hormones. Androgens were described to cause regression of female breast cancer (Nathanson, 1952) and oestrogen, which appeared to be necessary for the maintenance and growth of breast cancer in younger women, was found to be beneficial in older, postmenopausal women (Haddow *et al*, 1944; Carter, 1977; Henderson, 1987). Other agents used in additive hormone therapy include oestrogen antagonists such as tamoxifen (Cole *et al*, 1971; Ward, 1973).

2.1.2

The Role of Hormones in Breast Cancer

The mammary gland, which is not completely formed at birth, begins to develop in early puberty when the primitive ductal structures enlarge and branch. The branching of the ductal system becomes more complex and lobular structures form at the ends of terminal ducts to produce terminal lobular units (TDLUs) once menstrual cycles have begun (Anderson, 2002). The development of normal mammary tissue is the result of complex interactions between a number of

hormones and growth factors including steroids, insulin and various pituitary factors. Genomic mediated effects of steroids, which include induction of mitogenesis, occur following significant perturbations in the level of expression of a number of genes (Duval *et al*, 1983). Oestrogen and progesterone are important in alveolar formation, ductal branching (Murr *et al*, 1974; Bronson *et al*, 1975) and for full lobulo-alveolar development (Warner, 1978).

The ductal system of the human mammary gland is lined by a continuous layer of luminal epithelial cells surrounded by a layer of myoepithelial cells which are in direct contact with the basement membrane. The TDLUs are surrounded by delimiting fibroblasts and are embedded in an intralobular stroma. Primary breast carcinomas appear to arise from epithelial rather than myoepithelial cells of the glandular epithelium, presenting as either intraductal or intralobular lesions. Epidemiological evidence indicates that exposure of oestrogenic stimuli to the mammary gland is a potent factor in breast cancer risk (Henderson *et al*, 1988). Increased exposure to endogenous oestrogens may occur as a result of early menarche, late menopause (Pike *et al*, 1983) and late age at first full-term pregnancy (Henderson *et al*, 1988). Obesity has also been implicated in increased breast cancer risk possibly due to increased peripheral aromatization of circulatory androgens to oestrogen (Tartter *et al*, 1981; Ingram *et al*, 1989).

Oestrogen stimulated cell growth appears to require the presence of specific, high affinity, low capacity binding sites or receptors (Lippman *et al*, 1976). In mammary epithelial cells the "classical" oestrogen receptor (ERα), which is a nuclear receptor (King and Green, 1984; Netto *et al*, 1990), is thought to be the major

factor mediating the cellular response to oestrogens. The presence of ERα has been detected in stromal cells of developing breast tissue (Boyd *et al*, 1996; Keeling *et al*, 2000) but in normal mature human breast tissue stromal cells are clearly ERα negative (Boyd *et al*, 1996). A small proportion (6 – 7%) of epithelial cells is ERα positive (Petersen *et al*, 1987). Levels of ERα fluctuate with the menstrual cycle (Balakrishnan *et al*, 1987). The myoepithelial cells appear to be ERα negative (Jacquemier *et al*, 1990). ERβ has been observed in both epithelial and stromal tissue (Crandall *et al*, 1998; Palmieri *et al*, 2002).

The presence of ERα in breast tumours is an indication that these tumours may arise from within ERα positive epithelial cell populations of normal breast tissue (Clarke *et al*, 1990). Approximately two-thirds of all breast tumours express detectable levels of ERα (McGuire *et al*, 1975) and the majority of ERα positive tumours are also associated with adjacent non-neoplastic tissue which is also ERα positive (Netto *et al*, 1990).

There is already a marked heterogeneity of ERα expression by the time a breast tumour mass is detected (Van Netten *et al*, 1988) and this may be due to the dedifferentiation of ERα positive populations resulting in the formation of ERα negative populations. ERα negative breast tumour cells have been observed as less well differentiated than those that are ERα positive (Narbaitz *et al*, 1980) and poorly differentiated tumours (histological grade III) are more likely to be ERα negative when compared to well differentiated tumours (grades I and II) (Henry *et al*, 1988; Singh *et al*, 1988). Some predominantly ERα negative tumours may also

arise from within the normal ERα negative sub-populations (Brunner *et al*, 1990). The stages at which cells lose their dependence on oestrogen and their ability to express ERα and thus acquire resistance to endocrine manipulation are critical in the progression of the disease.

There has been a statistically significant association between ERα status and progesterone receptor (PR) status (Khan *et al*, 1994). PR synthesis is a known consequence of oestrogen-ER-DNA binding (Sherman *et al*, 1972; Faber *et al*, 1978) and therefore the determination of both ERα and PR may be used as an index of oestrogenic action, hormone responsiveness (Horwitz *et al*, 1975a; De Sombre *et al*, 1979; McGuire, 1980) and prognosis in breast cancer (Knight *et al*, 1977; Maynard *et al*, 1978; Cooke *et al*, 1979; Pichon *et al*, 1980; Clark *et al*, 1983; Saez *et al*, 1985).

2.1.2.1

Mechanism of action of ER

Recent evidence suggests that oestrogen can also bind ER located in or near the plasma membrane and rapidly activate other signalling pathways. Oestrogen action in the nucleus (genomic action) has now been termed nuclear-initiated steroid signalling (NISS) and oestrogen action at the plasma membrane (non-genomic action) is termed membrane-initiated steroid signalling (MISS) (Nemere *et al*, 2003).

2.1.2.1.1

Nuclear-initiated steroid signalling (NISS)

The hormone-regulated nuclear transcription factor, ERα, can induce expression of a number of genes such as PR (Horwitz and McGuire, 1978). On ligand activation, ERα binds to oestrogen response elements (EREs) in target genes, recruits a coregulator (CoR) complex, and regulates transcription specific genes (Figure 1, pathway a) (Beato, 1989; Klein-Hitpass *et al*, 1989). ERα may also act without DNA binding by modulating gene expression at regulatory sequences such as AP-1 (Kushner *et al,* 2000), SP-1 (Safe, 2001), and upstream stimulatory factor (USF) sites (Xing and Archer, 1998). (Figure 1, pathway b).

Coactivators and corepressors are coregulatory proteins that control ERα action and modulate chromatin structure to facilitate or repress gene transcription (Dobrzycka K.M. *et al*, 2003; Smith and O'Malley, 2004). The recruitment of coregulatory proteins to the receptor complex is influenced by the relative accessibility of the various coactivators and corepressors in a given tumour cell. Selective ER modulators (SERMs) such as tamoxifen, that have agonist/antagonist properties, may therefore have their effects modified according to the absolute and relative levels of ER coregulator proteins which has been associated with *de novo* and acquired resistance to endocrine therapy (Schiff *et al*, 2003).

The expression level of ERα is regulated through DNA methylation and chromatin condensation of promoter regions via hypermethylation of CpG islands in the ERα

promoter which is associated with a marked decrease in ERα mRNA expression among ERα-expressing cancer cell lines. The inhibition of DNA-methyltransferases reactivates ERα expression in these cell lines (Ottaviano *et al*, 1994).

ERα may also interact with other nuclear proteins involved with breast cancer. Interaction with BRCA1, which results in a reduction of ER activity (Fan *et al*, 1999), may be due to increased CpG methylation of the ERα promoters (Archey *et al*, 2002). Similarly, cyclin D1 may be regulated by ERα (Zwijsen *et al*, 1997; Liu *et al*, 2002).

2.1.2.1.2

Membrane-initiated steroid signalling (MISS)

There is growing evidence that oestrogen can have rapid cellular effects that occur long before its effects on gene transcription. This suggests other mechanisms of action (Levin, 1999). ERα can be detected in or near the plasma membrane, where it can directly interact with and modulate several signalling pathways. This type of signalling is known as membrane-initiated steroid signalling or MISS (Figure 1, pathway c).

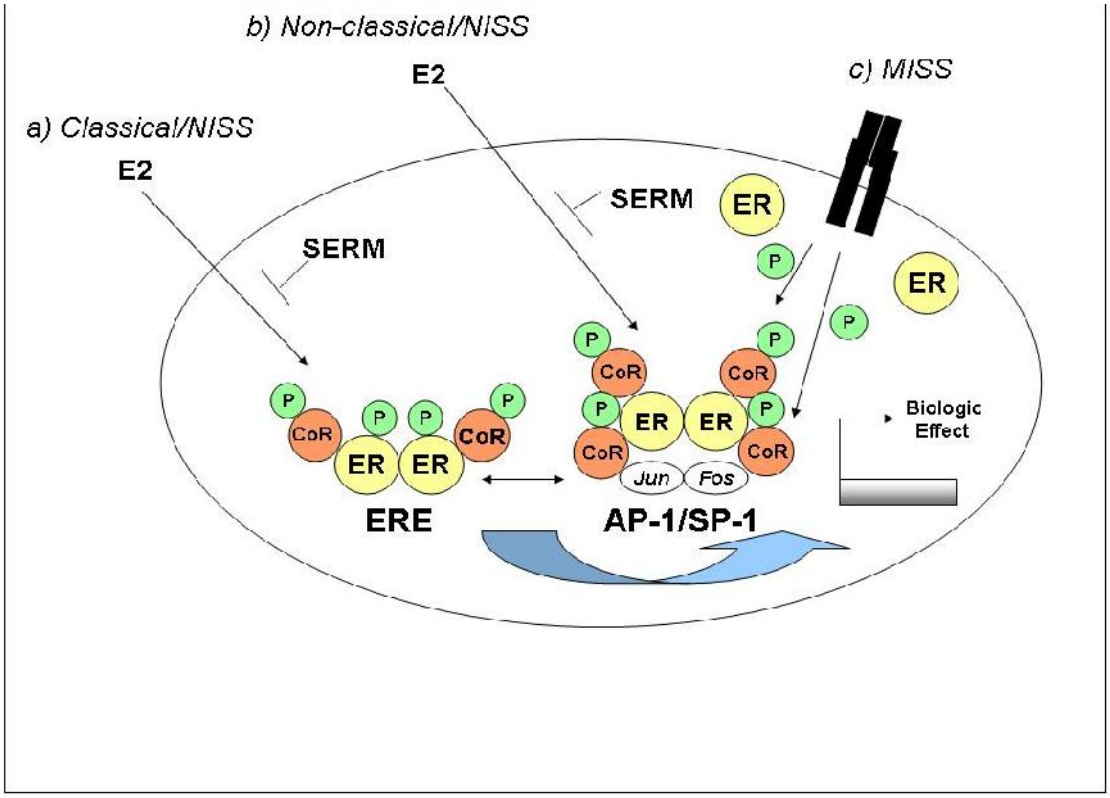

Figure 1. Oestrogen Receptor Action

(adapted from Cui *et al*, 2005).

NISS, nuclear-initiated steroid signalling; MISS, membrane-initiated steroid signalling; ER, oestrogen receptor; CoR, Coregulator; P, progesterone; ERE, oestrogen response element; E2, estradiol; SERM, selective oestrogen receptor modulator.

This membrane ER has been demonstrated by numerous biochemical, immunohistological and genetic methods (Levin, 2002; Pedram *et al*, 2002; Razandi *et al*, 2003a; Pedram *et al*, 2006) and can potentially interact with and activate via phosphorylation several membrane tyrosine kinases in breast cancer cells. Direct interaction of ERα with HER2 in the membrane has been shown to protect HER2-overexpressing breast cancer cells from tamoxifen-induced apoptosis (Chung *et al*, 2002). Interaction of insulin-like growth factor receptor (IGFR) and membrane ERα has been described which leads to the activation of IGFR downstream signalling that is stimulated by tamoxifen but can be blocked by either fulvestrant, a pure anti-oestrogen (Huynh and Pollack, 1993), or inhibitors of mitogen activated protein kinase (MAPK) (Kahlert *et* al, 2000).

Epidermal growth factor receptor (EGFR) can also be phosphorylated and activated by oestrogen-activated membrane ERα in a process involving activation of G-proteins, c-Src, and matrix metalloproteinases (MMPs)(Razandi *et al*, 2003b). Again this is stimulated by oestrogen and SERMs like tamoxifen but blocked by fulvestrant.

2.1.3

Prognostic Indicators in Clinical Breast Cancer

The steroid receptor content of human breast cancer has been found to be valuable both in providing prognostic information in patients with early breast cancer and in identifying patients with advanced disease that are likely to respond to endocrine treatments (De Sombre *et al*, 1979; Allred *et al*, 2001). There are also

other valuable prognostic indicators such as epidermal growth factor receptor, Her-2/*neu* oncogene, ploidy and S-phase fraction.

2.1.3.1

Oestrogen Receptor Status

ERα status is useful as a prognostic indicator of the course of the disease. Women with ERα positive breast tumours have a longer disease-free interval and better overall survival than those with ERα negative tumours, regardless of lymph node status (Rich *et al*, 1978). ERα positivity has also been linked to histologically well-differentiated tumours. The more poorly differentiated ERα negative tumours appear to recur earlier than poorly differentiated ERα positive tumours and have a very unfavourable prognosis (Maynard *et al*, 1978). Smaller tumours show a higher rate of ERα positivity (Clark *et al*, 1984). ERα status is significantly associated with age and menstrual status (Thorpe *et al*, 1987). Breast cancers arising in young women show significantly lower ERα mRNA (Anders *et al*, 2008). Post-menopausal women show the highest frequency of ERα positivity which increases with each decade of age after the menopause. A number of large studies have found the ERα to be an independent prognostic factor in both node positive and node negative early breast cancer (Maynard *et al*, 1978; Thorpe *et al*, 1987).

