The Role of the Steroid Nuclear Receptor Genes in Breast Cancer

By

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Abstract

Breast cancer is a great source of morbidity and mortality in the developed world, being the most common cause of cancer death in Australian women and affecting roughly 1 in 10 women. The development and progression of cancers is a multi-stage process, involving numerous perturbations to normal cellular functions, especially to those genes which control cellular growth, cellular differentiation and DNA repair. Over time, these alterations combine to change normal cells into cancerous ones that typically no longer respond to normal control stimuli and grow with great rapidity.

The nuclear receptors are a family of proteins which accept incoming signals from various molecules, and then alter gene expression or affect cell behavior directly. The steroid nuclear receptors receive transducer signals from hormones such as estrogen and testosterone and are intimately involved in affecting cellular growth and differentiation. Stimulation of the steroid receptors have been used as successful treatment avenues, so how these genes behave in cancer is of great interest. Accordingly, this study has examined the expression of various nuclear receptors and some related genes in a number of tissue samples derived from breast tumours and from surrounding areas, and examined how various pathological parameters affect their expression. Tissue samples were first microdissected to separate tumour tissue from the surrounding stroma and then subjected to RNA extraction procedures to allow gene expression to be quantitated. RNA was then converted into cDNA by reverse transcription and the genes of interest amplified and quantitated using semi-quantitative polymerase chain reaction. Expression data was then normalized by

expressing it as a ratio of the gene of interest to the 18S ribosomal gene and differences in expression analysed by analysis of variance (ANOVA) testing.

The first portion of the study dealt with the progesterone and glucocorticoid receptors in tumour tissue. Progesterone is an anti-mitogenic signal for breast tissue and the stimulation of the receptor is a useful part of combined hormonal therapy for breast cancer. The glucocorticoid receptor plays a similar role to the progesterone receptor, slowing breast tissue growth and promoting apoptosis. The complete tissue population of 25 samples from control, grade 1, grade 2 and grade 3 tumours underwent PCR for the glucocorticoid and progesterone receptor genes and was normalized using 18S. Analysis of the expression data by ANOVA showed that the progesterone and glucocorticoid receptors were more highly expressed in grade 3 tumour tissue ($p= 0.023$ and $p=0.00033$, for the progesterone and glucocorticoid receptors, respectively). Most advanced tumours cease being responsive to growth repressing hormones, so these results indicated that the control of the expression of these receptors in tumour tissue was more complicated than might have been expected. The increase in expression observed may be the result of intact growth preventing feedback mechanisms which have malfunctioned in some manner. There are several mechanisms the tumours may be using to escape an increase in progesterone or glucocorticoid sensitivity. The mRNA detected may simply not be being translated into completed proteins or be being spliced into isoforms of the receptors that favour tissue growth.

The second portion of the study dealt with the estrogen receptors alpha and beta in tumour tissue. The estrogen receptors control estrogen signaling and are highly

important in breast cancer, since estrogen is the primary growth inducing hormone for breast tissue. For the estrogen receptors alpha and beta, the complete tissue population of 25 samples from control, grade 1, grade 2 and grade 3 tumours underwent PCR and were normalized using 18S. ANOVA analysis found that the expression of the estrogen receptors did not change significantly in any grade of cancer ($p= 0.057$ and $p=0.738$, for ESRα and ESRβ, respectively) nor in tissues that are negative for the estrogen receptor alpha protein, a poor prognostic factor in breast cancer ($p= 0.794$ and $p=0.716$, for ESRα and ESRβ, respectively). These results indicated that the loss of estrogen receptor in advanced cancers is not controlled at the level of mRNA. The mRNA observed in this study may be being spliced into alternate and possibly inactive isoforms, or may be being degraded post transcriptionally, preventing estrogen stimulation in these tumours. This could prove an excellent area for further study, since if the mechanism that prevents or distorts ESR mRNA translation can be discovered, it would allow manipulation of one of the most important treatment avenues for breast cancer.

The third portion of the study dealt with the expression of the androgen receptor in tumour tissue. Androgens are a strong anti-growth signal in breast tissue and despite the side effects that androgens have on women, they have been used to treat breast cancer with some success. The complete tissue population of 25 samples from control, grade 1, grade 2 and grade 3 tumours underwent PCR for the androgen receptor gene and normalized using 18S. The results obtained for androgen receptor expression in tumour tissue showed that androgen receptor expression was significantly elevated in grade 2 and grade 3 tumour tissue, as well as in ESRα negative tumours ($p= 0.014$ and $p=0.025$, respectively). An increase in expression in late stage tumours would

seem to be unusual for an anti-mitogenic receptor, however many advanced breast tumours have been found to be receptive to androgen stimulation, even when they no longer respond to other hormones. The increased expression of AR may be a normal response to cellular over-growth, or it may be a mechanism by which the tumour prevents stimulation by other growth retarding hormones, by sequestering all available receptor co-enzymes with a receptor that is unlikely to be stimulated.

The fourth portion of the study examined the expression of the estrogen alpha, estrogen beta, progesterone, glucocorticoid and androgen nuclear receptors in stromal tissue derived from the tumours studied in previous chapters. Tumours have been observed in other studies to manipulate the activities of the cells that surround them through the release of cofactors and *vice versa*. These cofactors include the steroid hormones, among others, and hence the study of how the tumour and stroma interacts is a valuable extension to the results obtained in the previous sections. PCR was performed for all nuclear receptors, except for the estrogen receptor alpha, in the complete tissue population of 25 samples of tissue derived from the stroma of the grade 1, grade 2 and grade 3 tumours used in the previous studies as well as the control tissues. Due to difficulties in PCR optimization for estrogen receptor alpha, only three stromal samples from each grade and four controls were able to produce results, for a total population of 13 samples. Of all the receptors tested, only the progesterone and glucocorticoid receptors displayed significant changes in expression in stromal tissue, with PgR having significantly lower expression in all stromal samples compared to control, while GR was more highly expressed in stroma derived from high grade tumours ($p= 5.908x10^{-7}$ and $p=2.761x10^{-5}$, for PgR and GR, respectively). GR expression was also increased in stroma derived from ESRα

negative tumours ($p=5.85x10^{-5}$). These alterations reflect the kind of stimulation a tumour is likely to apply to the surrounding stroma, using progesterone to stimulate the cells into differentiating to provide a more suitable environment, hence the loss in PgR expression. The increase in GR expression may be the result of the high level of growth stimulating factors that tumours produce, priming the local cells to be more sensitive to growth suppressors, a situation that is also mirrored in results previously obtained for the tumour tissue.

The fifth part of the study concerned the expression of the nuclear receptor co-activator 1 and nuclear receptor co-activator 3 genes. These proteins are required for the activation and function of the nuclear receptors and both have been implicated in cancer development, being found to be over-expressed in several tissues and cell lines. As integral parts of the nuclear receptor pathway, their level of expression is important for determining how effective any nuclear receptors present will be when stimulated. The complete tissue population of 25 samples from control, grade 1, grade 2 and grade 3 tumours underwent PCR for both nuclear receptor co-activator genes and normalized using 18S. The result of ANOVA analysis on the NCoA data showed that NCoA3 expression remained unaltered in all grades of cancer and stroma and in both ESRα positive and negative tissue. NCoA1 however, was significantly up-regulated in grade 3 tumours as compared to grade 1 tumours and also in ESRα negative tumours. This increase in expression would seem to indicate that these tissues would be more capable of acting on any received hormonal stimulation. That this increase in expression occurs in more advanced cancers could be evidence that the nuclear receptor expression observed in prior sections is resulting in NR splice

variants that favour, rather than repress, growth, as advanced cancers usually do not respond normally to hormonal stimulation.

The final part of the study investigated the possibility of correlations between the expression of the nuclear receptors, between the nuclear receptor co-activators and between all of the tested genes and other pathological parameters, including tumour size, metastasis, site of tumour, carcinoma *in situ* invasiveness, age of patient and the presence of calcification. The data generated in the prior studies was analyzed using ANOVA for categorical data and correlation analysis for numerical data. The ANOVA and correlation analysis revealed a number of interactions between these factors, which provide additional information on the relationships between the tested genes. Expression of the progesterone receptor was found to be correlated with the expression of GR, AR and NCoA1 ($p= 0.022$, $p=0.003$, and $p=0.019$, respectively). Likewise the expression of GR and AR were found to be correlated ($p=0.029$). Additionally, AR was found to be associated with tumour size ($p=0.036$) while GR was found to be associated with both tumour size and metastasis ($p= 0.006$ and $p=7.6x10^{-6}$, respectively). ESRα and ESRβ expression were found to be negatively correlated ($p=0.044$), as were patient age and the amount of ductal carcinoma *in situ* invasion. Given the results of previous analyses, it is not surprising that PgR, GR, AR and NCoA1 expression are related, and the negative correlations between ESRα and ESRβ expression, as well as between age and ductal carcinoma *in situ* invasion have been documented in other studies. Hence, these results provide reinforcement for previous observations, as well as providing new information, particularly on AR and GR.

Most interesting is the correlation between AR and tumour size and GR for both metastasis and tumour size. In the case of AR, the increased expression in large tumours may be a reflection of the ability of AR to sequester nuclear receptor chaperones. Larger tumours which have more exposure to other anti-growth hormones may have the same requirements as advanced cancers to escape stimulation by those hormones. The association between GR and tumour size is possibly a reflection of the fact that larger tumours are often more advanced in their development. However, the additional association of GR to metastasis would seem to indicate that the high expression of GR is a requirement, or a result of, the processes that allow tumours to disrupt the extracellular matrix and both grow large and escape their initial site of development. It is this relationship that may partially explain why GR is highly expressed in both tumours and their surrounding stroma, while PgR remains highly expressed only in tumours.

Overall, this study has examined the expression of steroid nuclear receptor genes in a multiple tumours and their associated stroma and has uncovered several trends in expression based, on certain pathological characteristics. Most universal is the trend for the anti-mitogenic nuclear receptors to be highly expressed in advanced tumours. However, the relationships of AR and GR to tumour size and metastasis that were detected indicate that the outcome of this increased level of expression is highly specific within the complex nature of the nuclear receptor pathways. Additional study to identify the nature of these outcomes would greatly improve the knowledge of breast cancer physiology.

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Statement of Originality

The material presented in this report has not previously been submitted for a degree or diploma in any university, and to the best of my knowledge contains no material previously published or written by another person, except where due acknowledgement is made in the Thesis itself.

Robert Smith

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CHAPTER 1. Introduction.

1.1. Significance.

Breast cancer is one of the major health problems facing the world today, and is a massive cause of morbidity and mortality. Breast cancer is a problem principally in the first world, where infectious diseases are not so great a factor in rates of illness and death, but also in other countries, where screening facilities are fewer and treatment comparatively expensive. In many national populations, breast cancer is the most common cancer specific to women and the second most common cancer in women overall, following just behind lung cancer in frequency.

The world wide incidence of cancer is relatively low compared to infectious diseases, but roughly ten million new cancer cases were reported world wide in 2000 with 6.2 million deaths also reported. This does however represent a huge toll of morbidity and mortality, and is a significant drain on the health systems of many nations, costing Australia 2.76 billion dollars in 2000-2001, including just over 200 million dollars spent on cancer research. In addition to the purely monetary cost, cancer, and breast cancer specifically, has profound social consequences, removing valuable individuals from the workforce,

straining the fabric of families and causing emotional pain to sufferers substantially greater than most other forms of cancer.

As might be deduced from its status as the second most common form of cancer in women, the risk of developing breast cancer is relatively high, with a lifetime risk of 1 in 10 in the United States and a comparable rate of risk in most other developed nations. This level of risk is a result of the blending of a number of risk factors for the disease, which include both genetic and environmental factors. There are relatively few genes which have a serious influence on the risk of breast cancer, the most famous of these being BRCA1 and BRCA2. However, many genes appear to have small effects on the risk of breast cancer, being either protective or additive, depending on the nature of the genes and the alleles involved. There are also many known environmental risk factors for breast cancer, including age, obesity, age at menarche, age at menopause, diet, smoking and others. In developed nations, many of these environmental risk factors show increased rates compared to developing countries and this may contribute to the greater overall risk in wealthier nations.

There are numerous treatment regimes available for breast cancer, but the three oldest methods, surgery, chemotherapy (using cytotoxic drugs) and radiotherapy, are still in use and are still very effective. However, the problem with cancer treatment has always been greater because the cancer cells are part of the patient's own body and typically share the same metabolism, meaning that whatever effect is applied to the tumour must also be applied to any healthy tissues. Technology has allowed improvements in cancer targeting

for surgery, chemotherapy and radiotherapy, making them more cancer specific, however there are several other avenues of treatment available and in development that can be used to target tumours more directly. These treatments are often actually aimed at the still properly functioning systems of the body, such as anti-angiogenic treatments or the highly promising field of cancer vaccines, but many others operate by manipulating or disrupting still functioning growth control mechanisms in the cancer itself. In breast cancer, hormonal growth systems, specifically the estrogen pathway, are the most commonly targeted for treatment purposes.

Breast tissue is under constant hormonal manipulation, in preparation for its role in the nutrition of offspring, and is in a constant cycle of growth and proliferation depending on what stage the menstrual cycle is currently at. Estrogen is the primary growth inducing hormone in the breast and it continues to induce cellular proliferation in most breast cancers. Indeed, exposure to estrogens (either naturally occurring or taken in through environmental exposure) is recognized as a major risk factor for breast cancer development, forming part of the risk for age, age at menarche and age at menopause. Progesterone is the second major player in the breast, being the hormone which controls the normal cellular differentiation of breast tissue in preparation for milk production, antagonizing the effects of estrogen. Together these two hormones form the basis of the most commonly used hormonal treatment for breast cancer, which involves the administration of tamoxifen, which blocks the action of estrogen, as well as doses of progesterone to encourage tumours to cease growth and undergo differentiation. Of course, these are not the only hormones which affect breast tissue, with glucocorticoids,

androgens, vitamin D and others all making their contributions to rates of breast tissue and hence, breast cancer, growth. Yet, by some poorly understood mechanism, many tumours loose their sensitivity to hormonal manipulation, maintaining their growth even in the absence or blocking of growth inducers, requiring the use of more generalized treatments to control them.

With the significance of hormones in the treatment of breast cancer, and the as yet poorly understood mechanisms by which tumours begin to evade hormonal control, it is important that a more complete understanding of the action of hormones in breast cancer is obtained. Many of the functions of hormones in a cell are mediated by the nuclear receptors, a large family of proteins which accept the incoming hormone signals and induce changes in cellular metabolism and gene expression in response. Thus the genes which produce these proteins are an important area of study if a greater understanding of how breast cancers function and how they might be better treated is to be obtained. Many previous studies have examined the expression of hormone receptors, especially the primary estrogen receptor, but to date most of these studies have focused on the presence of the receptor itself and few have looked at how the levels of mRNA for the receptors is affected within human tumours as opposed to cell lines and animal models. Thus, the studies outlined in this thesis examined an area of breast cancer research which is still not well described and may prove to be highly useful in the treatment of both new and advanced breast tumours alike.

1.2. Research Aims.

The research that forms the substance of this thesis had several aims, however the most basic aim of the research was to examine the levels of mRNA expression for several influential members of the nuclear receptor superfamily, including the two receptors for estrogen, the progesterone, glucocorticoid and androgen receptor genes, as well as genes which assist and modulate the function of these receptors. Expression was to be determined both within tumours and normal breast tissue, and to search for any relationship between levels of expression of the genes and cancer grade. Expression would also be compared to other useful prognostic factors such as patient age, or tumour size. Knowledge of how expression is affected by, or affects, these factors might shed light on the mechanisms which govern cancer growth and sensitivity to hormones.

The expression of the nuclear receptors was also to be tested in stromal tissue surrounding the tumours. Stromal expression was studied to examine the role of the stroma in the expression of tumour nuclear receptors, and determine what level, if any, of cellular cross-talk was underway between tumours and their supporting tissues.

Additionally, the research aimed to determine if any of the tested nuclear receptors had detectable effects upon the expression of any other genes tested. The expression of the nuclear receptors were also to be compared to polymorphisms of the nuclear receptor genes, either their own polymorphisms or those of other nuclear receptors, to determine if

polymorphisms previously implicated in breast cancer had any relationship to nuclear receptor expression.

1.3. Research Hypothesis.

Since there have been a number of previous studies on the expression of nuclear receptors in breast cancer with their primary focus on the receptor proteins themselves, there was a relatively large amount of pre-existing data to form a basic hypothesis covering the expected levels of mRNA expression for the nuclear receptor genes tested.

For the primary estrogen receptor, estrogen receptor alpha (ESRα), most studies have shown that the expression of the receptor declines as tumours progress, affected by increased stimulation of the pathway and indeed may disappear altogether as tumours achieve highly advanced states. In addition to this, ESRα protein staining is a common practice in the diagnosis and pathology of breast cancers and this information was available at the beginning of the study. This data showed that many of the more advanced tumours no longer had any detectable expression of the receptor. Thus, it was anticipated that the expression of estrogen receptor alpha would decline as tumours advanced in grade. In stromal tissues, studies have shown that localized production of estrogen in the tumour area stimulates the estrogen receptor pathway in the stroma as well as the tumour, leading to drops in ESRα expression in stroma as well as tumour. On the basis of this,

estrogen receptor expression in stroma was also expected to decline in tumours, though possibly to a less severe extent.

For the secondary estrogen receptor, estrogen receptor beta (ESRβ) the picture in the literature was more complex than for the alpha receptor and not all studies were in agreement. The relative expression of estrogen receptor beta has been known to decrease in malignant cells, but also to increase as a particular isoform in later stage cancers as a means of anti-estrogen resistance, though this has been found to be uncommon in tumours not exposed to tamoxifen for extended periods. With this in mind, estrogen receptor beta was also expected to decline in advancing tumours, with the possibility that the trend might reverse iṇ any ESRα positive late stage tumours as these may have been tamoxifen treated. For stromal tissues ESRβ expression has not been determined by any prior studies to be significantly altered, so it was considered that similar results would be obtained for this study, save that the localized estrogen production within either tumour or stroma might result in stimulation of the pathway and a corresponding loss of ESRβ expression in stromal cells.

Prior studies on the androgen receptor (AR) have indicated that while the receptor is expressed in the breast, its level of expression is relatively unaffected by the development of breast cancer, despite its anti-growth and differentiation inducing effects, perhaps largely because of the relatively low level of androgen stimulation in the female body. However, previous studies have also noted that androgen receptor expression persists when other nuclear receptors cease expression, and remains a viable target for androgen

based therapies for a long time. It has also been found that some breast cancer cell lines and tumours respond to androgen stimulation by increasing rather than decreasing proliferation. It is unclear whether this effect is a result of alterations to the splicing of the androgen receptor, an increase in the alteration of androgens to estrogen via aromatase, a reduction of androgen receptor expression, or a combination of all of these events. The balance of data suggests, and this was the position hypothesized, that the androgen receptor should remain unchanged in most tumours, but that certain advanced tumours might reduce expression, perhaps in response to a specific histological variable.

The final two nuclear receptors in the study, the progesterone and glucocorticoid receptors (PgR and GR, respectively) are, like the androgen receptor, anti-mitogenic signals in breast cancer and have been used in breast cancer treatment. However, clinical data and previous studied have reported that breast cancers in advanced states of development show resistance to hormonal manipulation. Hence, it was hypothesized that both GR and PgR expression would decline as cancers acquired advanced characteristics. Stromal tissues are under a different set of signaling conditions than are tumours, previous studies showing that they can be manipulated by tumours into providing their normal support functions and are typically found to have majority of their signaling mechanisms intact. Thus, the data from prior studies would seem to indicate, and this was the hypothesized outcome, that the expression of the glucocorticoid and progesterone receptors would be reduced in stromal tissues and probably more so in the stroma of advanced tumours, as the tissue receives additional signals on the pathways for these

receptors from the tumour they support, driving them to undergo differentiation in order to keep the tumour well supplied with nutrients, growth and survival factors.

As for the correlation of nuclear receptor expression and histological parameters, the overall body of evidence for all nuclear receptors does not seem to indicate any clear pattern in this regard, with many parameters being associated with expression levels in one study, whilst being completely independent in others. Taking the diverse nature of previous studies into account, it was hypothesized that the expression of the nuclear receptors would not be directly affected by any single parameter, save each other, since a large body of evidence exists to indicate that stimulation of hormone pathways directly affects the expression levels of both the receptor of the hormone stimulated as well as other nuclear receptors.

1.4. Cancer.

1.4.1. Cancer, the Disease and an Historical Perspective.

Cancer is the second biggest killer in the first world, overall cancer deaths in developed countries only being surpassed by deaths caused by the more traditional killer of heart disease, though this is still tempered by local variation. In Australia in 1998, for example,

cancers overall accounted for 27% of all deaths, while cardiovascular diseases accounted for 40% of total deaths [1]. However it is strictly speaking inaccurate to consider all cancers (or indeed all cardiovascular diseases) together in this way. While all cancers display a similar set of gross symptoms, they arise in many different tissues from an array of various causes, so tumours in particular locations, and also, thought to a lesser extent, in particular individuals present sets of unique challenges to research and researchers.

Cancer is a disorder which is characterized by uncontrolled proliferation of cells in a specific location. The disorder progresses as the mass of uncontrolled cells grows, consuming the body's resources and impeding the function of nearby tissues by stealing or restricting blood supply or through disruption or outright crushing of nearby tissue, especially in the brain [2, 3]. Additionally, certain cancer cells may develop the capacity to separate from their original tumours, break through the barrier of the extracellular matrix and establish themselves at new locations. This ability, termed metastasis, makes cancers that possess it more hazardous and difficult to treat.

Cancer, despite being a disease of the affluent first world, is not a disease of lifestyle, though lifestyle factors modify cancer risk. Rather, it is a disease of age and strikes in the first world primarily because of the longer lifespan enjoyed by citizens of those countries. Nor is it a new disease, for cancer has been with the human race since the beginning of recorded history and for millennia before that. Cancer was known to ancient physicians and healers, the Egyptians writing about a primitive technique for the treatment of breast tumours by cauterization in approximately 1600 BCE [4]. The disease was also well

known to the ancient Greeks and Romans, with the coining of the term cancer for the disease attributed to Hippocrates, the "father of medicine", himself. In this era, and right through the middle ages to the Renaissance, the understanding of cancer was severely limited, with treatment of the disease consisting of only surgical removal of easily accessed tumours and the few herbal preparations believed to have some affect on cancer. Surgery of the age was highly dangerous and not always able to prevent recurrence of the tumour. Additionally, most of the medicines used had little reliable effect, though some of these traditional remedies do show potential anti-cancer properties in modern studies. For these reasons, cancer was widely considered to be incurable until surgical advances in the 19th century allowed for more radical and complete removal of tumours [4].

Just before the beginning of the 20th century, the discovery of X-Rays started a revolution in the treatment of cancer, with the use of radiation delivered by radium to kill cancer cells, a technique further refined by the delivery of doses of radiation over an extended period of time [4]. Shortly after the discovery of radiation as a cancer treatment, its ability to induce the formation of cancer was also discovered, primarily because early radiologists used little to no shielding for themselves and were routinely exposed to large doses of radiation from their own apparatus. Radiation was still useful as a treatment and remained, along with surgery, one of the only reliable treatments for cancer until the middle of the 20th century.

As with treatment, the understanding of the causes and processes of cancer were also extremely limited until relatively recent times, with most theories prior to the 18th century

making false assumptions on the causes of cancer, such as the theory that it was caused by a parasite, a theory that even had a Nobel Prize awarded in 1926 for a study that supported it [4]. More successful was the identification of certain carcinogens from observational evidence on the increased levels of cancer in people from certain professions or who were exposed to different environmental factors. It was, for example noted that the removal of the ovaries reduced the growth of breast tumours in some patients and the relationship between breast tissue and factors secreted by the ovaries was correctly theorized.

Despite these identifications, it was not until the 20th century when increases in the knowledge of the structure and function of DNA that a more complete picture of the mechanisms of cancer began to emerge. Chemotherapy treatments then began to appear, targeting specific cancer systems and disrupting known pathways of cellular proliferation [4]. Cancer detection has vastly improved, catching many cancers before they have a chance to spread and treatments have now become far more reliable, with combination therapies resulting in much greater survival rates. However, even with the modern understanding of cellular systems and cancer, much remains to be uncovered if the deaths from this disease are to be effectively eliminated.

Cancer is a genetic disease, arising in no single way and from no single process; instead, tumours form when several of a variety of damaging effects occur in an individual cell [5, 6]. These events, and the order in which they occur, may be unique even between two cancers arising in the same tissue, or even in the same individual. Despite this, it is

damage to DNA that will lead to the formation of cancer, though not all DNA damage suffered by a cell will result in cancer, as detailed in the following section [6].

1.4.2. Carcinogenesis.

1.4.2.1. The Genetic Model of Carcinogenesis.

1.4.2.1.1. DNA Damage in Cancer.

Cancers are principally generated by an alteration to a cell's DNA, the genetic material that stores all the inheritable information which directs the metabolism and activities of that cell. Indeed almost all cancers tested in mutation studies display some form of alteration to their DNA, alterations which are not part of the normal processes of the cell, resulting instead from some form of DNA damage [5]. There are a variety of ways in which DNA can be damaged, with most sources of DNA damage fall into one of two categories, chemical or radiation, though DNA damage through mechanical means is also possible. The double helix structure of DNA is relatively stable, but the metabolic environment of a cell is replete with reactive substances derived from the ongoing production of waste from energy usage, the uptake of new molecules and various intermediate molecules in the pathways of the cell [5]. Additionally, DNA is also

exposed to a constant amount radiation from the environment, even when there is no major source of radiation nearby. Thus, within the cell, DNA is always accumulating damage from these sources, leading to alterations to its structure and possibly changes to the genetic information it contains [7].

These changes occur when chemical reactions either directly alter DNA composition, through the destruction or removal of bases, or when structural alterations caused by the reactions cause the cell's DNA synthesis mechanisms to misread the base and change the gene structure and its resulting protein, leading to altered gene function [6]. Additionally, alterations to DNA make-up may occur when damage to an area has been so great that functional repair mechanisms replace damaged bases with incorrect ones. Changes may also occur through mutation, when DNA replication mechanisms insert the wrong base into the new sequence in error [6].

Factors that cause DNA damage are referred to as mutagens, while those substances that cause cancer are called carcinogens. All carcinogens are also mutagens, but not all mutagens are also carcinogens [6]. Carcinogens are generally electrophilic substances that react with DNA molecules, causing direct change to nucleotide subunits of the DNA, binding to the DNA, forming adducts that render the DNA unreadable or causing complete breakage of the DNA strand, the most destructive form of DNA damage [2]. Radiation can also be carcinogenic. Mostly implicated are UV light, X-rays and other high energy ionising radiations [2, 5], though recently low energy radiations, including microwaves, radio waves from mobile phones, electromagnetic fields and even visible

light have also come under suspicion [8, 9]. Radiation induces cell transformation into cancers by damaging the integrity of cellular DNA. Ultra-violet radiation is absorbed by aligned double bonds of stacked pyrimidines, which then undergo rearrangement to form dimers, while ionising radiation causes the formation of highly reactive radical species and may also directly break DNA in the cell [5]. Lower energy radiation is believed to cause damage by the heating of exposed tissue, leading to the break-up, denaturing, or rearrangement of DNA and other substances.

It is also possible for DNA to be damaged by another organism. There are numerous viruses, such as Rous Sarcoma Virus and the human Papillomaviruses that are known to transform cells that they infect into a cancerous state [10]. Such viruses are believed to cause this transformation in order that the infected cell will reproduce itself, and thus, new virions, at an accelerated rate. This is not DNA damage in the strictest sense, since it involves the normal function of viral genes, but the process of carcinogenesis by biological induction is an important one, accounting for the vast majority of cervical cancers (through Papillomaviruses) and it has been theorized that many other cancer types may have their origins in viral infection, particularly in tissues such as the brain which are not normally exposed to as high a level of carcinogen exposure as epithelial tissues. [11, 12].

For all the vast panoply of substances that can damage a cell's DNA and the sheer volume of damage that can occur within a few hours, most DNA damage is repaired by intrinsic mechanisms which are highly conserved from bacteria through to mammals.

These repair mechanisms clean up DNA damage but they are, however, not perfect and if DNA damage is great they may fail to repair all damaged DNA.

1.4.2.1.2. DNA Repair.

DNA repair is not a simple process and it has many stages and several forms that it can take. The repair mechanisms are comprised of multiple proteins that detect the damage, transducer proteins that communicate this news and proteins that perform the repair. Damage is recognised by direct detection by certain enzymes, distortion of chromatin structure by altered bases and when transcription or translation is blocked by an unreadable signal [13]. Different forms of DNA damage will then recruit the appropriate repair mechanisms through the transducer proteins, drawing them directly to the site so that the damage may be excised and replaced with appropriate normal DNA [13, 14].

There are six main mechanisms of DNA repair, each handling a different kind of DNA damage, which are direct reversal, base excision repair, nucleotide excision repair, mismatch repair, recombination repair and translesion repair [13, 14]. Direct reversal is the simplest method of repair and involves repair enzymes which directly scan DNA and remove any excess atoms that have bound to nucleotides, restoring the sequence without removing the offending base from the DNA. Base excision repair also deals with damage to individual bases, from sources as diverse as methylation, oxidation depurination and deamination. Since depurination occurs at rates as high as 104 bases per cell per day, this

repair mechanism is an extremely important one [7, 14]. Base excision repair consists of a vast array of repair enzymes, each recognizing and repairing a specific alteration to a base, but all operate by the specific protein remove the damaged base and having several common enzymes mediate the replacement of the appropriate replacement. Nucleotide excision repair functions in a similar manner, but deals with multiple base damage, operating through the removal of the bases, plus between 25 and 32 additional bases around them, and replacing the missing nucleotides using the remaining strand as a template.

The remaining repair mechanisms deal with forms of damage more complex than alterations to bases. Mismatch repair operates after DNA replication has occurred and deals with cases where DNA polymerase has added an incorrect base to the daughter strand. It strips out the incorrect bases and replaces them, using the presence of Okizaki fragments to detect which is the daughter strand and therefore the incorrect sequence [14]. In this regard, it could be considered an error-checker rather than a damage repair enzyme, but it nonetheless important in maintaining genomic integrity. Recombination repair deals with double stranded breaks, the most severe of DNA damage, and operates by two basic mechanisms. The most reliable of these is by recombination of a homologous DNA sequence, from another chromosome or other source, into the area of the break, completely replacing the damaged area with the undamaged sequence from that source. The second, and far less reliable method of repairing double stranded breaks is through non-homologous end joining, where severed DNA ends are simply flattened and joined together, altering the sequence of the area by removing, or possibly adding to,

the bases within [14]. The final method of DNA repair, translesion repair, is also prone to errors. It consists of DNA polymerases that are able to write new DNA sequences when the template strand contains dimerized bases which would stall the normal DNA polymerases. When these enzymes read dimerized bases, they have preferences as to what base they will place in the daughter strand, meaning that some errors in the template will be repaired but that others will result in an incorrect signal. This kind of error may seem to be counterproductive for a repair enzyme, but it is believed that the mechanism remains conserved because the rapid (if possibly erroneous) repair during DNA replication prevents the far more injurious double stranded breaks caused by the stalling of the normal DNA polymerase [14].

The errors that arise during repair and replication take many forms, including the replacement of nucleotides with incorrect or non-complimentary nucleotides. Addition or subtraction of nucleotides to the gene may also occur. All of these errors may alter the gene's product, the expression of the gene or the expression of other genes if the altered gene is involved in their regulation. This altered activity can result in new products, subtly altered or inactive products, vast amounts of the product or no product at all and these alterations in gene activity can lead to cancer [5]. Additionally, altered DNA may be simply missed by repair enzymes and remain in the genome, leading to changes in gene activity just like that produced by repair error in one or both daughter cells [15].

There are additional mechanisms to prevent the replication of cells whose DNA cannot be repaired, or has been subjected to massive damage. These functions include

senescence, halting the dividing of the cell, so that it will not replicate the errors contained within it. Alternatively, a highly damaged cell may undergo programmed cell death, or apoptosis, to prevent it, or its daughter cells from becoming malignant [16]. However, not all damaged cells that escape these mechanisms become tumours, though eventually a damaged non-malignant cell will succumb to them. Cancers do not arise from all DNA damage; instead, they are formed when damage occurs to particular kinds of genes.

1.4.2.1.3. Oncogenes and Tumour suppressors.

Since cancer is, at its root, a disruption of genetic order in cells, there are genes which influence the development and progression of tumours. These genes are collectively known as oncogenes. There are few genes that cause cancer directly, since this would simply select for the death of the organism that carried the gene (viral genes being an exception), so the majority of oncogenes arise from damage to precursor genes which are called proto-oncogenes. Whether or not a gene is a proto-oncogene depends upon its function. Damage to a gene which controls a vital metabolic function will more often simply be fatal to the cell while damage to less important genes may simply shut down a non-essential pathway. Oncogenes and proto-oncogenes typically control one of a specific set of functions within the cell. These functions all revolve around cellular reproduction, DNA repair, growth control and the disposal of carcinogenic compounds.

Significant alterations in gene function in any of these categories are likely to be the first step in cancer development [6, 17].

Oncogenes tend to arise spontaneously, but may be heritable, as evidenced by a strong history of malignancy in some families and ethnic groups. Heritable oncogenes are often found to be mutated versions of wild type proto-oncogenes, for example mutations in the p53, BRCA1 and BRCA2 genes [6, 18]. These mutations are occasionally unique to particular families or ethnic groups, but also often resemble the common mutations found in spontaneous tumours. Heritable oncogenes arise when damage to the original gene occurs in germ-line tissue, or tissue that later becomes germ-line, i.e. sperm and ova. Once a mutation has entered the germ line, they may be passed on to the carrier's subsequent offspring. However, since children receive only one copy of each gene from a parent, a correctly functioning gene inherited from the other parent may override the mutated version and the child may not develop cancer at all, unless the functioning gene is subsequently damaged [19]. In that case, recombination repair mechanisms may use the inherited oncogene gene as a template to repair the formerly functional gene, resulting in Loss of Heterozygosity, a common feature in cancers [6]. What this means is that individuals who inherit oncogenes are not assured of developing cancer, but that the chances of them doing so are significantly increased.

Whether or not a single copy of an oncogene is sufficient to cause cancer or not depends largely on the precise mechanism of action of the gene. In most cases, damage to a gene will result in an unreadable sequence, a non-functional protein, or a protein with altered

function. If the function of the gene it to prevent the cell from reproducing, loss of function may lead to cancer formation since the cell may begin to ignore further DNA damage, where loss of function in a growth induction gene would slow cellular growth [6]. An alteration of function in a gene is a more mixed event, depending on the precise nature of the change, since an altered gene may function more or less efficiently than the original gene. Indeed, the possession of multiple polymorphisms for a gene, presumably mutated from a single precursor some time in the past, has proved to be protective for certain gene functions [20, 21]. Unfortunately, not all oncogenes have a single function, making alterations to these genes a far more dangerous event. One of the most well known oncogenes, p53, plays a central role in the control of tumours and oversees a myriad of functions, involving growth suppression, DNA repair, and hormonal signalling [14, 22, 23]. With such an array of functionality, alterations to p53 might affect one of its duties and not another, but still result in a decreased ability of the cell to halt the change into cancer. Such is the importance of p53 in cancer control that an appreciable percentage of tumours display mutations in this gene [22, 23].

Even if both copies of a single gene are disabled or altered in such a way as to favour growth, cells do not automatically become cancerous. This is because the metabolism of cells is replete with feedback systems as well as multiple layers of growth limiting genes that prevent cells from growing out of control. However, genetic damage accumulates over the life of a cell and particularly if repair genes become damaged. The regulators of some of these control systems are tumour suppressor genes. Tumour suppressor genes are genes whose function is primarily to prevent cancer development, though they also

include multi-function genes such as the ubiquitous p53 [6]. Tumour suppression is typically accomplished through repressing cellular growth, increasing differentiation or by promoting apoptosis in a cell that cannot be otherwise rescued from a cancerous state [6]. Alterations to the function of tumour suppressors do not automatically induce cancer and even alterations to both copies of a gene in an individual may only increase cancer risk slightly [6]. However, deletions of both copies of a tumour suppressor gene are a common event in cancers, since they remove the influence of the gene entirely, increasing the risk of cancer development as for any other oncogene.

1.4.2.1.4. The Immune Response.

Once a cell has escaped normal growth control through DNA damage, it may be prevented from ever becoming an established tumour by the immune system. Indeed, the immune system is an integral part of the body's defence against cancer and there is evidence that long-term immune system depression, such as in AIDS and chronic stress, is a risk factor for many cancers, including breast cancer [24, 25]. Normally, the immune system ignores the body's own cells, but as tumours accumulate mutations they may begin to display aberrant proteins or types of protein on their cell surface. Tumours that retain the correct preventative mechanisms may also begin to release factors that attract and activate immune cells. In these cases, the cancer cells will no longer be recognised as self tissue by the immune system and will be eliminated by phagocytosis by macrophages, induction of apoptosis by T and B cells or direct killing by natural killer

cells or monocytes [25, 26]. In this way, tumour growth within an organism is prevented before it can become established, so long as the tumour displays a recognisably non-self signal. However, there is some evidence that the immune system is capable of detecting the presence of cancer without an obvious non-self signal, presumably by the detection of cancer-like cellular behaviour. Near such arising tumours, immune cells will begin to accumulate, mostly T-cells capable of reacting to various self antigens, as the beginning of a defence response. Unfortunately, the imperative of the immune system is to not recognise self antigens and though these cells continue to be produced, they do not constitute an effective response against a tumour that displays only self antigens. To make matters worse, there is some evidence that such accumulations may "warn" tumours of an impending immune response and increase selective pressure on them to develop means to evade and even subvert the immune system [25].

There are numerous mechanisms that tumours can use to evade the immune system once they have undergone sufficient change to be targeted by it. Many of these systems are similar to the ones used by pathogens to evade immune responses, while some are unique to tumours, utilising normal body signalling systems to ablate immune responses to the tumour. The simplest of these responses is to downregulate or entirely cease the expression of proteins that interact with the immune system, making recognition difficult and reducing the strength of immune response if they are detected [25, 27]. Changes to the structure of antigen presenting proteins provide another mechanism to prevent detection by the immune system, making it impossible for immune cells to detect mutated peptides and thus any lethal response to the tumour. Finally, since tumours possess all of

the genome's control genes, they may also secrete signaling molecules that directly affect immune cells, causing them to reduce their level of activity or even begin the process of apoptosis. This ability to control surrounding cells is also of use to the tumour in progression to more dangerous stages of cancer.

