HORMONE AND TRANSCRIPTION FACTOR REGULATION OF CYTOKINES IN THE MAMMARY GLAND

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DISCIPLINE OF SURGERY

SCHOOL OF MEDICINE

FACULTY OF HEALTH SCIENCES

THE UNIVERSITY OF ADELAIDE

15TH OCTOBER 2018

A thesis submitted to the University of Adelaide in fulfilment of the requirements for admission to the degree Doctor of Philosophy

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ABSTRACT

Increased number of menstrual cycles is associated with an increased lifetime risk for breast cancer, however the biological basis for this increased risk is not well understood. Previous research in mouse models suggest the immune microenvironment is critically regulated by fluctuations in circulating estrogen and progesterone across the menstrual cycle, which may affect breast cancer susceptibility. The work in this thesis aims to investigate hormonal regulation of transcription factors and cytokines that affect cells of the immune system in the mammary gland, using an array of approaches including primary human mammary epithelial organoid structures (n=6), human mammary epithelial cell lines (MCF7, T47D and ZR751), and mouse mammary gland tissues.

Firstly, primary mammary epithelial organoid cultures were treated with combinations of 17-beta estradiol and progesterone for 72 hours, and the abundance of messenger RNA encoding cytokines transforming growth factor beta 1 (TGFB1), tumour necrosis factor alpha (TNFA), signal transducer and activator of transcription 3 (STAT3), STAT5, interleukin-12 (IL12), E74-like factor 5 (ELF5), C-X-C motif chemokine ligand 12 (CXCL12), S100 calcium binding protein A8 (S100A8), S100A9, Forkhead box P3 (FOXP3), and Zinc Finger E-Box Binding Homeobox 1 (ZEB1) were analysed using real-time PCR. Moreover, lentiviral vectors were used to investigate the effects of FOXP3 overexpression on downstream cytokines in the human mammary epithelial organoids. In the second approach, human mammary epithelial cell lines were treated with combinations of 17-beta estradiol and progesterone at different time courses and the abundance of mRNA encoding the cytokines of interest was analysed using real-time PCR. Further, the expression of ELF5, a mammary epithelial morphogenesis transcription factor, was transiently silenced by small interfering RNA oligos in T47D mammary epithelial

cell lines to investigate the role of ELF5 in progesterone-mediated cytokine expression. Lastly, to investigate the effects of Foxp3 heterozygosity on mammary ductal morphogenesis, C57BL6 wildtype and Foxp3 heterozygous female mice were tracked over a period of 28 days by histological analysis of vaginal smears. The 4th pair of the mammary glands from each mouse was collected at each of the four stages of the cycle for cytokine expression studies and whole-mount analysis.

In primary organoid cultures, there was high variability in cytokine expression between patients; the only consistent result was that combined estradiol and progesterone treatment significantly attenuated TGFB1 mRNA expression ($p<0.05$). Also, overexpression of FOXP3 in these cells resulted in an insignificant increase in the mRNA expression of *ZEB1*. Hormone treatments of cell lines at different time courses resulted in different expression of mRNA encoding the cytokines of interest. However, silencing of the *ELF5* gene in T47D cells resulted in induced mRNA expression of *S100A9* and *CXCL12* by 50% compared to non-silenced cells ($p<0.05$). Finally, analysis of whole-mount images revealed that mammary ductal morphogenesis in Foxp3 heterozygous mice was similar to that of wildtype mice.

These results suggest that estrogen and progesterone variably regulate the cytokine synthesis by mammary epithelial cells, depending on the hormone receptor profile of the cells. In this case, ELF5 transcription factor might moderate the effects of progesterone on pro-inflammatory cytokines. Moreover, it seems that Foxp3 heterozygosity does not have any significant effect on mammary glands morphogenesis in mice.

DECLARATION

I, Vahid Atashgaran, declare that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university and that to the best of knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text. I also certify that no part of this work will be used in a submission in my name, for any other degree in any other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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ACKNOWLEDGEMENTS

Foremost, I would like to thank all the patients and surgeons at The Queen Elizabeth Hospital who kindly provided the tissue samples for my research and I am grateful to the School of Medicine at the University of Adelaide for all their support in the past four years. I have been fortunate to receive Adelaide Graduate Research Scholarship to support me financially throughout my PhD studies.

I would like to express my sincere gratitude to my principle supervisor Associate Professor Wendy Ingman for her motivation and immense knowledge throughout the years. Her guidance helped in all aspects of my research project and her patience and support gave me confidence in working in the laboratory and learning different skills. I would like to thank my co-supervisor Dr Pallave Dasari for the mentoring and technical expertise that I received. She gave me sound advice and great ideas about my future career and helped me improve my communication and networking skills. I would also like to thank my other co-supervisor Associate Professor Simon Barry for his time and great technical advice during my candidature.

My sincere thanks also go to the Breast Biology and Cancer Unit, in particular to Leigh Hodgson and Joe Wrin for their assistance with immunohistochemistry and Western blot techniques. I would like to extend a gracious thank you to Dr Dinny Graham for her time and help associated with this project. My special friend Dr Mahnaz Ramezanpour, helped me with some technical aspects of the project and supported me through all the problems I faced with. Harshani Pedige, Niko Mitkas, and Aneta Zysk also deserve an enormous thank for their invaluable help and support throughout these years. I wish to thank Sue Lester and Stuart Howell who helped me with the statistical analysis of data and advised me on my project.

Last but not the least; I want to thank my lovely family for their great support, encouragement and unconditional love. I could not have persisted with and completed my studies without them. In particular, my late father, who always supported me and believed in my ability to be successful in life. I am where I am because of him and I am sorry that he has not lived to see me graduate.

PUBLICATION ARISING FROM THIS THESIS

(See Appendices)

Atashgaran, V., et al. (2016). "Dissecting the Biology of Menstrual Cycle-Associated Breast Cancer Risk." Frontiers in Oncology 6: 267.

ABBREVIATIONS

Bp: Base pair

CXCL12: C-X-C motif chemokine 12

DAB: 3,3 diaminobenzadine

DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride

ELF5: E74-Like ETS Transcription Factor 5

ER: Estrogen receptor

FKBP51: FK506-binding protein 51

FOXP3: Forkhead box P3

HRP: Horseradish peroxidase

IL12: Interleukin-12

Kb: Kilo base

MPRL19: Homo sapiens mitochondrial ribosomal protein L19

mRNA: Messenger Ribonucleic acid

PGR: Progesterone receptor

qRT-PCR: Quantitative Real-time Polymerase Chain Reaction

siRNA: Small Interfering Ribonucleic Acid

STAT3: Signal transducer and activator of transcription 3

STAT5: Signal transducer and activator of transcription 5

TEB: Terminal End Bud

TGFB1: Transforming growth factor beta 1

TNFA: Tumour necrosis factor alpha

CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

Breast cancer is a complex disease characterized by abnormal growth of cells, leading to invasion into the surrounding tissue, metastasis to distant sites in the body and, at times, death. It is the most common cancer diagnosed in females; in Australia, 1 in 8 women will be diagnosed with breast cancer before the age of 85 (1). A number of risk factors are associated with breast cancer, including family history of breast cancer, increasing age, high breast density, and increased number of years of menstrual cycling (1-3). In the past, breast cancer was known as "The Nun's Disease" because Catholic nuns had more prevalence and higher death rates from it compared to ordinary women (4). This observation was initially linked to the marital status and the presence or absence of sexual and reproductive factors. However, significant number of epidemiological studies have found strong correlations between cumulative number of menstrual cycles and the development of breast cancer in women (1, 5, 6). For example, for each year younger a girl commences menstrual cycling, there is a 5% increase in lifetime risk of breast cancer. Similarly, for each year older at the time of menopause, there is a 3.5% increased breast cancer risk (6). This indicates that fluctuations in ovarian hormones associated with menstrual cycling affect breast cancer susceptibility. However, the biological basis for this increased risk is not well understood.

1.2. OVERVIEW OF MAMMARY GLAND DEVELOPMENT

The mammary glands are bilateral organs present in females of all mammalian species which produce milk essential for the nourishment of infants. It is a unique organ, in which the vast

majority of mammary gland development occurs postnatally, during puberty, pregnancy, and the postpartum period. This development is regulated through complex interactions between hormones, growth factors, and cytokines.

