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Analysis of Progesterone-regulated Target Genes in Mammary Gland and Uterine Development

Claire A. Walsh

Technological University Dublin

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by

Claire A. Walsh

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Dublin Institute of Technology

Kevin Street,

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Declaration:

I certify that this thesis, which I now submit for examination for the award of M.Phil. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract:

Progesterone is an ovarian steroid hormone which plays a very important part in the regulation of pregnancy-related changes. Progesterone functions by binding to its specific nuclear receptor, PR, which regulates defined genes in a ligand dependent manner. The aim of my thesis is to explore and elucidate some of the important mediators of progesterone action with regards to pregnancy-related mammary gland development and also uterine development. Knockout mouse models and comparative wild type mice were used in order to explore the expression patterns of specific genes of interest. Quantitative PCR using cDNA derived from mammary gland tissue was used to analyse the differences in gene expression of progesterone's downstream target molecules. Established target genes such as RANKL, Wnt4 and Amphiregulin, TGF-β1, Sfrp2 and Mig6 were analysed to determine their mRNA expression levels in the late stages of pregnancy where growth and proliferation is at its most intense. Comparisons were then made between the mammary gland and the uterus, in order to assess the tissue specificity of progesterone function. The results clearly indicate how progesterone is able to mediate both proliferative and anti-proliferative signals to ensure normal structural development within both the mammary gland and the uterus. A novel target gene; BDNF; was shown to be strongly regulated by PR and was also analysed in terms of the proliferative target genes to determine what functional role it may have in the signalling pathways. The findings of this study will help to further understand the intricacies of progesterone dependent signalling in both normal and tumourigenic mammary gland development.

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Acknowledgements:

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1. Introduction

1.1. Mammary Gland Structure:

The mammary gland consists of two tissue compartments: the epithelium and the stroma. The epithelium of the mature non-pregnant mouse resembles a branching tree-like ductal structure that develops during puberty and milk-producing alveolar cells which develop during pregnancy (Molyneux et al, 2007; Hennighausen et al, 2001). The primary ducts branch into decreasingly smaller secondary and tertiary ducts. These ductal branches of the mammary gland are encased in myoepithelial cells on the exterior and lined with ductal luminal cells along its length (Buono et al, 2006). The ductal branches terminate in lobules; which are composed of alveoli, which in turn consist of secretory epithelial cells that undergo functional differentiation in pregnancy (Hennighausen et al, 2001). The alveoli have a more open network of myoepithelium than the characteristic continuous layer of the ducts. These alveolar cells also contain smooth muscle actin and contract in response to oxytocin stimulation, which results in milk release (Hennighausen et al, 2001). A basement membrane separates this extensive system of ducts and alveoli from the surrounding fatty stromal matrix in which they are embedded (Molyneux et al, 2007; Hennighausen et al, 2005). The stroma also known as the mammary fat pad consists mainly of adipocytes but fibroblasts, blood vessels and neurons are also present (Hennighausen et al, 2005).

The cellular structure of the mammary gland is dependent on distinct cell lineages. It has been proposed that cells forming the epithelial compartment of the mammary gland are derived from mammary stem cells which have the capacity to self renew and give rise to committed epithelial precursor cells (EPCs). The progeny of the EPCs are then restricted to either a ductal or alveolar fate (Hennighausen et al, 2005). The ductal precursor cells form basal cells and luminal cells, the two cell types that constitute ducts. During pregnancy, alveoli are generated from alveolar precursors which give rise to basal and luminal cells, the differentiated milk-producing cells. The presence of stem cells is the basis of the profound capacity for alveolar renewal in each subsequent pregnancy (Hennighausen et al, 2005).

1.2. Mammary Gland Development:

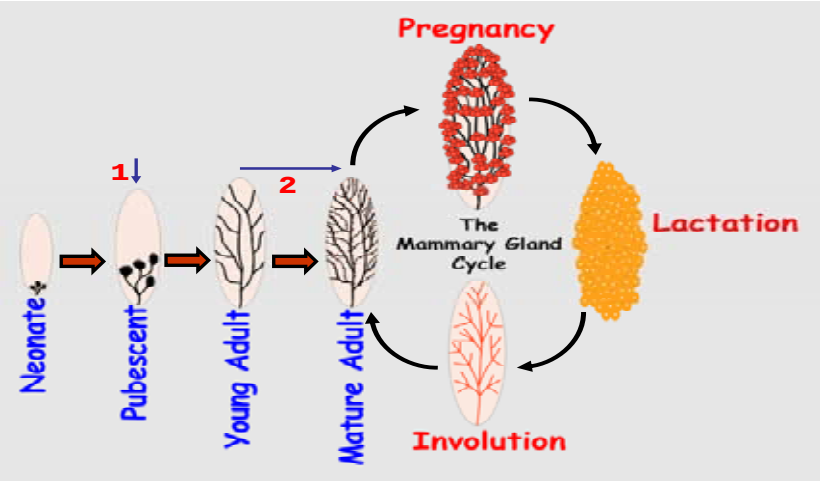


Fig 1.1: A diagram which depicts the stereotypical morphological changes that occur during murine postnatal mammary gland development, the mammary gland cycle of development begins with pregnancy and concludes with the completion of the involution process (Fernandez-Valdivia et al, 2005).

Development of the mammary gland occurs in defined stages that are connected to sexual development and reproduction (fig.1.1). These stages are essentially conserved between rodents and humans and are referred to as embryonic, prepubertal, pubertal, pregnancy, lactation and involution (Hennighausen et al, 2001).

1.2.(i)Embryonic and Prepubertal Mammary Gland Development:

The initial stages of mammary development in the embryo occur independently of steroid hormone regulation. In the mouse embryo, there are five pairs of glands which develop along a line that that runs slightly ventral to the limb buds, whereas only one pair develops in the thoracic region in humans (Hennighausen et al, 2005). In mice, five ectodermal placodes appear between embryonic days 10 and 11. The placodes form buds which slowly increase in size up to embryonic day 15. Cell proliferation intensifies at the tip of the bud and leads to the formation of a primary sprout which grows out toward the prospective mammary fat pad. Continued proliferation and branching leads to the formation of a small ductal tree at birth (Hennighausen et al, 2001). This primitive rudimentary gland grows just enough to keep up with normal body growth until pubertal stage of growth can begin (Hennighausen et al, 2005).

1.2.(ii) Pubertal Mammary Gland Development:

The bulk of mammary gland development occurs postnatally in two distinct growth phases that are initiated at the onset of puberty and pregnancy respectively (Conneely et al, 2003). During puberty, cyclical production of ovarian estrogen and progesterone accelerates ductal outgrowth and branching (Hennighausen et al, 2005). Growing epithelial ducts in pubescent animals have conspicuous club-shaped structures at their distal ends which are known as terminal end buds (TEBs) (Conneely et al, 2003). The TEBs consist of two morphologically distinct cell types, an inner layer of body cells and an outer layer of cap cells (fig.1.2). It is likely that there is stem cell activity within the cap cells (Molyneux et al, 2007). Under the regulation of estrogen and other locally acting growth factors; these cells divide at a high rate and proliferate to promote ductal elongation and dichotomous secondary side branching (Conneely et al, 2003).

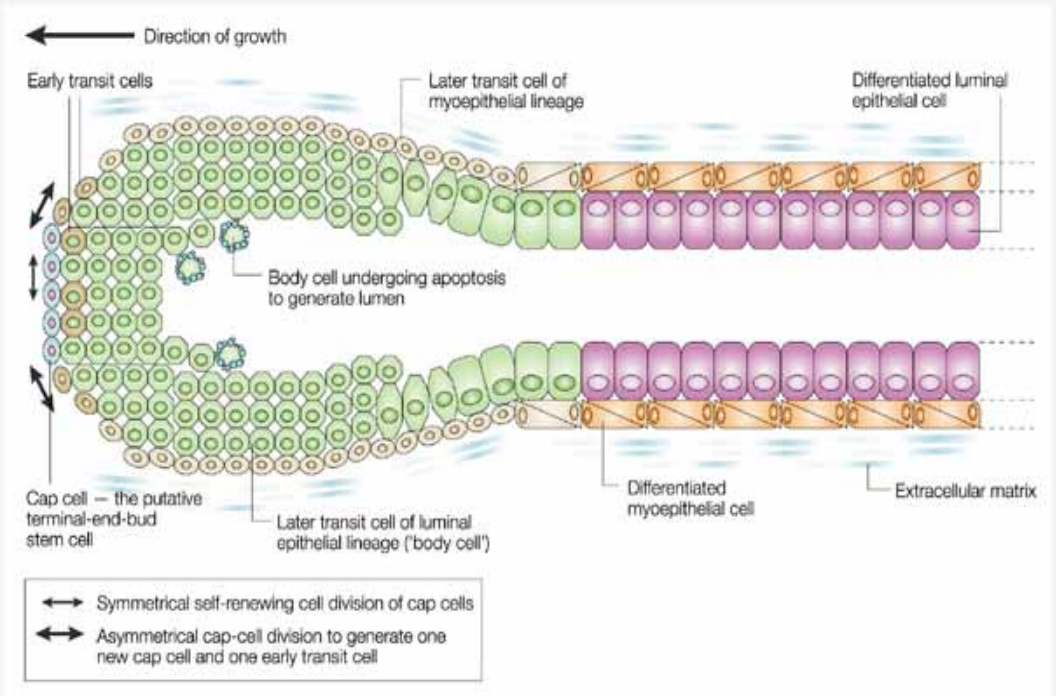


Fig 1.2. The ducts of the developing mammary gland are established as the terminal end buds (TEBs). The TEBs move through the mammary fat pad, they consist of an inner luminal epithelial cell layer and an outer myoepithelial cell layer. It is thought that the cap cells at the tip of the TEB generate transit cells of a myoepithelial lineage on the outer side of the TEB and generate transit cells — known as 'body cells' — of a luminal epithelial lineage to form the central TEB mass. The ductal lumen is formed as central body cells apoptose and outer cells differentiate into luminal epithelial cells. Extracellular-matrix enzymes degrade the stroma in front of the TEB to enable it to move through the fat pad, but it is unclear how the structures actually 'move' through the gland. It might simply be that progressive cell division building up cell bulk at the front of the mass of body cells, coupled with progressive apoptosis degrading cell bulk at the back of the body cell mass, creates the illusion of forward movement (Smalley et al, 2003).

The ducts grow into the mammary fat pad, branching at regular intervals to create a network of primary and secondary ducts. During lactation, these primary and secondary ducts form continuous channels for the transport of milk from the secretory alveoli to the nipple (Molyneux et al, 2007). Once the fat pad has been filled with glandular tissue, the TEBs will regress, and are then converted to terminal ducts (Conneely et al, 2003; Molyneux et al, 2007).

At adulthood, the virgin gland becomes relatively quiescent with the exception of small side-branches which form and disappear on primary and secondary ducts as a result of the cyclic rise of ovarian steroids during the estrous cycle (Molyneux et al, 2007, Hennighausen et al, 2005). Precursors of the secretory alveolar structures may also appear and regress during the estrous cycle (Molyneux et al, 2007).

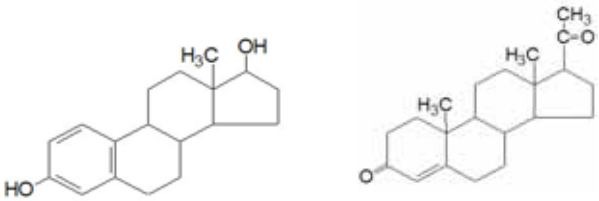
1.2.(iii) Pregnancy-Associated Mammary Gland Development:

During pregnancy, a surge of hormones results in major structural changes in the mammary gland (Molyneux et al, 2007). Exposure to progesterone and prolactin results in extensive epithelial proliferation, increased side branching of both secondary and tertiary ducts and differentiation of milk-filled alveolar lobules that uniformly fill the interductal spaces by late pregnancy (Conneely et al, 2003). The combined action of both progesterone and prolactin dependent signalling pathways is essential to achieving the proper pregnancy-associated ductal and alveolar morphogenesis of the mammary gland (Conneely et al, 2003). In humans, the alveoli are localised in clusters which resemble bunches of grapes and are called Terminal Ductal Alveolar Units (TDLUs) (Molyneux et al, 2007). Nearing parturition, alveolar tight junctions close and milk and colostrum proteins move into the alveolar lumen in preparation for active milk secretion (Oakes et al, 2006). During suckling, milk is expelled by contractile myoepithelial cells which form a basketlike network to the nipple (Molyneux et al, 2007). At the end of lactation, the loss of suckling stimuli and the pressure

build-up on cessation of milk removal initiates a remodelling programme called involution. This causes massive cell death; it causes the collapse of the alveoli and the remodelling of the epithelial compartment to restore a simple duct structure which resembles the prepregnant mammary gland. A new developmental cycle of alveolar expansion, maturation, lactation and involution is initiated with the next pregnancy (Hennighausen et al, 2005).

1.3. The Ovarian Steroid Hormones:

Hormones are signalling molecules which travel through the bloodstream and interact with cells in a variety of target tissues via specific receptors. The ovarian steroid hormones are derived from a cholesterol precursor and contain four rings of 17-carbon atoms (Fig 1.3) (Kleinsmith et al, 2007). The side chains attached to the carbon rings enable the hormone molecules to confer specificity for appropriate receptors.



Estradiol **Progesterone**

Fig 1.3: The chemical structures of the two ovarian steroid hormones; estradiol (E) and progesterone (P)

1.3.(i): The Steroid Hormone Receptors; ER and PR:

This project focuses on two ovarian steroid hormones; estrogen (E) and progesterone (P), because of their specific and overlapping interactions in both the breast and the uterus. Steroid hormones mediate their cellular effects

through their specific steroid receptors. Estrogen or estrogen-related molecules bind specifically to the estrogen receptor (ER) whilst progesterone or progesterone-related molecules bind specifically to the progesterone receptor (PR). Both ER and PR are members of the nuclear receptor superfamily and therefore they share the structural homology and the key functional domains that are common to this group (Lee et al, 2006). ER and PR have three major functional domains; the ligand-binding domain, the DNA binding domain, and an activation domain. The ligand-binding domain confers specificity to the receptor for a particular ligand that regulates its transcriptional activation (Lee et al, 2006). The DNA-binding domain determines which DNA sequences the receptor will recognise while the activation domain links transcriptional activity of the receptors to the core transcriptional complexes (Lee et al, 2006).

Both ER and PR are intracellular receptors therefore in order for activation to occur, a specific hormone ligand must enter the cell. Estrogen and progesterone are both small, lipophilic, hydrophobic molecules which can easily diffuse through the phospholipid bilayer of the cell membrane and into the cytoplasm. Once in the cytoplasm, the ligand binds to and induces a conformational change in the specific receptor, the ligand-receptor complex is then activated and can then translocate to the nucleus. In the nucleus, the ligand-receptor complex interacts with the genome and binds to particular response elements which are situated near the target genes. Progesterone-bound PR seems to bind as a homodimer to cis-acting progesterone response elements that are usually present in the 5' flanking regions of the specific genes (Lee et al, 2006). This ligand

dependent signalling enables the ligand-receptor complex to interact with the cell genome to activate or to silence the transcription of downstream effector pathways (Lee et al, 2006). It is the induction or suppression of these pathways by the ovarian steroid hormones that produce the physiological responses observed in the breast and the uterus (Fig 1.4).

In the absence of any ligand-binding, the steroid receptors remain transcriptionally inactive and are unable to interact and influence the DNA. Under such circumstances, PR is known to associate with a large complex of chaperone proteins including heat shock proteins in the cytoplasm. Only specific ligand binding can release PR from the chaperone complex so that it can undergo the conformational change which will enable it to translocate to the nucleus (Lee et al, 2006).

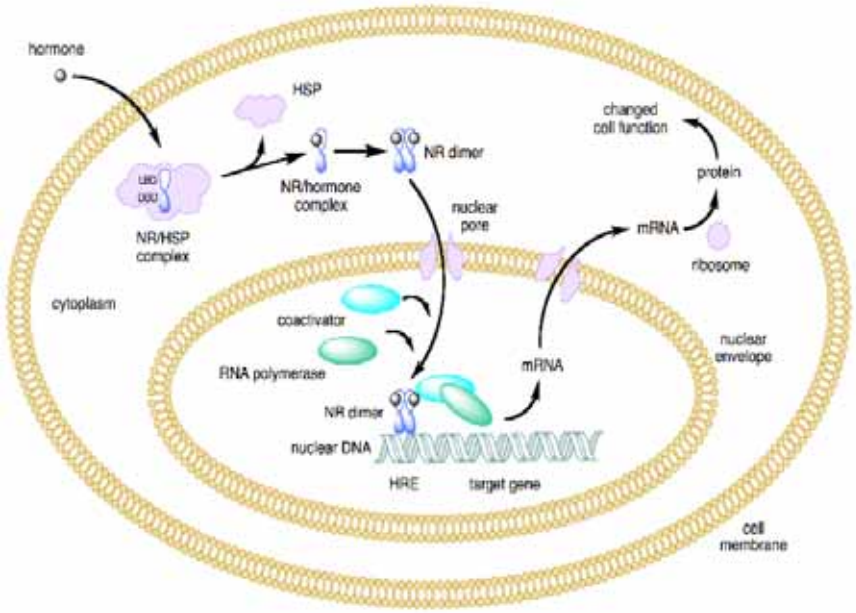


Fig 1.4: Schematic diagram depicting the ligand dependent signalling of steroid hormone receptors (NR) within the cell once they are bound by the steroid hormone in the cytoplasm. In the absence of steroid hormone, the steroid receptor is in an inactive state and is bound to a complex of chaperone proteins such as heat shock protein 90 (HSP) in the cytoplasm. The steroid hormone interacts with the receptor and changes its conformation which dissociates it from the chaperone complex so activation can occur. The newly formed ligand-receptor complex dimerizes and translocates to the nucleus where it recruits other coactivator proteins and interacts with cells genome to regulate transcription (http://en.wikipedia.org/wiki/Nuclear_receptor - 3rd September 2008).

1.3.(ii) The Estrogen Receptor Isoforms; ERα and ERβ:

Estrogen and estrogen-related molecules can bind to two distinct receptors; ERα and ERβ, which are encoded by two separate genes. ERα and ERβ function as transcription factors when bound to the steroid hormone. PR is a known transcriptional target of the ERs in the reproductive tissues, including the mammary gland (Hennighausen et al, 2001; Conneely et al, 2003).

In puberty, ductal branching within the mammary gland requires growth hormone and also estrogen; which signals predominantly through ERα. A deletion of ERβ has no adverse effects on ductal and alveolar development, whereas both stromal and epithelial ERα seem to be required for normal ductal elongation and outgrowth during puberty (Hennighausen et al, 2005; Conneely et al, 2003).

1.3.(iii) The Progesterone Receptor Isoforms; PR-A and PR-B:

Progesterone (P) has been referred to as the 'hormone of pregnancy' as its hormonal effects are seen to dominate development during pregnancy.

There are two distinct PR isoforms; PR-A and PR-B (Lessey et al, 1983). Both isoforms arise from alternative transcription start sites in the single PR gene (Molyneux et al, 2007). The isoforms are transcribed at two distinct promoters and translated at two alternative AUG signals (Conneely et al, 2001). PR-B contains a short additional stretch of amino acids, termed the B-upstream sequence (BUS) which is located at the N-terminus of the receptor. The BUS

domain contains the transactivation function (AF-3) which is not present in the PR-A isoform (Fernandez-Valdivia et al, 2005).

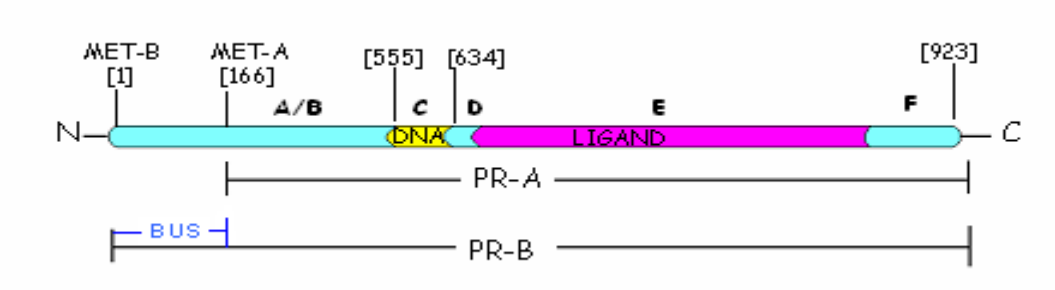


Fig 1.5: Diagram depicting two specific PR isoforms; PR-A and PR-B. There is a third PR isoform; PR-C but there is currently little information on its function. PR-B has the additional BUS domain attached to its N-terminal domain.

Both the A and B protein of PR are capable of binding P, dimerizing and interacting with P responsive elements and the transcriptional machinery to regulate gene expression. When expressed in equimolar ratios in cells, the A and B proteins can dimerize and bind DNA as three species: A:A or B:B homodimers or A:B heterodimers (Conneely et al, 2001). It is generally thought that PR-A and PR-B are functionally different, that they regulate different physiological target genes and that they have different transactivation capabilities in different target tissues. Results have previously shown that if agonist bound PR-A is inactive, then it can act as a dominant repressor of PR-B activity; this repressor capability appears to be a selective property of the PR-A protein and has been shown to extend to other steroid receptors through competition for common limiting coactivators (Conneely et al, 2001). PR-A and PR-B also respond differently to progestin antagonists, PR-A is rendered inactive whilst PR-B is converted to a strongly active transcription factor (Conneely et al, 2001).

1.3.(iv) The PRKO Mouse Model:

As a way of directly delineating progesterone's involvement in general reproductive endocrinology, a progesterone receptor knockout (PRKO) mouse was generated in which both isoforms of PR were functionally ablated through gene targeting approaches (Ismail et al, 2003). Abrogation of the P signal resulted in an overt female infertility phenotype in which normal ovarian-, uterine- and behavioural-functions were severely compromised (Ismail et al, 2003). Despite these reproductive phenotypes, the PRKO mammary epithelium responded to the pubertal rise in serum levels of E to generate a normal ductal architecture almost indistinguishable from that of the wild-type (Ismail et al, 2003). The normal development of the PRKO pubertal gland is in stark contrast to the estrogen receptor-α knockout (ERKO) pubertal gland which exhibited ductal outgrowth defects.

In PRKO mice, there is no development of tertiary ductal side branching and no differentiation of the epithelial cells which confirms a specific role for P in pregnancy associated mammary gland development (Conneely et al, unpublished). In order to learn more about the actions of P in the mammary gland during pregnancy, isolated PRKO mammary epithelial cells (MECs) were transplanted into the cleared mammary fat pad of a wild-type nulliparous host (Ismail et al, 2003). Experimental techniques such as these were developed in order to circumvent the block to pregnancy presented by the PRKO animal (Ismail et al, 2003).

1.3.(v) Progesterone's Role in the Pregnant Mammary Gland:

Progesterone action in the mammary gland strongly induces increased tertiary side-branching and along with prolactin instigates the proliferative phase of alveolar morphogenesis for milk production. Administration of E plus P to age-matched wild-type virgins and PRKOs associated the absence of progesterone with a block in hormone-induced cellular proliferation (Ismail et al, 2003). During pregnancy, there is massive tissue remodelling within the mammary gland. Cell differentiation becomes dominant from mid-pregnancy as the gland moves into the secretory initiation phase. The developing alveoli cleave and the alveolar cells become polarised and form a sphere-like single layer of epithelial cells that envelopes a circular lumen, connected to the ductal network via a single small duct (Oakes et al, 2006). Tissue localisation and epithelial recombination experiments demonstrate that progesterone in the mammary epithelium, not the stroma, was essential for epithelial cell proliferation and lobuloalveolar development, although stromal PR may play a role in tertiary branching (Oakes et al, 2006; Sternlicht et al, 2006). PR-expressing cells are evenly spaced in the ducts of young mice but their distribution becomes mosaic in mature virgin mice and during early pregnancy. In early pregnancy, PR-positive MECs are found closely apposed to proliferating epithelial cells (Hennighausen et al, 2005). This segregation of PR-positive cells from proliferating cells is a conserved feature in normal rodent and human mammary tissue. An expression pattern such as this suggests that the regulation of epithelial cell proliferation by P may occur through a paracrine mechanism. PRs

residing in non-proliferating cells may induce expression of a proliferative signal that can promote proliferation of neighbouring receptor negative cells (Conneely et al, unpublished). PR has been identified as a transcriptional target of ERα in reproductive tissues and the mammary gland, however absence of ERα does not result in the loss of PR expression in mammary epithelium. If ERα null mammary cells are supplemented with P and prolactin, they respond by inducing lateral side-branching and lobular alveolar differentiation (Conneely et al, unpublished).

1.4. A Role for Progesterone in Mammary Tumourigenesis:

The pivotal role played by the progesterone proliferative signal in normal mammary gland development raised the possibility of whether progesterone may also affect mammary gland tumourigenesis (Ismail et al, 2003). Ablation of PR expression in PRKO mice resulted in a significantly reduced incidence of mammary tumour growth in response to carcinogen challenge relative to the wild-type littermates underscoring a specific role for PR as essential regulators of intracellular signalling pathways that are essential for the initiation of murine mammary tumours induced by carcinogens (Conneely et al, 2003). Also in many human breast tumours, the majority of ER and PR positive cells undergo proliferation. This observation suggests a switch in steroid dependent regulation from a paracrine to autocrine mechanism as populations of proliferating MECs

which are positive for ER and PR emerge in response to carcinogen challenge (Conneely et al, 2003).

The following sections; 1.4(i) to 1.4(v), summarise the research efforts which have been made in recent years to link progesterone with breast related tumourigenesis. Research such as this has encouraged the work in this thesis and is essential if new and more effective treatments are to be made available for the many women who develop breast cancer around the world.

1.4.(i) Reproductive History-Associated Risk:

Reproductive history is a consistent risk factor for human breast cancer. Whilst an early first pregnancy has a strong protective factor against breast cancer, the highest incidence of breast cancer still occurs in the premenopausal reproductive years (Conneely et al, 2003). It is at this biological time-point that the MECs are most reliant on the ovarian steroid hormones for proliferation therefore it is important to investigate the relationship between hormone dependent MEC proliferation and the acquisition of cancer causing genetic mutations. The average woman undergoes hundreds of cycles of breast cell division and cell death repeated over a span of roughly forty years, from puberty to menopause (Fig 1.6).

Although the steroid hormones do not seem to directly cause the DNA mutations, proliferation of normal cells from exposure to these hormones creates a vulnerability to spontaneous somatic DNA mutations, some of which may represent a first step on the pathway to cancer.

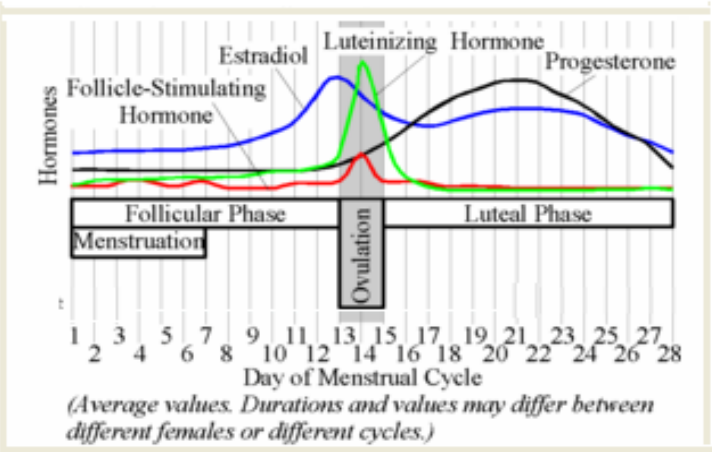


Fig 1.6: A chart depicting the hormonal changes associated with the human female 28 day menstrual cycle. Estrogen spikes just before ovulation whilst progesterone spikes in the middle of the luteal phase. However, in the absence of pregnancy, progesterone levels drop off towards the end of the cycle (http://upload.wikimedia.org/wikipedia/commons/thumb/f/f0/MenstrualCycle.png/300px-MenstrualCycle.png - 2nd August 2008).

Alternatively if one or more breast cells possess a DNA mutation that increases the risk of developing cancer, then these mutant cells will proliferate along with the normal breast cells in response to the hormone stimulation and also increase the risk of breast cancer development (Kleinsmith et al, 2007).

Research with progesterone receptor knockout (PRKO) mouse models has indicated that progesterone is specifically involved in inducing epithelial proliferation. The luminal compartment of mammary epithelium has been shown to be the target of this progesterone-induced proliferation, this compartment also happens to be the site of mammary epithelial tumourigenesis

1.4.(ii) Progesterone in mouse mammary carcinogenesis

In 1999, Lydon et al studied the functional relevance of progesterone-initiated intracellular signalling in mammary gland tumourigenesis by using the PRKO mouse model in the context of an established carcinogen-induced mammary tumourigenesis system. At five weeks, female wild type (WT) and PRKO mice received pituitary isografts in order to induce a high incidence of mammary

tumours with a short latency period. At eight weeks, the mice were administered 1 mg weekly of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) for three consecutive weeks. The control group, who received no pituitary isografts, were administered 1 mg weekly of DMBA for six consecutive weeks. In DMBA-treated pituitary-isografted mice, a reduction was observed in the mammary tumour incidence in PRKO mice as compared with isogenic WT mice. Mammary tumours developed in 60% of the WT mice but only 15% of the PRKO mice by 44 weeks after DMBA treatment. With no pituitary isograft, mammary tumours were observed in 20% of the WT mice and 20% of the PRKO mice up to 47 weeks. When the carcinogen was administered, the proliferative index of the pituitary-stimulated WT mammary gland was four-fold higher than similarly treated PRKO glands. This study indicates a specific role of the PR as a mediator for intracellular signalling pathways that are essential for the initiation of the majority of murine mammary tumours induced by DMBA. The data strongly supports a role of progesterone in a murine model of carcinogen-induced mammary gland tumourigenesis (Lydon et al, 1999).

1.4.(iii) The Women's Health Initiative (WHI) HRT Study

One high profile study in 2002 proposed a link between progesterone and breast cancer in menopausal women undergoing hormone replacement therapy (HRT) (Roussouw et al, 2002). HRT is aimed at women who have passed through menopause and as a result produce significantly reduced amounts of estrogen. The lack of estrogen in these women is linked to several health problems.

Estrogen has positive effects on blood vessels and on bones therefore after menopause, women are at increased risk for heart disease and osteoporosis. To counteract such potential problems, some postmenopausal women undergo hormone replacement therapy (HRT) and take hormone pills containing estrogen to strengthen bones and help control other menopausal symptoms. Unfortunately there are some serious risks associated with HRT; the potentially harmful proliferative effects of estrogen have been well established, namely an increased risk for invasive breast cancer and for uterine cancer in women with an intact uterus. Estrogen blockades have been developed as both a prophylactic tool to prevent cancer development and as chemotherapy in early stages of breast cancer (Roussouw et al, 2002). Studies which were done in the 1980's suggested that adding progesterone to estrogen could offer a protection against the harmful proliferative effects of estrogen on the uterus. HRT which combined both estrogen and progesterone became very common however, only women who had an intact uterus benefited from the addition of progesterone whilst those women who had undergone a hysterectomy continued to receive HRT with estrogen alone (Kleinsmith et al, 2007).

In July 2002, the National Institutes of Health (NIH) halted a large, in-progress study examining the effects of a widely used type of HRT medication called Prempro®, which combines the hormones estrogen and progestin. The study was scheduled to run until 2005 and was to determine whether estrogen plus progestin could prevent heart disease and hip fractures in women between ages 50 and 79 with an intact uterus. Preliminary studies found a 26 percent increase

in breast cancer in women receiving the hormones compared with women receiving a placebo. Those figures represent the *relative incidence* of the findings however when the data is examined in terms of *relative risk* for the individual woman, the results and the associated risks appear far less sinister (Roussouw et al, 2002).