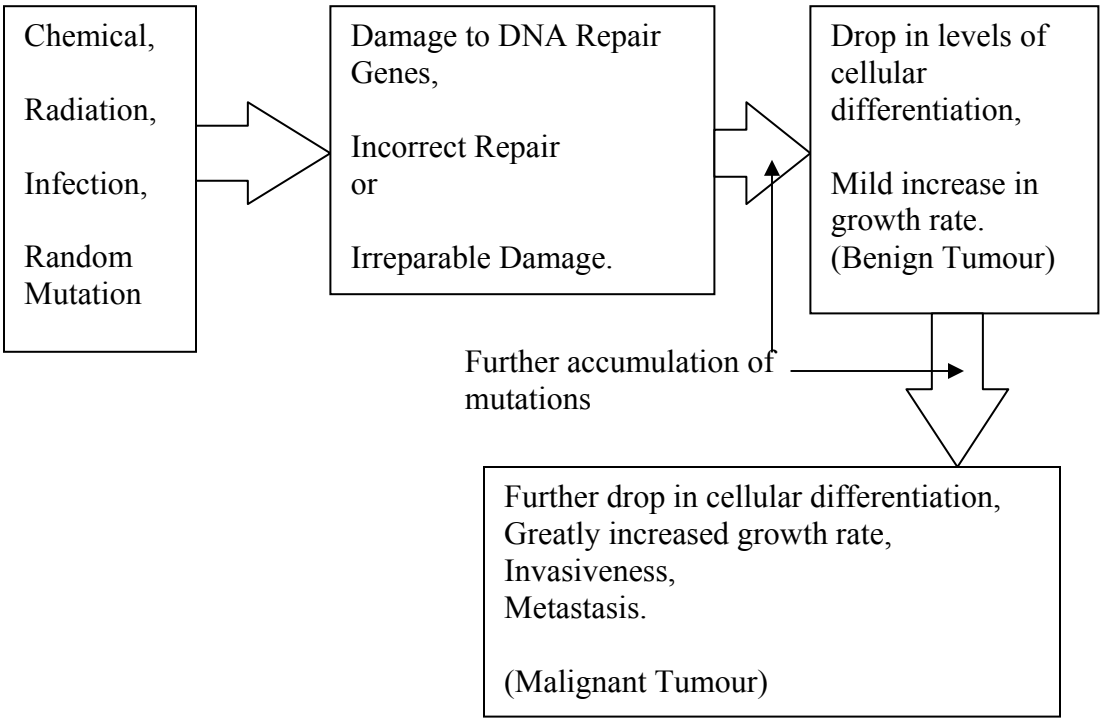

Figure 1.1. Events Leading to Cancer Formation. Derived from [6].

1.4.2.2. The Epigenetic Model of Carcinogenesis.

The epigenetic model of carcinogenesis is not a competing model of carcinogenesis, recognizing that genetic changes are nigh universal in carcinogenesis, but rather seeks to compliment the genetic model by explaining those events which have an impact on carcinogenesis, but are not genetic in nature.

There are a number of epigenetic factors which affect the behavior of the cell and these also may or may not be heritable, though they are not part of the genetic sequence contained within the nucleus. The most well described of the epigenetic factors is DNA methylation, a modification of cytosine which was long associated with gene silencing. Though this is part of its function, DNA methylation is also involved in the definition of the boundaries between active and inactive chromatin [28]. The makeup of chromatin is another major epigenetic influence on a cell, especially the methylation, phosphorylation and acetylation of histone proteins, particularly the H3 and H4 variants, which help stabilize DNA structure and modify regulation of genes. These states are believed to be retained during DNA replication [28]. Genomic imprinting, the state where one chromosome's expression profile is altered depending on which parent the chromosome is inherited from, is another major epigenetic factor implicated in cancer development, specifically where proper gene silencing is lost and an increase in expression for growth enhancing genes occurs.

These epigenetic factors are less stable than DNA and have fewer repair mechanisms, so are more vulnerable to alterations from endogenous and exogenous factors within the cell and occur somewhat more frequently [29]. Since many epigenetic factors influence how genes behave, alterations to them may have effects similar to those of a full blown mutation, particularly if a tumour suppressor or similar gene is silenced [30]. Indeed, alterations to epigenetic factors may explain some of the less than 100% concordance of certain disorders in monozygotic twins [28]. Principally, proper epigenetic control is lost with time, either degenerating as DNA replication chips away at histone modifications, is altered by reactive metabolic factors or genetic factors such as the proteins which control DNA methylation and the modification of chromatin and histone proteins [29]. These are ultimately the same influences that can induce genetic changes, but like genetic mutation, a single event of epigenetic alteration would not have the power to push a cell into a cancerous state. However, together with genetic changes, they modify the risk for cancer development, and in line with Knudson's classic "two-hit" theory of cancer development, a cell will be more likely to develop cancer with both kinds of change present than with only one [28, 29, 31].

Finally, there are numerous transcription factors, inhibitors and other signaling molecules, both endogenous and exogenous, which can assist in the development of cancer and may be considered as epigenetic factors. Typically, these factors affect the transcription of genes associated with cellular growth and differentiation, increasing the rate of growth of a cell in a temporary manner and are not usually heritable [29]. These signals are usually part of the normal life of the cells and are required for proper

organization of the body. While cells have feedback mechanisms that normally prevent such signaling from going awry, any increase in the proliferation of cells can increase the risk of developing a genetic mutation, as DNA replication is a very vulnerable time for the genome. A classic example of this kind of mechanism is estrogen in breast cancer, where lifetime exposure to estrogen has been determined as a risk factor for breast cancer [32].

1.4.3. Cancer Progression.

Once the process of carcinogenesis is underway and the cell has escaped normal growth control mechanisms, it must develop several traits if it is to continue to survive and become, eventually, a hazard to the life of the organism. These characteristics include immortalization, evasion of the immune system, self-sufficiency of growth factors, the ability to induce angiogenesis and the ability to cross extracellular barriers and invade new tissues. If the tumour fails to develop these characteristics, it will eventually reach limits in its growth, cease expansion and may also die.

1.4.3.1. Immortalization.

Part of the nature of tumour cells is that they grow at accelerated rates, and this causes a problem for tumour cells. DNA reproduction is one of the most vulnerable times for the genome, for errors arise in the DNA sequence and chromosomes lose bases from their

ends, being shortened by the process. Normal cells have DNA repair mechanisms to deal with sequence errors and long repeating DNA sequences called telomeres at the end of chromosomes. Telomeres serve to prevent any loss of genetic information when bases are deleted from the chromosome ends and are also implicated in cellular senescence and aging [33]. DNA repair mechanisms are usually compromised in a tumour, so mutations will accumulate more rapidly in tumours than in normal cells, though some repair mechanisms will remain, such as the proofreading ability of DNA polymerase. However, telomeres are a different matter. With such a high rate of reproduction, telomeres in tumours are rapidly lost, and once they have been depleted genes will begin to be damaged, which will reduce the cell's ability to survive and will eventually be fatal.

In normal cells which are dividing rapidly, as during healing and childhood, the telomeres are expanded during each round of replication through the action of an enzyme called telomerase [33]. As an organism ages, the rate of telomerase activation decreases, probably through epigenetic means, and a normal cell's ability to activate it at the time that most cancers develop is low. Therefore, if a tumour is to survive and continue reproduction, it must reactivate telomerase to a level sufficient to compensate for the loss of telomeres as they grow [33]. Telomerase, however, is not the only mechanism that allows cells to prevent this damage. Other genes which are not typically used in normal cells may also expand the telomeres through distinct mechanisms that are not as effective as telomerase, but if sufficiently expressed, will serve the same function [33]. These aberrant telomere extension mechanisms are indicative of tumours and may serve as prognostic markers.

Once the tumour has developed the ability to resist chromosomal damage from cellular replication, the cells become immortalized and no longer suffer age related senescence or loss of growth ability over time [32, 33]. Indeed there are cancer cell lines derived from humans which have been under culture for decades showing no sign of degeneration due to age.

1.4.3.2. Growth Factors, Angiogenesis and the Stroma.

All cells in a multicellular organism require a series of growth factors to make them undergo proliferation, as well as a number of survival signals to keep them from committing to apoptosis while doing so [29]. In the normal body, these factors are secreted by various tissues at various times and are under careful control, to keep the body's cells operating in the correct manner. Because these factors are under the control of other tissues, a tumour may not receive enough to allow it to grow at its maximum rate. If the tumour is to grow at a rate greater than that of the surrounding normal tissue, it must obtain the growth factors for somewhere else [29].

Cancers have two main mechanisms that they use for this. The first is simply to produce the required factors themselves, through changes in regulation of the genes that produce them, and use autocrine signaling to supply themselves [29]. Cancer cells, in particular advanced tumours, may also use a different form of self-stimulation where messenger

proteins are mutated or spliced in such a manner that they are constitutively activated and no longer require the signaling molecule to induce cellular growth. The second major mechanism cancers use to obtain growth factors is to subvert the metabolism of nearby cells to provide them. Many epithelial cells do this normally, and supporting cells are already primed to provide requisite growth factors, albeit at slower rates than the cancer requires [27]. With relatively minor changes to the cancer's own signaling rate, it can cause the local tissues to increase their release of growth factors to sufficient levels to supply the tumour. This is also related to the tumour's ability to escape immune system control, for immune cells can also provide growth and survival factors if properly stimulated, and if the tumour can avoid being recognized as non-self, it may be able to cause the immune cells to likewise support its continued growth [27].

A cancer that is able to grow to its full potential will soon reach an additional barrier to its continued survival. Cells at the core of a tumour that reaches a certain size will begin to be starved of oxygen and other factors due to the limitations of diffusion from the blood vessels. Thus, if a cancer is to continue to grow, it must be able to induce angiogenesis, the recruitment and growth of new blood vessels, to ensure a continued supply of necessary materials.

These kinds of reactions are common among healthy cells, for example, cells healing an injury must be able to signal blood vessels to grow to supply the new tissue, but interactions between the tumour and the stroma are also common, and may be necessary for the survival of the tumour [34, 35]. In many cases, these interactions are more or less

the same as those taking place in healthy tissues, such as signaling to provide growth factors, but disruptions to regulatory mechanisms in the tumour make these interactions take place at unusual times and rates. As tumours develop however, they may begin to send different signals to the stroma, causing the local microenvironment to change to suit the current requirements of the tumour, thus facilitating progression of the disease [34]. These changing signals may induce the stroma to produce enzymes to degrade the extracellular matrix and reduce binding to nearby cells, allowing tumour cells to escape their current location, and with only minimal metabolic cost to the tumour itself [34]. Thus, as tumours develop, their surrounding stroma will begin to manifest changes to cellular behavior, particularly in receptors that control the release of cellular growth factors, cell adhesion and cell proliferation. This makes the stroma an important consideration in cancer research and a potential area for targeted cancer therapies [34, 36].

1.4.3.3. Invasion.

The ability for tumours to invade other tissues, or metastasize, is one of the factors that make cancers so life threatening and difficult to treat. The ability to migrate to other tissues which may be more important to the organism's continued life increases the rate at which the tumour can absorb the body's resources. It also increases the chance that it will begin to impinge on vital functions of the body by disrupting the functions of the organs it grows in. Coincidentally, it also makes treatment more difficult, since it makes

removal of the tumour by surgery more difficult, and requires a much broader spectrum of chemo- or radiotherapy to treat them, involving greater side effects. However, unlike other factors, the ability to metastasize is not absolutely required for tumour survival and it tends to be amongst the last of the characteristics developed by tumours, making the treatment of tumours less problematic if they can be detected early [34, 37].

There are numerous steps involved in the gaining of metastatic potential. First, the cancer must be able to break down the extracellular matrix, a series of cross-linked proteins and other substances which provide support for most tissues, especially the epithelial tissues from which most cancers are derived. Mechanisms to remodel the extracellular matrix already exist and are used in healing and embryonic implantation [34]. There are numerous genes implicated in the development of metastasis, particularly the matrix metalloproteinases (MMPs), which break down proteins of the extracellular matrix and the tissue inhibitors of metalloprotinases (TIMPs) which serve to keep the MMPs in check. Dysregulation of these genes has been shown to exist in numerous metastasis capable tumours and cancer cell lines, and appears to be one of the major mechanisms by which tumours achieve metastasis [34].

The second of the required mechanisms for a tumour to metastasize is motility, and the ability to survive without cell-cell contacts. In tumours, these mechanisms are based around the same cytoskeletal elements used in muscle and in motile immune cells. Indeed, many tumour cells undergoing metastasis adopt similar phenotypes to immune cells, in a sense masquerading as a different cell type to avoid detection [25]. Generally

speaking, only very few cells in a tumour will develop motility, since there is little selective pressure to be able to do so and less than for ECM breakdown, which allows a tumour to expand beyond the original confines of its tissue. However, once these few cells have the ability and escape, additional metastases become more likely, since all of the daughter cells of the escapee will retain the ability.

Metastatic cells may escape into the circulation, and thus are capable of being carried by the blood to many parts of the body. Often though, metastatic cells make their way into the lymphatic system, where the slower movement of fluids in the lymph nodes makes for ideal sites of initial colonization. Few escaping cells are viable however, so the colonization of local tissues forms an intermediate step, allowing tumours to further develop the characteristics required to survive in other tissue types [38].

1.4.4. Treatment.

1.4.4.1. Surgery.

Surgery is the oldest of the treatments for cancer, and remains as a very effective method, since there is little a cancer can do to resist simply being cut out of the body. Metastasis, of course, is the major problem with surgery, since cells that have escaped from the main tumour will also escape any surgical intervention on that tumour. Likewise, if even a single cell is missed during surgery, the entire tumour can re-grow in a relatively short

period of time [6]. After the advent of anesthesia, surgical methods were improved, but tended to be radical, removing as much tissue as possible in an attempt to get the whole tumour. However, as pathological techniques have also improved, surgery has become less radical, doing little damage to the surrounding tissue, and often being combined with radio- or chemotherapy to eliminate any cells that have been missed during the procedure [39].

Despite the simple nature of surgical intervention, numerous improvements have been made and are continuing to be made in this field of cancer treatment. Keyhole surgical techniques can remove the tumour with minimal damage to surrounding tissues, while improvements in imaging technology have allowed even very small tumours to be identified before and during surgery [37, 40]. By minimizing damage to surrounding tissue, the risks that any remaining tumour cells will escape through the breech in the extracellular matrix before it heals are lessened greatly. Greater clearance of tumour cells is the reason for the procedure in the first place, though these improvements also lessen the requirements and dosage of additional therapies, which can have dangerous side effects.

1.4.4.2. Radiation Treatment.

High-energy ionizing radiation was found to have anti-cancer properties not long after it was discovered, and has been used in the treatment of cancer ever since. Though ionizing

radiation is fatal to all cells at certain dosages, treatment with radiation has several benefits. Firstly, cancer cells are more vulnerable to radiation, due to the pre-existing damage to DNA repair mechanisms, and secondly, it is easier to target radiation to given areas than it is to target a drug [37].

Radiation does have several disadvantages as a treatment mechanism, however. The primary problem with radiation as a cancer treatment is that radiation is itself a carcinogen, since it causes DNA damage [6]. While cancer cells will accumulate mutations and eventually die under radiation treatment, any healthy cells in the treatment area may not die when treated with radiation, due to their still competent repair systems. Once treatment is discontinued, the DNA damage done to these surviving cells may be sufficient to cause them to become secondary tumours and the entire regime must be repeated some years later [37]. Radiation's second major disadvantage as a treatment regime is that its damaging nature makes it unsuitable for use in treating multiple metastases. A body or even organ wide dose of radiation high enough to kill a tumour may lead to heavy side effects of radiation sickness, hair loss, possible carcinogenesis and perhaps even death [6, 37, 40].

However, as with surgery, advances in recent years have been made in radiation treatment. These changes revolve around the use of lower energy radiations and increases in target specificity, with the aim of reducing the exposure of nearby healthy tissues to radiation, reducing side effects and the possibility of secondary carcinogenesis [41]. This has been achieved through implantation of very low tissue penetration radiation sources

at the site of primary surgery as well as through specialized beam focusing techniques that minimize the profile of the radiation.

1.4.4.3. Chemotherapy.

Chemotherapy is a particularly broad area of cancer treatment, incorporating many treatment types that use multiple different approaches to arrest and destroy cancer cells. There are, however, two main mechanisms of chemotherapy, cytotoxic drugs and cell signaling.

Cytotoxic drugs work in a similar manner to radiation, attacking the metabolism of the cancer cells directly. These may work by DNA fragmentation, disruption of major metabolic pathways disruption of cell structure and so on [40]. Like radiation, these drugs also affect normal cells and rely on the fact that the healthy cells will be better able to cope with the stress than the already damaged cancer cells, which will die in greater numbers, while healthy cells will simply be replaced by healing. The global nature of cytotoxic drugs means that they can have heavy side effects as they destroy cells in all parts of the body. This also means that such treatments must often be discontinued for periods to allow the patient to recover from these side effects [40]. Unfortunately, this also gives the tumour a chance to recover, and this period of remittance may allow cancer cells that have developed partial resistance to the drugs the chance to grow and further develop their immunity. Certainly many cancers develop resistance to such drugs and

dosages must increase to control the tumour, increasing the side effects and eroding the patient's general health as a result.

The second major mechanism of chemotherapy is via cell signaling. In these treatments, known metabolic control systems within the cell are manipulated, in an attempt to slow growth, increase differentiation or induce apoptosis. The side effects of these drugs are typically less than for cytotoxic drugs, though the signals may have consequences in other cell systems that interpret the drugs differently [18]. Tamoxifen, a blocker of the estrogen based growth pathway, is one of the classic drugs of this type. However, like the cytotoxic drugs, tumours may begin to develop resistance to these forms of treatment, often as part of their progression towards independence of growth signals [18]. These drugs can be used for longer periods than cytotoxins, with fewer side effects and are a good alternative, especially for newer tumours, and as additional therapy after surgery.

More recent advances in chemotherapy have mainly involved the discovery and development of new drugs, with fewer side effects or greater strength. Additionally though, efforts have also focused on specialized mechanisms for drug delivery, such as by attaching the drugs to specialized molecules to ensure uptake by cancers preferentially [40]. This confines the drugs to target areas and minimizes the side effects produced so that the drugs can be used for longer periods. Additionally, a newer method of treatment, termed metronomic therapy has recently been pioneered. The drugs used are cytotoxic, but are given in lower doses than is customary, just below the level where side effects become apparent. The treatment is then continued indefinitely, the level of the drug never

allowed fall so that the tumour is under constant bombardment and has no respite to build on any resistance it might possess or develop. The treatment has an improved success rate than the heavier but more periodic doses and has the additional benefit of producing none of the debilitating side effects usual in chemotherapy, which improves patient health and quality of life while undergoing treatment.

1.4.4.4. Recent Developments.

While surgery, chemotherapy and radiation treatment remain the primary forms of treatment for cancer, there are numerous other areas being explored in cancer treatment, which promise effective and relatively side-effect free regimes. A few of these have already completed clinical trials and have been added to the arsenal of treatments available to clinicians, even while other approaches on the same lines are still being developed.

Anti-angiogenesis is one of those methods which have reached the stage of availability. Tumours cannot supply themselves with nutrients, and if they are to grow, they need these in abundance. This is ensured by their ability to induce blood vessels to grow into them, just like any growing tissue [42]. However, unlike the cancer, the blood vessels are operating normally and thus will respond to signals that instruct it not to grow. Anti-angiogenic drugs prevent the growth of new blood vessels into a tumour, with the result that growth is slowed, halted or even reversed if there is sufficient distance between the

blood and the tumour [40, 42]. Used in conjunction with other treatments, it is extremely useful in preventing remaining cancer cells from regaining their foothold. The treatment does have some side effects, slowing wound healing, but the burden on the patient is low and the treatment can be kept localized in a similar manner to other chemical treatments if required.

Another approach that has shown considerable potential is cancer vaccination. There are several ways this can be achieved. One is through challenging the patient with one, or a mix of, common cancer antigens to sensitize the immune system to cancer. Alternately, the antigens may be derived from the patient's own cancer cells. Both methods have certain limitations. The common antigens may not serve to sensitize against the patient's own tumour and the personal antigens may not sensitize against all possible metastases that may have escaped removal. However, the methods are still being refined, and the benefits of the treatment are great. Once properly sensitized, the immune system will seek and destroy all cancer cells in the body, including metastases that begin to grow anywhere in the body [25]. The protection is lasting and can be boosted, allowing the immune system to clear up recurrent cancers or subsequently developing cancers that have the same antigens. The treatment is difficult for tumours to adapt to and side effects are minimal in comparison to other treatments. A similarly based treatment involves the preferential infection of cancer cells by genetically tailored viruses, inducing an immune response through the virally induced changes to the tumour cell. This method has similar advantages, but is limited by the ability to make the virions tumour specific, and for them to induce the proper changes in the cancer cells. Yet another form of immune treatment

involves monoclonal antibodies for common cancer antigens, which can be produced in large quantities and given to the patient, binding to those antigens and inducing an immune response [40].

The final type of newer therapy also involves the use of tailored viruses, as well as other types of specialized vector, to insert copies of tumour suppressor genes and other control systems into cancer cells. This is termed gene therapy and it is also being developed to treat heritable genetic defects and other ailments. By reintroducing control genes that may be lost or inactivated in the tumour, the growth of the cancer may be slowed, or stopped [40]. Cancers treated in this way may also return to a differentiated phenotype and become indistinguishable from normal cells. Gene therapy has few, if any, side effects, since control mechanisms accidentally introduced into a normal cell will not affect the cell's function. However, depending on the mechanisms used, the growth rate of any accidentally altered cell may be radically slowed, reducing its ability to respond to wounding [40]. Gene therapy has proved difficult to make work reliably, but it has been used with some success in other disorders and has a great deal of potential to aid in the fight against cancer.

CHAPTER 2. Background.

2.1. Breast Cancer.

2.1.1. The Breast.

The breast is a secretory organ originally derived from sweat glands and modified to secrete milk to feed mammalian offspring. The breast is a relatively complex organ, containing several types of cells to facilitate the synthesis, storage and transmission of milk. These cells include fat bearing cells to store energy, secretory cells for the synthesis of the milk constituents, and ductal cells for the storage and transmission of the milk to the offspring [3, 43]. The structure of the breast is shown in Figures 2.1 and 2.2, below.

Since the breast is only required to function when an offspring is in the initial stages of life, it remains in an undeveloped state until the hormonal changes of pregnancy signal the tissues to proliferate and differentiate into the required structures [3, 43]. Prior to first pregnancy, the system of ducts in the breast remains small and the alveoli which produce milk are few in number. During the hormonal changes that take place in the human menstrual cycle, the ductal system undergoes small cycles of expansion and reduction, as the optimal time for fertilization approaches and recedes [43]. Once fertilization occurs, the ductal system expands greatly and side branches form, eventually differentiating into alveoli. After lactation is concluded, the system undergoes a certain amount of atrophy,

but still remains more extensive than it was previously and still undergoes the cyclical growth and reduction phases with changes in hormone levels [43]. As breast tissue is under a constant cycle of growth and remodeling due to hormonal control, it is vulnerable to DNA mutations and is a prime site for carcinogenesis in women.

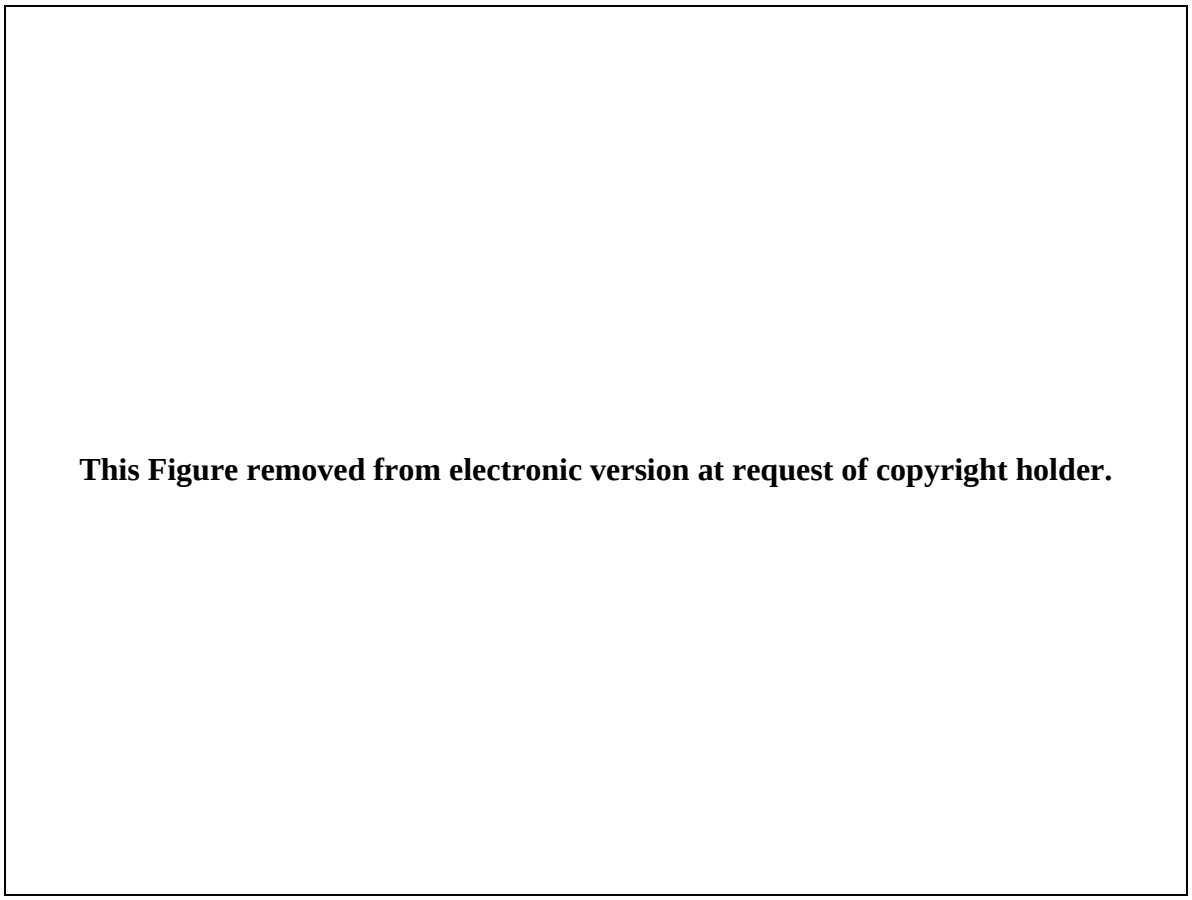

Figure 2.1. Breast Anatomy. The breast is organized into up to 25 lobes, each of which contains many lobules, which in turn contain alveoli, the functional units of milk excretion. The lobules empty into the terminal ducts, which in turn empty into larger ducts and eventually arrive at the nipple, where milk collects in the lactiferous sinus, just below the nipple. In women who are not lactating this system is rudimentary but increases slightly as the menstrual cycle progresses [43].

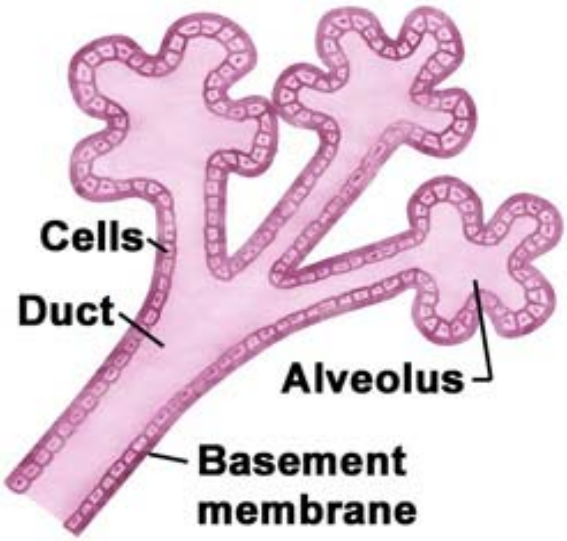

Figure 2.2: Lobular Anatomy. The breast lobules are organized into clusters, which are themselves formed by clusters of alveoli. As can be seen, the alveoli are formed by a layer of secretory cells, which blend into the epithelial cells of the adjacent ducts. These are the cells that proliferate under hormonal control and the ductal and lobular cells form the vast majority of breast cancers [43, 44]. Diagram from YourSurgery.com [45].

2.1.2. Incidence and Risk.

The most common form of cancer for women is breast cancer, which accounted for 16% of all cancer mortality for women in Australia in 2004, though this is only a few percent more than lung cancer or melanoma. [46]. The lifetime risk of developing breast cancer has been estimated at 1 in 10 for the USA, [47] while women in Australia currently have an estimated 1 in 11 chance of developing breast cancer [21]. Incidence of breast cancer before the age of 20 is extraordinarily rare and remains so up to the age of 25 [44], after

which, the risk of developing breast cancer begins to steadily increase until menopause is reached. After menopause the risk still increases, but the rate of risk increase drops. While the chance of developing breast cancer increases throughout life, those cancers that develop later in life are, in general, not as malignant as a cancer that appears in a woman's early years [48]. Breast cancer also occurs in men, but it is extremely rare, accounting for 1% or less of diagnosed breast cancers. Because the growth of male breast tissue is minimal over the lifetime of men, sporadic male breast cancer is in the minority, with most male breast cancers being associated to familial mutations of the BRCA2 gene [6, 49].

Definitive causes for breast cancer have not been discovered, beyond the simple exposure to carcinogens that characterizes most cancer development. However, many additional risk factors that may contribute to the development of the disease have been identified. These tend to fall into several groups, reproductive, diet/environmental/lifestyle, familial and genetic [50]. Absolute age is also a risk factor, of course, and the one with the most utilized predictive value. Reproductive risk factors include early age at menarche, late age at menopause, overall length of reproductive span, giving birth to the first child late in life, having no children at all and the use of hormone replacement therapy (HRT) after menopause [50, 51]. Lifestyle factors include obesity, dietary fat intake, dietary alcohol intake, dietary intake of estrogen like compounds, the use of the contraceptive pill (particularly its early incarnations) smoking and exposure to other carcinogens due to personal activities, such as diet or work. Familial risk factors include a strong family history of breast cancer (first degree maternal relative with breast cancer), weak family

history of breast cancer (more distant relatives affected) or any previous breast cancers in the individual [50]. The genetic risk factors are genes which have specifically been identified to have a role in sporadic breast cancer, but may also be the same genes implicated in familial cancer. Like most implicated genes, the possession of specific alleles or mutations of the gene constitute the additional risk factor, as many of them actually have anti-cancer roles in their original states [5, 6, 16]. Additionally, there are only a few known genes which significantly increase breast cancer risk on their own, those producing the greatest risk being alterations of the NEU, BRCA1 and BRCA2 genes [6]. Other genetic risk factors may have small additive effects on breast cancer risk, despite having been identified for various other cancer types, such as the GSTM1 gene for skin cancer and the H-ras gene in oral cancers [52]. As mentioned above, these, and other genetic risk factors like them, exert far less powerful effects than the dominant BRCA genes and often exert these effects in conjunction with other risk factors, both genetic and environmental.

A further risk factor, and one which provides evidence of further genetic and environmental risk factors, is ethnicity and/or country or origin. As an example, the United States, Canada and several European countries, such as Denmark and Italy all have rates of breast cancer incidence higher (in some cases much higher) than Australia [44]. In contrast to this, countries like Japan, China and India have much lower incidences of breast cancer [44]. To some extent, these statistics may actually reflect the availability and usage of breast screening. However, these statistics may also be influenced by environmental and genetic factors within the country. Certainly Japan, as

an industrialized nation, might expect rates of breast cancer similar to Western nations, but it does not [32]. Incidence rates in an ethnic population relates to the general distribution of risk genes as well as tumour suppressors and cultural factors (like diet) which can influence cancer development [32]. One outstanding example of this is Iceland, which has extremely high incidence rates for many cancers, including breast cancer, due to the fact that the Icelandic population is descended from only a few families, who immigrated to the island several hundred years ago. These families carried several strong risk genes, including mutations to the BRCA genes, thus leading to the increased cancer rates in modern Iceland [19].

Many risk factors for breast cancer, especially age and the reproductive risk factors, are actually related to the length of time an individual is exposed to the hormone estrogen. Estrogen is one of the female sex hormones and is thus most prevalent in women, though it is found in small quantities in males. Estrogen is primarily generated by the ovaries but can also be formed in concentrations of adipose tissue, thus, being overweight increases the risk for development of breast cancer and other estrogen influenced cancer types [53]. The principle mechanism for this is the action of an enzyme called aromatase, which synthesizes estrogen from cholesterol based precursors, including androgens produced in the adrenal gland. Despite this risk increase, estrogen is a normal substance in the body and governs a number of functions in various tissues. In breast tissue, its primary function is to stimulate the proliferation of cells in order to prepare for a possible upcoming pregnancy. While estrogen is not itself carcinogenic, it is among the most important growth factors for breast tissue and directs cells to proliferate, regardless of how strongly

a given cell reacts to it [6, 49]. Proliferation is one of the more vulnerable times for the cell's DNA, hence, the longer breast tissue is exposed to estrogen, then the greater the chance that a benign but over-proliferating cell may accumulate a mutation that causes it to become malignant.

Other hormones and growth factors, both self-generated by the cell and from external sources, also play a role in the development of breast cancer. These growth factors may enhance the effects of certain mutations, may block them entirely or the mutations may function only with the assistance of these factors. These factors will often also affect each other. As an example, stimulation of the estrogen receptor induces an up-regulation of the progesterone receptor and thus an increased sensitivity to progesterone, which is an antagonist of estrogen in many tissues [54]. This effect can also be seen in the action of different forms of hormone replacement therapy (HRT). Estrogen only HRT is heavily associated with endometrial cancers, while combined estrogen/progesterone HRT eliminates this effect, but unfortunately increases the risk of breast cancer [55].

2.1.3. Familial and Sporadic Breast Cancer.

As indicated in section 2.1.2 above, breast cancer can be split into two main categories, those arising from inherited mutations, or familial breast cancer, and those arising from spontaneous mutations, or sporadic breast cancer. While family history of breast cancer is

a risk factor itself, familial breast cancer refers to heavy incidences of breast cancer in a family, which are usually attributable to inherited mutations in one of a few specific genes, including BRCA1, BRCA2 and p53 [19, 56]. Sporadic breast cancer may involve mutations in these genes, but such mutations occur within cells as they develop into tumours and are not pre-existing.

Familial breast cancers, which account for between five and ten percent of breast cancers, show several characteristics that differentiate them from sporadic breast cancer [56, 57]. Familial breast cancers normally arise much earlier in life than sporadic breast cancers, because an inherited deleterious mutation means that fewer mutation events are needed to initiate cancer than in an intact cell [19]. Familial breast cancers often occur in both breasts or multiple locations simultaneously, where sporadic breast cancers normally occur in single locations [56, 57]. Familial cancers also tend to be more aggressive and fall into similar pathways among different members of the family, unlike sporadic cancers, which show less aggression and greater heterogeneity in pathological characteristics [19, 56, 57].

2.1.4. Pathology.

Breast cancers are complicated in both their development from various molecular mechanisms and are also complex in the end result of their development, as can be seen in the large variety of possible tumours. Many different tumour types can result from

causes that are apparently the same [58]. Like almost all other cancers, breast cancers arise from damage to genes governing functions such as cellular growth, tumour suppression, DNA repair and so on [6, 10, 16, 59]. Two of these genes are the BRCA1 and BRCA2 genes. Risk increasing polymorphisms of both of these genes are primarily associated with familial breast cancers, although they also operate in sporadic cancer cases [49]. Research indicates that the BRCA1 gene is a transcription factor that acts as a tumour suppressor, while there is little evidence of BRCA2's function, save a homology to BRCA1 [49]. Risk increasing BRCA polymorphisms typically alter the gene so that it produces no functional product [60]. In most cases this is not disastrous, since two copies of the gene are present in all cells and only one functional gene is necessary for the BRCA gene to function correctly. However, individuals who have inherited a mutant, inactive, gene from one parent only require a single mutation to inactivate their single functional gene and thus abolish the gene's effects entirely [6]. Other genes are certainly involved in breast cancer development, though few of these would be absolutely restricted to breast cancer since many of these genes serve a similar function in many cells.

Breast cancers may be derived from one of several cell types present in breast tissue. The two most common forms of breast cancers are derived from ductal or lobular cells, with ductal being the more common, by far, of the two [44, 49]. Other cell types, including medullary and papillary tissue may become cancerous. These last two, along with lobular cells, rarely actually become cancerous and when they do are typically less malignant, so most research is concerned with ductal cancers [44]. Absolute rates of cellular breast

cancer incidence can be seen in Table 2.1. Ductal cells form the channels that carry milk produced in the lobules to the nipple, where it is collected for excretion. Ductal cells are subjected to cyclical proliferation and atrophy when estrogen is present or absent, in a way similar to the uteral lining [43, 51]. Due to these higher levels of proliferation (and hence DNA reproduction) ductal cells have a greater sensitivity to carcinogens and a greater rate of random mutation than other cells in the breast, which proliferate less. The higher level of proliferation also implies a greater sensitivity to growth factors, such as estrogen, which allows a new cancer cell to replicate quickly on even low levels of growth factors.

Table 2.1. Absolute Incidence of Breast Cancer by Cellular Origin (Australia, 1986-1990) modified from Kricker [44].