The mammary gland is composed of a number of different cell types that together form complex interactive networks required for the normal development and function of the tissue (7). Morphologically, there are numerous lobules connected to the nipple through a series of branched ducts (2) (Figure 1.1). The lobules contain epithelial structures surrounded by mammary stroma that provide physical support for the overall architecture of the ductal epithelium. At the histological level, the epithelial structures are comprised of two types of mammary epithelial cells: myoepithelial cells found as a thin layer above the basement membrane, and luminal epithelial cells that produce milk during lactation. These different types of mammary epithelial cells are organised together to form the ductal structures of the mammary gland. It is important to note that the majority of breast carcinomas originate from mammary epithelial cells (8). The stroma of the mammary gland supports epithelial cell morphogenesis and function and is comprised of an extracellular matrix containing an abundance of collagen fibres, as well as fibroblasts, endothelial cells, macrophages, T lymphocytes and other immune cells (7) (Figure 1.2).

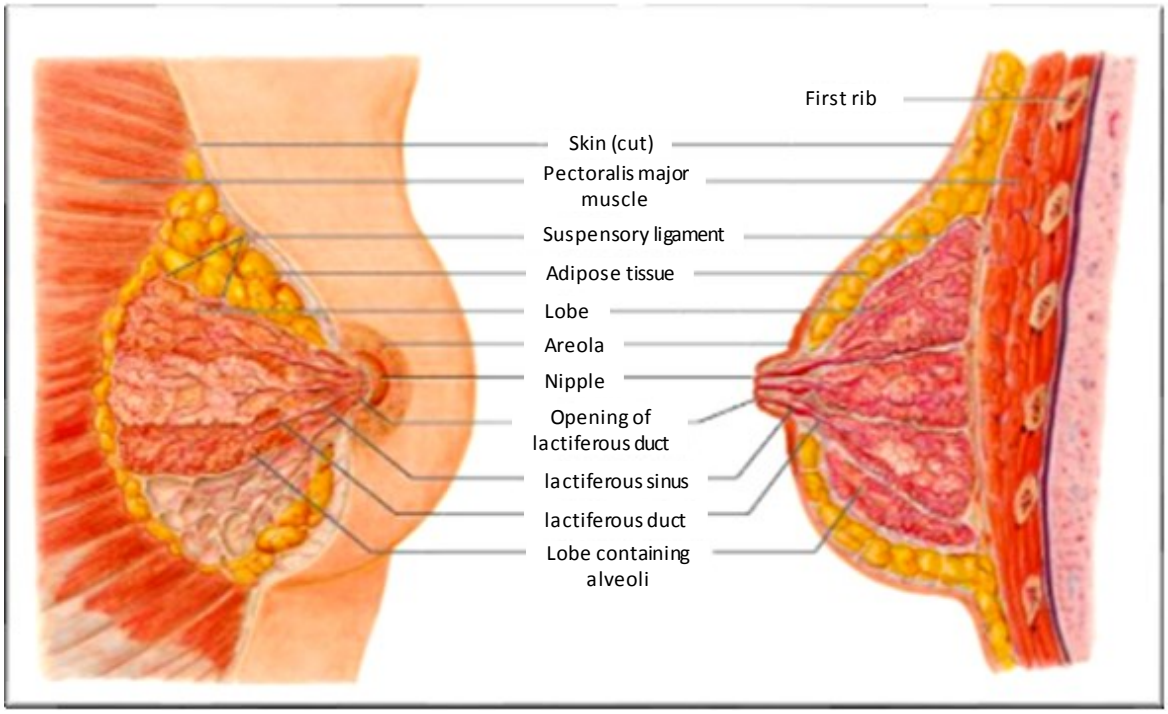


Figure 1.1: Diagrammatic representation of the main architectural parts of normal mammary gland in human female (9).

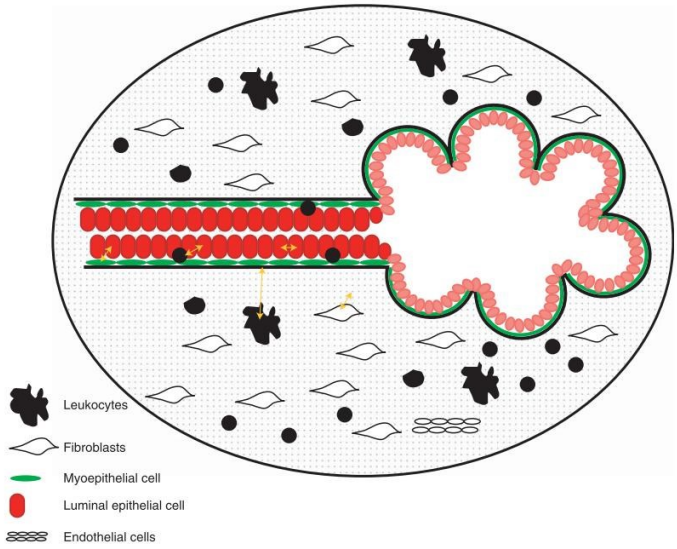


Figure 1.2: Schematic of normal mammary duct and lobule supported by their stroma (7).
Myoepithelial cells in the duct are located as a complete layer around the luminal epithelial cells, whereas they form a more fenestrated layer in the alveoli. The yellow arrows represent potential interactions between the different cell types and/or their surrounding matrix, important to support mammary gland morphogenesis.

1.3. MAMMARY GLAND DEVELOPMENT

At birth, the structure of the mammary gland is a simple rudimentary epithelial duct present in both female and male infants. With the onset of puberty, the surge of estrogen in females drives the primary epithelial ducts to proliferate and develop into club-shaped structures known as terminal end buds (TEBs) (2). Further proliferation of these structures gives rise to smaller projections called alveolar buds that form bundles eventually becoming organized into a sphere-like structure termed a lobule. This process of pubertal development occurs slowly, with lobules developed over the course of two or more years.

During adulthood, the lobules can further differentiate into four types; type I and II lobules are branching primary and secondary ducts with minimal alveolar budding, type III lobules have greater abundance of alveolar buds sprouted into tertiary structures, and type IV lobules which form during pregnancy, and have the highest intensity of budding and lobule formation. By the end of pregnancy, the breast tissue is considered fully developed, enabling commencement of lactation. Upon weaning of the infant, the mammary epithelial cells undergo programmed cell death (i.e. apoptosis) causing the mammary gland to remodel back to its basic architecture (Figure 1.3) (2, 10).

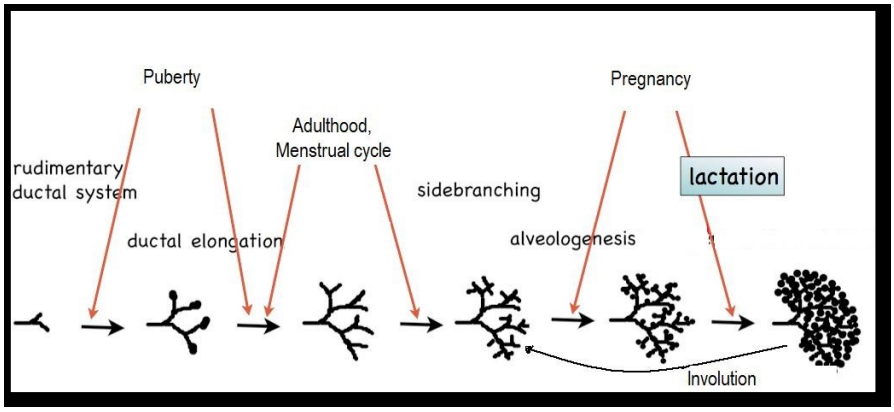


Figure 1.3: Schematic representation of mammary gland development during different stages (11). The structure of mammary gland is a simple rudimentary epithelial duct observed at birth, which proliferates and undergoes dramatic morphological changes during different stages of life.

1.4. THE MENSTRUAL CYCLE AND MAMMARY GLAND

While there has been much interest in the cellular and molecular interactions directing mammary gland development during pregnancy, surprisingly little is understood of the biological mechanisms that promote development during the menstrual cycle. Although the developmental changes that occur during the menstrual cycle are less dramatic than those during pregnancy, understanding the mechanisms that regulate these developmental processes will shed light on the increased breast cancer risk associated with increased number of years of menstrual cycling. It is suggested that ovarian hormone-regulated proliferation of mammary epithelial cells with each successive menstrual cycle causes increased likelihood of progressive somatic mutations occurring in the cell-lineage, leading to increased risk of breast cancer (12, 13). However, what is less extensively investigated is the possible hormone-dependent role of cell-to-cell interactions between epithelium and the surrounding stroma in affecting DNA mutation rate and the DNA damage response. Therefore, understanding the cellular interactions that regulate mammary epithelial morphogenesis during the menstrual cycle may reveal new strategic directions to treat or prevent breast cancer.

1.4.1. THE MENSTRUAL CYCLE

Hormonal fluctuations over the course of the menstrual cycle in women are necessary to prepare the mammary gland for pregnancy and lactation. The duration of the menstrual cycle is 28 – 32 days (14) and can be categorized into three main phases:

1- The Follicular Phase: Immature oocytes in the ovaries, each within a sac-like structure called a follicle, grow as a result of hormonal secretions from the pituitary gland. One of these follicles become dominant and continues to mature.