2.1.3.2

Progesterone Receptor Status

Progesterone receptors (PR) are ligand-activated transcription factors of the steroid hormone family of nuclear receptors which include ERs. There are two isoforms of PR in human cells, PR-A and PR-B. The molecular weights of these forms are 95000 and 120000 respectively and they appear to be derived from two alternative translation start sites on the same RNA transcript (Kastner *et al*, 1990). Human PR-B is 933 amino acids in length and contains a unique activation function AF3 (Sartorius *et* al, 1994a). PR-A lacks the 164 N-terminal residues that contain AF3 and is 769 amino acids in length. The breasts of normal women express equimolar ratios of PR-A: PR-B (Mote *et al*, 2002).

The inclusion of total PR measurements has been observed to improve the predictive values of steroid receptor analysis in breast cancer further. In particular the ERα- positive/PR-negative tumour type is less likely to respond to therapy than tumours that are positive for both receptors. Primary tumours exhibiting high ERα and PR contents are also associated with favourable survival of patients (Howell *et al*, 1984; Shek and Godolphin, 1989; Koenders *et al*, 1992; McCormack *et al*, 2007). Elevated PR levels significantly and independently correlate with increased probability of response to tamoxifen, longer time to treatment failure, and longer overall survival in patients with metastatic disease (Ravdin *et al*, 1992; Stendahl *et al*, 2006). Response is positively and directly related to PR levels as is observed with ERα (Elledge *et al*, 2000). During tamoxifen therapy, levels of both PR and ER decrease but PR levels decrease more dramatically than ER levels and it has

been observed that up to half of the tumours lose PR expression completely as they develop tamoxifen resistance (Gross *et al*, 1984). PR-A excess has also been reported to be associated with resistance to tamoxifen (Hopp *et al*, 2004) and poor clinical outcome (Mote *et al*, 2002). PR-Bs, on the other hand, are strong transcriptional activators and increased production of PR-B, resulting from a functional promoter polymorphism, is associated with an increased risk of breast cancer (De Vivo *et al*, 2003). PR isoform ratios may therefore also be important predictive factors for endocrine therapies.

2.1.3.3

Epidermal Growth Factor Receptor (EGFR)

The EGFR belongs to the erbB family of four closely related cell membrane receptors, namely, EGFR (HER1 or erbB1), c-erbB-2 (HER2), c-erbB-3 (HER3), and c-erbB-4 (HER4), and is believed to play an important role in breast cancer development (Cohen *et al*, 1998; Gullick and Srinivasan, 1998; Klapper *et al*, 2000; Holbro *et al*, 2003). These receptors are transmembrane glycoproteins consisting of an extracellular ligand-binding domain, a single transmembrane pass and a cytoplasmic tail that includes a tyrosine kinase domain for signal transduction. EGFR activation occurs when a ligand, such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α) or amphireulin, binds to its extracellular domain, causing the receptor to dimerize. Tyrosine kinase activity and tyrosine autophosphorylation then occur, leading to mitogenic signalling and other cellular activities (Alroy and Yarden, 1997).

Activation of EGFR has been shown to enhance processes responsible for tumour growth and progression, including the promotion of proliferation, angiogenesis, and invasion/metastasis, and inhibition of apoptosis (Woodburn, 1999; Wells, 2000). Overexpression of EGFR has been associated with enhanced metastatic potential of some breast cancer cell lines (Fitzpatrick *et al*, 1984; Roos *et al*, 1986) and has been correlated with disease progression, poor survival and poor response to therapy in clinical studies (Sainsbury *et al*, 1987; Toi *et al*, 1991; Anders *et al*, 2008).

2.1.3.4

HER-2/*neu* oncogene

The HER-2/*neu* oncogene codes for a transmembrane tyrosine kinase and has been suggested as an etiologic factor in several kinds of cancer. Human epidermal growth factor receptor 2 (HER-2)-positivity has been found in up to 25% of women with early breast cancer and has been associated with aggressive disease, a higher likelihood of recurrence after initial treatment, and poor prognosis (Baselga *et al*, 2006). A number of studies have also shown that increased expression of HER-2/*neu* has significant prognostic value for decreased survival in node-negative breast cancer (Rilke *et al*, 1991; Allred *et al*, 1992; Rosen *et al*, 1995; Press *et al*, 1997; Reed *et al*, 2000; Anders *et al*, 2008). HER-2/*neu* is routinely measured in patients to predict the likelihood of their response to Trastuzumab (Herceptin®, F. Hoffmann-La Roche Ltd., Basel, Switzerland), a monoclonal antibody directed against the extracellular domain of HER2, should they relapse after standard adjuvant therapy. HER-2/*neu* positivity may also predict response to

other adjuvant therapy regimes. HER-2/*neu*-positive tumours treated with doxorubicin (Paik *et al*, 1998) or paclitaxel (Baselga *et al*, 1997) have shown a significantly improved response.

2.1.3.5

Ploidy and S-phase Fraction

Analysis of tumour ploidy (DNA content) and S-phase fraction (SPF) have been demonstrated to be important as markers for neoplastic progression and as differential descriptors of benign *versus* malignant tumour tissues with high tumour proliferation indicated as an adverse prognostic factor in surgically treated cancers (Barlogie *et al*, 1980; Kute *et al*, 1981). Previous methods of cell kinetic analysis using tritiated thymidine labelling were expensive and time consuming. The use of DNA flow cytometry is now the powerful technique used to evaluate the DNA content and cell proliferation of breast cancer (Spyratos, 1993). Cell material obtained by fine needle aspirate is particularly suitable for flow cytometric DNA analysis as it contains a lower proportion of non-tumour cells than tissue samples (Gazic *et al*, 2008). Knowledge of the cell cycle is necessary in order to explain ploidy and SPF.

Cells which are not involved in cell division are referred to as being in the G_0-phase. Once cell division is triggered the cells enter the G_1-phase of the cell cycle, during which the amount of RNA increases and certain proteins essential for replication of DNA are made. As the cells start to synthesize new DNA they enter the synthetic phase, or S-phase of the cell cycle. During this phase the DNA

content of the cells increases until it has doubled. It is this latter phase that reflects the proliferative capacity of the cells. DNA synthesis ceases as the cells enter the G_2-phase. Finally, the cells divide by mitosis during the M-phase and, if cell division is to be sustained, return to the G_1-phase. The cells return to the G_0-phase if they are to quiesce (Figure 2).

Cells in the G_0- and G_1-phase have diploid (2n) DNA content and are collectively designated the $G_{0/1}$-phase. Cells in the G_2- and M-phases have tetraploid (4n) DNA content and are referred to as G_2M-phase cells. Cells in S-phase have a DNA content varying between these two extremes. The mean DNA content of diploid $G_{0/1}$-phase cells is a measure of the ploidy of the cell population. Aneuploidy is the term used for an abnormal number of chromosomes which is reflected by a change in content of DNA. Tumours with a chromosomal number less than 2n are considered hypodiploid whereas greater than 2n is termed hyperdiploid.

Figure 3 shows a wide variety of DNA histogram types in human breast cancer tumours analyzed by flow cytometry (Coulson *et al*, 1984). Normal human mammary tissue (Figure 3A) and diploid breast cancer cells (Figure 3B) have no aneuploid $G_{0/1}$ peaks. The first peak represents the $G_{0/1}$ nuclei containing the 2n (2c) complement of DNA. The smaller peak represents the G_2M nuclei containing 4n (4c) amounts of DNA, with the population between these peaks being S-phase nuclei. Peaks which are greater or less than these $G_{0/1}$ and G_2M peaks contain aneuploid nuclei.

Diploidy, near-hyperdiploidy, and tetraploidy have been associated with a better survival (Toikkanen *et al*, 1989; Fisher *et al*, 1991). A significantly poorer survival has been observed in breast cancer patients with hypodiploid, hypotetraploid, and hypertetraploid tumours (Beerman *et al*, 1990; Fernö *et al*, 1992; Balslev *et al*, 1994). Hypodiploidy and hypotetraploidy are associated with large tumour size, high histological grade, and low expression of ER and PR (Fernö *et al*, 1992, Balslev *et al*, 1994). Hypertetraploidy has been associated with large tumour size and advanced tumour stage (Beerman *et al*, 1990) and the worst clinical outcome (Pinto *et al*, 1999). High SPF has been found to indicate an increased risk of recurrence and mortality in patients with breast cancer (Merkel *et al*, 1993; Stål *et al,* 1993; Camplejohn *et al*, 1995; Gazic *et al*, 2008) and is associated with nuclear grade, tumour size, ER, PR, lymph node metastasis, and age (Fisher *et* al, 1991; Fernö *et* al, 1992; Stål *et* al, 1993).

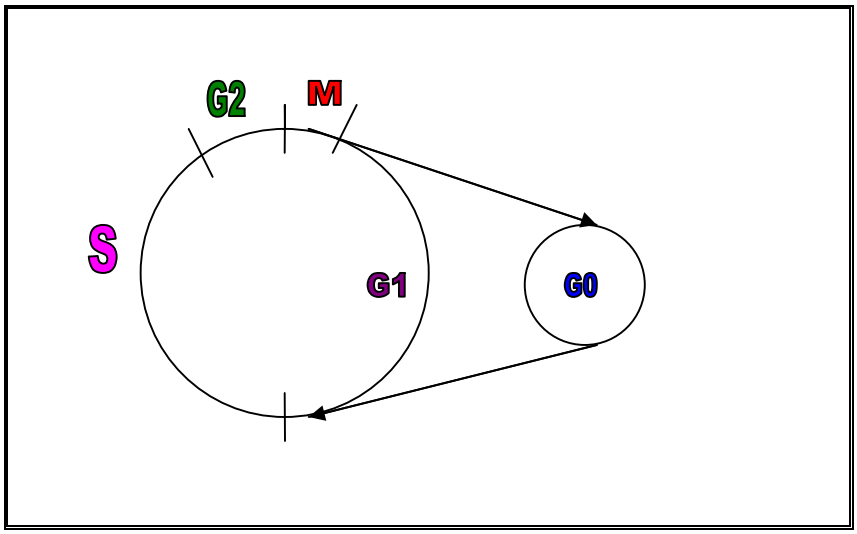

Figure 2. Diagram Illustrating the Cell Cycle

(adapted from Ormerod, 1990).

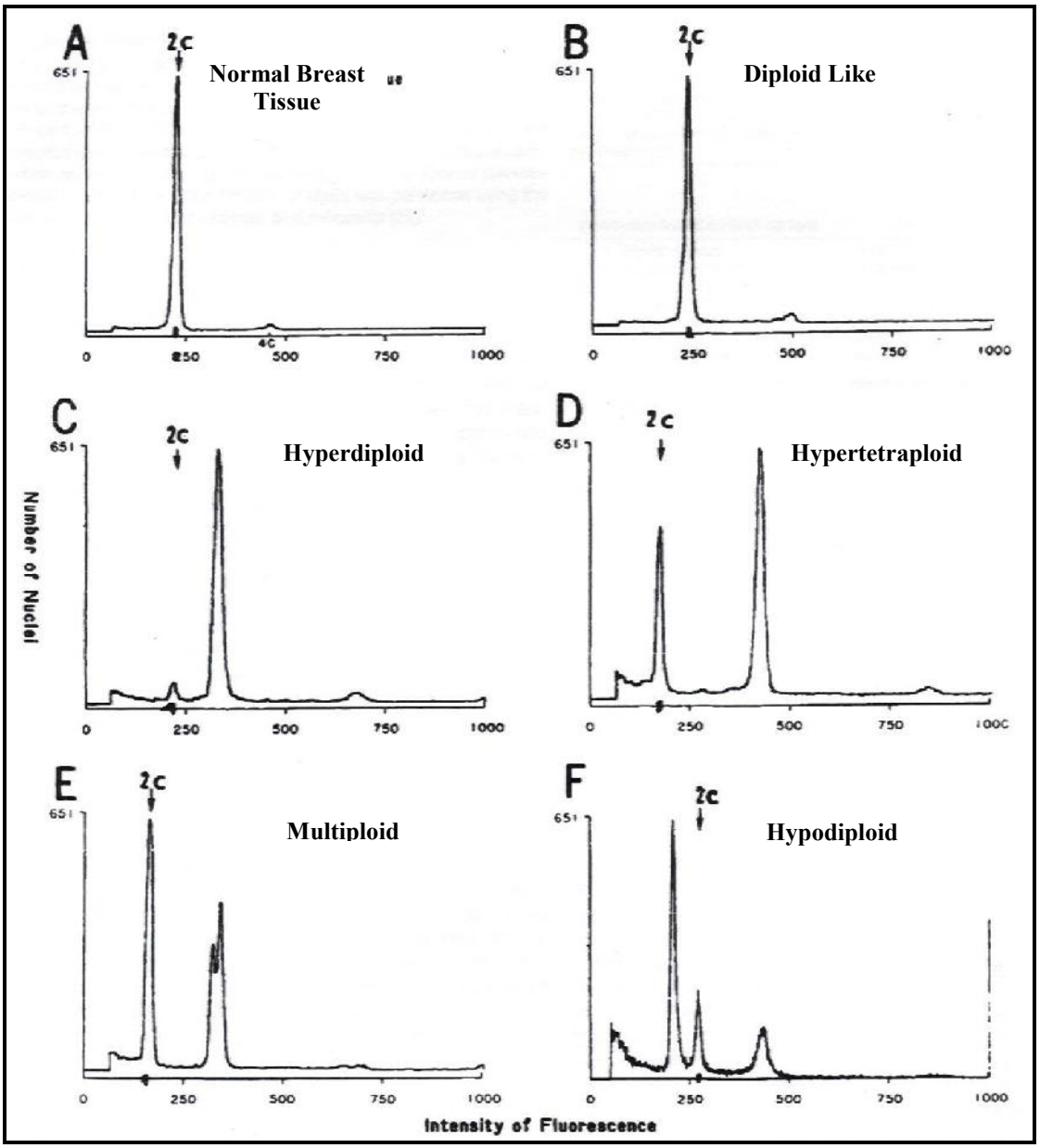

Figure 3. DNA flow cytometer histograms of mammary tissue

(Coulson *et al*, 1984).