Cell of Origin	**Percent of Breast Cancers**
Ductal	80.9
Lobular	7.7
Mucinous	1.8
Medullary	1.7
Papillary	0.5
Tubular	1.2
Paget's Disease	1.3
Undifferentiated	0.5
Other	1.3
Unknown	3.2
Total	100

As far as prognosis for a cancer victim is concerned, one of the most important factors in breast cancer is the level of invasiveness a tumour has. Invasiveness is the ability of cancer cells to undergo metastasis, or migrate, across cellular and extracellular barriers and lodge in new areas of the body [49]. A tumour which is non-invasive will remain in a localized area and thus, is far easier to treat and less likely to leave scattered cells which can cause a relapse. Invasiveness is not an inherent trait of cancers; instead it is acquired when mutations develop in genes that control cellular differentiation or cellular binding, as well as the ability to degrade barrier and basal proteins of the extracellular matrix. There are several ways to estimate the level of invasiveness in a tumour, based mostly on evidence of accumulated mutations. These include the degree of differentiation of the tumour, non-uniformity of cells, high mitotic counts and a low percent of the tumour being composed of tubular structures [48]. Mutations in differentiation genes cause the cell to begin to lose its differentiation from other cells and thus the function it once performed, causing a greater stress on the body as it consumes more and does less [49]. Mutations in binding or adherence genes allow the cells to grow without being anchored to other cells or to the extracellular matrix, which allows the cell to float free. These mutations will allow a cancer cell to move to nearby areas of the same organ, but not outside it. Some cancers may develop mutations which allow them to break down both cellular and extracellular barriers and thus escape into the blood or lymphatic systems, which may move a cancer cell to almost any part of the body [49]. If conditions at this site are favourable, then the cancer cell will begin to grow again and will likely repeat the degradation of nearby barriers to move into the surrounding tissue.

Individual breast tumours are placed into one of three histological grades. These grades are representative of the degree of loss of differentiation and the acquisition of advanced metastatic characteristics. However, tumour grades are a rough guide and are based on phenotype rather than molecular changes [49]. Grade 1 tumours will closely resemble normal breast tissue and are still typically organized into ducts, but show some irregularity and an increased rate of growth. Grade 2 tumours still appear somewhat duct-like but are now obviously growing rapidly and are displaying aberrant or irregular growth patterns. Changes in the density of nuclear material also begin to show here. Grade 3 tumours grow in a pattern totally unlike breast tissue, if any pattern is present at all. Grade 3 tumours also display irregular amounts of nuclear material as well as irregularly shaped nuclei [49]. Examples of a tumour from each grade can be found in Figure 2.3.

At diagnosis, breast cancers are also classified into stages, from I to IV, which combine several traits into a general picture of the seriousness of the individual disease. Stage I tumours are 2 cm or less and possess no extensions or nodal metastases. Stage II tumours are up to 5 cm long, with no metastases or with metastases that show no binding to surrounding tissue. Stage III tumours are over 5 cm in length, with or without nodal metastasis. Stage III also refers to any tumour with metastases sufficient to cause adherence to nearby structures, or adherence to muscle or fascia not located on the chest wall. Stage IV tumours are any tumours with fixation to the chest wall or breast skin, or any metastasis outside of the local area [49]. These and other prognostic factors, such as

the mitotic count of the tumour, are important for determining the level and intensity of treatment required.

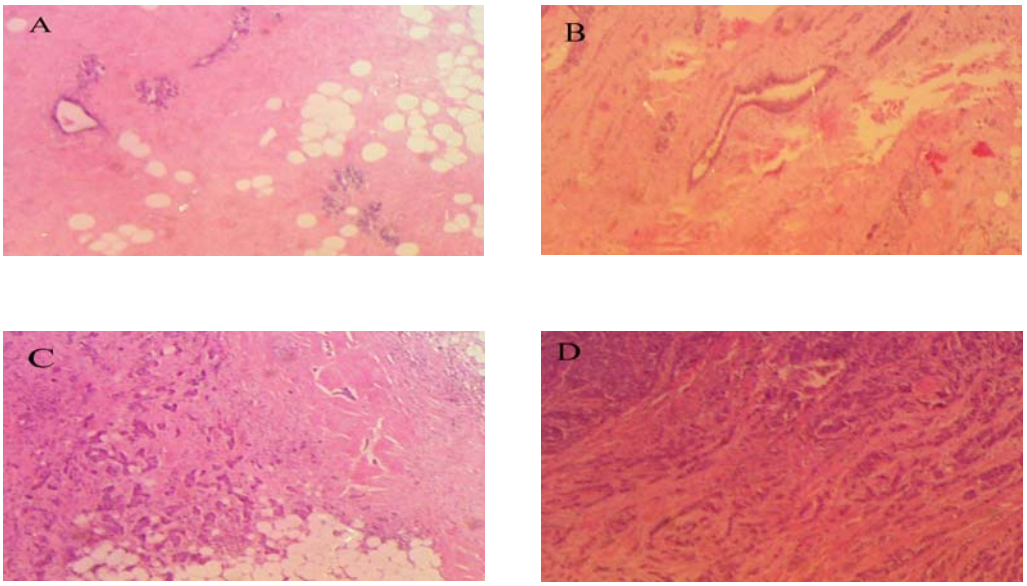

Figure 2.3. Benign Breast Tissue and Breast Tumours, Grades 1 to 3. Figure 2.3A shows benign breast tissue. The left hand side of the figure shows a section of a lactiferous duct as a purple ring. Other concentrations of alveolar and duct tissue can be seen as the purple areas, with the rest of the figure being taken up by adipose tissue. Figure 2.3B shows grade 1 tumour tissue. There is little difference between this and benign tissue, save for a greater concentration of ductal and alveolar cells, seen as concentrations of purple. Figure 2.3C shows grade 2 tumour tissue. Changes from the normal become more apparent. Ducts and alveoli still exist, but are extremely distorted. Concentration of ductal and alveolar cells increases. Figure 2.3D shows grade 3 tumour tissue. Ducts and alveoli are almost completely absent and concentration of proliferating cells is extremely high.

2.1.5. Detection and Treatment.

Treatment of breast cancers is similar to therapies used for other cancers, including radiation therapy, tumour suppression drugs, hormones and of course, surgery. However,

one of the most important factors in breast cancer treatment is detection. Since cancers can take a long time to develop and continue to accumulate mutations that make them increasingly malignant and difficult to successfully treat, detection of tumours while they are still small and relatively benign is important. Originally, the only means of detecting a breast tumour was palpation, but this method of detection can only discover relatively large tumours that are already in a state of advancement [61]. Even with this limitation, it is a useful method that can be carried out with minimal training in any place, enabling women to check themselves for possible cancer development. A more sensitive and the most commonly accepted current method of medical screening is the mammogram. Mammograms are able to detect the increased density of a developing tumour very early in its development, enabling early treatment and an improvement in patient survival [61]. Other mechanisms of detection also exist, but are not as well developed or funded as mammography. These methods include ultrasound and detection of cancer through the detection of aberrant proteins shed from the tumour and circling in the blood [62, 63].

Many industrialized nations, Australia included, have launched large initiatives to make breast screening by mammography more readily available to all women. Due to this increased usage of screening methods, more and more cancers are being discovered before they progress into more malignant and difficult to treat forms. Indeed, some 70% of breast cancers diagnosed in the United States are only Stage I at diagnosis (less then 2 cm, no metastases) [49]. Increases in general rates of breast cancer have been reported in industrialized nations in recent years and there is debate about whether or not these

numbers represent an actual increase in incidence or simply an increase in reporting rates due to the increased take-up of screening.

The increasing prevalence of early detection of breast cancers is certainly a factor in the increasing success rates in treating the disease. However, the increasing sophistication and effectiveness of the treatments themselves should not be discounted. A refinement of pre-existing drugs is an important, evolving part of breast cancer treatment. As an example, tamoxifen is an anti-estrogenic drug that is currently one of the most common drugs given to women whose tumours are hormone sensitive. Tamoxifen prevents the proliferative effects of estrogen by preventing it from binding with the estrogen receptor [18]. On the surface this would seem to be an ideal solution, but the drug can have unpleasant side-effects, including premature onset of menopause, problems with sexual function, sleep disturbance, and an increased risk of endometrial cancer [18]. Thus research is underway to alter the drug to retain its anti-estrogenic properties while eliminating (or at least reducing) the side-effects. This commonly occurs with many drugs, especially with popular and highly useful drugs such as tamoxifen. Molecular techniques have also provided therapeutic options, through the identification of physiological markers such as the estrogen receptor. The presence of this receptor generally indicates that a tumour will respond better to hormonal manipulations [59]. Identification of other alterations in tumours, both to genes and their expression, can be an invaluable aid when deciding which treatments to utilize, as the presence or absence of certain markers may indicate that a particular treatment will be useful, or useless. This also is an evolving part of breast cancer treatment, as research identifies new mechanistic

disturbances in breast tumours that alter how the cancer cells behave towards certain drugs.

Surgery is still amongst the most common treatment responses to cancer. It has the advantage of being able to remove a tumour almost in its entirety, with the few cells left behind more vulnerable to chemo- and radiotherapy. In recent years a great deal of breast cancer surgery has become breast conservative, rather than radical, as was the fashion in the past. There are several reasons for this. The first reason is that less radical surgery is far less likely to result in further damage to the body, lowering the risk of a secondary infection in a surgical wound. This is particularly important in an age when antibiotic-resistant bacteria are becoming more common. Secondly, improvements in mammography and other scanning methods enable surgeons to more easily ensure complete removal of the tumour with minimal disturbance to healthy tissue. Thirdly, a total mastectomy can be a traumatic event for a woman, the perceived loss of femininity leading to depression, lowering of self-esteem and possibly the break-up of relationships. These are conditions to be avoided for sick people and especially those with cancer, since emotional stress is known to affect health [64]. Incidentally, this kind of psychological problem can also be a barrier to obtaining screening for the cancer in the first place, as many people do not wish to entertain the possibility that they may have developed cancer. The techniques of radiotherapy have also improved over time. The ability to more selectively target tumour tissue with focused beams of radiation, as well as the use of advanced medical scanning methods all increase the effectiveness of radiotherapy and reduce its side effects [65].

2.2. The Nuclear Receptors.

2.1.1. Family and Structure.

The nuclear receptor genes are an extremely large family of genes that code for individual, but similar, molecules, which bind to various messenger molecules, usually hormones, and are typically found at or near the nuclear membrane [66]. Originally, the nuclear receptor proteins were thought to be bound to the membrane surface of the nucleus, but have been found to exist free in the cytoplasm [66]. There is also a family of more distantly related receptors, which bind the same targets as the nuclear receptors, but are found at the external membrane of the cell [67]. The steroid nuclear receptors are a sub-family of the nuclear receptors, which bind, specifically, to steroid hormones. The structure of a steroid receptor consists of a DNA binding domain, a ligand binding domain and a hinge region, connecting the two. The DNA and ligand binding domains are highly conserved to provide specificity for the target hormone and genes, while the hinge region is subject to a fair amount of alteration [54]. While these are the major domains shared by all the nuclear receptors, there are also areas on the receptor which are binding and interaction sites for the multiple accessory proteins that assist and modulate nuclear receptor function. These accessory factors typically bind to the areas whose

function they affect, such as the chaperone HSP90 binding to the ligand binding domain to keep it in the correct conformation for hormone binding. Some transcription factors are known to interact partially or fully with the hinge regions of the nuclear receptors, however, so this region serves as more than a mere connection between the ligand and DNA binding domains.

Steroid receptors only function once their specific ligand, usually a hormone, binds to them, although there are known to be several co-activator molecules [68]. Once the receptor's specific hormone binds to it, the receptor molecule moves across the membrane, into the nucleus and binds to its specific hormone response element on the genes it targets, as well as the gene’s DNA [66]. Once bound to its target genes the receptor complex alters the transcription of the target genes usually up-regulating them, however down-regulation of genes is also possible.

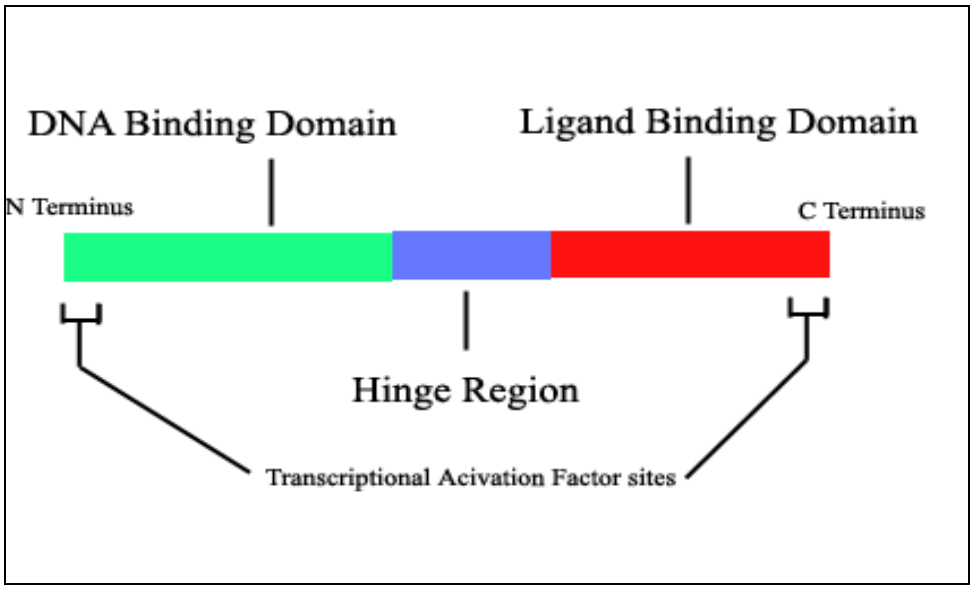

Figure 2.4. Nuclear Receptor General Structures, Based on ESRα/β. [69].

2.1.2. Function and Functional Mechanisms.

The members of the steroid receptor family have numerous functions, but are associated with the control of cellular growth and differentiation in many tissues and hence are inherently important to cancer research. The overall actions of any one of the pathways affected by steroid receptor paths are rarely simple, with many other signaling systems increasing, decreasing or modifying the final overall effect of hormone stimulation [59]. The steroid receptors also rarely perform the same function in different tissues, as can readily be seen by the presence of receptors for estrogen in both the uterus and the brain, indeed, estrogen receptors are even found in males [59]. Despite the capacity for the

pathways they mediate to be modified by other signaling pathways, the primary mechanism of action for these receptors remains the same. When activated by ligand binding, they will form dimers, attempt to migrate to their specific DNA binding sites and once there will alter the transcription of target genes [6].

The nuclear receptors act to promote or repress the transcription of certain genes though several different mechanisms, which are related to the receptor's structure as well as the structure of its targets. In their inactive state, the steroid receptors are bound to heat shock proteins, including HSP90 [70]. As mentioned above, the heat shock proteins function to keep the receptor molecule in a state to accept their specific ligand, and if the receptor is not bound to such a chaperone, it will not be able to bind its ligand. Once a steroid molecule, from whatever source, binds the receptor, the heat shock protein is released and the receptor is activated. The now activated receptor then moves across the nuclear membrane and binds with another activated receptor to form a homodimer, or a heterodimer with another isoform of the receptor or a different receptor altogether [70]. The receptor homodimer then typically binds to a specific target sequence on the chromosome, called a steroid response element, which is specific for the hormone, though there is a deal of overlap between them [71]. Once bound to the response element, the expression of the gene to which the receptor is bound is altered.

Response element bound steroid receptors are known to additionally bind hBRG1, which is a chromatin remodeling complex, suggesting that the mode of action for the steroid receptors, in this case, is via the rearranging of the structure of the chromosome [72]. Up-

regulation of genes by the steroid receptors is usually facilitated by altering the chromosome structure so that the promoters and DNA polymerase binding sequences of the gene are exposed to the enzymes that initiate transcription. Down-regulation of genes by the steroid receptors is achieved in a different manner. In down-regulated genes, the response element overlaps the TATA box, the DNA sequence that acts as a recognition site for the TATA binding protein (TBP), which is one of the initiators of transcription [70]. When the receptor binds to the response element it displaces the TBP, thus preventing the transcription of the gene. Down-regulation of genes may also occur through altering chromatin structure in such a way that the TATA box or other promoter regions necessary to begin transcription are sequestered away from the transcriptional machinery [72].

As an alternative mechanism of down-regulation, activated receptors may interfere with the DNA binding of other transcription factors, in for example, the collagenase gene, by binding DNA without initiating transcription, or by binding to the transcription factor itself [72]. Finally, steroid receptors may act as part of a sort of genetic switch, by interacting with other molecules. As an example, in the mouse proliferin gene a glucocorticoid receptor homodimer along with a homodimer of a protein called Jun bound to the gene's promoter results in a very high level of transcription. Conversely, transcription of the proliferin gene is completely stopped if the Jun homodimer is replaced by a heterodimer of Jun and another protein, Fos [70]. In order for either effect to work, an activated glucocorticoid receptor must be bound to the gene's promoter region. All responses to steroid receptor stimulation are dependent on the local

concentration of the receptor [72]. A clarification of some of these mechanisms of nuclear receptor activity can be found in Figure 2.5.

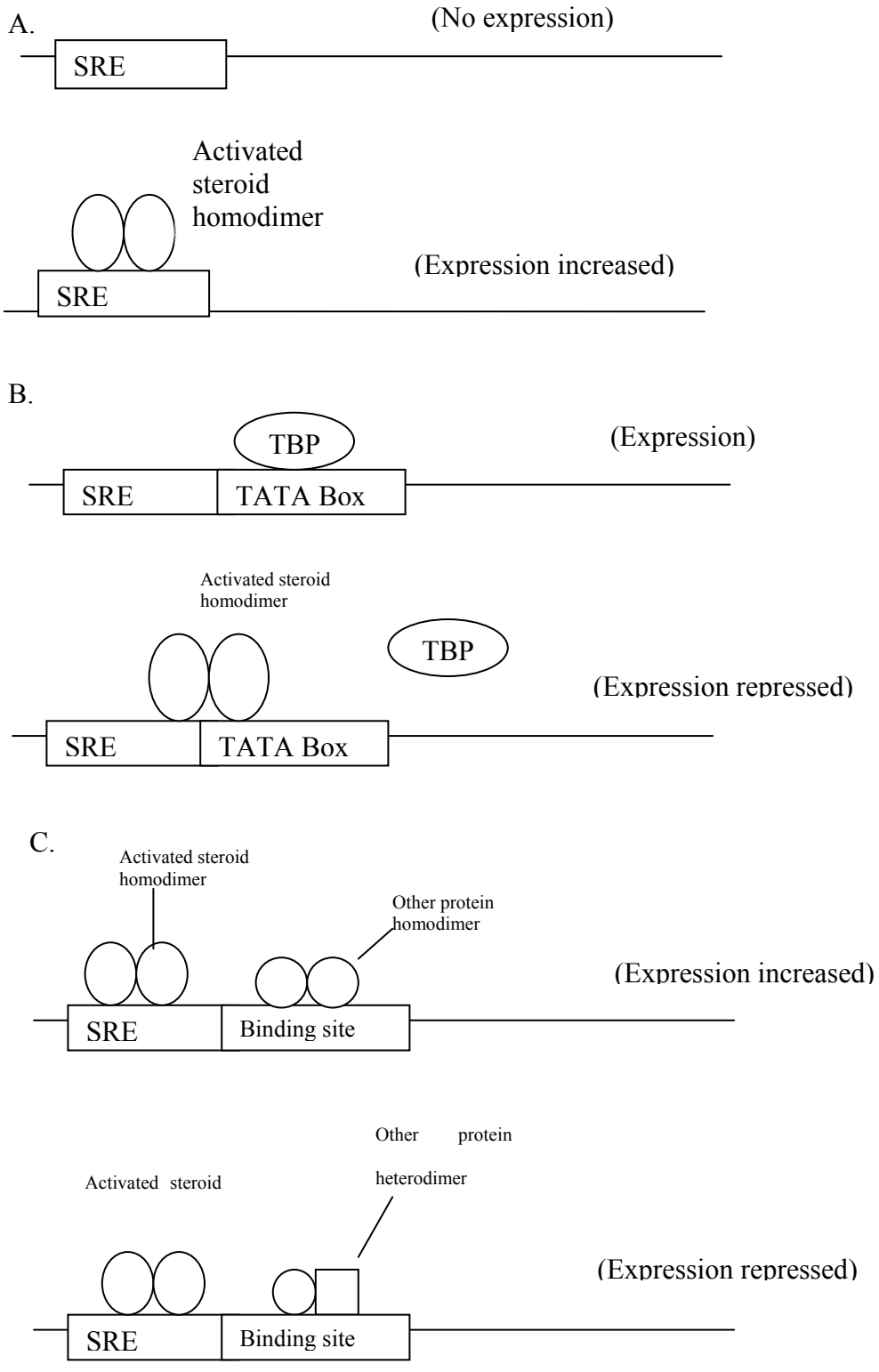

Figure 2.5. Mechanisms of Nuclear Receptor Action (Modified from [53]).

Figure 2.5A shows the primary method of steroid receptor expression induction. An activate steroid receptor homodimer binds to the steroid response element (SRE) in the gene promoter and induces a conformational change exposing the rest of the promoter to induce transcription [70]. Figure 2.5B shows one means of steroid receptor mediated repression of gene expression. In this case the SRE overlaps the TATA box, which is bound by the TATA binding protein (TBP) to initiate transcription. When the activated steroid receptor binds to the SRE it displaces the bound TBP and prevents initiation of transcription [70]. Figure 2.5C shows a steroid receptor gene expression switch. In this case, the action of the activated steroid receptor is influenced by the proteins bound at another site in the gene's promoter. If this second binding site is bound by a homodimer of another protein then gene transcription is increased, but if the site is bound by a heterodimer, then transcription is halted [70].

Nuclear receptors may also alter transcription without direct interaction with DNA. These non-genomic effects of steroids stimulation are far more rapid than those produced from genomic regulation, taking only a few minutes to effect changes rather than a few hours [73]. These interactions are believed to take place through protein interactions that may also take in transient DNA binding. Activated nuclear receptors may bind directly to other signaling proteins that are already bound to DNA, modulating the function of these already bound proteins, typically resulting in down-regulation of the targeted gene [73]. The nuclear receptors may also function as a form of second messenger, by binding to other signaling molecules, such as SRC kinases, and inducing their activation and subsequent effects on an entirely different set of genes than are affected by the traditional

DNA binding effects [74]. This mechanism has not been conclusively proven, but interactions with the SRC kinases have been shown to occur [74].

The specific effects of the nuclear receptors may also be modulated by changes to the binding of particular cofactors to the activated receptor, splicing of mRNA prior to translation, or modification to the translated protein [54, 75]. The population of cofactors present within the cell will alter the results of hormone stimulation, as changes in what proteins are bound to the receptor dimer will alter the complex's affinity for hormone response elements. Additionally, the binding of certain cofactors may be required for the dimer to bind to response elements, displace inhibitor proteins or commence the DNA remodeling required to alter gene expression [75-77].

The alternative splicing of nuclear receptor exons into different receptor isoforms represents one of the major methods within the cell to modulate the effect of hormone stimulation. These receptor isoforms may have altered affinity for the receptor's ligand, other receptors, hormone binding elements, and receptor co-factors, thus altering the response of the cell to introduction of hormones. One of the more common changes in an isoform is a reduced affinity for the receptor ligand, resulting in a receptor that does not respond to hormonal stimulation [54]. Such isoforms usually function to repress the primary isoform by interfering with the formation of dimers and the recruitment of cofactors [54, 75].

Finally, post translational modifications to nuclear receptor proteins can result in significant changes to the response of cells to hormone stimulation. Post translational modification is the addition of small additional molecules to the protein at specialized sites by specific enzymes or enzyme complexes, which in turn alter the behavior of the protein [75, 78, 79]. Such modifications include phosphorylation, acetylation, sumoylation, sulfation and ubiquitination, among others. Phosphorylation is particularly common and typically increases the activity of the ligand bound receptor, with phosphorylation of multiple sites corresponding to greater activity [75]. In contrast, ubiquitination is a signal for degradation mechanisms to remove the tagged protein to the proteosome for degradation, thus ablating the effect of hormone stimulation [75]. Other post translational modifications may repress or increase receptor function either directly, or through encouraging the binding of specific cofactors to receptor complexes [75, 79].

2.1.3. Nuclear Receptors in Breast Cancer Treatment.

One of the major ways in which hormone receptor systems are manipulated in breast cancer treatment is through the use of receptor antagonists. Antagonists usually work by binding to inactive receptors, blocking the hormone binding domain and preventing activation of the receptor. The most widely used of these in breast cancer treatment is tamoxifen, an antagonist of estrogen. However, it should be noted that some of these antagonists bind to several receptors and are not always wholly antagonistic to the

receptors to which they bind. Some antagonists, like the actual hormones, may also initiate different responses in different tissues. It is believed that the composition of the gene promoter regions may influence how a gene will react to being bound by a nuclear receptor which has been itself bound by an antagonist [66]. An example of this behaviour is the drug RU486, which was originally developed as a progesterone antagonist, but was also found to have anti-glucocorticoid properties [80]. RU486 has been found to act as a glucocorticoid antagonist in breast cancer cells, but to act as a mild GR agonist in osteosarcoma cells [81]. Additionally, the presence or absence of certain cofactors may also modulate how the antagonist functions within the same tissue type. Significantly for breast cancer, tamoxifen has been known to cause growth induction rather than repression in certain tumours. This alteration in function is linked to an increase of estrogen receptor beta expression, along with modulation from the activator protein AP-1 [69].

Antagonism of the nuclear receptor pathways is not the only mechanism used to treat breast cancer. Many of the nuclear receptors repress breast tissue growth when stimulated by their proper ligands, including the glucocorticoid, progesterone and androgen receptors [24, 70, 71, 82, 83]. Progesterone in particular is favoured as a breast cancer treatment, being an endogenous hormone with few side effects. Progesterone encourages a slowing of growth and differentiation in breast tissue as part of the normal processes that prepare the breast for milk production, effects which are partly exerted through down-regulation of the estrogen receptor. This cross-talk with the estrogen receptor means that progesterone treatment is typically coupled with tamoxifen to maximize the

overall effects of the treatment [18]. Androgens, which likewise limit breast tissue growth, are also used in breast cancer treatment. However, the direct application of male hormones can have significant side effects on women, so this treatment is reserved for those tumours which show poor response to other hormonal treatment regimes.

There are limitations with hormonal treatments however, and these principally revolve around the expression and modification of the receptors. Many advanced cancers lose the expression of various nuclear receptors, for a variety of reasons [18]. For anti-mitogenic receptors, there is a selective pressure for tumours to lose this expression. For mitogenic nuclear receptors this loss of expression may be a result of becoming either independent of the growth factor, or by producing mutant, constituently active, receptors that need no ligand to stimulate them. Alternate isoforms of the receptors may also result in a receptor that performs an antagonistic function in response to normal ligand stimulation [54, 84]. An additional problem with steroid treatments is that stimulation of the receptors usually results in a down-regulation of the receptor, reducing sensitivity to the ligand. Thus the treatments must be discontinued or other drugs given to restore tissue sensitivity, which may give tumours a chance to develop resistance.

CHAPTER 3. Methodology.

3.1. Method Design.

The principle focus of this PhD study was the determination of the expression of the hormone nuclear receptors and allied genes in different breast cancer grades as well as in control tissue. The levels of mRNA present within the tissues were compared to one another, to the alleles of breast cancer candidate genes and to pathological markers found within the tissues, to determine what factors, if any, are associated with breast cancer progression from one grade to another. Several methods were utilized to determine these factors, the theories behind them being described in the following sections.

3.1.1. Polymerase Chain Reaction.

Polymerase chain reaction, or PCR, is an invaluable tool for genetic studies and is used in a wide variety of applications. PCR allows a researcher to amplify specific areas of DNA through the utilization of a thermostable DNA polymerase originally derived from a hot water dwelling bacteria [85]. There are several steps required for the completion of a PCR. First, double stranded DNA must be denatured by heating to allow short sequences

of DNA called primers to bind to the boundaries of the area or areas to be amplified. These primers match exactly the DNA sequence found at the boundary sites and so will only bind to these sites. The primers then serve to signal the DNA polymerase to bind to and begin replication of the target area, utilizing individual nucleotides as raw material. Once the area is amplified, the process can be repeated by reheating the DNA to remove the newly synthesized strands and allow another set of primers to bind, each time the cycle is repeated doubling the amount of target DNA [59]. This exponential and predictable increase in DNA quantity for a specific amplicon allows PCR to be used in the variety of applications, including allelic determination and the detection of gene expression.

3.1.1.1. Semi-Quantitative PCR.

Due to the predictable rate of amplification in PCR, the method can also be used to determine the original amount of DNA or RNA present in a sample derived from a cell or cells. The brightness of a DNA band on an imaging gel, either by fluorescent primer tagging or basic UV fluorescence, is directly proportional to the amount of DNA present, and can be quantitated by software or dedicated imaging instrumentation [85]. While the rate of DNA amplification in PCR is theoretically predictable, in practice, there are variations in the rate in individual PCRs, so to obtain an accurate quantitation of the amount of DNA present, a technique known as real time PCR is used, which examines

the amount of DNA present at the end of every cycle, allowing a curve to be generated, from which a true number of original copies can be determined [86].

However, it is known that cancer cells often show generalized increases in mRNA levels, which are not representative of changes to the regulation of any specific gene [86]. Therefore, if any meaningful comparison of mRNA levels for specific genes among samples is to be made, the general variation of RNA levels between different cancer tissues and normal tissue must be controlled for. This can be done by performing a real time PCR of a ubiquitous gene which is expressed at a basal level in all cells, and is indicative of the general metabolic rate of the cell [59, 85]. The abundance of the ubiquitous gene is then compared to the abundance of the gene of interest. This gives a relative abundance, but unless the reactions can be performed simultaneously, error due to variations in the efficiency of the PCR reactions is introduced. While methods exist to allow simultaneous real time PCR, the required instruments were not available for performing the present study.

Since the measure of mRNA expression for this study only required a relative measure of expression between the control and gene of interest, the level of accuracy provided by real time PCR was not required. Instead, semi-quantitative PCR was used. This involves performing a PCR which simultaneously amplifies the gene of interest and a ubiquitous control gene to control for variations in the efficiency of the individual PCRs. The PCR is not allowed to run to the point of depleting reagents, being allowed only 25 replication cycles, which was insufficient for reagent depletion using 60 μg of genomic DNA. After

the PCR is completed, the relative expression of the gene of interest is determined, by expressing it as a ratio of the amount of the gene of interest to the positive control gene. The method does not allow a direct quantitation of mRNA expression for either the gene of interest or the ubiquitous control, but the relative ratio allows comparison of mRNA expression across multiple cancer grades and samples.

3.1.2. cDNA Synthesis.

However, polymerase chain reactions cannot amplify RNA directly, so if mRNA is to undergo PCR, it must first be changed into a form that the DNA polymerase in the PCR reaction is capable of amplifying. This can be accomplished by using the enzyme reverse transcriptase, which polymerases DNA, using RNA as a template. The process is similar to PCR, except that RNA is single stranded, so no temperature cycling is required, save a single increase in temperature to cause the RNA to disassociate any self-binding that has occurred [85]. The cDNA is transcribed using individual nucleotides as raw materials and are assembled once the reverse transcriptase is bound to the single stranded RNA.

As with DNA in PCR, RNA must be primed for reverse transcriptase to begin the process of cDNA transcription, a function performed by either short repeats of thiamine nucleotides (oligo dTs), or short sequences of random nucleotides. Oligo dTs bind to the sequences of adenine nucleotides, termed poly A tails, found at the terminal sequences of mRNA, which are signals to cellular machinery to translate the RNA into a protein [2,

85]. The randomized nucleotides will bind to, and cause the transcription of, any kind of RNA. RNA is relatively fragile, and embedding in paraffin causes some level of damage to RNA by cross-linking it to other molecules, even as it preserves it by stabilizing its structure. This means that mRNA derived from paraffin embedded tissue may have lost its poly A tail and not be able to be transcribed using an oligo dT primer. Hence, random primers were used in this study to ensure that the maximum possible amount of mRNA would be transcribed, controlling for varying levels of damage caused to different RNA sequences.

3.1.3. Statistical Analysis.

Analysis of variance, or ANOVA, is a parametric method used to examine changes in quantitative values in multiple categories, using an F-curve random distribution. ANOVA is robust against low population sizes and large outliers, and so is useful for this study, which had a limited number of tissue samples available for analysis. Despite ANOVA's robustness against outliers, it is a parametric test, so in the case of a significantly abnormally distributed population of expression data, the Kruskal Wallis test for independent samples was used. The Kruskal Wallis test is a non-parametric test that utilizes the same basic method of ANOVA. However, since it is not subject to the same limitations, it is a valid method to check the veracity of ANOVA results where those limitations are breached.

3.2. Method Application.

3.2.1. Tissue Population.

The tissue population for this study was comprised of 25 archived breast tissue sections embedded in paraffin and fixed with 10% buffered formalin on slides. The sections were 10 microns thick and were supplied with H&E stained slides as a reference for tumour location. All tumour samples were diagnosed as infiltrating ductal carcinoma, the most common form of breast cancer. There were 6 samples from tumour grade 1, 7 samples each from grades 2 and 3 and 5 samples of benign breast tissue taken from unaffected patients as the control population. The average age of the individuals from whom the biopsies were obtained were 56.88 years, 59.18 years, 60.45 years and 55.93 years for the control and grades 1, 2 and 3 groups, respectively. The archival breast tissue samples were obtained through collaboration with the Pathology Department of the Gold Coast Hospital, with relevant ethical approvals. For consistency, cancer grade for each sample was determined by a single pathologist from the Gold Coast Hospital's Pathology Department. Samples selected had no indication in medical history of a familial basis for the breast cancer. There was a variety of pathological data available for the population, the most important of which was immunohistochemical staining to detect ESRα proteins

and a summary of the population's ESRα status can be found in Table 3.1. Samples were considered to be ESRα positive if the tumour cells themselves had stained positive for ESRα. Samples where tumour cells had no staining or a clinically insignificant amount of staining were considered ESRα negative.

Table 3.1. Population ESRα Immunohistochemical Staining Status.

Tumour Grade	ESRα Positive	ESRα Negative	Total
Control	5	0	5
Grade 1	6	0	6
Grade 2	5	2	7
Grade 3	2	5	7

3.2.2. RNA Extraction.

RNA extraction was performed once for each tissue sample for all PCR based methods. Tumour tissue was first separated from the stroma and then from proteins, DNA and other contaminants, processed to protect the RNA for eventual cDNA synthesis and finally purified ready for semi-quantitative PCR.

3.2.2.1: Microdissection.

Prior to further processing, tumour tissue had to be separated from the surrounding stroma. This was done in order to avoid contaminating tumour RNA with RNA from the

stroma, which is known in many cases to have different genes in operation [35]. Separation of tumour and stroma was also performed to allow for distinct analysis of the expression levels in tumour and stroma. Separation of tumour tissue was achieved by cutting tumour tissue away from the slide using a scalpel blade under a stereo microscope. The H&E stained section was used to determine the location of the border between tumour and stromal tissue. Tumour tissue was differentiated by the increase in nuclear density and therefore purple stain on the H&E slide. Though the tissue was examined by a pathologist at the Gold Coast hospital to ensure there was little stromal involvement with the tumours, the inability to use laser-capture microdissection on these tissue samples does raise the possibility that some stromal elements were removed along with tumour tissue. An example of a microdissected slide with the tumour removed next to its H&E slide can be found in Figure 3.1, below. Control tissue did not undergo microdissection, instead the entire slide was removed by scalpel for RNA extraction. Due to difficulties in obtaining accurate measurements for the tumour samples, tissue was not weighed.

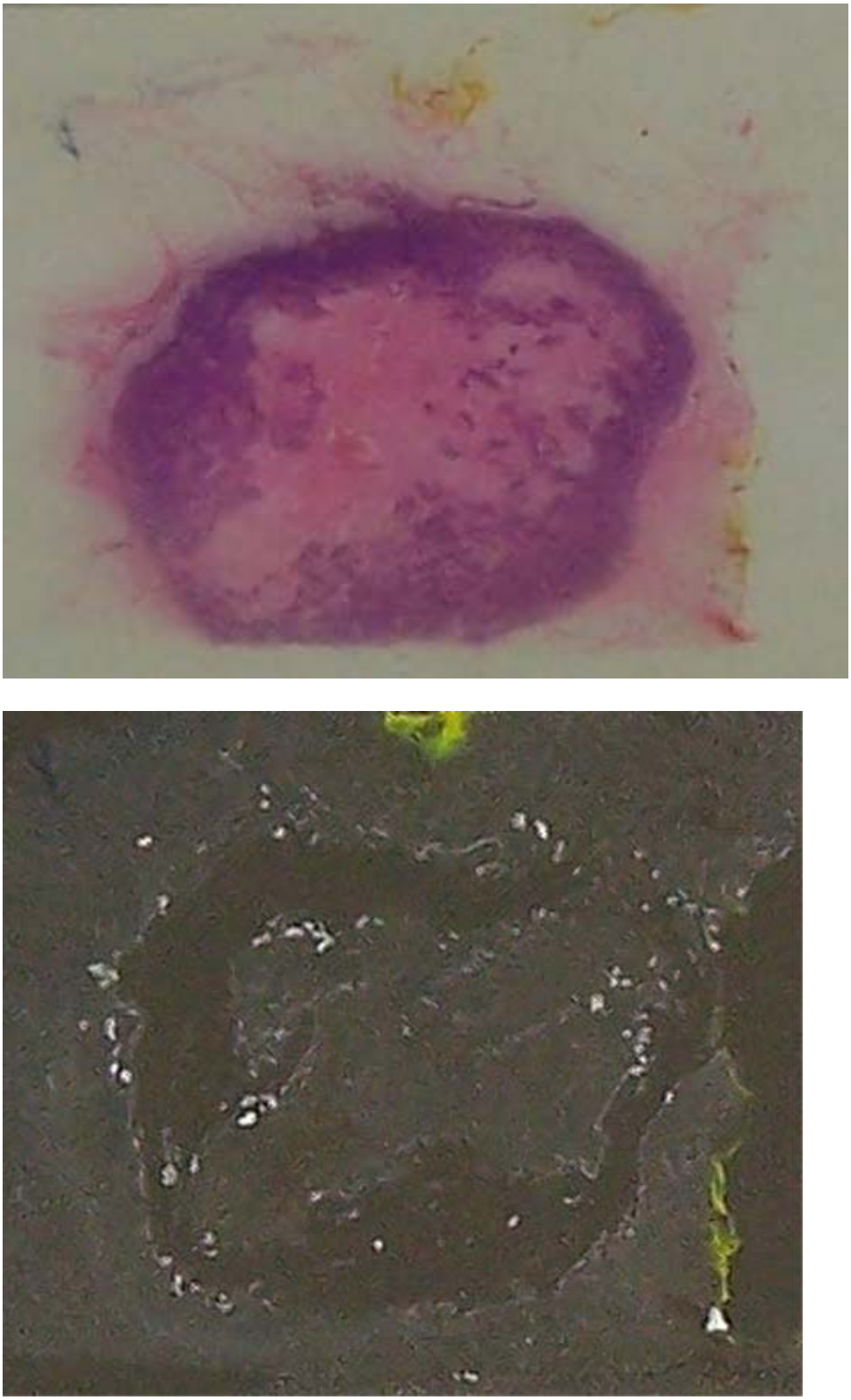

Figure 3.1. Microdissected Tissue. Tumour tissue can be seen as the highly dense purple stain on the H&E slide above. Below the H&E stained slide is a microdissected tissue slide, showing that only the tumour tissue has been removed.