2- The Ovulatory Phase: The fully developed dominant follicle releases its mature oocyte into the fallopian tube.

3- The Luteal Phase: Following ovulation of the mature oocyte, inhibitory factors present in the follicle dissipate, and the remnant follicle differentiates into a structure known as the corpus luteum. This structure produces and secretes progesterone in preparation for implantation (15). If pregnancy does not proceed, the corpus luteum begins to break down, and declining progesterone secretion initiates menstruation and the next follicular phase.

The phases of the menstrual cycle are driven by interactions between the pituitary gland hormones, follicle-stimulating hormone and luteinizing hormone, and the ovarian hormones, estrogen and progesterone. During the follicular phase, follicle-stimulating hormone stimulates follicle development and oocyte maturation. When the oocyte is nearly matured, the high concentration of circulating estrogen produced by the dominant follicle stimulates the pituitary to produce luteinizing hormone. This weakens the wall of the mature follicle and causes it to release the mature oocyte into the fallopian tubes (15).

Clinical studies on premenopausal women indicate that the concentration of circulating estrogen increases during the follicular phase and is reduced during the luteal phase until menstruation, where the lowest serum level of estrogen is observed (16, 17). During the follicular phase, circulating progesterone concentration is low, and begin to rise following ovulation. In the luteal phase, the corpus luteum secretes progesterone and the circulating concentration of progesterone continues to rise, peaking in the mid-luteal phase. If implantation does not occur, progesterone decreases gradually towards the end of the luteal phase. Circulating concentration of estrogen and progesterone are at a minimum during menstruation (16, 18). Figure 1.4 illustrates the hormonal changes during different stages of the menstrual cycle and categorizes the events occurring in the breast, ovaries, and uterus.

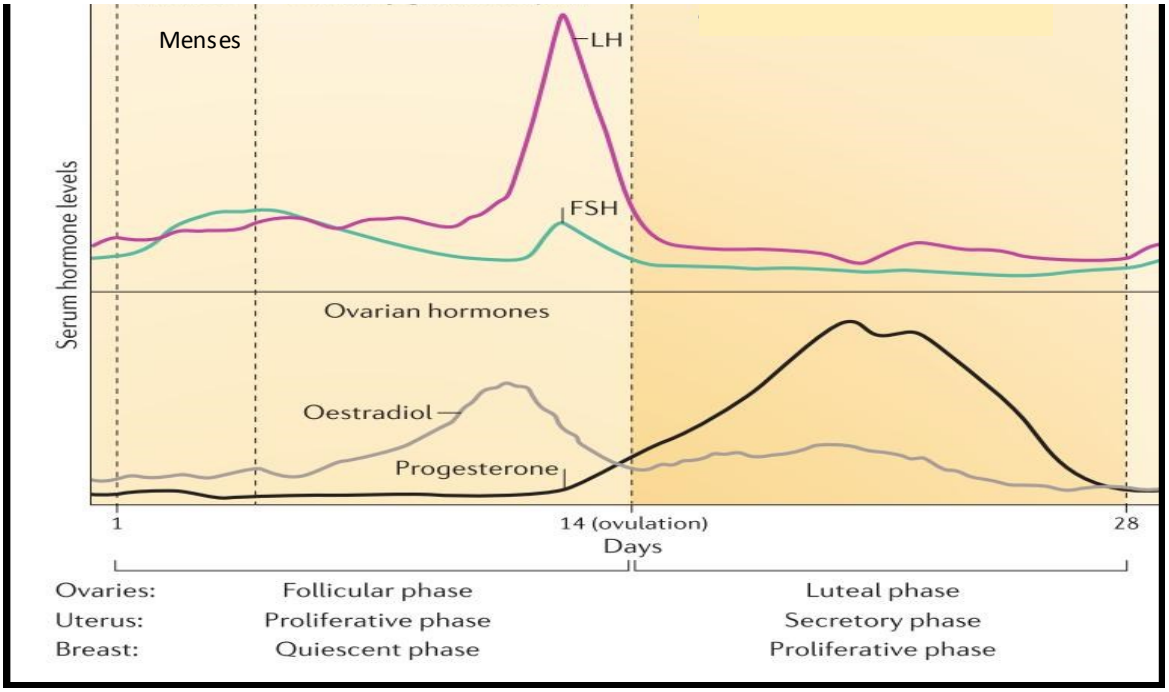


Figure 1.4: Serum hormone fluctuations during a typical menstrual cycle along with changes in the breast and uterus (16).

Fluctuations in the ovarian hormones, estrogen and progesterone across the menstrual cycle are associated with morphological changes in the breast tissue. Note that, the proliferative phase on the uterine cycle is considered as the time the endometrium builds up before ovulation. However, in the breast, cell proliferation occurs post ovulation and during luteal phase of the menstrual cycle.

1.4.2. CHANGES IN MAMMARY EPITHELIAL CELLS DURING THE MENSTRUAL CYCLE

Fluctuations in circulating estrogen and progesterone during the menstrual cycle affect mammary gland epithelial cell morphology. Mammary epithelial cell proliferation is mediated by estrogen and progesterone in the early to mid-luteal phase, whilst declining progesterone drives epithelial cell apoptosis and mammary gland regression in the late luteal phase (12). Progesterone is the key hormone likely to promote mammary gland development, as a murine study conducted by Fata, Chaudhary (19) demonstrates that epithelial cell proliferation has a positive correlation with the serum concentration of progesterone, but not estrogen. Similarly, Chua, Hodson (20) demonstrated that during the ovarian cycle there is a positive correlation between the percentage of alveolar epithelial ducts in the mammary gland and the concentration of serum progesterone, but not with serum estradiol.

Analysis into the effect of the different stages of the estrous cycle on development of alveolar epithelial cells in the mammary glands of mice found that the highest percentage of alveolar epithelium were observed during the diestrus phase, where the concentration of serum progesterone is maximal (Figure 1.5). Furthermore, a progesterone receptor knockout mouse model suggested that progesterone receptor expressed by mammary epithelial cells is necessary for epithelial cell proliferation (21). The role of estrogen in the mammary gland during the menstrual cycle appears primarily to be to upregulate expression of the progesterone receptor (18). Conversely, epithelial cell apoptosis is greatest during the menstrual phase with rapidly decreasing estrogen and progesterone levels (18).

Several histological studies have demonstrated differences in breast tissue morphology during different stages of the menstrual cycle in women (22-24). In a study of 30 women with healthy breast tissue, the epithelial and myoepithelial layers were observed as two moderately separate

layers during the follicular phase, whereas in the luteal phase there was a prominent distinction between epithelial and myoepithelial layers; this suggests that the architecture of the breast tissue was more developed during the luteal phase (23). This study also reported that the highest level of mitotic and apoptotic activity in mammary epithelial cells occurred during the luteal phase of the cycle. However, not all literature is consistent with this observation. Vogel, Georgiade (25) reported that the highest level of mitotic and apoptotic activity was observed during the follicular phase (days 8-14), while there were high levels of apocrine secretions during the luteal phase (days 15-20).

Fluctuations in hormones and mammary gland morphology associated with menstrual cycling are indeed cyclical in nature, and occur continuously, such that each cycle merges into the next. There are also degrees of variability between different women. This makes it difficult to match the morphological stage with the exact date or phase of the menstrual cycle (23). Consequently, the literature offers some conflicting results about the correlation of mammary gland histological characteristics with the different stages of the menstrual cycle. However, the majority of the literature on menstrual cycle-associated changes in women is consistent with mouse literature, and suggests that the main proliferative phase is the early luteal phase of the menstrual cycle, during which time circulating progesterone and estrogen are both high, and epithelial alveolar buds begin to form. The high level of mitotic activity in this phase suggests progesterone is associated with a proliferative action (22). Conversely, the late luteal phase or the menstruation phase could be considered as the regression phase of mammary gland epithelium. During this time, the concentration of circulating ovarian hormones decrease significantly and the newly formed alveolar buds undergo apoptosis and the breast tissue reverts to its basic architecture (20).