The WHI results suggest that during one year, among 10,000 postmenopausal women with a uterus [as opposed to those who had a hysterectomy] who are taking estrogen plus progestin, eight more will have invasive breast cancer, than will a similar group of 10,000 women not taking these hormones. This is a relatively small annual increase in risk for an individual woman. The WHI study did however spur further investigations into the nature of PR and the potential threat that it may pose to normal mammary gland development (Jovanovic Poole et al, 2006).

1.4.(iv) Hormone Dependence in Premalignant Mammary Progression

This marked dependence on progesterone-mediated signalling for tumourigenesis was examined in another study in 2003 by Medina et al. They looked at a model of mouse mammary tumourigenesis; BALB/c p53-null mammary epithelium. This mouse model has a deletion of the tumour suppressor gene p53 which results in enhanced tumourigenic risk. The model progresses through ductal hyperplasia and then ductal carcinoma in situ before becoming invasive breast cancer. The p53-null mammary cells exhibit the same

properties of hormone dependence as p53 wild-type cells but they are highly sensitive to the tumour-promoting effects of the ovarian hormones. The study examined the effect of chronic administration of progesterone on the tumourigenic potential of p53-null mammary epithelial cells over a 14 month period. Mice with p53 wild-type mammary epithelium were not evaluated because previous experiments had shown that chronic hormone stimulation did not convey a tumourigenic stimulus over these time periods.

The importance of normal signalling of progesterone was demonstrated by removing PR function in the p53-null epithelial cell. The group cross-bred p53-null mice with PRKO mice to generate p53-/-,PR-/- mammary ducts. Mammary tumourigenesis in hormone-stimulated p53-null epithelial cells was markedly reduced in the absence of progesterone signalling from 84% to 32%. The removal of progesterone-stimulated proliferation in the mammary gland decreased the number of tumours by almost two-thirds in the p53-null mice thus suggesting that progesterone has significant tumourigenic potential under such circumstances.

1.4.(v) Prevention of BRCA1-Mediated Mammary Tumourigenesis in Mice by a Progesterone Antagonist

In 2006, a paper by Poole et al demonstrated that a progesterone antagonist mifepristone (RU486) was able to prevent mammary tumourigenesis in a *BRCA1*/p53 conditional knockout mouse model. This was the first paper to produce a very strong and substantial data set for the critical role of PR function

in mammary carcinogenesis. *BRCA1* (breast cancer 1) is a human gene that belongs to a class of genes known as tumour suppressors, which maintains genomic integrity to prevent uncontrolled proliferation. The BRCA1 protein is implicated in DNA damage repair, ubiquitination, cell cycle checkpoint control and transcriptional regulation. Mutations in the gene have been implicated in a number of hereditary cancers, namely breast, ovarian and prostate.

The specific suppression of breast and ovarian carcinogenesis by the BRCA1 tumour suppressor has been attributed to its regulation of ERα and the two PR isoforms. BRCA1 interacts with ER and PRs directly and modulates ligand-dependent and ligand–independent transcription. The study by Jovanovic Poole et al, attempted to address the specific roles of ER and PRs in *BRCA1*-mediated tumourigenesis by studying a *BRCA1*/p53 conditional mammary gland knockout mouse model and also a p53 mammary gland knockout mouse model. Inactivation of both *BRCA1* and p53 genes mimics the majority of human *BRCA1*-associated tumours, which also harbour p53 mutations. The mammary gland morphology of the mature, nulliparous *BRCA1*/p53 knockout model was similar to that of a wild-type pregnant mouse suggesting that proliferation of mammary epithelial cells (MECs) was altered. Proliferation of the MECs is regulated by the ovarian hormones. MEC proliferation was measured by 5-bromo-2-deoxyuridine (BrdU) staining and showed a five fold increase in proliferation in the *BRCA1*/p53 knockout compared to wild-type mice. The BrdU staining also showed that the paracrine action of PR was maintained in at

least most cases of the hyperplastic *BRCA1*/p53 knockouts (Jovanovic Poole et al, 2006).

This study also evaluated PR protein expression and demonstrated elevated expression of PR-A in *BRCA1*/p53 conditional knockout mammary glands. Overexpression of the short isoform, PR-A has been associated with abnormal mammary gland development and ductal hyperplasia. The results suggest a correlation between *BRCA1* deficiency and PR accumulation.

Since progesterone is a potent mitogen for *BRCA1*/p53 MECs, the group tested whether a blockade of PR activity by a progesterone antagonist could prevent or delay mammary carcinogenesis in the *BRCA1*/p53 conditional knockout mouse. Mice were treated with a placebo pellet or with a pellet containing mifepristone (RU 486). The median tumour latency established for the *BRCA1*/p53 knockout mice was 6.6 months with complete penetrance; however no palpable tumours were detected in the mifepristone-treated mice at 12 months of age, thus producing a 100% reduction in mammary carcinogenesis at that particular time point. Five weeks of mifepristone treatment substantially reduced branching and suppressed alveolargenesis in the mammary glands of *BRCA1*/p53 knockout mice.

This study is very significant as it presents strong evidence that PR function is critical for *BRCA1*-mediated mammary carcinogenesis and that antiprogesterone treatment can prevent or delay mammary carcinogenesis in the *BRCA1*/p53 conditional knockout mice. Up to now, the therapeutic options for women with *BRCA1* mutations were very limited with a bilateral mastectomy the being the

most common method of both treatment and prevention, however the results of this creates an opportunity to investigate the use of antiprogesterones as a potential chemopreventive strategy and offer hope for the many women who carry a *BRCA1* mutation (Jovanovic-Poole et al, 2006).

This thesis and its investigations of progesterone-mediated signalling is heavily weighted on studies such as those discussed in section 1.4 because of the clear associations they have made between progesterone and tumourigenesis.

1.5. Investigation of Signalling Mechanisms in Mammary Gland Development:

Although, significant progress has been made over the last ten years towards understanding the contribution of P to the proliferation and differentiation of the normal mammary gland, the signalling mechanisms that mediate these P-dependent responses remain poorly understood (Conneely et al, 2001). This section highlights a number of important investigations which were implemented in order to explore, identify and elucidate the downstream targets of P-regulated pathways in this thesis. With better knowledge of its functional mediators, progress can be made to better understand how and where a signalling pattern can become corrupted and cause abnormal or malignant mammary gland development.

1.5.(i) Cre/loxP Transgenic Mouse Models

Transgenic mouse models were used heavily in this thesis to generate experimental data. The extensive homology between the murine and the human form ultimately make transgenic animal studies an extremely worthwhile research tools. In this instance, it was specifically PRKO mice which were generated. The Cre/lox targeting approach was used to generate these valuable PRKO mouse models.

Cre recombinase is an enzyme derived from the bacteriophage P1 that specifically recognizes loxP sites. Cre has been shown to effectively mediate the excision of DNA located between loxP sites by binding to each of the two palindromic halves of the loxP sites (Franco et al, 2008). After the excision event, the DNA ends recombine leaving a single loxP site in place of the intervening sequence.

To use this system in the mouse, the Cre recombinase needs to be expressed at a sufficiently high level either as a transgene or a 'knock-in' approach. An additional mouse is needed in which the gene of interest is flanked by loxP sites generating a floxed mouse. The resulting Cre mouse can then be crossed to the floxed mouse to generate a mutant mouse in which a gene can be ablated or overexpressed (Fig 1.7).

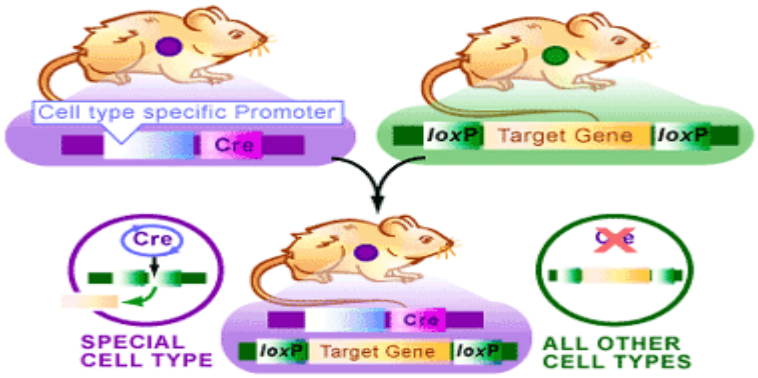


Fig 1.7: A schematic diagram illustrating the gene targeting technique Cre/lox. Mice with the Cre protein expressing in a specific cell type (eg MECs) are bred with mice that contain a target gene (eg PR) surrounded by loxP sites. When the mice are bred, the cells carrying Cre will cause the specific cells to lose the floxed target gene and thus produce conditional knockout models of that specific knockout gene. *(*http://www.scq.ubc.ca/targeting-your-dna-with-the-crelox-system/)

The manipulation of the gene of interest will be determined by the spatiotemporal expression of the Cre recombinase allowing for conditional alteration of the gene of interest (Franco et al, 2008).

For this thesis, MMTV/Cre mice were cross bred with mice in which the PR gene was floxed thus producing mutant mice in which the PR gene had been 100% ablated in the mammary gland. MMTV-Cre mice are a transgenic strain of mice in which the P1 Cre recombinase is under the transcriptional control of the mouse mammary tumour virus (MMTV) promoter/enhancer. The MMTV/Cre transgene has high levels of recombination in the virgin and lactating mammary gland with specificity for the luminal mammary epithelial cell compartment. This strain was deemed highly appropriate as the PR gene could be deleted specifically in the virgin and lactating mammary gland.

1.5.(ii) DNA Microarray Technology

An Affymetrix DNA microarray was carried out prior to the commencement of this thesis (Fig 1.8). The microarray technology used DNA extracted from the mammary gland tissues of the PRKO transgenic mouse models. The DNA microarray analysed the differential expression of several hundred genes in a wild type mouse and concurrently compared these wild-type expression levels to the expression of the same genes in a PRKO mouse model which was conditional for the murine mammary gland.

In this instance, the primary intentions of the microarray were to observe the regulation of the mouse genome in response to progesterone in pregnancy associated mammary gland development. The mammary glands of both the wild-type and PRKO mouse models were manipulated into a full-term pregnant state by treating both sets of mice with low doses of estrogen and progesterone over a three week period. It was important to control and standardise the amount of administered exogenous ovarian hormone circulating within the mice therefore all mice underwent an ovariectomy to ensure that no endogenous hormone was being produced which could potentially interfere with the results.

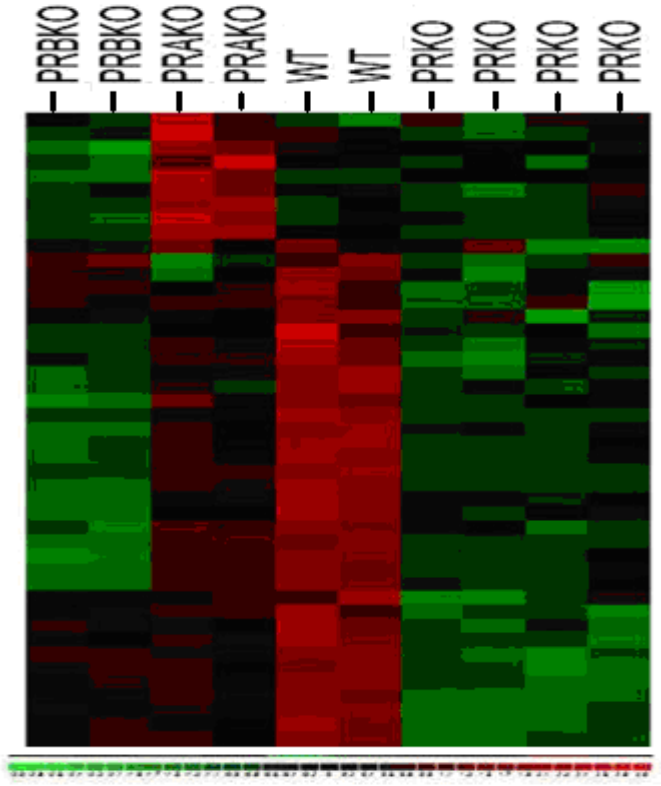


Fig 1.8: An example of a heat map from the Affymetrix GeneChip® Mouse Genome Array. Expression values were visualised as colour maps where the log of the expression intensity values for each progesterone-treatment group were first centred on the mean values of the corresponding vehicle control (WT). In this array, the wild-type genes were the control and were compared to three different forms of the conditional progesterone knockout mouse model; PRKO, PRAKO and PRBKO. Green signifies downregulation whilst red signifies upregulation of the target gene (Conneely et al, unpublished data).

The results of this microarray essentially formed the basis for the investigations undertaken by this thesis. The microarray heat map produced some predicted results for the commonly documented and well established downstream target genes of P however it also showed strong PR-regulation of some unexpected mammary gland targets (Fig 1.9). The intention of this thesis was to examine some of these well known genes and assess what role, if any, that they may have in mediating the structural and functional changes that occur in the mammary gland during pregnancy, i.e. tissue remodelling and alveolar differentiation. In this thesis, the role of lesser known targets genes are also investigated in the same mouse models to elucidate what kind of role they may be playing in development at this specific time point.

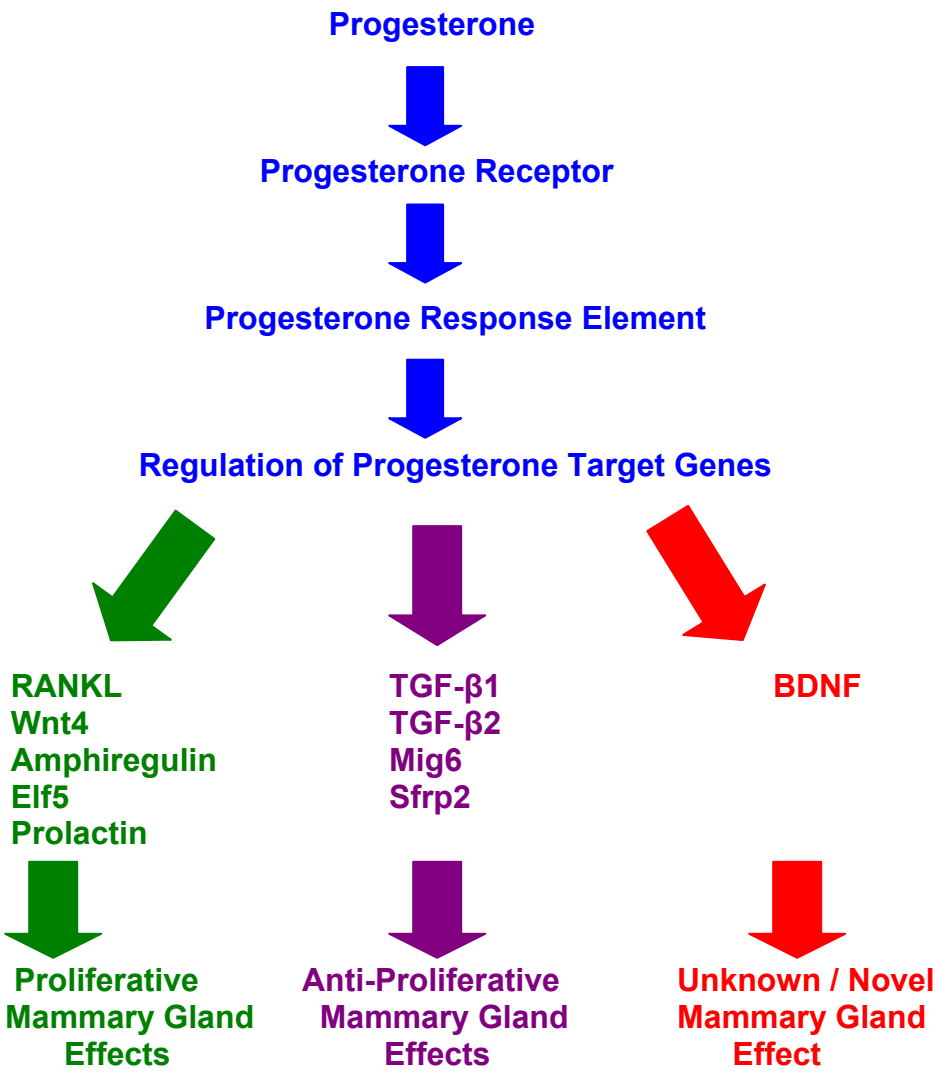


Fig 1.9: An introductory diagram of target genes that are analysed as potential mediators of P signalling in this thesis. These targets were chosen on the basis of their expression profiles in the DNA microarray which was carried out prior to this work. RANKL, Wnt4, Amphiregulin and Elf5 are thought to have a proliferative effect on the mammary gland development whilst TGF-β1 and TGF-β2, Mig6 and Sfrp2 are thought to be anti-proliferative. BDNF was also identified on the microarray as being strongly regulated by P yet little is known about its role in mammary gland development.

1.5.(iii) Validation of Targets via quantitative PCR technology

The results obtained from a DNA microarray help identify potential mediators of the progesterone signalling pathways however, it is common with an microarray to get a significant number of 'false hits' therefore it is important

that all of the genes which appear to be regulated by progesterone on the heat map are in fact confirmed as bone fide targets of PR action using qPCR technology. In the case of the mammary gland, priority is given to factors that are known to promote proliferation and also to those factors which are known secreted mediators as they in particular may have a role in the paracrine action of PR in the mammary epithelial cells. Some genes which appear to be of particular interest to the lab may merit further research by the lab and it is at this point that the generation of specific conditional knock out mouse models can be addressed.

1.6. Mediators of Progesterone Action in the Mammary Gland:

1.6.(i): Prolactin

Prolactin expression is largely responsible for the production of milk during lactation in the mammary gland. Whilst the hormone itself is not a direct mediator of P, the prolactin receptor has been shown to be upregulated by P (Oakes et al, 2006). Nevertheless, P and prolactin demonstrate a strong co-functional relationship as they work together to prepare the mammary gland during pregnancy. Massive tissue remodelling occurs within the mammary gland during pregnancy, resulting in the formation of the lobuloalveoli that are capable of milk secretion. It is important to emphasise that the formation of these alveoli is highly dependent on the synergy between prolactin and P (Oakes

et al, 2006). Pituitary prolactin stimulates ovarian P enabling P to maintain its required levels during early pregnancy. In addition, upregulation of P expression by prolactin and prolactin receptor expression by P suggests that these hormones interact in a synergistic manner to control alveolar development (Oakes et al, 2006). P and prolactin signalling pathways have many similarities in their mediated effects during early pregnancy. Many of the target genes that are of interest to this study have shown evidence of prolactin regulation as well as P regulation which suggests cooperation and a possible convergence between the two hormonal pathways.

The Proliferative Effectors of Progesterone Signalling:

1.6.(iii) RANKL:

As previously mentioned, PR positive cells can act on their neighbouring PR negative cells in a paracrine fashion. Grafts of PRKO epithelium or stroma in combination with PR WT stroma or epithelium demonstrated that mammary epithelial cells can give rise to alveoli when placed in close vicinity to PR WT epithelial cells, indicating that progesterone does not need to act directly on the alveolar cells and instead can orchestrate the morphogenetic and proliferative events of alveolargenesis by affecting nearby cells in the mammary epithelium (Brisken et al, 1998). A secreted protein known as RANKL (receptor activator of nuclear factor κB – ligand) is known to be regulated by PR and is one of these paracrine mediators of alveolar proliferation and survival (Fig 1.10) (Kleinsmith et al, 2007). RANKL is a member of the tumour necrosis factor

family and is an important regulator of osteoclast development. In terms of mammary gland research, RANKL-null mice are unable to nurse their young because of an inhibition of alveolar development during pregnancy. These mice have decreased activation of the anti-apoptotic molecule AKT/PKB, they exhibit an increase in alveolar cell death during pregnancy and a decrease in cellular proliferation. Alveolar buds do still form in the RANKL-null mice which suggests that RANKL is required for the later steps of alveolar development i.e. differentiation (Hennighausen et al, 2005). Once RANKL is upregulated by PR signalling, it signals downstream to induce expression of its target NF-κB. NF-κB is required for the activation of the cell cycle regulator; cyclin D1 via the kinase IκB in neighbouring proliferative cells. The RANKL/NF-κB/Cyclin D1 pathway is crucial for the formation of the alveolar structures during pregnancy (Oakes et al, 2006). NF-κB is a transcription factor which is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival thus it promotes the P/RANKL-driven proliferation within the alveoli whilst also blocking apoptosis occurring (Gonzales-Suarez et al, 2007). It is generally understood that NF-κB expression and activity are tightly regulated during mammary gland development. This strict regulation appears to be critical for mammary gland development and tumourigenesis as genetically modified mice carrying alterations in proteins of the NF-κB pathway show marked defects at different stages of pregnancy and elevated NF-κB DNA-binding activity has been documented in both mammary

cell lines and in primary human breast cancer tissues (Gonzales-Suarez et al, 2007).

1.6.(iv) Wnt4:

Wingless-related MMTV integration site 4 (Wnt4) is one of the 19 members of the Wnt family of proteins. These Wnt proteins regulate a wide range of cellular processes including proliferation and differentiation (Roarty et al, 2007). Wnts participate in branching of other tissues, their transgenic overexpression affects mammary branching and mammary targeted expression of an inhibitory form of a Wnt receptor that blocks Wnt signalling suggests that at least one Wnt signalling pathway is involved in mammary ductal development (Sternlicht et al, 2006). Wnt4 is a well-known secreted glycoprotein that is regulated by progesterone. It is a downstream mediator of the progesterone response and has been shown to be coexpressed in PR positive cells (Hennighausen et al, 2005). Mammary transplants of Wnt4-/- epithelium have demonstrated that Wnt4 acts in a paracrine fashion to stimulate epithelial ductal side branching during early pregnancy (Oakes et al, 2006). However, Wnt4 does not act alone in this as these Wnt4 null mice are able to overcome the defects in late pregnancy hence other PR-dependent signalling pathways also have a role to play in this response (Hennighausen et al, 2005). Upon stimulation by the progesterone receptor, Wnt4 can bind and upregulate the activity of its target; β-catenin. β -catenin has specific actions in both the luminal and myoepithelial compartments of the epithelium (Fig 1.10) (Oakes et al, 2006). Once upregulated via Wnt4, β-catenin

will begin to accumulate in the cytoplasm. The accumulated β-catenin then translocates into the nucleus, where it interacts with the DNA-binding T-cell factor complex and can act as a transcriptional activator (Wong et al, 2002). In addition to its role in transcription, B-catenin is also a component of cell-cell junctions and appears to have a role in signalling to luminal epithelial cells (Oakes et al, 2006). Teuliere et al 2005 showed that activation of β-catenin within the basal epithelial cells results in premature differentiation of the luminal epithelium during pregnancy and persistent proliferation resulting in tumours. These tumours consisted predominantly of undifferentiated basal cells, which were amplified in response to β-catenin activation, thereby implicating this molecule in cell fate decisions in the mammary gland (Oakes et al, 2006).

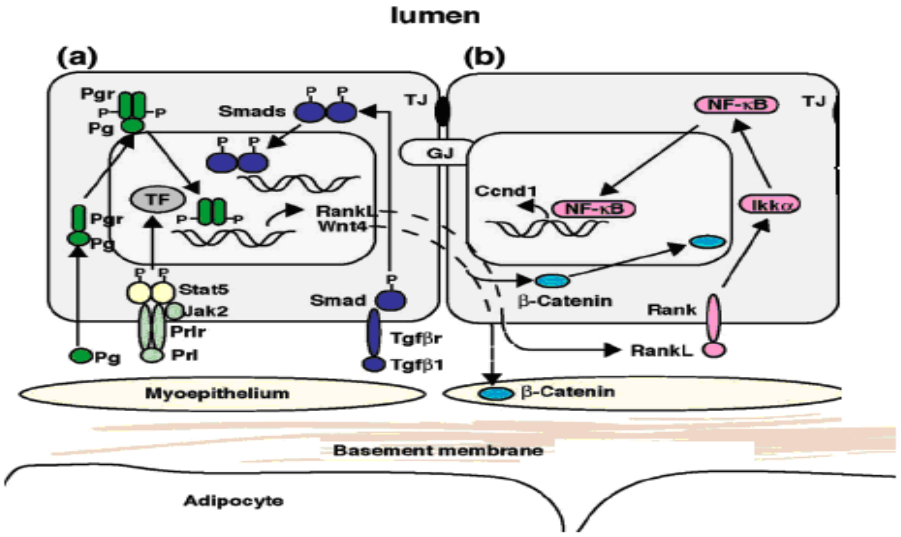


Fig 1.10: Molecular control of alveolar morphogenesis. Signalling from the progesterone receptor (PR) and prolactin receptor (Prlr) is essential for alveolar morphogenesis in pregnancy. Increases in serum progesterone (P) and prolactin (Prl) result in luminal cell proliferation during early pregnancy, which continues throughout gestation. (a,b) Heterogenous receptor patterning is essential for complete alveolar morphogenesis. (a) Wnt4 and RANKL are transcribed in response to Pgr signalling, probably in cooperation with Prl signalling, and appear to stimulate proliferation of neighbouring cells via paracrine mechanisms. (b) RANKL binds to its receptor RANK in a neighbouring cell and activates the RANKL/nuclear factor (NF)-.B pathway, resulting in cyclin-D1 (Ccnd1) transcription and proliferation. Wnt4 binds and activates its target ß-catenin, which has specific roles for both luminal and myo-epithelium for cell fate decisions involving both proliferation and differentiation (Oakes et al, 2006).

1.6.(v) Amphiregulin:

Amphiregulin is a gene that exhibited downregulation in the PRKO samples on the Affymetrix microarray heat map that was carried out by the lab (Fig 1.8). Amphiregulin is established as a promoter of mammary gland development but it is upregulated during puberty when its expression is strongly induced by estrogen (Sternlicht et al, 2006). Amphiregulin is an epidermal growth factor receptor (Egfr) ligand and is exclusively expressed in the epithelium. EGFR is a receptor tyrosine kinase that activates multiple intracellular pathways that are often associated with cell proliferation and survival. EGFR activity is elicited when it binds one of seven EGF-related ligands and then dimerizes with another EGFR monomer or one of three related receptors ErbB2, ErbB3 and ErbB4. Amphiregulin is the only EGFR agonist that is absolutely required for mammary development as ductal outgrowth is impaired in Amphiregulin-null mutant mice but not in mice lacking one or more alternative EGFR ligands. The Amphiregulin deficient mice are unable to form a competent ductal tree that fills the entire fat pad but instead form a small ductal outgrowth of insufficient size to nourish pups (Sternlicht et al, 2006).

Amphiregulin is only expressed on mammary epithelial cells yet it has to bind and activate EGFR on nearby but separate stromal cells. Amphiregulin is expressed as a transmembrane precursor therefore for Amphiregulin to activate adjacent stromal cells it has to be shed from the epithelial cell surface. ADAM 17 is a transmembrane metalloproteinase which proteolytically sheds Amphiregulin from the epithelial cell surface to activate EGFR on the nearby

stromal cells. EGFR phosphorylation can only occur when ADAM 17 and Amphiregulin are expressed on mammary epithelial cells and EGFR is present in the stroma (Fig 1.11). This signalling pathway is very important for epithelial-stromal cross talk which acts to regulate mammary development (Sternlicht et al, 2006).

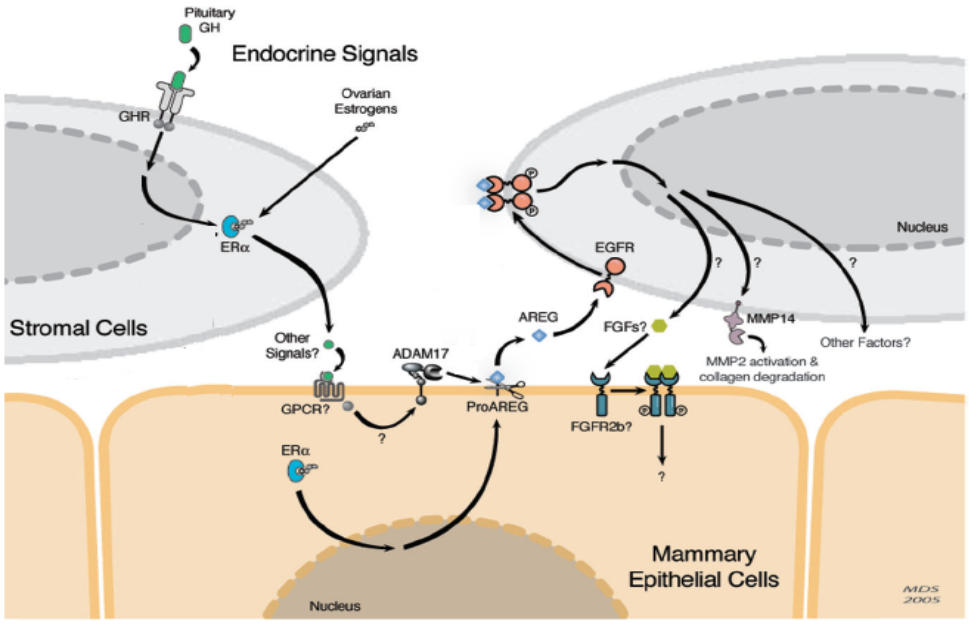


Fig 1.11: A model diagram depicting Amphiregulin (AREG), Epidermal Growth Factor Receptor (EGFR) and some of the other key endocrine and paracrine signals that regulate mammary branching morphogenesis. Amphiregulin is expressed exclusively on the mammary epithelial cells but it binds to EGFR on the adjacent stromal cells ADAM 17 is a metalloproteinase required to activate AREG and enable it to interact with EGFR in the stroma and stimulate branching within the gland (Sternlicht et al, 2006).

1.6.(vi) Elf5:

Elf5 is a member of the Elf subfamily of Ets transcription factors. Ets transcription factors regulate gene expression during the differentiation of multiple tissues including vascular, lymphoid, muscle and bone. Elf5 is expressed specifically in the luminal cells of the mammary tissue and its expression is increased dramatically during pregnancy to levels that far exceed

those seen in other tissues (Oakes et al, 2006). Elf5-null foetuses die at embryonic day 7.5 which points to a crucial function during embryogenesis. Mice that have only one functional Elf5 allele show abrogated pregnancy mediated alveolargenesis (Hennighausen et al, 2005). The levels of Elf5 are reduced in prolactin receptor (PRLR) heterozygote mice but there is no similar reduction in the expression of prolactin receptor in Elf heterozygote mice thus indicating that Elf5 is downstream of the prolactin receptor (Oakes et al, 2006). Retroviral re-expression of Elf5 in PRLR-/- MECs was able to completely compensate for the loss of the PRLR signalling cascade. It is hypothesised by Oakes et al, 2006, that Elf5 is a key mediator of structural and functional development of lobuloalveoli and that it appears to be a master regulator of the transcriptional cascade controlling alveolar morphogenesis (Oakes et al, 2006). Since so much emphasis has been placed on Elf5's importance in alveolar morphogenesis, it was decided to analyse whether or not there was any progesterone regulation involved in the induction of Elf5 in pregnancy thus qPCR analysis was carried out on the Elf5 gene for this study.

The Anti-Proliferative Effectors of Progesterone Signalling:

Each of the branching agonists outlined have their own negative regulators (Sternlicht et al, 2006). Negative regulators can be as important to normal functional mammary gland development as their positive counterparts are.