3.2.2.2: Paraffin Removal.

Embedded in the paraffin wax of the slides the RNA within the tissues was preserved, but before the RNA could be used, it needed to be unbound from the paraffin and the paraffin removed. This was achieved by placing the microdissected tissue in 1mL of xylene in an Eppendorf tube, allowing that to dissolve the paraffin for two minutes. The samples were then centrifuged for 5 minutes at 12,500 RPM to drive the tissue and RNA to the bottom of the tube, allowing for the removal of the xylene and dissolved paraffin. This xylene wash step was then repeated to remove any residual paraffin from the sample. After this, 1mL of cold ethanol was added to the tissue to protect the RNA and dilute any remaining xylene to prevent interference with the following enzymatic processes. The samples were then centrifuged again at 12,000 RPM for 5 minutes to drive the tissue to the bottom and allow removal of the ethanol and residual xylene. Finally, the ethanol wash step was repeated, using 95% ethanol in DEPC treated water, to rehydrate the samples for separation and protection.

3.2.2.3: RNA Separation and Protection.

With the paraffin removed, the RNA now needed to processed to remove excess tissue proteins and cell membrane fragments so that enzymes to protect the RNA could be applied and preliminary purification could occur.

First, 500 μL of Trizol reagent (from Invitrogen) was added to the tissue and the tissue homogenized, by drawing the tissue in the Trizol repeatedly through an 18 gauge needle to break up any remaining large pieces and maximize the surface area available for the Trizol to work on. After homogenization, an additional 500 μL of Trizol was added and allowed to incubate at room temperature for one hour to digest the tissue fragments.

When digestion was completed, 200 μL of chloroform was added to the samples, which were then vigorously shaken to dissolve the RNA in the chloroform. The samples were then centrifuged at 12,000 RPM for 15 minutes to separate the solution into layers, with the RNA remaining dissolved in the upper phase. This upper phase was then removed and the remainder of the solution discarded.

The solution containing the RNA was then added to 500uL of isopropanol alcohol and incubated at room temperature for 30 minutes to allow the RNA to precipitate out of solution. The solution was then centrifuged for 15 minutes at 12,000 RPM to drive the RNA to the bottom of the tube and allow for removal of the isopropanol and chloroform. 1 mL of 75% ethanol in DEPC treated water was then added to the samples and mixed

briefly to rehydrate the samples and remove residual chloroform and centrifuged again at 10,000 RPM for 15 minutes. The rehydration step was repeated to ensure the cleanliness of the RNA. Following this, the RNA pellet was allowed to air-dry briefly.

The RNA was then resuspended in 100 μL of pure DEPC treated water, and 2.5 μL of Rnasin (from Promega), an inhibitor of RNases added to the RNA and the solution incubated for 10 minutes at 55 degrees Celsius to protect it for the following procedures. After Rnasin incubation, another 40 μL of DEPC water was added to each sample, along with 20 μL of Dnase buffer, 20 μL of 100 mM DTT and 5 μL of Dnase I (from Promega). The samples were then incubated at 37 Celsius for 30 minutes to digest any genomic DNA that remained in the samples.

3.2.2.4: RNA Purification.

After DNA digestion, the RNA was purified by using Rneasy Mini-Kits from Qiagen, which bind RNA to a glass membrane, allowing contaminants to be washed away in solution.

First, 200 μL of ethanol was added to each sample and mixed briefly. Then 200 μL from each sample was placed in an Rneasy mini column (2 per sample) and incubated for 5 minutes at room temperature to allow the RNA to bind to the glass membrane. After this, the columns were centrifuged for 20 seconds at 12,000 RPM to remove the ethanol and

larger contaminants. The columns then had 700 μL of the supplied RW1 buffer added and the columns then centrifuged at 12,000 RPM for 20 seconds to wash away additional contaminants.

The columns were washed a second time using 500 μL of the provided RPE buffer, centrifuged at 12,000 RPM for 20 seconds to remove the solution and this step was then repeated, with centrifugation occurring for 3 minutes to remove any final contaminants. After this, a new collection tube was placed on each column and 30 μL of Rnase free water was added to the columns and incubated at room temperature for 2 minutes to allow the RNA to unbind from the membrane. The column was then centrifuged for 1 minute at 12,000 RPM to elute the RNA into the collection tube. This step was repeated a second time to elute any RNA that might remain on the membrane. The split samples for each individual tissue slide were then pooled for cDNA synthesis.

3.2.3. cDNA Synthesis.

cDNA synthesis for the semi-quantitative PCR portion of the experiment simply required the purified RNA to be subjected to reverse transcription, using random primers to transcribe RNA that may be fragmented due to being bound into paraffin wax. cDNA synthesis was performed by heating the master mix in table 3.2 below to 65 Celsius for five minutes to dissociate any secondary structures formed by the RNA and then

incubating the mix for 2 hours at 42 Celsius, the working temperature of the reverse transcriptase.

Table 3.2 cDNA Synthesis Protocol.

Reagent	Concentration	Quantity per Reaction
Reverse Transcriptase Buffer	10x	2 μL
dNTPs	5μM	0.5 μL
Random Primers	3μg/μL	3 μL
Reverse Transcriptase	200U/μL	0.2 μL
Rnase free Water	-	As Required
RNA Template	2ng	As Required
Total	-	25 μL

3.2.4. Polymerase Chain Reaction.

3.2.4.1: PCR Conditions.

Each PCR reaction in this study utilized a unique set of chemical conditions, which are described fully in the chapters covering the individual experiments. However, all semi-quantitative PCR reactions included two common components, the cDNA for the reaction and the DNA primers for the 18S ribosomal RNA, a ubiquitous RNA signal used to provide the control for any general increase in cancer mRNA expression. The amount of

cDNA per reaction was a total of 6ng, with volume adjusted for the cDNA concentration of each individual sample. 18S was chosen as the ubiquitous control in these studies for a variety of reasons. Firstly, prior work in our laboratory has found that β-actin, a control gene often used in other studies, shows tremendous variation in cancer cells, and so is not suitable as an internal control in a cancer study [87]. Other commonly used ubiquitous RNA, including GAPDH and even 18S itself also show a degree of variation in cancer cells. However, studies have shown that 18S has a relatively low deviation of expression between different cell lines, a situation similar to this study, which is examining expression in tumours derived from different sources, as cell lines typically are [88]. All forward primers for semi-quantitative PCR were labeled with a fluorescent dye, which allowed the PCR product to be visualized in an ABI 310 Genetic Analyzer to allow quantitation. Details for the 18S primer are shown in Table 3.3, below.

Table 3.3. Primers for 18S ribosomal gene.

Primer Name	**Sequence (5'-3')**
18S-A#	CTTAGAGGGACAAGTCGCG
18S-B	GGACATCTAAGGGCATCACA

= Primer labeled with HEX at 5'

All of the primers used in this study were designed using published mRNA sequence data from the NCBI sequence database. Exon boundaries were determined using a human BLAT search and suitable primers designed by hand. Primers were checked for non-specific binding by subjection to a BLAST-N search at NCBI

(http://www.ncbi.nlm.nih.gov/BLAST/) and rejected if binding an E value less than 1 was obtained to any human DNA/mRNA sequence not that of the target gene.

Polymerase chain reactions were optimized using genomic DNA and cDNA derived from T74D cells for all receptors where possible (ESRα, PgR, GR, NCoA genes) Where this was not possible, or failed (all other genes) PCR reactions were optimized using genomic DNA. Each PCR set also included genomic DNA to act as a positive control to ensure that the reaction had succeeded. PCRs for each sample were performed in triplicate and the genomic controls checked using an agarose gel before being pooled for quantitation of amplicons.

3.2.4.2: Thermal Cycles.

Because all PCRs for semi-quantitative PCR share the 18S primer to control for both PCR efficiency and baseline expression variation, with the primers for the individual nuclear receptors being designed to bind to DNA at the same temperature, all PCRs shared the same thermal cycling conditions, which are outlined in Table 3.4, below.

Table 3.4. Thermal Cycling Conditions for Semi-quantitative PCR.

Temperature (C)	**Time of Cycle (Mins)**	**Number of times Repeated**
95	5:00	1
95 57 72	1:00 1:30 1:00	25
72	7:00	1

3.2.4.3: Data Reading for Semi-quantitative PCR.

Each PCR for semi-quantitative PCR was performed in triplicate and the individual PCR products placed in an ABI 310 Genetic Analyzer for quantitation. The genetic analyzer produces a histogram of each labeled DNA fragment for a PCR reaction, ranking them by size and the dye used, as depicted in figure 3.2, below. The histogram records both height and area of the peaks, both of which reflect the amount of PCR product present. Analysis indicated that peak height and peak area were directly related, so peak height alone was used as the measure of quantitation. Peak height for the gene of interest was divided by the peak height for 18S and the ratios averaged between the triplicates to give the final determination of NR expression.

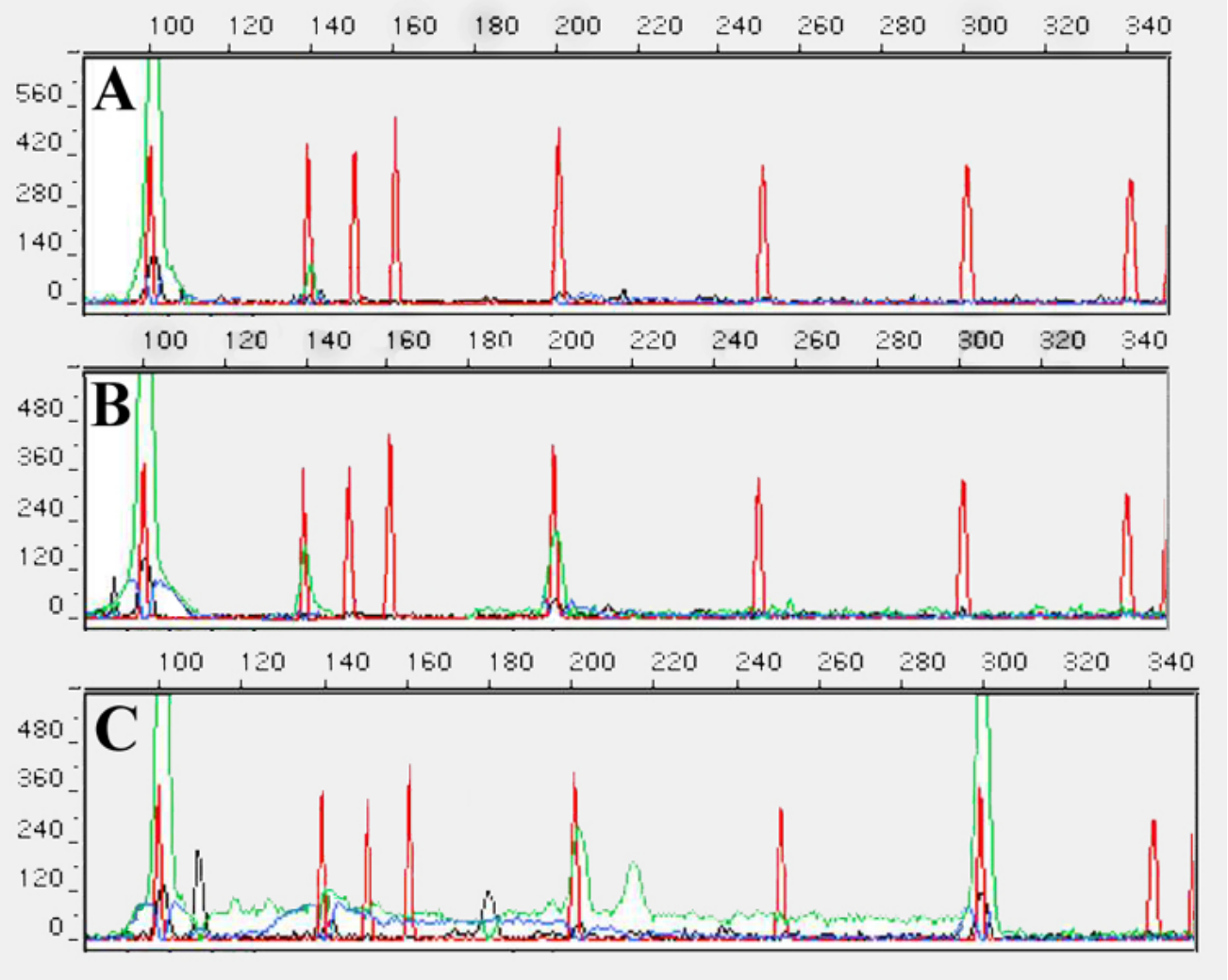

Figure 3.2. Sample Genetic Analyzer Output. The red peaks are the size standard fragments for the base pair sizes indicated on the top of each readout, while the green peaks are those from the produced from the nuclear receptor gene (GR). The 18S fragment is 60 base pairs in size, and is off frame to the left. Figure 3.2A shows a standard output. Figure 3.2B shows an output with noise generated by the size standards. Figure 3.2C shows an output from a genomic DNA sample, with some sample noise and the genomic peak at 300 base pairs.

CHAPTER 4. The Progesterone and Glucocorticoid Receptors.

4.1. The Progesterone Receptor.

4.1.1. Background.

The progesterone receptor, as for many transcription activating factors, has different effects on different tissues, a function partially mediated by the existence of several different isoforms of the receptor. These isoforms differ slightly in their structure and the accessory molecules attached to them, which help to act on the DNA, initiating or blocking transcription [54]. The primary form of the progesterone receptor (PgRα) acts as an antagonist to several other members of the steroid receptor including, most importantly in breast cancer, the alpha estrogen receptor [54]. This helps to explain why cancers expressing both the progesterone and estrogen receptors are more likely to respond to hormone-based treatments. In such cases, not only can the action of the estrogen receptor (which induces cell growth) be blocked by anti-estrogens, but also by the use of progesterone. Interestingly, expression of the progesterone receptor is actually up-regulated by estrogen stimulation [89], though this sort of relationship is actually quite

common as a feedback mechanism to keep stimulation by both steroids in check. Having this particular link intact is part of what makes ESRα/PgR positive tumours sensitive to hormone treatments.

Like most of the other nuclear receptors, the progesterone receptor has other functions, beyond its interference with the estrogen receptor, though many of these additional functions are related to this major one. Almost all of these additional functions are related to reproduction [82]. The progesterone receptor gene itself is located at 11q22-23, contains eight exons and is approximately 90kb long [90]. The alpha isoform of the progesterone receptor (PgRα) is a 98 kDa protein. As mentioned above, the primary role of PgRα as a repressor of ESR and other nuclear receptors, including the beta PgR isoform, though it also functions in the activation of certain genes [84]. The beta isoform of the progesterone receptor (PgRβ) is larger than the alpha isoform at 116 kDa and, in general, acts as an activator of gene expression [84]. While the two isoforms of PgR have opposing effects, there appears to be only a little overlap in the genes they target, each isoform regulating a sub-set of genes, despite their shared DNA binding domains [91]. Both the PgR isoforms are derived from the same gene, though appear to be activated by different promoters. PgRα is does not contain any additional exons as compared to PgRβ and is a truncated version of this isoform [92].

Many of the progesterone receptor's functions focus on reproduction, particularly in the uterus [82]. Progesterone has, in tandem with estrogen, an important role on the

proliferating endometrium, the transformation from proliferating to secretory state and once in that state, the maintenance of the endometrium [82]. In these roles, the progesterone receptor is vital to the implantation of a new fetus in the endometrium and thus, the initiation of pregnancy in general [82, 84, 90]. The other major function of the progesterone receptor in pregnancy is prevention of early labour by keeping the musculature of the uterus in a quiescent state [84]. Interestingly, progesterone levels continue to rise throughout pregnancy, so the onset of labour is controlled by some means which prevents the expression or activation of the progesterone receptor [84]. There is some thought that this mechanism is, in fact, the expression of the PgRα isoform, since it is known to repress the action of the beta isoform, but because of the difficulty in distinguishing the two, this has not been established for certain [92]. Finally, aside from these uteral functions, the progesterone receptor has a role in breast tissue. Partially by interfering with the estrogen receptor and partially by the direct induction of differentiation, the progesterone receptor slows the growth of breast epithelial tissue. This is part of the normal function of the tissue, and is in preparation for milk production [3, 91]. The function of the progesterone receptor as an estrogen receptor inhibitor is important in breast cancer, where it can be utilized to block the growth signals produced by exposure of breast tissue to estrogen [53]. This role in combinational therapy is increased by progesterone receptor's ability to increase the expression of ESR, helping to keep a supply of the receptor available to be bound by estrogen modulators.

4.1.2. Method.

To investigate how the expression of the progesterone receptor is affected in cancer tissue, semi-quantitative PCR was used. Fluorescently labelled primers recognising a sequence in exon 4 of the progesterone receptor gene were designed, in order to obtain a sufficiently specific amplicon for quantitation of the receptor. These primers were not able to distinguish the different forms of the receptor and so only gross expression results were obtained. In order to control for the presence of genomic DNA contamination in the samples, a third primer, recognising an intronic sequence following the exonic sequence, was designed and run in the same PCR. Control for PCR efficiency and reference for the relative quantitation was provided by the multiplex PCR of fluorescently labelled 18S RNA primers as outlined in Chapter 3. See Table 4.1 for the sequence of the primers used and Table 4.2 for the chemical conditions of the PCR. After PCR, quantitation was performed by an ABI 310 genetic analyser, and then normalised by expressing it as a ratio of PgR/18S for that sample. Normalised PgR expression data was then analysed by ANOVA as detailed in Chapter 3.

Table 4.1. Primers for Progesterone Receptor PCR.

Primer Name	**Sequence (5'-3')**
PgREX4-F1*	ATTGATGACCAGATAACTCTCCAT
PgREX4-R1	CTGACGTGTTTGTAGGATCTC
PgREX4-R2	GTAGTTAATTTACTGCATAGAGTG
18S-A#	CTTAGAGGGACAAGTCGCG
18S-B	GGACATCTAAGGGCATCACA

*= Primer labeled with TET at 5' #= Primer labeled with HEX at 5'

Table 4.2. Chemical conditions for PgR PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix D (From Epicentre)	5x	5.5 μL
PgR Forward Primer	5μM	2 μL
PgR Reverse Primer	5μM	2 μL
PgR Intronic Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	2 μL
18S Reverse Primer	5μM	2 μL
BE polymerase	1/5 dilution	0.3 μL
cDNA	-	2 μL
Total	-	17.8 μL

4.1.3. PgR Results.

The normalised data for the PgR gene showed a specific trend. Expression levels of PgR increase from normal to cancer tissue, and continue to increase as cancer grade increases (see table 4.3 for PgR data). After analysis by ANOVA, a significant difference was found between tumour grades for PgR expression. However, it was noted that several outlier values were present in the data, especially in grade 3 tissue. Considering the heterogeneous nature of breast cancer, it was decided that these outliers were valid data and in order to help clarify the results, the data was re-analysed using the non-parametric Kruskal-Wallis test for K independent groups. Kruskal-Wallis analysis confirmed a significant difference and post-hoc tests were then applied to discover where the significant difference lay (see table 4.4 for ANOVA and K-W results).

Table 4.3. Data for PgR Expression Across Tumour Grade.

Tumour Grade	Average Ratio (PgR/18S)	Std Dev. (PgR)
Grade 1 Samples	0.913	0.458865
Grade 2 Samples	1.051	0.216025
Grade 3 Samples	10.384	11.67608
Control Samples	0.565	0.171585

Table 4.4. ANOVA and Kruskal-Wallis Results for PgR.

Results for ANOVA		**Results for K-W Test**	
Statistic	Significance	Statistic	Significance
F= 3.9	0.023	χ= 15.869	0.001

Post-hoc tests indicated that the expression of the progesterone receptor was significantly elevated in grade 3 tumour tissue, compared to control and all other tumour grades. The results for the progesterone receptor are illustrated in figure 4.1.

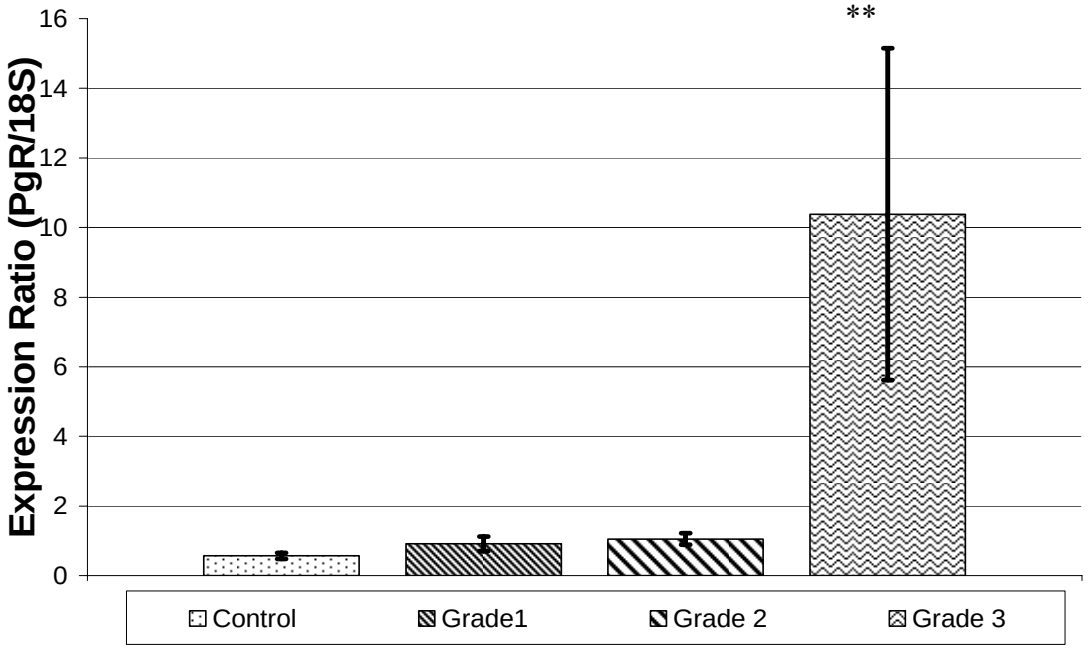

Figure 4.1. Expression Results for Progesterone Receptor.

Analysis was also carried out for PgR with respect to ESRα protein status. Initial observations indicated that expression of PgR was higher in ESRα negative tissue, and after ANOVA analysis, this was found to be the case. PgR data for ESRα protein status is

summarized in Figure 4.2, while expression ratios can be found in Table 4.5 and ANOVA results can be found in Table 4.6.

Table 4.5. Data for PgR Expression by ESRα Status.

	Ratio (PgR/18S)	**Std. Dev.**
ESRα Positive Tissue	1.259563	1.494204
ESRα Negative Tissue	9.382208	12.29238

Table 4.6. ANOVA and Kruskal-Wallis Results for PgR, with Respect to ESRα Status.

Results for ANOVA		**Results for K-W Test**	
Statistic	Significance	Statistic	Significance
F= 8.097	0.009	χ= 5.015	0.025

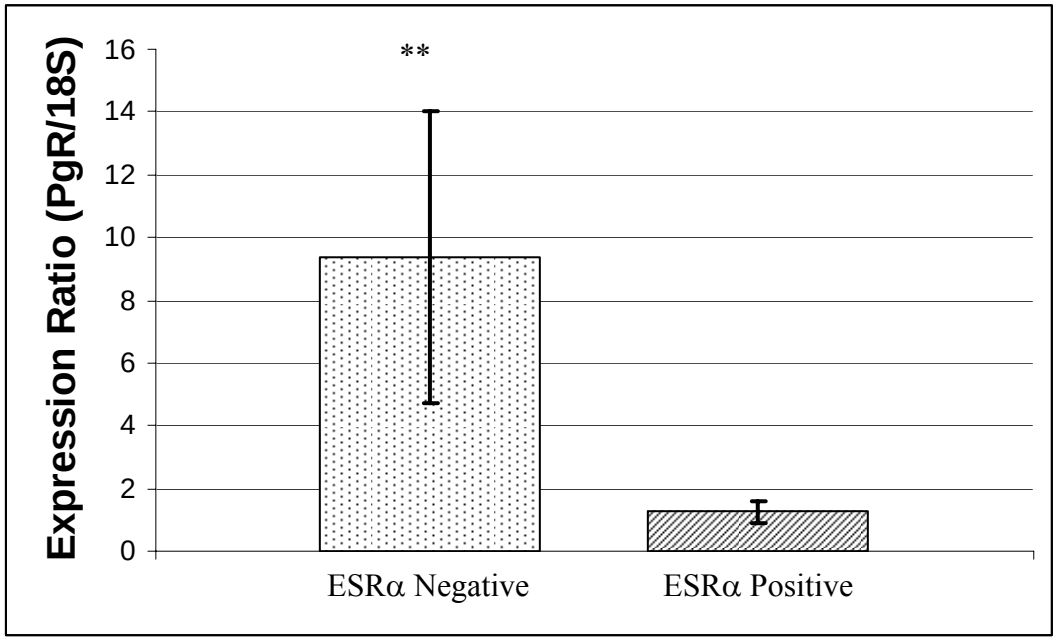

Figure 4.2. Expression Results for Progesterone Receptor, with Respect to ESRα Status.

The results for the progesterone receptor indicate that PgR undergoes significant changes in expression in multiple tumour types, specifically in late-stage and ESRα negative tumours. These results will be discussed in section 4.3, below.

4.2 The Glucocorticoid Receptor.

4.2.1. Background.

The glucocorticoid receptor, like most other nuclear receptors, has a huge array of multiple functions. The receptor is known to both promote and repress the transcription of certain genes, some of which have extracellular effects, such as the corticotropin-releasing hormone, which the glucocorticoid receptor represses [70]. As with the estrogen and progesterone receptors, the glucocorticoid receptor has two possible forms. The first, termed glucocorticoid receptor alpha (GRα) is composed of 777 amino acids and performs the receptor's initiation and repression functions [54]. The second form of glucocorticoid receptor is termed glucocorticoid receptor beta (GRβ), and is 742 amino acids long, differing in structure to the alpha variant through the loss of some of its hormone binding domain [54]. GRβ acts as a repressor of GRα activity, probably through

binding to the same DNA targets and failing to cause conformational change in the chromatin to prevent activation of those genes by GRα.

As mentioned above, the glucocorticoid receptor has a large array of functions in various tissues. Unlike the related estrogen and progesterone receptors, however, it has no particular direction in which these functions trend, like mitogenesis for ESR or interference with other receptors, like PgR. As far as breast cancer is concerned, one of the glucocorticoid receptor's major functions is an anti-proliferative effect on breast tissue [81]. Part of this anti-proliferative effect may be due to an additional positive role in differentiation, since activated glucocorticoid receptor has been observed to induce differentiation in murine cancers [93]. Additionally, the glucocorticoid receptor has been found to be a general antagonist for estrogen in breast tissue [81].

These effects indicate that glucocorticoid treatment is suitable for use in breast cancer, and indeed, it is one of the more common hormonal treatments for breast cancer, though some individuals show resistance to it. It has been observed that estrogen acts to down-regulate the expression of the glucocorticoid receptor gene [81]. This may contribute to the resistance some tumours show to glucocorticoid treatment. Although it is known that the estrogen down-regulation of GR is affected through the estrogen receptor, it is unknown whether proliferating ESRα negative cells also decrease the expression of GR. Another form of glucocorticoid resistance may come from the enzyme 11β

hydroxysteroid dehydrogenase, which inactivates glucocorticoids in the cell. Over-expression of this enzyme would result in lower sensitivity to glucocorticoids and indeed suppression of it has shown an increase in glucocorticoid response in tumour cells [80].

The glucocorticoid receptor has a number of other effects on various tissues, several of which are important from a treatment point of view. First, the glucocorticoid receptor actually down-regulates itself in a dose and time dependant manner, so doses of glucocorticoids have to be spaced out to remain effective. Secondly, the glucocorticoid receptor is an immunosuppressant, as well as an anti-inflammatory [70] and has been known to induce cytolysis [72]. This means that prolonged exposure to the hormone may cause problems with general infections and may even cause damage to the immune system itself. Thirdly, possibly in its role as a general estrogen antagonist, glucocorticoid exposure has been linked with loss of bone density [66]. Fourth, glucocorticoid stimulation has been linked with muscle protein wastage during trauma and sepsis, though some evidence refutes that it is responsible for the same effect in late cancer development [94]. Finally, increasing evidence shows that the glucocorticoid receptor interacts with several other nuclear receptors aside from estrogen, and notably, the vitamin D receptor [70]. Since polymorphisms of the vitamin D receptor have been associated with breast cancer [95], then the expression of the glucocorticoid receptor may have implications on breast cancer development.

4.2.2. Method.

The method used to analyse the role of the glucocorticoid receptor (GR) in cancer tissue was, like that used for PgR and the other nuclear receptors, semi-quantitative PCR. The Glucocorticoid receptor primers corresponded to a sequence in exon 2 of the glucocorticoid receptor gene. As for the progesterone receptor, these primers were not able to distinguish the different isoforms of GR and so only gross expression results were obtained. In order to control for the presence of genomic DNA contamination in the samples, a third primer, recognising an intronic sequence following the exonic sequence was designed and run as a multiplex with the quantitative PCR. As for the progesterone receptor, control for PCR efficiency and normalisation of data was provided by multiplexing 18S RNA primers with the GR primers. See Table 4.7 for the sequence of the primers used and Table 4.8 for the chemical conditions of the GR PCR. Quantitation and data analysis were performed in exactly the same way for the glucocorticoid receptor as for the progesterone receptor. That is, PCR product was quantitated by an ABI 310 genetic analyser, data normalised by expression as a ratio of GR/18S and then analysed using ANOVA. This method is similar to the method used for other NR genes and is described in more detail in Chapter 3.

Table 4.7. Primers for Glucocorticoid Receptor PCR.

Primer Name	Sequence (5'-3')
GREX2-F1*	GAGTACCTCTGGAGGACAGA
GREX2-R1	GCTTCTGATCCTGCTGTTGA
GREX2-R2	ATGTCCATTCTTAAGAAACAGGA
18S-A#	CTTAGAGGGACAAGTCGCG
18S-B	GGACATCTAAGGGCATCACA

*= Primer labeled with TET at 5' #= Primer labeled with HEX at 5'

Table 4.8. Chemical Conditions for GR PCR.

Reagent	Concentration	Quantity per Reaction
Master Amp Premix D (From Epicentre)	5x	5.5 μL
GR Forward Primer	5μM	2 μL
GR Reverse Primer	5μM	2 μL
GR Intronic Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	2 μL
18S Reverse Primer	5μM	2 μL
BE polymerase	1/5 dilution	0.3 μL
cDNA	-	2 μL
Total	-	17.8 μL

4.2.3. GR Results.

The normalised data for the GR gene showed a similar trend to that experienced by PgR, though the ratios are generally lower than those obtained for PgR. Expression levels for GR increase from normal to cancer tissue, and continue to increase as cancer grade increases (see table 4.9 for GR data). After analysis by ANOVA, a significant difference was found between tumour grades for GR expression. Like the data obtained for PgR, several outliers were noticed in the expression data for GR, although the magnitude was not as great. Since the data for PgR had also been re-analysed using the non-parametric Kruskal-Wallis test, the similar GR data was also re-analysed. Kruskal-Wallis analysis for GR confirmed a significant difference and the same post-hoc tests were then applied to discover where the significant difference lay (see table 4.10 for ANOVA and K-W results).

Table 4.9. Data for GR Expression Across Tumour Grade.

Tumour Grade	Average Ratio (GR/18S)	Std Dev. (GR)
Grade 1 Samples	0.263	0.089978
Grade 2 Samples	0.236	0.069009
Grade 3 Samples	1.007	0.581517
Control Samples	0.222	0.118726

Table 4.10. ANOVA and Kruskal-Wallis Results for GR.

Results for ANOVA		**Results for K-W Test**	
Statistic	Significance	Statistic	Significance
F= 9.644	0.00033	χ= 7.908	0.048

However, the post-hoc results for GR were quite different to those obtained for PgR. Post-hoc tests indicated that the expression of GR in grade 3 tumour tissue was significantly different from GR expression in grade 1 and control tissue (α = 0.05). However, expression in grade 3 tissue was not significantly different from expression in grade 2 tissue, nor was grade 2 tissue significantly different from grade 1 or control tissue. This result may well be a consequence of the relatively low sample size hampering the power of the post-hoc test. The expression data for the glucocorticoid receptor is depicted in Figure 4.3.

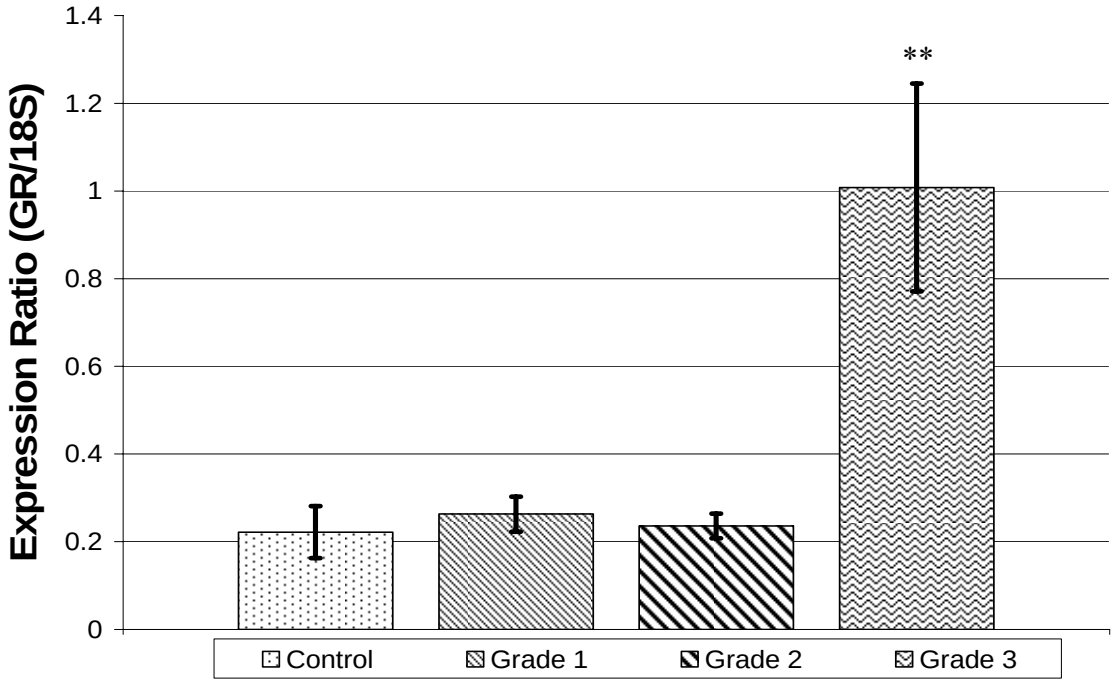

Figure 4.3. Expression Results for the Glucocorticoid Receptor.

Analysis was also carried out for GR with respect to ESRα protein status. Initial observations indicated that expression of GR was higher in ESRα negative tissue, as was PgR. However, after ANOVA analysis, this was not found to be the case, though the data was close to significance, with a p value of 0.058. GR data for ESRα protein status is summarized in Figure 4.4, and Table 4.11, while ANOVA results can be found in Table 4.12.

Table 4.11: Data for GR Expression by ESRα Status.

	Ratio (GR/18S)	**Std. Dev.**
ESRα Positive Tissue	0.347101	0.333316
ESRα Negative Tissue	0.734987	0.642352

Table 4.12. ANOVA and Kruskal-Wallis Results for GR, with Respect to ESRα Status.

Results for ANOVA		**Results for K-W Test**	
Statistic	Significance	Statistic	Significance
F= 3.996	0.058	χ= 2.11	0.146

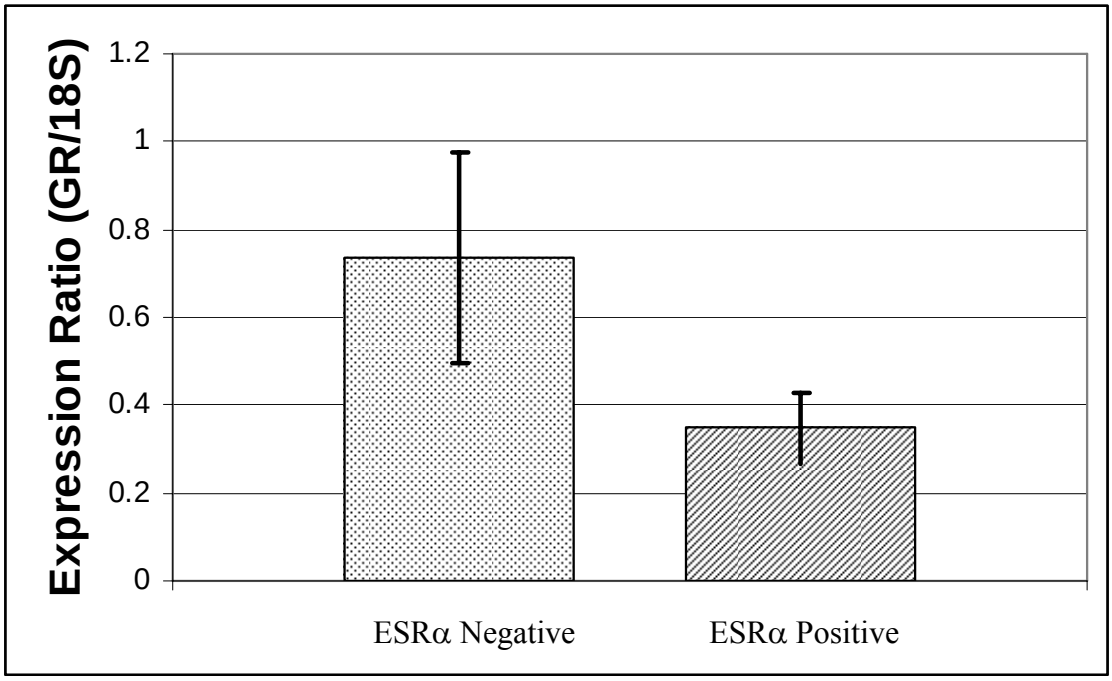

Figure 4.4. Expression Results for Glucocorticoid Receptor, with Respect to ESRα Status.