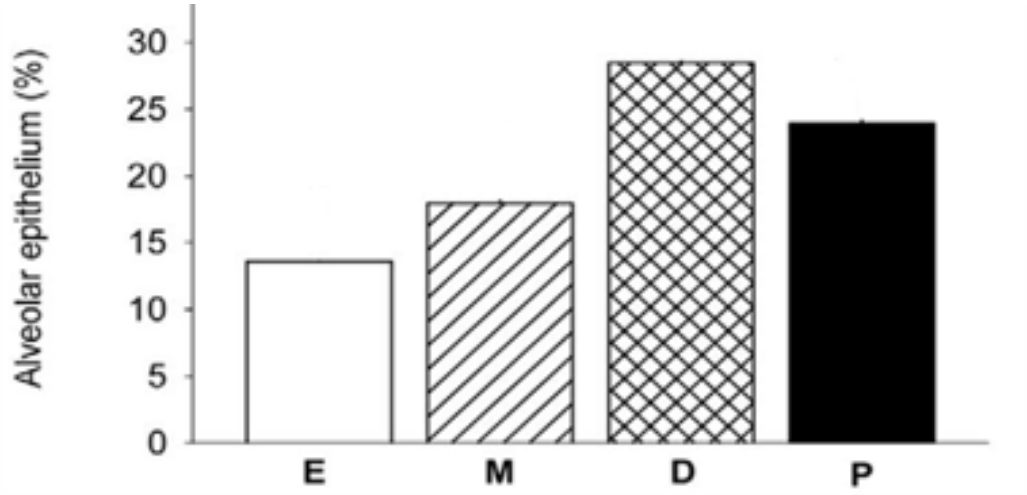


Figure 1.5: The percentage of development of alveolar epithelial cells in the mouse mammary gland during estrous cycle (20).

The percentage of alveolar epithelial cells fluctuates across the course of estrous cycle and it is correlated positively with serum progesterone levels. The highest levels of alveolar epithelial cells are found at diestrus compared to other phases of the cycle (E, estrus; M, metestrus; D, diestrus; P, proestrus).

1.5. THE IMMUNE MICROENVIRONMENT IN THE MAMMARY GLAND

It is well established that mammary gland development and function is dependent on the dynamic interactions between hormonally responsive mammary gland epithelium and the immune microenvironment in the stroma. Immune cells localise to different sites during the various stages of mammary gland development and contribute to multiple effector functions (26) and may also affect cancer risk and development. According to Hanahan and Weinberg (27), there are ten hallmarks, or vital biological processes, required for cancer to be established and metastasize into other tissues. Two hallmarks are related to the immune system; they are 1) evading the immune detection and destruction and 2) promotion of tumour growth by inflammation (28). Indeed, the failure of immune cells in recognition and elimination of transformed cells throughout life can lead to cancer development. In the mammary gland, the immune cells primarily present are macrophages, mast cells, eosinophils, and T cells, which together have essential roles in hormonally-driven mammary gland development (29), and may also promote or protect the breasts against cancer (30). Considering their roles in the immune evasion of the tumour in the breast (31) and their crosstalk with mammary epithelial cells, it is possible that these cells affect menstrual cycle-associated breast cancer risk.

1.5.1. MACROPHAGES

Macrophages are immune cells involved in the generation and execution of immune responses. However, over the past 20 years, new roles for macrophages in developmental processes have been discovered. These functions in development appear to be particularly significant in reproductive tract tissues, which undergo considerable morphological changes over the course of adult life (32). In the mammary gland, macrophages are a significant component of the stroma and have diverse roles in cell proliferation, phagocytosis, and tissue remodelling (20). There is high plasticity in macrophage phenotypes to accomplish specific immunological and

developmental requirements in different tissues. Activated macrophages are classified into classically activated macrophages and alternatively activated macrophages, each of which respond to and produce specific cytokines and have particular functions within the tissue (32). Classically activated macrophages function as part of cell-mediated immune system responses, which are involved in host defense against intracellular pathogens and produce pro-inflammatory cytokines. Alternatively activated macrophages produce anti-inflammatory cytokines and have roles in tissue repair, immune tolerance, and wound healing (33).

Due to their high plasticity, macrophages can change from one class to another class dependent on certain signals within the microenvironment (34). Previously it was thought that macrophages only protect the tissues from cancer by phagocytosing the apoptotic cell debris or presenting tumour-associated antigens to T cells. However, more recent studies suggest that these cells can also be involved in breast tumorigenesis, progression and metastasis, depending on their functional phenotype (35, 36).

Macrophages are required for normal ductal epithelial development, and they have been found to be in direct contact with ductal and alveolar mammary epithelium, suggesting that there is a paracrine singling network between these cell types (20, 37-39). Also, the number of macrophages fluctuates over the course of the estrous cycle in mice; increasing at metestrus, peaking at diestrus, and decreasing rapidly at proestrus (20, 40). As mammary gland macrophages are physically associated with hormonally responsive mammary epithelial cells, ovarian hormones are likely to indirectly affect the function of macrophages through the production of cytokines by mammary epithelial cells. Therefore, it is important to understand the cytokine microenvironment that directs macrophage function within the mammary gland.

1.5.2. T LYMPHOCYTES

T lymphocytes are another class of immune cell that play central roles in cell-mediated immunity through cytokine signals and cell-to-cell interactions, and mediate humoral and immunoregulatory immune responses. T cells express unique receptors (known as TCRs) on their surface and are subdivided into different groups based on their lineage markers and functional activities (41). A major T cell lineage includes T helper cells (i.e., identified by surface cluster of differentiation CD4), which can differentiate into various effector subsets such as Th1, Th2, Th17 and regulatory T cells (Tregs), based on the signals that they receive from particular cytokines. The different subsets of Th cells have very different functions, they can promote or inhibit inflammation, or dampen the immune response. Recent studies illustrate the importance of T cells and their mediators in various stages of mammary gland development (42, 43); however, studies into their function in the mammary gland are limited.

Cytokines usually associated with different T cell responses appear to be involved in mammary development associated with lactation. Induction of mammary epithelial cell differentiation to milk-secreting cells is accompanied by a switch from production of Th1 cytokines (such as TNFA, IFNG, and IL12) to Th2 cytokine (such as IL4, IL10, and IL13) by mammary epithelial cells (42). Interestingly, progesterone has been shown to regulate Th1/Th2 phenotypes in the mammary gland (44). Th1 cytokines are more effective in producing antitumor immunity and tumour rejection, whereas Th2 cytokines are mostly produced by tumours and they are involved in increasing humoral protumouringenic responses (45, 46). Therefore, an imbalance of normal Th1/Th2 ratios in the mammary gland microenvironment could cause a major dysfunction in the cytotoxic T cell responses to foreign invaders, hinder immune surveillance, and promote tumour growth.

1.5.3. REGULATORY T CELLS (TREGS)

Tregs play critical roles in the prevention of autoimmunity, dampen excessive inflammation, downregulate the amplitude of an immune response, and regulate immunological tolerance (41, 47, 48). Naturally arising Tregs develop in the thymus and account for 1-2% of peripheral CD4+ T cells in healthy humans (49). However, these cells are also present in non-lymphoid organs, and they are postulated to mediate suppressive mechanisms during mammary gland development.

It has been suggested that estrogen and progesterone promote immune suppression via Tregs and cytokines (50, 51), which may assist incipient tumours evade immune detection. Exogenous estradiol promotes proliferation of T cell receptor-activated Tregs isolated from healthy individuals and enhances their suppressive function *in vitro* (50). Moreover, progesterone induces naïve T cells to differentiate into immune suppressive FOXP3+ Tregs and promotes immune tolerance in foetal cord blood (52). However, Tregs require activation before hormones can enhance their suppressive functions (50, 53). It is far from clear what stimuli activate Tregs during the menstrual cycle; however, infections or altered cell signalling pathways may play a role.

In 2007, Arruvito, Sanz (53) stated that Treg abundance in the peripheral blood of healthy women increases during the follicular phase of the menstrual cycle, correlates with serum estrogen concentration, and decreases dramatically during the luteal phase. Therefore, fluctuations of the ovarian hormones over the course of the menstrual cycle could affect the modulation of tolerance by Tregs and impose immunosuppressive effects in the mammary gland microenvironment, increasing the risk of breast cancer development.

1.5.4. MAST CELLS AND EOSINOPHILS

Other type of immune cells that contribute to mammary gland development and homeostasis are eosinophils and mast cells (29). Eosinophils are phagocytic leukocytes implicated in combating multicellular parasites, helminth infections, and allergic reactions (54). Mice genetically deficient in eosinophils exhibit mammary gland retardation, and have altered estrous cyclicity (55). In the rat uterus, the abundance of eosinophils fluctuates across the estrous cycle with the lowest abundance observed during diestrus, and the greatest abundance during the estrus phase (56).

Mast cells are tissue-resident leukocytes that play roles in allergic reactions, wound healing, inflammatory disorders, and immune tolerance (57). These cells are present in mouse mammary stroma at all stages of mammary gland development, regulate mammary epithelial ductal branching during puberty, and are localized around the ductal epithelium and the TEBs (58). An increase in the number of mast cells is linked to mammary epithelial lobule regression during estrous cycling and involution (59). Like eosinophils, the abundance of lobule-associated mast cells is also hormone-dependent and fluctuates in cycling rats (59).