1.6.(vii) TGF-β1:

Transforming growth factor beta (TGF-β) controls proliferation, cellular differentiation and other functions in most cells. TGF-β is a secreted protein that exists in three isoforms called TGF-β1 TGF-β2, TGF-β3. The cytokine TGF-β1 is an important regulator of mammary cell proliferation during pregnancy (Oakes et al, 2006). TGF- β1 is a member of the transforming growth factor beta superfamily which includes three TGFβs; activins, inhibins and bone morphogenic proteins (BMPs) (Monks et al, 2007). TGF-β family members can modify cell behaviours via autocrine, paracrine and endocrine mechanisms of action (Ewan et al, 2005). TGF-β1 is restricted to the luminal epithelial cells and can control cell proliferation via phosphorylation of Smad following TGF-β receptor activation. The SMAD pathway is a classical signalling pathway that TGF-β family members signal through to trigger apoptosis. TGF-β1 is a well known negative regulator of mammary gland branching as it limits epithelial proliferation and stimulates extracellular matrix production (Sternlicht et al, 2006).TGF-β1 heterozygote mice display accelerated lobuloalveolar development due to increased proliferation indicating that the expression of TGF-β1 restricts alveolar cell proliferation. Epithelial cell proliferation was increased more than 15-fold in TGF-β1 null ovariectomized animals treated with E&P compared to wild-type mice (Oakes et al, 2006). Previous studies have revealed that activated TGF-β colocalizes with steroid-receptor containing MECs thus suggesting that TGF-β may be one of several factors for preventing

steroid-receptor containing MECs from entering the cell cycle and preventing proliferation (Fernandez-Valdivia et al, 2005).

There is evidence from many species that both progesterone and TGF-β suppress milk secretion during pregnancy however no formal link has yet been made between the two. In this study, the expression of TGF-β is analysed in the same manner as the previous positive regulators of proliferation to assess its response, if any, to progesterone after three weeks of E&P treatment.

1.6.(viii) Sfrp2:

In addition to TGF-β, two other genes were observed in this study to further explore the anti-proliferative effects that are in play in the mammary gland during pregnancy. The first of these genes is known as Sfrp2 or secreted frizzled-related protein 2. Secreted frizzled-related proteins (Sfrp) are a family of secreted proteins that contain a region homologous to the extracellular cysteine-rich domain of the Frizzled (FZD) family proteins (Wong et al, 2002). As previously mentioned the Wnt genes encode a family of highly conserved secreted proteins which modulate cell fate and behaviour in cells through activation of receptor mediated signalling pathways (Wong et al, 2002). Members of the Wnt family interact with seven-transmembrane receptors of the Frizzled (FZD) family. Diverse signaling pathways are activated upon Wnt/FZD binding. The Wnt ligand/FZD receptor interaction has been shown to induce the phosphorylation of scaffolding proteins such as dishevelled, an event which was reported to be a component of all Wnt-induced signaling pathways (Fig 1.12).

The canonical Wnt signaling pathway leads to stabilization of its target β-catenin through inactivation of a protein complex consisting of, amongst others, the tumor suppressors APC and Axin. This protein complex is destructive and normally triggers rapid β-catenin phosphorylation, inducing its ubiquitination and degradation. In the presence of canonical Wnt ligands, β-catenin is stabilized, binds transcription factors of the LEF-1/T-cell factor (TCF) family, and stimulates target gene transcription (Schlange et al, 2007).

The secreted frizzled-related proteins (Sfrps) are considered to be negative regulators of the Wnt signaling pathway; they are extracellular inhibitors of Wnt signaling, which compete with FZD receptors for ligand binding. Sfrps contain a cysteine-rich domain (CRD) which is homologous to the putative Wnt-binding domain, but lacks the transmembrane domain of frizzled proteins (Wong et al, 2002). Studies have suggested that deregulation of the Wnt pathway by a loss of expression of the Sfrp proteins could lead to the development of breast cancer (Schlange et al, 2007).

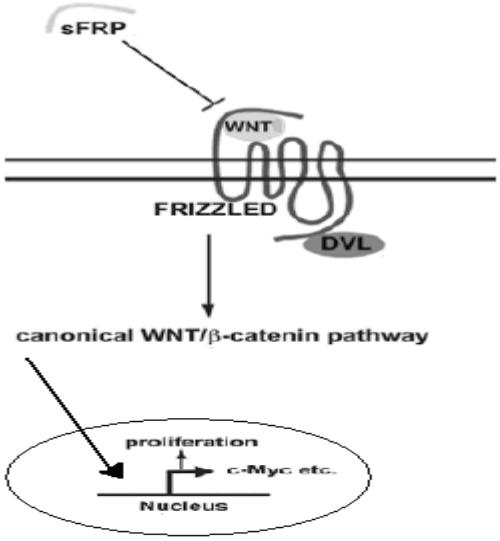


Fig 1.12: A simple schematic diagram to illustrate the inhibition of Wnt signalling pathway via Secreted Frizzled-Related Protein (Sfrp), to prevent transcription of the proliferative signals i.e. Wnt, within the mammary gland. Wnt signalling can activate a common Wnt pathway component; the scaffolding protein dishevelled (DVL) (Schlange et al, 2007).

1.6.(ix) Mig6:

The additional anti-proliferative gene observed in this study was mitogen-inducible gene 6 (Mig6). Mig6 is also known as several other names; ERBB receptor feedback inhibitor 1(*Errfi1*), receptor associated late transducer (RALT) and gene 33 (Xu et al, 2006).Mig6 is present in higher-order species but not in relatively less complex organisms suggesting that Mig6 has been acquired during evolution for the needs of more-complex signaling circuits (Zhang et al, 2007). Mig6 is an immediate early-response gene whose expression can be rapidly and robustly induced by a variety of external stimuli such as growth factors, cytokines and stress factors indicating that Mig6 may play a critical role in the early regulation of many cellular responses (Zhang et al, 2006; Ferby et al, 2006). Mig6 has been established as a tumour suppressor gene and is found to be downregulated in various human cancers however the reasoning behind the inclusion of Mig6 in this study arises from its role as a negative regulator of epidermal growth factor signaling. Mig6 is a negative regulator of the EGFR (Ferby et al, 2006). The EGFR is the receptor through which the proliferative mediator; Amphiregulin, signals thus Mig6 may be a possible negative regulator of proliferation during pregnancy-associated mammary gland development, hence it became a gene of interest in this study. Ferby et al 2006 showed that a deletion of the mouse gene encoding Mig6 caused hyperactivation of endogenous epidermal growth factor receptor (EGFR) and sustained signaling through the mitogen-activated protein kinase (MAPK) pathway, resulting in overproliferation and impaired differentiation of epidermal

keratinocytes (Ferby et al, 2006). Mig6 can be transcriptionally induced by EGF and it then acts as a negative feedback inhibitor of EGFR signaling by downgrading EGFR tyrosine phosphorylation and the subsequent activation of other downstream pathway targets. As well as the EGFR, Mig6 associates in situ with its related receptors; members of the ErbB family and inhibits signaling from these receptors as well; it also reduces the abilities of mitogens in the Erb family to trigger cell proliferation and also to enter the cell cycle in cultured cells and in vivo (Xu et al, 2006). Recently it has been reported that downregulated expression of the Mig6 gene is observed in human breast carcinomas, which correlates with reduced overall survival of breast cancer patients. However since no mutations in the Mig6 gene have been detected in human breast carcinoma, it was important to observe a normal Mig6 response in relation to the ovarian steroids as well as the simulated pregnant state in the hope of gaining a greater understanding of how and where it is exerting its effects (Zhang et al, 2006).

As previously mentioned, much of the work in this study has revolved around the original DNA microarray. The gene targets which have already been discussed are mostly established targets of progesterone signalling yet their expression in the mammary gland was being examined for the first time after 21 days of E&P treatment to determine their activity at this later and established stage of pregnancy.

1.7.The BDNF Study: A Novel Target of Progesterone Signalling in the Mammary Gland

This section focuses on one particular novel target gene which has never before been associated with the mammary gland or with pregnancy. This gene came to the attention of the lab for exhibiting a strong P response in the microarray and moreover because it was a well known neuronal gene. Brain derived neurotrophic factor (BDNF) was significantly upregulated in the EP-treated wild-type samples and its expression then decreased drastically in the EP-treated PRKO sample, in which PR had been totally ablated.

1.7(i) The Established Neuronal Functions of BDNF:

BDNF is a member of the neurotrophic family which are a group of small, basic, secreted proteins that aid in survival and maintenance of specific neuronal populations (Sohrabji et al, 2006). BDNF is widely distributed throughout the brain and significantly impacts neuronal survival and function through a variety of cell types to include neurons, astrocytes, oligodendrites, microglia and endothelial cells. BDNF mRNA is localised to the cortex, hippocampus, midbrain, hindbrain, cerebellum, olfactory bulb, spinal cord and the hypothalamus. BDNF also promotes regeneration of adult sensory neurons, retinal ganglion cells and basal forebrain cholinergic neurons following injury (Sohrabji et al, 2006).

The neurotrophins and their genes share homologies in sequence and structure. The protein product of each gene includes a signal sequence and a prodomain followed by a mature neurotrophin sequence. Each gene product must be processed by proteolysis to form a mature protein (Reichardt, 2006). The BDNF gene consists of 5 exons although the mature protein is entirely encoded by one exon; exon number 5. All untranslated exons possess individual promoters, resulting in a complex pattern of tissue and cell-specific expression of this protein (Sohrabji et al, 2006).

The neurotrophins interact with two distinct classes of receptors. P75 was identified as a low affinity receptor for all of the neurotrophins whilst the members of the Trk subfamily of receptor tyrosine kinases were identified as high affinity receptors for the neurotrophins. Trks are transmembrane receptors with conserved intracellular domains that mediate several well-characterised signalling pathways (Tapia-Arancibia et al, 2004). BDNF selectively binds to TrkB by dimerizing the TrkB receptors which results in activation through transphosphorylation of the kinases present in their cytoplasmic domains (Reichardt, 2006). BDNF and TrkB mRNA expression varies as a function of development, age and cognitive performance. In hippocampal cortical or cerebellar granule neurons, BDNF can downregulate TrkB protein and/or TrkB mRNA levels thus serving as some form of negative feedback mechanism (Tapia-Arancibia et al, 2004).

1.7.(ii) BDNF and the Ovarian Steroid Hormones:

Hormonal status can greatly influence the expression of BDNF. Estrogen replacement in young adult, ovariectomized female rats increases BDNF expression in the olfactory bulb, hippocampus, cortex, amygdale and the septum. However other reports showed that high endogenous estrogen levels during the estrus cycle are associated with decreased BDNF mRNA in the hippocampus and prefrontal cortex. The estrogen-BDNF interactions remain poorly understood and will require many more efforts to resolve. It is important that attention is also given to the other ovarian hormones which may significantly influence estrogen-BDNF interactions. It was noted that the timing of high progesterone levels during the estrus cycle may be a critical switch for increased or decreased BDNF expression (Sohrabji et al, 2006). Franklin and Perrot-Sinal note that estrogen and progesterone act to impact BDNF levels in the hippocampus as certain regions of the hippocampus express both progesterone receptor mRNA and estrogen receptor mRNA (Franklin et al, 2006). Estrogen may also modulate BDNF protein levels through an estrogen response element that has been identified in the BDNF gene in rats although it is not clear if it is functional. The mechanism through which progesterone might alter BDNF levels is still unclear. Progesterone might directly interact with PRs present in the hippocampus or it may be on neurons in other areas that contain PRs and project to the hippocampus (Franklin et al, 2006).

Unlike the other previous genes examined in this study, there is currently no information or any publications on what role BDNF might play in the mammary

gland. Even though, BDNF has been well established and documented as a neuronal gene, in terms of mammary gland development it is still regarded as a novel target gene. Interestingly, there has been some work done on BDNF in the uterus where sympathetic neurite innervation is suppressed by high estrogen levels. It appears that BDNF may mediate this neurite suppression. High levels of estrogen induce BDNF expression in the uterus, which can be abolished by blocking antibodies to BDNF thus suppressing sympathetic neurite outgrowth (Sohrabji et al, 2006).

1.7.(iii) The BDNF Mammary Gland Hypothesis:

The strong expression profile produced by BDNF on the heat map came as an unexpected surprise to the lab and immediately caused special interest. The strong regulatory response of this secreted neuronal protein encouraged the lab to hypothesise on what BDNF may be contributing to the progesterone regulated mechanisms that seem to govern so much of the pregnancy-associated mammary gland development and structure. In order to confirm and further validate the microarray result, which demonstrated significant regulation by P in many specific genes (Fig 1.8), the lab carried out qPCR analysis using similar samples to those used in the microarray i.e. wild-type mice and PR mice of varying genotypes that had been treated with E&P for three weeks (Fig 1.13a). The lab also performed immunofluorescent staining techniques on wild-type and PRKO mammary glands to determine whether there was co-localisation of BDNF to PR within the gland. Their staining showed co-localisation of BDNF

and PR in the ductal epithelium in the wild-type gland. As expected, the PRKO mammary gland shows no functional PR activity and as a possible consequence of this, no BDNF activity is seen either (Fig 1.13b).

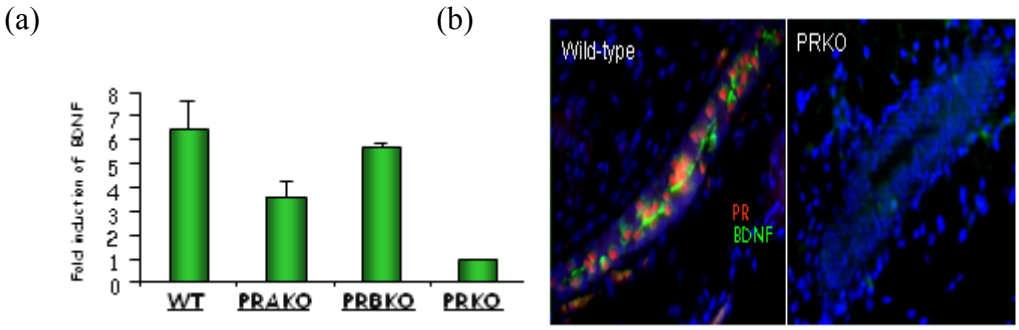


Fig 1.13: (a) qPCR analysis showing BDNF expression in the mammary glands of wild-type (WT), PRAKO, PRBKO and PRKO mouse models after three weeks of E&P treatment and (b) immunofluorescent data produced by the Conneely lab which confirms BDNFs regulation by PR in the mammary gland as BDNF is localised to PR in the luminal epithelium in the wild-type model but is absent in the PRKO model when there is no functional PR present.

Once the regulation of BDNF by progesterone was further confirmed, the lab generated BDNF conditional knockout transgenic mice in order to further investigate where the effects of BDNF were being expressed downstream of the progesterone receptor. The CRE-lox gene targeting approach was used in order to specifically ablate expression of BDNF in the mammary glands of C55/129 mice. A heterozygous and homozygous conditional knockout for BDNF was produced for the purposes of this study. In an attempt to understand the response of BDNF to progesterone, the lab examined what happened to the mammary gland in the absence of BDNF and then compared this to what happened in the mammary gland in the absence of PR to try and functionally connect the two.

The role of BDNF as hypothesised by our lab therefore arises from the two opposing phenotypes that were seen in the PR-/- mice and BDNF-/- mice generated by the lab (Fig 1.14).

Fig 1.14: Schematic diagram depicting the phenotypical characteristics of mammary gland development i.e. branching and alveolar differentiation in three mice models with varying genotypes (a) PR homozygous knockout, (b) normal murine model, (c) BDNF homozygous knockout.

When PR is removed, no side-branching or alveolar differentiation can occur during pregnancy in the absence of a progesterone signal. It is hypothesised that the proliferative signals; Amphiregulin, RANKL and Wnt4, may be downregulated in the absence of functional PR therefore they may be involved in the loss of proliferation within the mammary gland.

When functional BDNF is conditionally removed from the mammary gland, a very different phenotype is produced. Despite its own regulation by progesterone, the BDNF knockouts do not affect branching in the same manner as progesterone knockouts. In the absence of BDNF, there is a hyperbranching phenotype with increased ductal side branching and alveolar differentiation (Fig 1.15a). The BDNF knockout models also demonstrate reduced cellular polarity, which is often associated with the initial stages of tumour formation (Fig 1.15b).

The hypothesis suggests that progesterone simultaneously upregulates BDNF expression along with its upregulation of the proliferative signals except that the presence of BDNF is there to limit and control the proliferation induced by Amphiregulin, RANKL and Wnt4.

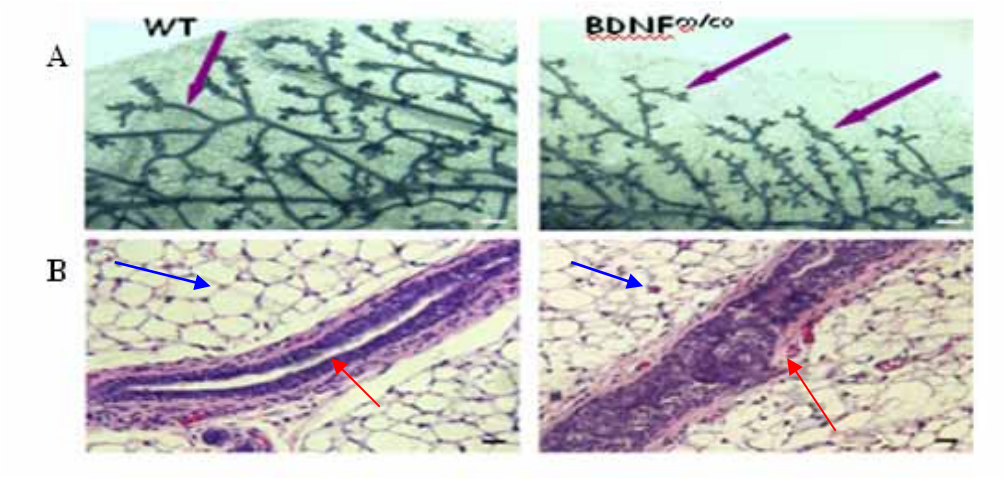


Fig 1.15: (A) Comparison of mammary gland morphogenic responses in wild-type (WT) and BDNF-/- ovariectomized mice treated for 21 days with pregnancy levels of E & P. In the absence of BDNF, the mammary gland exhibits hyperbranching of secondary epithelial ducts compared to the normal ducts in the WT (purple arrows). (B) This staining shows the cellular structure of the WT and the BDNF knockout samples, the lumen seems to be filled with cells suggesting the normal cellular mechanisms for moving dead cells out of the lumen may be inhibited (red cells) and there also seems to be a loss of cell-matrix interactions in the stroma (blue arrows)

It is important that these powerful signals can be maintained and curtailed in order to preserve the proper structure and spacing of the ductal branches as they develop within the gland. BDNF is believed to monitor and curb the proliferation within the developing gland. Without BDNF, the ductal branches may grow and expand uncontrollably which can create potential for aberrant growth patterns and tumour formation. The hypothesis of BDNF regulation by PR is illustrated in Fig 1.16.

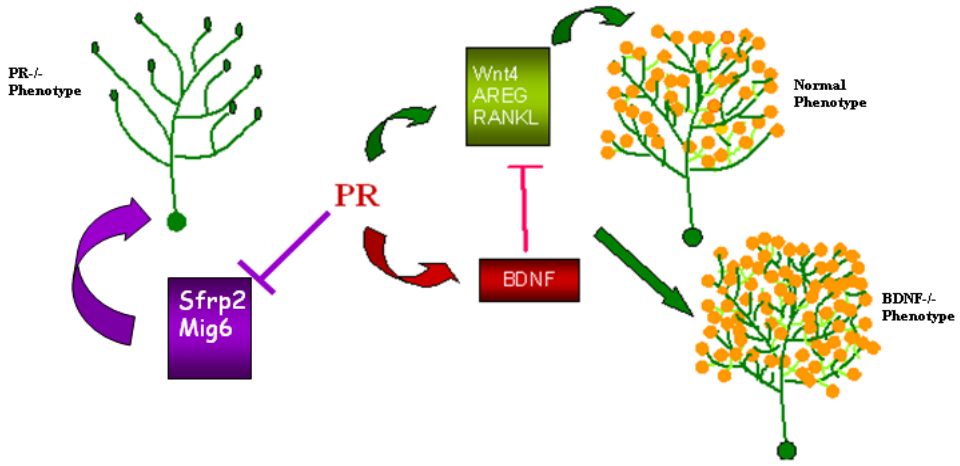


Fig 1.16: Schematic diagram of the hypothesis of BDNF regulation by the progesterone receptor (PR) and its functional role in relation to mammary gland development

1.7.(vi) BDNF and the Progesterone Response Element (PRE):

The sequence of the BDNF gene was analysed to determine whether or not it had a progesterone response element in its promoter region as previous work in the BDNF field has identified the presence of an estrogen response element. The 5' sequence flanking exon1 of the BDNF gene was analysed for potential PRE sites and showed very high sequence homology between the human and the mouse (Fig 1.17). The progesterone response element sequence is a palindromic sequence separated by three variable nucleotides; 5'TGTTCTnnnTCTTGT 3'. A half-site PRE was found within the BDNF promoter region and was conserved between both species. Since the estrogen receptor is capable of binding to half sites, it is possible that progesterone receptor could be able to do the same. The presence of a conserved PRE on the BDNF promoter region strongly infers that BDNF transcription can be driven directly by PR.

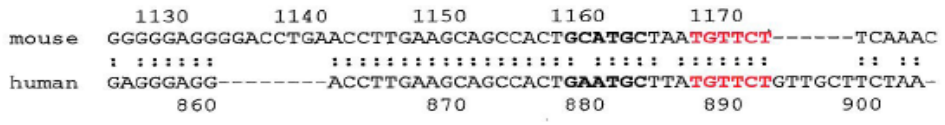


Fig 1.17: Identification of the PRE half site on the 5' sequence flanking exon1 of the BDNF gene of both the mouse and human. The half site was found at position 893 on the mouse gene and on position 1172 on the human gene – the presence of the PRE half site confirms the regulation of BDNF by PR in the mammary gland

1.8. Progesterone Receptor Signalling Targets in the Uterus

Progesterone and its role in mammary gland development has been the main focus of this study up to now however the uterus is also a known target of progesterone action therefore it was chosen to use as a comparison to determine if progesterone operates in a similar or dissimilar way in other important reproductive tissues when compared to the mammary gland. These comparative observations of progesterone tissue specific function should aid in a greater understanding of this very powerful ovarian steroid hormone.

1.8.(i) The Uterus:

The endometrium functions as a lining for the uterus; it is susceptible to many hormonal changes and is the focal point of this uterine study. The histology of the endometrium consists of a single layer of columnar epithelium, resting on a layer of connective tissue, which varies in thickness according to hormonal influences – this is known as the stroma (Lee et al, 2006). Simple tubular uterine glands reach from the endometrial surface through to the base of the

stroma, which also carries a rich blood supply of spiral arteries. In a woman of reproductive age, two layers of endometrium can be distinguished. The functional layer is adjacent to the uterine cavity. This layer is built up after the end of menstruation during the first part of the previous menstrual cycle. Proliferation is induced by estrogen (follicular phase of menstrual cycle), and later changes in this layer are engendered by progesterone from the corpus luteum (luteal phase). It is adapted to provide an optimum environment for the implantation and growth of the embryo. This layer is completely shed during menstruation. The basal layer, adjacent to the myometrium and below the functional layer, is not shed at any time during the menstrual cycle, and from it the functional layer develops. In the absence of progesterone, the arteries supplying blood to the functional layer constrict, so that cells in that layer become ischaemic and die, leading to menstruation (Lee et al, 2006).

Embryo implantation is a critical uterine event in which the blastocyst attaches to the luminal epithelium of the endometrium and then invades into the stroma (Franco et al, 2008). During the menstrual cycle or the estrous cycle, the endometrium grows to a thick, blood vessel-rich, glandular tissue layer. During pregnancy, the glands and blood vessels in the endometrium further increase in size and number. Vascular spaces fuse and become interconnected, forming the placenta, which supplies oxygen and nutrition to the potential embryo and fetus. The stromal cells surrounding the blastocyst undergo a proliferation and differentiation program to become decidual cells (Franco et al, 2008). The decidua is the mucous membrane which lines the uterus and undergoes

modification during pregnancy (Franco et al, 2008). Embryo implantation will only occur when the developmental progression of the hatched embryo to the activated blastocyst stage is precisely synchronised with the differentiation of the uterus to the receptive state. This restricted time period is termed the window of receptivity and its onset is controlled by the sequential and synergistic actions of ovarian estrogen and progesterone (Fernandez-Valdivia et al, 2005). In the mouse, the periimplantation period can be divided into prereceptive, receptive and refractory with implantation occurring at day 4 of pregnancy. Uterine development of the prereceptive period is under the influence of progesterone stimulation; progesterone levels increase at this point as progesterone is released form the newly formed corpus luteum (Takamoto et al, 2002; Franco et al, 2008). During this progesterone-dominated period, a nidatory period surge of estrogen is required to confer endometrial receptivity to the sensitized uterus, facilitating the implantation (Takamoto et al, 2002). Embryo attachment is followed by a characteristic endometrial transformation, decidualisation, which involves both differentiation and cell proliferation. PR action is indispensable whereas ERα is not essential. In the mouse, progesterone appears to prime the endometrium for before embryo attachment/implantation and thus is one of the essential functions regulated by progesterone during the periimplantation period (Takamoto et al, 2002). In the uterus, progesterone is also involved in the suppression of uterine proliferation which is induced by the effects of estrogen (Franco et al, 2008). Just as in the mammary gland, PR operates in a paracrine fashion in the uterus. Progesterone projects its influence

indirectly via paracrine molecular pathway: stromal to epithelium in the uterus and intraepithelial in the mammary gland (Fernandez-Valdivia et al, 2005).

1.8.(ii) PR Knockout Mouse Models and the Uterus:

As with the mammary gland, PR knockout mouse models were used to study the role of PR in the uterus. Female PRKO mice are infertile with defects in multiple reproductive tissues. These mice had an abnormal response to E&P treatment, they failed to ovulate and their uteri failed to support implantation as was demonstrated by an inability to undergo an artificially induced decidual response (Lee et al, 2006; Franco et al, 2008). Studies which used selective ablation of specific PR isoform indicated that PR-A is the functional isoform in the uterus and the major mediator of progesterone signalling (Fernandez-Valdivia et al, 2005). PRAKO mice developed normally to adulthood but were infertile, their uteri also failed to undergo the artificially induced decidualisation reaction and some progesterone target genes associated with uterine receptivity were downregulated (Franco et al, 2008). PR-B expression was not increased in these mice indicating that there was no compensation however selective activation of PR-B in the PRAKO mice resulted in an abnormal P-dependent induction of epithelial proliferation. This gain of proliferative activity by removal of PR-A indicated that PR-A is required not only to inhibit E-induced hyperplasia of the uterus but also to limit potentially adverse proliferative effects of the PR-B isoform (Fernandez-Valdivia et al, 2005). Observations from the PRBKO mice indicate that they also developed normally, they were

fertile and carried successful pregnancies and they did demonstrate the antiproliferative effects of progesterone upon treatment with E&P (Franco et al, 2008). The specific PR isoform knockouts seem to indicate that the two isoforms have discrete and non-overlapping functions as PR-A is the predominant form of PR in the uterus whilst PR-B remains the predominant isoform in the mammary gland (Lee et al, 2006).

Effectors of PR Action in the Uterus:

Although progesterone seems to regulate the uterus and the mammary gland in distinctly different ways, the goal of this comparative section aims to seek out some common threads between the two whilst also elaborating on the differing signalling mediators in the uterus. Amphiregulin, RANKL and Wnt4 were all targeted in the uterus to determine what expression pattern may be produced.

1.8.(iii) Amphiregulin:

As in the mammary gland, Amphiregulin binds to the EGFR and was found to be transiently expressed in the luminal epithelial cells of the murine uterus at the time of implantation. Areg-/- mice were viable and fertile suggesting that there must be redundancy among the EGF family of ligands working within the uterus (Franco et al, 2008).

1.8.(iv) Wnt4 and BMP2:

Wnt4 expression increases with decidualisation in both mouse and human. Wnt4 expression seems to be localised to the stromal cells of the uterus and knockdown of Wnt4 in the human endometrial stromal cells prevented in vitro decidualisation (Franco et al, 2008).

In the uterus, Wnt4 has been identified as a downstream target of BMP2. Bone morphogenetic protein 2 is a member of the BMP family of morphogens and has been shown to be expressed in the uterine stroma at the site of attachment (Franco et al, 2008). Conditional ablation of BMP2 in the uterus produced infertile female mice. Embryos were able to attach to the uterine luminal epithelium but were unable to implant due to a failure of decidualisation (Franco et al, 2008).

1.8.(v) Indian Hedgehog:

Indian hedgehog (Ihh) has been identified as an important effector of PR action in the uterus. Ihh is a member of the Hedgehog (Hh) family of morphogens. In general, Hh signalling regulates both cell proliferation and differentiation by either short range or long range actions as Hh protein is diffusible. Ihh has been identified as a downstream target of PR in the uterus (Takamoto et al, 2002). Ihh is acutely induced in the luminal epithelium and glandular epithelium by P with an expression profile that peaked right before the time of implantation and decreased immediately after implantation (Takamoto et al, 2002; Franco et al, 2008). The expression of Ihh preceded the expression of other P target genes.

Known Hh target genes Patched-1 (Ptc-1), Hedgehog Interacting Protein 1 (HIP1) and Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII) were shown to be regulated in a temporal manner similar to Ihh. Ptc-1 and HIP1 were found in the luminal epithelium and the stroma whereas COUP-TFII was found only in the stroma thus suggesting that Ihh may be a major effector of PR signalling in the uterus (Franco et al, 2008). Also with Ihh expressed in the epithelium and its targets expressed in the stroma suggests that Ihh may act as a mediator of epithelial-stromal cross-talk in the uterus (Franco et al, 2008).

Previous studies which used the Cre/lox system of gene targeting highlighted the importance of Indian hedgehog to PR signalling in the uterus. A floxed allele of Ihh was generated and the resulting mouse was crossed to a PR-Cre mouse. The resultant phenotype of these mice was almost identical to that of the PRKO uterus. The uteri of $Ihh^{d/d}$ mice were unable to support embryo implantation and they were unable to undergo the artificially induced decidualisation reaction (Franco et al, 2008).

1.8.(vi) Hoxa10:

Another effector of P-regulated action in the uterus is the transcription factor Homeobox A10 (Hoxa10) (Lim et al, 1999). Hoxa10 is a member of the AbdB-like Hoxa gene family that have an important role in development. Hoxa10 is expressed in the luminal and glandular epithelium on day 0.5 to day 2.5 of pregnancy. It is also strongly expressed in stroma and decidua of the pregnant

mouse uterus (Lim et al, 1999). Hoxa10 expression then persists through day 4.5 to day 7.5 in the developing decidua. Hoxa10 knockout female mouse models were subfertile (Franco et al, 2008). At the time of implantation, most embryos were small and degenerating with almost half of them unattached. The embryos that had attached resulted in abnormal implantation sites on days 6.4 to 8.5 suggesting that Hoxa10 is required for successful embryo implantation (Franco et al, 2008). Also decidualisation of the uteri in the Hoxa10 knockouts was severely compromised during embryo implantation demonstrating an additional role for Hoxa10 in early pregnancy (Franco et al, 2008). Previous studies have shown that Hoxa10 is induced in the mouse uterine stroma within 4 hours of a P injection in a protein-synthesis dependent fashion and that the upregulation of Hoxa10 by P is inhibited by the PR antagonist RU-486 suggesting a requirement for PR for this induction. These studies imply that Hoxa10 is a primary responsive gene and is involved in implantation as a direct mediator of P action (Lim et al, 1999).

1.9. General Aims of the Study:

- To uncover downstream mediators of the progesterone response in the pregnant mammary gland using the results obtained from an Affymetrix microarray previously carried out by the lab on PRKO mice versus wild-type mice.