4.3. Discussion of PgR and GR Results.

This study investigated the expression of the progesterone and glucocorticoid receptor genes in different grades of breast cancer tissue. The pattern of results obtained for both PgR and GR are fairly similar, though the glucocorticoid receptor's expression levels are generally lower. Results indicated that there was significant statistical evidence of a relationship between cancer grade and expression of PgR and GR within the tested population.

Specifically, post-hoc tests indicated that expression of the PgR gene increased in late stage breast cancer, as well as in ESRα negative tissue. It is possible that the progesterone receptor may be being expressed in response to constant stimulation of estrogen mediated pathways, since stimulation of the estrogen receptor and related pathways is known to up-regulate PgR expression [96]. These results resemble those of a study by Il'icheva *et al*, which found that expression of the progesterone receptor in breast cancer cells may be up to 2.5 times higher than expression in healthy cells [97]. It is known that higher grade tumours display a marked level of differences in their expression of PgR, with some high grade tumours failing to express it altogether [98].

Stimulation of the estrogen receptor is known to increase the expression of PgR, but expression of the estrogen receptor may be lost in higher grade cancers and indeed, the majority of the samples from grade 3 and many of the grade 2 samples used in the study were estrogen receptor negative. Since expression of PgR in estrogen receptor negative

samples was not reduced and indeed, was significantly increased, other factors must be coming into play to keep the tumours expressing PgR. This is certainly not unknown, but ESR-/PgR+ tumours are less likely to respond to hormone based treatments [54, 89]. The interference that PgR causes to other nuclear receptors may also be a reason for the advanced and ESRα negative tumours to over-express it. Once the ESRα pathway has been circumvented, stimulation of PgR would not slow tumour growth appreciably and would interfere with pro-apoptotic signaling from the glucocorticoid receptor or anti-growth signaling from the androgen receptor [91].

A similar relationship is unlikely for GR, since it is known to be down-regulated by estrogen receptor stimulation [81]. However, it is unknown if the loss of ESRα expression, as in many advanced cancers, would affect GR expression. However, a study by Smirnova *et al* did scrutinise glucocorticoid receptor expression in different histological grades of breast cancer [99]. The study found a similar relationship to that discovered here, that is, that expression of GR increases with cancer grade, positively correlating with ESR and PgR expression [99].

Because both GR and PgR are estrogen antagonists and anti-mitogenic agents in breast tissue, a high level of expression for these genes in an advanced cancer seems unusual. It may be that the tumour cell is expressing these genes in response to its rapid proliferation, as a tumour repressing mechanism, but that another pathway in the cell is preventing translation of GR and PgR mRNA, preventing the receptor from slowing cellular proliferation. It is also possible that the GR and PgR genes are being expressed

exclusively as alternate isoforms of the receptors, which cannot be distinguished from the standard isoforms by the methods used in this study. Alternate isoforms are known for both receptors, with the alternative GR, GRβ, acting as a repressor of anti-estrogenic GRα activity [54] and the PgR alternative, PgRβ, acting as a general gene activator rather than the standard PgRα isoform, which is generally a transcription inhibitor [84]. Both of these roles seem more recognizable in advanced cancers, which are often resistant to hormonal treatments. There is also some evidence that the ratio of receptor isoforms in a cell will affect how a given hormone influences cellular behavior [54, 100]. Thus, it is also possible that some mechanism in the cell is not preventing translation, but influencing the transcription or splicing of the receptors and favouring the production of one isoform over another, giving a ratio of receptors that keeps cellular proliferation high.

CHAPTER 5. Estrogen Receptors.

5.1. Background.

Amongst the most important receptors in breast cancer is the estrogen receptor (ESR). This receptor plays a large role in cellular metabolism and especially in the breast. The primary effect of the estrogen receptor is to act as a mitogenic factor, which is common in many tissues and especially the breast [100]. The receptor has a number of other effects, including maintaining bone density in both men and women [101]. Receptors for estrogen are found in many areas of the body, including the brain, though the precise effects of estrogen in these tissues is not well understood. The estrogen receptor has two isoforms, or versions, of the receptor an α isoform and a β isoform [54]. These isoforms are actually the products of completely separate genes, located at chromosome 6q25.1 for ESRα and chromosome 14q22-24 for ESRβ, with ESRβ believed to have originated in the distant past as a copy of ESRα [69, 100, 101]. ESRβ is slightly smaller than ESRα however, encoding a protein that is only 530 amino acids long, compared to ESRα at 595 amino acids. There is a large degree of homology between the two genes, especially in the ligand binding domain, where there is only one base pair of difference. This homology is at its least at the ends of the genes, in the trans-activation factor domains, which modulate the functions that the receptors perform, accounting for the differing

effects the receptors perform [69]. A comparison of ESR structure can be found in Figure 5.1.

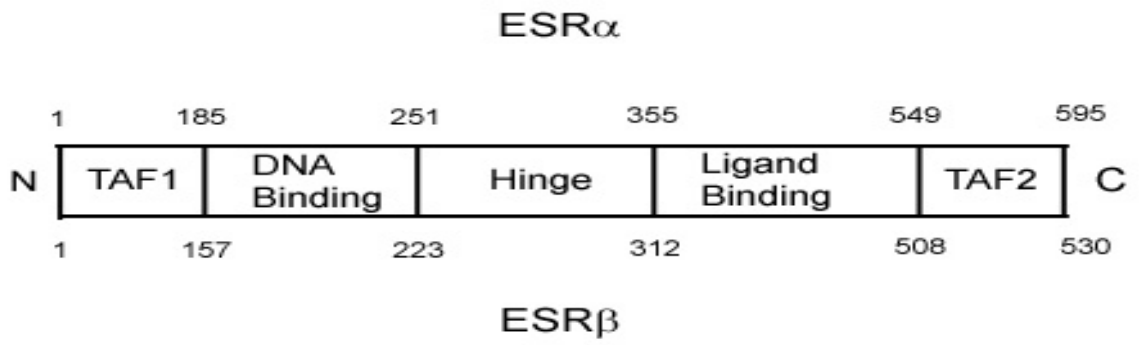

Figure 5.1: Comparative Structure of the ESRs (Numbers given are amino acid lengths for domains. Derived from.[69]).

Despite the difference in their structures, the expression of both ESR genes seem to be broadly affected by the same factors [100]. Of course, both proteins bind to estrogen, though different specificities have been reported and the receptors operate on similar genes, as well as subsets of genes unique to each receptor [54, 69, 100]. ESRα is the receptor which functions primarily as a mitogenic factor in breast tissue, while ESRβ in general antagonises the functions of the alpha receptor [69, 100]. The ESR proteins do not merely form dimers with their own kind, but also form heterodimers with each other after activation, which may induce altered transcriptional events [54]. Additionally, various cofactors will also modulate the effect of an activated receptor, be the receptors in homo- or heterodimers. Such cofactors may even reverse the general function of the receptor, such as the activator protein 1, which provides an alternative transcription

pathway to the normal estrogen response element and results in ESRα restricting breast tissue growth when stimulated by estrogen, and ESRβ inducing cellular growth when stimulated with tamoxifen, normally used as a treatment for breast cancer [69].

Not all of the effects of the estrogen receptor are due to direct genomic binding of the activated receptor. There are a number of protein-protein interactions that activated ESR will undergo, that also result in altered transcriptional events. These include interactions with the Sp1, Ap1, Cyclin D1 and NFκB proteins, as well as with the well known ras, raf, MEK pathway [100, 102]**.** These protein-protein interactions produce highly rapid effects that can be seen within minutes rather than hours, tending however, to be complimentary to the receptor's genomic functions.

While many breast cancers respond normally to estrogen, some advanced cancers will lose expression of ESRs, particularly ESRα, either because the gene becomes disabled and the cells already produce the needed growth factors themselves, or else the receptor is spliced or mutated into a permanently active state [53]. In these breast cancers, the cell does not respond to estrogen stimulation and will often respond poorly to other drugs that rely on the manipulation of estrogen mediated pathways, like tamoxifen. It is already known that expression of the progesterone and estrogen receptors together indicates a greater chance for responsiveness to certain treatments [53] and this is due to the actions that these receptors have on gene expression.

5.2. Method.

5.2.1. Samples

The sample population was comprised of the standard 25 archived breast tissue sections embedded in paraffin as used in all the semi-quantitative PCR studies in this thesis, as outlined in Chapter 3.

5.2.2. Expression Assay.

cDNA made using the method outlined in Chapter 3 underwent PCR to amplify portions of cDNA that corresponded to the mRNA for ESRα and ESRβ with fluorescently tagged primers. Individual samples underwent PCR in triplicate for each gene before results were pooled.

Because of the large size of introns in the NR genes, intron spanning primers would be unlikely to amplify in a PCR optimized for small fragments, therefore triple primer sets were designed so that genomic DNA contamination would be indicated by amplification from a second reverse primer, placed a short distance into the intron. However, due to

difficulties in obtaining a functional triple ESRα primer set for the detection of DNA contamination, primers from a previous study, corresponding to an intronic/exonic fragment of the glucocorticoid receptor gene were used for the purpose. No genomic DNA contamination was observed in any sample. Primer details appear in Table 5.1. The fragment amplified for both receptors was approximately 100 base pairs long, allowing cDNA derived from partially degraded RNA to be fully utilized. Fragments produced by any contaminating genomic DNA present was 150bp in length for samples using the GR primers and 161 for the ESRβ intronic primer. As outlined in Chapter 3, the nuclear receptors were also multiplexed with the ribosomal 18S gene, to control for PCR efficiency and variations in basal expression. Chemical conditions for PCR are outlined in Tables 5.2.1 and 5.2.2. Gene expression was quantified using an ABI 310 Genetic Analyzer, utilizing peak height as the measure of expression.

Table 5.1. Primer Details for ESRs.

Primer Name	**Sequence (5'-3')**
GREX2-F1*	GAGTACCTCTGGAGGACAGA
GRIntron2	ATGTCCATTCTTAAGAAACAGGA
ESRaEX1-F1*	CCAAAGCATCTGGGATGGCC
ESRaEX1-R1	GGATCTTGAGCTGCGGACGG
ESRbEX2-F1*	CCAACACCTGGGCACCTTTC
ESRbEX2-R1	CCAGGGACTCTTTTGAGGTTC
ESRbIntron2	TGGCTAGCAACTATAATTCAGAATGAA

*= Primer labeled with TET at 5'

Table 5.2.1 Chemical Conditions for ESRα PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix E (From Epicentre)	5x	5.5 μL
ESRα Forward Primer	5μM	2 μL
ESRα Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	1 μL
18S Reverse Primer	5μM	1 μL
GR Forward Primer	5μM	1 μL
GR Intronic Reverse Primer	5μM	1 μL
BE polymerase	1/5 dilution	0.3 μL
Water	-	0.7 μL
cDNA	-	2 μL
Total	-	16.5 μL

Table 5.2.2 Chemical Conditions for ESRβ PCR.

Reagent	**Concentration**	**Quantity per Reaction**
MgCl	25mM	2 μL
PCR Buffer	10x	2 μL
ESRβ Forward Primer	5μM	2 μL
ESRβ Reverse Primer	5μM	2 μL
ESRβ Intronic Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	1 μL
18S Reverse Primer	5μM	1 μL
dNTPs	5mM	0.8 μL
BE polymerase	1/5 dilution	0.5 μL
Water	-	1.5 μL
cDNA	-	2 μL
Total	-	17.8 μL

5.2.3. Statistical Analysis.

Expression data obtained for all receptors was normalized by expressing it as a ratio of the expression of the receptor to 18S, as outlined in Chapter 3. Normalized final data was analysed using One-way Analysis of Variance to determine if there was a significant

difference of expression between tumour grades. A second ANOVA test was performed to determine whether expression of the nuclear receptors was significantly affected by ESRα status, rather than breast cancer grade. Homogeneity of variance tests were also performed to discover whether non-parametric tests would be required in addition to the original ANOVA. Appropriate post-hoc tests were subsequently performed to elucidate any differences found. The conventional α-level of 0.05 was specified as the significance threshold. The software package SPSS version 10.1 was employed for all statistical analyses.

5.3. Results.

The expression levels for ESRα and ESRβ were determined for all archival tissue samples, with the normalized data summarized in Tables 5.3 and 5.4 for cancer grade and ESRα status, respectively. Despite initial observations of the data for ESRα indicating a drop in expression for grade 1 tumours (see Figure 5.1) ANOVA results determined that this change was not significant ($p= 0.057$). Data for ESRβ indicated that there was little variance in ESRβ expression, regardless of what stage of cancer the tissue is derived from (see Figure 5.2). This was borne out by ANOVA testing which returned an insignificant result for ESRβ ($p= 0.622$). A statistical summary for all ANOVA analyses can be found in Table 5.3.

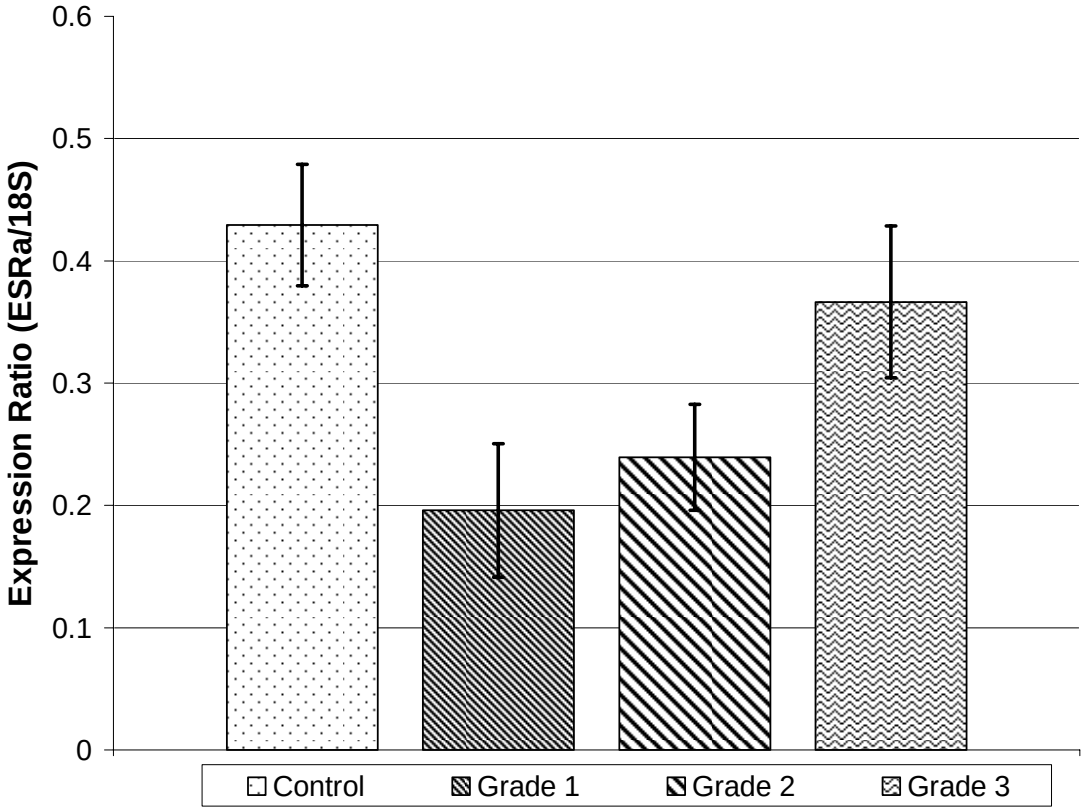

Figure 5.2: ESRα Expression Across Different Cancer Grades.

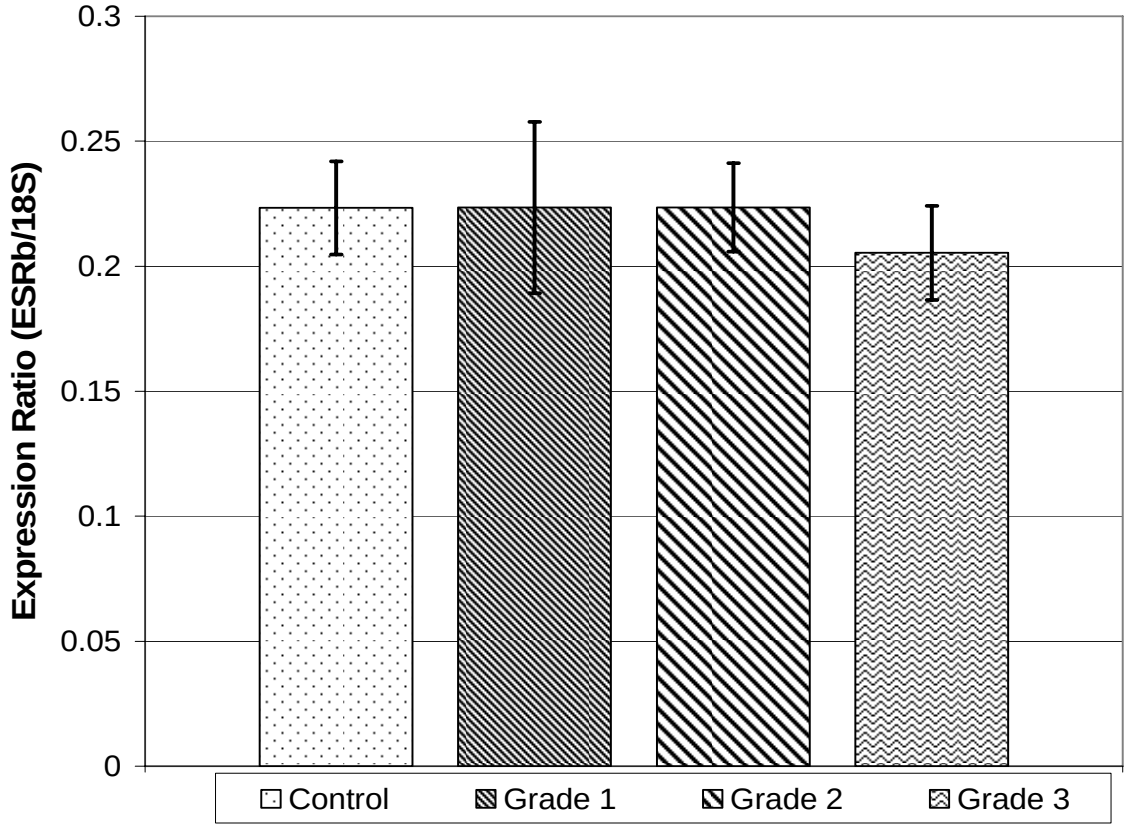

Figure 5.3: ESRβ Expression Across Different Cancer Grades.

Table 5.3. Expression Data for Nuclear Receptors by Tumour Grade.

Tumour Grade	**Average Ratio (ESRa/18S)**	**Std Dev. (ESRα)**	**Average Ratio (ESRβ/18S)**	**Std Dev. (ESRβ)**
Grade 1 Samples	0.195848	0.0546	0.223501	0.03424
Grade 2 Samples	0.239395	0.0433	0.223477	0.01767
Grade 3 Samples	0.366408	0.0621	0.205338	0.01877
Control Samples	0.429228	0.0495	0.223349	0.01865

Table 5.4. Expression Data for Nuclear Receptors by ESRα Status.

	Average Ratio (ESR/18S)	**Std Dev.**
ESRα, ESRα Positive Tissue	0.356534	0.140583
ESRα, ESRα Negative Tissue	0.317748	0.176262
ESRβ, ESRα Positive Tissue	0.245033	0.056206
ESRβ, ESRα Negative Tissue	0.224409	0.032618

Table 5.5. Summary of Statistical Results for Nuclear Receptors.

Nuclear Receptor	**Results for BC Grade**		**Results for ESRα Status**	
	Statistic	Significance	Statistic	Significance
ESR Alpha	F= 2.628	0.057	F= 0.071	0.794
ESR Beta	F= 0.738	0.622	F= 0.135	0.716

After testing expression in cancer grades, the expression levels for ESRα and ESRβ were compared against ESRα protein status, a useful prognostic factor in breast cancer, as determined by immunohistochemical staining (see Figure 5.4). Initial observation of results indicated that ESRα expression was decreased in ESRα negative tissues and ESRβ expression increased in ESRα negative tissues. However, ANOVA results for ESRα and ESRβ indicated that there was no significant relationship between ESRα

protein status and the mRNA levels present for either estrogen receptor (p= 0.794 and p=0.716, respectively).

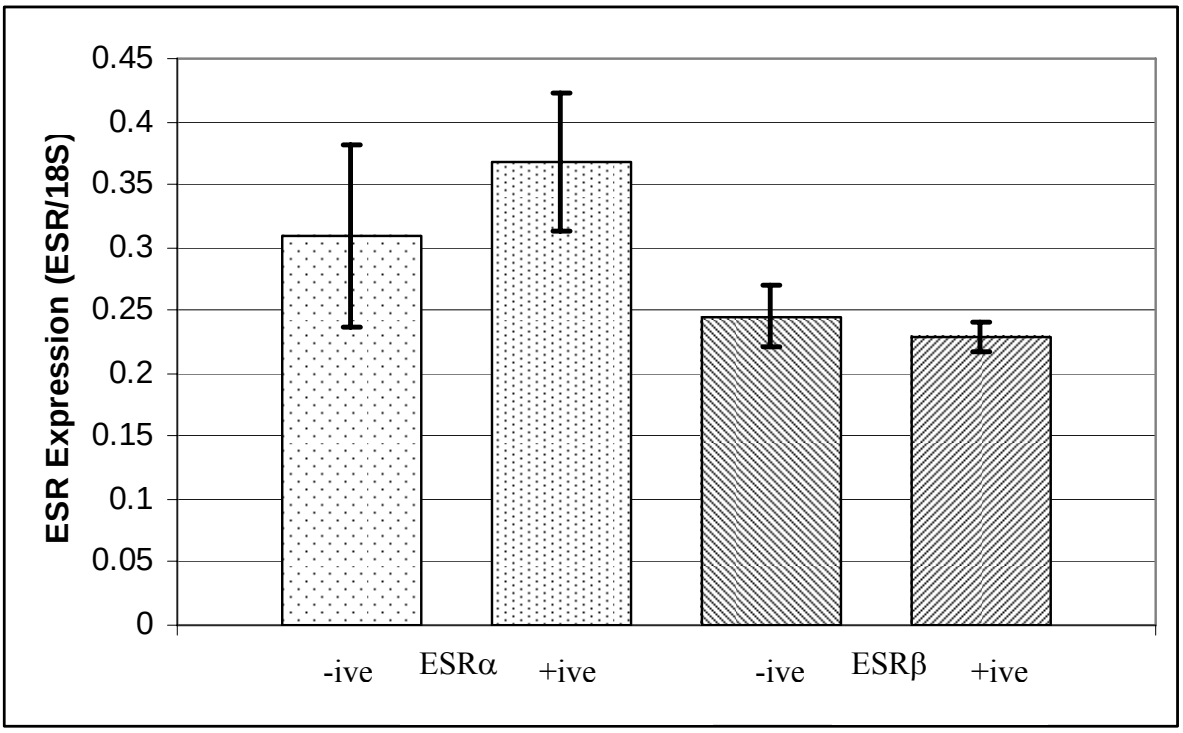

Figure 5.4: ESR Expression by ESRα Status. Both genes are arranged as ESRα negative - ESRα positive.

5.4. Discussion.

The estrogen alpha and estrogen beta receptor genes are members of the steroid receptor sub-family of the nuclear receptors superfamily. This study investigated the expression of these genes in different grades of breast cancer tissue. The results obtained from one way ANOVA within the tested population indicated that there was no significant statistical

evidence of a relationship between the expression of either ESR and cancer grade or ESRα protein status.

Data from this study has found that the expression of both estrogen receptors did not change in the tested population, at least at the level of mRNA, for either cancer grade or ESRα status. The initial drop off in ESRα expression seen in grade 1 tumours, though not significant, would be consistent with the self down-regulation of ESRα in response to high levels of stimulation of relatively normal cellular growth pathways in the early tumour. Though the difference in the data is not significant, it is close to significance, and it is possible that the relatively low number of samples in this study has insufficient power to detect the change in expression. By contrast, ESRβ showed no obvious trend of any kind, its expression static for all grades. Although some studies have shown an increase in ESRβ expression in some breast cancers, the effects of ESRβ are partially mediated through isoforms. Hence, the relatively unchanged gross expression of ESRβ observed here does not rule out significant effects for this gene in the progression of breast cancer in these cells [16, 69].

Immunohistochemical data for each sample indicated that there is no detectable ESRα protein in two out of the seven grade 2 samples and five out of the seven grade 3 samples. Many advanced breast tumours no longer express detectable ESRα or express altered and non-functional receptors and this is a poor prognostic indicator, since it indicates that the tumour may be unresponsive to treatments that manipulate the estrogen pathway, such as

tamoxifen [18]. However, none of the individual samples in this study failed to express ESRα mRNA, nor was there any significant difference in mRNA levels between ESRα positive and negative tissues. Previous studies have shown strong correlations between ESRα mRNA and ESRα protein levels, though other studies have found that the levels can vary considerably, even to the point of mRNA being present without protein expression [53, 54]. This would indicate that PCR techniques alone cannot be used to determine whether or not tumours will be responsive to hormonally based treatments. It is also interesting to note that ESRα mRNA levels were higher in ESRα positive than in ESRα negative tissue in this study, though the difference was not statistically significant.

Since all tissues in this study maintain expression of ESRα mRNA, tissues that fail to produce detectable levels of the finished receptor may be undergoing some post-transcriptional event which prevents final assembly of the ESR. This event may be rapid degradation of transcribed mRNA, the removal of any translated proteins or alternative splicing of ESRα mRNA into proteins undetectable with standard ESRα antibodies and which favour cellular growth. If the specific mechanism which prevents the detected mRNA from being translated into detectable ESRα can be discovered, it may be able to be blocked, causing tumours to re-express the protein and become responsive to hormonal treatments again. However, due to the heterogeneity of cancer tissue, it seems probable that not all tumours failing to express ESRα, but maintaining the mRNA, will be doing so by exactly the same mechanism. Therefore therapies based on this premise might have to be tailored to individual tumours.

With no immunohistochemical data for ESRβ, the data obtained from this study does not verify the presence of completed ESRβ proteins derived from the mRNA that is expressed in the tested population. Since isoforms of ESRβ are known to modulate the effects of ESRα, it seems likely that advanced tumours have skewed expression of any ESRβ proteins to isoforms that favour cellular growth. When examining the expression of ESRβ by ESRα status however, it can be noted that ESRβ expression increases as ESRα decreases. While the differences for both genes are not significant, this behavior is in keeping with previously observed negative feedback relationships between the two ESRs. [18, 69]

5.5. Conclusion.

The results of this study indicate that the expression of the estrogen receptor alpha and beta genes remain relatively stable in breast tumours. Additionally, breast tumours that no longer produce ESRα proteins at levels detectable by immunohistochemistry maintain the production of statistically normal amounts of ESRα and ESRβ mRNA. The precise implications of this information for breast cancer treatment are not fully clear, due to the inability of this study to scrutinize post transcriptional activity in the tumours, but it is possible that the continuation of ESRα mRNA expression in tissue that lacks the ESRα protein may provide an additional angle of treatment for these advanced tumours, especially those lacking functional expression of other NRs.

CHAPTER 6. Androgen Receptor.

6.1. Background.

The androgen receptor (AR) gene is located at Xq11-12 and is some 90kb long with eight exons. The androgen receptor plays a role in a number of tissues, most obviously in the development of male traits in animals [103]. Like the estrogen receptors, the progesterone receptor and the glucocorticoid receptor, AR has alternative isoforms that assist in mediation of its function. The two isoforms, ARα and ARβ are splice variants produced from two separate promoters on the AR gene and are of 87 and 110 kDa, respectively [104]. The precise activities of the two isoforms are not entirely clear and ARα was at first thought to be inert, though some more recent evidence disputes this [104].

As is the case for the other nuclear receptors, the exact effect of stimulation of the AR is modified depending on precisely which tissue it is found in, from differentiation to proliferation [104]. As far as breast cancer is concerned, the androgen receptor is extremely important, mostly because of the effects of androgens on breast tissue. Exposure to androgens inhibits the proliferation of mammary ductal cells *in vivo* and androgen exposure is an effective tool in the treatment of breast cancer. This anti-proliferative effect is believed to be dependant on AR itself, rather than interaction with

estrogen or the estrogen receptors, due to the fact that combined androgen and anti-estrogen therapy is more effective than either treatment alone [104, 105]. However, in certain tumours and breast cancer cell lines, androgens have been found to induce a proliferative response, rather than suppression of cell growth [83, 104]. The nature of this role reversal is still poorly understood, but is believed to be caused by mutations to the AR gene, alterations to co-activator behaviour or the aromatase conversion of androgens to estrogen in the cells or surrounding tissue [83, 104].

Another factor making the androgen receptor important in breast cancer is also a possible link to the occasional cause of androgen mediated cellular proliferation in breast tissue. The androgen receptor has a CAG repeat in its first exon. This CAG repeat has a variable length and at extremes of this variation it has been linked with several disorders, including cancers of the breast, prostate and colon, male infertility and motor neurone diseases [104, 106]. Most tellingly for breast cancer, several studies have linked AR alleles, including the CAG repeat to an increased risk of breast cancer in those who also carry a breast cancer associated BRCA1 allele [105]. Additionally, a later study also found that the BRCA1 protein acts directly on AR as a co-activator [104]. This involvement of the BRCA1 tumour suppressor, along with the androgen receptor's known major function of breast tissue growth suppression indicate that the alleles and expression of the AR may play an important role in the development and progression of sporadic breast cancer.

6.2. Method.

6.2.1. Samples

The sample population was comprised of the standard 25 archived breast tissue sections embedded in paraffin as used in all the semi-quantitative PCR studies in this thesis, as outlined in Chapter 3.

6.2.2. Expression Assay.

cDNA made using the method outlined in Chapter 3 underwent PCR to amplify portions of cDNA that corresponded to the mRNA for the androgen receptor with fluorescently tagged primers. Individual samples underwent PCR in triplicate for each gene before results were pooled.

Due to difficulties in obtaining a functional triple primer set for the androgen receptor, primers from a previous study, corresponding to an intronic/exonic fragment of the glucocorticoid receptor gene were used for the purpose, as for ESRα in Chapter 5. No genomic DNA contamination was observed in any sample. Primer details appear in Table 6.1. The fragment amplified for the androgen receptor was approximately 100 base pairs

long, allowing cDNA derived from partially degraded RNA to be fully utilized. Fragments produced by any contaminating genomic DNA present was 150bp in length. As outlined in Chapter 3, the nuclear receptors were also multiplexed with the ribosomal 18S gene, to control for PCR efficiency and variations in basal expression. Chemical conditions for PCR are outlined in Table 6.2. Gene expression was quantified using an ABI 310 Genetic Analyzer, utilizing peak height as the measure of expression.

Table 6.1. Primer Details for AR Experiment.

Primer Name	**Sequence (5'-3')**
AREX1-F1*	CCTGATGTGTGGTACCCTGG
AREX1-R1	CCGGAGTAGCTATCCATCCA
GREX2-F1*	GAGTACCTCTGGAGGACAGA
GRIntron2	ATGTCCATTCTTAAGAAACAGGA

*= Primer labeled with TET at 5'

Table 6.2. Chemical Conditions for AR PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix D (From Epicentre)	5x	5.5 μL
AR Forward Primer	5μM	2 μL
AR Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	2 μL
18S Reverse Primer	5μM	2 μL
GR Forward Primer	5μM	1 μL
GR Intronic Reverse Primer	5μM	1 μL
BE polymerase	1/5 dilution	0.3 μL
cDNA	-	2 μL
Total	-	17.8 μL

6.2.3. Statistical Analysis.

Expression data obtained for the androgen receptors was normalized by expressing it as a ratio of the expression of the receptor to 18S, as outlined in Chapter 3. Normalized final data was analysed using One-way Analysis of Variance (ANOVA) to determine if there

was a significant difference of expression between tumour grades. A second ANOVA test was performed to determine whether expression of the nuclear receptors was significantly affected by ESRα status, rather than breast cancer grade. Homogeneity of variance tests were also performed to discover whether non-parametric tests would be required in addition to the original ANOVA. Appropriate post-hoc tests were subsequently performed to elucidate any differences found. The conventional α-level of 0.05 was specified as the significance threshold. The software package SPSS version 10.1 was employed for all statistical analyses.

6.3. Results.

The expression level for the androgen receptor was determined for all archival tissue samples, with the normalized data summarized in Tables 6.3 and 6.4 for grade and ESRα status, respectively. Data obtained for AR (see Figure 6.1), showed that the average AR/18S expression ratio was elevated in both grade 2 and grade 3 tumours. One way ANOVA indicated that the differences between tumour grades were significant, with a p value of 0.014. There was considerable variation among the ratios for some samples and a test for homogeneity of variance was performed to test for a violation of ANOVA assumptions. However, this test proved not to be significant, with the p value being 0.078.

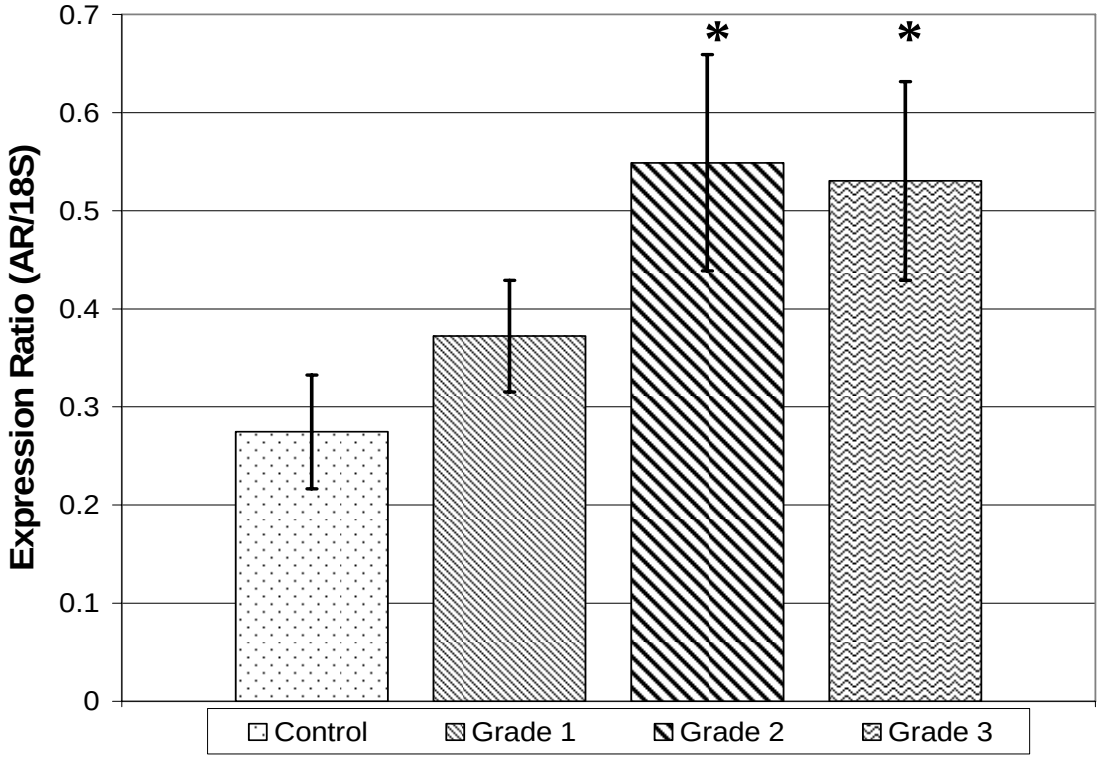

Figure 6.1. AR Expression Across Different Cancer Grades.

With the existence of a significant difference between groups indicated in the original analysis, post hoc testing was performed to identify the precise effect of cancer grade on AR expression. The results of the post-hoc analysis for the androgen receptor confirmed the initial impression that expression ratios were elevated in grade 2 and grade 3 tumour tissues as compared to control tissue ($p < 0.05$). Post hoc tests indicated that the grade 1 tumour tissue formed an apparent intermediate state between control and advanced tumours, not being significantly different from these extremes.

Table 6.3. Expression Data for Androgen Receptor.

Tumour Grade	Average Ratio (AR/18S)	Std Dev. (AR)
Grade 1 Samples	0.372213	0.05684
Grade 2 Samples	0.548775	0.1102
Grade 3 Samples	0.530409	0.1012
Control Samples	0.274586	0.058

Table 6.4. Expression Data for AR Expression by ESRα Status.

	Ratio (AR/18S)	Std. Dev.
ESRα Positive Tissue	0.340636	0.194891
ESRα Negative Tissue	0.483674	0.241341

After testing expression in cancer grades, the expression levels for androgen receptor were compared against ESRα protein status, a useful prognostic factor in breast cancer, as determined by immunohistochemical staining (see Figure 6.2). ANOVA results indicated that there was a significant relationship between ESRα protein status and AR expression ($p= 0.025$), with expression increasing in ESRα negative samples. A statistical summary for all ANOVA analyses can be found in Table 6.5.