Although eosinophils and mast cells are required for expansion of mammary epithelial cells in rodents, there is little known about their roles in the human breast. Moreover, both of these cell types are capable of producing inflammatory cytokines and chemokines that contribute to the metastatic potential of tumours. Therefore, it would be of interest to understand the associations of eosinophils and mast cells in human mammary gland during the menstrual cycle, and whether they are linked to breast cancer risk during this period.

1.6. KEY TRANSCRIPTIONAL REGULATORS OF MAMMARY GLAND DEVELOPMENT AND FUNCTION

The mammary gland has adopted a number of coactivators, transcription factors, and signalling pathways for its development and function. Through experimental mouse genetics and mammary gland transplantation techniques, researchers have identified some of the key signalling molecules that function along with the ovarian hormones to promote and regulate mammary epithelial cell proliferation, differentiation and regression (60). Of particular significance are the signal transducer and activator of transcription (STAT) family of proteins which play a wide range of functions in mammary gland development and activate diverse genetic programs (61, 62). STATs usually reside in the cytoplasm where they can be activated by tyrosine phosphorylation, dimerize and then translocate to the nucleaus. In the nucleus, they act as transcription factors by binding to DNA in order to regulate gene transcription (63). Two highly homologous STAT family members, STAT5 and STAT3 are essential for mammary alveolar development and tissue remodelling (64). Conditional knockout studies in mice showed that STAT5 is essential for proliferation and differentiation of mammary epithelium, and that its loss in differentiated alveolar cells causes rapid cell death (64, 65). On the other hand, STAT3 plays a pivotal role in mammary gland involution by inducing mammary epithelial cell death, removing the apoptotic cells, and regulating the immune cell microenvironment within the mammary gland (62, 66, 67).

STAT3 and STAT5 are activated by estrogen and progesterone and they are often inappropriately activated in a variety of human malignancies, including breast cancer (68-70). These proteins are progesterone-dependent as their expression has been found to be induced by progesterone in human and mouse breast cancer cells in a PR-dependent manner (69, 70). Proietti, Salatino (71) noted that transcriptional activation of STAT3 is essential for progestin-stimulated breast cancer growth *in vitro* and *in vivo*. However, estrogen is known to have

inhibitory effects on IL-6 induced STAT3 activation in breast cancer cells, an effect that is reversible with ER antagonist, Tamoxifen (72). Considering their essential roles in mammary epithelial cell proliferation and regression as well as in breast cancer, they are ideal candidates for studying the effects of estrogen and progesterone on mammary epithelial cell function. Nonetheless, the activity of these transcription factors is not only regulated by hormones, but other transcription factors and cofactors could also be involved. For example, the activity of STAT5 in governing the mammary alveolar differentiation program is suggested to be mediated by E74-like factor 5 (ELF5) transcription factor (73).

Recent studies suggest that transcription factors are critical elements in controlling the function of immune cell signalling and they may affect complex interactions between immune cells and the hormonally-regulated network of epithelial cells in the breast (74). It is not clear how transcription factors contribute to epithelial-stromal crosstalk in the breast and affect menstrual cycle-associated breast cancer risk. However, it is likely that alterations of any cellular events in the mammary gland affect the proliferation rate of mammary epithelial cells which might increase DNA mutations and consequently lead to breast cancer in women. In the current study, we will utilise a variety of approaches *in vitro* and *in vivo* to investigate the expression of specific transcription factors in the mammary epithelium to investigate whether they are hormonally regulated and their relationship with the immune microenvironment.

1.6.1. ROLE OF ELF5 IN MAMMARY GLAND DEVELOPMENT AND BREAST CANCER

ELF5 (also known as ESE-2) is an epithelial cell-specific member of ELF subfamily of Ets transcription factors, found in the lung, kidney, placenta, and most prominently in the mammary gland (75, 76). In the mammary gland, ELF5 has roles in mammary epithelial cell proliferation and promotes transcription of genes involved in alveolar morphogenesis (77). ELF5 specifies

alveolar cell fate and is mainly expressed by the luminal progenitor cells in the mammary gland (78). Studies in Elf5 null mutant mouse models demonstrate that these animals are either unable to lactate due to failed alveolar development, or exhibit impaired functional secretory units due to improper differentiation of alveoli. This indicates that ELF5 is a crucial transcriptional mediator required for structural and functional morphogenesis of lobuloalveoli (77).

The role of ELF5 in breast cancer is controversial. It has been suggested to be either a tumour suppressor gene (79, 80), suppressor of epithelial-to-mesenchymal transition (EMT) and breast cancer metastasis (81), or even a mediator of mammary tumour's metastasis into the lungs in mouse models (82). The chromosome on which ELF5 is located (human chromosome 11p 13-15) is known to have a loss of heterozygosity in some breast cancer cases (83). Moreover, high expression of ELF5 correlates with more aggressive basal cancers and resistance to anti-estrogen cancer therapies (84).

ELF5 is a direct transcriptional target of the progesterone receptor, and its expression is increased by progestin treatment *in vivo* in mice and *in vitro* in T47D human breast cancer cell lines (85). Little is known of the role of this transcription factor in directing epithelial cell-specific cytokine secretion. However, ELF5 is essential for progesterone-mediated RANK ligand production (75). RANK ligand is a tumour necrosis factor-like cytokine that promotes alveolar development during the ovarian cycle and pregnancy and is responsible for progestin-mediated mammary cancer risk in a mouse model (86). Hence, ELF5 can be considered an essential mediator of progesterone-regulated mammary epithelial cell proliferation and differentiation and might affect cancer susceptibility through the production of cytokines that affect interactions between the mammary epithelium and surrounding immune cell populations.

1.6.2. ROLE OF FOXP3 IN MAMMARY GLAND DEVELOPMENT

The Forkhead box 3 (FOXP3) gene is located at the short arm of the X chromosome, region 11.23 and contains 11 coding exons and three non-coding exons (87). FOXP3 is a member of forkhead/winged family of transcription factors and functions as an essential regulator of CD4+CD25+ Tregs development (88). Thymic CD4+CD25+ T cells express FOXP3 and become Tregs in order to temper immune responses (47, 48). Null mutation in FOXP3 in humans leads to a deficiency in the population of CD4+CD25+ Tregs, which leads to severe inflammation. This manifests as an X-recessive autoimmune disease called immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) in males (89). These patients develop enteropathy, dermatitis, thyroiditis, and nail dystrophy, and usually die in the first 1-2 years of life due to severe infections (90). An analogous disease develops in male scurfy mice ($Foxp3^{sf/Y}$) which are characterised by scaly and ruffled skin, reddened eyes, enlarged spleen and lymph nodes, and these mice die approximately 3-4 weeks after birth (91, 92).

FOXP3 is predominantly expressed in the thymus and the spleen from where Tregs are derived. However, some reports have shown its expression in the epithelial cells of specific tissues such as the breast, lung, and prostate (93, 94). The expression of FOXP3 in the epithelium suggests that it may play a broad function outside of Tregs. Although epithelial-intrinsic function of FOXP3 has not been well-investigated, most studies suggest that FOXP3 acts as a tumour suppressor gene in breast cancer (94-96). As a transcription factor, it can bind to approximately 700 genes (97) and act as either a transcriptional repressor (e.g. by directly repressing the S-phase kinase-associated protein 2 (SKP2) and HER2 oncogenes (95, 98), or a transcriptional activator (e.g. by maintaining the expression of the p21 tumour suppressor in the mammary epithelium) (99).

In 2007, Zuo, Wang (94) analysed the expression of FOXP3 in normal and cancerous tissues and noted its expression to be present in only 20% of human breast cancer samples (mostly the HER2− or ER+ phenotype), whereas 80% of normal breast tissues expressed this protein. These researchers also found that Foxp3 heterozygous female mice develop mammary carcinomas spontaneously at a high rate as they age (94). In addition, when Foxp3 heterozygous female mice were challenged with the chemical carcinogen DMBA in conjunction with progesterone, a significant increase in the susceptibility to mammary cancer development was observed (94). Further studies in human breast cancer samples showed that a high rate of FOXP3 somatic mutations in breast tumours (100, 101), suggesting that FOXP3 defects play a role in breast cancer susceptibility.

The biological function of FOXP3 in the mammary gland and whether it is regulated by estrogen and progesterone is not well-investigated. However, *in vivo* and *in vitro* studies on mouse models showed that estrogen at physiological doses expands the number of CD4+CD25+ T cells in different lymphoid tissues in estradiol-treated ovariectomised mice and that it induces the expression of FoxP3 gene (102). Therefore, it is possible that hormone regulation of FOXP3 expression in the mammary gland is associated with breast cancer risk.