- To hypothesise a functional role for the novel target; BDNF, in pregnancy-associated mammary gland development.

- To examine the progesterone regulated response of genes in another reproductive tissue; the uterus and compare these results to those observed in mammary gland.

1.9.(i) Specific Aims of the Study:

- Analyse known progesterone-regulated target genes from the Affymetrix microarray to observe their differential expression between wild-type and PRKO mammary glands using quantitative PCR techniques. The target genes are divided into proliferative genes; RANKL, Wnt4, Amphiregulin and Elf5, and also into anti-proliferative genes; TGF-β1, TGF-β2, Mig6 and Sfrp2.

- Analyse these known progesterone-regulated target genes after 21 days of estrogen and progesterone treatment to assess their differential expression profiles in the mammary gland during a simulated pregnant state

- Use quantitative PCR techniques to elucidate the hypothesised role of the novel progesterone target gene; BDNF as proposed by the lab.

- Analyse the expression of well-known progesterone-regulated target genes in conditional BDNF knockout mouse models to further elucidate how BDNF may be functioning in the mammary gland. The proliferative target genes; RANKL, Wnt4 and Amphiregulin were analysed.

- Compare the proliferative and differentiating role of progesterone in the uterus and the mammary gland using the differential expression profiles of commonly expressed target genes; RANKL, Wnt4, Amphiregulin, Mig6

- Use quantitative PCR techniques to further investigate the expression profiles of the non-comparative, rapidly induced genes by progesterone in the uterus; Indian hedgehog, BMP2, Hoxa10.

2. Materials and Methods

2.1. Mouse Models and Hormone Treatments:

The strain of mice used in this research is known as C55/129. The PRKO mouse model has been used for a long time to investigate the role of progesterone in the mammary gland; it is regarded as an indispensable tool for any signalling pathway investigations. The PRKO mouse model is generated by ablation of both isoforms of PR using gene targeting techniques (Ismail et al, 2003). The resultant mice are usually bred from a heterozygous female and a homozygous or heterozygous male to produce both PRKO and PRKO+/- genotypes, both of which were used in the uterus study. Constitutional ablation of PR function did not affect embryonic and postnatal development of female and male PRKOs because both sexes progressed to adulthood at normal Mendelian frequencies (Fernandez-Valdivia et al, 2005). Due to the viability of the PRKO models, there was no need to use the sophisticated Cre/lox targeting approach. However, the Cre/lox system was also used to generate the isoform specific knockout mouse models; PRAKO and PRBKO which were used in the uterus study and were generated in house. The Cre/loxP-gene targeting approach was used in embryonic cells to introduce a point mutation into the PR gene at the ATG codon encoding Met1 in order to specifically ablate expression of the PR-B protein and then at the ATG encoding Met166 in order to specifically ablate expression of PR-A.

The BDNF mice were also generated by crossing a MMTV-Cre mouse with a floxed BDNF mouse; a floxed BDNF mouse is one in which the BDNF gene is flanked by lox sites which will be ablated when crossed with the Cre mouse. The BDNF knockout mice were conditional knockouts hence the cohort of mice had a specific ablation of the BDNF gene in the mammary gland. These mice were also selectively bred to produce both homozygous and heterozygous knockouts.

All animals were generated, reared and housed at the Baylor College of Medicine Animal Facility. All of the mice were weaned at three weeks of age and genotyped. At six weeks of age, the mice underwent surgical ovariectomies followed by ten days of rest. The surgery curtailed any endogenous production of ovarian steroid hormones which could adversely affect the results of the various studies.

The 'E&P treated' mice which, were used for the mammary gland studies, received daily subcutaneous injections of 1mg of progesterone and 250ng of estrogen in 50µL of corn oil. The 'P treated' mice which, were used for the uterus study, received one subcutaneous injection of 1mg of progesterone in 50µL of corn oil. The 'control treated' mice were treated with 50µL of corn oil alone. All of the mice were sacrificed with CO_2 once their term of treatment concluded and the tissues of interest were surgically removed by a technician. The tissue samples were then labelled and stored in eppendorf tubes at -80ºC for RNA extraction.

2.2. Protocol for RNA Extraction from Frozen Tissue

2.2.(i) Materials:

- PowerGen* Model 125 Homogeniser (Fisher Scientific, USA)
- Beckman J2-HS Floor Model Centrifuge *C0706037* (Beckman Coulter, USA)
- Trizol® Reagent, *Cat# 15596-018 Lot#50300417* (Invitrogen, USA)
- Chloroform (C298-1) *Lot# 982778* (Fisher Scientific, USA)
- 2-Propanol (A416-1) *Lot# 992220* (Fisher Scientific, USA)
- Portable Pipet Aid *Serial# 0152299* (Drummond Scientific Co. USA)
- Pipetman® p200 *M10627C* (Gilson Inc, France)
- Pipetman® p1000 *E11507C* (Gilson Inc, France)
- DEPC Water (Made in-house, see Appendix)
- 75% DEPC Ethanol (Made in-house, see Appendix)

2.2.(ii) Preparation for the day of extraction:

The allocated bench space was cleared of anything unrelated to the protocol. The bench and all labware and materials were sprayed with RNase Zap and left for 15 minutes. All the tubes and tips used in the RNA work were RNase-free and all were autoclaved. Required aliquots of the necessary reagents were made up before beginning the extraction procedure. The homogenisation step required a number of 20ml DEPC water washes, the DEPC water was measured in 50 ml

Falcon tubes. There was one wash for each tissue sample, one extra wash for each change of genotype as well as six start washes and six end washes.

Other reagents required were aliquoted out as follows:

- Trizol® (2ml per gland with a max of 4mls)
- Chloroform (0.2mls per 1ml of Trizol®)
- 2-Propanol (0.5mls per 1ml of Trizol®)
- 75% DEPC ETOH (1ml per 1ml of Trizol®)

2.2.(iii) Preparation of frozen tissue for extraction:

Two plastic SARSTEDT tubes were labelled for each sample – the sample number was written on the lid and three times on the base as Trizol® can dissolve ink. The homogeniser was washed three times with DEPC water and then once with Trizol. The homogeniser was operated at 75% of its potential speed and each wash lasted for an average of 15 seconds. The frozen tissue samples were taken from the freezer and placed on ice. The autoclaved pipette tips were used as forceps to separate the frozen tissue and remove it from the eppendorf tube. This step must be done quickly to prevent the other additional tissue samples from thawing out. The tissue was immersed in a tube with 2mls of Trizol® and placed on ice. The tissue samples were homogenised until there were no clumps and the solution had a 'soupy' appearance. The tubes were then returned to ice. The homogeniser was washed with one 20ml DEPC wash between each sample and two washes were used between each genotype. When all samples had been homogenised, the homogeniser was washed three times

with DEPC water and once with 70% ethanol to clean off any fat or residue from the probe.

2.2.(iv) Extraction of the RNA from the homogenised tissue samples:

The tissue was removed from ice and placed in a rack at room temperature for 10 minutes. 0.2ml Chloroform per 1ml of Trizol® was pipetted into each tube and the tube was shaken vigorously by hand for about 30 seconds. The samples were left for 5 minutes at room temperature. The samples were then centrifuged at 2-8°C at 12,000 x g for 15 minutes. The aqueous phase was retained and transferred to a fresh labelled tube using a 1ml pipette. 0.5ml of 2-Propanol per 1ml of Trizol® was pipetted into the tube and swirled gently. The sample was centrifuged at 2-8°C at 12,000 x g for 10 minutes. The tube was checked for an RNA pellet and the supernatant was carefully poured off. The pellet was washed with 2mls of 75% ETOH (DEPC) – at least 1 ml of ethanol was added for every 1ml of Trizol®. The samples were centrifuged at 10,000 rpm for 5 minutes. This ethanol wash was repeated once more. The supernatant was then discarded – the lip of the tube was wiped with a kemiwipe. The tubes were inverted on a kemiwipe and the RNA pellets were allowed to air dry for about 5 – 10 minutes. The pellet was resuspended in 100μL of RNase free water. The samples were incubated on ice for 1 hour. The extracted RNA samples were stored at -80°C.

2.3. Protocol to Determine the Concentration of the RNA Samples

2.3.(i) Materials:

- Beckman Coulter DU800 Spectrophotometer (Beckman Coulter, USA)
- Pipetman® p200 *M10627C* (Gilson Inc, France)
- Reusable DU800 cuvettes (Beckman Coulter,USA)
- Double Distilled Water (Made in-house)

2.3.(ii) Analysis of the RNA using the DU800 Spectrophotometer:

The UV light of the spectrophotometer should be turned on for about 10 minutes before use. The cuvette was washed out with double distilled water and filled with 100µL of the sample blank – the sample blank must be the same as the diluent that was used to resuspend the extracted RNA i.e. Rnase free water. The cuvette was placed in the spectrophotometer in correct orientation to the UV light. The sample blank is named as 'blank', the 'blank' was then blanked and read. The cuvette was washed out with the double distilled water for reuse with the next sample. 5 µL of the RNA sample was diluted into 95µL of Rnase free water, this was pipetted into the cuvette, named and read. The cuvette was washed out again and subsequent samples are named and read. The results were then exported to a Microsoft Excel spreadsheet and saved (Fig 2.2).

UV spectroscopy is the traditional method for assessing RNA concentration and purity (http://www.ambion.com/techlib/tn/94/949.html). The absorbance of a

diluted RNA sample is measured at 260 and 280 nm. The nucleic acid concentration is calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

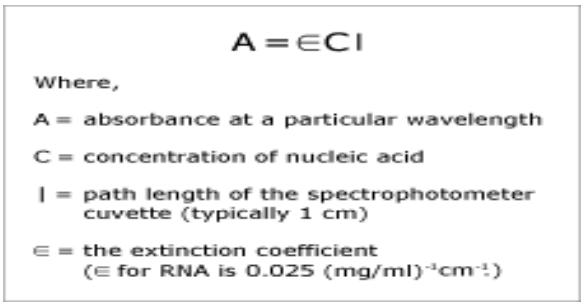


Figure 2.1: Beer-Lambert Law for calculating UV absorbance by nucleic acid (http://www.ambion.com/techlib/tn/94/949.html).

Using this equation, an A_{260} reading of 1.0 is equivalent to ~40 µg/ml single-stranded RNA. The A_{260}/A_{280} ratio is used to assess RNA purity and is dependent on both pH and ionic strength. As pH increases, the A_{280} decreases while the A_{260} is unaffected. This results in an increasing A_{260}/A_{280} ratio. Water often has an acidic pH, which lowers the A_{260}/A_{280} ratio. An A_{260}/A_{280} ratio between 1.8 and 2.1 is indicative of highly purified RNA when the RNA is diluted with Rnase free water at a pH of 6 – 7 (http://www.ambion.com/techlib/tn/94/949.html).

2.3.(iii) Formulas to determine the RNA concentrations:

- *Concentration of RNA in µg/ml:*
- 40 µg/ml x A_{260} x dilution factor

- *Concentration of RNA in µg/µL:*
- (40 µg/ml x A_{260} x dilution factor)/1000

- *Total amount of RNA present in the sample:*

RNA conc. x sample vol (mls or µls depending on the conc. units)

Sample	Gentoype	Treatment	Absorbance 260nm	Absorbance 280nm	Ratio 260/280
Blank			0	0	0
Pool	Wild-type	Control	0.6439	0.3591	1.793
8552	Wild-type	Control	0.7287	0.3867	1.8844
8555	Wild-type	Control	1.2	0.9301	1.2901
8150	Wild-type	Control	0.764	0.4081	1.872
8148	Wild-type	Control	0.579	0.2963	1.9541
8124	Wild-type	Control	1.5169	0.7985	1.8998
8107	Wild-type	Control	0.1416	0.0922	1.5355
8105	Wild-type	Control	0.2888	0.1864	1.549
P2418	Wild-type	EP 48hrs	1.6883	0.9376	1.8006
P2419	Wild-type	EP 48hrs	1.7438	0.9599	1.8167
P2429	Wild-type	EP 48hrs	3.0976	1.7261	1.7946
8208	Wild-type	EP 48hrs	1.0859	0.6225	1.7442
8643	Wild-type	EP 48hrs	1.5403	0.8699	1.7707
8642	Wild-type	EP 48hrs	1.3606	0.7562	1.7992
8640	Wild-type	EP 48hrs	1.3586	0.7343	1.8503
8639	Wild-type	EP 48hrs	1.4692	0.7978	1.8417
8609	Wild-type	EP 48hrs	1.3418	0.7306	1.8366
8146	Wild-type	E 48hrs	1.214	0.6728	1.8045
8147	Wild-type	E 48hrs	1.3367	0.7466	1.7905
8149	Wild-type	E 48hrs	1.5619	0.8781	1.7787
P2446	PRKO+/-	EP 48hrs	2.3076	1.2661	1.8225
P2448	PRKO+/-	EP 48hrs	1.5363	0.8528	1.8015

Fig 2.2: Example of a read-out from the UV spectrophotometer which calculates the absorbance of the RNA samples at 260nm. The samples are identified by their original mouse numbers. Absorbance is measured at 280nm and 260nm as nucleic acids absorb UV light at these wavelengths. The 260:280 ratio measures RNA purity, a value between 1.8 and 2.1 indicates high quality RNA. The data is then exported into an Excel sheet to determine RNA sample concentration using Beer Lamberts Law.

2.4. Protocol to Determine the Quality of the RNA Samples

2.4.(i) Materials:

- EC 400 Power Source (E-C Apparatus Corporation, USA)

- Ultrapure™ Agarose 500gm Cat# 15510-027 Lot# D030507 (Invitrogen, USA)
- Stovall Hybridization Water Bath (Life Science Inc, USA)
- Sanyo Microwave
- Orange sample denaturing buffer containing ethidium bromide (Made in-house, see Appendix)
- 10X TBE buffer solution
- Double Distilled Water
- Bromophenol Blue Loading Solution (Promega Corporation, USA)
- Loading buffer (made in-house)
- Pipetman® p20 Z53746B (Gilson Inc, France)
- Pipetman® p200 *M10627C* (Gilson Inc, France)

2.4.(ii) Preparation of a 0.8% agarose gel:

The water bath was turned on and set to 65ºC. 0.48g of agarose powder was weighed out onto an electronic scale and was tipped into a conical flask. 6mls of 10X TBE running buffer was added to the conical flask. The gel solution was then made up to 60mls with 54mls of double distilled water. The gel solution was then boiled in the microwave for 2 minutes. The gel solution was then left to cool to 65ºC. The agarose gel was then poured into the plastic mould. It is important to ensure there are no air bubbles present. The gel is left to set for 30 minutes.

2.4.(iii) Sample preparation for loading onto the agarose gel:

3µl of each RNA sample was pipetted into 20µl of orange sample denaturing buffer in a 0.5ml eppendorf tube. Samples were then heated at 65ºC in the water bath for 15 minutes and then placed on ice. 2µl of a bromophenol blue loading solution was added to each sample to track the migration of the mRNA during electrophoresis. Samples were spun for a few seconds before loading onto the gel.

2.4.(iv) Loading of samples onto the agarose gel:

One litre of 1X TBE buffer was made up using double distilled water. The set agarose gel was placed horizontally onto the platform of the gel container. The container was filled with 1X TBE (~400mls) ensuring that the surface of the gel is flooded. The full sample amount (25µl) was loaded into each well of the gel. Electrodes were attached to the container at either end – the black electrode is always attached at the back. The gel was run with 1X TBE at 110V for 30 minutes (the gel should not be run any longer as it may cause the RNA to degrade). After 30 minutes, the gel was photographed using an Alphamager camera and software – the presence of two clear bands of ribosomal RNA; 28S and 18S indicate good quality RNA.

Alternative Agarose Protocol with Ambion Glyoxal Load Dye:

Make a 1% agarose gel with 1µL of 0.5µg/ml ethidium bromide. Mix the RNA sample 1:1 with the loading dye and heat at 55ºC for 30 minutes. Load onto the gel and run for 30 minutes.

2.5. Protocol for Reverse Transcription PCR of Extracted RNA to Generate cDNA

2.5.(i) Materials:

- DNA Thermal Cycler 480 (Perkin Elmer)
- High Capacity cDNA Reverse Transcription Kit *Lot# 0710032* (Applied Biosystems, USA)
- Ribonuclease Inhibitor (cloned) *Cat# 15518-012 Lot# 283324* (Invitrogen,USA)
- Pipetman® p20 Z53746B (Gilson Inc, France)
- Pipetman® p200 *M10627C* (Gilson Inc, France)
- Pipetman® p1000 *E11507C* (Gilson Inc, France)
- Nuclease-Free Water (Ambion, USA)

2.5.(ii) The Reverse Transcriptase PCR (RT-PCR):

Before beginning, it was very important to determine a balance between the final volume of cDNA that was needed for the number of qPCRs that were

expected to be run and how many µg's of the extracted RNA that can be used in preparing the cDNA. The minimum amount of RNA that can be used in a RT-PCR reaction is 0.002µg/µl whilst the maximum amount of RNA that can be used in a RT-PCR reaction is 2µg/µl. The actual amount of RNA that is used generally depends on how abundant the resultant target sequence is expected to be.

The concentration of the RNA included in the RT-PCR reaction was 6µg RNA/60µl sample solution. The sample solution therefore contained 6µg of RNA, Rnase free water and Rnase inhibitor which was made up to a final volume of 60µl (Fig 2.3).

A: RT RNA Sample Solutions

Tube	RNA conc. ug/uL	RNA vol to get 6ug uL	Nuclease Free H_2O uL	RNase Inhibitor (stock: 20U/uL) uL	Total vol
RTnoRNA	0	0.0	54.0	6	60.0
STD (i)	1.000	12.0	42.0	6	60.0
STD (ii)	1.000	12.0	42.0	6	60.0
STD (iii)	1.000	12.0	42.0	6	60.0
Wild-type C1	0.395	15.2	38.8	6	60.0
Wild-type C2	0.373	16.1	37.9	6	60.0
Wild-type C3	0.398	15.1	38.9	6	60.0
Wild-type EP1	0.544	11.0	43.0	6	60.0
Wild-type EP2	0.403	14.9	39.1	6	60.0
Wild-type EP3	0.512	11.7	42.3	6	60.0
Wild-type EP4	0.444	13.5	40.5	6	60.0
Wild-type EP5	0.372	16.1	37.9	6	60.0
Wild-type EP6	0.434	13.8	40.2	6	60.0
BDNF+/- EP1	0.549	10.9	43.1	6	60.0
BDNF+/- EP2	0.635	9.4	44.6	6	60.0
BDNF+/- EP3	0.400	15.0	39.0	6	60.0
BDNF+/- EP4	0.402	14.9	39.1	6	60.0
BDNF+/- EP5	0.429	14.0	40.0	6	60.0
BDNF+/- EP6	0.388	15.5	38.5	6	60.0
BDNF-/- EP1	0.563	10.7	43.3	6	60.0
BDNF-/- EP2	0.441	13.6	40.4	6	60.0
BDNF-/- EP3	0.367	16.3	37.7	6	60.0
BDNF-/- EP4	0.374	16.0	38.0	6	60.0
BDNF-/- EP5	0.436	13.8	40.2	6	60.0
PRKO EP1	1.119	5.4	48.6	6	60.0
PRKO EP2	1.665	3.6	50.4	6	60.0
PRKO EP3	1.752	3.4	50.6	6	60.0

B: NRT RNA Sample Solutions

Tube	RNA conc. ug/uL	RNA vol to get 4ug uL	Nuclease Free H_2O uL	RNase Inhibitor (stock: 20U/uL) uL	Total vol
RTnoRNA	0	0.0	36.0	4	40.0
STD (i)	1.000	8.0	28.0	4	40.0
STD (ii)	1.000	8.0	28.0	4	40.0
STD (iii)	1.000	8.0	28.0	4	40.0
Wild-type C1	0.395	10.1	25.9	4	40.0
Wild-type C2	0.373	10.7	25.3	4	40.0
Wild-type C3	0.398	10.1	25.9	4	40.0
Wild-type EP1	0.544	7.4	28.6	4	40.0
Wild-type EP2	0.403	9.9	26.1	4	40.0
Wild-type EP3	0.512	7.8	28.2	4	40.0
Wild-type EP4	0.444	9.0	27.0	4	40.0
Wild-type EP5	0.372	10.8	25.2	4	40.0
Wild-type EP6	0.434	9.2	26.8	4	40.0
BDNF+/- EP1	0.549	7.3	28.7	4	40.0
BDNF+/- EP2	0.635	6.3	29.7	4	40.0
BDNF+/- EP3	0.400	10.0	26.0	4	40.0
BDNF+/- EP4	0.402	10.0	26.0	4	40.0
BDNF+/- EP5	0.429	9.3	26.7	4	40.0
BDNF+/- EP6	0.388	10.3	25.7	4	40.0
BDNF-/- EP1	0.563	7.1	28.9	4	40.0
BDNF-/- EP2	0.441	9.1	26.9	4	40.0
BDNF-/- EP3	0.367	10.9	25.1	4	40.0
BDNF-/- EP4	0.374	10.7	25.3	4	40.0
BDNF-/- EP5	0.436	9.2	26.8	4	40.0
PRKO EP1	1.119	3.6	32.4	4	40.0
PRKO EP2	1.665	2.4	33.6	4	40.0
PRKO EP3	1.752	2.3	33.7	4	40.0

Fig 2.3: An example of a Microsoft Excel spreadsheet showing the volumes of RNA that are required to make up the RNA sample solutions required to generate cDNA. Fig 2.3A shows the "reverse transcriptase" (RT) sample solutions and fig 2.3B shows the "no reverse transcriptase" (NRT) solutions. The RT sample solutions have a RNA concentration of 6µg/60µl whilst the NRT sample solutions have a RNA concentration of 4µg/40µl. The remaining solution volume is made up with the Rnase free water and Rnase inhibitor. Equal amounts of the RT/NRT RNA sample solution will be added to the appropriate RT/NRT mastermix to generate the required volume of cDNA. The RNA standards (STD) are required to generate a standard curve for the qPCR. The stock concentration of these standards is 1µg/µl. 12µl of the RNA standards are added to the sample solution, this creates a concentration of 12µg/120µl, when added to the mastermix thus their final concentration for the qPCR is 100ng/µl.

The RNA samples were prepared separately to the RT-PCR mastermix. A second set of the RNA sample solutions were also prepared to be used as negative controls for the experiment, these samples were also added to an equal volume of mastermix but their mastermix lacked any of the reverse transcriptase enzyme hence these RNA samples could not undergo the RT-PCR reaction thus no DNA was expected to be generated in these samples. These samples were known as "no reverse transcriptase" (NRT). The final volume of the sample solution for the NRT samples was 40µl as opposed to 60µl; this was done in order to conserve reagents.

Other RNA samples which were also prepared for RT-PCR included an RNA standard which was prepared in triplicate and also an RTnoRNA sample which served as another negative control to ensure that no of the RT reagents were contaminated. The standard RNA samples were selected to undergo RT-PCR and generate cDNA so that they could be used in the qPCR to generate a standard curve in order to quantify the other cDNA samples. Mouse Universal RNA is commercially bought and used as a standard for the qPCR experiments. Universal RNA refers to total RNA controls which are derived from whole tissue sources. Since mice are the animal model of choice in this study, the species of the universal RNA is also mouse and it is made by pooling the total RNA extracts from a collection of different murine tissues, thus providing the broadest coverage of expressed genes in the mouse. The stock concentration of the universal RNA is 1µg/µl, a serial dilution of this was sufficient for the RT-

PCR. The concentration for the RT-PCR step was therefore 12µg of Universal Mouse RNA for the RT standards samples and 8µg for the NRT standard samples. The final concentrations of RNA standards that underwent the RT-PCR reaction were therefore 12µgRNA /120µl and 8µg RNA/80µl which equates to 100ngRNA /µl of cDNA standard.

The RT-PCR mastermix kits had to be thawed out on ice for at least one hour. Two separate RT-PCR mastermixes were prepared for the RT samples and the NRT samples as follows: An equal volume of mastermix had to be added to each sample solution hence the RT mastermix had a final volume of 60µl whilst the NRT mastermix had a final volume of 40µl. In the NRT mastermix, RT is substituted with Rnase free water.

Each mastermix (RT and NRT) were prepared in a 2ml eppendorf tube – extra volume was added to allow for pipetting error.

Vol of reagents for 1 RT reaction	
10X RT buffer	*12µl*
25X dNTPs	*4.8 µl*
10X oligo dTs	*12µl*
Rnase free water	*25.2 µl*
Reverse Transriptase	*6 µl*

Vol of reagents for 1 NRT reaction	
10X RT buffer	*8 µl*
25X dNTPs	*3.2 µl*
10X oligo dTs	*8 µl*
Rnase free water	*16.8 µl*
Rnase free water	*4 µl*

The RT-PCR master mix is bought commercially and comes as 2X but addition of the RNA sample solution dilutes it to 1X. The final volume for the reaction was established at 120µl (60µl of sample + 60µl of mastermix) for the RT samples and 80µl (40µl of sample + 40µl of mastermix). The sample solutions and the appropriate mastermix were mixed together in 0.5ml eppendorf tubes,

the samples were then briefly centrifuged to spin down the contents and eliminate any air bubbles. The reaction tubes were placed in the PCR machine for a 2hr cycle:

- 25ºC for 10mins
- 37ºC for 120mins
- 85ºC for 5secs
- 4ºC for ∞

Once the RT-PCR reaction was complete, the samples were stored overnight at 4ºC. The remaining RNA samples were stored in the -80ºC freezer.

2.6. Protocol for the Analysis of the cDNA Samples via quantitative PCR Methodology.

2.6.(i) Materials:

- MicroAmp™ Fast Optical 96 Well Plate with Barcode 0.1ml *Part# 4346906* (Applied Biosystems, USA)
- Optical Adhesive Covers *Part# 4360954* (Applied Biosystems, USA)
- StepOnePlus Real Time PCR System (Applied Biosystems,USA)
- Taqman® Gene Expression Master Mix Part# 4369016 Lot# 0802023 (Applied Biosystems, USA)
- Taqman® Gene Expression Assays (Applied Biosystems, USA)
- Nuclease Free Water (Ambion, USA)
- Pipetman® p20 Z53746B (Gilson Inc, France)

- Pipetman® p200 *M10627C* (Gilson Inc, France)
- Pipetman® p1000 *E11507C* (Gilson Inc, France)

2.6.(ii) Preparation of cDNA Standards for use in the qPCR Reaction:

For each gene that was amplified by qPCR, a set of standards had to be amplified for that gene in the same reaction. This resulted in the generation of a standard curve from which the quantity of the gene expressed in the experimental samples could be quantified.

The Mouse Universal RNA underwent RT-PCR and the resultant cDNA was used to generate a set of standards which could be used for each qPCR. One of the experimental samples was also chosen to generate a second set of standards that could be used for those genes that have quite low expression – it is best to pick the experimental sample that is likely to have the highest expression of your gene of interest.

A standard range of six serial dilutions was selected – *such a dilute range should cover any genes that are abundantly expressed*

ST1	ST2	ST3	ST4	ST5	ST6
100 ng/µl	10 ng/µl	1 ng/µl	0.1 ng/µl	0.01 ng/µl	0.001 ng/µl

Yeast RNA is added to nuclease free water before preparing the standard dilutions as this prevents very dilute RNA i.e. ST5 and ST6 from sticking to the tubes.

6µl of 100ng/µl stock of the yeast RNA was pipetted into a fresh 2ml eppendorf tube containing 594µl of Rnase free water in order to make 1ng/µl dilution of yeast RNA. 30µl of undiluted standard sample i.e. Universal Mouse cDNA was pipetted into a fresh tube labelled ST1. 90µl of the RNase free water containing yeast RNA was pipetted into fresh tubes labelled ST2 to ST6 for a 1:10 serial dilution. 10µl of ST1 was pipetted into ST2 and mixed. 10µl of ST2 was pipetted into ST3 and mixed. This was repeated through the remaining standards until ST6 had been mixed. These six serial dilutions are the set of standards that will be used in along with each gene that undergoes qPCR in this study.

2.6.(iii) Preparation of the qPCR Mastermix:

The qPCR mastermix is bought commercially from Applied Biosystems' Taqman.

The Taqman® Gene Expression Kit and the Taqman® Gene Expression Assays i.e. the 'primers and probe' mix must all be stored on ice. The 'primers and probe' mix must be defrosted and should be wrapped in tin foil as it is photosensitive.

The number of reactions required to analyse all samples, standards and controls during one qPCR run was calculated to determine how much master mix must

be made up. It is important to include extra master mix to account for pipetting error.

Eg: 50 reactions = **3 RT samples**
3 NRT samples (+RNA, H_2O instead of RT)
6 standards (Mouse Universal cDNA)
1 RT no RNA (H_2O instead of RNA, +RT)
1 NTC (H_2O instead of cDNA)
14
x 3 (samples done in triplicate)
42 reactions
+ 8.4 (20% error)
50 reactions

The following qPCR reaction recipe was used for each gene – this recipe represents the volumes present in one well of the 96 well reaction plate

Taqman Gene Expression Master Mix	12.5µl	**qPCR Mastermix (20µl)**
Taqman Gene Expression Assay (Specific Primers and Probe)	1.25µl	
Rnase free water	6.25µl	
cDNA	5µl	
Total	**25µl**	

2.6.(iv) Preparation of the 96 well qPCR reaction plate:

Accuracy is pivotal to the success of qPCR hence all samples are pipetted in triplicate using ART pipette tips.

5µl of each standard was pipetted into three wells. 5µl of each cDNA sample were pipetted into three wells. 5µl of the NTC control was pipetted into one well. 5µl of the RTnoRNA control was pipetted into one well. 20µl of the qPCR master mix was then added to each well. The plate was then covered with an

optical adhesive cover and centrifuged. The plate was wrapped in tin foil and stored at 4ºC until its analysis on the qPCR machine. The plate was run as on the Applied Biosystems StepOnePlus Real Time PCR Machine in accordance with the standard protocol. The qPCR results for each gene were analysed using the accompanying StepOnePlus software and bar charts were generated to visualise gene expression.

RANK-L exp in BDNF WT, heterozygote for the conditional knockout and homozygote for the conditional knockout and PRKO.
Treated with C (cohort 2)or E+P (cohort 1 and 2) for 3 weeks. PRKO are E+P treated only.
Using cDNA 1/11/08

		1	2	3	4	5	6	7	8	9	10	11	12
	A	WT STD1	WT STD1	WT STD1	WT STD2	WT STD2	WT STD2	WT STD3	WT STD3	WT STD3	WT STD4	WT STD4	WT STD4
C	B	WT 1.1	WT 1.2	WT 1.3	WT 2.1	WT 2.2	WT 2.3	WT 3.1	WT 3.2	WT 3.3			
	C	WT 1.1	WT 1.2	WT 1.3	BDNF+/- 1.1	BDNF+/- 1.2	BDNF+/- 1.3	BDNF-/-1.1	BDNF-/-1.2	BDNF-/-1.3	PRKO 1.1	PRKO 1.2	PRKO 1.3
	D	WT 2.1	WT 2.2	WT 2.3	BDNF+/- 2.1	BDNF+/- 2.2	BDNF+/- 2.3	BDNF-/-2.1	BDNF-/-2.2	BDNF-/-2.3	PRKO 2.1	PRKO 2.2	PRKO 2.3
E&P	E	WT 3.1	WT 3.2	WT 3.3	BDNF+/- 3.1	BDNF+/- 3.2	BDNF+/- 3.3	BDNF-/-3.1	BDNF-/-3.2	BDNF-/-3.3	PRKO 3.1	PRKO 3.2	PRKO 3.3
	F	WT 4.1	WT 4.2	WT 4.3	BDNF+/- 4.1	BDNF+/- 4.2	BDNF+/- 4.3	BDNF-/-4.1	BDNF-/-4.2	BDNF-/-4.3		RT no RNA	RT no RNA
	G	WT 5.1	WT 5.2	WT 5.3	BDNF+/- 5.1	BDNF+/- 5.2	BDNF+/- 5.3	BDNF-/-5.1	BDNF-/-5.2	BDNF-/-5.3		NTC	NTC
	H	WT 6.1	WT 6.2	WT 6.3	BDNF+/- 6.1	BDNF+/- 6.2	BDNF+/- 6.3						

Fig 2.3: An example of a 96-well plate Excel spreadsheet from the BDNF study. The 96 wells are replicated in Excel so that it can be used as a map when filling the plate. Each well is named with regards to the assigned individual sample or standard triplicate. The negative controls RT no RNA and NTC are also included. WT STD refers to the series of standard dilutions that are used in order to generate the standard curve for these particular samples. WT refers to the wild-type samples, BDNF+/- refers to the samples that are conditional for the heterozygous BDNF knockout whilst BDNF-/- refers to those samples that are homozygous for the conditional BDNF knockout. PRKO samples are those lacking any PR. qPCR plates set up for other target genes in this project are all based on this template.