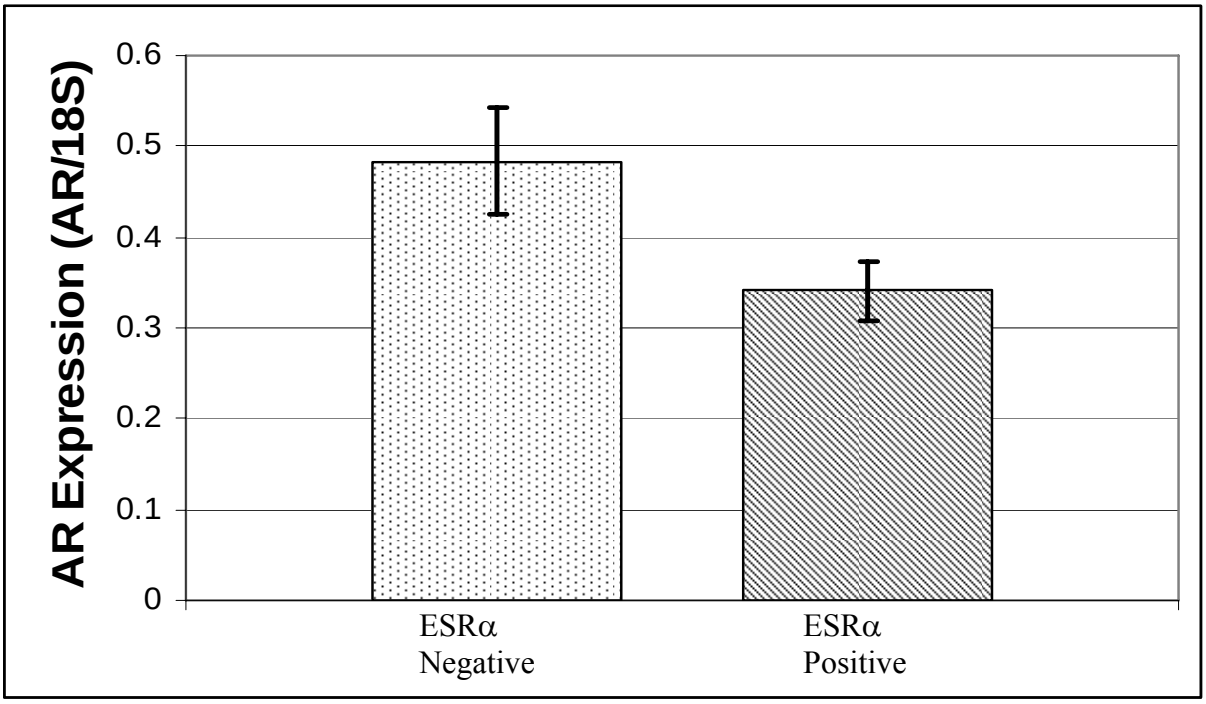

Figure 6.2: AR Expression by ESRα Status.

Table 6.5: ANOVA Results for AR.

Results for BC Grade		**Results for ESRα Status**	
Statistic	Significance	Statistic	Significance
F = 3.130	0.014	F = 5.743	0.025

6.4. Discussion.

The results obtained from one way ANOVA within the tested population indicated that there was significant statistical evidence of a relationship between AR expression and cancer grade, as well as AR expression and ESRα status. Post-hoc tests indicated that the

difference in AR expression involved the significant up-regulation of AR in grade 2 and grade 3 tumour tissues in comparison to control tissue and in ESRα negative tissues in comparison to ESRα positive tissues.

In contrast to the levels of the estrogen receptors as outlined in Chapter 5, which remained unchanged in the studied population, the androgen receptor showed a significant up-regulation in late stage tumours, as well as in ESRα negative tissue. As for PgR and GR, this result may be due to the fact that all ESRα negative tissues in this study are either grade 2 or 3 tumours, or it may be a direct link between the loss of ESRα protein and the expression of AR. An additional ANOVA comparing ESRα positive and negative tissue in grades 2 and 3 only, showed no significant differences however ($p = 0.336$), so it seems likely that the higher AR expression in ESRα negative tissue is not a direct result of the loss of ESRα expression.

It is known that many breast tumours continue to express AR even when they fail to express ESR or PgR, so these tumours remain sensitive to androgen stimulation even after other endocrine therapies become useless [48]. However, the elevated levels of AR in late-stage breast tumours would indicate that these tumours would be more sensitive to androgen stimulated growth arrest than early stage tumours. This would seem to be at odds with the high proliferation rate of advanced tumours, but the increase in expression may be due to the influence of the estrogen receptor pathways, which are known to have effects on the behavior of AR in the breast [48]. Such an increase in AR expression may

be an aspect of normal growth arrest mechanisms, which remain active within the tumour cells. As in the previous results outlined in Chapter 4, a similar pattern of expression also occurs within the same population for the progesterone and glucocorticoid receptors, both anti-mitogenic in the breast [19]. Circulating levels of androgens in female breast cancer patients are likely to be fairly low, considering losses for aromatase activity in adipose and tumour tissue. Hence, the effects of the increase in AR expression may not be particularly marked, compared to the *in vivo* effects derived from GR and PgR [15]. At the same time, this lack of effect may be precisely the reason that AR expression is known to continue in tumour cells, as the cells are under little selective pressure to cease expression or become AR resistant. This means that AR may remain a viable target for hormone treatment strategies even in late stage tumours.

However, it is known that advanced tumours often become insensitive or completely independent of hormone stimulation or manipulation of steroid receptor pathways. It is possible that the increased androgen receptor expression observed in this study may not result in increased sensitivity to androgens due to disruption of normal AR pathway function. Such disruption could come from a number of sources. As often occurs for the alpha estrogen receptor, it is possible that the AR mRNA observed in this study is not being translated into a final protein, preventing any androgenic stimulation of the cell. Alternatively, the mRNA may be undergoing post transcriptional splicing to inactivate the resultant protein or to produce an alternate isoform of AR that is ineffective, antagonizes, or suppresses the primary form. Restriction of AR effectiveness might also be achieved through regulation of the various NR co-activator and co-repressor proteins,

restricting or increasing availability as needed to prevent AR stimulated growth restriction. Interestingly, prostate cancer has shown similar behavior for AR in advanced cancers. Though in contrast with breast cancer, higher expression in late stage prostate cancers typically indicates resistance to hormonally stimulated pathways. This resistance has been linked to mutations in AR, which reduce receptor specificity or constitutively activate the receptor, mechanisms that may also be at play in the test population [107].

Finally, it is also possible that the increased AR expression observed in this study is a mechanism for retarding the effectiveness of other anti-mitogenic nuclear receptors. The androgen receptor's normal functions do not include antagonism of PgR or GR, but AR has been known to interfere with other NRs through competition for common chaperone proteins and cofactors [44]. This competition, combined with low levels of *in vivo* androgen stimulation, would reduce the overall effect of other anti-mitogenic hormones in the breast tumour; though likely not abrogate them entirely. Thus, the increased AR levels in this study may reflect such a situation, sequestering available chaperones and preventing stimulation from increased levels of progesterone and glucocorticoid receptors.

6.5. Conclusion.

The results of this study indicate that the expression of the androgen receptor gene is significantly related to cancer grade, showing an increase in later stage (grades 2 and 3) breast tumours. The results of this study also indicate that breast tumours that no longer produce ESRα proteins show increased AR mRNA levels. The precise implications of this information for breast cancer treatment are not fully clear, due to the inability of this study to scrutinize post transcriptional activity in the tumours, but it is possible that the enhanced expression of AR in late stage breast tumours may provide an additional angle of treatment for these advanced tumours, despite the possibility that the increase in AR expression may be being used by the tumours as a mechanism to avoid growth retardation by other hormones.

CHAPTER 7. Stromal NR Expression.

7.1. Background.

Early studies into cancer concentrated on the cells in a tumour itself, considering the effect of various cancer causing agents and the behavior of those cells which were obviously no longer functioning correctly. However, more recent studies are showing that there is significant cross-talk between cancer and apparently healthy cells and that the stroma of a tumour also plays a role in cancer development and progression. These studies have also shown that this apparently healthy stromal tissue may have suffered damage itself [36, 108]. Stromal tissue may show changes which are characteristic of tumour growth well before there is any sign of abnormal morphology or behavior in the tissue that will eventually form the tumour [109]. Experiments where stroma showing altered characteristics has been transplanted into other animals has shown a significant increase in tumour development in the tissues surrounding the transplants [109]. In a similar manner, cancer cells that were transplanted without their associated stroma show a reduced propensity to form tumours in recipient animals, indicating that certain clonal types within a tumour are incapable of influencing stromal behavior and that the altered stroma is necessary for their survival [109].

Previous studies have indicated that the expression of genes in the stroma of a tumour may differ very significantly from expression in normal, healthy tissue. These changes include the unusual expression of genes involved in wound healing and inflammation, such as desmin, smooth muscle α-actin and myosin, collagenases, matrix metalloproteinases and other remodeling proteins [34, 35, 108, 109]. In tumours that are more recognizable, stromal cells may show more extreme perturbations to gene expression, characterized by alterations in growth patterns and rates. These alterations may also be present prior to tumour formation and may be one of the mechanisms that assist in tumour formation [109]. It has still not been determined if the relationship between tumour and stroma is initiated by the stroma as a result of exposure to chemical or radiological factors, if tumour cells initiate it through the abnormal release of signaling molecules that induce such changes, or if the process does not require one to come first. It is also possible that the events that begin the path to tumour development must happen in both tumour and stromal tissue nearly simultaneously in order for the relationship to develop [35, 109].

There are numerous ways in which stroma and tumour can influence one another, including the release of molecules that remodel the extracellular environment directly, as well as releasing signaling molecules that affect the transcription of genes in nearby cells [34, 35, 108, 109]. These transcription factors include the production and release of hormones, both in an autocrine and paracrine fashion, enabling tumours to signal the nearby stroma to undergo increased growth to increase support networks, or differentiation to improve delivery of essential nutrients. Studies on stromal signaling

have found that estrogen in particular can be produced and released from breast stroma into nearby tissue, inducing growth in the tumour [110]. Estrogen can be produced directly from cholesterol in adipose tissue, or may be formed from androgens and other hormones by the action of aromatase enzymes.

7.2. Method.

7.2.1. Samples

The sample population was comprised of the standard 25 archived breast tissue sections embedded in paraffin as used in all the semi-quantitative PCR studies in this thesis, as outlined in Chapter 3. Controls for the stromal samples were the same control tissue as used in the tumour studies.

Due to difficulties with the PCR reaction for ESRα however, only three of the stromal samples from grades 2 and 3 of tumour tissue and two from grade 2 produced reliable results. The average ages for this population were 57.66, 56.0 and 59.66 years for grades 1, 2 and 3 stroma, respectively.

7.2.2. Expression Assay.

cDNA made using the method outlined in Chapter 3 underwent PCR to amplify portions of cDNA that corresponded to the mRNA for the ESRα, ESRβ, PgR, GR and AR nuclear receptors with fluorescently tagged primers. Individual samples underwent PCR in triplicate for each gene before results were pooled.

As indicated in Chapters 5 and 6 above, there were difficulties in obtaining functional triple primer sets for ESRα and AR. Again in this instance, primers for the intronic/exonic fragment of the glucocorticoid receptor gene were used to detect genomic DNA contamination. No genomic DNA contamination was observed in any sample. Primer details appear in Table 7.1. The fragment amplified for the nuclear receptors was approximately 100 base pairs long, allowing cDNA derived from partially degraded RNA to be fully utilized. Fragments produced by any contaminating genomic DNA present was 150bp in length for samples using the PgR or GR primers and 161 for the ESRβ intronic primer. As outlined in Chapter 3, the nuclear receptors were also multiplexed with the ribosomal 18S gene, to control for PCR efficiency and variations in basal expression. Chemical conditions for each PCR are outlined in Tables 7.2.1 to 7.2.5. Gene expression was quantified using an ABI 310 Genetic Analyzer, utilizing peak height as the measure of expression.

Table 7.1: Primer Compositions.

Primer Name	**Sequence (5'-3')**
ESRaEX1-F1*	CCAAAGCATCTGGGATGGCC
ESRaEX1-R1	GGATCTTGAGCTGCGGACGG
ESRbEX2-F1*	CCAACACCTGGGCACCTTTC
ESRbEX2-R1	CCAGGGACTCTTTTGAGGTTC
ESRbIntron2	TGGCTAGCAACTATAATTCAGAATGAA
PREX4-F1*	ATTGATGACCAGATAACTCTCCAT
PREX4-R1	CTGACGTGTTTGTAGGATCTC
PREX4-R2	GTAGTTAATTTACTGCATAGAGTG
GREX2-F1*	GAGTACCTCTGGAGGACAGA
GREX2-R1	GCTTCTGATCCTGCTGTTGA
GREX2-R2	ATGTCCATTCTTAAGAAACAGGA
AREX1-F1*	CCTGATGTGTGGTACCCTGG
AREX1-R1	CCGGAGTAGCTATCCATCCA

*= Primer labeled with TET at 5'

Table 7.2.1 Chemical Conditions for ESRα PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix E (From Epicentre)	5x	5.5 μL
ESRα Forward Primer	5μM	2 μL
ESRα Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	1 μL
18S Reverse Primer	5μM	1 μL
GR Forward Primer	5μM	1 μL
GR Intronic Reverse Primer	5μM	1 μL
BE polymerase	1/5 dilution	0.3 μL
Water	-	0.7 μL
cDNA	-	2 μL
Total	-	16.5 μL

Table 7.2.2 Chemical Conditions for ESRβ PCR.

Reagent	**Concentration**	**Quantity per Reaction**
MgCl	25mM	2 μL
PCR Buffer	10x	2 μL
ESRβ Forward Primer	5μM	2 μL
ESRβ Reverse Primer	5μM	2 μL
ESRβ Intronic Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	1 μL
18S Reverse Primer	5μM	1 μL
dNTPs	5mM	0.8 μL
BE polymerase	1/5 dilution	0.5 μL
Water	-	1.5 μL
cDNA	-	2 μL
Total	-	17.8 μL

Table 7.2.3. Chemical Conditions for AR PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix D (From Epicentre)	5x	5.5 μL
AR Forward Primer	5μM	2 μL
AR Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	2 μL
18S Reverse Primer	5μM	2 μL
GR Forward Primer	5μM	1 μL
GR Intronic Reverse Primer	5μM	1 μL
BE polymerase	1/5 dilution	0.3 μL
cDNA	-	2 μL
Total	-	17.8 μL

Table 7.2.4. Chemical Conditions for PgR PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix D (From Epicentre)	5x	5.5 μL
PgR Forward Primer	5μM	2 μL
PgR Reverse Primer	5μM	2 μL
PgR Intronic Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	2 μL
18S Reverse Primer	5μM	2 μL
BE polymerase	1/5 dilution	0.3 μL
cDNA	-	2 μL
Total	-	17.8 μL

Table 7.2.5. Chemical Conditions for GR PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix D (From Epicentre)	5x	5.5 μL
GR Forward Primer	5μM	2 μL
GR Reverse Primer	5μM	2 μL
GR Intronic Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	2 μL
18S Reverse Primer	5μM	2 μL
BE polymerase	1/5 dilution	0.3 μL
cDNA	-	2 μL
Total	-	17.8 μL

7.2.3. Statistical Analysis.

Expression data obtained for all receptors was normalized by expressing it as a ratio of the expression of the receptor to 18S, as outlined in Chapter 3. Normalized final data was analysed using One-way Analysis of Variance (ANOVA) to determine if there was a significant difference of expression between tumour grades. A second ANOVA test was

performed to determine whether expression of the nuclear receptors was significantly affected by ESRα status, rather than breast cancer grade. Homogeneity of variance tests were also performed to discover whether non-parametric tests would be required in addition to the original ANOVA. Appropriate post-hoc tests were subsequently performed to elucidate any differences found. The conventional α-level of 0.05 was specified as the significance threshold. The software package SPSS version 10.1 was employed for all statistical analyses.

7.3. Results.

The expression levels for ESRα, ESRβ, PgR, GR and AR were determined for all archival tissue samples (with the exception of ESRα, as detailed in the Samples section above), with the normalized data summarized in Tables 7.3 and 7.4 for grade and ESRα status respectively. Comparisons were then made using the grade of tumour the stroma was derived from and the tumour's ESRα protein status as grouping variables. Initial observations of the data indicated that several possible alterations in expression for the various groups could be occurring. ESRα expression for grade 1 stroma (see Figure 7.1) showed an increase in expression compared to control breast tissue and a decrease in grade 3 stroma, PgR (Figure 7.3) showed a drop in expression for all stromal tissues, while GR and AR (Figures 7.4 and 7.5) showed increased expression in later stage stromal tissues. By comparison, the data for ESRβ indicated that there was little variance in ESRβ expression, regardless of what stage of cancer the tissue is derived from (see

Figure 7.2).

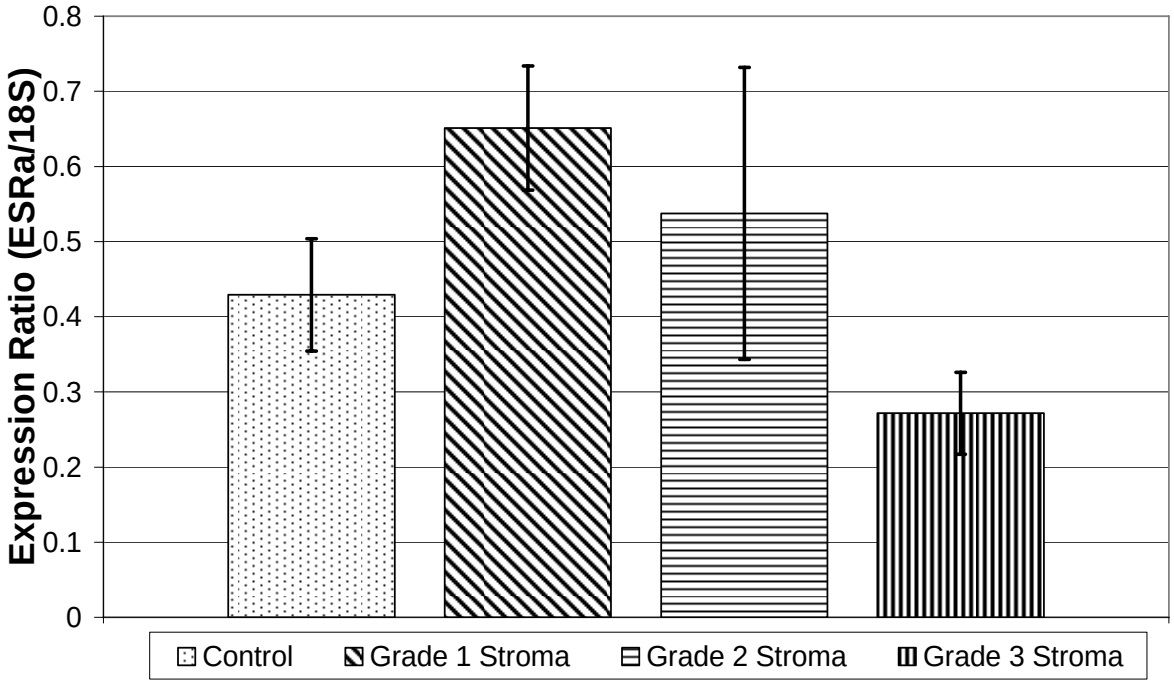

Figure 7.1: Expression of ESRα in Stroma Derived From Different Tumor Grades.

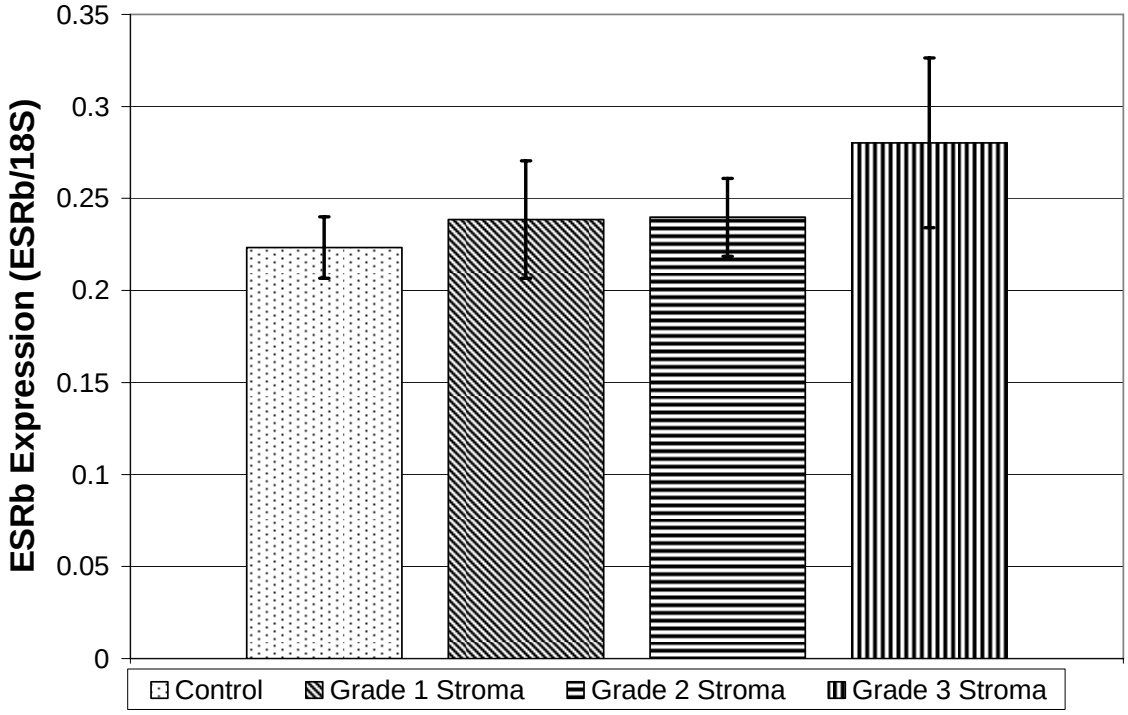

Figure 7.2: Expression of ESRβ in Stroma Derived From Different Tumor Grades.

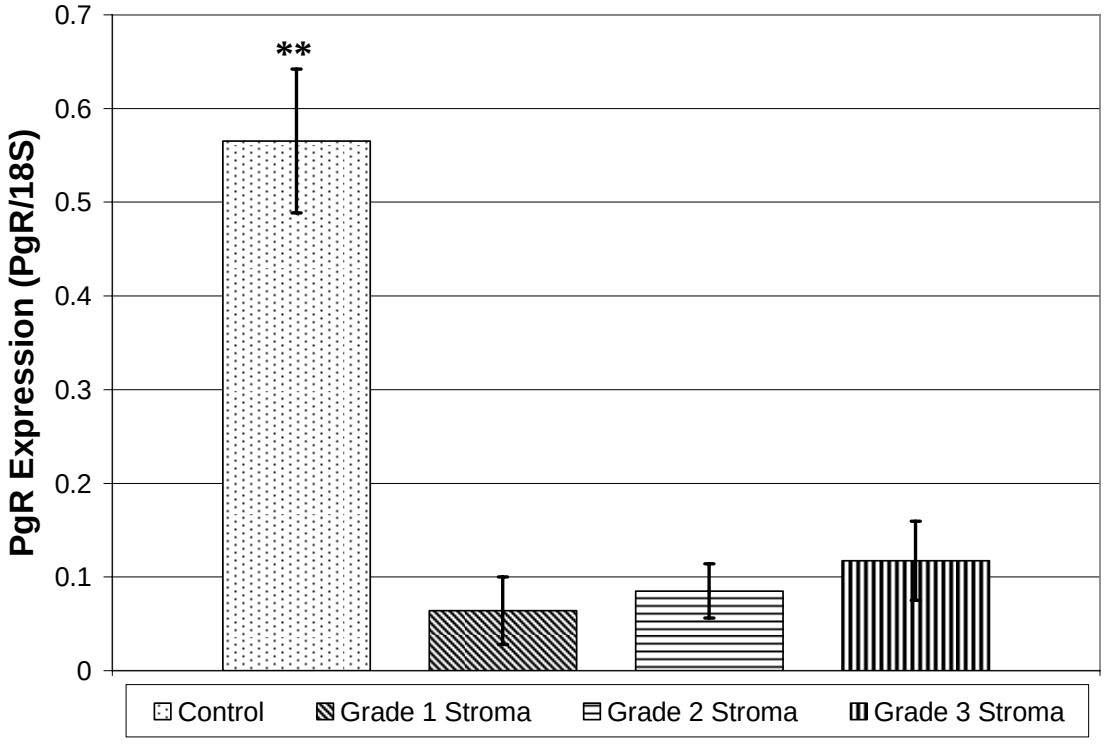

Figure 7.3: Expression of PgR in Stroma Derived From Different Tumor Grades.

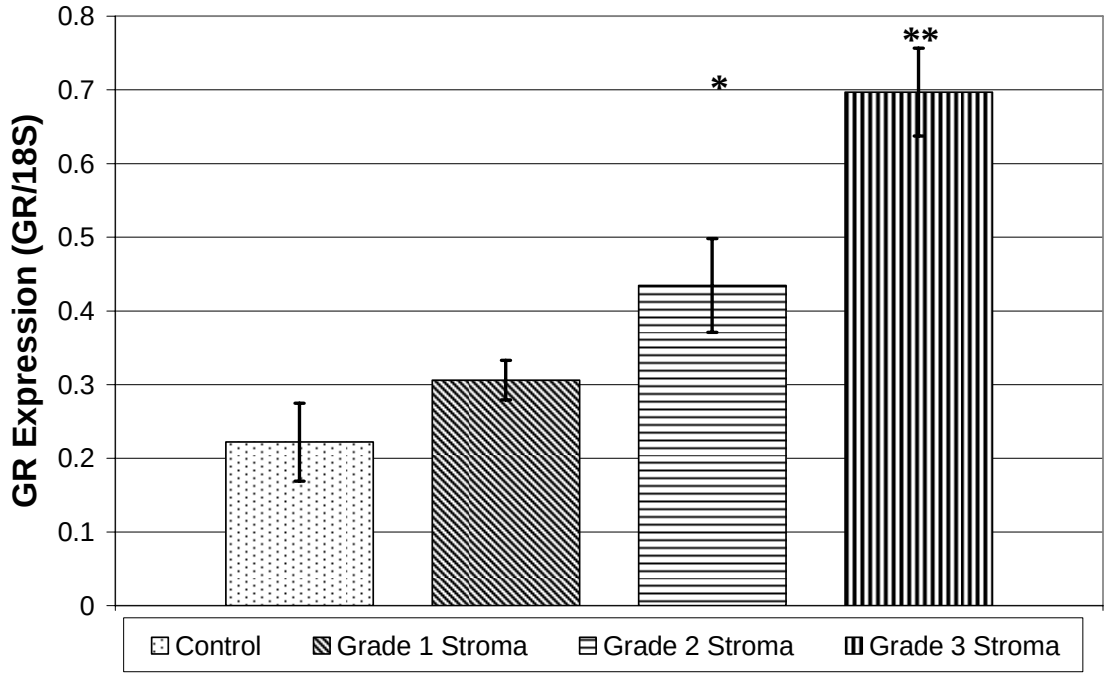

Figure 7.4: Expression of GR in Stroma Derived From Different Tumor Grades.

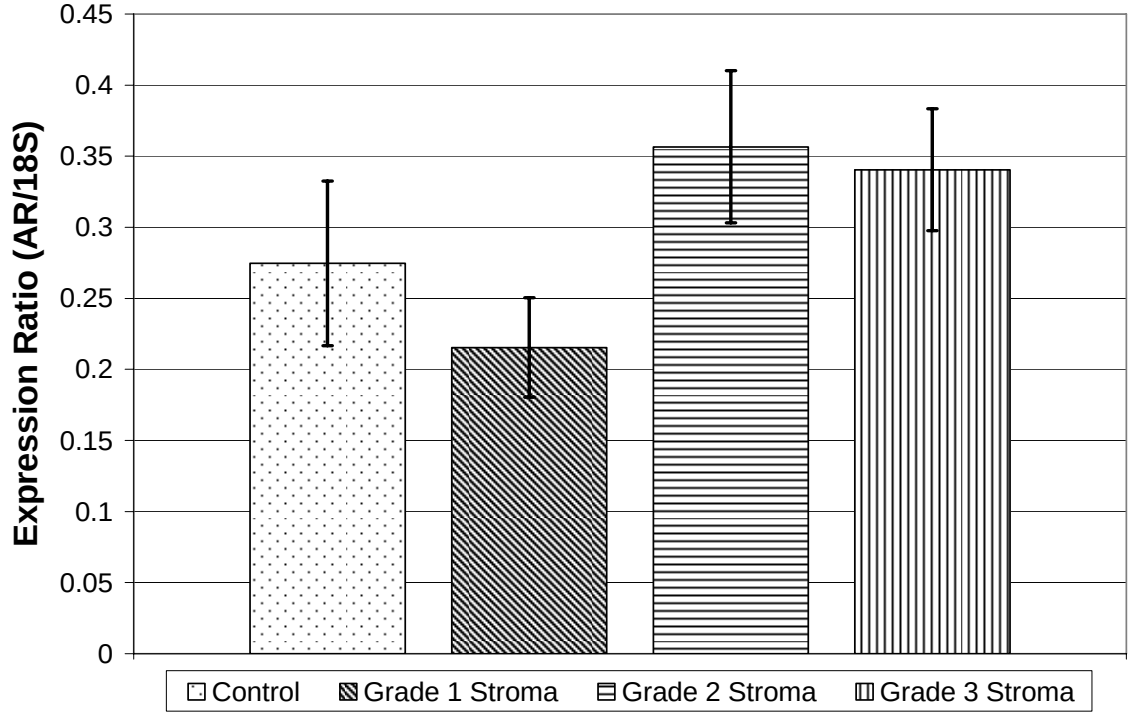

Figure 7.5: Expression of AR in Stroma Derived From Different Tumor Grades.

However, when ANOVA testing was applied, significant results were only returned for the progesterone and glucocorticoid receptors (both $p< 0.0001$), with the alterations shown in ESRα and AR being insignificant ($p= 0.372$ and $p=0.174$, respectively). Changes in ESRβ were, as the preliminary data indicated, insignificant, with a p value of 0.655. Post-hoc tests indicated that the expression of PgR was significantly lower in all stromal tissues compared to normal breast tissue. The expression of GR was increased in stroma from grades 2 and 3 tumour tissue, with grade 1 forming an intermediate step between control and grade 2 stroma.

Table 7.3: Normalized Data for Stromal NR Expression by Tumour Grade (NR/18S).

Tissue	**ESRα**	**ESRβ**	**PgR**	**GR**	**AR**
Control	0.429228	0.223349	0.56524	0.221925	0.274586
Grade 1 Stroma	0.651124	0.238575	0.064177	0.306093	0.215329
Grade 2 Stroma	0.537429	0.239743	0.085006	0.43453	0.356536
Grade 3 Stroma	0.25739	0.280238	0.117354	0.696899	0.340419

Table 7.4: Normalized Data for Stromal NR Expression by ESRα Status (NR/18S).

Tissue	**ESRα**	**ESRβ**	**PgR**	**GR**	**AR**
ESRα positive	0.533501	0.246548	0.226968	0.33677	0.278677
ESRα negative	0.376379	0.266598	0.077481	0.686333	0.361058

The initial data for the comparison by ESRα protein status showed some relatively minor changes in all samples, but the two most pronounced differences were for PgR and GR, showing a drop and a rise in receptor expression, respectively. After ANOVA analysis however, only the difference observed in GR expression proved to be significant ($p = 0.00005$), with all other differences in the tested receptors being insignificant. A summary of all ANOVA results, for both grade and ESRα protein status comparisons can be found in Table 7.5.

Table 7.5: ANOVA Results for NR Expression by Tumour Grade and ESRα Status.

Nuclear Receptor	**Tumour Grade**		**ESRα Protein Status**	
	F Statistic	Significance	F Statistic	Significance
ESRα	1.249	0.372	0.532	0.487
ESRβ	0.547	0.655	0.533	0.473
PgR	23.799	5.908×10^{-7}	2.425	0.133
GR	14.228	2.761×10^{-5}	24.093	5.85×10^{-5}
AR	1.822	0.174	2.228	0.149

With expression data for both tumour and stroma available, the expression data from Chapters 4, 5 and 6 have been graphed below in Figures 7.6 to 7.10, together with the stromal expression obtained in this Chapter to gain an overview of NR expression in different cancer grades.

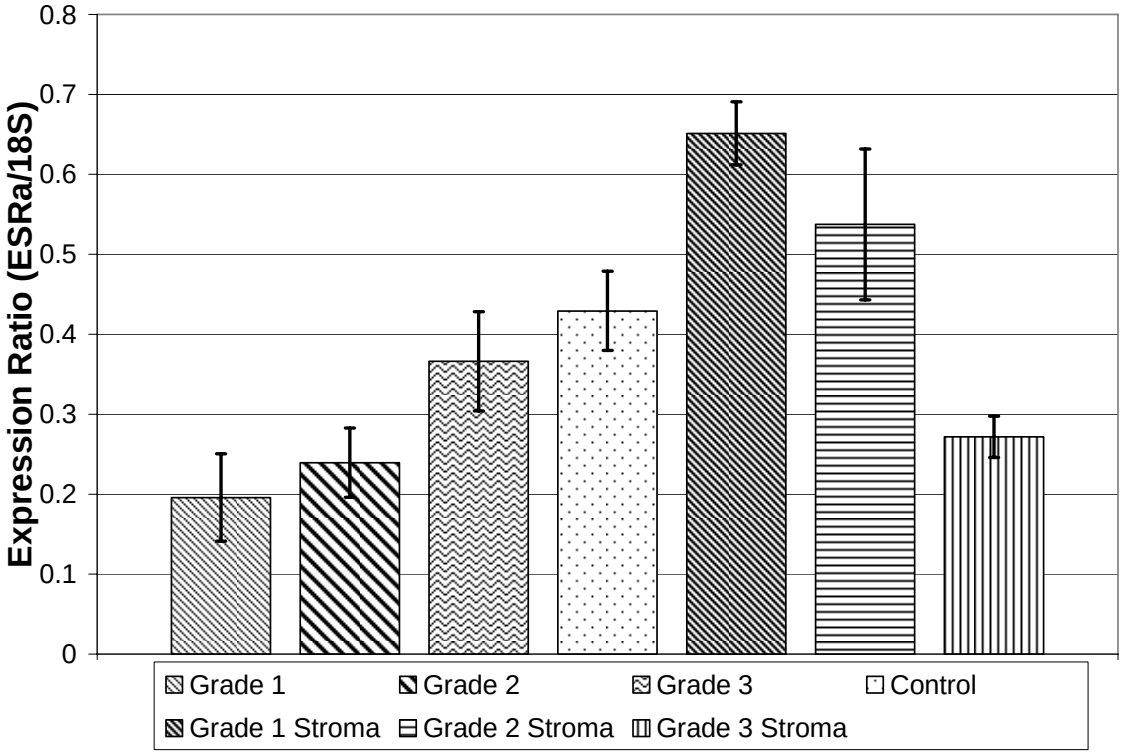

Figure 7.6: Expression of ESRα in Tumour and Stroma.

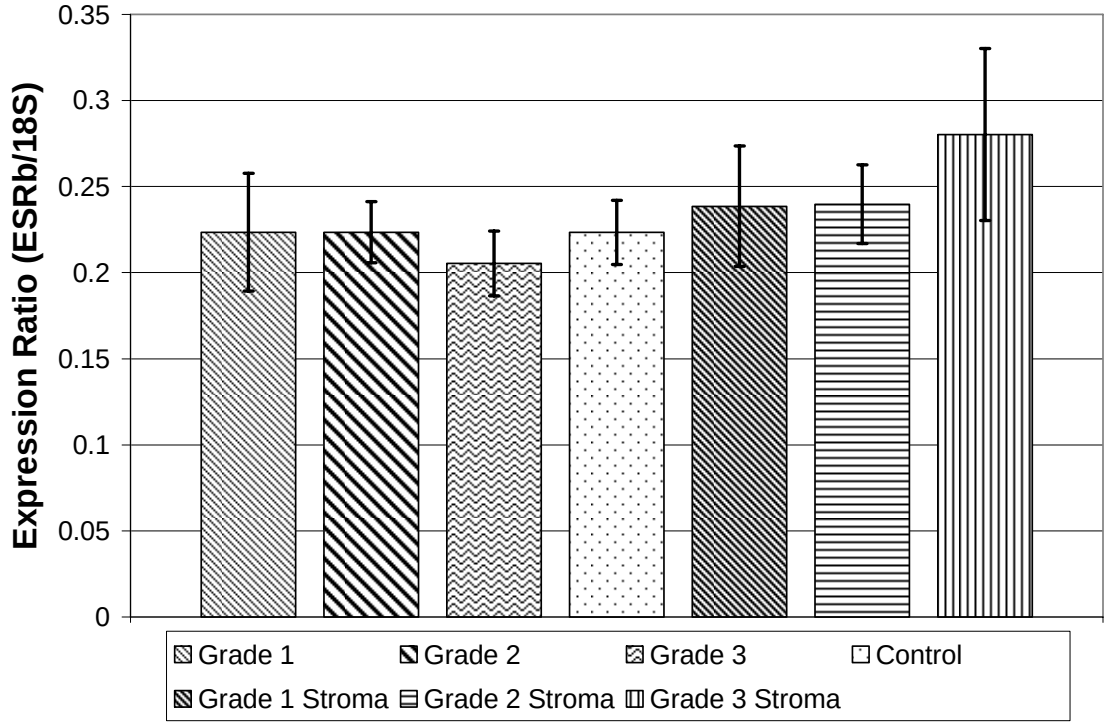

Figure 7.7: Expression of ESRβ in Tumour and Stroma.