Each section represents the number of nuclei measured (Y-axis) *versus* the relative intensity of fluorescence (DNA content) per nucleus (X-axis).
2c = 2n and 4c = 4n (in the text).

2.1.4

Choice of Treatment

2.1.4.1

Oestrogen Receptor Antagonists

The presence of ER has been shown to assist in the identification of patients likely to respond to endocrine manipulation (Osborne *et al*, 1980). ERα positive tumours respond to endocrine therapy 50 to 70% of the time while ERα negativity is associated with a response rate of less than 10% (McGuire *et al*, 1977). The incidence of response to hormone therapy increases with increasing ERα levels (Wittliff, 1984). The non-steroidal oestrogen antagonist, tamoxifen, is a triphenyl-ethylene derivative (Figure 4) and is frequently used as first line endocrine therapy for breast cancer as it interrupts the stimulatory effects of oestrogen by binding to and blocking the ER (Encarnacion *et al*, 1993) (Figure 5). The response to this drug has been shown to depend on the presence of ERα (Rose *et al*, 1985; Glauber and King, 1992) and therefore reduces the incidence of ER-positive tumours but has no effect on ER-negative tumours. Tamoxifen also decreases circulating cholesterol which is thought to greatly improve patient prognosis. However, tamoxifen has shown oestrogen agonist properties in the uterus with an increased risk of endometrial cancer in postmenopausal women (Fisher *et al*, 1998).

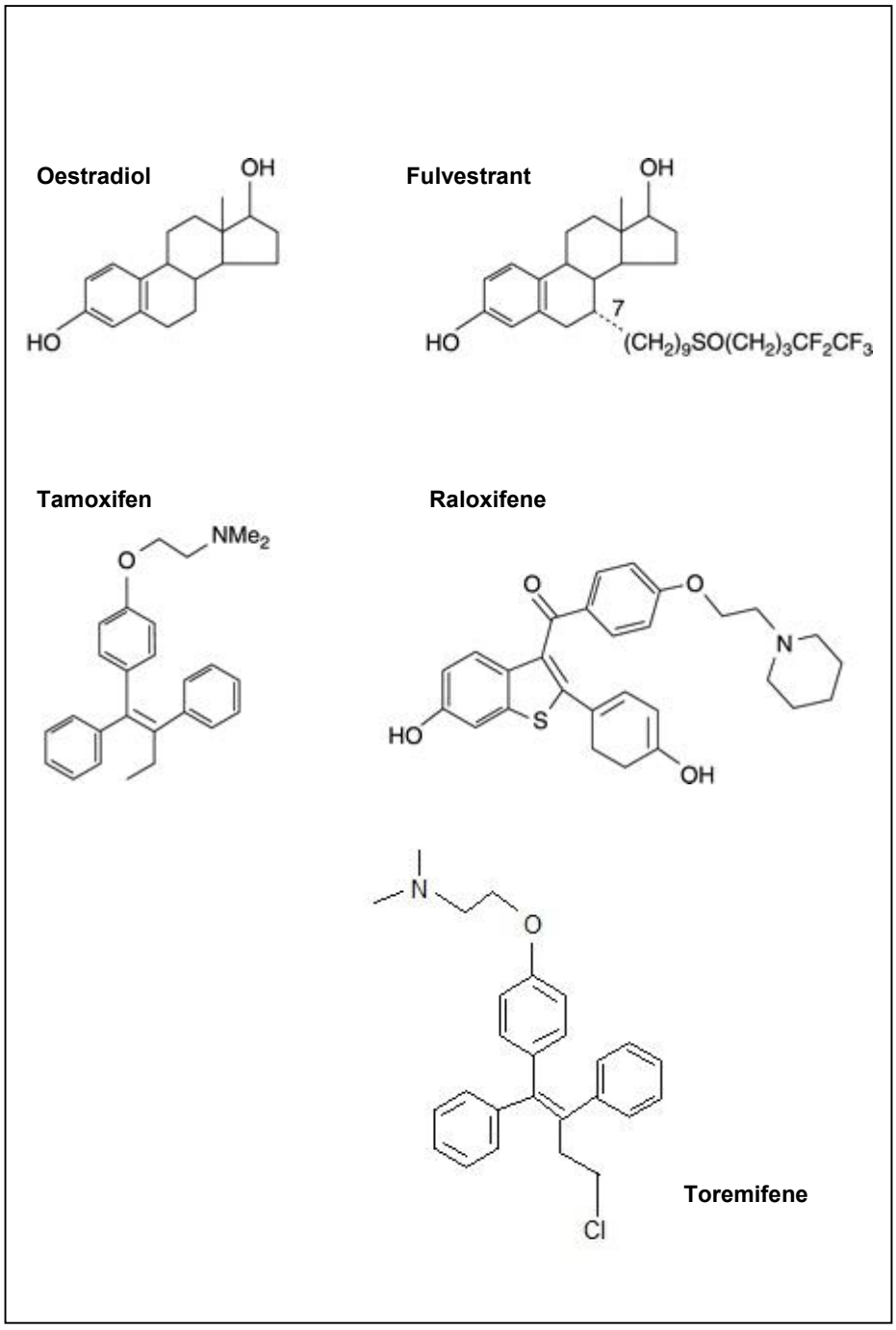

Figure 4. Chemical structures of oestradiol, tamoxifen, fulvestrant, raloxifene and toremifene.

Clinical resistance to antioestrogens like tamoxifen is a major problem in the treatment of hormone-dependent breast cancers. It has been hypothesized that mutations in the ER could be one mechanism by which breast tumours evolve from a hormone-dependent to a hormone-independent phenotype (Karnik *et al*, 1994). It has also been suggested that the resistance to tamoxifen occurs due to an increase in cell surface signalling through the HER2/neu/EGFR or Insulin like growth factors that promote phosphorylation of the ER and its coactivators (Jordan, 2004; Sengupta and Jordan, 2008) which in turn activates breast cancer cell growth.

First-generation SERMs, such as toremifene (Fareston®), a chloro-tamoxifen (Figure 4), droloxifene and idoxifene, were based on the structure of tamoxifen but showed no benefits over tamoxifen and are all partial agonists (Wakeling, 2000). A second generation SERM, raloxifene (Evista®), a 'fixed-ring' benzothiophene derivative (Figure 4) exerts anti-oestrogenic activity in the breast and uterus and agonistic activity in bone and the cardiovascular system (Thiebaud and Secrest, 2001). Initially raloxifene (originally named keoxifene) was developed for breast cancer treatment (Clemens *et al*, 1983) but its use was abandoned in the late 1980's as clinical trials indicated no activity in tamoxifen-resistant patients (Buzdar *et al,* 1988). According to the more recent Study of Tamoxifen and Raloxifene (STAR) trial the two SERMs are equivalent in the prevention of invasive breast cancer, but tamoxifen is slightly better in preventing non-invasive breast cancer. Raloxifene, however, has fewer side effects than tamoxifen (Vogel *et al*, 2006).

The discovery of an additional ER subtype (ERβ) (Kuiper *et al*, 1996; Mosselman *et al*, 1996), which is highly homologous with the "classical" ER (now designated ERα) and has been shown to bind oestrogens with an affinity similar to that of ERα, has added a new dimension to the problem of tamoxifen resistance and progression of the disease (See 2.3).

A more recent oestrogen receptor antagonist used in the treatment of advanced ERα-positive breast cancers is fulvestrant (Faslodex®), a 7α-alkylsulphinyl analogue of 17β-oestradiol that is structurally distinct from the non-steroidal oestrogen antagonists (Figure 4). The C7 side chain of fulvestrant differentiates it from oestradiol and its position, length and flexibility determine its pure antagonistic activity (Bowler *et al*, 1989). Fulvestrant, which competitively binds to the oestrogen receptor with a much greater affinity than tamoxifen (Wakeling *et al*, 1991), prevents oestrogen receptor dimerization, inhibits oestrogen receptor DNA binding and leads to down-regulation of oestrogen-regulated genes (Wakeling *et al*, 2000) (Figure 6). Fulvestrant may help to circumvent hormone resistance via pathways of receptor cross-talk by reducing cellular ER levels (Hutcheson *et al*, 2003). After fulvestrant treatment tumour sensitivity to subsequent endocrine therapies appears to be maintained (Vergote *et al*, 2003; Robertson *et al*, 2005).

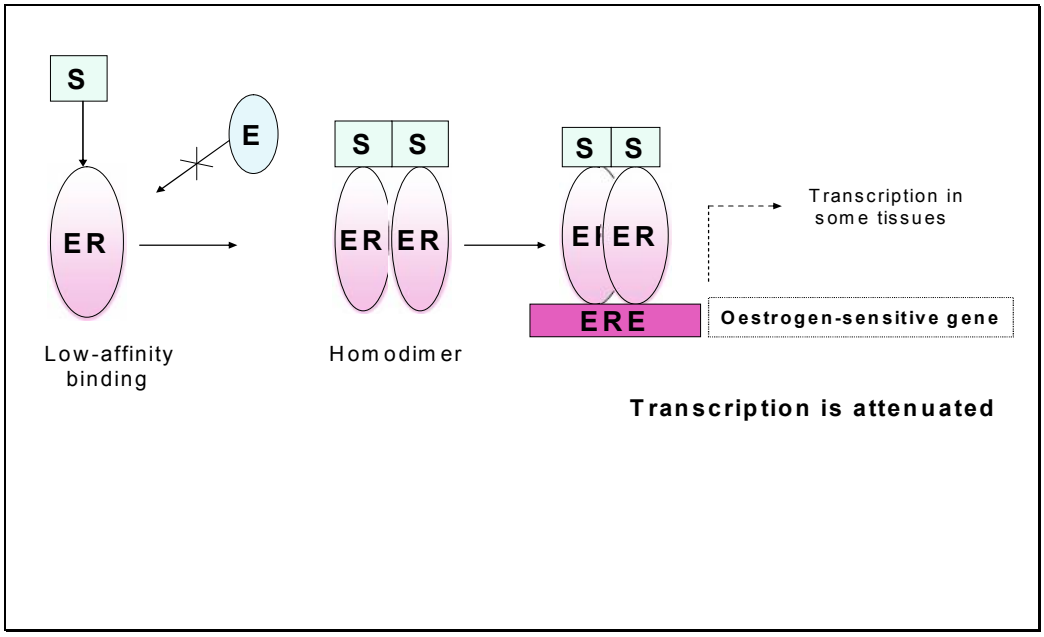

Figure 5. SERMs action on ER in breast cancer

(adapted from Morris and Wakeling, 2002).

SERMs (S) compete with oestrogen (E) for binding to the ER and inhibit transcription of oestrogen sensitive genes. ERE, oestrogen response element.

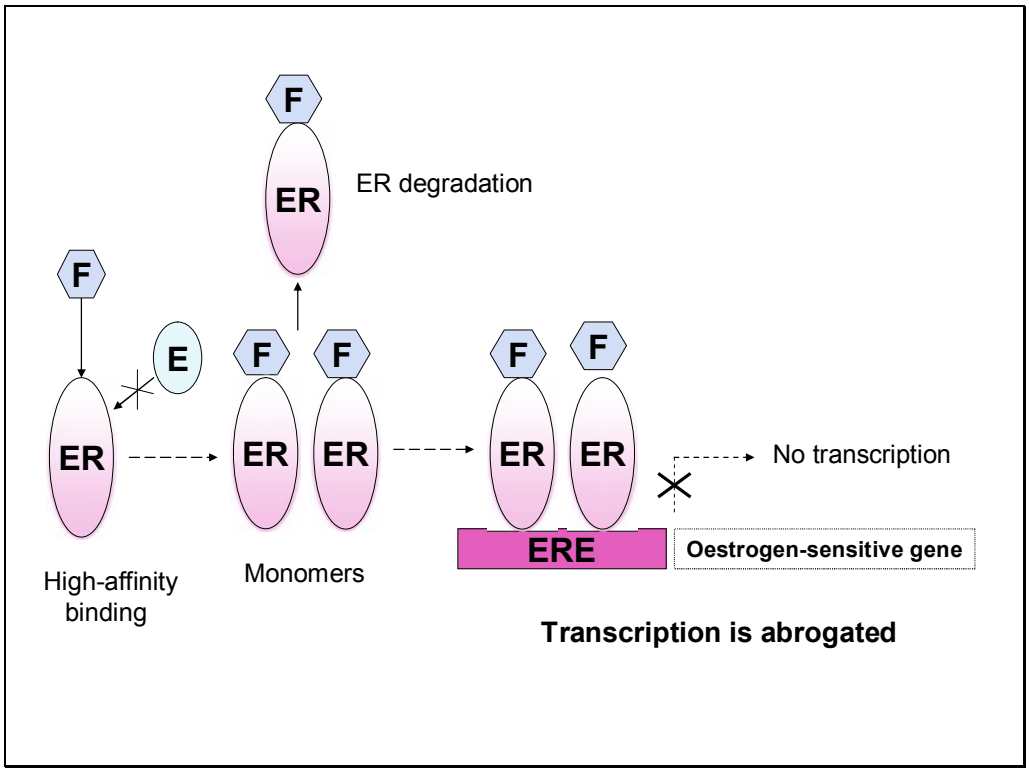

Figure 6. Fulvestrant action on ER in breast cancer

(adapted from Morris and Wakeling, 2002).