2.7. Detection of the qPCR product on a 1% agarose gel

6g of agarose powder was added to 6mls of 10X TBE buffer in a conical flask. 1µL of 0.5µg/µl ethidium bromide was also added to the conical flask. The solution was then made up to 60mls with double distilled water. The agarose gel solution was then heated for 2 minutes in the microwave to dissolve the agarose powder. The solution was left to cool for 5 minutes and then poured into the mould and left to set for 1 hour. 2µL of the loading buffer; TrackIt™ Cyan/Orange Buffer was added to the 25µL DNA sample in the wells of the qPCR reaction plate. 15µL of the mixture was then loaded onto the gel for electrophoresis. 10µL TrackIt™ DNA ladder was loaded onto the gel in order to analyse the size of the qPCR product.

2.8. Statistical Analysis

All qPCR reactions were performed in triplicate. Graphs were generated in Microsoft Excel and the data was expressed as the fold induction of the mean values, relative to the wild-type control ± SD. Statistical analysis was carried out using Analysis ToolPak software and a Student's t-test was used to demonstrate the statistical significance of the comparisons that were made between various sample genotypes and sample treatments. P values less than 0.05 were considered significant.

3. Project Results:

3.1. qPCR Analysis of Microarray Target Genes:

3.1.(i) The Experimental Cohort for qPCR Analysis of Microarray Target Genes:

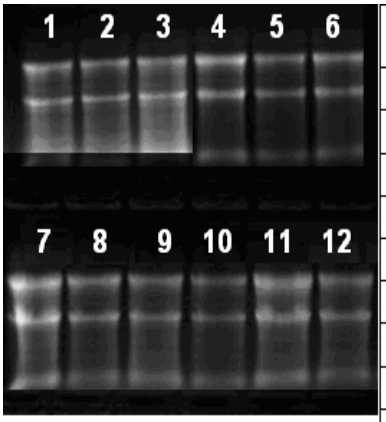
Quantitative PCR analysis was used to determine the expression pattern of the target genes of interest that had been identified in the microarray. Twelve wild-type mice were selectively bred and treated for this study by a lab technician. The 12 wild-type mice underwent ovarectomies at 6 weeks of age and were left to rest for 10 days post surgery. Six of the mice were then selectively treated for three weeks with a control vehicle and six of the mice were treated with estrogen and progesterone to again simulate a pregnant proliferative state within the mammary gland. The treatment consisted of daily subcutaneous injections; the control treated animals were injected with 50µL of corn oil alone whilst the hormone treated animals were injected with 50µL of corn oil containing 250ng of estrogen and 1mg of progesterone. The 12 wild-type mice were sacrificed by inhalation of CO_2 and their mammary glands were surgically removed and stored at -80ºC. The mouse model may have four or more mammary glands but for the purpose of this work only one from each was used to generate cDNA for quantitative PCR. Three additional mice which were homozygous for the conditional PR knockout were also used; however these samples were in RNA form and had been archived by the lab at -80ºC for the past three years.

3.1.(ii) RNA Extraction for the qPCR Analysis of Microarray Target Genes:

RNA extraction was performed for the 12 mammary glands that were to be included in the study. Once an RNase free work area had been prepared as per Materials and Methods 2.2(ii), the mammary glands were homogenised with Invitrogens Trizol reagent. Trizol maintains the integrity of the RNA while disrupting cells and dissolving cell components (13). This procedure was carried out as per Materials and Methods 2.2.(ii). The newly isolated RNA along with the archived PRKO RNA, was accurately quantified by measuring its absorbance in a spectrophotometer. The optical density of RNA was measured at its maximum absorbance wavelength of 260nm. To assess whether any DNA contamination has occurred during the extraction process, a sample of the extracted RNA is then electrophoresed on a denaturing agarose gel and stained with ethidium bromide.

In this study, to further ensure the quality of the extracted RNA, the RNA was cleaned and purified using a Qiagen RNeasy Mini Kit. This protocol can be used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labeling) or to desalt RNA samples. Buffer RLT (contains guanidine thiocyanate) and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

The newly cleaned RNA was once again analysed on the spectrophotometer (Fig 3.2) and these purer concentrations were used to calculate the volume of the RNA that would be required for the reverse transcriptase PCR to yield enough cDNA for the quantitative PCR analysis. All of the RNA samples were electrophoresed again onto a 0.8% agarose gel. Both the 28S ribosomal RNA and the 18S ribosomal RNA should appear as two sharp bands.



Lane	Laboratory Mouse no.	Mouse Model Genotype	Treatment administererd over 21 days	RNA extraction date	
1	130	Wild-Type	Control Treated	11/15/07	WTC1
2	133	Wild-Type	Control Treated	11/15/07	
3	134	Wild-Type	Control Treated	11/15/07	WTC2
4	72	Wild-Type	Control Treated	11/20/07	
5	77	Wild-Type	Control Treated	11/20/07	WTC3
6	89	Wild-Type	Control Treated	11/20/07	
7	136	Wild-Type	Estrogen & Progesterone	11/20/07	WTEP1
8	C366	Wild-Type	Estrogen & Progesterone	11/20/07	
9	C367	Wild-Type	Estrogen & Progesterone	11/20/07	WTEP2
10	C370	Wild-Type	Estrogen & Progesterone	11/20/07	
11	C372	Wild-Type	Estrogen & Progesterone	11/20/07	WTEP3
12	C373	Wild-Type	Estrogen & Progesterone	11/20/07	

Figure 3.1: Gel electrophoresis results for the extracted RNA of the 12 wild-type mice. The wild-type mice have been treated with either a control or with estrogen and progesterone for three weeks. A legend to identify each lane on the gel is included. The concentrations of these RNA samples were determined using Beer Lamberts Law (Fig 2.1) and the RNA samples were then pooled together before they underwent the RT-PCR reaction WTC1, WTC2, WTC3, refer to the names assigned to the pooled wild-type control RNA samples. WTEP1, WTEP2, WTEP3 refer to the names assigned to the pooled RNA wild-type E&P treated RNA samples. Each pool contains two individual RNA samples.

In order to reduce the work load and accommodate the size restrictions of the qPCR 96-well plate, the RNA samples were pooled together according to genotype and treatment. The wild-type control treated RNA samples were pooled together and the wild-type EP treated samples were pooled together.

Each pool contained two individual RNA samples and there were a total of three pools for each treatment group i.e WTC1, WTEP1 (Fig 3.1/3.2). The three PRKO samples retained their individual sample status as they were the only samples of this genotype available and for statistical purposes a minimum of three samples was required.

A Microsoft Excel spreadsheet was designed to calculate the concentration and volume that was required from each pooled sample to generate a sufficient amount of cDNA for use in quantitative PCR analysis (Fig 3.2). It was important to make more than the minimum to ensure that there was enough cDNA left in case it was needed at a later date for repeat analysis of the data.

Mouse no.	Sample Genotype	Sample Treatment	RNA extraction date	RNA concentration ug/uL	Sample volume required to get 15 ug RNA	Final volume of pooled samples 30 ug RNA	Original sample volume remaining ul.	Pooled samples diluted 1:20 for spec *5uL in 95uL nuclease free H_2O*				Volume of pooled sample required to get 10 ug RNA	Pooled Sample Name
								Absorbance 260nm	Absorbance 280nm	Purity 260/280	Conc. ug/uL		
130	Wild-Type	Control 21	11/20/07	0.433	34.6	70.5	40.4	0.4489	0.2369	1.895	0.395	25.31	WTC1
133	Wild-Type	Control 21	11/15/07	0.418	35.9		39.1						
134	Wild-Type	Control 21	11/20/07	0.411	36.5	74.2	38.5	0.4240	0.2215	1.914	0.373	26.80	WTC2
72	Wild-Type	Control 21	11/15/07	0.398	37.7		37.3						
77	Wild-Type	Control 21	11/20/07	0.420	35.7	70.9	39.3	0.4522	0.2360	1.916	0.398	25.13	WTC3
89	Wild-Type	Control 21	11/15/07	0.426	35.2		39.8						
136	Wild-Type	E&P 21	11/20/07	0.453	33.1	64.2	41.9	0.5043	0.2682	1.880	0.444	22.53	WTEP 1
C366	Wild-Type	E&P 21	11/20/07	0.482	31.1		43.9						
C367	Wild-Type	E&P 21	11/20/07	0.391	38.4	75.7	36.6	0.4232	0.2232	1.896	0.372	26.85	WTEP 2
C370	Wild-Type	E&P 21	11/20/07	0.402	37.3		37.7						
C372	Wild-Type	E&P 21	11/20/07	0.463	32.4	67.1	42.6	0.4928	0.2590	1.903	0.434	23.06	WTEP3
C373	Wild-Type	E&P 21	11/20/07	0.432	34.7		40.3						

Fig3.2: The Excel spreadsheet used to determine the concentration of RNA samples that were to be pooled together for the RT-PCR reaction. All of the samples had been treated for three weeks with either control or EP. Six individual wild-type control treated RNA samples were pooled together into three pooled samples with two individual RNA samples in each (WTC1, WTC2, WTC3). The same was done for the six wild-type EP treated RNA samples (WTEP1, WTEP2, WTEP3). Each pool contains 30µg of RNA, 15µg from each individual sample. The newly pooled samples undergo spectrophotometry again and the new RNA concentration µg/µl is determined. 10µg of pooled RNA is required for the RT-PCR reaction, 6µg is required for the RT sample solution and 4µg is required for the NRT sample solution (Fig2.3) to generate cDNA for the qPCR analysis.

3.1.(iii) Reverse-Transcriptase PCR:

Single-stranded cDNA was generated from the total RNA using the Applied Biosystems High-Capacity cDNA Reverse Transcriptase Kit. The protocol was carried out as per protocol Materials and Methods 2.5. Negative controls were set up for this step of the study to ensure that neither the sample nor the reagents were in any way contaminated. One control consisted of all the RT-PCR reagents required but did not contain any RNA sample to ensure that none of the reagents used were contaminated with any genetic material that may be amplified in the reaction and produce a false positive. The RT-PCR reaction was also controlled to ensure the integrity and purity of the RNA samples used. For each RNA sample that underwent the RT-PCR, the same sample was set up again without the reverse transcriptase enzyme to ensure that any DNA that was generated was the result of the RT-PCR reaction and not some form of sample contamination. Once the cDNA had been created, it was stored at 4ºC for quantitative PCR analysis.

3.1.(iv) Quantitative PCR Analysis (qPCR)

In order to carry out qPCR analysis on the cohort samples, a standard curve had to be generated against which the samples could be quantified. Mouse Universal RNA was used to create a standard curve. This RNA was commercially bought and had a concentration of 1µg/µL. The Universal RNA underwent RT-PCR alongside the experimental samples, as per Materials and Methods 2.5.(ii), to produce a final cDNA concentration of 100ng/µl. Six serial dilutions were then

made from the Mouse Universal RNA and the standard curve was made as per Materials and Methods 2.6.(ii). In order for a standard curve to be accepted, the slope of the curve had to be 3.3 ± 0.5 (Fig3.3).

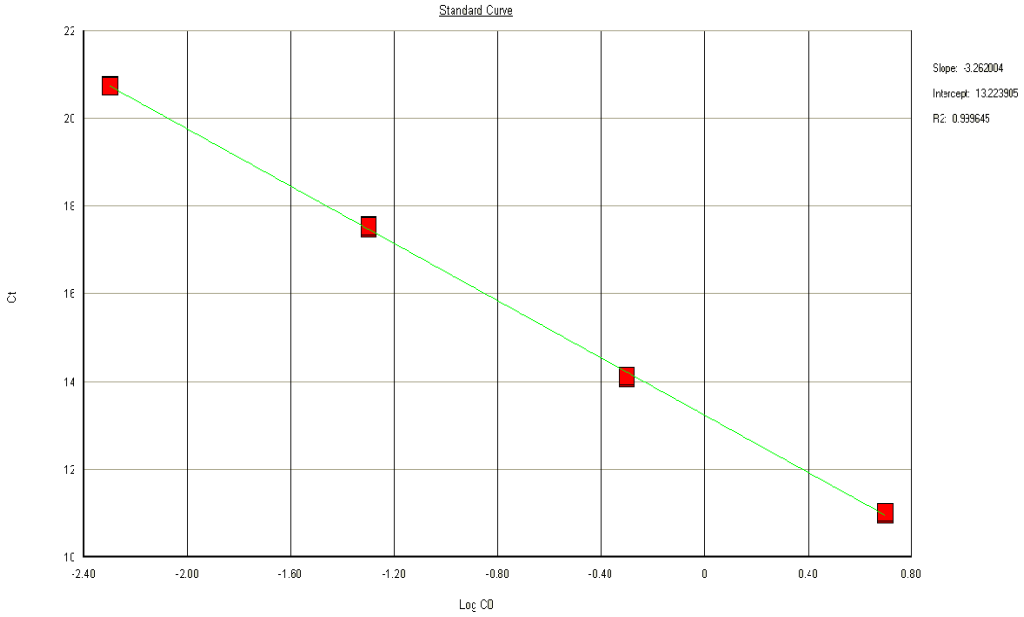


Figure 3.3: A standard curve generated using the Applied Biosystems SDSShell qPCR software. This curve incorporates four serial dilutions of the Mouse Universal RNA and has an acceptable slope of -3.26. This particular standard curve was generated for qPCR analysis of 18S, the housekeeping gene that was used in all the qPCR analysis performed in this project. 18S was used to normalise the target genes analysed

The qPCR reagent mastermix and the cDNA samples for the 96 well PCR plate were set up for each gene as per Materials and Methods 2.6.(iv). Taqman reagents were used for the reaction and were all commercially bought from Applied Biosystems. To reduce the effects of human pipetting error, each cDNA sample was analysed in triplicate on the PCR plate (Fig 3.4).

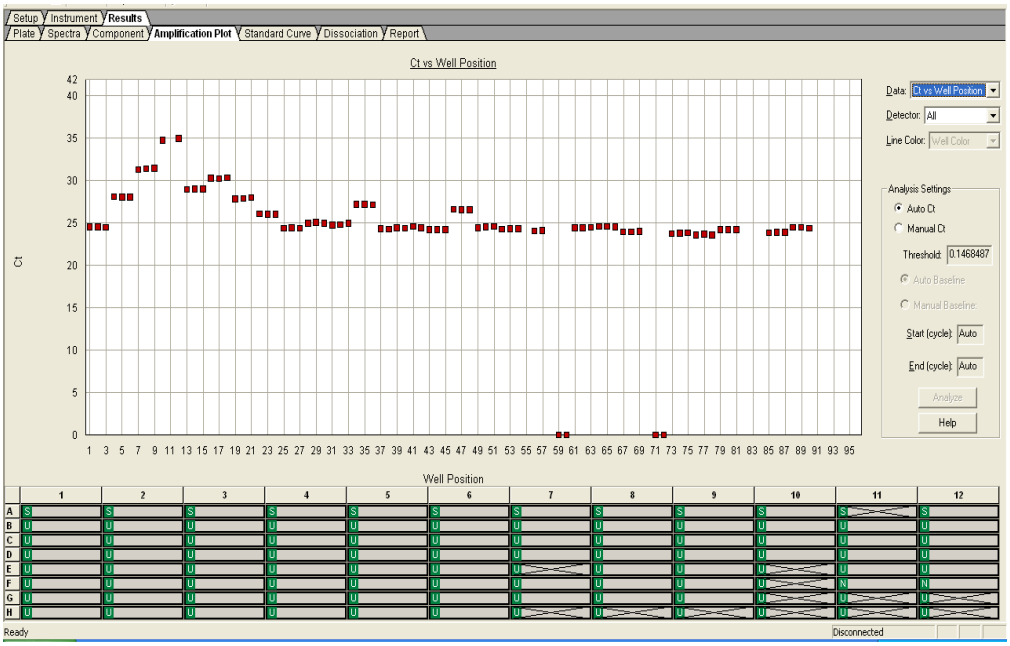


Figure 3.4: An Applied Biosystems SDSShell qPCR 96-well plate template. qPCR analysis on the RNA samples were performed in triplicate and are represented as the small red squares. Well positions 1 -12 account for the standards whilst positions 59 and 60 as well as 71 and 72 account for negative controls.

The quantification of gene targets in this study used the relative quantification comparative method. The relative target quantity was measured in relation to standard curves of both standard and reference. A housekeeping gene known as 18S is highly expressed in all cells and it was quantified in each of the cDNA target samples to be used as an internal endogenous standard against which the target gene could be normalised. 18S can be referred to as an active reference as its use can determine changes in the amount of a given sample relative to another internal sample (Lanz, 2008).

3.1.(v) qPCR Analysis of the Microarray Target Genes:

The target genes RANKL, Wnt4 and Amphiregulin are three well established genes with regards to mammary gland literature. The microarray carried out by

the lab, showed that all three genes were upregulated following E&P treatment for three weeks in the wild-type mouse model. Incidentally, the same three genes were all downregulated in the PRKO mice following the same treatment, suggesting that these genes are targets of the progesterone signalling pathway in the mammary gland and may be the paracrine factors responsible for the cellular proliferation in steroid receptor negative cells. Therefore it was of interest to confirm the microarray and study the mRNA expression after three weeks of E&P treatment to determine whether the genes were still regulated at what is essentially day 12.5 of pregnancy. The qPCR protocols as per Materials and Methods 2.6. were employed to analyse gene expression within the mammary gland.

3.1.(vi) Proliferative Mediators of the Progesterone Response:

RANKL:

RANKL is known to have a role in mammary gland development during pregnancy. RANKL is thought to co-localise with progesterone receptors in response to pregnancy levels of estrogen and progesterone. RANKL is absent in the virgin gland, it is upregulated during pregnancy and decreases after day 18.5 of a murine pregnancy (Gonzales-Suarez et al, 2007). Their experiments also show that the highest levels of RANKLs ligand; RANK correlates with the peak of mammary epithelial proliferation at day 15.5 of pregnancy (10). In this study, RANKL mRNA levels were analysed after 21 days of estrogen and

progesterone treatment to observe its expression at this simulated late stage of pregnancy and proliferation (Fig 3.5).

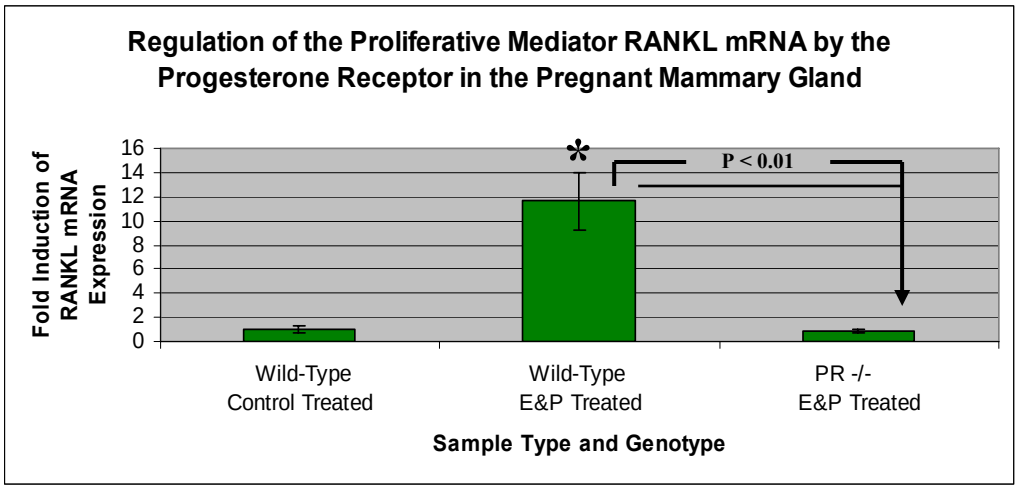


Figure 3.5: Quantitative PCR analysis of RANKL mRNA expression in wild-type and conditional PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The upregulation of RANKL mRNA in the wild-type E&P sample is significant when compared to the wild-type control sample as determined by a paired Student's t-test *($p<0.01$). Statistically significant downregulation of RANKL mRNA is also seen in the conditional PR-/- treated sample when compared to the wild-type E&P treated sample where $p<0.01$.

The results in fig 3.5 show a 12-fold increase in RANKL mRNA expression when treated with estrogen and progesterone for three weeks. Statistical analysis confirms that this increased induction of RANKL is statistically significant ($p< 0.01$). There is a significant difference between the wild-type E&P-treated samples and the PRKO samples ($p<0.01$) thus suggesting that RANKL expression in the E&P-treated mammary gland is strongly dependent upon progesterone. From this data set, it can be inferred that RANKL has an active and functioning role to play in the late stages of pregnancy where there is intense growth and proliferation due to the its upregulation during this time.

Wnt4:

Wnt4 is a secreted glycoprotein which is coexpressed in PR positive cells and is essential for regulating ductal branching via paracrine mechanisms of proliferation (Conneely et al, 2001). Wnt4 upregulates the activity of its target β-catenin which is hypothesised to have a role in cell fate decisions in the mammary gland. The paracrine action of Wnt4 has been said to occur earlier in pregnancy than that of RANKL. Wnt4 expression was analysed in this study after three weeks of the E&P treatment in order to assess the strength of its signalling abilities at this later stage of pregnancy.

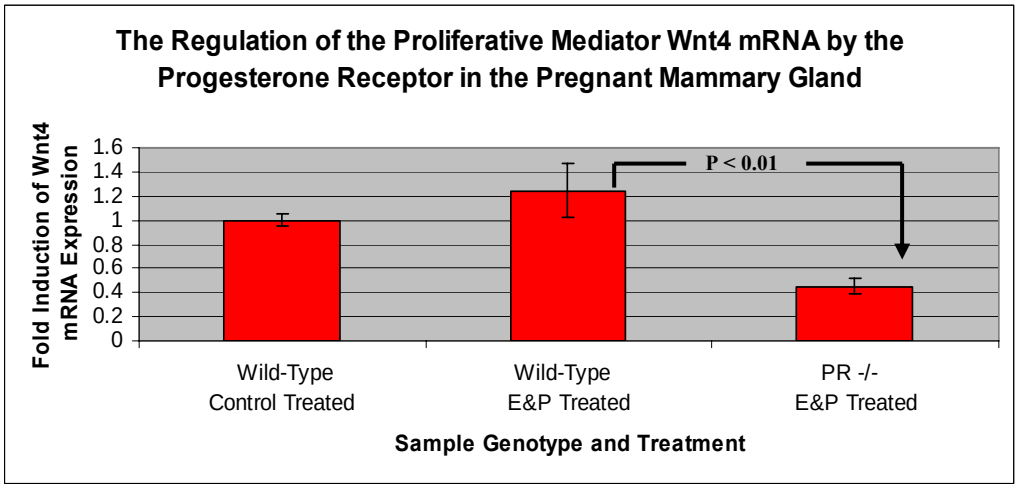


Figure 3.6: Quantitative PCR analysis of Wnt4 mRNA expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The data was analysed using a paired Student's t-test and statistically significant downregulation of Wnt4 mRNA ($p<0.01$) by PR was observed when the EP-treated wild-type samples were compared to the PRKO EP-treated samples.

Figure 3.6 shows that Wnt4 mRNA expression is only slightly upregulated in the E&P treated wild-type when compared to the wild-type control model which correlates with the previous microarray data, however statistical analysis of the data has shown that this increase is not significant thus suggesting that at day

12.5 of pregnancy, there seems to be no significant increase in the activity of Wnt4. This data set does seem to concur with the suggested early role of Wnt4 action in mammary gland development. At what is presumed to be ~day 12.5 of pregnancy-associated proliferation, it is likely that Wnt 4 activity levels may have already peaked therefore this data set may be showing Wnt4 downregulation possibly induced by some kind of a negative feedback mechanism. Knockout models of Wnt4 have shown that the consequences of its absence fade in late pregnancy therefore other parallel pathways may begin to contribute thus allowing Wnt4 levels to drop off and return to normal (Sternlicht et al, 2006). The PRKO sample does confirm the regulation of Wnt4 mRNA by the progesterone receptor as Wnt4 mRNA is further reduced by 0.8 fold in the absence of PR compared to the wild-type EP-treated samples and this downregulation is shown to be statistically significant ($P<0.01$). The results show that there is still some Wnt4 mRNA present in the PRKO samples but it is possible that this is due to the cooperative regulatory powers of prolactin signalling.

Amphiregulin:

Amphiregulin is best documented in terms of pubertal mammary gland development where estrogen is the primary regulating hormone however since it was shown to be strongly regulated by progesterone in the microarray (Fig 1.10), its expression mRNA levels during the intense period of pregnancy-associated growth and proliferation were deemed to be worthy of further

analysis. This study wanted to assess the potential of Amphiregulin alongside RANKL and Wnt4 as another paracrine mediator of progesterone signalling during late pregnancy.

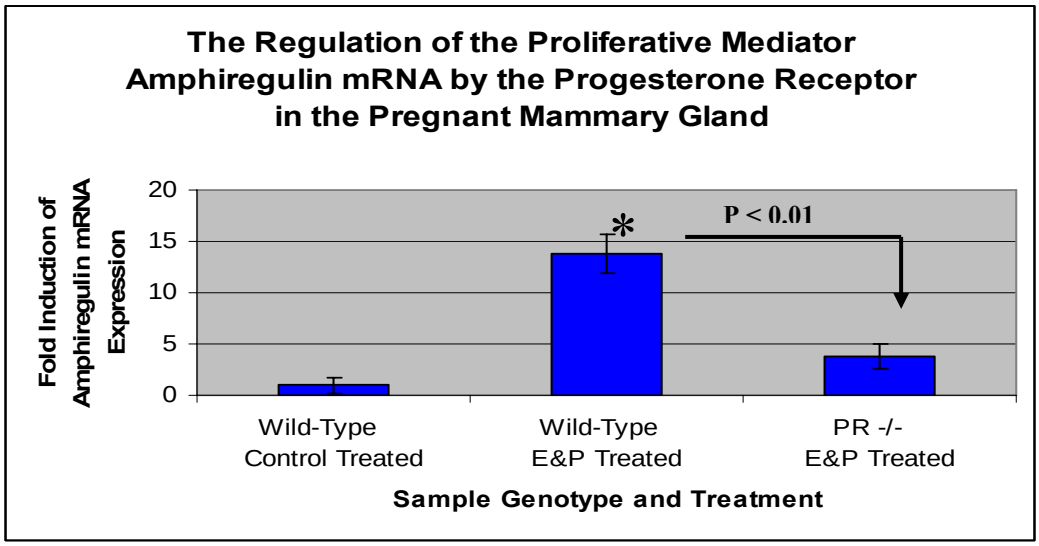


Figure 3.7: Quantitative PCR analysis of Amphiregulin mRNA expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. Amphiregulin mRNA is significantly upregulated (*$p<0.01$) in the wild-type mouse upon treatment with EP for three weeks, when compared to the wild-type control samples. Amphiregulin is significantly downregulated ($p<0.01$) in the absence of PR as determined by the 10 fold downregulation of Amphiregulin mRNA in the PRKO sample. The PRKO samples was also treated for three weeks with EP and statistical analysis was determined with a

Amphiregulin mRNA expression exhibits a 13-fold increase in activity compared to its wild-type control treated expression. This upregulation induced by the hormonal treatment is deemed statistically significant ($p<0.01$). The results in figure 3.7 do suggest that Amphiregulin is a transcriptional target of the progesterone receptor as its expression in the EP treated sample is downregulated by 10-fold when PR is ablated. The difference between Amphiregulin mRNA expression in the wild-type EP-treated sample and the

PRKO sample is also statistically significant as determined by the student's t-test ($p < 0.01$). However, Amphiregulin's expression in the absence of PRKO is still greater than that in the control model when treated with E&P thus it is most likely that Amphiregulin is still regulated by estrogen to some extent which accounts for the retained activity.

Elf5:

Elf5 is a transcription factor that signals downstream of the prolactin receptor. Its expression is highly upregulated during pregnancy and is thought to have significant implications for alveolar morphogenesis in the mammary gland. Since progesterone and prolactin signalling pathways can often cooperate and even converge downstream of their respective receptors, it is not unlikely that progesterone could exert some effect on Elf5 mRNA expression thus qPCR analysis was carried out on the Elf5 gene for this study.

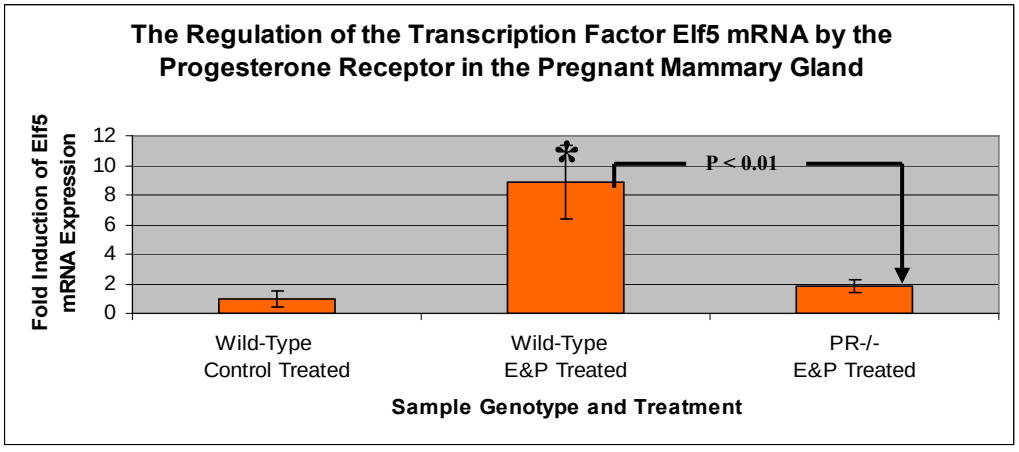


Figure 3.8: Quantitative PCR analysis of Elf5 mRNA expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. Elf5 mRNA expression is significantly upregulated in wild-type mice upon treatment with EP for three weeks when compared to the control treated wild-type mice (*p<0,01). In EP-treated wild-type mice, Elf5 is also significantly downregulated ($p<0.01$) when PR is ablated in the PRKO sample thus suggesting that Elf5 is strongly regulated by PR. Statistical analysis was determined by a paired Student's t-test.

In figure 3.8, Elf5 mRNA expression is significantly raised during a simulated pregnant state; it has an 8-fold increase when compared to the wild-type control ($p<0.01$). Elf5 mRNA also shows strong regulation by the progesterone receptor at this stage of pregnancy as evidenced by the Elf5 mRNA levels measured in the PRKO sample. There is a strong and statistically significant decrease in Elf5 mRNA expression between the wild-type EP-treated samples and the PRKO EP-treated samples. This seems to suggest a lack of compensation from the prolactin signalling cascade in terms of Elf5 regulation.