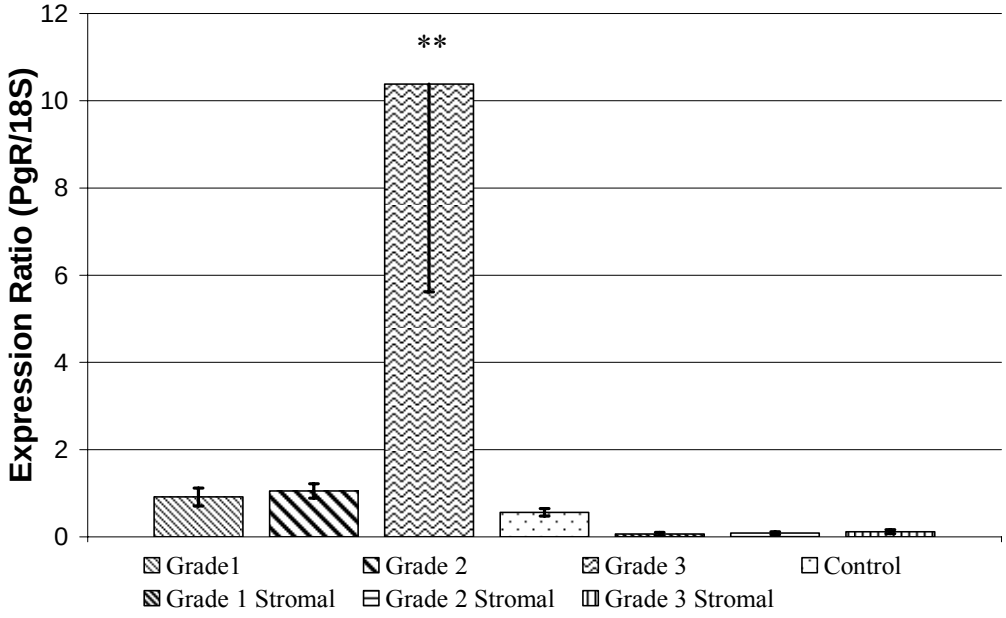

Figure 7.8: Expression of PgR in Tumour and Stroma.

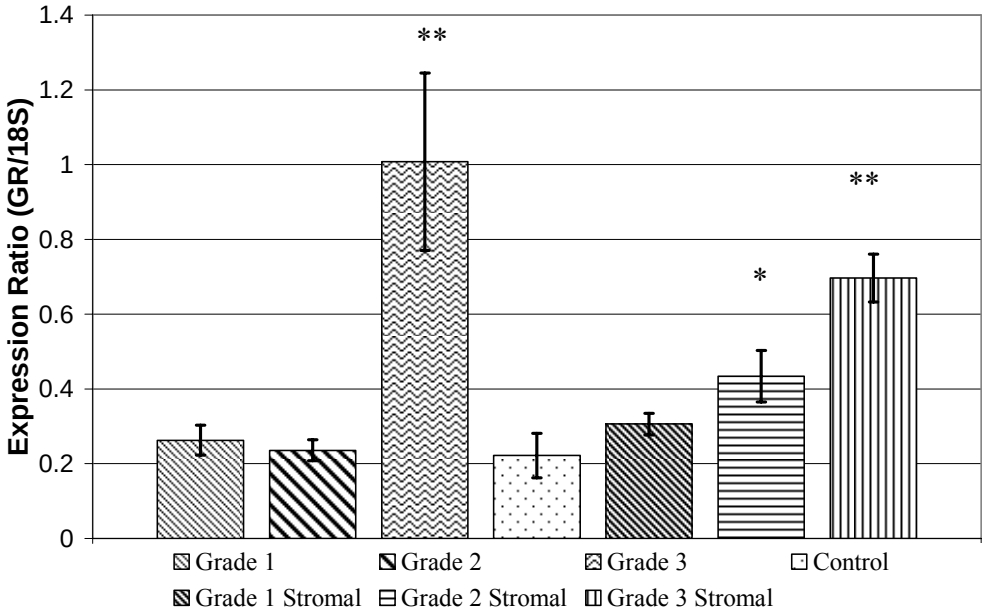

Figure 7.9: Expression of GR in Tumour and Stroma.

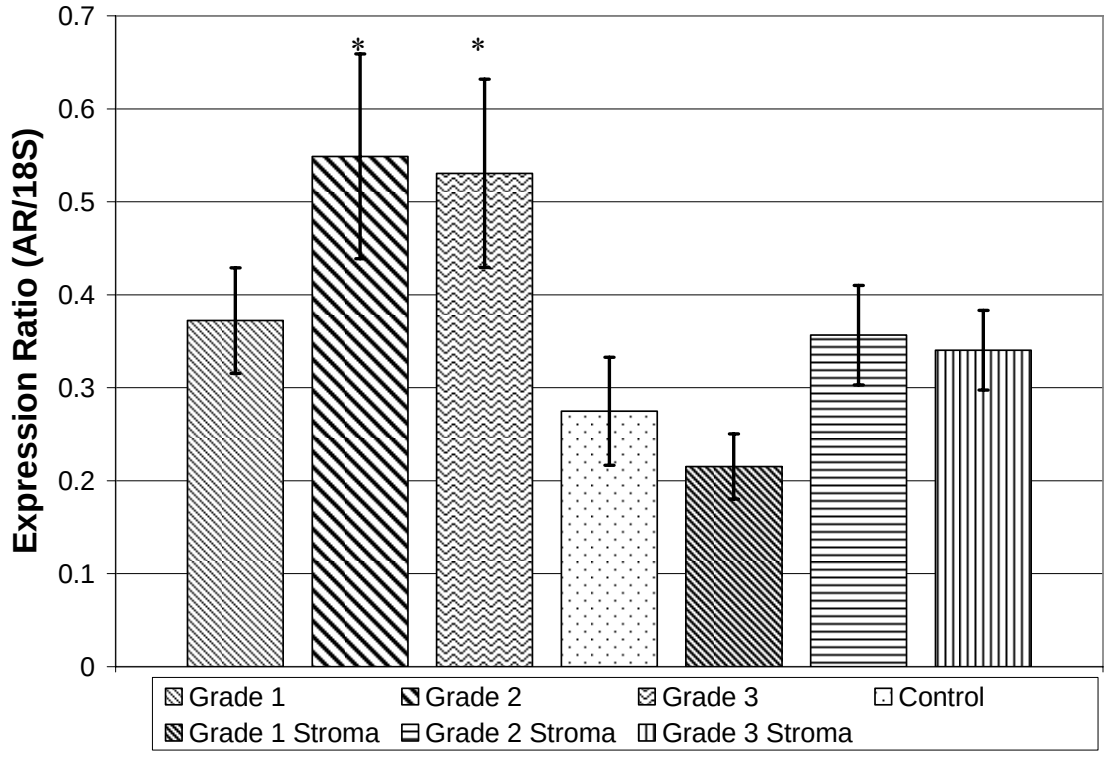

Figure 7.10: Expression of AR in Tumour and Stroma.

7.4. Discussion.

The results of these experiments have shown that there were some significant differences in gene expression between normal breast tissue and the stromal tissue of tumours derived from different cancer grades and between normal breast tissue and stromal tissue when analysed by ESRα protein status. Both the progesterone and glucocorticoid receptors showed significant alteration to their expression, depending on the grade of tumour that the stroma was supporting.

For the progesterone receptor, this manifested in a significant drop in expression for all grades of cancer when compared to control. This is in marked contrast to the mRNA pattern observed in the tumours, which showed increasing expression of PgR as cancer grade increased, with a significant difference in grade 3 as compared to control (see Chapter 4). These expression patterns for the stromal samples indicate that something significant is happening to the signals they receive after the time of carcinogenesis and that the tumour itself may be the culprit. It is, of course, in the interest of the tumour that the tissue that supports it will be fully differentiated and capable of performing all of the various functions the tumour requires. Hence, it is possible that the tumours are signaling the stromal tissue through the progesterone pathway, resulting in a reduction of PgR expression as is normal. However, if this were the case we would have expected to see a

rise in ESRα expression in response to such PgR stimulation, which was not present. This could be explained by the lower number of samples that were able to produce successful results for ESRα, as there is a rise in ESRα expression in early tumours in the data, but this was not significant. Alternately, the tumours may be signaling the pathway at a point which causes the down-regulation of PgR, but not affecting ESRα.

The glucocorticoid receptor also showed a significant alteration to its expression in the tested tissues, when both grade and ESRα protein status were examined. Expression of GR was increased in stromal tissue supporting grade 2 and 3 tumours as compared to control tissues and grade 3 stroma compared to all tissue types. Expression of GR was also significantly elevated in stroma derived from ESRα negative tumours. It is important to note that ESRα negative tumours in this population are restricted completely to grades 2 and 3, though these grades are not universally ESRα negative, as indicated in Table 3.1. Thus, it is possible that these results may be measuring the same effect, and that the increase in GR expression may be an increase in stress signals on the cells as the tumours develop, or it may be the result of an alteration in tumour signaling which occurs at or near the time when ESRα expression is lost. If the increase is a result of cumulative signaling on the stroma, it may be a defensive response by the stroma to increase its susceptibility to glucocorticoid induced apoptosis. Alternately, the expression could be the result of signals to the tumour for the same reasons, since the GR results in Chapter 3 showed an increase in GR expression in the same tumours. It is also possible that the increased GR mRNA levels observed in the grades 2 and 3 tissues may not reflect an actual increase in GR protein in the cell, with mRNA being degraded prior to translation,

truncated to produce a non-functional protein or spliced into the alternate isoform of GR which antagonizes the primary form, as may also be occurring in the tumours.

There is also some perturbation to expression of ESRα, ESRβ and AR across cancer grades, but these changes are insignificant and no real conclusions can be drawn from them. It is interesting to note however, that AR expression was found to be significantly affected by cancer grade within the tumours themselves in the previous experiment in chapter 6, yet remains unchanged in the stromal tissue. Like both PgR and GR, the expression of AR in these tumours was found to increase along with cancer grade. As postulated in the discussion for that experiment, the elevated expression of AR in the tumour tissue might be a self-induced event, used as a means of side-stepping normal sensitivity to the anti-mitogenic nuclear receptors. Highly expressed AR would sequester available chaperone molecules common to the nuclear receptor family and thus render the high levels of PgR and GR observed in the tumours non-functional. The data from this experiment lends some credence to this theory, since AR expression is only changed within the tumour itself, with the stroma maintaining a normal level of AR expression. However, it is also possible that the stroma is causing this up-regulation of AR expression in the tumours through some signaling mechanism, either as part of the aforementioned de-sensitization to PgR and GR, or as part of a normal growth arrest mechanisms to attempt to slow the growth of the tumour. The data offers no solid conclusions on this theory, but it does indicate that the tumour is not affecting AR expression in the stroma, excepting the possibility that signals from the tumour are keeping it unchanged.

The data for both estrogen receptors does not show any significant alteration in their expression for any cancer grade, nor when ESRα protein status of the tumour is considered. However, the tumours themselves also showed no change in expression, yet many of the advanced tumours in this population had no detectable ESRα protein present within them. Therefore the control of ESRα expression, if not that of ESRβ which also remains static regardless of grade, would appear to be controlled at a level after mRNA transcription. Since the stroma shows no significant alteration in expression, it is possible that changes to ESR expression in the stroma also occur at a post-transcriptional level. This could be, perhaps, in the splicing of the mRNA into the various alternate isoforms of ESR alpha and beta, which modulate the effect of estrogen stimulation on the cell. It is worth considering, however, that the ESRα population in this study was smaller than the others, due to PCR difficulties, and that if a larger population had been examined, the alterations present in the data may have shown a significant result.

7.5. Conclusion.

The data from this experiment indicated that there are significant effects on the expression of PgR and GR in tumour stroma. PgR showed a drop in mRNA levels for PgR in the stroma of all grades of cancer, while GR showed an increase of mRNA levels in stroma derived from grades 2 and 3 tumours as well as ESR α protein negative tissues.

The expression of ESRα, ESRβ and AR remained unchanged. The precise implications of these results are unclear, as this data does not elucidate the ratios of the various isoforms of these receptors in the tissues. However, it is possible that the stromal tissues of advanced tumours would show an increased sensitivity to glucocorticoids and thus an increased rate of apoptosis if faced with GR stimulation.

CHAPTER 8. Nuclear Receptor Co-Activators.

8.1. Background.

The nuclear receptors do not function alone, requiring the presence of numerous co-enzymes which promote, assist and otherwise regulate their ability to transmit signals and change gene expression. Among these co-enzymes are the nuclear receptor co-activators, a family of genes with three known members in humans, which allow the nuclear receptors to bind to one another and recruit further protein complexes vital for nuclear receptor function.

Members of the nuclear receptor co-activator (NCoA) family has a number of additional names, according to their putitative functions or protein associations, most notably NCoA3, which has the alternative name AIB1, short for Amplified in Breast Cancer. As this implies, the over-expression of NCoA3 and the other NCoA family members has been found to increase the transactivation function of several nuclear receptors, and experiments have found them to be required for the function of the nuclear receptors [77]. The NCoA proteins interact with the ligand binding domains of activated nuclear

receptors, binding both receptors in a dimer through LXXLL amino acid motifs present on both the NCoA and NR proteins, with binding observed to be taking place near the AF-2 domain of the nuclear receptor [76, 77].

Once bound to the activated nuclear receptor, the NCoA protein is able to recruit the CBP/p300 and p/CAF protein complexes, both of which have an acetyltransferase activity, which results in the acetylation of histone proteins, which is required for the de-repression of the genes controlled by the nuclear receptors [77, 111]. Members of the NCoA family are also known to have a weak histone acetyltransferase activity, but it is unknown as to exactly how this comes into play and if the NCoA proteins are able to increase transcription on their own. Binding to the additional cofactors also appears to be mediated by the LXXLL amino acid motifs, a theory reinforced by crystal structure analysis and the conservation of these motifs in the genes, indicating that they perform some important function in the overall complex [77, 111, 112].

It is currently poorly understood if the NCoA genes have specificity for the nuclear receptors they bind to or are controllers of the type of additional cofactors they recruit and thus the final function of the NR complex. However, some evidence from peroxisome proliferator activated receptor gamma binding assays indicates that the specific NCoA bound to a NR complex does affect the binding of additional cofactors. The specificity of the NCoA gene may, however, be mediated through amino acids that border the LXXLL motifs mediating NR binding [76, 77]. Other studies support this, indicating that NCoA1 activity is restricted to the nuclear receptors that accept hormone

signals [111]. However, some additional studies with NCoA ablation have shown that loss of NCoA1 function does not remove hormonal signaling, cells responding by up-regulation of NCoA2, indicating that the NCoA family may not be entirely locked down for receptor specificity, functions perhaps mediated by NCoA isoform splicing [77, 111].

8.2. Method.

8.2.1. Samples

The sample population was comprised of the standard 25 archived breast tissue sections embedded in paraffin as used in all the semi-quantitative PCR studies in this thesis, as outlined in Chapter 3. Due to difficulties with the PCR reaction for NCoA3, only 5 stromal samples from grade 3 produced reliable results.

8.2.2. Expression Assay.

cDNA made using the method outlined in Chapter 3 underwent PCR to amplify portions of cDNA that corresponded to the mRNA for the nuclear receptor co-activator 1 and nuclear receptor co-activator 3 genes. Individual samples underwent PCR in triplicate for each gene before results were pooled.

Unlike the primers for members of the nuclear receptor family in prior chapters, the primers for the NCoA genes were intron spanning, meaning that the primers lay on opposite sides of an intron. As a result, any genomic DNA contamination would produce larger amplicons than those produced from cDNA, which have the introns spliced out. However, because the introns are kilobases in length and unlikely to amplify in a PCR optimized for small fragments, the intronic primers for GR were multiplexed into the NCoA PCRs to detect genomic DNA. No genomic DNA contamination was observed in any sample. Primer details appear in Table 8.1. Also, as outlined in Chapter 3, the nuclear receptor co-activator genes were also multiplexed with the ribosomal 18S gene, to control for PCR efficiency and variations in basal expression. Chemical conditions for each PCR are outlined in Tables 8.2.1 and 8.2.2. Gene expression was quantified using an ABI 310 Genetic Analyzer, utilizing peak height as the measure of expression.

Table 8.1: Primer Compositions.

Primer Name	**Sequence (5'-3')**
NCoA1-F1*	CATTCCCCGGGAAGCTAC
NCoA1-R1	TTCACACCTGGGAGACTTCTT
NCoA3-F1*	TGGTTAGCCAGTTGCTGATGT
NCoA3-R1	GCAATTTGCGTTTTCGTGA
GREX2-F1*	GAGTACCTCTGGAGGACAGA
GREX2-R2	ATGTCCATTCTTAAGAAACAGGA

*= Primer labeled with TET at 5'

Table 8.2.1. Chemical Conditions for NCoA1 PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix C (From Epicentre)	5x	5.5 μL
NCoA1 Forward Primer	5μM	2 μL
NCoA1 Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	1 μL
18S Reverse Primer	5μM	1 μL
GR Forward Primer	5μM	1 μL
GR Intronic Reverse Primer	5μM	1 μL
BE polymerase	1/5 dilution	0.2 μL
Water	-	0.3 μL
cDNA	-	2 μL
Total	-	16 μL

Table 8.2.2. Chemical Conditions for NCoA3 PCR.

Reagent	**Concentration**	**Quantity per Reaction**
Master Amp Premix L (From Epicentre)	5x	5.5 μL
NCoA3 Forward Primer	5μM	2 μL
NCoA3 Reverse Primer	5μM	2 μL
18S Forward Primer	5μM	1 μL
18S Reverse Primer	5μM	1 μL
GR Forward Primer	5μM	1 μL
GR Intronic Reverse Primer	5μM	1 μL
BE polymerase	1/5 dilution	0.2 μL
Water	-	0.3 μL
cDNA	-	2 μL
Total	-	16 μL

8.2.3. Statistical Analysis.

Expression data obtained for NCoA1 and NCoA3 was normalized by expressing it as a ratio of the expression of the receptor to 18S, as outlined in Chapter 3. Normalized final

data was analysed using One-way Analysis of Variance (ANOVA) to determine if there was a significant difference of expression between tumour grades. A second ANOVA test was performed to determine whether expression of the nuclear receptor co-activators was significantly affected by ESRα status, rather than breast cancer grade. Homogeneity of variance tests were also performed to discover whether non-parametric tests would be required in addition to the original ANOVA. Appropriate post-hoc tests were subsequently performed to elucidate any differences found. The conventional α-level of 0.05 was specified as the significance threshold. The software package SPSS version 10.1 was employed for all statistical analyses.

8.3. Results.

The expression levels for the NCoA1 and NCoA3 genes were determined in all samples, both tumour and stroma, with the normalized data being shown in Table 8.3, below. Comparisons were then made using the grade of tumour and the tissue's ESRα protein status as grouping variables, with the normalized data being shown in Table 8.4, below. Initial observations of the data indicated that some changes in expression for the NCoA genes were occurring for both grades and ESRα status. Expression ratios for NCoA1 (Figure 8.1) appeared to drop in early tumours and then rise to much higher levels in late grade tumours, while increasing in early stage stroma and dropping away to levels similar to control in grade 3 stroma. This trend was continued in the ESRα expression data

(Figure 8.3), which showed a higher level of expression in ESRα negative tissues. For NCoA3, expression appeared to lower in grade 1 and 2 tissue compared to control, and increase again to higher levels in grade 3 tumours. Stromal expression appeared to drop in grade 1 stroma, rise slightly for grade 2 stroma and drop again in grade 3 stroma.

Table 8.3: Normalized Data for NCoA Expression by Tumour Grade (NCoA/18S).

	Tumour Data		**Stromal Data**	
Tissue	**NCoA1**	**NCoA3**	**NCoA1**	**NCoA3**
Control	0.269270	0.531451	-	-
Grade 1	0.150102	0.425304	0.374664	0.476234
Grade 2	0.285357	0.326563	0.322915	0.535601
Grade 3	0.546922	0.608348	0.279531	0.494251

Table 8.4: Normalized Data for NCoA Expression by ESRα Status (NCoA/18S).

Tissue	**NCoA1**	**Std Dev**	**NCoA3**	**Std Dev**
ESRα positive	0.263354	0.195698	0.4945	0.222377
ESRα negative	0.466086	0.131074	0.468332	0.20894

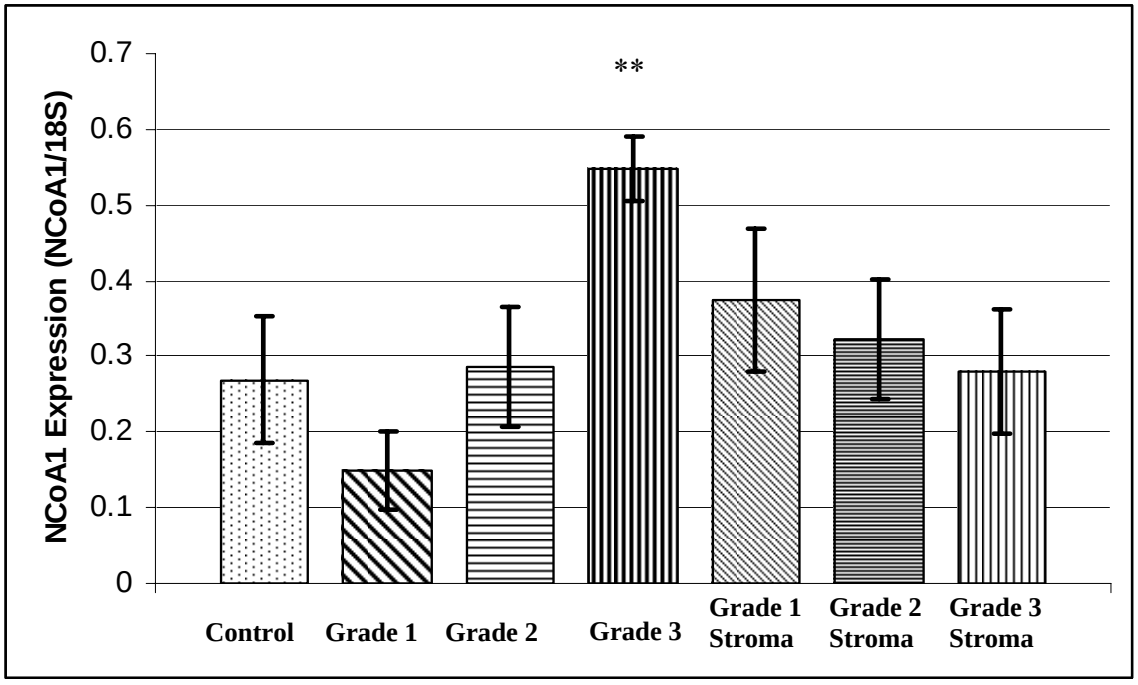

Figure 8.1: Expression of NCoA1 in Tissue Derived From Different Tumor Grades and Surrounding Stroma.

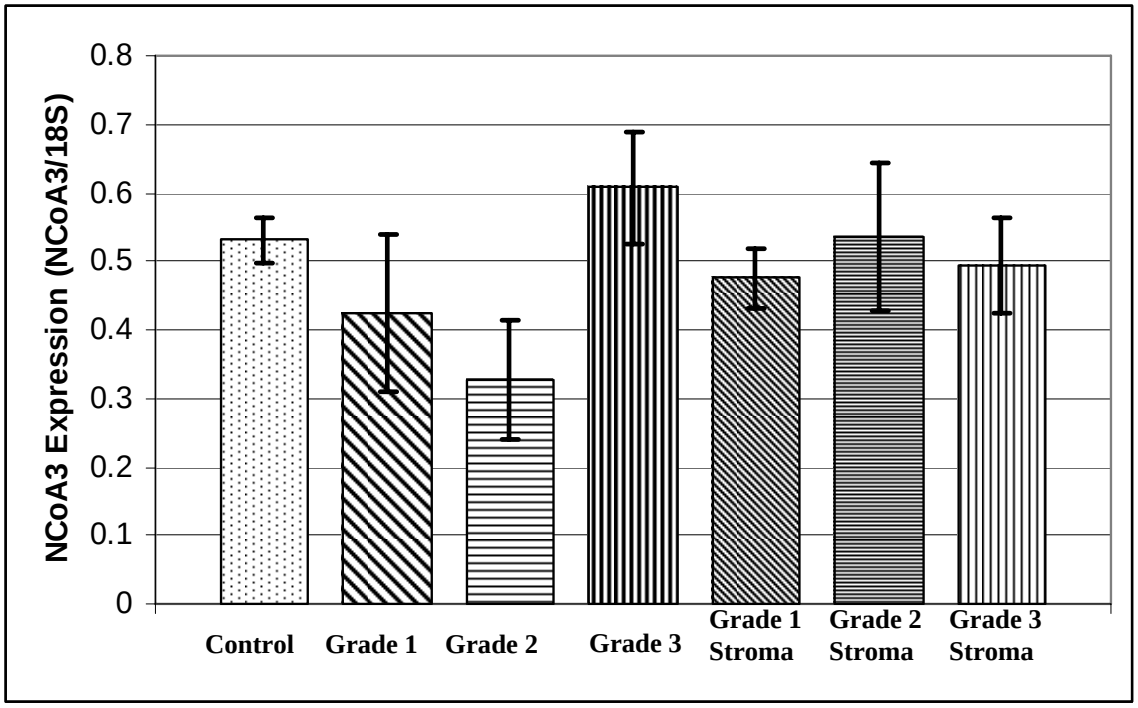

Figure 8.2: Expression of NCoA3 in Tissue Derived From Different Tumor Grades and Surrounding Stroma.

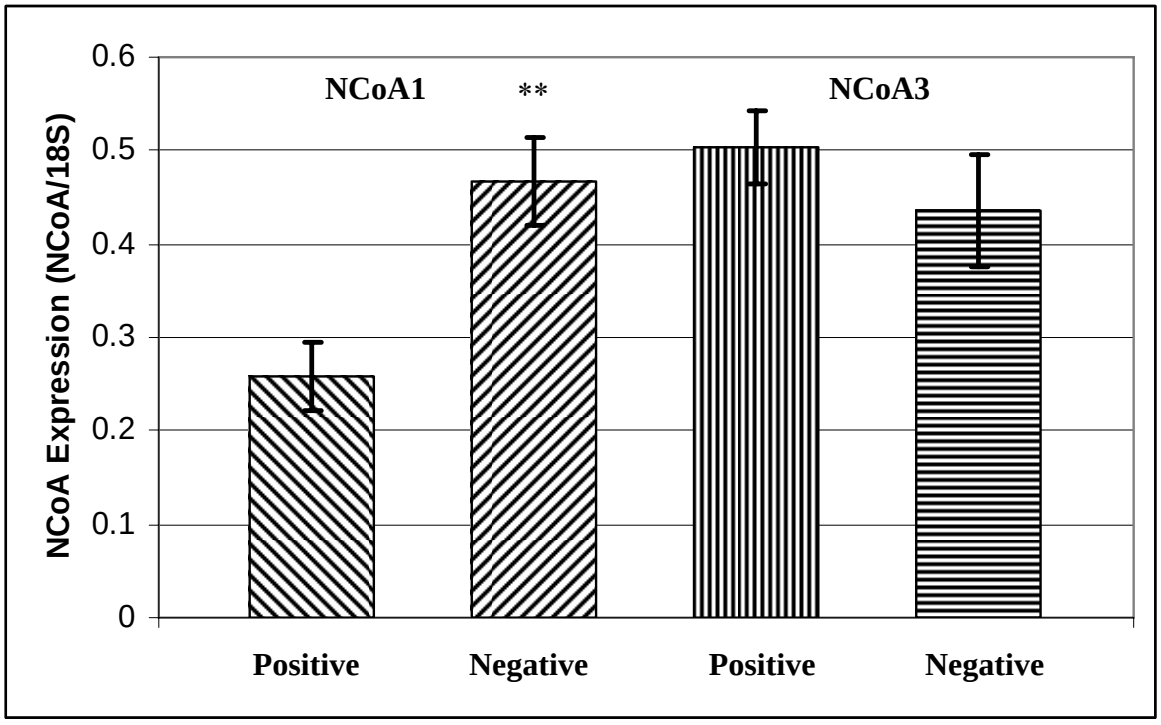

Figure 8.3: Expression of NCoA1 and NCoA3 in Tissue Derived From Different Tumor Grades and Surrounding Stroma, Compared by ESRα status.

After the expression data was analysed using ANOVA, the differences in these preliminary observations proved to be significant only for NCoA1 with a p value of 0.028 for cancer grade and 0.001 for ESRα status. By contrast, the p values for NCoA3 were 0.322 for the cancer grade analysis and 0.365 for the ESRα protein analysis. The full results for the ANOVA tests are summarized in Table 8.5. Post-hoc tests on the NCoA1 data indicated that the expression of NCoA1 was elevated in grade 3 tumours, but only in comparison to grade 1 tumours. For the ESRα protein status analysis, the expression of NCoA1 significantly increased in ESRα negative tissues, both tumour and stroma derived from those tumours, compared to ESRα positive tissues. Both NCoA1 and

NCoA3 proved to be normally distributed, with p values from the homogeneity of variances test being 0.089 and 0.107, respectively.

Table 8.5: ANOVA Results for NCoA Expression by Tumour Grade and ESRα Status.

Gene	**Tumour Grade**		**ESRα Protein Status**	
	F Statistic	Significance	F Statistic	Significance
NCoA1	2.712	0.028	11.624	0.001
NCoA3	1.214	0.322	0.837	0.365

8.4. Discussion.

The results of this experiment have shown that the expression of NCoA1 is significantly affected by both cancer grade and ESRα protein status, while NCoA3 expression remains unchanged in all tumour grades, surrounding stroma and in ESRα protein negative tissues compared to positive tissues.

The NCoA family are transcription factors that bind to various signaling proteins and recruit further proteins to the signaling complex, modulating the effects of incoming cellular messengers, including the steroid hormones and other factors such as transforming growth factor β [76, 77, 113]. As such, they have a role to play in multiple

processes, including cellular growth, function and differentiation, making their regulation important in the development and progression of cancer [76, 77, 114].

The results for NCoA1 indicated that the expression of that gene is increased in late stage breast cancer. This result was not entirely unexpected, as the expression of NCoA family members, including NCoA1, have been found to be up-regulated in many cancers, including breast tumours and cell lines [114-116]. Such up-regulation increases the transcriptional activity of a large range of target genes and can affect the cells so doing in multiple ways. Proteins that both promote and repress cellular division are assisted in their function by NCoA1, which is associated with the steroid hormones, meaning that an increase in expression may not have any specific effect on the tumour cells, contributing to the behavior of the cells in concert with other co-activators and the signaling milieu the cells find themselves in.

The results for NCoA1 also indicated that the expression was increased in grade 3 tumours in comparison to grade 1 tumours only, which themselves are not significantly different to any other class of tumor, nor are they different to control or stromal tissue. Hence, although the expression of NCoA1 was high in grade 3 tumours, it was not significantly higher than the expression of NCoA1 in control tissue. This is perhaps not a reflection of a difference between grade 1 tumours and grade 3, but rather simple variation between the different tumours, as there are considerable differences within the grades, as can be seen from the error bars in Figure 8.1. This, however, is consistent with results from other studies, which indicate that over-expression of the NCoA family is not

universal for all cell lines or tumours, despite the fact that it does occur [114-116]. More likely to be the result of a specific effect was the result of the ESRα protein analysis, which indicated that the expression of NCoA1 was higher in tissues that are ESRα negative. With a p value of 0.001, this association was stronger than cancer grade's p value of 0.028 and has lower variation in the groups, reinforcing the idea that the loss of the ESRα protein may correlate to an increase in NCoA1 expression. It is also possible, however, that the difference observed was because all the ESRα negative tissues are advanced grade 3 tumours.

There are numerous effects that the increase in NCoA1 expression could be having within the cells, particularly in view of the results previously obtained for the nuclear receptors. Most basic is the possibility that the expression of NCoA1 is increasing to meet the requirements of increased PgR, GR and AR expression in the later stage tumours. This could either be as part of the normal anti-proliferative function of these genes or as a requirement for any isoform mediated growth stimulatory effect. In either case, a member of the NCoA family would need to be present to allow NR genomic functions to occur. In view of this and the strong association of NCoA1 expression increase and ESRα protein negativity, NCoA1 may be playing a role in estrogen independence. If estrogen independence in these tissues is being mediated through the production of truncated ESRα proteins which are not detectable using the standard antibodies and longer require estrogen to stimulate them, then an NCoA family member would still be required to allow stimulation of the target genes. Thus, to maintain a high level of growth, the cells would require higher than normal amounts of NCoA.

For NCoA3, the data indicated that there was no significant association between the expression of this gene and either cancer grade, or ESRα protein status. In part, this was an unexpected result, because one of the additional names NCoA3 bears is AIB1, or amplified in breast cancer. As this suggests, NCoA3 has been found to have increased expression in breast cancer, both in cell lines and biopsies [114, 115]. As mentioned for NCoA1 above, however, this trend is not universal, and other studies have found that the expression of NCoA3 remains unchanged in some cell lines [114, 116]. It is worth noting, however, that the general level of expression for NCoA3 was higher than NCoA1 and that the expression of NCoA3 in grade 3 tumours is higher than in control tissues, though this was not significant. NCoA3 may not be being over-expressed in the tumours because the particular alterations to growth induction pathways that these tumours are using to grow do not require NCoA3. It is also possible that the cells are not experiencing a significant increase in NCoA3 expression because the NCoA requirement of the cells is being fulfilled by the increase in NCoA1 expression. Finally, as for the nuclear receptors, it is possible that post transcriptional modification of the NCoA3 mRNA is modulating its function, removing the necessity to increase expression to achieve a specific function.

8.5. Conclusions.

The data from this experiment indicated that there are significant effects on the expression of NCoA1 in late stage tumours and in tissues that are negative for the ESRα protein, showing an increase in expression in these tissues. In contrast, the expression of NCoA3 remained unchanged in all tissue types. Precisely what these results indicate is not fully clear and the role of NCoA3 and the increase in NCoA1 expression are likely to be part of a larger picture of nuclear receptor cofactor levels within the cells. The continued expression of the NCoA family members either at the control tissue baseline or at increased levels would seem to indicate, however, that these genes are not participating in any kind of anti-proliferative drive by the cell, except that they may be participating in the signaling from PgR, GR, AR or other anti-proliferative signaling proteins.

CHAPTER 9. Correlation of Pathological Characteristics and Gene Expression.

9.1. Background.

There are two main methods of determining cancer severity at time of diagnosis, termed stage and grade, as outlined in section 2.13, with stage running from I to IV and based on size and metastatic characteristics while grade ranges from 1 to 3 and is based primarily on cell morphology. Both these methods use various physical characteristics to gauge the advancement and probable future development of the tumour and are useful clinical tools in cancer treatment.

There are also numerous molecular tools that are able to assist in the diagnosis and treatment of cancer, such as immunohistochemistry, which can obtain a determination of the presence or absence of certain proteins within a tumour. This will typically be performed for known markers of extreme proliferation like HER2, further fine-tuning the clinician's ability to estimate the state of development of the tumour. The technique can

also be utilized for other molecules, most importantly for breast cancer ESRα and PgR, for a tumour that ceases expression of one or both of these receptors will respond poorly or not at all to tamoxifen, the primary breast cancer drug [18, 49].

Continuing studies on the development of cancer have shown that while there are multiple unique events in the progression of individual tumours, there are also a number of factors common to stages of cancer development that can be used to further prognosticate on future development. Research into aging has indicated that certain tumour types developing in older individuals show less aggression and are often diagnosed at a lower level of severity [2, 3, 17, 117]. Likewise, the probability of metastasis can be linked to the level of infiltration of cells into the surrounding ECM and the level of angiogenesis near the tumour. Indeed, the work in the previous chapters have linked the expression of PgR, GR and AR to cancer grade and ESRα protein status in tumours and PgR and GR to cancer grade in stroma.

Accordingly, this portion of the study sought to compare the expression data obtained for the nuclear receptors and nuclear receptor co-activators with pathological characteristics for the individual tumours and find any correlations that might exist between the expression of these genes and those pathological characteristics.

9.2. Method.

9.2.1. Samples

The sample population was comprised of the standard 25 archived breast tissue sections embedded in paraffin as used in all the semi-quantitative PCR studies in this thesis, as outlined in Chapter 3.

9.2.2. Pathology.

Pathological characteristics, such as age at diagnosis, presence of metastasis, size of tumour, ESRα staining, calcification in the tumour, which side the tumour originated on and the presence of *in situ* carcinoma were determined at the Pathology department of the Gold Coast Hospital, which also provided and graded the samples. These determinations were collated and organized into a complete data set and subjected to statistical analysis as outlined below.

9.2.3. Statistical Analysis.

Comparison of qualitative parameters was carried out using One-way Analysis of Variance (ANOVA), as for the expression data. Quantitative parameters were subjected to correlation testing, using Pearson's correlation and Spearman's correlation for abnormally distributed populations to determine to what degree any significant differences observed were able to be related between the parameters. Homogeneity of variance tests were also performed to discover whether a non-parametric test would be required in addition to the original ANOVA and correlation. Appropriate post-hoc tests were subsequently performed to elucidate any differences found. The conventional α-level of 0.05 was specified as the significance threshold. The software package SPSS version 10.1 was employed for all statistical analyses.

9.3. Results.

9.3.1. Site of Tumour.

Some differences in expression for the genes was evident depending on where the tumour was taken from, with NCoA1, PgR, AR and ESRβ having higher expression in breast tissue derived from the left side of the chest, NCoA1 and ESRα having higher expression

in controls and GR alone having higher expression in tissue derived from the right side. However, none of these observed trends proved to be significant, with the lowest p value being for GR, at 0.11. The data for the populations is summarized in Figures 9.1 and 9.2 and the ANOVA analysis data can be found in Table 9.1, below.