In the study by Zuo, Wang (94), 40% of aged Foxp3 heterozygous mice with breast cancer developed lung metastasis, suggesting that Foxp3 is involved in metastatic mechanisms (94, 103). Also, using human and mouse breast cancer cells, Zhang, Zhang (103) found that FOXP3 regulates the transcriptional activity of mircroRNA (miR)-200s, which are promising biomarkers of breast tumour progression and metastasis. On the other hand, transcription factor Zinc Finger E-Box Binding Homeobox 1 (ZEB1), which promotes the EMT in various human tumours (104, 105), has been shown to inhibit the transcription of miR-200 clusters (105, 106). Since FOXP3 and ZEB1 are linked together through the regulation of miR-200, we hypothesise

that regulation of FOXP3 by ovarian hormones could influence the expression of ZEB1in the mammary epithelial cells.

1.7. CYTOKINE NETWORKS IN MAMMARY GLAND IMMUNE MICROENVIRONMENT

The events involved in mammary gland development require not only steroid hormones but also signalling by an array of other proteins, including cytokines (107). Cytokines are a group of small proteins (5-20 kDa) that are involved in cell signalling processes, cell fate determination, and act as intercellular mediators in generating immune responses. These molecules are usually secreted transiently and locally and exert their effects in a paracrine or autocrine manner (108). In the mammary gland, cytokines are produced and secreted by a broad range of cells, including epithelial cells, fibroblasts, adipocytes, macrophages or other immune cells (74). More importantly, some of these cytokines have been ascribed to be involved directly in mammary gland development and tissue remodelling during different stages of development (55, 74, 109).

The cytokine microenvironment in the mammary gland can be affected by estrogen and progesterone during different stages of development (39). The function of these cytokines can be pro-inflammatory, anti-inflammatory, tissue growth promoting, or immunoregulatory. Pro-inflammatory cytokines promote systemic inflammation, while anti-inflammatory cytokines are immunoregulatory proteins that limit pro-inflammatory cytokine responses (110). In a study on ovariectomised mice, Dasari and colleagues reported that the concentration of specific pro-inflammatory cytokines fluctuates in the mammary gland across the estrous cycle and is hormonally regulated. They observed that estradiol induces cytokine production in the mammary gland during the estrus phase, which is actively suppressed by progesterone during the other phases of the cycle (111). They suggested that fluctuations in the cytokine

microenvironment in the mammary gland regulates the phenotypes of resident leukocytes and affect mammary gland tumour susceptibility (111).

In order to assess the effect of menstrual cycle hormones on the immune microenvironment and breast cancer risk, we have identified leading cytokine candidates to investigate in *in vitro* and *in vivo* studies. These candidate cytokines are expressed by mammary epithelial cells and have known roles in breast cancer development and progression. These cytokines are: Transforming Growth Factor Beta 1 (TGFB1), Tumour Necrosis Factor Alpha (TNFA), Interleukin 12 (IL-12), Chemokine C-C receptor ligand 12 (CXCL12), S100A8 and S100A9.

TGFB1 is an important multifunctional cytokine that has roles in a diverse range of processes including mammary epithelial cell proliferation, differentiation, apoptosis, and immune system responses and affects local mammary macrophage population (112, 113). TNFA is a pro-inflammatory cytokine produced by many cell types including mammary epithelial cells and is proposed to play a role in mammary ductal branching morphogenesis (114, 115). The serum levels of IL12, which is the main regulator of Th1 differentiation is higher in breast cancer samples and correlates with tumour progression (116). CXCL12 is a homeostatic chemokine for lymphocytes and monocytes and has roles in breast cancer progression (117, 118). It is primarily expressed in hormone receptor-positive luminal cells (119) and its expression is strongly upregulated in estrogen-treated MCF7 breast cancer cell lines (120). The calcium-binding proteins S100A8 and S100A9 are expressed by neutrophils, macrophages, and activated monocytes (121) and their expression is associated with estrogen receptor loss in breast cancer cell lines (122). S100A8 and S100A9 are overexpressed in breast cancer and regulate inflammatory responses, suggesting that they might play a potential role in tumorigenesis (123). Although these inflammatory cytokines have roles in breast cancer, the

molecular mechanisms through which they are involved in tumorigenesis in the mammary gland is not well understood.

1.8. CONCLUSION

The cumulative effects of fluctuating ovarian hormones on mammary epithelial cell proliferation, differentiation and apoptosis are considered as a highly significant factor leading to increased risk of breast cancer (124). Furthermore, early age at menarche, late age at menopause, and short cycle length are all factors that increase lifetime risk of breast cancer, and contribute to prolonged exposure to ovarian hormones (6, 125). Hence, it is important to study the precise cellular and hormonal mechanisms involved in regulating mammary gland development during the menstrual cycle.

From the literature, it is clear that estrogen and progesterone promote mammary gland development through direct effects on epithelial cells and indirect effects through macrophages, T cells and other immune cells in the mammary stroma. Although the mechanisms through which estrogen and progesterone direct these functions is not known, it is likely to involve crosstalk with the hormonally responsive mammary epithelium. Mammary epithelial cells secrete a variety of cytokines, and mouse models suggest these epithelial cell-derived cytokines affect the surrounding microenvironment. However, the crosstalk between mammary epithelial cells and the immune microenvironment has not been previously investigated in human breast. In this context, ELF5 and FOXP3 may be key mediators of hormone-regulated cytokines produced by human mammary epithelium. Dissecting the interaction between hormonally responsive mammary epithelial cells and their cytokine expression in the breast will shed light on how fluctuations in ovarian hormones during the menstrual cycle affect breast cancer risk.

1.9. HYPOTHESIS AND AIMS

The experiments described in this thesis aim to address the following hypotheses:

- Estrogen and progesterone regulate the expression of specific immune-related mammary epithelial cytokines in different human cell culture models.
- Transcription factors ELF5 and FOXP3 are key regulators of hormone-induced cytokine production by human mammary epithelial cells.
- Foxp3 heterozygosity affects the ovarian cycle regularity and mammary gland development in pubertal and adult mice.

These experiments will address the following aims:

- To explore the effects of estrogen and progesterone in the regulation of gene expression in different human cell culture models.
- To explore the role of estrogen and progesterone in the regulation of ELF5 and downstream cytokine production by mammary epithelial cells.
- To explore the role of estrogen and progesterone in the regulation of FOXP3 and downstream cytokine production by mammary epithelial cells.
- To investigate the physiological role of Foxp3 in regulating mammary gland development using Foxp3 heterozygous mice.

1.10. RESEARCH PLAN

To investigate the role of estrogen and progesterone in regulating the cytokine expression in the mammary gland, healthy non-neoplastic mammary epithelial organoids, human breast tissue explants, human breast cancer cell lines, and mouse mammary glands will be utilised.

When primary mammary epithelial cells are cultured in single cell 2 dimensional (2D) monolayers, they lose their responsiveness to estrogen and progesterone (126). Therefore, these

studies seek to utilise new approaches to cell culture using mammary epithelial cells obtained from women at surgery that retain their cell-to-cell associations. Organoids are fragments of mammary alveolar epithelial structures which retain some of their *in situ* 3 dimensional (3D) structure, and can provide understanding of hormonal regulation of normal breast structure and development (127). To investigate the role of estrogen and progesterone on human mammary epithelial cytokine production, primary human organoids were cultured in Matrigel in a 3D model before being treated with hormones. This *in vitro* culture model, recently developed and described by Graham, Mote (126), can preserve the proliferative capacity and progenitor cell complement of normal breast tissue and retain the hormone responsiveness of mammary epithelial cells up to 14 days; hence it is suitable for studies related to hormone action in the normal breast (126). Moreover, it does not favour the growth of a specific epithelial cell type over the other, unlike most primary culture models (128). Indeed, when primary cells are embedded into 3D Matrigel cultures, both luminal and myoepithelial cells grow in the culture in a balanced manner. In contrast, when these cells are cultured in a 2D monolayer, bipotent epithelial progenitors lose their complex inter-relationships, their ability to give rise to both luminal or myoepithelial cells, and their hormone responsiveness within three days of culture (126). Nevertheless, this 3D model recapitulates the effects of estrogen and progesterone on human mammary epithelium, thus providing a reliable model system to represent the *in vivo* actions of these hormones on cytokine production.