Fulvestrant (F) competitively inhibits the binding of oestrogen (E) to the ER, prevents dimerization, promotes degradation and prevents transcription of oestrogen-sensitive genes. ERE, oestrogen response element.

2.1.4.2

Aromatase Inhibitors

The aromatase inhibitors (AIs) act by blocking aromatase, the enzyme that catalyzes the final and rate-limiting step in the synthesis of oestrogens. Aromatase is an enzyme complex consisting of a cytochrome P450 haemoprotein and NADPH cytochrome P450 reductase (Buzdar and Howell, 2001). Aromatization of the androgen, androstenedione, proceeds via three successive oxidation steps resulting in the formation of the oestrogen product, oestrone (Figure 7) and oestradiol.

The expression of aromatase is tissue specific and is controlled by several different promoters. The most significant activity of aromatase in premenopausal women is in the ovaries where it is regulated by the gonadotropins luteinising hormone (LH) and follicle-stimulating hormone (FSH). Aromatase is also expressed at lower levels in nonovarian tissue such as muscle, skin and adipose tissue in both premenopausal and postmenopausal woman. It is in postmenopausal women that these nonovarian tissues become the most dominant sources of oestrogen where the expression of aromatase is not regulated by LH or FSH (Hemsell *et al*, 1974). Aromatase has also been measured in the stromal cell component of normal breast and breast tumours. The expression of aromatase is highest in or near breast tumour sites (Miller *et al*, 1997; Suzuki *et al*, 2005).

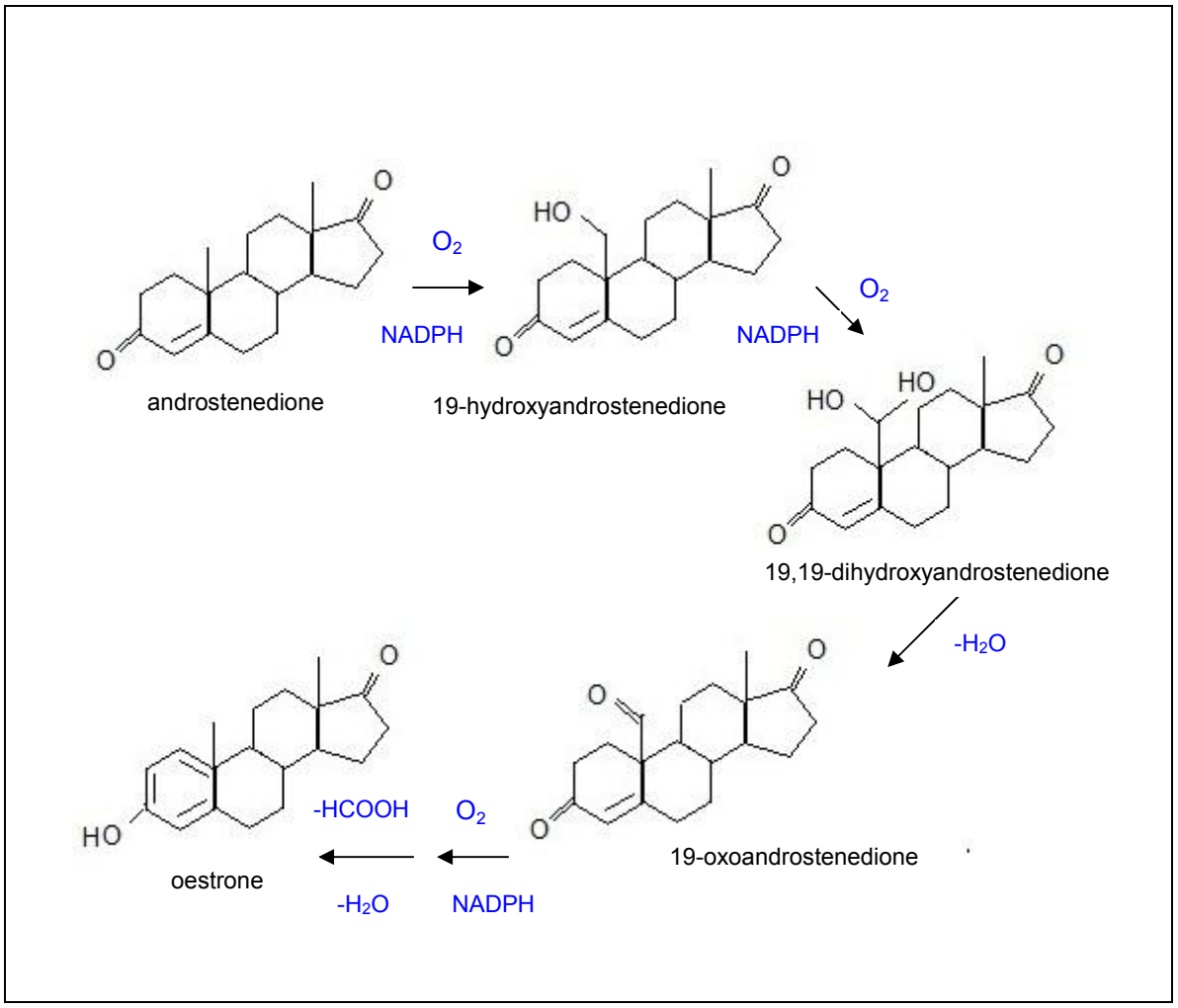

Figure 7. Aromatase enzyme reaction

(from Brueggemeier *et al*, 2005).

Aromatase inhibitors block the conversion of androstenedione to oestrone and testosterone to oestradiol (Figure 8) and lower the level of oestrogen in the tumour. This is particularly relevant in postmenopausal women as the primary oestrogen source is derived from the conversion of androstenedione (produced by the adrenals) to oestrone and oestradiol in the peripheral tissues. In premenopausal women AIs induce increased gonadotrophin secretion resulting in ovarian stimulation and potential increase in ovarian size which may result in ovarian cysts. AIs are therefore not indicated in premenopausal women (Nabholtz, 2008).

Other steps in the synthesis pathway of steroid hormones are catalyzed by enzymes related to aromatase. Early aromatase inhibitors, such as aminoglutethimide, had limited selectivity over these other enzymes and thus treatment of patients with these inhibitors required coadministration of corticosteroids with resulting toxicities. A second-generation AI, 4-hydroxyandrostenedione (formestane) and fadrozole, had increased selectivity and fewer side-effects than aminoglutethimide but did not show any benefit over tamoxifen (Wiseman and McTavish, 1993).

Aromatase inhibitors are categorized by generation and by mechanism of action. There are two types of third-generation aromatase inhibitors, irreversible steroidal activators and reversible nonsteroidal imidazole-based inhibitors (Figure 9). Both types interfere with the final step of oestrogen biosynthesis but they do by different mechanisms (Figure 8). Steroidal agents, such as exemestane (Aromasin®), have an androgen structure and compete with the aromatase substrate

androstenedione. They bind irreversibly to the catalytic site of aromatase causing loss of enzyme activity. Before oestrogen biosynthesis can resume more aromatase enzyme must be produced. This is reflected by the decrease in aromatase activity observed when tested in *in vitro* systems (Miller, 2000). These steroidal agents are often referred to as suicide inhibitors or aromatase inactivators. Due to their steroidal structure, exemestane and its 17-hydroexemestane metabolite have potentially androgenic effects.

Nonsteroidal imidazole-based agents, which include anastrozole (Arimidex®) and letrozole (Femara®), bind reversibly to the haem portion of the aromatase molecule (Buzdar and Howell, 2001) and are associated with an increase in aromatase activity when tested in *in vitro* systems. This may occur as the biosynthesis of oestrogens in the presence of reversible steroidal agents does not require synthesis of new aromatase (Miller, 2000). This increase in aromatase activity may be important to the development of tumour resistance, particularly in long-term treatment. Both anastrozole and letrozole have shown efficacy advantages over tamoxifen in postmenopausal patients as first-line therapy (Bonneterre *et al*, 2000; Nabholtz *et al*, 2000; Mouridsen *et al*, 2001).

Aromatase inhibitors are supplanting tamoxifen as the most widely used hormonal agent in the treatment of breast cancer particularly in postmenopausal women as they improve disease-free survival and decrease the risk of endometrial cancer (Coombes *et al*, 2004; Boccardo *et al*, 2005).

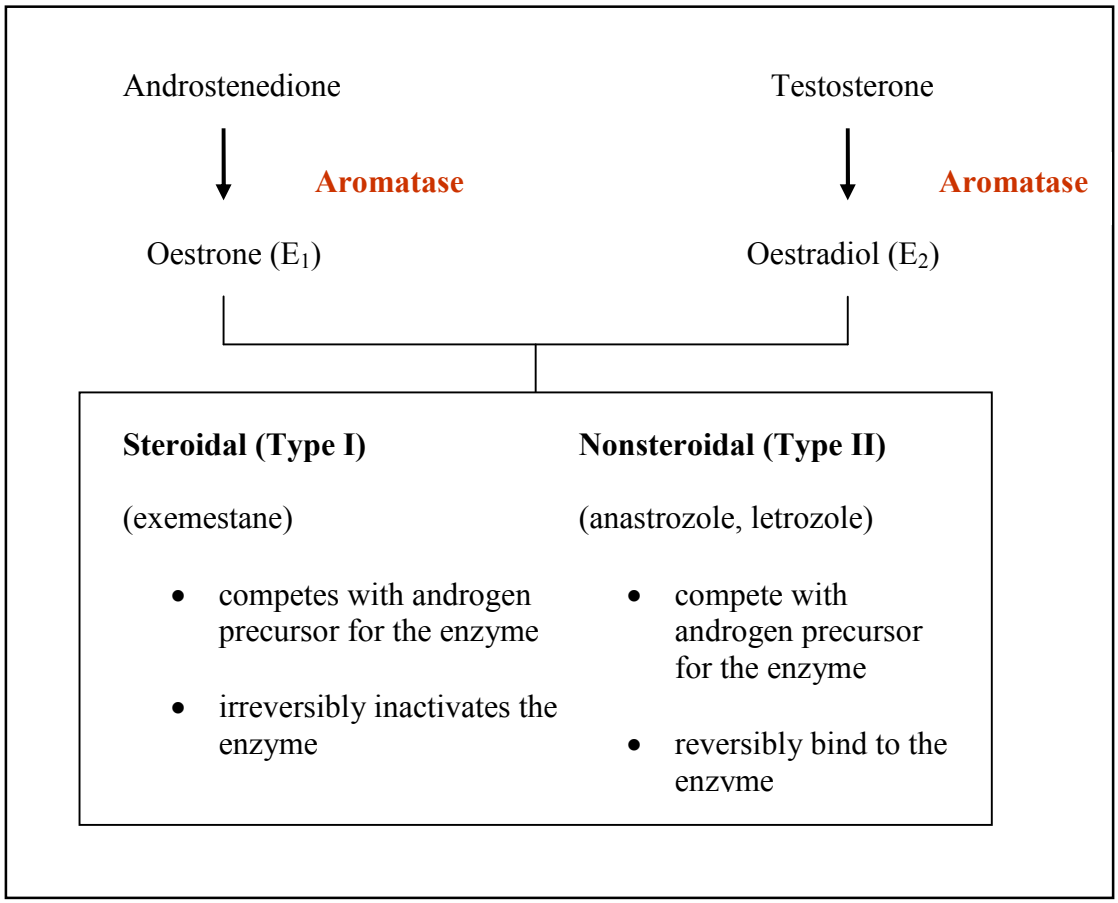

Figure 8. Third-generation aromatase inhibitors: mechanism of action

(adapted from Lake and Hudis, 2002).

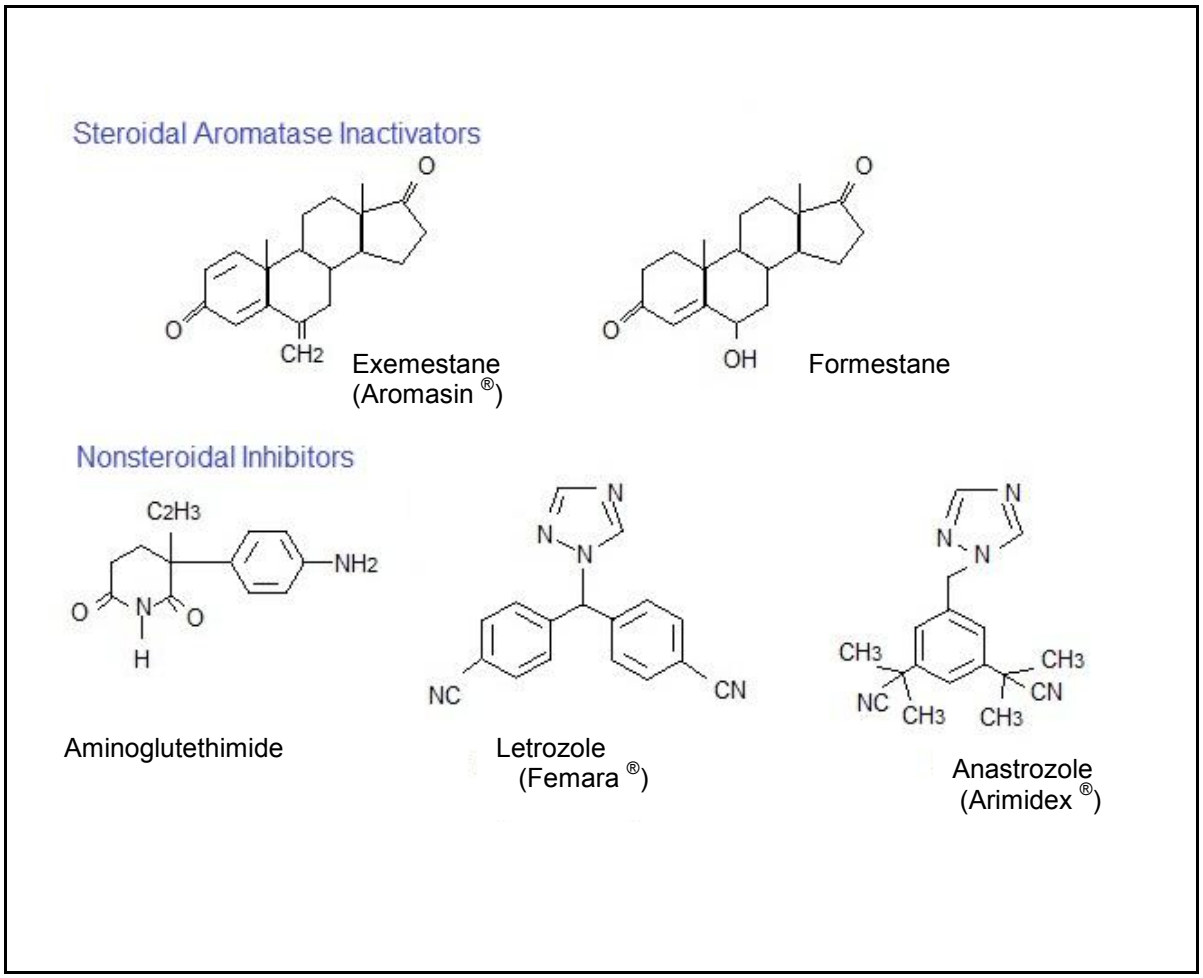

Figure 9. Chemical structure of different aromatase inhibitors

(adapted from Lonning, 2004; Brueggemeier *et al*, 2005).