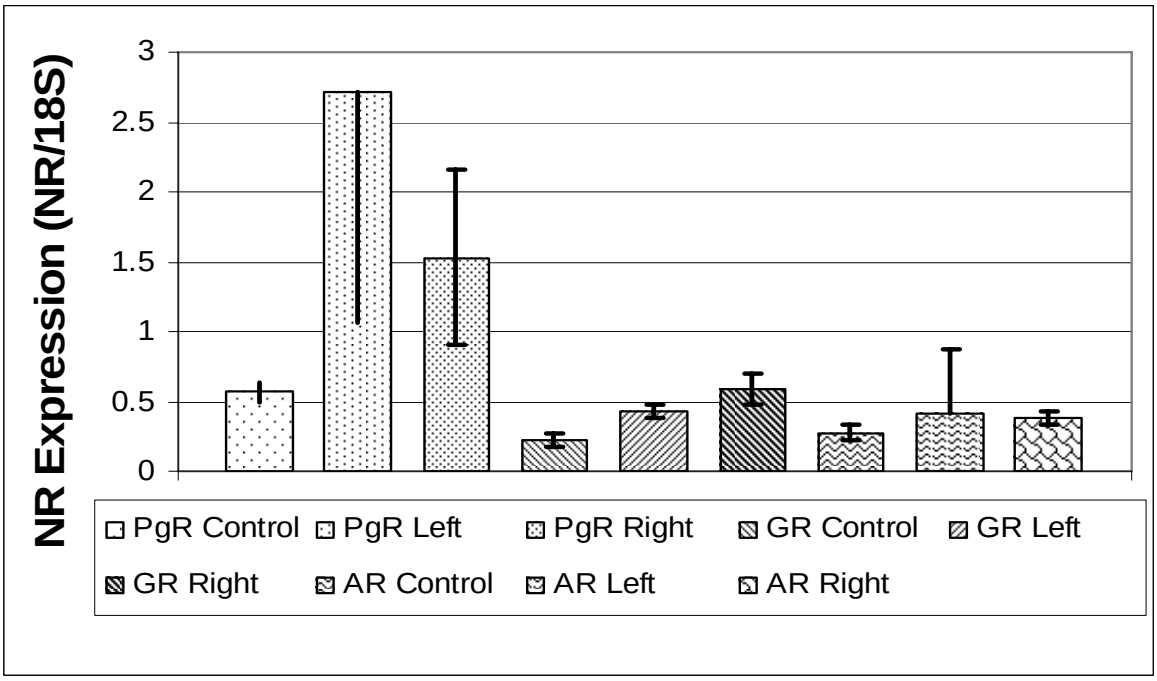

Figure 9.1: Expression of PgR, GR and AR Genes, Sorted by Side of Tissue Collection.

All genes are organized in the following order: Control (No side information), Left, and Right.

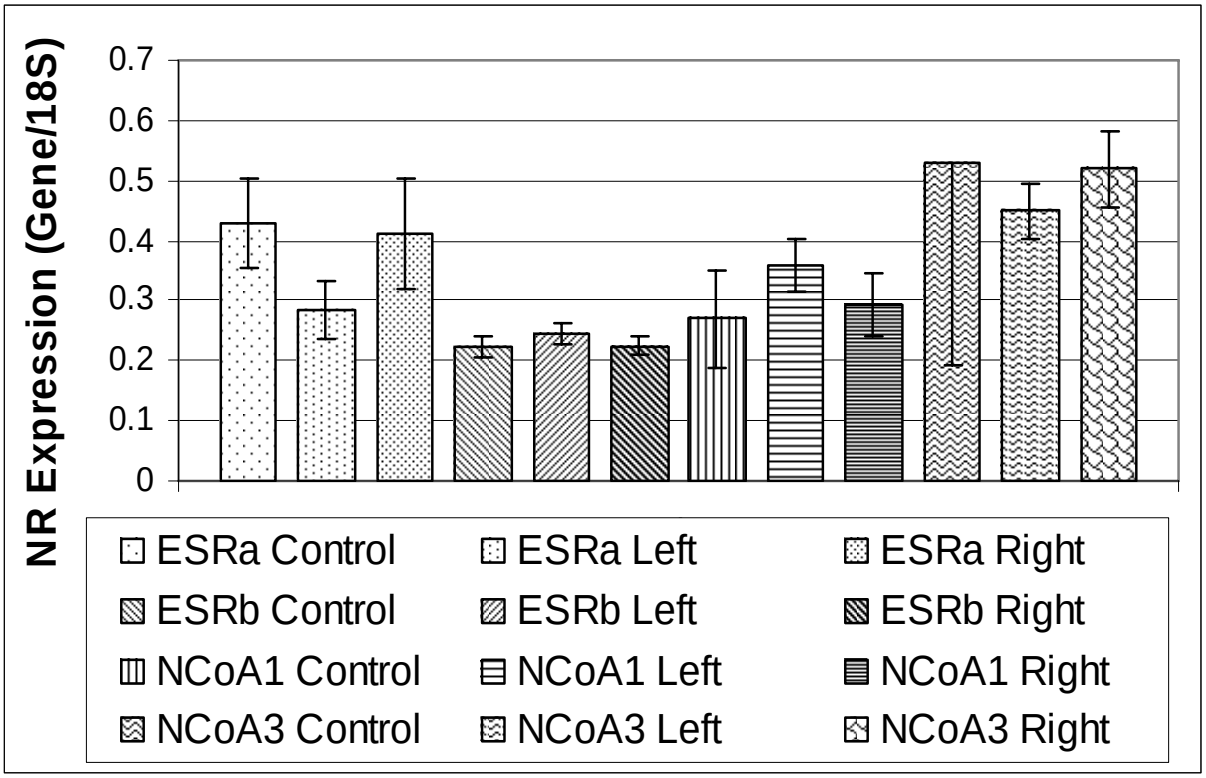

Figure 9.2: Expression of ESR and NCoA Genes, Sorted by Side of Tissue Collection. All genes are organized in the following order: Control (No side information), Left, and Right.

Table 9.1: ANOVA Data for Side of Tumour Analysis.

Gene	F Statistic	Significance
ESRα	1.142	0.339
ESRβ	0.407	0.668
PgR	0.391	0.679
GR	2.331	0.110
AR	0.915	0.408
NCoA1	0.604	0.551
NCoA3	0.638	0.534

9.3.2. Tumour Calcification.

As observed in the side of tumour analysis, there were also some preliminary trends appearing in the initial data for the presence of calcification in the tumours, often a sign of localized necrosis in the tumour tissue. Calcification was classified as any presence of calcium nodules within the tumour or detected in stromal elements nearby. All genes, except ESRα, showed a higher expression in tissues which had no calcification compared to those that had. However, ANOVA analysis showed that this trend was not significant for any of the genes tested, though NCoA1 had a p value close to significance at 0.066. This might be an indicator that dying or apoptotic cells naturally express NCoA1 at lower levels, but not at levels that are significantly lower then cells that are continuing to survive. The data for the populations is summarized in Figures 9.3 and 9.4 and the ANOVA analysis data can be found in Table 9.2, below.

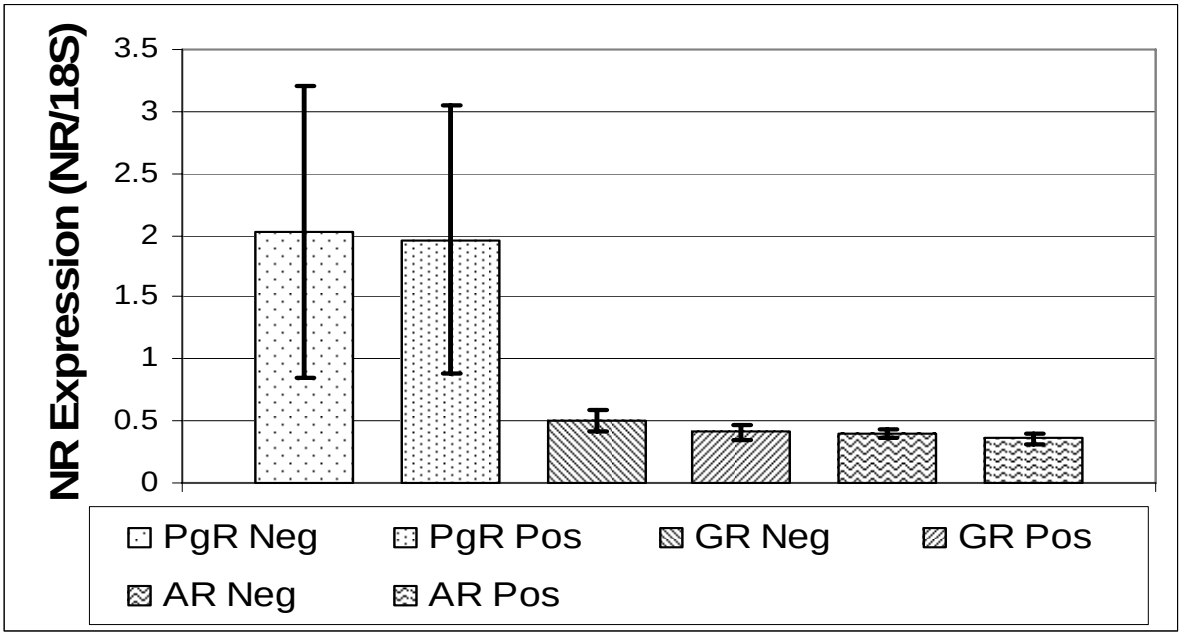

Figure 9.3: Expression of PgR, GR and AR Genes, Sorted by Calcification. All genes are organized in the following order: No Calcification, Calcification.

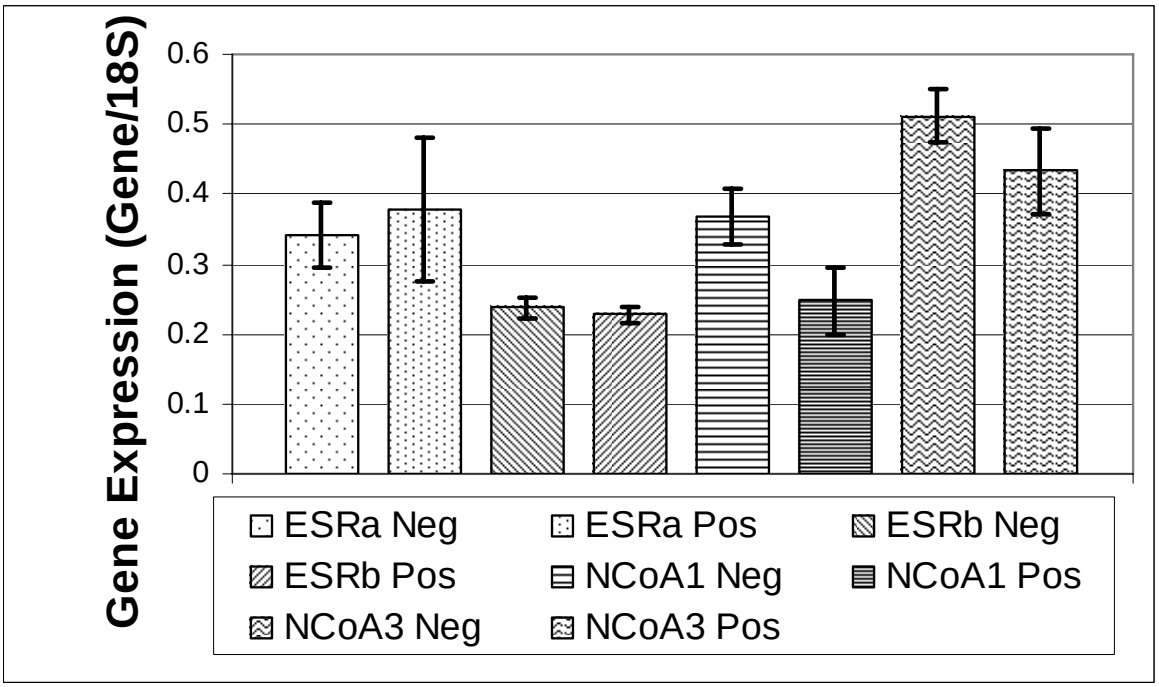

Figure 9.4: Expression of ESR and NCoA Genes, Sorted by Calcification. All genes are organized in the following order: No Calcification, Calcification.

Table 9.2: ANOVA Data for Calcification Analysis.

Gene	F Statistic	Significance
ESRα	0.145	0.707
ESRβ	0.197	0.659
PgR	0.001	0.972
GR	0.638	0.429
AR	0.445	0.508
NCoA1	3.559	0.066
NCoA3	1.268	0.267

9.3.3. Lymph Node Metastasis.

The initial data for the metastasis analysis also showed some trends, with the expression of ESRα, ESRβ and NCoA1 being higher in non-metastatic tissue, while the expression of PgR, GR, AR and NCoA3 was higher in primary tumours where metastatic offshoots had also been detected. These differences were particularly pronounced for PgR and GR. When the ANOVA analysis was conducted, the results indicated that the alterations observed in expression were significant only for the glucocorticoid receptor, with a p value of 7.6×10^{-6}. This indicated that the glucocorticoid receptor plays a significant role in some facet of metastasis, possibly through tumour manipulation of surrounding tissue

(See Chapter 8). The data for the populations is summarized in Figures 9.5 and 9.6 and the ANOVA analysis data can be found in Table 9.3, below.

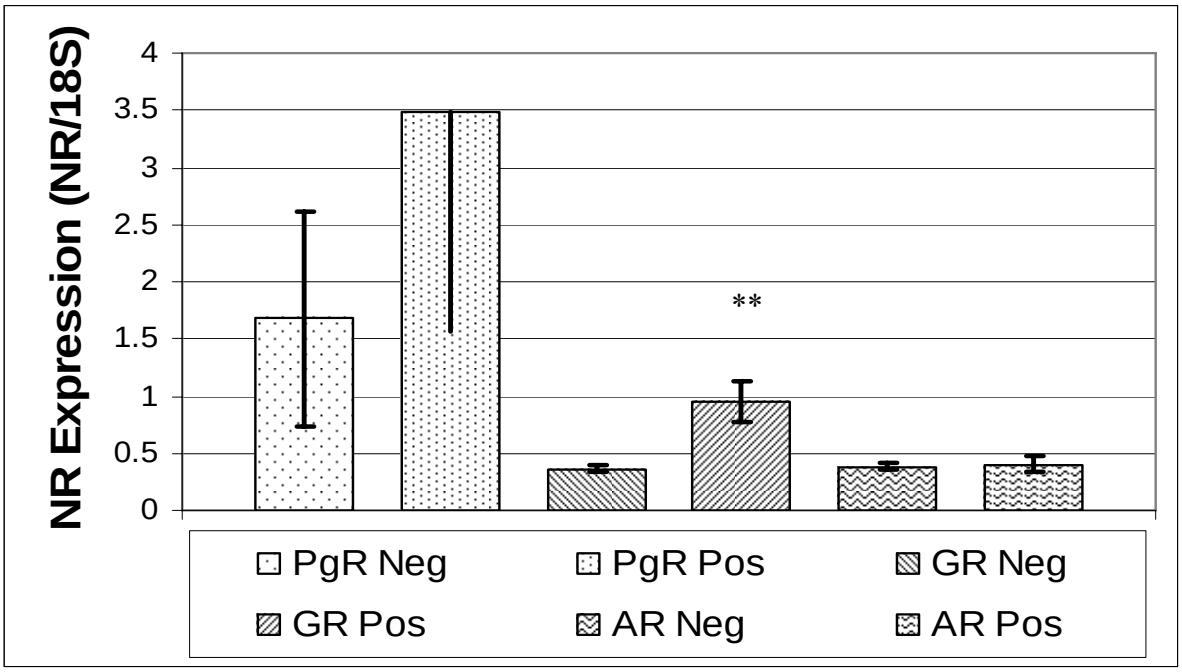

Figure 9.5: Expression of PgR, GR and AR Genes, Sorted by Lymph Node Metastasis. All genes are organized in the following order: No Metastasis, Metastasis. Only GR showed a significant difference in expression, being elevated in metastatic tissue.

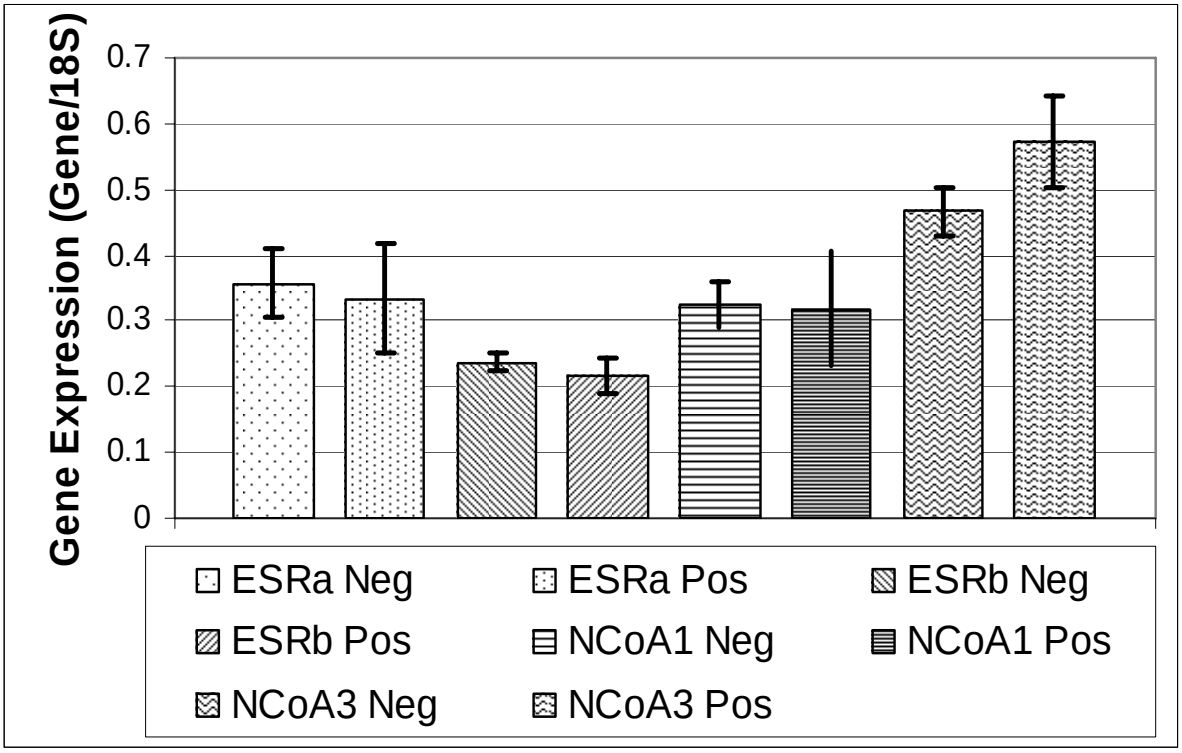

Figure 9.6: Expression of ESR and NCoA Genes, Sorted by Lymph Node Metastasis. All genes are organized in the following order: No Metastasis, Metastasis.

Table 9.3: ANOVA Data for Lymph Node Metastasis Analysis.

Gene	**F Statistic**	**Significance**
ESRα	0.041	0.841
ESRβ	0.599	0.443
PgR	0.668	0.418
GR	25.747	$7.6x10^{-6}$
AR	0.081	0.778
NCoA1	0.007	0.934
NCoA3	1.413	0.241

9.3.4. Quantitative Value Correlations.

The remaining clinical parameters available, namely age, tumour size and the amount of ductal carcinoma *in situ* (if any) present, were all quantitative parameters, and thus unsuitable for ANOVA analysis, which compares means in distinct groups. However, such parameters are suitable for correlation analysis, to determine if the level of one quantity affects the level of another. Thus, all the genes tested were submitted to correlation analysis for these three parameters, as well as one another. Two forms of analysis were used, the Pearson correlation coefficient, which is a parametric measure of correlation, and the Spearman's rho coefficient, which is a non-parametric measure of correlation, and used for those population which were abnormally distributed, to gain further insight into the relationships observed. It is important to note that these tests reveal only linear relationships, and more complicated effects remain obscured. The initial analysis revealed a number of correlations between different factors, particularly between the nuclear receptors and the NCoAs, though there were interactions between the purely pathological factors as well.

After the full correlation analysis was applied, many of these correlations proved to be significant, with positive relationships between PgR and GR, AR and NCoA1, GR and AR and tumour size. Significant negative relationships were observed between ESRα and

ESRβ and patient age and the amount of ductal carcinoma *in situ* (DCIS) present. All significant relationships are recorded in Table 9.4.

Table 9.4: Significant Correlations Observed in Tissue Population Using Pearson Correlation.

Relationship	**Pearson Correlation**	**Significance**
PgR and GR	0.342	0.022
PgR and AR	0.428	0.003
PgR and NCoA1	0.353	0.019
GR and AR	0.326	0.029
GR and Tumour Size	0.405	0.006
ESRα and ESRβ	-0.424	0.044
Age and DCIS	-0.39	0.008

When the non parametric Spearman's test was applied, not all of these relationships remained significant. PgR lost its relationship with GR and NCoA1 and GR were no longer correlating with tumour size. All other relationships remained significant. Additionally, a new correlation appeared, a positive relationship between AR expression and tumour size. The values obtained for the Spearman's test are recorded in Table 9.5.

Table 9.5: Significant Correlations Observed in Tissue Population Using Spearman's Correlation.

Relationship	Spearman's Correlation	Significance
PgR and AR	0.480	0.001
GR and AR	0.332	0.026
AR and Tumour Size	0.314	0.036
ESRα and ESRβ	-0.446	0.03
Age and DCIS	-0.432	0.003

9.4. Discussion.

The results of this study have shown that there were numerous relationships between the expression of the nuclear receptor genes and each other, as well as correlations with some pathological phenotypes, reflecting the complex and intertwining nature of the steroid signaling pathways. Despite this inherent complexity, the relationships indicated by the analyses performed shed some additional light on how these genes behave in breast cancer and has provided some direction to theories derived from the expression analysis of the genes.

The ANOVA analysis for both site of tumour and calcification showed no significant association between these factors and the expression of any of the tested NR or NCoA genes. Certainly for site of tumour, there has been no previous linkage with either NR or

NCoA expression, nor has there been evidence of serious differences in behaviors from one site of tumour to another. Calcification, however, was a much more promising line of inquiry, having been associated with certain disease states, such as diabetes and hypertension, as well as poor survival in breast and other cancers, though its value as a prognostic factor is debated [118-121]. The lack of any association between calcification and the expression of the NR or NCoA genes was not entirely unexpected, since they do not directly affect calcium metabolism, although with its role in apoptosis, it was thought that GR expression might have some association. It is interesting to note that NCoA1 was close to significance in this analysis, perhaps reflecting a drop in expression in dying cells, or those cells recruiting large amounts of calcium, though the localization of the expression of any of the genes tested has not been tested histologically and the drop in NCoA1 expression was not at levels significantly different than otherwise normal cells.

Unlike tumour site and calcification, the lymph node metastasis analysis did show one significant association, an increase of glucocorticoid receptor expression in metastatic tumours. Lymph node metastasis is a known poor prognostic factor, used in conjunction with tumour size and other characteristics to derive cancer stage, and as such is itself associated with more advanced cancers [29, 108, 120]. Although the Glucocorticoid receptor was also associated with more advanced cancers (see Chapter 4) it was also associated with the stroma of higher grade tumours. Given the role that it plays in breast tissue differentiation, it would seem that GR, or the factors that increase GR expression, may be affecting the susceptibility of the surrounding stroma to apoptosis by glucocorticoid stimulation, this removing impediments to tumour escape, though the

tumours themselves would require some degree of glucocorticoid resistance if this were the case. Alternatively, the tumours may be manipulating the surrounding cells to produce factors that break down the basement membrane and facilitate metastasis of the tumour. Since glucocorticoids also have a role in immune suppression and apoptosis, it may also be a defense mechanism required for the tumour to be able to successfully escape into the remainder of the body, making immune system cells more likely to undergo apoptosis or cease activity.

The correlation analysis also showed a number of significant associations between the various NR and NCoA genes and the quantitative pathological parameters. The progesterone receptor in particular was associated with several other genes, being positively associated with GR, AR and NCoA1, though for the Spearman's analysis only the relationship with AR remained significant. It was perhaps to be expected that PgR would be correlated with GR, AR and NCoA1, since all of these genes were found to have increased expression in late stage breast cancer (see Chapters 2, 4 and 7). Additionally, stimulation of the estrogen receptor pathways, the major pathway through which breast tissue growth is signaled, has been known to increase the expression of all these genes [68, 71, 74, 77, 82, 99, 100]. That the majority of the relationships do not remain significant when the non-parametric Spearman's test is applied may be a reflection of the large variability in the progesterone receptor results, rather than a complete invalidation of the relationships observed, which were in agreement with the results obtained in the previous chapters.

In addition to its relationship to PgR, the glucocorticoid receptor was also significantly positively correlated with both AR and tumour size. As for the PgR relationship, the correlation of AR and PgR may be an indirect relationship due to their both being highly expressed in late stage tumours, or perhaps a more direct relationship through their similar regulation by growth pathways. That the relationship is not significant if analyzed non-parametrically would seem to indicate an indirect relationship due to their common expression.

More interesting was the positive correlation between GR and tumour size. While this too can be correlated with advanced cancers that have had sufficient time to grow, it does have a secondary link to the significant association of GR and metastasis, since in order to grow beyond a certain size, a tumour must be able to remodel the local ECM and recruit blood vessels, both of which are also requirements for cancer to metastasize. The relationship does not survive non-parametric analysis however, so this may not be a direct relationship, perhaps again an indirect link with high tumour grade. However, the GR results do show a high degree of variation, thus obscuring the relationship between GR and tumour size when testing through non-parametric means. The additional link GR has with metastasis does throw some credence behind the original parametric analysis, though additional testing in this area is warranted.

The androgen receptor also has an association with tumour size, though it only does so through the non-parametric Spearman's analysis. The androgen receptor also has a role to play in breast tissue differentiation and in halting the growth breast tissue, which are

requirements that tumours have for their surrounding stroma, forcing them to cease growth and develop a suitable environment for the tumour to grow. However, AR expression was significantly elevated in later stage breast tumours, but not in the surrounding stroma, so the linkage observed in the Spearman's analysis here may be the same relationship that AR has to high grade cancers, since high grade tumours were generally larger in size than the lower grade ones. It is possible that the same mechanism that AR is surmised to perform in these late stage tumours is also at play in the larger sized tumours of lower grade. That is, that a high expression of AR may result in the pool of NR chaperones being sequestered by AR proteins. This makes the tumours unable to have their growth slowed through stimulation by the also up-regulated PgR and GR, whose ligands are more common in a woman's circulation than androgens. Large tumours in lower grades would have similar needs to prevent PgR and GR stimulation, so the correlation seen here may reflect the same change. The fact that AR expression in the stroma of high grade tumours is not significantly up-regulated reinforces this idea, since it is a tumour-specific event.

The estrogen receptors also displayed a significant correlation, one that stands up to both parametric and non-parametric analysis, though this was to be expected, since the degree of variation in ESRα and ESRβ is much lower than the other NR genes. The correlation between ESRα and ESRβ is negative, and quite strong at a correlation coefficient of -0.428 and -0.446 for Pearson's and Spearman's analysis, respectively. However, due to the relatively static nature of ESRβ expression this relationship was not immediately apparent. ESRα and ESRβ are antagonistic to one another and it has been well observed

that the stimulation of one increases the expression of the other, with the relative ratio between them being one of the primary determinants on how a cell will react to the estrogen receptor[69, 71, 100, 114]. Therefore the relationship observed in this analysis was not unexpected and indicates that, at least at the mRNA level, the control of the ESR genes is intact, even in those tumours that are no longer expressing the ESRα protein.

Finally, a negative correlation was observed between age and the percent of carcinoma *in situ* infiltration present, which remained for both parametric and non-parametric analysis. As with the ESR relationship, this particular correlation was not entirely unexpected, since there is some evidence that cancers developing in older individuals tend to be less aggressive and invasive than those developing in younger individuals [3, 43, 117]. The data obtained here would seem to support this, with the relatively strong negative correlation revealed by both Pearson and Spearman's correlation analysis.

9.5. Conclusion.

There were a number of relationships observed between the NR and NCoA genes and the pathological factors studied in this analysis. These relationships include linkages between the expression of PgR, GR and AR, linkage between PgR and NCoA1 expression, an association between GR expression and both metastasis and tumour size, AR expression and tumour size and negative correlations between ESRα and ESRβ and between age and carcinoma *in situ* invasiveness. For the most part, these relationships seem to further

develop and support some of the conclusions made about the various nuclear receptors, including their possible roles in the development, progression and metastasis of breast tumours.

CHAPTER 10. Conclusions.

10.1. The Progesterone and Glucocorticoid Receptors.

The progesterone and glucocorticoid receptor genes are members of the steroid nuclear receptor family of genes, which accept incoming signals to cells and elucidate changes to the expression of other genes. PgR and GR themselves are closely involved in the growth and development of cells, both being antagonistic to estrogen in breast tissue, PgR encouraging differentiation and GR additionally being involved in apoptosis [72, 91].

The study on the progesterone and glucocorticoid receptors found that they were more highly expressed in advanced tumours ($p= 0.023$ and $p= 0.00033$ for PgR and GR, respectively). Only PgR was more highly expressed in ESRα negative tissues than ESRα positive tissues, however, ($p= 0.009$ and $p=0.058$ for PgR and GR, respectively), though this result could be a reflection of the relationship between the PgR and late stage cancer. This result was somewhat unexpected, as it would seem to indicate that these tumour tissues would be more responsive to stimulation by progesterone or glucocorticoids, a condition not usually seen in late stage breast cancer. This increase in expression may be from stimulation of the estrogen growth pathways, or part of an intact negative feedback

mechanism still in operation in the tumours. In order to maintain hormone insensitivity, it is possible that the tumours are splicing the mRNA detected into ineffective or antagonistic isoforms. It is also possible that the high levels of expression observed do not result in finished proteins, the mRNA being degraded before translation can take place.

In addition, this study found that the expression of PgR and GR in stroma were significantly affected by the cancer grade the stroma was derived from ($p= 5.908x10^{-7}$ and $p=2.761x10^{-5}$, for PgR and GR, respectively). Expression of PgR lowered in all stroma samples and expression of GR increased in higher grade stroma. Expression of GR was also higher in stroma from ESRα negative tumours ($p= 5.85x10^{-5}$), though this may be measuring the same effect as high grade expression.

The lowering of PgR expression in the stroma would seem to be consistent with the tumour signaling the stroma to maintain a working state and provide increased support for the tumour. This is also in marked contrast to the tumours, which display higher expression as they progress, possibly as a remnant of normal control mechanisms which make cells more sensitive to anti-mitogenic signals when stimulated to grow at increased rates. That GR expression increases in stroma may also reflect PgR signaling, as the two receptors do antagonize one another to some extent. It may also reflect other signals being released by the tumour to affect either itself (as the tumours also show increased GR expression) or the stroma, making both types of tissue more sensitive to glucocorticoids. Whether or not this translates into the tumours being more sensitive to

GR stimulation is unknown, as the mRNA could be being degraded or spliced into growth inducing isoforms in the stroma, as may also be happening in the tumour.

The correlation analysis produced a number of results which reinforced and further expanded on the conclusions reached above. First, PgR, GR and AR expression were found to be significantly correlated with each other, not unexpected considering their high expression in advanced tumours ($p= 0.022$, $p=0.003$, and $p=0.029$, for the PgR/GR, PgR/AR and AR/GR relationships, respectively). Increased GR expression was also significantly associated with both tumour size and lymph node metastasis ($p= 0.006$ and $p=7.6x10^{-6}$, respectively). This would seem to indicate that in order to grow and metastasize, tumours require local stroma to be GR sensitive, possibly as a means to induce apoptosis or escape immune system surveillance. Alternatively, this increase in GR expression could be a result of stimulation of GR antagonistic pathways, resulting in an increased GR expression as part of negative pathway controls.

10.2. The Estrogen Receptors.

The estrogen receptors are major players in the control of cellular growth and division, particularly in breast tissue. As the estrogen pathway is amongst the major targets for breast cancer treatment, the study of how these receptors behave in the development and progression of breast cancer is important to increase our understanding of this complex disease.

This study found a number of interesting relationships between the estrogen receptors and each other, the estrogen receptors and cancer grade and the estrogen receptors and the continuing expression of ESRα proteins. At first glance, the results obtained seem to indicate no relationship, with insignificant differences between both ESRs when compared by cancer grade or ESRα protein status ($p= 0.057$, $p=0.794$, $p=0.622$ and $p=0.716$, for cancer grade and ESRα status for ESRα and ESRβ, respectively). However, these insignificant differences do indicate that despite the loss of detectable ESRα expression or increase in cancer grade, which is often associated with low responsiveness to hormonal treatments, the levels of mRNA for the estrogen receptors remain unchanged. Additionally, correlation analysis showed that the expression of ESRα and ESRβ in these tissues is significantly negatively correlated ($p=0.044$), which is itself a reflection of normal control mechanisms within cells.

Given the results in this study, it is possible that the normal controls for ESR mRNA expression remain intact within the studied cells, even in highly advanced cancers, raising the possibility that if whatever mechanism preventing detectable ESRα expression could be discovered and sufficiently understood, it may be possible to disrupt it. This may return ESRα negative cells to responsiveness for hormone treatments, allowing more effective treatment of advanced breast cancers. Further study on the relationship between ESRα mRNA and protein, and possible modification to either may well prove highly fruitful for both increasing the understanding of the mechanisms involved in expression controls and breast cancer treatment.

10.3. The Androgen Receptor.

The androgen receptor, like the other nuclear receptors, is heavily involved in the control of cellular growth and differentiation. In breast cancer, stimulation of the androgen receptor has a strong anti-mitogenic effect, and the hormone has been used in some advanced breast cancers despite the side-effects it can bring, since breast tumours often remain sensitive to androgen stimulation even while other hormonal signals are ignored.

The results obtained for the androgen receptor indicated that the expression of AR is elevated in grade 2 and 3 tumours ($p= 0.014$), as well as in ESRα negative tissues ($p= 0.025$), while remaining unchanged in stromal tissue derived from all grades. This would seem to indicate that more advanced tumours are more sensitive to AR than normal tissue, perhaps as a response to stimulation of estrogen based growth pathways, and as part of normal growth control mechanisms. It is also possible, however, that the tumours are increasing expression of AR as a form of defense mechanism to prevent stimulation by other anti-mitogenic receptors. This might occur because a high expression of AR would sequester available chaperone molecules and be stimulated only to low levels by the low concentration of androgens in a woman's body. That the trend is restricted to tumours only and is also significant in ESRα negative tissue, which may not require chaperone proteins for ESR modulated growth pathways, would seem to provide weight to this theory. It is also consistent with the evidence that late stage tumours are often

androgen sensitive, and further indicates that the AR mRNA detected in these studies is being spliced into normal isoforms.

As related in section 10.1 above, the correlation analysis for AR indicated that AR expression is positively related to both PgR and GR expression ($p= 0.003$ and $p=0.029$, respectively), but AR expression also showed positive correlation with tumour size ($p= 0.036$). This relationship may be related to the same factors that increase AR expression in high grade and ESRα negative tumours, large sized tumours being more exposed to the possibility of anti-growth stimulation than smaller tumours. The results obtained from these tissue samples provide some confirmation for the previously observed sensitivity to AR by many breast tumours. They also indicated that, paradoxically, this sensitivity may provide some explanation for the ability of some advanced tumours to ignore the anti-growth signals of other hormones.

10.4. The Nuclear Receptor Co-activators.

The nuclear receptor co-activator family of genes code for proteins which are required for proper nuclear receptor function. They facilitate the formation of activated receptor dimers and recruit further cofactors required for chromatin remodeling and the alteration of gene expression.

The results from the ANOVA analysis of the NCoA genes indicated that the expression of the NCoA1 gene was increased in grade three tumours, but only in comparison to grade 1 tumours, and also in ESRα negative tissue (p= 0.028 and p= 0.001, respectively). NCoA1 has been previously associated with steroid receptor signaling and has also been found to be amplified in cancers, so this result was not entirely unexpected. The correlation analysis also indicated a significant positive correlation between PgR and NCoA1 expression (p= 0.019). In earlier analyses it was suspected that highly advanced and ESRα negative tumours may be splicing the large quantities of anti-mitogenic NR mRNA observed into growth inducing isoforms instead of the normal receptors and the relationship between PgR and NCoA1 may reflect just such an occurrence. Once ESRα expression has ceased, a high quantity of NCoA1 would be useful to a tumour only if the stimulation that it allowed was not growth repressive. However, the increase in expression could be part of a more general response to the large and general increase of NR expression in the advanced tumours.

10.5. Future Directions.

This study has found a number of relationships between the nuclear receptors and the nuclear receptor co-activators with one another, as well as with various pathological parameters including cancer grade and ESRα status. While these results do answer a number of questions on the behavior of the nuclear receptor pathways in breast cancer,

those pathways are highly complex and the data from this study raises additional questions that provide further avenues for research.

First, the apparent retention of normal ESRα mRNA controls in even highly advanced cancers warrants further investigation. If this trend is widely replicated in breast cancers, it could provide a mechanism for making some tamoxifen resistant cancers responsive to the drug again, as long as the mechanism by which the mRNA transcripts are destroyed can be understood. This could involve research into how mRNA clearance systems are functioning with respect to ESR and other NR mRNA in tumours, or the expression of specific ESR isoforms that are not detectable by antibodies and are either constituently active or able to pass on estrogen signals.

The relationship between GR expression and advanced cancers, tumour size and metastasis is another area that could prove a fruitful area of investigation. This relationship may be either a directed trend of expression by tumours to control nearby stroma through GR stimulation, or the symptom of a different stimulatory pathway. Studies into cell lines and the expression of related genes would throw additional light on the processes involved. If the mechanisms could be more clearly understood, methods of blocking the growth and metastasis of breast tumours might be developed that would provide a great reduction in mortality and morbidity.

Finally, the general increase in anti-mitogenic NRs in advanced breast cancers is an area that should be investigated to determine if the receptors are being produced, and if they are, whether or not these receptors are being spliced into growth inducing isoforms. This could be relatively easily obtained with staining to detect cellular proliferation, data which was not available for this study. Related to this, investigations into additional cofactors of the nuclear receptor pathways and nuclear receptor chaperones would need to be performed, so that the conclusions reached in both this study and any further studies on nuclear receptor isoforms can be fully understood in the complex web of interactions between these factors and the nuclear receptors.

10.6. Conclusion.

Breast cancer is a great source of morbidity and mortality in the developed world and will affect roughly 1 in 10 women in their lifetimes. Hormones play a significant role in the progression and development of breast cancer and form a significant portion of current breast cancer treatments. Hence, the study of the genes that mediate hormone signals, such as the nuclear receptors and their cofactors, is an important part of the ongoing research into breast cancer biology and treatment. This study has examined how those genes behave in breast tumours and control tissue derived from biopsy slides and compared that behavior to one another, as well as a number of pathological characteristics to determine what relationships, if any, existed between them. The results obtained by this study have built on the results of pre-existing studies as well as filled

gaps in these prior studies, which have primarily concentrated on a single protein, gene or cell line. The more comprehensive approach taken in this study has brought to light a number of relationships between the nuclear receptors, their allied genes and various important prognostic factors. These relationships point the way to avenues of future research that may refine the knowledge of breast cancer biology as well as its treatment.

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