3.1.(vii) Anti Proliferative Mediators of the Progesterone Response:

For the purpose of this study, it is important to consider the role that the progesterone receptor may play in controlling the expression of genes that are known to inhibit the proliferative signals which produce much of the ductal branching and alveolar differentiation associated with pregnancy.

TGF-β1:

TGF-β1 has been identified as a negative regulator of branching morphogenesis in the mammary gland. TGF-β1 deficient mice exhibit significantly accelerated but morphologically normal ductal development. It has been hypothesised that TGF-β1 may aid in the maintenance of proper ductal spacing by enabling neighbouring ducts to avoid one another (Sternlicht et al, 2006).

There is a significant difference between TGF-β1 mRNA levels in the control and EP-treated wild-type samples. TGF-β1 mRNA expression is decreased (0.4 fold) during the simulated late pregnancy model when compared to the wild-type control ($p<0.01$) (Figure 3.9).

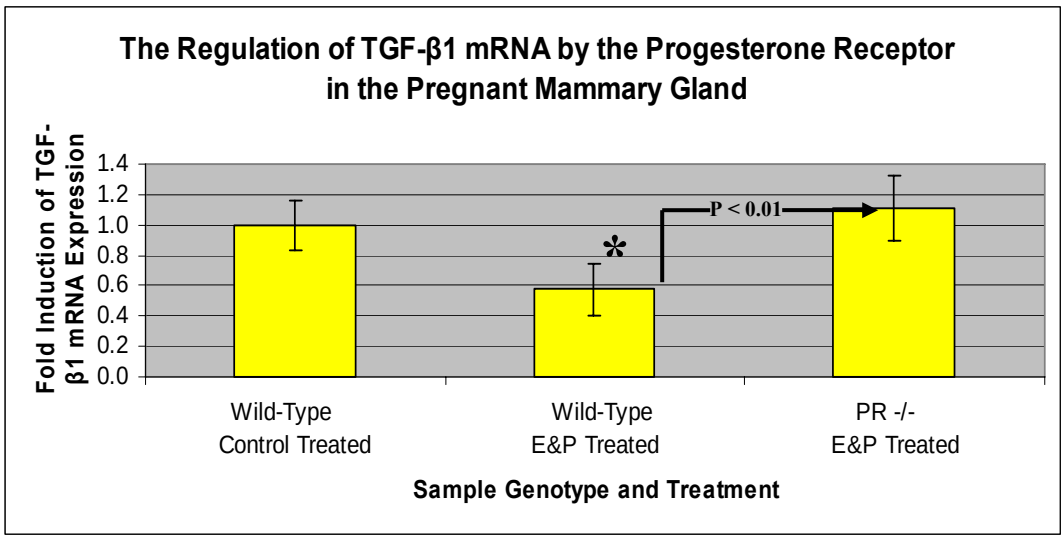


Figure 3.9: Quantitative PCR analysis of TGF-β1 mRNA expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The Student's t-test is used to determine the statistical significance of these results when $p < 0.01$. TGF- β1 is significantly downregulated in the wild-type samples upon treatment with EP for three weeks (*$p<0.01$). TGF- β1 also demonstrates significant upregulation ($p<0.01$) by progesterone as its expression increases again in the EP-treated PRKO samples where PR is absent when compared to the EP treated wild-type samples.

This data concurs with the reports that TGF-β1 activity falls during mid- to late pregnancy. The activity of TGF-β1 may have an earlier transcriptional response to the increased proliferative state induced by pregnancy and so its stronger effects may occur prior to this later stage of the pregnant mouse model. It should be also noted that at the time point of this data set, TGF-β1 mRNA expression also exhibits significant regulation by the PR, which is demonstrated in the PRKO samples where TGF- β1 mRNA expression increases again to mimic wild-type control expression levels thus suggesting that progesterone

seems to negatively regulate TGF- β1 to minimise its effects during such a proliferative phase of pregnancy within the mammary gland.

TGF-β2

In a system as complex as the mammary gland, it may be difficult to discern the intermediaries in the transduction pathway therefore as a point of interest, qPCR analysis was also performed on TGF-β2, another TGF-β family member. TGF-β2 expression was shown to be low in the virgin gland; it increases linearly through pregnancy, begins to fall at day 17 of pregnancy and decreases through lactation (Monks et al, 2007).

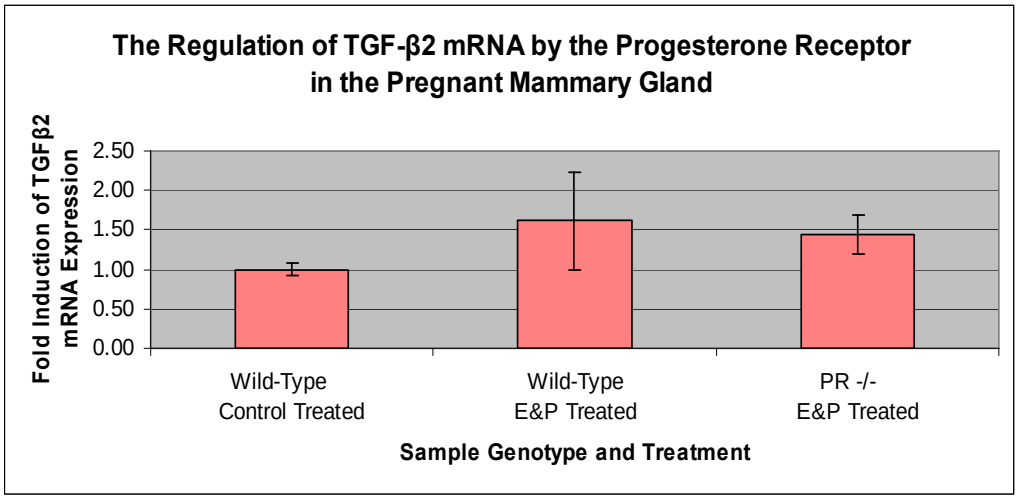


Figure 3.10: Quantitative PCR analysis of TGF-β2 expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The data was analysed for statistical significance using a student's t-test however no comparisons made between these samples were deemed statistically significant when $p < 0.01$ nor when $p < 0.05$. The larger error bar on the EP-treated wild-type samples makes statistical analysis more difficult.

The profile for TGF-β2 showed a slight increase in TGF-β2 mRNA expression in the wild-type following treatment with E&P for three weeks. If TGF-β2 is

only supposed to be decreasing after day 17 of pregnancy, then it is likely that at day ~12.5 of the simulated pregnancy, TGF-β2 would still be increasing compared to the wild-type control as it is a thought to be a more prominent marker in late stage pregnancy than TGF-β1. The TGF-β2 results show no significant regulation by the progesterone receptor upon treatment with EP (Fig 3.10). Its expression in the absence of progesterone is almost the same as its expression in the wild-type counterpart which suggests that there is no regulation by progesterone occurring at this time point. It could be possible that TGF-β2 is an estrogen regulated gene which may explain its upregulation upon EP treatment. Statistical analysis of this data set in Fig 3.10 showed that none of these results were statistically significant.

Mig6 and Sfrp2:

Another form of anti-proliferative regulation is carried out by the progesterone receptor which was investigated during the course of this study. It is thought that PR contributes to the normal development and proliferation of the pregnant mammary gland by downregulating the anti-proliferative factors which are capable of inhibiting the effects of progesterone. The progesterone receptor acts to target the inhibitors of two of its strongest paracrine proliferative factors. Mig6 and Sfrp2 are the two anti-proliferative factors that were examined. Mig6 is a known inhibitor of the epidermal growth factor receptor (EGFR) which is the receptor that Amphiregulin signals through and Mig6 appears to be downregulated in response to progesterone.

Sfrp2 is the second anti proliferative factor that was observed, it is an inhibitor of the Wnt signalling pathway hence it would be expected to be decreased during times of proliferation so as to allow proliferative signalling of progesterone via Wnt4.

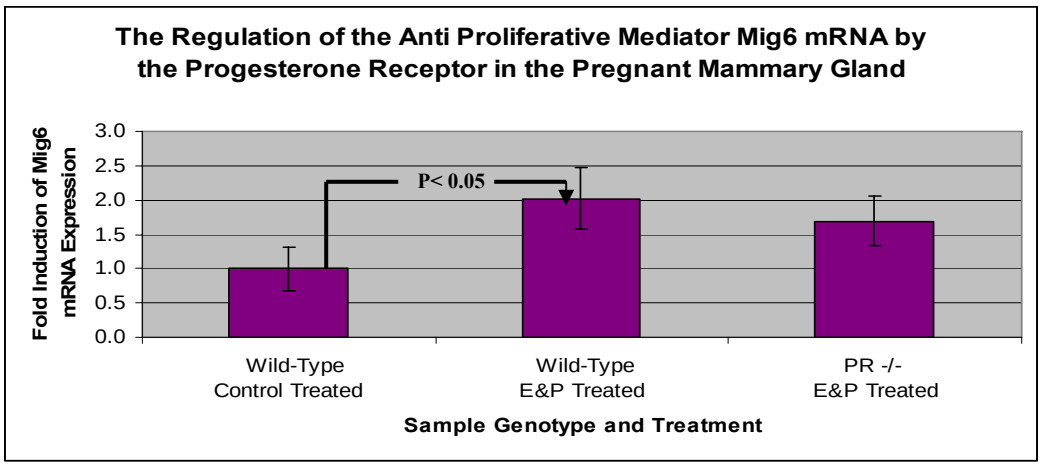


Figure 3.11: Quantitative PCR analysis of Mig6 mRNA expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. Statistically significant regulation is determined by a Student's t-test and noted by the presence of p-values when $p < 0.05$.

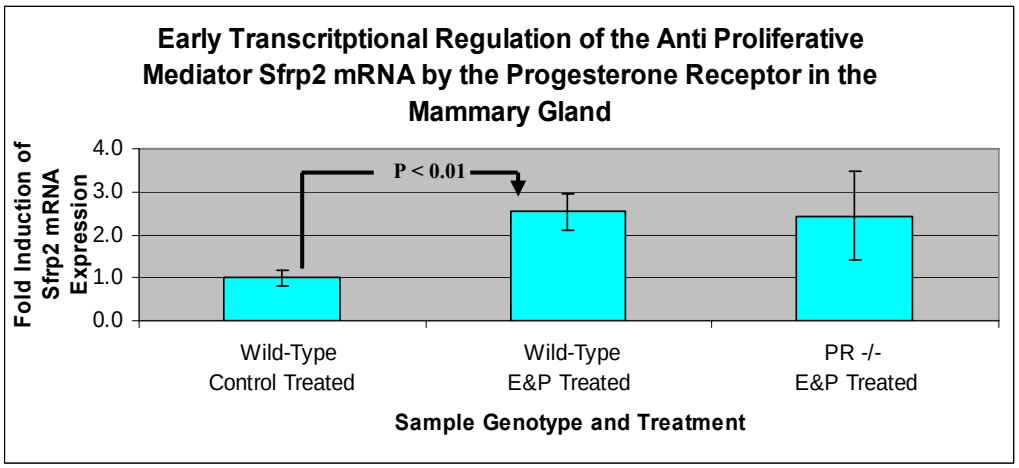


Figure 3.12: Quantitative PCR analysis of Sfrp2 mRNA expression in wild-type and PR-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The data has been analysed using a student's t-test and deemed statistically significant by the presence of p-values when $p < 0.01$

cDNA from the same cohort as the previous proliferative signals was used to assess the expression of Mig6 and Sfrp2 after three weeks of E&P treatment. The results show that after three weeks of treatment, the gene expression profiles for both genes are remarkably similar. There is an approximate 2-fold increase in mRNA expression of the E&P treated wild-type model when compared to the wild-type control which suggests that the anti-proliferative genes are increasing at this late stage of pregnancy. Mig6 mRNA shows significant upregulation ($p<0.05$) in Fig 3.11 whilst Sfrp2 exhibits more of a significant difference in Fig 3.12 ($p<0.01$). However, there seems to be no significant regulation of either of the genes at this time point by the progesterone receptor which is indicated by the mRNA levels observed in the PRKO samples. It seems unlikely that progesterone action is directly responsible for the increase of these genes in the simulated pregnant state since their expression profiles are hardly changed in its absence when compared to the wild-type EP treated samples.

It is important to mention that other qPCR analysis was carried out throughout this study which analysed the anti proliferative genes, Mig6 and Sfrp2 after 48 hours of treatment with estrogen and progesterone as opposed to the current three week treatment period (Fig 3.13, 3.14).

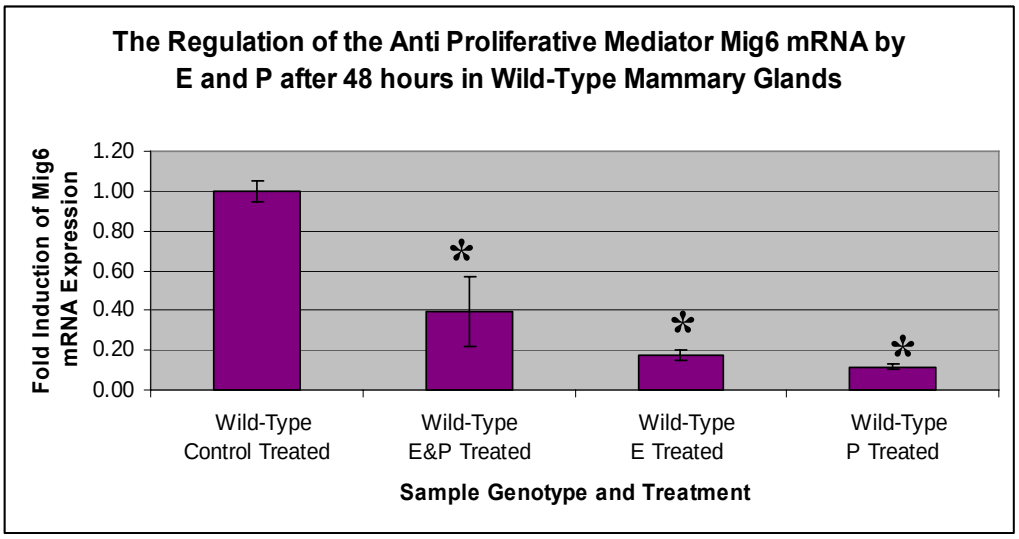


Figure 3.13: Quantitative PCR analysis of Mig6 mRNA expression in wild-type mice which have been either control treated, treated with E&P, treated with E alone or treated with P alone for 48 hours to determine if they exhibit an early transcriptional response to the ovarian steroid hormones. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The data has been analysed using a student's t-test and deemed statistically significant; * $p < 0.01$

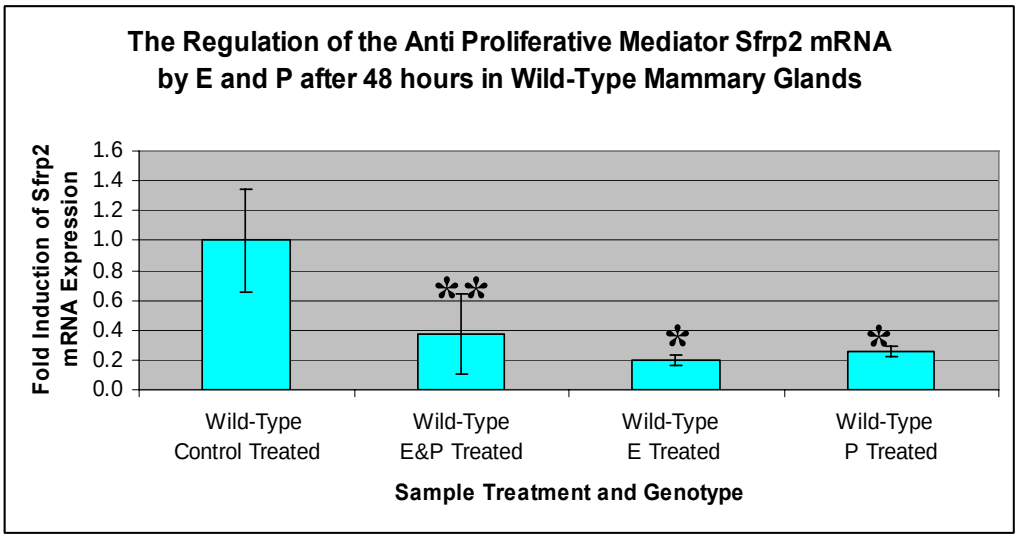


Figure 3.14: Quantitative PCR analysis of Sfrp2 mRNA expression in wild-type mice which have been either control treated, treated with E&P, treated with E alone or treated with P alone for 48 hours to determine if they exhibit an early transcriptional response to the ovarian steroid hormones. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. The data has been analysed using a student's t-test and deemed statistically significant; ** $p < 0.05$, *$p<0.01$

The data sets obtained showed that the E&P treated wild-type mice had significant downregulation of both anti-proliferative genes when compared to

the wild-type control mice. Unfortunately there was no PR knockout sample to accompany these data sets and confirm that it is progesterone that is specifically inducing their downregulation therefore any inference made on their part is considered negligible in terms of statistical significance.

3.2. The BDNF Study: A Novel Target of Progesterone Signalling in Pregnancy-Associated Mammary Gland Development

These results address the novel target gene BDNF with a view to proving its hypothesised function in the mammary gland which was proposed in section 1.7(iii). Previous investigations were carried out on this gene (Fig 1.13 / Fig 1.15) which encouraged the lab to invest in BDNF knockout mouse models to further their research in this area. Mammary gland tissue from these BDNF knockout mouse models were used to generate experimental data for this section of results.

Since the previous study had shown the established signals of proliferation; RANKL (Fig 3.5), Wnt4 (Fig 3.6) and Amphiregulin (Fig 3.7), to be functionally active at the same developmental time point, these signals were suitable targets to be measured in the BDNF knockout models to validate the hypothesised role of BDNF. The expression of all three proliferative signals were analysed in terms of a wild-type control sample, a wild-type hormone-treated sample, a BDNF heterozygous hormone-treated sample and also a BDNF homozygous hormone-treated sample. The data sets produced following

the quantitative analysis of each proliferative target proved to be very similar in their respective expression patterns (Fig 3.18).

3.2.(i) The BDNF Experimental Cohort:

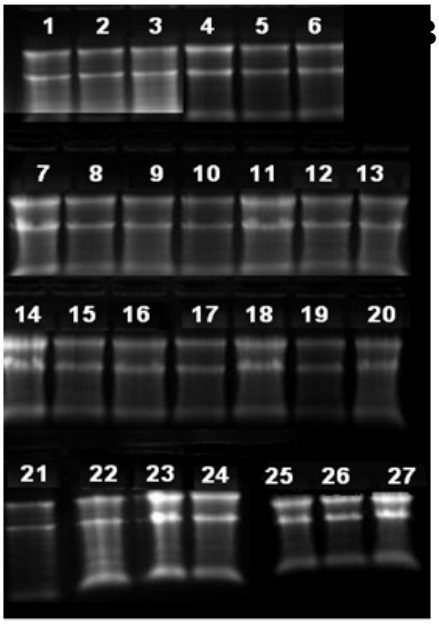
The CRE-loxBDNF gene targeting approach was used in order to specifically ablate expression of BDNF in the mammary glands of C55/129 mice. All of the transgenic techniques and breeding were carried out at the in-house Baylor College of Medicine Animal Facility. A cohort of mice was once again selected from these animals to accommodate the genotyping requirements for this experiment. Twenty-seven mice were used in total to produce the BDNF data sets. For this study, there were twelve wild-type mice, seven mice which were heterozygous for the conditional BDNF knockout and eight mice which were homozygous for the conditional BDNF. The mice all underwent ovarectomies and after ten days of rest, they were treated with either control or with estrogen and progesterone for three weeks in the same manner as the animals involved in the previous study outlined in section 3.1(i). After a period of treatment, the mice were all sacrificed using CO_2 and their tissues of interest were surgically removed and frozen at -80ºC.

3.2.(ii) Techniques of the BDNF Study:

The mammary glands from the BDNF study were all treated in the same manner as the mammary glands previously dealt with in section 3.1. The techniques were all carried out in an identical fashion, from RNA extraction to reverse

transcriptase PCR to quantitative PCR analysis. The BDNF study does not include a PRKO sample as all of the targets which were analysed using the BDNF knockouts have already been analysed in terms of the PRKO sample in the PR study therefore this expression pattern was not repeated here. Once the cDNA was generated from the extracted RNA, selected gene targets could be amplified within each sample.

A:



B:

Lane	Laboratory Mouse no.		Mouse Model Genotype	Treatment administered over 21 days	RNA extraction date
1	130	Bdnf 36	Wild-Type	Control Treated	11/15/07
2	133	Bdnf 37	Wild-Type	Control Treated	11/15/07
3	134	Bdnf 38	Wild-Type	Control Treated	11/15/07
4	72	Bdnf 12	Wild-Type	Control Treated	11/20/07
5	77	Bdnf 13	Wild-Type	Control Treated	11/20/07
6	89	Bdnf 14	Wild-Type	Control Treated	11/20/07
7	136	Bdnf 24	Wild-Type	Estrogen & Progesterone	11/20/07
8	125	Bdnf 21	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
9	145	Bdnf 27	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
10	139	Bdnf 30	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
11	C366	EP-1	Wild-Type	Estrogen & Progesterone	11/20/07
12	C367	EP-2	Wild-Type	Estrogen & Progesterone	11/20/07
13	C370	EP-3	Wild-Type	Estrogen & Progesterone	11/20/07
14	C372	EP-4	Wild-Type	Estrogen & Progesterone	11/20/07
15	C373	EP-5	Wild-Type	Estrogen & Progesterone	11/20/07
16	C359	EP-6	BDNF +/- Knockout	Estrogen & Progesterone	11/20/07
17	C360	EP-7	BDNF +/- Knockout	Estrogen & Progesterone	11/20/07
18	C365	EP-8	BDNF +/- Knockout	Estrogen & Progesterone	11/20/07
19	C375	EP-9	BDNF +/- Knockout	Estrogen & Progesterone	11/20/07
20	C363	EP-10	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
21	C364	EP-11	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
22	C369	EP-12	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
23	C371	EP-13	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
24	C377	EP-14	BDNF -/- Knockout	Estrogen & Progesterone	11/20/07
25	C154		BDNF +/- Knockout	Estrogen & Progesterone	01/10/08
26	C151		BDNF +/- Knockout	Estrogen & Progesterone	01/10/08
27	C129		BDNF +/- Knockout	Estrogen & Progesterone	01/10/08

Figure 3.16: (A) Agarose gel electrophoresis results for the extracted RNA of the 27 wild-type and BDNF knockout mice. The mice have been treated with either a control or with estrogen and progesterone for three weeks. **(B)** A legend to identify each lane on the gel is included.

3.2.(iii) Proliferative Mediators of Progesterone in BDNF Knockout Mammary Gland Tissue:

The hypothesis suggests that the secreted BDNF protein acts in the mammary gland under the control of PR, to curb the proliferation induced by the major

proliferative signals and by doing this aids in maintaining the proper spacing and structure of the ductal branching networks as they grow within the mammary gland. The data sets obtained using BDNF conditional heterozygous and homozygous knockout mouse models suggest concurrence with the proposed hypothesis (Fig 3.18).

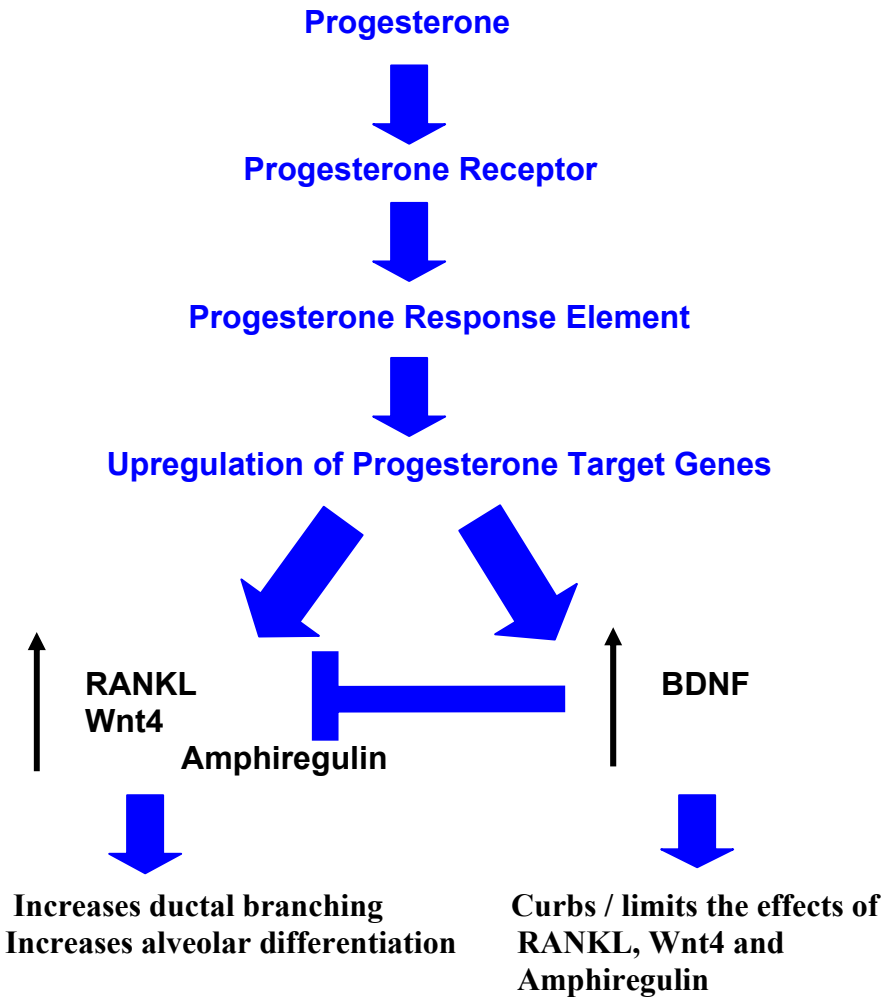


Fig 3.17: Schematic flowchart of the Conneely hypothesis of BDNF regulation by PR and its functional role with regards to controlling the proliferative genes to maintain normal mammary gland development

Previous qPCR results for the proliferative signals RANKL, Wnt4 and Amphiregulin (Fig 3.5- 3.7), following PR ablation showed dramatic downregulation of their expression. However in the qPCR data presented in Fig 3.18 below; the expression of these proliferating genes is maintained in the partial or complete absence of BDNF, thus their expression and proliferation continues to be promoted thus causing the hyperbranching phenotype. Whilst the qPCR data does not provide any definitive evidence of how BDNF may be working in the mammary gland, this data infers that BDNF does not directly regulate the proliferative signals but that its absence does indeed produce a hyperbranching which is most likely caused by the uncontrolled activity of the major proliferative signals that are known to be operating in the gland at this time point. The statistical analysis also supports this statement as there were no statistical differences observed between the EP-treated wild-type samples and the EP-treated BDNF knockout models for all three target genes. Amphiregulin and RANKL did exhibit significant upregulation in the wild-type EP-treated samples when their expression was compared to those observed in their wild-type control counterpart samples, this was also observed in the previous study (Fig 3.5 and 3.6).

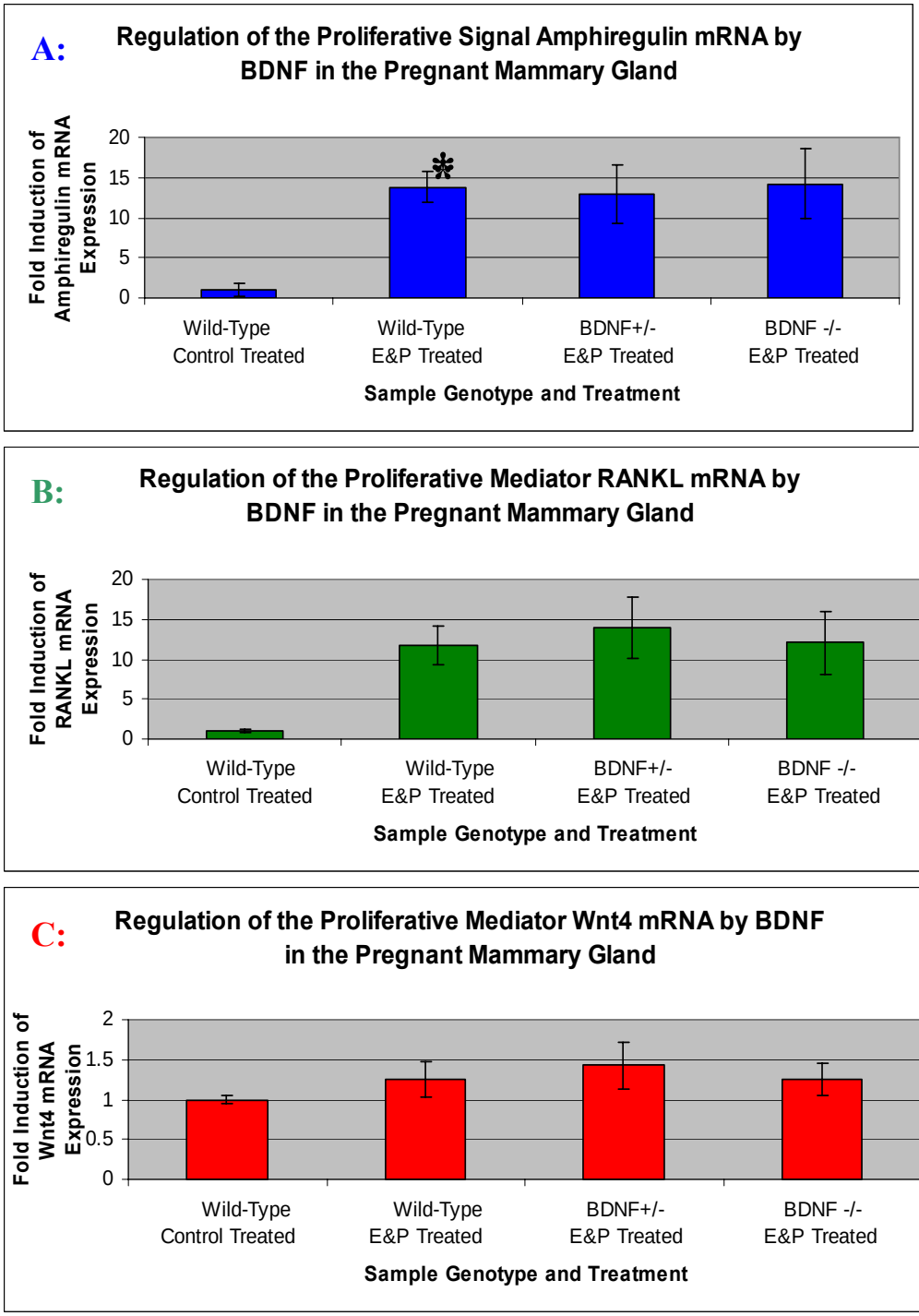


Figure 3.18: Quantitative PCR analysis of **(A)** Amphiregulin, **(B)** RANKL and **(C)** WNT4 expression in wild-type, BDNF+/- and BDNF-/- mice which have been either control treated or treated with estrogen and progesterone for three weeks in order to simulate a pregnant murine state. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. Statistical significance is noted by the presence of p- values; *p<0.01. Figure 3.18A exhibits a significant difference between the wild-type control samples and the upregulation of the EP-treated samples .Just like figure 3.18A, figure 3.18B exhibits a significant difference between the wild-type control samples and the upregulation of the EP-treated samples however 3.18C has no statistical differences between any of the samples analysed. There was no significant difference between the wild-type EP treated samples and both of the BDNF knockout samples

Figure 3.18A and 3.18B suggest that Amphiregulin and RANKL may be regulated more strongly than Wnt4 (Fig 3.18C), as Wnt4 showed no significant statistical differences between its differentially treated wild-type expression profiles. It is important to note the absence of any significant p-values for any of the three targets when their induction in the EP-treated wild-type samples was compared their induction in both of the conditional BDNF knockout samples which were also EP-treated. This lack of statistical significance seems to correspond with our hypothesised role of BDNF in the mammary gland in that the absence of BDNF allows the proliferative signals to continue supporting growth and differentiation in the mammary gland. There was no statistical significance for the three genes noted between the expression profiles induced in the heterozygous BDNF knockout model and those induced in the homozygous BDNF knockout model. This may suggest that a partial BDNF knockout may incur the same consequences as a total knockout in the mammary gland however such a conclusion would require much more detailed investigation.