Current data suggest that there is a lack of cross-resistance between steroidal and nonsteroidal agents as clinical benefits may occur when a nonsteroidal AI is prescribed following a steroidal agent, and *vice versa*. Anastrozole given after exemestane (Lonning *et al*, 2000) shows benefit as does exemestane after anastrozole (Bertelli *et al*, 2005). This allows for another hormonal therapy option before switching to chemotherapy in women with advanced breast cancer.

Recently, new synthetic AIs obtained by modifications in the A and D-rings of androstenedione, 5α-androst-3-en-17-one and 3α,4α-epoxy-5α-androstan-17-one, have shown to be strong inhibitors of aromatase (Cepa *et* al, 2005) and breast cancer cell proliferation *in vitro* (Cepa *et al*, 2008).

2.1.4.3

Progestagens

Although anti-oestrogens have been the first choice of hormonal manipulative therapy for human breast cancer, progestagen treatment of hormone-dependent breast cancer has also been used. Progestagens (progesterone and synthetic progesterone-like compounds) signal mainly through the progesterone receptor which has been described in Chapter 2.1.3.2. Five different progestagens have been used historically in various concentrations, routes of administration, schedules and lengths of treatment. These include progesterone (Figure 10), 17-hydroxyprogesterone, norethisterone acetate (NA), medroxyprogesterone acetate (MPA), and megestrol acetate (MA) (Sedlacek and Horwitz, 1984). The synthetic progestagen, medroxyprogesterone acetate (Figure 10), is a 17α-

hydroxyprogesterone derivative and was used frequently until the early 1990s as a second-line hormonal therapy for metastatic breast cancer (Parazzini *et al*, 1993). Following the advent of tamoxifen and aromatase inhibitors the use of MPA declined but there has been renewed interest in alternative hormonal treatments, including MPA, for use both in early disease and in the advanced setting when conventional therapies fail (Focan *et al*, 2004; Zaucha *et al*, 2004). Their appeal is limited, however, by the occurrence of weight gain and oedema, as well as cardiovascular and thromboembolic side effects. Recent studies indicate that MPA and other progestagens may have an impact on the progression of PR positive breast cancer by enhancing the ability of cancer cells to interact with the extracellular environment and to invade the surrounding tissue *in vitro* (Fu *et al*, 2008). These results suggest that PR activation may play a role ER positive/ PR positive breast cancer progression.

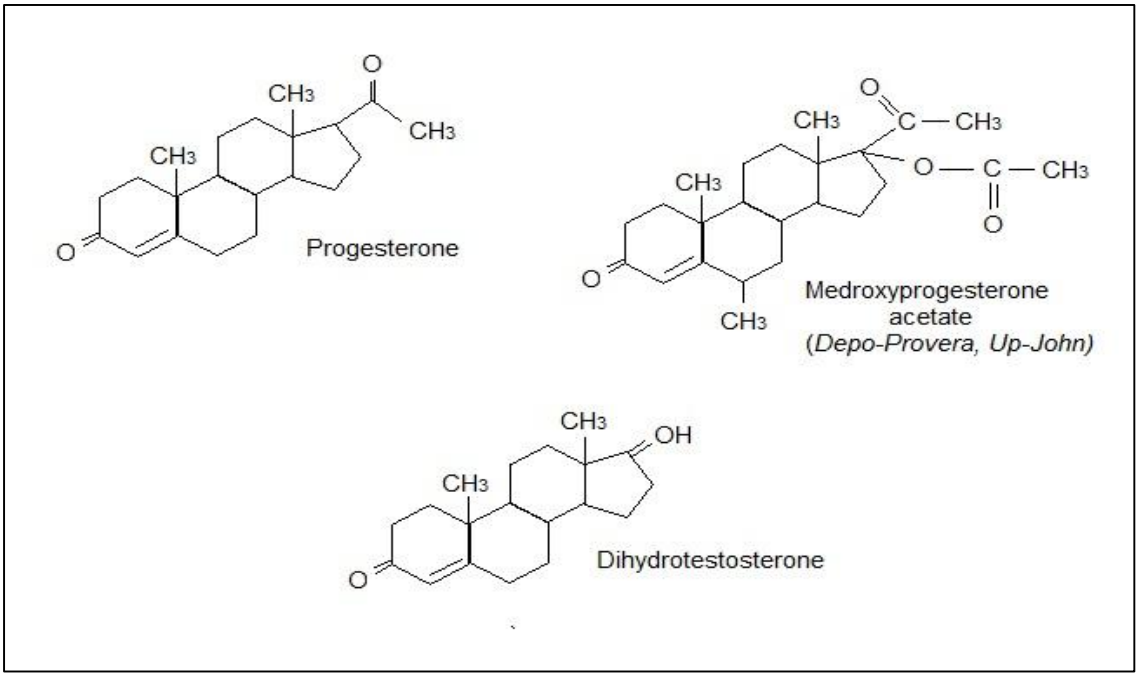

Figure 10. Chemical structures of progesterone, MPA and DHT

(adapted from Ghatge *et al*, 2005).

DHT, dihydrotestosterone; MPA, medroxyprogesterone acetate.

The pharmaceutical name and manufacturer are shown in italic text.

2.2

Oestrogen Receptor α

The "classical" ER, now termed ERα, was cloned by Walter and co-workers in 1985. The cDNA sequence was determined in 1986 by Green *et al* and the genomic organization was described by Ponglikitmongkol *et al* in 1988. ERα is a ligand-activated transcription factor which binds to specific *cis*-acting hormone-responsive DNA elements which behave as enhancers (Yamamoto, 1985). The ERα gene has been localized to human chromosome 6q24-27 (Walter *et al*, 1985).

2.2.1

Molecular Structure of the ERα Gene

ERα is composed of several domains important for hormone binding, DNA binding and activation of transcription. The wild-type ERα is a 66 kDa protein coded by a 6.6 kb mRNA, comprised of six functional domains derived from eight exons (Figure 11).

Region A (encoded by part of exon 1) and region B (encoded by part of exons 1 and 2) constitute the amino (NH_2) terminal part of the receptor in which one of the transactivation function elements (AF1) is located (Kumar *et al*, 1987). In addition, the A/B region contains a co-regulatory domain, which binds various ER coactivators and corepressors that modulate ER-mediated transcriptional activity.

Region C is a 55-amino acid region which has the potential to form at least two "zinc-finger" motifs which are responsible for binding the ER to its DNA recognition site called the oestrogen responsive element (ERE) (Klein-Hitpass *et al*, 1989). This region is encoded by exon 3 and parts of exons 2 and 4 (Green and Chambon, 1987). It also contains a nuclear localization signal so that the protein is targeted to the nucleus after translation.

Exons 2 and 3 are separated by an intron of greater than 16 kb which is located between the two zinc fingers (Ponglikitmongkol *et al*, 1988). Point mutations replacing two cysteines by two histidines in the first DNA binding finger have been shown to prevent the activation of gene transcription.

The replacement of the 66 amino acid region of the ERα with that of human glucocorticoid receptor (hGR) has resulted in the expression of a glucocorticoid-inducible gene, not an oestrogen inducible gene, in the presence of oestradiol (Green and Chambon, 1987) thus indicating that not only is region C important for activation of transcription but it also determines the receptor's specificity of target genes.

Region E, encoded by exons 5 to 7 and parts of exons 4 and 8, is the hormone-binding domain and is involved in several other functions including dimerization and hormone-dependent transcriptional activity (AF2). This large hydrophobic domain folds to form a hydrophobic pocket, which binds the hormone specifically (Green *et al*, 1986). The presence of an unoccupied hormone-binding domain results in the masking of the DNA-binding domain. Oestrogen binding has been

shown to cause phosphorylation and induction of conformational change in the ER (Beekman *et* al, 1993; Kraus *et al*, 1995). This change releases the heatshock proteins and allows ER homodimerization, DNA binding, and association with proteins called co-activators and the histone acetylase, p300/CBP, to mediate activation of transcription. Antioestrogens do not cause this conformational change and are therefore unable to fully activate the receptor.

The hinge (D) domain contributes flexibility to the DNA- versus the ligand binding domain and has been shown to influence the DNA-binding properties of individual receptors. It may also serve as an anchor to certain co-repressor proteins (Beato *et al*, 1995). Part of the ligand-dependent, transactivation domain AF-2a is located in this region and lies between amino acids (aa) 282-351 (Norris *et al*, 1997). A portion of the ER nuclear localization signal is also located in this region.

Region F contains the 42-amino acid carboxy-terminal region of ERα which, together with region E, is involved in receptor dimerization, the binding of coregulatory proteins, and the binding of chaperone proteins such as heat shock proteins (hsp) 70 and 90 (Chambraud *et al*, 1990). Region F differs from region E in that it is not well conserved among different vertebrate ERα species. Montano and co-workers (1995) suggest that the F domain of ERα has a specific modulatory function that affects the agonist/antagonist effectiveness of anti-oestrogens and the transcriptional activity of the liganded ERα in cells.

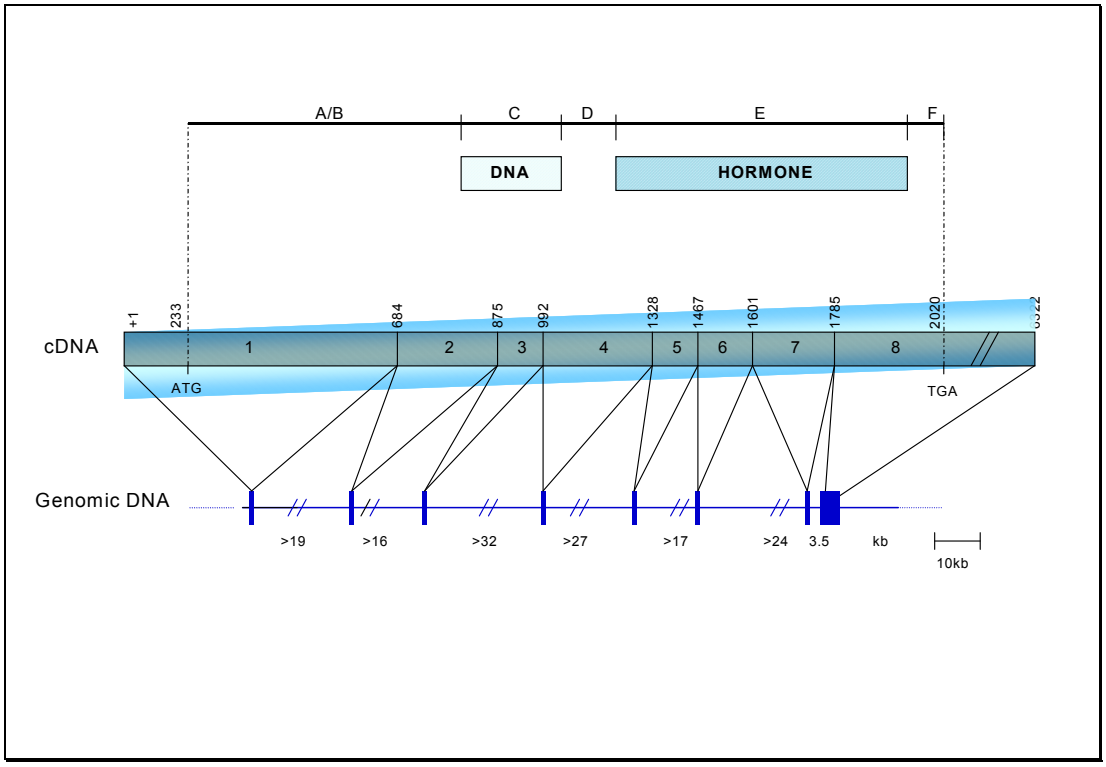

Figure 11. The Oestrogen Receptor α Gene

(adapted from Ponglikitmongkol *et al*, 1988).