To determine the effects of estrogen and progesterone on epithelial cell cytokine expression in *ex vivo* human breast tissue explants, small fragments of human breast tissue are cultured. This model enables investigation of the mammary epithelium in the context of its natural microenvironment, as each tissue fragment is composed of the mammary epithelium embedded within the breast extracellular matrix and surrounded by stromal cells including fibroblasts and macrophages. Studies from our laboratory demonstrate that breast explant cultures undergo a

process of cellular rearrangement when first cultured, with the orientation of epithelial and stromal tissue compartments re-established within the first 7 days (Dasari et al., in preparation). Importantly, the epithelial cells maintain estrogen and progesterone responsiveness, and the cellular components of stroma are observed directly adjacent to epithelial cells in these cultures, remaining stable for at least 7 days following the initial 7-day establishment phase. Therefore, this model is an ideal approach to investigate the paracrine signalling between hormonally responsive mammary epithelial cells and the surrounding microenvironment.

To complement studies on human primary mammary cells, which are obtained at the time of surgery and are therefore greatly limited in abundance, studies on human breast cancer cell lines were also conducted as part of this work. Cell lines are cost-effective, valuable *in vitro* tools which can compensate for experimental problems with primary mammary epithelial cells, and have advanced our understanding of breast biology. For this study, ATCC human mammary epithelial cell lines T47D, ZR751, and MCF7 were used as each of these express receptors for estrogen and progesterone (129, 130) and they have retained the luminal epithelial phenotype of breast cells (131). It should be noted that we are focusing on the overall actions of hormones on cytokine production by mammary epithelial cells in different conditions, and we do not translate the findings from breast cancer cell lines into a context of normal cycling mammary gland. Moreover, to reflect our gene expression studies in the context of naturally occurring cycling mice, and validate our results from the *in vitro* human cell culture models, mouse mammary glands were analysed at different stages of the estrous cycle.

CHAPTER TWO

MATERIALS AND METHODS

2.1. HUMAN BREAST TISSUE COLLECTION:

Healthy non-neoplastic breast tissues were collected from women undergoing surgery for breast cancer, prophylactic mastectomy or reduction mammoplasty at The Queen Elizabeth Hospital. Human ethics committee approval was obtained from The Queen Elizabeth Hospital (TQEH Ethics Approval# 2011120) and informed consent was obtained from patients. In patients with a past or present history of breast cancer, breast tissues were taken to the pathology laboratory at the Queen Elizabeth Hospital to confirm the tissues are clear of malignancy. Inclusion and exclusion criteria for this study are as follows:

Inclusion criteria:

- Women attending TQEH for breast surgery
- Between 18 and 75 years of age
- Capable of giving informed consent

Exclusion criteria:

- Pregnancy
- Patients highly dependent on medical care who may be unable to give consent.

2.2. PROCESSING OF BREAST TISSUE FOR HISTOLOGY, EPITHELIAL ORGANOIDS AND EXPLANTS

2.2.1. ISOLATION OF HUMAN MAMMARY EPITHELIAL ORGANOIDS

Breast tissue was dissected with scalpel blades into small pieces, discarding excess fat and digested with 100U/ml of Hyaluronidase (Sigma Aldrich, St Louis, USA; Cat# H3506) and 480U/ml of Collagenase (Sigma Aldrich; Cat# C0130) in Advanced DMEM/F12 medium (Life

Technologies, Australia; Cat# 12491015); supplemented with 2.5mg/ml Fungizone (Life Technologies; Cat# 15290018), 1X Penicillin/Streptomycin (Life Technologies; Cat# 15240062), 10mM HEPES buffer (Life Technologies; Cat# 15630080), and 1X L-Glutamine (Life Technologies; Cat# 25030081). The digestion flask was placed in a 37ºC shaking water bath for 16 hours to dissociate the stromal and epithelial components of the tissue. It was then centrifuged at 180 x g for 10 minutes to separate undigested tissue fragments from the lipid layer. Advanced DMEM/F12 media was added to the pellet, mixed and gravity settled for 2 hours. Fragments of mammary alveolar epithelial structures, known as organoids, were isolated from the supernatant through gravity settling. Media was removed and the cells were digested again with collagenase (300U/ml) for 2 hours and centrifuged at 480 x g for 5 minutes. The organoid pellet was treated with red blood cell lysis buffer (BD Bioscience, USA; Cat# 555899) (4ml for 15 minutes) to remove red blood cells. Organoids were then filtered through 100µm and 40µm cell strainers (Sigma-Aldrich; Cat# CLS431750) and collected in a Falcon tube. Cells were centrifuged at 480 x g for 5 minutes, resuspended in Advanced DMEM/F12 medium and stored in liquid nitrogen until required (refer to section 2.4. for freezing and thawing protocol).

2.2.2. DISSECTION AND CULTURE OF BREAST TISSUE INTO EXPLANTS

Breast tissue was dissected into small tissue fragments (approximately 5mm) using scalpel blades. Tissue fragments were cultured on dental sponges (Ferrosan, Denmark; Cat# MS0005) as explants, maintained in 500µl RPMI1640 phenol red-free media (Life Technologies; Cat# 32404014) (supplemented with 10% low-hormone Foetal Calf Serum (FCS) (Thermo Fisher Scientific, USA; Cat# 10099141), 1X Penicillin/Streptomycin, 10µg/ml Hydrocortisone (Sigma-Aldrich; Cat# H4001), and 10µg/ml insulin (Sigma Aldrich; Cat# I6634). Explant cultures were incubated at 37°C CO_2 incubator for one week to allow tissue to recover before they are treated with combinations of hormones for one week. Figure 2.1 summarises the tissue processing protocol used to obtain human mammary epithelial organoids and explants.

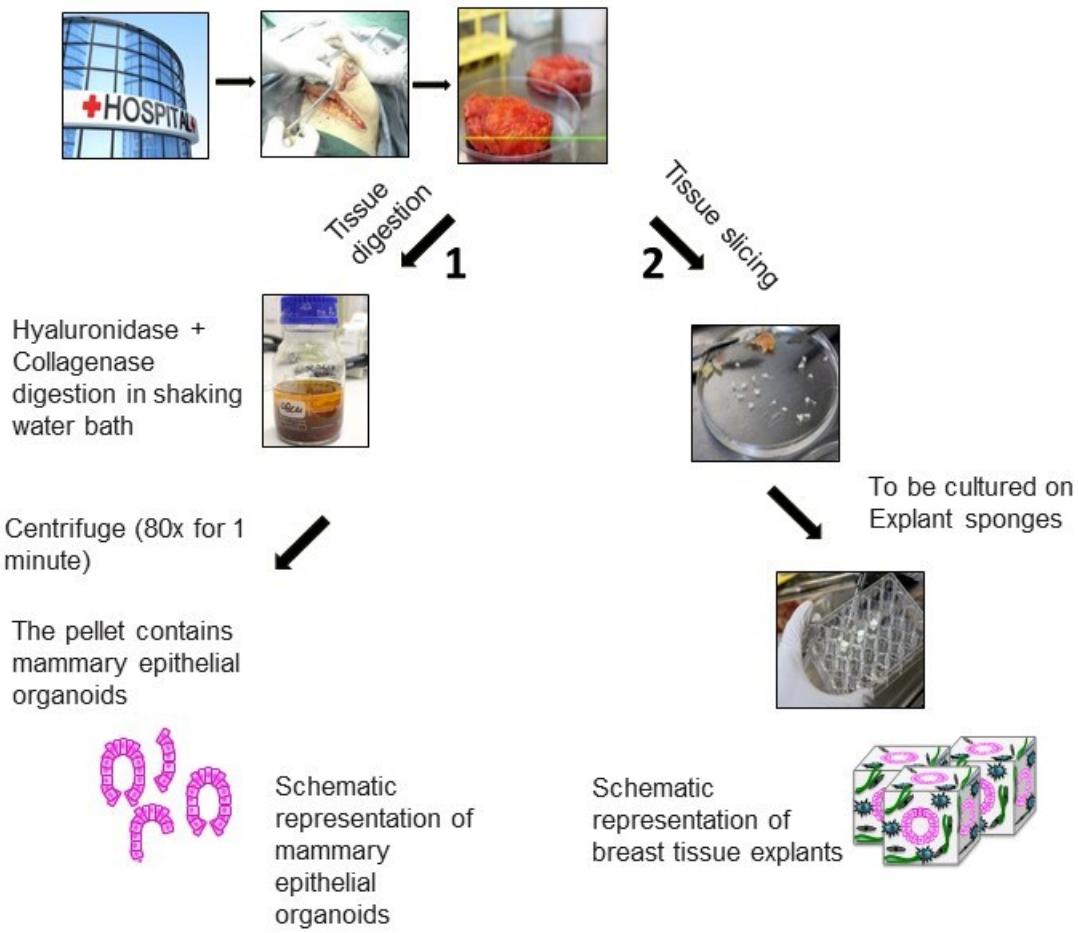


Figure 2.1: Human breast tissue processing protocol.
Human breast tissues were collected from patients and prepared for culture as organoids or explants: 1) A large portion of breast tissue was digested with enzymes for 16 hours in shaking water bath. Through several steps of gravity settling, the mammary epithelial organoids were obtained. 2) Fragments of the breast were cut into small pieces and cultured on top of dental sponges as explants.