3.2.(iv) Additional qPCR Analysis of BDNF Function:

Another qPCR data set that was produced for BDNF examined BDNF mRNA expression at an early treatment time of 48 hours. This small experiment looked at BDNF expression in wild-type mice only which were differentially treated with hormones for 48 hours only. The mice were treated with either control, E&P, P alone or E alone. The purpose of this work was to examine how BDNF

was being regulated at an earlier stage of proliferation within the mammary gland and so the treatment time was scaled back to reflect this. This experiment only used wild-type mice as it was performed simply a preliminary enquiry into BDNF activity and its results can not carry much weight due to the absence of the PRKO sample form this data set. Due to breeding difficulties, no PRKO were available within the time frame of this experiment.

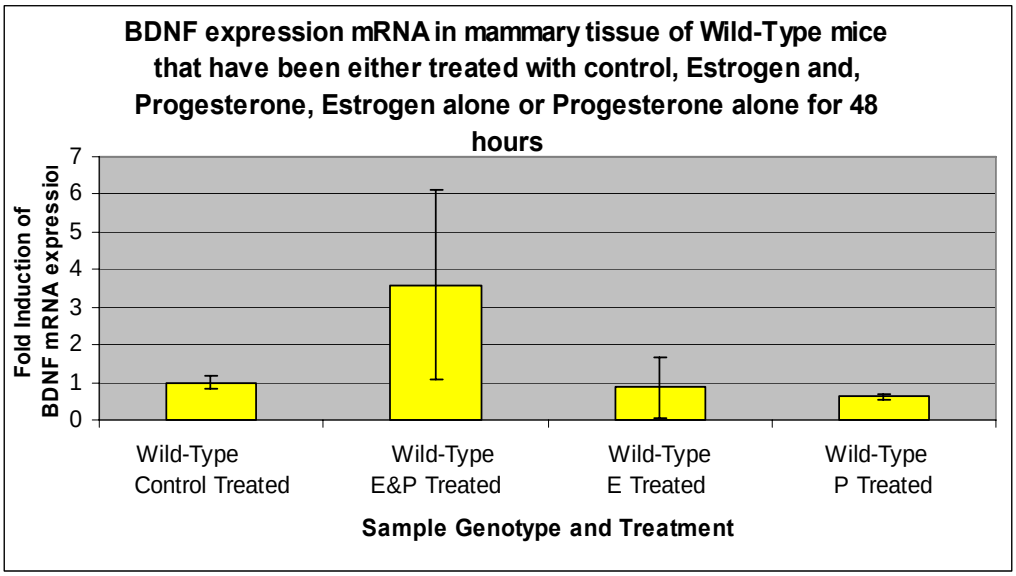


Fig 3.19: Quantitative PCR analysis of BDNF expression in wild-type mice which have been either control treated, treated with estrogen and progesterone, treated with estrogen alone or treated with progesterone alone for 48 hours in order to assess early transcriptional regulation of BDNF. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A student's t-test was performed in order to assess the statistical significance of the results obtained. In the case of this data set, no regulation of any statistical significance could be reported.

However, the 48 hour data infers that BDNF does have an early transcriptional response to E&P treatment. No real upregulation of transcription is observed when treated with either of the hormones alone which would suggest that the role of BDNF again seems to be pregnancy-associated mammary gland development. BDNF does seem to have a PRE sequence therefore it is expected that its expression would increase directly upon treatment with P yet this did not

occur at 48 hours. It is important to note that due to the large error bar on the wild-type EP-treated sample in figure 3.19, no statistical significance can be applied to this set of BDNF results.

3.3. Progesterone Regulated Target Genes of the Uterus

The third and final study in this project incorporated work on the uterus with a view to comparing progesterone signalling between it and the mammary gland. The uterus was of particular interest with regards to progesterone signalling due to the contrasting effects it elicits in both tissues. Just as progesterone was observed in the mammary gland at a particular time-point, i.e. after three weeks of E&P treatment, the uterus was also subjected to a particular course of treatment which emphasised the point during reproduction that progesterone is most potent in its uterine actions. The mouse models used for the uterine investigations were treated with progesterone alone for six hours progesterone is thought to prime the endometrium for implantation of the blastocyst in the early stages of pregnancy.

qPCR technology is employed to compare genes analysed in the uterus to those already analysed in the mammary gland. Some other more uterine-specific progesterone regulated genes were also analysed using qPCR to better gauge the effects that progesterone is stimulating in a differing reproductive environment to the mammary gland.

3.3.(i) The Experimental Cohort for the Uterus Study:

For the preparation of uteri samples in this study, thirty-five C55/129 mice were used in this uterine study. Eight wild-type control mice were used along with various PR knockout mouse models of varying genotype. The following PR genotypes used in this study were all treated with progesterone only for 6 hours. These genotypes included nine wild-type mice, nine PR isoform A knockout mice (PRAKO), five PR isoform B knockout mice (PRBKO), four PR knockout (PRKO) mice and three PR heterozygous knockout (PRKO+/-) mice. As with the previous cohorts of mice, all of these mice were weaned at three weeks, underwent ovarectomies and were allowed to rest for ten days before the administration of treatment. Treatment for this cohort consisted of a single subcutaneous injection of 1mg of progesterone; the mice were then sacrificed six hours later. This acute form of treatment serves to upregulate the expression of any rapidly induced genes that are regulated by progesterone within the uterus. It is these genes that are of interest to this study as they are the genes most likely to partake in the decidualisation reaction during the periimplantation period of pregnancy. Preparing for implantation is the most critical role of progesterone in the uterus therefore analysing various genes at this time point may infer which, are the major mediators of progesterone action.

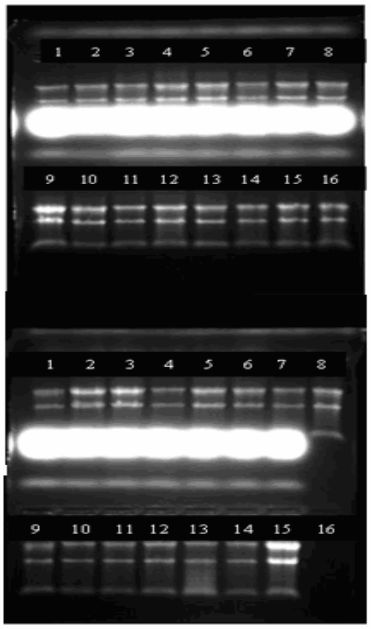
It should be noted that the uterine cohort includes PRAKO and PRBKO samples which were not included in the other studies due to breeding difficulties with various mice and also due to the questionable integrity of some of the archived samples. Unfortunately one of the three PRKO+/- samples was wrongly labelled

as a PRAKO+/- when the genotyping records were checked and was therefore unable to be included in the study. Since there were only two PRKO+/- samples remaining, they also had to be excluded as error bars could not be generated for any data produced by them. Nevertheless, the use of PRAKO and PRBKO in the uterus study enables much more information to be gathered from the qPCR analysis with regards to the mechanisms of progesterone regulation for each target gene.

3.3.(ii) RNA Extraction for the Uterus Study:

Once the animals were sacrificed, their uteri were frozen at -80ºC for RNA extraction. Only one horn of the uterus was used from each animal for RNA extraction. The frozen uteri were thawed and homogenised in Trizol just as the mammary gland tissues were. The RNA was extracted as before and analysed for quality and purity (Fig 3.20). To accommodate the size limitations of the qPCR plate, the extracted RNA of these individual mice were then pooled within their genotypes to make two or three pooled samples in a manner similar to Fig 3.2. The concentrations of these newly pooled samples was determined and it was on these ’pooled’ genotypes that the gene expression assays were performed

A:



B:

Lane	Sample	Extracted	Genotype
1	7027	8/8/00	Wild-type C
2	7104	8/9/00	Wild-type C
3	8601	1/24/01	Wild-type C
4	7857	11/30/00	Wild-type C
5	5871	5/12/00	Wild-type C
6	8646	2/14/01	Wild-type C
7	6708	8/22/00	Wild-type C
8	6583	8/9/00	Wild-type C
9	3243		Wild-type P
10	6661	8/22/00	Wild-type P
11	7072	8/8/00	Wild-type P
12	8194	1/24/01	Wild-type P
13	3322	5/19/99	Wild-type P
14	7117	8/9/00	Wild-type P
15	6574	8/9/00	Wild-type P
16	3272	5/6/99	Wild-type P

Lane	Sample	Extraction	Genotype
1	3424	6/17/99	PRAKO
2	3189		PRAKO
3	6700	8/22/00	PRAKO
4	8597	1/24/01	PRAKO
5	7868	11/30/00	PRAKO
6	3264	5/6/99	PRAKO
7	6646	8/9/00	PRAKO
8	8673	2/14/01	PRAKO
9	3301	5/19/99	PRAKO
10	7041	8/8/00	PRBKO
11	7131	8/9/00	PRBKO
12	8159	1/24/01	PRBKO
13	8183	1/24/01	PRBKO
14	8286	2/14/01	PRBKO
15	P190	8/22/00	PRKO

Fig 3.20: (A) Agarose gel electrophoresis results for the extracted RNA of the 31 wild-type mice. The wild-type mice have been treated with either a control or with progesterone alone for 6 hours. **(B)** A legend to identify each lane on the gel is included

3.3.(iii) Techniques of the Uterus Study:

Reverse-transcriptase PCR was carried out on the pooled RNA samples as per Materials and Methods 2.5 and cDNA was generated successfully and stored at 4ºC for quantitative PCR analysis. The qPCR techniques were carried out as per Materials and Methods 2.6 in the same manner for the uterus samples as they had been for the previous mammary samples. The calculations were made for each reaction and qPCR Excel templates were designed for the analysis of each target gene. Each uterus sample had an NRT negative control sample as well which was included in every reaction. The NTC and the RTnoRNA negative controls were also included in PCR reactions to ensure no contaminations had occurred throughout the experimental preparations. Universal mouse RNA was

also used as the standard to generate the standard curve for the uterus qPCR reactions. Again 18S was used as the normalising gene for the study.

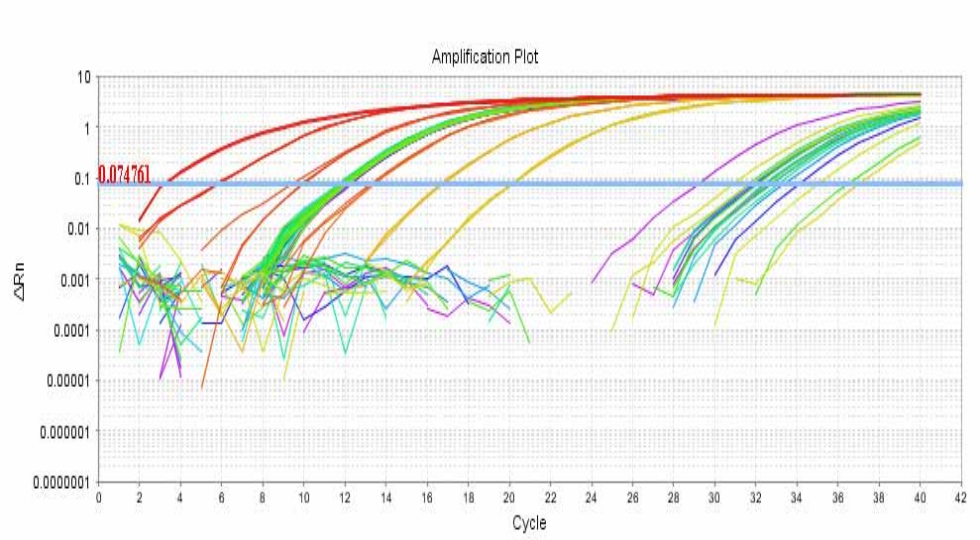


Fig 3.21: A logarithmic plot from the qPCR analysis of 18S expression in the uterus samples. The negative controls are the samples expressed to the far right of the graph. The threshold value is represented by the blue line.

3.3.(iv) The Comparative Uterus Target Genes:

The first three targets which were selected for analysis in the uterus were Amphiregulin, Wnt4 and RANKL. Since qPCR had been successfully performed on these targets in the other studies, they became ideal candidates for a comparative study with the uterus. Amphiregulin and Wnt4 are quite well published with regards to a functional role in the uterus however less is known about RANKL but its uterine expression was analysed nevertheless.

Amphiregulin:

Amphiregulin is a well established target of progesterone signalling and this is reflected in the uterus as well as in the mammary gland. Amphiregulin is expressed in the luminal epithelium of the uterus and its expression after six hours of treatment suggests that it has a functional role to play in the implantation of the embryo into the stroma. PR is mostly localised to the stromal cells of the uterus however it could once again employs paracrine action to upregulate Amphiregulin. The Amphiregulin data set shows very strong (a 120-fold induction) and statistically significant upregulation of gene expression in the wild-type models upon treatment with P (Fig 3.22). There seems to be total progesterone-dependent regulation of the gene after six hours of P-treatment as is inferred by the drastic downregulation of Amphiregulin in the PRKO sample when compared to the wild-type P-treated sample.

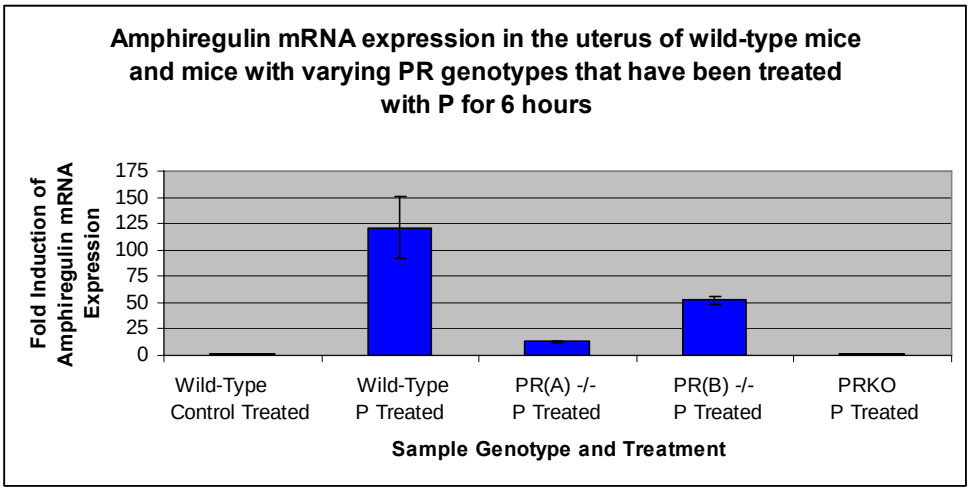


Fig 3.22: qPCR analysis of Amphiregulin mRNA expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A Student's t-test was used to statistically analyse the data where *:$p < 0.01$ was deemed to be significant. There was significant upregulation of Amphiregulin in the wild-type samples after treatment with P for six hours (*;$p<0.01$). Amphiregulin then shows further statistically significant behaviour in the wild-type P-treated samples; it is significantly downregulated when the PR-A isoform is selectively ablated and also when there is a total knockout of PR in the PRKO samples ($p<0.01$). There is downregulation of Amphiregulin in the PRBKO samples too but this is less significant than that observed in the other comparisons.

The statistics show that there is a significant regulation of Amphiregulin by PR-A upon treatment with P as its expression is obviously downregulated in the absence of PR-A thus PR-A seems to be the dominant receptor for Amphiregulin signalling in the uterus (Fig 3.22). There is also downregulation of Amphiregulin seen in the absence of PR-B after the six hour treatment. This is not as significant as the decrease observed in the PRAKO sample; nevertheless P-B does seem to be supporting the role of PR-A in mediating Amphiregulin expression.

Wnt4:

Wnt4 is stated as being expressed in the endometrial stromal cells and is thought to increase during the decidualisation reaction. Wnt4 is strongly regulated by P in the mammary gland but this regulation does not seem to translate to the uterus (Fig 3.23). The data presented by the qPCR analysis shows an increase in Wnt4 activity in the uterus of the wild-type mice after a six hour treatment of P. Whilst the fold induction is not as large as that seen for Amphiregulin in Fig 3.22, the increase is still considered statistically significant ($p<0,01$). No results of any statistical significance can be recognised with regards to the regulation of Wnt4 by progesterone when the P-treated wild-type samples were compared to the other P-treated samples of varying PR genotype.

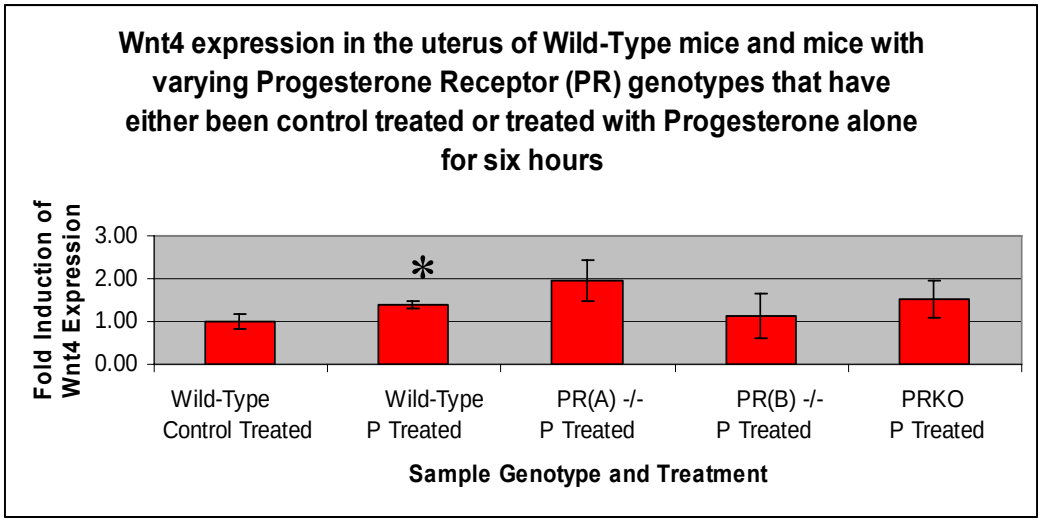


Fig 3.23: : qPCR analysis of Wnt4 expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A student t-test was used to statistically analyse the data where *; $p < 0.01$ was deemed to be significant. Wnt4 expression is significantly upregulated in the wild-type sample after a six hour treatment of progesterone when compared to the wild-type control however none of the EP-treated samples show any significant regulation with regards to the various PR isoform knockouts.

Any regulation of Wnt4 by progesterone in the uterus is certainly not as clear as the regulation observed in the case of Amphiregulin. Wnt4 has a differing expression profile in the PRKO uterus samples when compared to that already seen in the PRKO mammary gland samples (Fig 3.6) however the literature states that in the uterus Wnt4 operates as a downstream target of BMP2 which itself is regulated by progesterone therefore this could go some way to explaining the lack of regulation seen at 6 hours. It could be possible that Wnt4 is too far downstream in the progesterone signalling pathway to be classed as a rapidly induced gene.

RANKL:

RANKL has been established as an important proliferative and differentiating signal in the mammary gland therefore it was feasible that RANKL may also participate in the proliferation and differentiation that occurs in the uterus during the periimplantation period. RANKL does not exhibit any more significant regulation by progesterone than Wnt4 did, its mRNA uterine expression profile is also not as strong as that observed in the mammary gland.

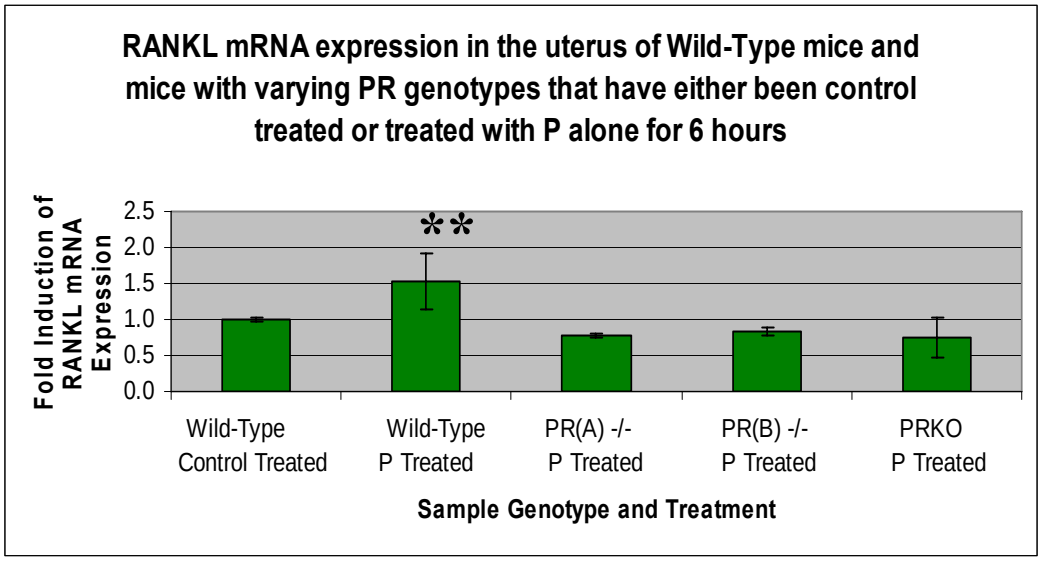


Fig 3.24: qPCR analysis of RANKL expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A student t-test was used to statistically analyse the data where $p < 0.01$ was deemed to be significant. The data set produced by the analysis of RANKL contains no results of such statistical significance however the upregulation of RANKL in the wild-type samples following treatment is significant when **;$p < 0.05$ which suggests some increased uterine activity for RANKL based on this six hour treatment.

Also RANKL is expressed in the epithelial cells of the mammary gland whereas whilst RANKL is clearly expressed in the uterus, it is not commonly known whether RANKL is localised to the stromal cells along with PR or whether it remains an epithelial target. The data in figure 3.24 shows that there is a significant increase in wild-type expression of RANKL when treated with

progesterone for six hours, (**;$p<0.05$) so whilst it is not as significant as some of the other results, there is still a definitive upregulation occurring upon treatment. The comparisons made between the other genotype samples which were treated with progesterone for six hours lack any statistical significance and therefore reveal very little about how RANKL may be functioning in the uterus during the periimplantation period or how its activity is being regulated.

Mig6:

Previous studies have shown that animals with disruption of Mig6 by gene targeting develop epithelial hyperplasia thus implying that Mig6 is a potential tumour-suppressor gene (Zhang YW et al, 2007). Mig6 interacts with the EGFR family of receptors and negatively regulates EGF signalling thereby providing through negative feedback a fine tuning of EGF signalling shortly after its activation (Zhang et al, 2006). It has been suggested that Mig6 may provide a checkpoint for normal cell proliferation in certain tissues (Zhang et al, 2007); it is quite possible that this statement applies to the uterus.

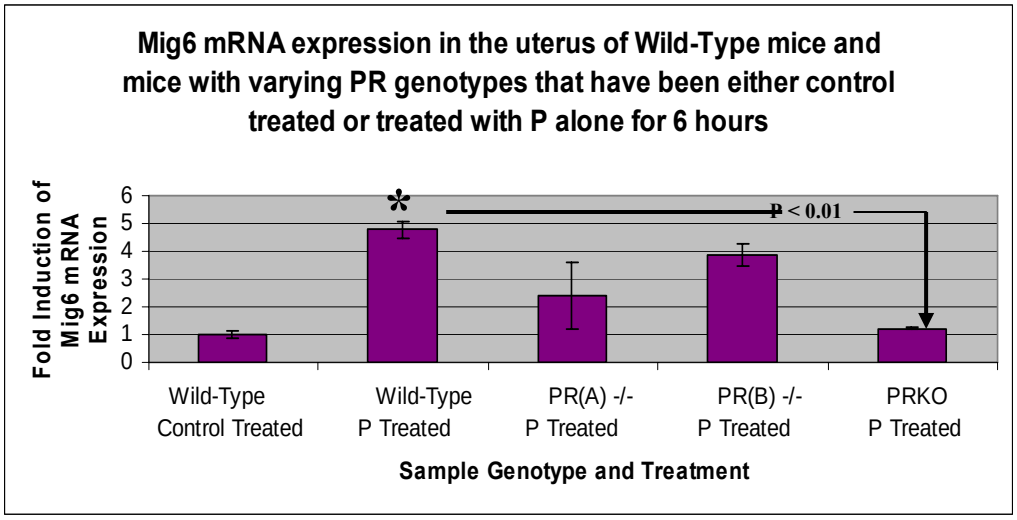


Fig 3.25: qPCR analysis of Mig6 mRNA expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A paired Student's t-test was used to analyse the data for statistical significance. Mig6 shows strong upregulation in the uterus when the wild-type samples are treated with progesterone for six hours (*;$p<0.01$). The other significant difference which occurs in Mig6 expression occurs with its downregulation in the PRKO samples when compared to its expression in the wild-type P-treated samples ($p<0.01$) – this suggests that Mig6 is regulated by progesterone in the uterus. There is no regulation of Mig6 of any statistical significance with regards to the specific PR isoforms; PR-A or PR-B.

Mig6 mRNA expression may be upregulated in a precautionary manner during the proliferative periimplantation period to curb any aberrant growth within the uterus. Mig6 mRNA expression in the wild-type samples after six hours of progesterone treatment is clearly upregulated by 4-fold when compared to the wild-type control treated samples, this upregulation of Mig6 upon treatment is deemed to be statistically significant. In addition, Mig6 expression falls drastically in the P-treated samples where PR has been specifically ablated; this infers that Mig6 is subject to progesterone regulation within the uterus. Again this downregulation of Mig6 in the PRKO sample is statistically significant as $p < 0.01$. With regards to the PR isoforms, PR-A appears to exert most influence over the expression of Mig6 but there does seem to be some redundant

regulation by PR-B in the absence of PR-A. It could be possible that Mig6 is regulated by a PR A:B heterodimer

.

3.3.(v) The Non-Comparative Uterus Target Genes:

Indian Hedgehog:

The qPCR data obtained for Indian hedgehog in figure 3.24 was analysed using the student's t-test to assess the statistical significance of the results obtained.

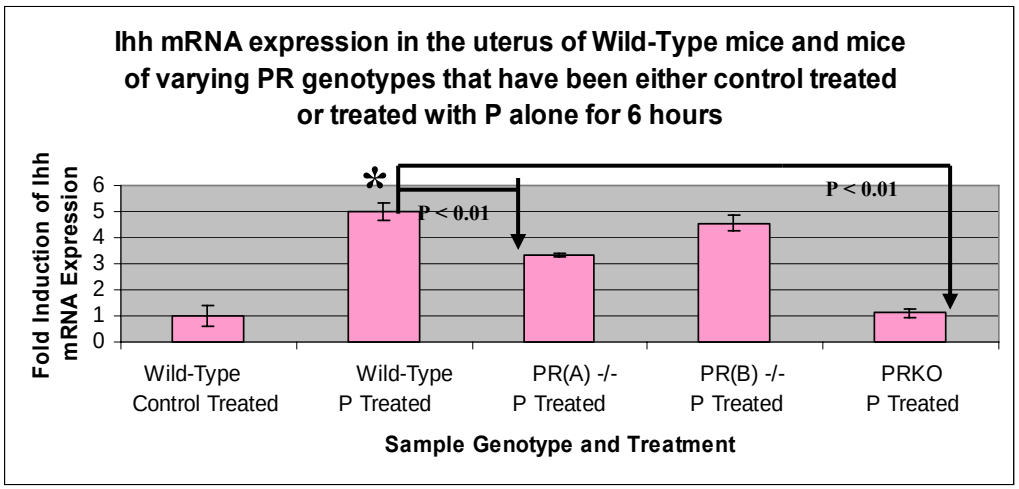


Fig 3.26: qPCR analysis of Indian Hedgehog expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A student's t-test was used to analyse the data for statistical significance and produced several significant results for this data set. Indian hedgehog expression increases in the wild-type samples once they are subjected to six hours of progesterone treatment. Indian hedgehog also shows regulation by progesterone in the P-treated samples. Indian hedgehog is significantly in the PRKO sample when PR is totally ablated but also when the PR-A isoform is ablated thus suggesting that Indian hedgehog is regulated by progesterone; in particular PR-A.

After exposure to progesterone for six hours, the expression of Indian hedgehog in the wild-type P-treated samples increased significantly when compared to the expression levels observed in the wild-type control samples. Indian hedgehog has been identified as an important mediator of progesterone action in the uterus and figure 3.26 does confirm its regulation by progesterone in the P-treated

samples. This is demonstrated by comparing the expression of Indian hedgehog in the wild-type P-treated samples to firstly the PRKO sample where there is a total knockout of PR, and then comparing the wild-type expression to the PRAKO sample where there is a partial ablation of PR function. The results show significant downregulation of Indian hedgehog in both these samples which infers that Indian hedgehog mRNA expression is positively regulated by PR and in particular PR-A. Whilst PR-A appears to be the predominant isoform with regards to Indian hedgehog regulation in the uterus, fig 3.26 suggests that PR-B does act to induce some upregulation when PR-A is knocked out.

BMP2:

Studies have shown that in human endometrial stromal cells, addition of BMP2 to the hormone regime enhanced the in vitro decidualisation process whereas siRNA to BMP2 prevented decidualisation in vitro (Franco et al, 2008).

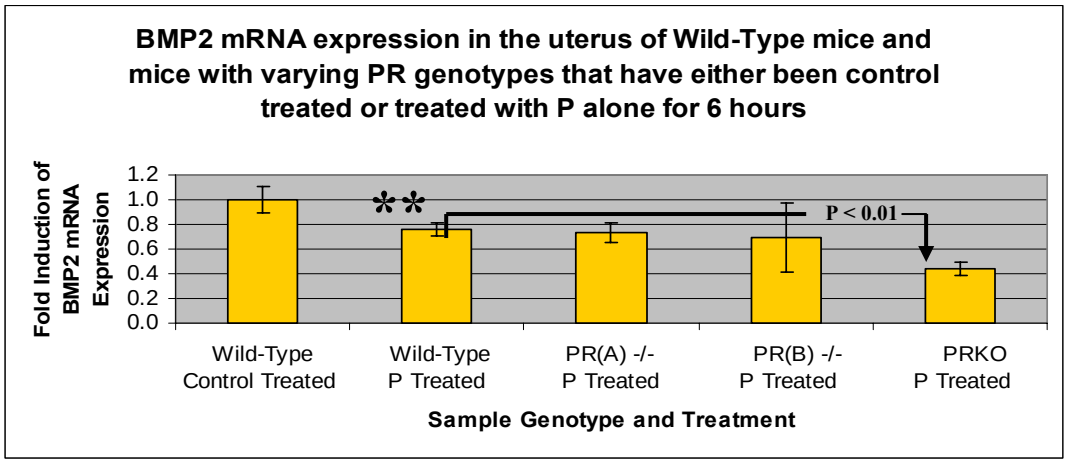


Fig 3.27: qPCR analysis of BMP2 expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. BMP2 is downregulated in the wild-type samples after treatment with progesterone for six hours. Statistics showed that the decrease in BMP2 after treatment is not as significant as other previous results as it is only deemed significant when **; $p < 0.05$. BMP2 shows more significance with regards to the P-treated samples, a comparison of BMP2 expression in the wild-type and the PRKO treated samples indicate BMP2 is regulated by progesterone as its expression is downregulated in the P-treated samples once PR is ablated ($p<0.01$). No statistical data of any significance could be reported for the specific PR isoforms.