The solid boxes (genomic DNA) represent the eight exons of the human ERα gene with the minimum size of each intron indicated between each box. The ERα cDNA is shown above with the corresponding positions of the eight exons (1-8). The numbers at the borders refer to the nucleotides (Green *et al*, 1986). The position of the translation initiation (ATG) and termination (TGA) codons are indicated. The division of the human ERα protein into six regions (A-F) together with the location of the DNA- (region C) and hormone- (region E) binding domains are shown above the cDNA.

Their data indicates that the conformation of the receptor-ligand complex is different with oestrogen as compared to anti-oestrogen and with wild-type as compared to region F deleted ERα such that the potential for interaction with protein cofactors or transcription factors in these cases is markedly different.

2.2.2

Oestrogen Receptor α Variants

Several ERα variants have been identified in breast cancer cell lines and breast tumours. These variants are generally found to co-exist with wild-type (wt) ERα and may interfere with its normal function (Zhang *et al*, 1996). Two main structural patterns of ERα variant mRNAs have been consistently identified: the truncated ERα mRNAs (Dotzlaw *et al*, 1992) and the deleted ERα mRNAs (Fuqua *et al*, 1993; Poola *et al*, 2000) (Figure 12). Cancer tissues appear to exhibit a higher total number of splice variants and have more multiple exon deletions compared with normal breast (Taylor *et al*, 2010).

2.2.2.1

ERα exon 2 deletion (ERαΔ2)

The deletion variant, ERαΔ2, is prematurely terminated after exon 1 and therefore missing the DNA binding domain. The resulting 17 kDa protein lacks transcriptional activity (Wang and Miksicek, 1991) (Figure 12). Only the A and B domains of ERα where AF-1 is located is encoded. It also lacks the dimerization

domain and therefore does not affect wild-type signalling (Wang and Miksicek, 1991). Experiments using tamoxifen-resistant and tamoxifen-sensitive MCF-7 cell lines have found no differences in expression of this variant suggesting that ERαΔ2 does not play a role in tamoxifen resistance (Madsen *et al*, 1997). ERαΔ2 has been identified in both normal and diseased tissue (Leygue *et al*, 1996b).

2.2.2.2

ERα exon 3 deletion (ERαΔ3)

The ERαΔ3 splice variant is missing part of the DNA binding domain resulting in an inability to bind DNA and therefore results in a 61 kDa protein with dominant-negative activity which is unable to suppress oestrogen-induced transcriptional activity (Wang and Miksicek, 1991; Han *et al*, 2004). This dominant negative activity may serve to reduce normal oestrogenic signalling and thereby influence tumour progression and growth. This variant has been detected in the majority of ER-positive, PR-negative tumours (Zhang *et al*, 1996; Taylor *et al*, 2010).

2.2.2.3

ERα exon 4 deletion (ERαΔ4)

ERαΔ4 is one of the most abundant ERα deleted variants (Leygue *et* al, 1996a; Pfeffer, 1996) and results in an in-frame deletion encoding a protein lacking a nuclear localization signal, the AF-2a activation domain and part of the ligand binding domain (Figure 12). This variant is unable to bind DNA and ligand and

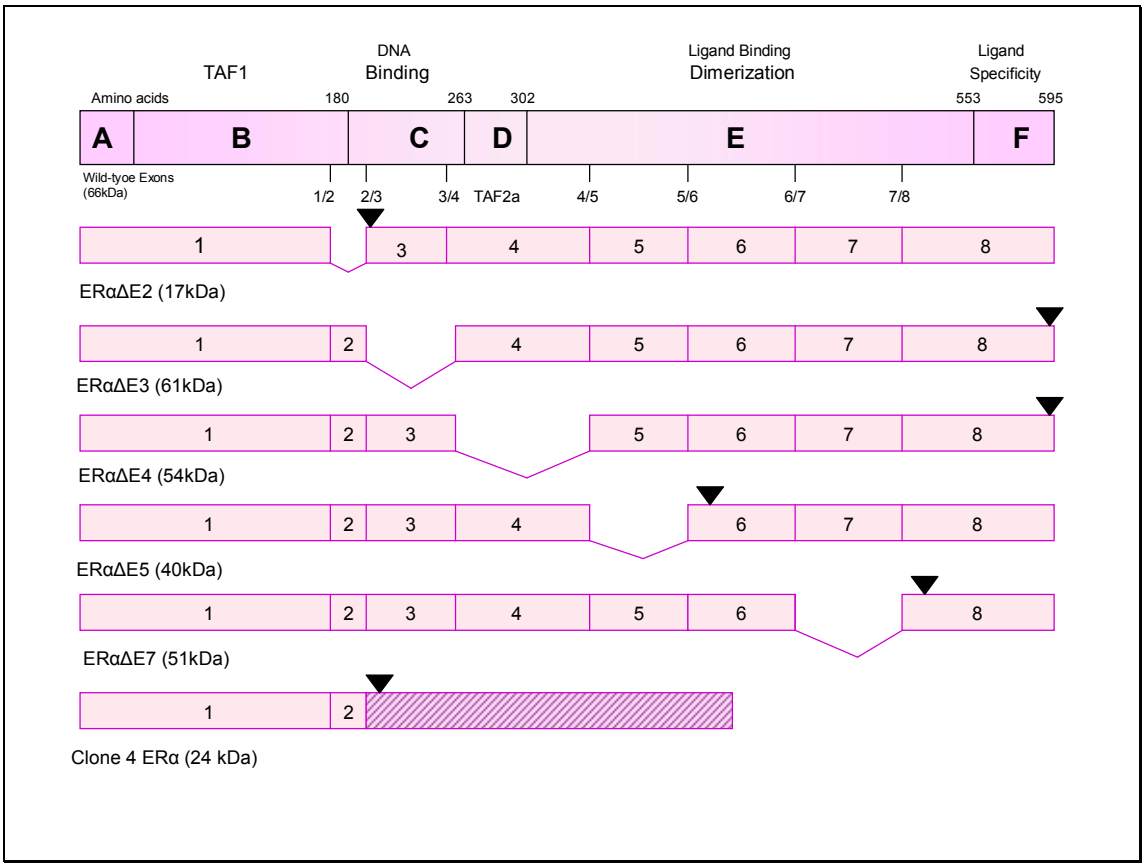

Figure 12. Schematic diagram of ERα exon deletions

(adapted from Ferguson and Davidson, 1997; Murphy *et al*, 1998).

A-F represent the structural and functional domains of the ERα protein. 1-8 represent the 8 different exons within the ERα cDNA. The arrowhead indicates the termination codon, and the estimated molecular mass (kDA= kiloDaltons) of each open reading frame is shown in brackets.

therefore has no basal or oestrogen-induced transcriptional activity and does not appear to interfere with wild-type ERα activity (Koehorst *et al*, 1994). The RNA expression of this variant has, however, been associated with low grade breast cancer and high PR levels which are biomarkers of a more favourable clinical outcome (Leygue *et al*, 1996a; Zhang *et al*, 1996). ERαΔ4 is also more common in PR-positive breast cancer (Leygue *et al*, 1996a; Zhang *et al*, 1996).

2.2.2.4

ERα exon 5 deletion (ERαΔ5)

The ERαΔ5 isoform was the first ER splice variant described and lacks exons 5 to 8, which includes the hormone binding domain. The truncated protein has a predicted molecular weight of 40kDa (Fuqua *et al*, 1991) (Figure 12). AF-1 activity and DNA binding ability is retained and therefore the encoded protein remains constitutively active (Castles *et al*, 1993). ERαΔ5 can form heterodimers with wild type ERα when coexpressed in the same cell. A number of studies have attempted to correlate expression of ERαΔ5 with tamoxifen resistance in clinical samples. Madsen *et al* (1997) found similar levels of ERαΔ5 mRNA in both tamoxifen-resistant and tamoxifen-sensitive MCF-7 cells. Daffada *et al* (1995) and Zhang *et al* (1996) supported these results *in vivo*. In contrast, Fuqua *et al* (1993; 1995) have shown that overexpression of ERαΔ5 in MCF-7 cells confers relative tamoxifen resistance. ERαΔ5 has also been found in normal breast tissue and is expressed at increased levels in breast cancer metastases (Leygue *et al*, 1996b; Zhang *et al*, 1996; Desai *et al*, 1997). No association has been shown between

ERαΔ5 levels and clinical prognostic indicators such as ER or PR status, tumour size, or SPF (Zhang *et al*, 1996).

2.2.2.5

ERα exon 6 deletion (ERαΔ6)

There have been few reports of an ERα exon 6 deletion variant (Poola *et al*, 2000) although duplications are more common in this region of the ERα gene (Murphy *et al*, 1996). This region encodes part of the hormone binding and dimerization domains and may play an important role in ERα signalling.

2.2.2.6

ERα exon 7 deletion (ERαΔ7)

ERαΔ7 is the most frequently observed variant in breast cancer regardless of the ER status of the tumour (McGuire *et al*, 1991; Zhang *et al*, 1996; Poola *et al*, 2000). This variant encodes a 51kDa truncated protein (Wang and Miksicek, 1991; Fuqua *et al*, 1992) (Figure 12) lacking the AF-2 domain and part of the ligand binding domain. ERαΔ7 has not been found to be constitutively active but has been demonstrated to act as a potent dominant-negative isoform for ERα and ERβ (Fuqua *et al*, 1992; Garcia Pedrero *et al*, 2003) and may contribute towards disease progression. This variant also does not appear to play a significant role in tamoxifen resistance (Zhang *et al*, 1996; Madsen *et al*, 1997).

2.2.2.7

Multiple ERα exon deletions

A number of multiple exon deletion variants have also been identified. ERαΔ4 and –Δ7, ERαΔ3-5, and ERαΔ5-7 were identified by Pfeffer *et al* in 1995. Double, triple and quadruple exon deletion mRNAs, ERαΔ2 and –Δ4; ERαΔ2 and –Δ5; ERαΔ2 and –Δ4-5; ERαΔ7 and -Δ4; ERαΔ7and -Δ3-4; ERαΔ7 and -Δ3-5, have been detected in normal and neoplastic tissues (Poola and Speirs, 2001). The double exon deletion variants, ERαΔ3 and –Δ7 and ERαΔ2-3, and the triple exon deletion variant ERαΔ2-3 and –Δ5 has been observed in breast tumour tissue (Poola and Speirs, 2001).

2.2.2.8

ERα insertions and exon duplications

Studies have shown a number of ERα variants with exon duplications of exon 6 (Murphy *et al*, 1996). Such duplication results in a truncation immediately after the duplicated exon and produces a 50 kDa protein that lacks the AF-2 and dimerization domains (Murphy *et al*, 1996). Duplication of exons 6 and 7 has been found to result in an 80 kDa protein that lacks the ability to bind ligands, such as oestrogen or tamoxifen (Pink *et* al, 1995; Pink *et al*, 1996b; Pink and Jordan, 1996). A 69 nucleotide insertion, resulting from a point mutation in the intron, between exons 5 and 6 has also been identified in three of 212 breast cancer tumours analyzed (Murphy *et al*, 1996).

2.2.2.9

Truncated ERα transcripts

These altered ERα-like transcripts are significantly smaller than the wild-type ERα mRNA. Dotzlaw *et al* (1992) identified truncated transcripts that are truncated from the 3' end compared to the wild-type ERα mRNA. These 3' truncated transcripts contain entire exon sequences of at least two of the 5' ERα exon sequences and then diverge into ERα unrelated sequences. Several different truncated ERα mRNAs have been cloned, some of which were found to be expressed in a single breast tumour eg. Clone 24 truncated ERα mRNA (Dotzlaw *et al*, 1992). One clone, clone 4 truncated ERα mRNA, has been found to be commonly expressed in human breast tumours. Clone 4 encodes a protein of 220 amino acids with a molecular mass of 23.9 kDa (Dotzlaw *et al*, 1992) (Figure 12). The functional significance of these variant ERs is not known.

2.3

Oestrogen Receptor β

Since the cloning of the ER in 1986 (Green *et al*, 1986; Greene *et al*, 1986), it was believed that only a single receptor was responsible for mediating the effects of oestrogens on target tissues. Recently, the cDNA of a second oestrogen receptor, oestrogen receptor β (ERβ), was cloned and sequenced from the rat (Kuiper *et al*, 1996), the human (Mosselman *et al*, 1996) and the mouse (Tremblay *et al*, 1997).

2.3.1

Molecular Structure of the ERβ Gene

Like ERα and other members of the steroid hormone receptor family, the human ERβ gene has been shown to be encoded by eight exons (Enmark *et al*, 1997) (Figure 13). The longest open reading frame of human ERβ encodes proteins of 530 amino acid residues with a molecular weight of 59.2 kDa (Ogawa *et al*, 1998a). This contrasts with the 66 kDa size of ERα (Green *et al*, 1986). The ERβ gene has been mapped to human chromosome 14q22-24 (Enmark *et al*, 1997). The ERβ cDNAs in humans, rats and mice have all shown significant sequence homology. ERβ is also highly homologous to ERα at the DNA (96%) and ligand binding (58%) domains, whereas the A/B domain, hinge region, and F region are not well conserved (Mosselman *et al*, 1996) (Figure 14).

ERβ has been shown to bind oestrogen specifically and with high affinity as well as to stimulate transcription of an ER target gene in an oestrogen-dependent manner (Kuiper *et al*, 1996; Kuiper *et al*, 1997). The classical (ERα) oestrogen response element (ERE) is composed of two inverted hexanucleotide repeats. The ligand-bound ER binds to the ERE as a homodimer.