The data set produced by the qPCR analysis of BMP2 expression in the uterus produced some unexpected results. BMP2 is known to have an important role in the decidualisation process therefore it would be expected that its expression would increase during the periimplantation period whereas the results from figure 3.27 clearly show a significant downregulation of the gene in the wild-type samples following the six hour treatment of progesterone. BMP2 does however exhibit progesterone regulation with regards to the PRKO samples as it is significantly downregulated in the P-treated samples in absence of PR. Unfortunately, figure 3.27 could not provide any statistically significant results as to how BMP2 is being regulated by PR or which isoform is the dominant receptor, there could of course be heterodimeric regulation of both PR-A and PR-B but again this cannot be conclusively determined on the basis of figure 3.27's results.

Hoxa10:

Analysis of Hoxa10 mRNA, in figure 3.28, produced a similar uterine expression pattern to that of Indian hedgehog. Statistical analysis does show that HoxA10 increases significantly in the wild-type samples following the prescribed treatment of progesterone for six hours ($p<0.01$). Hoxa10 is downregulated in the P-treated PRKO samples when PR is totally ablated, it is also downregulated in the absence of the PR-A isoform in the PRAKO sample suggesting that any of the positive regulatory effects of progesterone on HoxA10 are mediated through the PR-A isoform. Whilst some mRNA induction

was observed in the PRBKO samples, the large error bar meant that no statistically significant comparisons could be made with the P-treated samples.

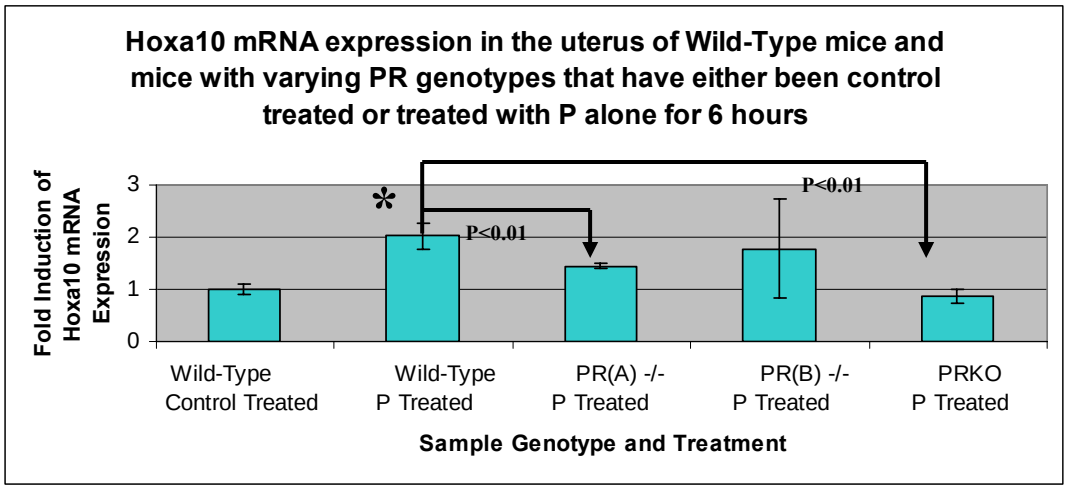


Fig 3.28: qPCR analysis of Hoxa10 expression in the uterus of wild-type mice, PRAKO, PRBKO, PRKO mice that have either been treated with control or treated with progesterone for six hours. Gene expression is analysed in terms of fold induction relative to the wild-type control and error is assessed by standard deviation. A paired Student's t-test was used to analyse the qPCR results in terms of statistical significance. There is significant upregulation of HoxA10 in wild-type samples which have been treated with progesterone for six hours as opposed to those which have been control treated (*;$p<0.01$). HoxA10 also shows regulation by progesterone as it is significantly downregulated in the P-treated PRKO samples and also in the P-treated PRAKO samples. Both decreases are considered to be statistically significant when compared to the expression profile of HoxA10 in the wild-type P-treated samples (p,0.01).

4. Discussion

Progesterone is a very powerful ovarian steroid hormone and its central role in female fertility has been well established since the early half of the 20th century however the definitive mechanisms of action by which progesterone functions remain elusive despite many years of research in this area. Our lab, along with many other labs, has focused its time, resources and efforts on progesterone investigations to try and decipher the specific signalling pathways of progesterone within the various reproductive tissues. The ultimate goal of these many and varied investigations is to generate a clear and concise account of how progesterone mediates its actions at a molecular level and whether there are common threads governing how it mediates its specific actions in the different target tissues.

4.1. qPCR Analysis of the Microarray Target Genes:

This work focuses on how progesterone mediates its effects in pregnancy-associated mammary gland development therefore a critical part of the project was to simulate a pregnant environment of intense growth and active proliferation within the mammary gland. A three week course of treatment consisting of low doses of estrogen and progesterone produced this desired effect within the mouse as it was equivalent to day 12.5 of the murine pregnancy. It was thought that this would be an optimum time point for the

activity of many of the proliferative target genes governing ductal branching and alveolar differentiation that our lab wanted to look at in the mammary gland. Any directly regulated target genes should exhibit very strong and clear expression profiles with regards to the wild-type and PRKO samples after receiving the three week course of treatment. The second reason for using three-week E&P treatment stems from the observation that the protective effects of an early first pregnancy observed in humans is mimicked with this form of treatment in rodents. Therefore results obtained from analysis at this time point may be more indicative of a protected, healthy, normal mammary gland environment which could be useful if a comparative analysis was to be made in a tumourigenic model at a later stage.

The purpose of the first study was simply to continue the investigative efforts of a previously performed microarray. The microarray was an effective strategy in identifying the full spectrum of target genes of mammary PR during pregnancy. The microarray provided a mammary transcriptome that was generated by progesterone exposure and which contains a vast amount of information about the genetic network that comprises and established the developed mammary gland. The target genes that were looked at in this represent only a small percentage of the parallel and interconnected signalling cascades required for normal, healthy mammary gland development, many of them need to be further investigated to map out their individual pathway.

The data obtained from the microarray outlined a number of genes which showed strong regulation by progesterone; these genes were further analysed

using new mouse models and quantitative PCR technology. Amphiregulin, Wnt4 and RANKL were all well known proliferative genes which have a history of mammary gland expression therefore our lab thought it would be worthwhile to assess their expression during a simulated pregnant state and determine their activity with regards to that particular environment. RANKL (Fig3.5) and Amphiregulin (Fig3.7) showed stronger regulation by progesterone than Wnt4 (Fig3.6) and their transcriptional response to the administered treatment was also more pronounced and sensitive than that seen in Wnt4. RANKL exhibited a 12-fold induction in mRNA expression in the wild-type sample following treatment with E&P (Fig3.5) whilst Amphiregulin mRNA expression also increased by 13-fold in the wild-type E&P-treated samples (Fig3.7). Both target genes also demonstrated significant downregulation in the absence of PR. RANKL appears to play a significant role in the later stages of pregnancy. It is suggested that the proper development of the lobuloalveolar structures requires an initial wave of proliferation signalled via RANKL that allows expansion of the alveolar bud epithelium followed by a decrease in the RANKL signal which is critical for the lobuloalveolar structures to differentiate to highly vacuolated and secretory alveoli (Gonzales-Suarez et al, 2007). At 12.5 days into a murine pregnancy, the mammary gland is preparing for lactation and alveolar development would be largely underway thus high levels of RANKL mRNA seen in figure 3.5 would correlate well with the expected observation. It would have been of interest to quantify RANKL mRNA

expression at a later time point to determine if its expression levels did drop off to accommodate the differentiation of the alveolar lobules.

Although Amphiregulin demonstrates regulation by P in both the microarray and in the subsequent qPCR analysis (Fig 3.7), current literature (Sternlicht et al, 2008, McBryan et al, 2008) regards it as a regulator of estrogen-induced proliferation since Amphiregulin is mostly known as a mediator of ductal branching in puberty rather than pregnancy. Pregnancy is recognised as a period of increased ductal branching and outgrowth therefore an important mediator of branching such as Amphiregulin would be expected to be involved. In puberty, the proposed mechanism of action of Amphiregulin in breast epithelial cell proliferation is that ER-positive cells, in response to stimulation by estrogen, secrete paracrine factors such as Amphiregulin which then act indirectly to stimulate neighbouring ER-negative cells (McBryan et al, 2008). Active Amphiregulin then binds to EGFR in the stroma of the mammary gland. This induces proliferation of nearby epithelial cells, by a possible paracrine mechanism, resulting in ductal outgrowth. This paracrine mechanism of signalling is identical to that of PR therefore it is possible that Amphiregulin can be regulated by both ovarian steroid receptors but at different stages of development (McBryan et al, 2008). It is also possible that the 13-fold induction of Amphiregulin in figure 3.7 could be the result of strong synergy between ER and PR during pregnancy. There is also evidence that Amphiregulin expression is reduced in prolactin receptor knockout mice, indicating that Amphiregulin

might also be regulated by prolactin in mammary epithelial cells during pregnancy (McBryan et al, 2008).

qPCR analysis of Wnt4 mRNA expression produced less regulation by P than RANKL or Wnt4. Figure 3.6 demonstrates negligible induction in the wild-type samples following three week E&P treatment however Wnt4 expression is significantly downregulated in the PRKO samples thus confirming its status as a target of progesterone regulation in the mammary gland. Wnt4 is a secreted paracrine mediator which is active in early pregnancy (Oakes et al, 2006) therefore 12.5 days of pregnancy could be due to too late a time point to observe a strong induction. Wnt proteins regulate a wide range of proliferative cellular processes and it is likely that there is more than one Wnt signalling pathway functioning in the mammary gland thus it is possible that Wnt4 is not a direct target of PR and may mediate its effects further downstream than RANKL or Amphiregulin (Sternlicht et al, 2006).

In a recent paper by Fernandez-Valdivia, 2008, Wnt4, RANKL and Amphiregulin was analysed in the murine mammary gland and the genes were found to be transcriptionally induced by acute exposure to progesterone (within 76 hours) in the mammary gland of ovariectomised mice. It would have been advantageous to the analysis of these genes if this study had incorporated various time points of treatment to monitor the expression profiles as the mammary gland progresses through pregnancy. Inclusion of such data would give a better indication of where in pregnancy-associated development each gene is most potent in its effects. A better characterisation of each gene in these

terms may aid in understanding the signalling mechanisms that are in place during this proliferative and differentiating time.

Elf5 was also downregulated in the microarray following E&P treatment in PRKO mice. Elf5 was of particular interest as it is commonly associated with prolactin signalling however the results obtained in this study did produce a strong progesterone-regulated profile for Elf5 (Fig 3.8). Elf5 mRNA expression did increase by 8-fold upon treatment with E&P for three weeks ($p<0.01$) and it did decrease to wild-type control-treated levels in the absence of PR ($p<0.01$). However, Elf5 is always referred to as a prolactin target gene rather than a progesterone target gene thus these results could be indicative of convergence or redundancy between these two hormonal pathways in a pregnant mammary gland that is preparing for lactation. Recent literature states that ERα, PR and PrlR may be colocalised to the same ductal luminal cells (LaMarca, 2008). If progesterone and prolactin are in such tight proximity to one another, it is possible that there may be some overlap and compensation in play.

It was important that the microarray study should also include genes that have to be downregulated in order to accommodate the proliferation and differentiation which is so synonymous with pregnancy-associated mammary gland development. They also require regulation by progesterone that is just as essential as that incurred by the proliferative markers and their inclusion in the study emphasised that.

Administration of exogenous TGF- β1 into actively branching mouse mammary glands using slow-release pellets resulted in reversible growth inhibition and

involution of mammary gland end buds (Gorska et al, 1998). It is suggested that TGF-β1 is a negatively regulated progesterone target gene yet it could still be an important mediator of progesterone action in the mammary gland (Monks et al, 2007). TGF-β1 production and activity are differentially regulated during mammary gland development such that periods of proliferation were accompanied by decreased TGF-β1 activation in most cells (Ewan et al, 2005). TGF-β1 is highest in the virgin and early pregnant gland, when PR levels are lowest or only beginning to rise. TGF-β1 expression falls during mid-pregnancy and remains low until involution (Monks et al, 2007). Figure 3.9 concurs with the downregulation of TGF- β1 at 12.5 days of pregnancy in the mouse model as its expression is decreased by half in the EP treated wild-type mice compared to the control-treated wild-type mice. Once PR is ablated, TGF- β1 mRNA expression returns to wild-type control treated levels. Progesterone seems to be suppressing the inhibitory effects of TGF-β1 during pregnancy so that the mammary gland can proliferate and develop to prepare for lactation.

TGF β2 was analysed for its anti-proliferative effects in the mammary gland because of its associations with TGF- β1. It is not a well established mammogen but its expression is known to increase during pregnancy and it is localised to the epithelial ducts and the alveoli. The qPCR analysis of TGF-β2 showed no significant regulation in the wild-type mouse following the three weeks of E&P treatment and no regulation by PR was observed. However, TGF- β2 is regarded as a marker of late pregnancy and it is expected to increase linearly to day 17 of pregnancy therefore it is impossible to tell from the data (Fig 3.10) whether

TGF-β2 would continue to increase if treatment was prolonged or whether figure 3.10. represents the maximum induction of TGF-β2 under such conditions. Again, analysis at various time points of treatment would have offered a better insight into TGF-β2s expression during pregnancy.

Mig6 is a gene which appeared in the microarray and exhibited negative regulation by PR. Most of the Mig6 literature focuses on the liver, the skin or the lungs, however in terms of the mammary gland; Mig6 is hypothesised to have an anti-proliferative effect. Mig6's role as an inhibitor of the EGFR signalling pathway coupled with its potential as a tumour suppressor made it an important candidate gene for negative regulation in terms of progesterone and pregnancy associated mammary gland development. Sfrp2 is another anti-proliferative factor capable of inhibiting the effects of progesterone during pregnancy as it inhibits the Wnt signalling pathway. qPCR analysis of both genes after three weeks of E&P treatment produced very similar expression profiles (Fig 3.11 and 3.12). Both genes increased in the wild-type samples following treatment and the increase in each was ~2-fold. Neither gene demonstrated any regulation by PR when analysed in the PRKO sample therefore it is unlikely that Mig6 or Sfrp2 are directly regulated by PR at this time point. The analysis of the two genes at the earlier time point of 48 hours to determine whether they exhibited an early transcriptional regulatory response to progesterone produced a differing expression profile to that seen at three weeks (Fig 3.13 and 3.14). The results at 48 hours, suggests that Mig6 and Sfrp2 did

have an early yet strong transcriptional response to the hormonal surge of the simulated pregnant state.

Consideration of the opposing regulatory profiles produced at the two separate time points enables the data presented in figure 3.11 and 3.12. to be better explained. It is hypothesised that progesterone may exert a strong and significant effect on Mig6 and Sfrp2 and downregulate their expression early on in pregnancy in order to curb any anti proliferative effects they may have on the initial proliferative processes within the mammary gland (Fig 3.13, 3.14). The effects of progesterone on these anti-proliferative genes are then reduced as pregnancy progresses and the proliferation has become more established and stable. At this stage the anti proliferative effects may be considered negligible and so their expression may rise without threat to the structure of the mammary gland (Fig 3,11, 3,12).

4.2. The BDNF Study: A Novel Target of Progesterone Signalling in Pregnancy-Associated Mammary Gland Development

The second of the three studies in this project revolved around the neuronal gene; brain derived neurotrophic factor (BDNF) which was a novel target gene in terms of mammary gland development. This study was more exploratory as there was no published literary findings with regards to any role BDNF may have had in the mammary gland or in general mammalian reproduction. There are overlapping features between this study and the first study, as BDNF first

came to the attention of our lab because of the regulation with regards to progesterone it demonstrated in the microarray. Our lab was genuinely intrigued by the BDNF results and thought that it would be of interest to further pursue it as a novel target of progesterone regulation in pregnancy-associated mammary gland development. BDNF is a secreted protein, which belongs to a class of growth factors, that are capable of signaling particular cells to survive, differentiate, or grow therefore it was thought it may replicate this in some form if active in the developing mammary gland. On the basis of the microarray data (Fig 1.10) and other data (Fig 3.15), the decision was made to generate conditional BDNF knock-out mouse models to investigate the phenotype produced in its absence so that a hypothesis could be formed as to where in the mammary gland BDNF was having an effect.

The BDNF mouse models and the other mouse models included in this cohort also received the three week E&P treatment because as with the first study, pregnancy-related activity was also the principle interest for BDNF investigations. Hyperbranching was rampant in the BDNF knockout mammary gland (Fig 1.17A) therefore it was hypothesised that BDNFs function was to curb or limit the proliferative signals which are enforced during pregnancy. Analysis was carried out on the three major proliferative signals which demonstrated mRNA regulation by progesterone in the microarray (Fig 1.10). RANKL, Amphiregulin and Wnt4 were all analysed in conditional BDNF heterozygous knockout models and in conditional BDNF homozygous knockouts to determine whether they were regulated by BDNF. The three genes

demonstrated very similar transcriptional responses in the BDNF knockout models. RANKL (Fig 3.18A) and Amphiregulin (Fig3.18B) once again undergo significant upregulation in the wild-type samples when treated with E&P for three weeks, this upregulation is then maintained in the partial or complete ablation of BDNF. Wnt4 mRNA expression does not increase to the same extent as RANKL and Amphiregulin following the hormonal treatment (Fig 3.18C) however the upregulation that does occur is also maintained in the two BDNF knockout models. As mentioned, the phenotypic consequences of BDNF ablation is hyperbranching and whilst the three genes retained their proliferative profiles in the absence of BDNF, there was no increase of mRNA expression in any of the three. It, therefore must be considered that if the hypothesis was to hold true, then mRNA expression of the proliferative mediators should actually increase if BDNF is not there to curb their effects. This was not evident from the qPCR data in figure 3.18 therefore it could be possible that BDNF is just one factor of many that might be working cooperatively to negatively regulate the proliferative mediators in pregnancy-associated mammary gland development. It might also be necessary to analyse other potential markers of proliferation as opposed to the proliferative signals themselves. Some cell cycle markers of proliferation may indicate the expected increase in proliferation when BDNF is ablated in a faster and more efficient manner.

BDNF mRNA expression was analysed in wild-type samples which had been differentially treated to determine whether BDNF exhibited an early transcriptional response to hormonal stimuli. The resultant data set (Fig 3.19)

did not produce any significant analysis. BDNF mRNA expression did appear to increase but there was large error bar present on the wild-type E&P-treated samples because of the animal-animal variation in that particular cohort. BDNF has been shown to have a PRE present in its genome hence it should be directly regulated by progesterone however the P-treated wild-type sample in Fig 3.19 showed very little upregulation after 48 hours. The data thus infers that BDNF does not have an early transcriptional response to progesterone but that there may be some synergy between estrogen and progesterone that can increase its expression. PR is a transcriptional target of ER therefore maybe together estrogen and progesterone create a more potent regulatory force than progesterone alone can after only 48 hours.

Using qPCR analysis in this study failed to produce any definitive results with regards to BDNF function in the mammary gland. The use of PRKO and PR isoform conditional knockout models would have enhanced the BDNF data set (Fig 3.19) enormously. Initial data produced by the lab incorporated PR-A and PR-B knockout models and observed that PR-A is the dominant isoform in the progesterone mediated regulation of BDNF. A staggered treatment time would also be of interest in elucidating how and when BDNF is being transcribed with regards to progesterone.

4.3. Progesterone Regulated Target Genes of the Uterus

The uterus was of particular interest with regards to progesterone signalling due to the contrasting effects it elicits in both tissues. Progesterone has proliferative effects in the mammary gland but in the uterus, it appears to be anti-proliferative as it counteracts the proliferative effects of estrogen hence why progestins are administered in conjunction with estrogen for menopausal women on HRT.

The mouse models used for the uterine investigations were treated with progesterone alone for six hours as this was thought to mimic the priming effect that progesterone has on the endometrium during the periimplantation period. Exposure to progesterone at this early stage is essential for the decidualisation reaction which allows embryo implantation to occur successfully.

The three proliferative target genes, RANKL, Amphiregulin and Wnt4 are revisited again in this study and it is on the basis of their expression profiles that comparisons can made between the uterus and the mammary gland. A huge advantage in the analysis of the uterus genes is the inclusion of the PR-isoform knockout samples; PRAKO and PRBKO. Using these knockout models give a more specified regulatory profile for each target gene and generally improves the quality of the data obtained from the qPCR.

Amphiregulin displays its strongest regulation by PR in the uterus with a 150-fold induction of mRNA expression after only six hours of progesterone

treatment. Such a powerful response at such an early time point is quite unusual and infers that Amphiregulin is a direct target of P signalling and has an important role to play in the uterine implantation process. Byun et al, 2008 examined the uterine expressions of the epidermal growth factor (EGF) family to elucidate their exact role in rat pregnancy. EGF and its receptors' (EGF-R) mRNA levels increased significantly at implantation after which their expression gradually decreased. Amphiregulin was strongly expressed around implantation and also at mid-pregnancy. Treatment of pregnant rats with the progesterone antagonist; RU486, at gestational day 5 blocked the expression of all the EGF genes including Amphiregulin, and administration of immature rats with progesterone induced the uterine expression of all the genes. The major role of Amphiregulin is cell proliferation (McBryan et al, 2008) thus it makes sense that it would be so strongly upregulated during the periimplantation period as that is a time of intense cell growth to thicken the uterine wall in preparation for receiving the blastocyst. As well as the cellular growth that is involved in the decidualisation reaction, there is also a huge amount of cellular differentiation occurring, which Amphiregulin could also be involved in. The PR isoforms in figure 3.22 infer that PR-A is the dominating regulator of Amphiregulin mRNA expression however since neither PR-A or PR-B can upregulate Amphiregulin to the same degree as the wild-type sample, there may be some synergy between the two to effectively stimulate Amphiregulin's expression. The highly proliferative effects mediated by Amphiregulin stand in stark contrast to the progesterone's anti-proliferative action in the uterus. It

seems that in the case of pregnancy-related regulation in the uterus, progesterone is required to prime the cells of the endometrium so that they are prepared for implantation. It seems that this priming action requires progesterone to promote growth, proliferation and differentiation therefore demonstrating how it can mediate its signalling pathways very eloquently to accommodate the differing and immediate requirements of the specific tissue in which it is functioning.

Wnt4 and RANKL were also analysed in the uterus but their fold inductions were not as high as Amphiregulin are not as definitive as those induced in the mammary glands. Wnt4 shows only a slight increase in its wild-type mRNA expression following the six hour treatment. The data derived from the PRAKO samples suggests that the PR-B isoform may be increasing Wnt4 mRNA expression, even more so than the wild-type P-treated sample which may infer an inhibitory role for PR-A in Wnt4 signalling in the uterus (Fig 3.23). Despite the lack of clear regulation seen here, Wnt4 is still established as an important mediator of the decidualisation reaction. It is most likely that Wnt4 is not a direct target of PR in the uterus and that it lies too far downstream to display an acute response to progesterone regulation. qPCR data for RANKL mRNA expression did not elicit any significant results. Unlike in the mammary gland, RANKL does not appear to have a very important role in the uterus at this particular time point. Thus in conclusion, only one out of the three proliferative mediators of progesterone; Amphiregulin, came close to replicating its effects in both the uterus and the mammary gland (Fig 3.7 and Fig 3.22).

Mig6's role as a mammary anti-proliferate made it an important choice for the uterine comparison to determine whether it was regulated in the same manner in both tissues. Mig6 was the only anti-proliferative target gene examined in both the mammary gland and the uterus because of the inhibitory effect that it has upon the EGFR and its signalling pathway. It may be of interest to determine the Mig6 mRNA expression profile in the uterus so as to contrast it to the mRNA expression profile which was so strongly induced by Amphiregulin; a major ligand of the EGFR. Mig6 does exhibit a significant and acute response to the six hour progesterone-treatment with a 4-fold increase in its mRNA levels. The PR-A isoform appears to be the more dominant isoform receptor and there is also significant downregulation of Mig6 in the absence of PR (Fig 3.25). The qPCR analysis infers that Mig6 is induced in the uterus at the same time as Amphiregulin and both appear strongly regulated by progesterone. It may be possible that PR is able to upregulate the proliferative factor Amphiregulin whilst at the same time upregulate its indirect inhibitor in an attempt to control the proliferation. This is a similar hypothesis to that proposed for the BDNF action in the mammary gland.

This study relied heavily on literary findings therefore other previously researched progesterone-regulated signalling pathways were explored which were not directly relevant to the mammary gland yet were still informative with regards to the differential action of progesterone in various tissues.

Indian hedgehog is one of these genes, it is expressed in the luminal and glandular epithelium of the uterus and its expression profile peaks before the

implantation of the blastocyst occurs and then decreases almost immediately after. The PR-Cre/lox-Ihh knockout points to Ihh as a major affector of PR action in the uterus since its phenotype mimics that of the PRKO mouse (Franco et al, 2008). Indian hedgehog is upregulated after treatment with P in the wild-type mice (Fig 3.26) which further confirms its acute response prior to implantation.

Bone morphogenic protein 2 (BMP2) is localised to the stromal cells in the endometrium and has an important role to play in the decidualisation reaction. qPCR analysis of BMP2 mRNA expression failed to display any significant regulation. BMP2 expression decreased upon treatment with P in the wild-type samples but did show regulation by PR with the PRKO samples (Fig 3.27). The literature states that BMP2 is a downstream effector of COUP-TFII which is a known target gene of Indian hedgehog signalling. Just like Wnt4, BMP2 may be too far downstream of PR following six hours of treatment to show any rapid induction; its expression may continue to rise beyond the time limitations imposed by this study.

The final target analysed for the uterus was HoxA10. Hoxa10 is a transcription factor thought to have a role in cell proliferation during the decidualisation reaction and also a mediator of prostaglandin signalling in response to PR (Lim et al, 1999).

HoxA10 mRNA expression does increase 1-fold following treatment of wild-type mice with progesterone for six hours. There is strong literary support for Hoxa10 as an important mediator of P action in the uterus but the initial data

obtained from the uteri samples in this study does seem to reflect this evidence for Hoxa10 as a critical target of progesterone regulation in the uterus.

4.4. Future Works:

The use of the 48 hour data, whilst limited due to the lack of a PRKO model, was nevertheless helpful in terms of analysis of the microarray target genes. Three weeks of E&P treatment did provide the required proliferative environment but it would have been hugely advantageous to this study to have had shorter concurrent treatment time points so that more of the genes could be investigated as direct effectors of progesterone signalling. The proliferation within the mammary gland after three weeks is quite intense and many pathways may be already activated which makes the sequence of such pathways increasingly difficult to decipher. Immunostaining for the localisation of the target genes and PR would be an advantage for further investigations into the paracrine signalling mechanisms of PR. Protein analysis for some of the genes may also have been valuable to discerning gene activity during the simulated pregnancy, this could have been performed using Western blotting techniques. With regards to the BDNF study, it is my opinion that for the pioneer investigations of novel targets, it would be easier to carry out initial studies on mammary cell lines rather than mouse models. Cell culture would allow more experiments to be done on conditional BDNF knockouts in larger numbers, in a more timely fashion and at less cost to the lab. Larger data sets would also eliminate the problem of animal-animal variability that is often associated with

the smaller sample sizes. When more information has been gathered on a novel target using cell lines, animal models could then be used more efficiently and effectively to enhance and continue previous investigations. Using ChIP analysis would also be of interest to determine if the PR homodimer is binding directly to the BDNF promoter sequence which would thus further confirm BDNF as a direct target of PR.

The final study of the uterus was able to instigate a screening of potential target genes which would be active in the uterus during decidualisation. Although this study was able to use specific PR-isoform knockout models, no target gene was singled out for further analysis to discover where in the signalling pathway it was positioned. It would be a significant advantage for this study to follow through on some of the results and perhaps monitor their mRNA expression at numerous time points to gauge their progesterone regulatory expression as the uterus develops past the decidualisation reaction.

A study on the ovary would also be important to assess how PR controls progesterone signalling on a third reproductive tissue to try and better understand the differential regulatory powers of progesterone and how it adapts its signalling pathways to suit the specific environment.

5: Conclusion:

As previously mentioned, the fundamental concept driving these experiments is how endeavours to better understand normal mammary branching morphogenesis are essential to explaining the abnormal; in the case of the mammary gland to determine how and where progesterone-regulated signalling pathways may become vulnerable to corruption which may induce tumour formation and malignancy. This concept is of greater significance in the mammary gland because a normal healthy mammary gland progresses through the same general principles, from proliferation to invasion, which are also seen to occur in malignant disease.

The purpose of these studies was the elucidation of progesterone-regulated target genes. The studies have looked at many such genes in differing treatment scenarios as well as differing tissue environments. This project has provided a deeper insight into the regulation of each gene on an individual basis when subjected to either a pregnant state or in the case of the uterus a periimplantation state. These genes were analysed at two critical times of reproduction and the responses they elicited may be useful for future research to decipher the inherent pathways upon which they operate. For example; Amphiregulin, Wnt4 and RANKL were hailed as major proliferative signals throughout this project and were grouped together beneath this umbrella term, however the work that was carried out on them highlighted that there were significant differences

between them especially with regards to how and where they were susceptible to progesterone regulation.

In conclusion, the importance of progesterone and PR to mammary gland development has been proven many times over yet much work remains to be done so that a molecular context can be applied to the activity and consequences of PR action in the developing mammary gland. Whilst this project has introduced many genes that might be important in establishing such sought-after signalling pathways, it has still only offered an introduction to the possibilities that they represent for progesterone-related molecular research thus more techniques and resources need to be employed to uncover the numerous molecular mechanisms in play within the reproductive tissues.

6. Appendix

Protocol for preparation of DEPC water for RNA extraction:

- This protocol for DEPC water used 0.2mls of DEPC per 100mls of water

  DEPC = Diethylpyrocarbonate

- A 500ml glass bottle was filled with 400mls of double distilled water using a 500ml graduated cylinder – room was left in the bottle as the volume may expand during autoclaving

- In a fume hood, 800µl of DEPC was added to the 400mls of double distilled water

- The bottle was shaken vigorously to ensure that the DEPC went into solution

- The bottle was then left overnight at room temperature

- The bottle was then autoclaved for 15 minutes

Protocol for preparation of 75% ethanol with DEPC

- This solution is to be made fresh for each RNA extraction, for this experiment a 100ml solution was made up

- In the fume hood, 75mls of 100% ethanol was measured using a 100ml graduated cylinder

- 25mls of DEPC water was then added to the graduated cylinder

- The solution was then transferred to a glass bottle and was thoroughly shaken

- The 75% DEPC ethanol was then stored in a fume hood until ready for use

- The glass bottle was clearly marked ‘RNA use only’ and was sprayed with RNaseZap

Protocol for preparation of a sample denaturing buffer i.e. orange buffer

- The solution preparation was carried out in a fume hood
    - 10X denaturing buffer 720µl
    - 98% formamide 3600µl
    - 37% formaldehyde 1170µl
    - Ethidium bromide (10µg/µl) 24µl
    - Double distilled water 490µl
- The above mix was then filter sterilised into a clean fresh 15ml Falcon tube
- The buffer was then stored at -20ºC, the solution however does not freeze

Protocol for preparation of RNA loading buffer

- 80% (v/v) formamide
- 1mM EDTA pH8.0
- 0.1% (w/v) bromophenol blue
- 0.1% (w/v) xylene cyanol

- The loading buffer was made up in a 0.5ml RNase free eppendorf tube and stored at 4ºC

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