ERα and ERβ both activate the transcription of target genes through similar EREs (Pace *et al*, 1997) and may form heterodimers with each other (Pace *et al*, 1997; Ogawa *et al*, 1998a). This heterodimer complex has been shown *in vitro* and *in vivo* irrespective of ligand binding. Differential activation of ERα and ERβ by the

anti-oestrogen 4-hydroxytamoxifen has been shown with ERE-regulated reporter genes (Watanabe *et al,* 1997) and because heterodimerization of ERα and ERβ has been demonstrated, putative cross-talk of the two signalling pathways has been suggested (Cowley *et al*, 1997). There may thus be three possible pathways of oestrogen signalling. In those tissues that exclusively express either ERα or ERβ, signalling would be via the specific receptor, whereas in those tissues expressing both subtypes, signalling would be mediated by ERα/ERβ heterodimers (Speirs *et al*, 1999). ERα and ERβ proteins may interact leading to differential responses to oestrogens or anti-oestrogens.

The ER also mediates gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation (Gaub *et al*, 1990; Umayahara *et al*, 1994). In transactivation experiments, the anti-oestrogen, tamoxifen inhibits the transcription of genes that are regulated by a classical ERα ERE, but like the natural oestrogen, 17β-oestradiol, tamoxifen activates the transcription of genes that are under the control of an AP1 element (Webb *et al*, 1995). ERα and ERβ respond differently to certain ligands at an AP1 element. At an ERα-AP1 site oestrogens act as transcription activators and anti-oestrogens inhibit the transcription of genes. ERβ-AP1 elements show the reverse ie. antioestrogens act as transcription activators and oestrogen as inhibitors (Paech *et al*, 1997). This suggests different regulatory functions for the two ER subtypes.

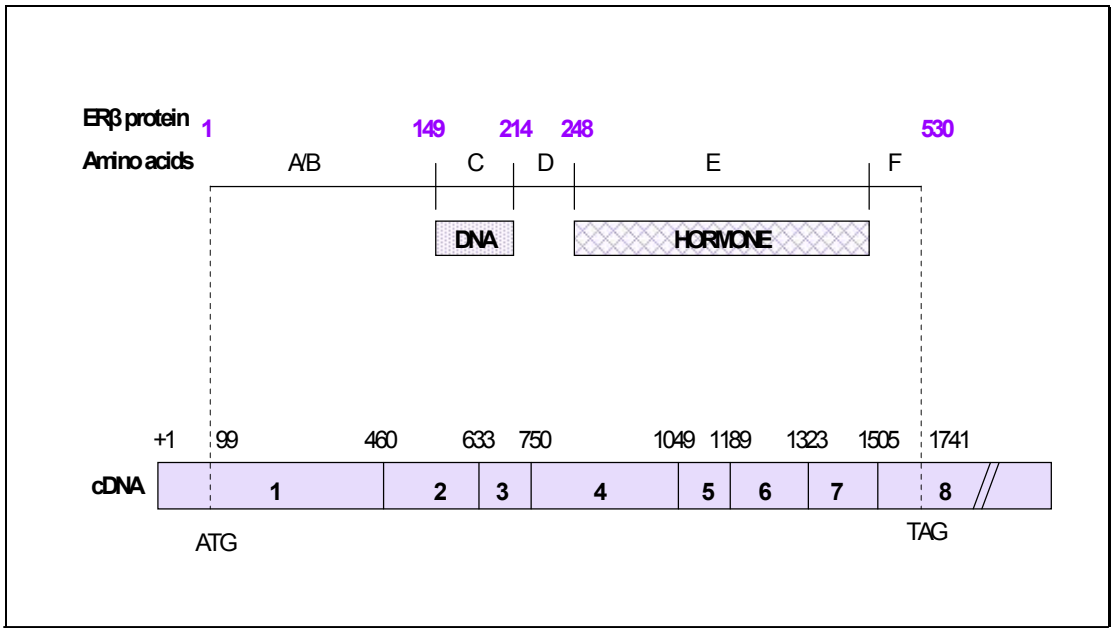

Figure 13. The Oestrogen Receptor β Gene

(adapted from Mosselman *et* al, 1996; Enmark *et al*, 1997; Ogawa *et al*, 1998a).

The ATG start codon and TAG stop codon are indicated below the ERβ cDNA (blocked region). Exons are numbered 1-8 with the nucleotide number above. The protein domains are labelled A-F with the relative positions of the DNA and Ligand-Binding domains indicated below the solid line. Amino acids are indicated by coloured numbers above the solid line.

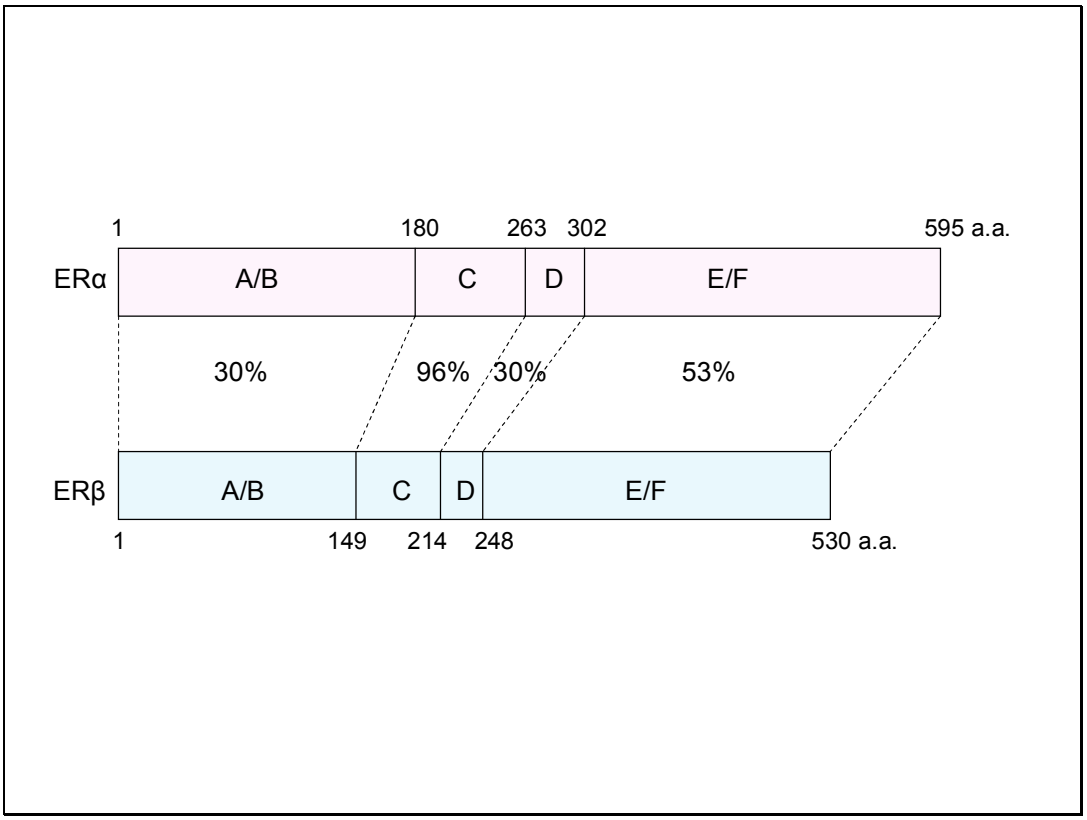

Figure 14. Comparison of the structures between ERα and ERβ

(from Ogawa *et al*, 1998a).

The functional A to E/F domains are schematically represented, with the number of amino acid (a.a.) residues indicated. Percentage of amino acid identity is depicted.

The demonstration of ERβ expression in both human breast tumours (Dotzlaw *et al*, 1997; Enmark *et al*, 1997; Vladusic *et al*, 1998) and normal breast tissue (Enmark *et al*, 1997; Lu *et al*, 1998) suggests that the well documented role of oestrogen in breast tumourigenesis (Henderson *et al*, 1982) may also involve both receptors. Using RT-PCR the ratio of ERα:ERβ has been shown to be significantly higher in breast tumours than it is in adjacent normal tissues. This ratio appears to alter during carcinogenesis, suggesting that ERα and ERβ specific pathways may have definitive roles in this process (Leygue *et al*, 1998). When ERs are coexpressed, it has been observed that ERβ exhibits an inhibitory action on ERα-mediated gene expression (Liu *et al*, 2002; Lindberg *et al*, 2003).

2.3.2

ERβ in clinical breast cancer

It has been estimated that only 7 to 10% of the epithelial cells in the normal human breast express ERα, and it has been shown that this expression fluctuates with the menstrual cycle (Petersen *et al*, 1987; Markopoulos *et al*, 1988; Ricketts *et al*, 1991). In contrast, ERβ expression is relatively high in the normal breast, with 80% of the cells expressing ERβ (Palmieri *et al*, 2002). ERβ expression also does not appear to change during the menstrual cycle (Critchley *et al*, 2002; Shaw *et al*, 2002). The potential role of ERβ in breast cancer progression is highly controversial with some studies suggesting that ERβ expression is a favourable prognostic indicator due to the inverse correlation with proliferation (Iwao *et al*, 2000a; Roger *et al*, 2001) on an RNA level, or its correlation with known biomarkers such as low histological grade, ERα and PR expression, longer

disease-free survival, and response to tamoxifen on a protein level (Jarvinen *et al*, 2000; Mann *et al*, 2001; Omoto *et al*, 2001; Omoto *et al*, 2002; Fuqua *et al*, 2003). Other studies suggest that ERβ expression is associated with known markers of poor clinical outcome such as EGF receptor expression and high tumour grade, and an inverse correlation between ERβ expression and PR status (Dotzlaw *et al*, 1999; Speirs *et al*, 1999; Iwao *et al*, 2000b). According to Speirs and coworkers (1999), most breast tumours express ERα, alone or in combination with ERβ. Those tumours that coexpressed ERα and ERβ were node positive and tended to be of higher grade. Dotzlaw *et al* (1999) found no significant correlation between ERβ mRNA levels and ER status (as determined by ligand- binding assays). They did, however, observe a significant difference in ERβ mRNA levels in those tumours that were ER positive and progesterone receptor (PR) positive (lowest expression) and those tumours that were ER negative and PR positive (higher expression) which could be interpreted to mean that both ER status and PR status could influence ERβ mRNA expression. It has also been suggested that the individual levels of ERα or ERβ are not as clinically relevant as the ratio of ERα:ERβ that may change during tumorigenesis (Lazennec *et al*, 2001; Sotoca *et al*, 2008).

2.3.3

ERβ Variants

ERβ splice variants have been described in some breast tumours although significantly fewer than those described for ERα. Ten exon deletion variants are schematically represented in Figure 15.

2.3.3.1

ERβ2 or ERβcx

Ogawa *et al* (1998b) identified and characterized a novel human ERβ isoform, ERbetacx, which is truncated at the C-terminal region but has an extra 26 amino acids due to alternative splicing. ERβcx mRNA is missing the wild-type exon 8 sequence and contains extra sequences termed exon 9, which is located downstream of exon 8 on chromosome 14 (Peng *et al*, 2003). It has no ligand binding ability and exhibits reduced DNA binding ability. This study also indicated that this isoform potentially inhibits ERα-mediated oestrogen action. Omoto *et al* (2002), in a study analyzing ERβcx in normal and breast cancer specimens, revealed that 54% of breast cancers express ERβcx variant, whereas only 9% of normal breast express ERβcx. ERβcx expression in ERα positive PR negative tumours has been found to exhibit a poor response to tamoxifen (Saji *et al*, 2002a), whereas tumours lacking ERβcx expression respond well to tamoxifen (Saji *et al*, 2002b).

2.3.3.2

ERβ exon 2 deletion (ERβΔ2)

The most common ERβ splice variant has been found to be the deletion of exon 2 (Poola *et al*, 2002a). It is frequently associated with deletion of exons 5 or 6 (Figure 15). The deletion of exon 2 causes a frame-shift mutation resulting in a premature termination codon leading to a carboxy-truncated protein (Poola *et al*, 2002a). No significant differences in expression have been observed between breast cancer or matched normal breast from a distant site (Poola *et al*, 2002b) suggesting that ERβΔ2 may not play a significant role in breast tumourigenesis.

2.3.3.3

ERβ exon 3 deletion (ERβΔ3)

ERβΔ3 does not result in a disruption of the ERβ mRNA open reading frame but produces a protein with internal deletions that are included in the carboxyl-terminal half of the DNA binding domain, including the second zinc finger (Poola *et al*, 2002a). This variant was initially identified in normal ovarian tissue (Poola *et al*, 2002a) and although it has not been identified in breast cancer, it may play a role in differential interactions with other transcription factors.

2.3.3.4

ERβ exon 4 deletion (ERβΔ4)

The loss of only ERβ exon 4 does not result in a disruption of the open reading frame of ERβ mRNA but the translated protein lacks the nuclear localization signal and remains in the cytoplasm (Poola *et al*, 2002a). ERβΔ4 has been identified in breast cancer but no significant differences have been observed between normal breast and breast cancer (Poola *et al*, 2002b).

2.3.3.5

ERβ exon 5 deletion (ERβΔ5)

Vladusic and coworkers (1998) detected a deletion variant of ERβ corresponding precisely to the deletion of the entire exon 5 of ERα (Figure 15). This variant was coexpressed with wild-type ERβ in the ER-negative, oestrogen independent breast cancer cell line MDA-MB-231 and in malignant breast tumour specimens. Sequence analysis revealed the absence of 139 bp within the hormone-binding domain (exon 8). After amino acid 468 ERβΔ5 is predicted to encode 5 novel amino acids with the resulting protein having a predicted molecular mass of 53 kDa (Peng *et al*, 2003). The ERβΔ5 protein is localized to the nucleus and does not have any differential effects on basal activation of ERβ (Inoue *et al*, 2000). ERβΔ5 acts as a dominant negative receptor for oestrogen-induced transactivation of both ERα and ERβ in a dose dependent manner (Inoue *et al*, 2000; Peng *et al*, 2003).

Expression of ERβΔ5 could impair the normal functions of both ERs and thus contribute to altered oestrogen binding and, consequently, the lack of anti-oestrogenic response.

2.3.3.6

ERβ exon 6 deletion (ERβΔ6)

Vladusic *et al* (1998) also detected precise deletions of ERβ exon 6 and exons 5+6 (Figure 15). These variants would all be expected to encode proteins which are unlikely to bind ligand as they result in a truncated translation product (Poola *et al*, 2002a). Unlike ERα mRNA, ERβΔ6 appears to be one of the most frequently deleted exons of ERβ mRNA, and although it is found in slightly lower levels in breast cancer compared to normal breast, the differences have not been shown to be statistically significant (Poola *et al*, 2002b). ERβΔ6 may not play a significant role in breast tumourigenesis.

2.3.3.7

ERβ exon 7 deletion (ERβΔ7)

Deletion of exon 7 is rare in ERβ whereas deletion of exon 6 is rare in ERα (Poola *et al*, 2002a). This suggests that both exon 6 in ERα and exon 7 in ERβ may play important roles in ER function (Herynk and Fuqua, 2004).

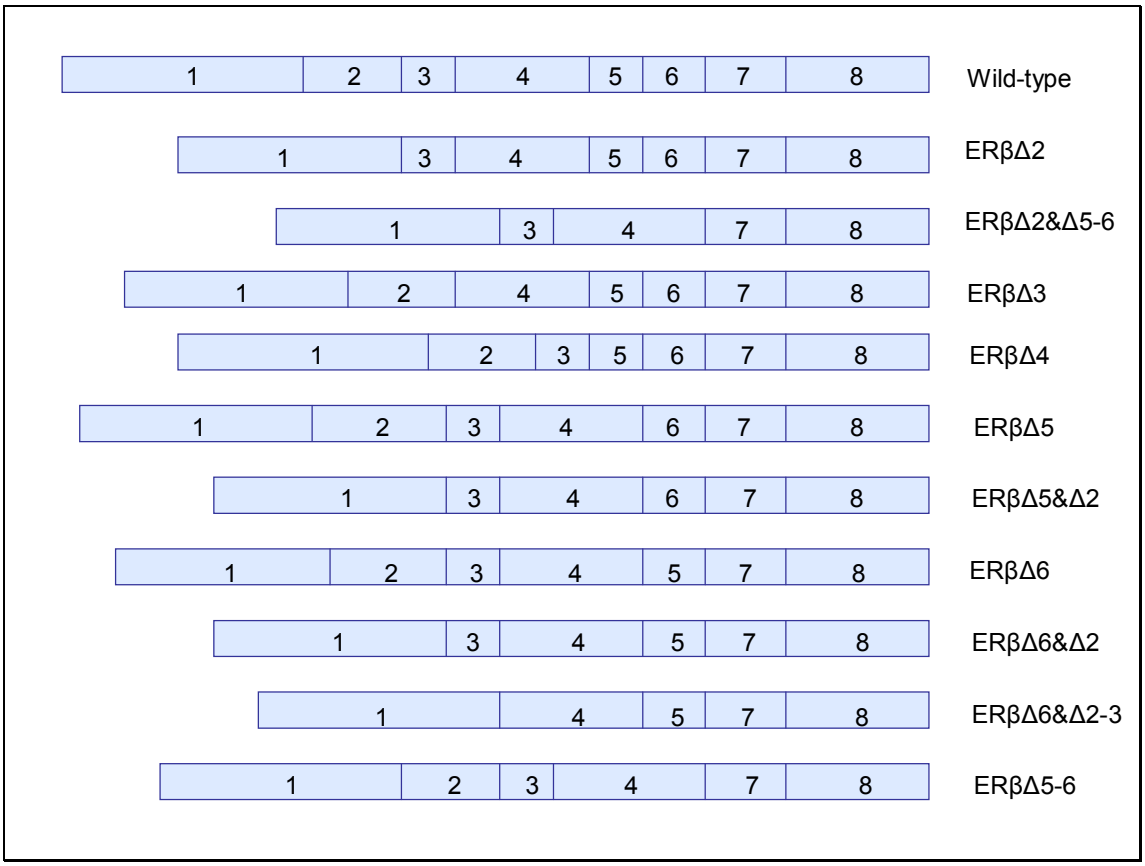

Figure 15. Schematic diagram of 10 human ERβ exon deletion variants

(adapted from Poola *et al*, 2002a).

ERβ mRNA exons are labelled 1-8.

2.4

Tissue Culture as a model of Tumour Progression and Oestrogen Action

2.4.1

Mechanisms of Tumour Progression

The alterations in cellular behaviour and morphology that accompany tumour progression have been well described (Foulds, 1975; Nicolson and Milas, 1984). Biochemical, cytogenetic, molecular genetic and immunological evidence indicates that most neoplasms arise from a single altered cell, with the progeny of that cell expanding as a neoplastic clone (Nowell, 1976; Fialkow, 1979; Arnold *et al*, 1983). It has also been hypothesized that clonal evolution might result from enhanced genetic instability within the tumour cell population. This would increase the probability of further genetic alterations and their subsequent selection (Cairns, 1975; Nowell, 1976; Klein, 1979; Sager, 1985). Most variants that arise in the tumour cell population do not survive but those that have an additional selective growth advantage become predominant subpopulations within the neoplasm and demonstrate characteristics of tumour progression. There is evidence supporting the clonal evolution concept from chromosome studies where advanced cancers show more extensive chromosomal aberrations than do early stages of neoplasia (Rowley, 1980; Nowell, 1982). It has also been shown that most neoplastic cells are more genetically unstable than comparable normal cells. Both *in vivo* and *in vitro* neoplastic cells may be more susceptible to chromosomal breakage, non-

disjunction and ploidy changes, sister chromatid exchange, and other genetic alterations such as translocations, deletions and inversions (Parshad *et al*, 1979; German, 1983; Ling *et al*, 1985; Sager, 1985). This genetic instability is a continuous process that lends to expansion and contraction of selected cell populations and a relentless remodelling of the tumour (Graham *et al*, 1990).

2.4.2

Tissue Culture as a Technique: a Brief History

Tissue culture was first devised at the beginning of the twentieth century (Harrison, 1907; Carrel, 1912) as a method for studying the behaviour of animal cells in a controlled environment. Initially the technique involved the maintenance of undisaggregated fragments of tissue and growth was restricted to the migration of cells from the tissue fragment, with occasional mitoses in the outgrowth. Embryonated hen's egg was used as a source of diverse cell types until techniques for the long term propagation of continuously growing rodent cell lines were established (Earle *et al*, 1943). The demonstration that human tumours could give rise to continuous cell lines (eg. HeLa: Gey *et al*, 1952) encouraged interest in human tissue. Since then many continuously growing cell lines of human origin have been described.

2.4.3

Human Breast Tumour Cell Lines

Cell lines established from human tumours provide an unlimited, self-replicating source of malignant cells free of contaminating stromal cells. As early as 1937 attempts to culture breast cells were reported (Cameron and Chambers, 1937), but it was not until 1958 that Lasfargues and Ozzello reported the first successful long-term culture of a breast tumour (BT-20). Since the establishment of the BT-20 cell line in 1958, 15 additional putative breast cancer cell lines from primary tumours have been established and a greater number of cell lines have been derived from solid tumours and pleural or ascitic effusions of patients with metastatic disease (Engel and Young, 1978). Breast cancer cell lines, such as MCF-7, ZR-75-1, MDA-MB231 and T-47D, are ideal model systems for studying the mechanisms of steroid action in tumours.

2.4.3.1

MCF-7 Human Breast Cancer Cell Line

The MCF-7 cell line was derived from a malignant pleural effusion taken from a 69-year old female patient with metastatic breast cancer (Soule *et al*, 1973). Soule *et al* (1973) established this cell line as the first hormone-responsive breast cancer cell line and cytogenetic studies indicated a distinct stem line of 88 chromosomes. The oestrogen receptor was first described in MCF-7 cells by Brooks *et al* (1973) by both Scatchard and sucrose density gradient analysis. Lippman and Bolan (1975) demonstrated that the anti-oestrogen tamoxifen inhibited the growth of

MCF-7 cells, but that this could be reversed by the presence of oestrogen. Receptors for glucocorticoids, progesterones and androgens were also identified in MCF-7 cells (Horwitz *et al*, 1975b). Some groups, however, were unable to show stimulatory effects of oestrogen on MCF-7 cells (Page *et al*, 1983; Soto and Sonnenschein, 1985). One factor causing this may have been the presence of growth factors within the foetal calf serum (FCS) (Page *et al*, 1983). Phenol red, the indicator used routinely in commercial cell culture media, also has a structure reminiscent of non-steroidal oestrogens first synthesized by Dodds (Dodds and Lawson, 1936) and the removal of this compound from the media was found to result in increased oestrogen sensitivity in MCF-7 cells (Berthois *et al*, 1986). Oestrogen receptor studies now utilize charcoal stripped FCS (Aakvaag *et al*, 1990) in a phenol-red free culture media.

Extensive research on MCF-7 cells has led to the development of monoclonal antibodies to the human ER (Greene *et al*, 1980) which are now used routinely for detection of ER in clinical cancer samples in order to predict the likely outcome of Tamoxifen therapy (Jordan *et al*, 1986; Mudduwa and Liyanage, 2009). This development also facilitated the cloning and sequencing of the ER (Walter *et al*, 1985; Green *et al*, 1986; Greene *et al*, 1986). Lippman *et al* (1987) utilized the MCF-7 cell line as a model for growth regulation of human breast carcinoma through growth factor secretion.

MCF-7 cells have the ability to exhibit anti-oestrogen resistance and tamoxifen has been observed to stimulate growth in this cell line (Wolf *et* al, 1993; Osborne *et al*, 1994; Wolf and Jordan, 1994). Over the years the use of the MCF-7 cell line has

led to the evolution of distinct MCF-7 lineages (Nawata *et al*, 1981; Westley *et al*, 1984; Bronzert *et al*, 1985). Documented differences include differential sensitivities to oestrogens and anti-oestrogens, differential expression of ER, PR and differences in tumorigenicity and proliferation rates (Osborne *et al*, 1987; Perry *et al*, 1995). These differences may be explained by the fact that MCF-7 is a polyclonal cell line consisting of heterogeneous cancer cells that are phenotypically and cytogenetically different (Resnicoff *et al*, 1987; Nugoli *et al*, 2003). Differential expression of ER mRNA was observed by Klotz *et al* (1995) and the ability to undergo DNA fragmentation by Gooch and Yee (1999).

MCF-7 human breast cancer cells continue to be used as a model for studying oestrogen-responsive breast cancer (Lacroix and Leclercq, 2004) although investigators should carefully document the source and identity of MCF-7 cells used in published experiments as it has been demonstrated by Osborne *et al* (1987) and Jones and co-workers (2000) that MCF-7 lines from different laboratories may have unique biological properties, despite having similar karyotype. MCF-7 cells from ATCC, in particular, exhibit entirely different properties to other MCF-7 sources.

2.4.3.2

T-47D Human Breast Cancer Cell Line

The T-47D line was originally isolated from a pleural effusion obtained from a 54-year old female patient with an infiltrating duct carcinoma of the breast (Keydar *et al*, 1979). It was found to have a modal chromosome number of 66 and contained

both ER and PR. The sensitivity of T-47D cells to the anti-oestrogen tamoxifen is similar to that of the other ER positive breast cancer cell lines (Reddel *et al*, 1984; Reddel *et al*, 1985). However, T-47D cells stand as a model of genetic instability among hormonally responsive cell lines as sublines have been found to be ER positive and oestrogen responsive (Chalbos *et al*, 1982), as ER positive and oestrogen resistant (Horwitz *et al*, 1982), and as ER negative (Chalbos *et al*, 1982). In addition to changes in steroid receptor levels and hormone sensitivity, the T-47D cell line has been shown to be genetically unstable as measured by changing DNA ploidy (Reddel *et al*, 1988; Graham *et al*, 1989; Fernandez *et al*, 1998) where spontaneous DNA duplication was observed to lead to conversion from the hyperdiploid state to a hypertetraploid state. Based on these observations it may be postulated that T-47D cells are in transition from the oestrogen-responsive, ER-positive state to the oestrogen-resistant, ER-negative state and may thus serve as a model for this process. T-47D breast cancer cells express high levels of PR and have therefore also become the major model to study the actions of progesterone and synthetic progestins in human cells (Sartorius *et* al, 1994b).

The regulation of ERα mRNA in T-47D cells has been reported to be different than that observed in MCF-7 cells. Oestrogen exposure in T-47D cells was observed to give rise to an increase in ERα mRNA whereas the effect of oestrogen on ERα mRNA levels in MCF-7 cells was dependent on the prior growth history of the cells (Read *et al*, 1989; Fernandez *et al*, 1998). Oestrogen withdrawal, on the other hand, causes a decrease in ERα expression in T47D cells (Pink *et al*, 1996a) in contrast to MCF-7 cells which show an increase in ERα expression following

oestrogen deprivation. The effects of oestrogen –like compounds on cell proliferation seem to be dependent on ERα:ERβ expression levels in T47D cells (Sotoca *et al*, 2008). T-47D cells may therefore be suitable for the study of oestrogens and anti-oestrogens and their effects on ERα and ERβ mRNA.