2.3. CELL CULTURE

2.3.1. CULTURE OF HUMAN MAMMARY EPITHELIAL ORGANOIDS IN MATRIGEL

Organoids were thawed and split evenly into four Eppendorf tubes to be cultured into the central wells of 8-well tissue culture chamber slide (Sarstedt, Germany; Cat# 94.6140.802). Medium 171 (Life Technologies; Cat# M171500) was supplemented with 1X Mammary Epithelial Growth Supplement (MEGS) (Life Technologies; Cat# S-015-5) for culturing the cells. Working quickly on ice, organoids (resuspended in approximately 40µl of the complete Medium 171, mixed with 160µl of Matrigel) were transferred into one of the centre wells of the 8-well chamber slide. After 5 minutes incubating at room temperature, chamber slide was transferred to the 37ºC incubator for 20-30 min to allow for complete solidification of the Matrigel. Once the Matrigel was solidified, it was overlaid with 400µl of complete medium. The cultures were returned to the incubator, and the media was replaced every 2-3 days. Cells were cultured for 5 days to allow organoids to stabilise and grow prior to hormonal treatments.

2.3.2. HUMAN MAMMARY EPITHELIAL CELL LINES

Human malignant mammary epithelial cell lines (MCF7, T47D, and ZR751) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell line were utilised as they express receptors for estrogen and progesterone (132), making them useful for analysing hormonal effects on mediating cytokine synthesis by mammary epithelial cells. However, hormone responsiveness is known to vary between mammary epithelial cell lines. T47D cell lines have naturally high levels of progesterone receptor and are highly responsive to progesterone, whereas MCF7 cells are mainly estrogen responsive, as they contain high affinity specific receptors of estrogen (133). On the other hand, ZR751 cells respond to both of these hormones effectively (134). All cells were grown in 6-well culture plates at the density of $2x10^5$ cells/well in complete RPMI-1640 (ATCC; Cat# 30-2001) (supplemented with 1X Penicillin/Streptomycin, 0.2 Units/ml bovine insulin (Sigma-Aldrich; Cat# I5500), 10mM

HEPES buffer and 10% FCS). The cells were incubated at standard culture conditions at 37°C in 5% CO_2 and 95% humidity.

2.4. CRYOPRESERVATION AND THAWING

To preserve mammary cells for later culture, human mammary epithelial organoids and ATCC mammary epithelial cell lines were resuspended in 50% FCS /Advanced DMEM/F12 medium, and 6% dimethyl sulfoxide (Sigma-Aldrich; Cat# D5879) and aliquoted into 1ml cryovials and stored in liquid nitrogen in storage facility at the Basil Hetzel Institute at The Queen Elizabeth Hospital.

To thaw, organoids and cell lines were collected from liquid nitrogen storage facility and thawed initially in a 37ºC water bath. The contents were then transferred to a Falcon tube where appropriate media was added to them drop-wise. Cells were then centrifuged at 300 x g for 5 minutes, and the pellet was resuspended in 1ml of appropriate media. The media used for thawing are as follows:

- Organoids: Medium 171 (Life Technologies; Cat# M171500) supplemented 1 in 100 (v/v) with 1 X Mammary Epithelial Growth Supplement (MEGS) (Life Technologies; Cat# S0155), 1X Penicillin/Streptomycin, and Fungizone at 1/1000 dilution.
- Cell lines: RPMI 1640 supplemented with 1X Penicillin/Streptomycin, 0.2 Units/ml bovine insulin, 10mM HEPES buffer and 10% FCS.

2.5. HORMONE TREATMENTS IN CELL AND TISSUE CULTURES

For hormone treatments of *in vitro* culture of organoids, cell lines and explants, stock hormones in ethanol of 10mM 17β-estradiol (Sigma-Aldrich; Cat# 491187) and 20mM progesterone (Sigma-Aldrich; Cat# P0130) were diluted in appropriate culture medium containing charcoal-stripped FCS. For hormone treatment of *in vitro* cultures, existing media was replaced with

media containing either ethanol control or the hormones (dissolved in ethanol 1/1000 dilution). Hormone treatments were performed every 24 hours using fresh media. The hormone treatment protocol was as following:

- Organoids and cell line cultures: 10nM estradiol and/or 100nM progesterone were diluted in 400µl of Medium 171 or in 2ml RPMI 1640 media, and the cultures were treated with hormones for 72 hours. These concentrations reflect the physiological serum hormone levels in women using combined oral contraceptives (135) and they have minimal toxicity effects on the cells *in vitro*.
- Explant cultures: 10nM estradiol and/or 10µM progesterone were diluted in 500µl of RPMI1640 media, and the cultures were treated with hormones for 72 hours.
- 0.1% ethanol diluted in sterile water was mixed into appropriate media and used as the control for all three models.

72 hours hormone treatment was chosen to follow the previous established protocols and also to be consistent with our cell line studies which showed that 72 hours hormone treatments results in higher hormone responses compared to 24 hours treatment.

2.6. BUFFERS AND SOLUTIONS

2.6.1. SIRNA SOLUTIONS

For transfection of cell lines, 20µM stock siRNA solutions were diluted in 1X siRNA buffer (Thermo Fisher Scientific; Cat# 13-002000-UB-100) to prepare 10µM working solutions.

2.6.2. PHOSPHATE BUFFERED SALINE

Commercial, sterile, 1X Dulbecco's phosphate buffered saline (PBS) (Thermo Fisher Scientific; Cat# 10010023) was used in all cell culture and immunostaining protocols.

2.7. TRANSFECTION OF HUMAN MAMMARY EPITHELIAL CELLS

2.7.1. ELF5 SIRNA TRANSFECTION OF CELL LINES

T47D cells were grown in T75 culture flasks until optimal density (i.e., 60-80% confluent); they were treated with 1ml trypsin and washed twice in antibiotic-free complete RPMI1640 medium (supplemented with 10% FCS). Cells were counted using Countess automated cell counter (Invitrogen, Australia), and diluted to 2.5 x 10^5 cells/ml for transfection in each well of a 6-well culture plate. 25pmol of SMARTpool human ELF5 siRNA (Dharmacon, Lafayette, USA, Cat# L-011265-02-0005) and non-targeting siRNA (Dharmacon, Cat# D-001810-01-05) solutions (at 10μM) were mixed with Lipofectamine RNAiMax transfection reagent (Life Technologies; Cat# 13778030) in 1:3 ratios for 5 minutes prior to transfection. Opti-MEM reduced-serum medium (Life Technologies; Cat# 31985062) was used as the transfection medium for initial dilution of ELF5 siRNA, non-targeting siRNA, and Lipofectamine RNAiMax. Next, cells were mixed with siRNA-transfection lipid mix. Transfected cells were then seeded into each well of a 6-well culture plate and 2ml antibiotic-free complete RPMI1640 media was added. Cells were incubated at 37°C in 5% CO_2 for 24 hours before hormone treatments.

2.7.2. PRODUCTION OF LENTIVIRAL VECTORS

To investigate the roles of FOXP3 and downstream cytokine production by mammary epithelial organoids, lentiviral vectors plvEIG-FOXP3 (previously described in (136)) were purchased from the Gene Silencing and Expression Facility (GSEx) at the University of Adelaide. These vectors had been generated by transient transfection of Human Embryonic Kidney cells (HEK 293T) with transfer vector (plvEIG) and packaging vectors pCMV-VSV-G (VSV-G), pMDL-g/p-PRE (gag/pol) and pRSV-REV (REV) in the presence of transfection reagent Lipofectamine 2000 (Invitrogen; Cat# 11668027) and Opti-MEM reduced serum medium. The

supernatant was harvested 72 hours post-transfection and the virus was concentrated to 25X using ultracentrifugation (22,000 rpm for 90min at 4°C).

Figure 2.2 illustrates the elements used for production of the lentiviral vectors. The construct, plvEIG, contains an expression cassette encoding the (EF)-1 alpha promoter, where FOXP3 has been inserted downstream followed by an internal ribosome entry site (IRES) which controls the reporter gene GFP. The vector is self-inactivating with a deletion in the 3'LTR. It also includes a Rev-Responsive Elemebt (RRE), Post-transcriptional Regulatory Element, Rous Sarcoma Virus promoter (RSV) and the central Polypurine Tract (cPPT) which enhances the transduction efficiency (136). These lentiviral vectors are capable of integrating into human mammary epithelial cells and subsequently overexpress FOXP3 in the cells. Also, pLVEIG-GFP lentiviral constructs which contain an additional GFP gene in place of FOXP3, were used as the control.

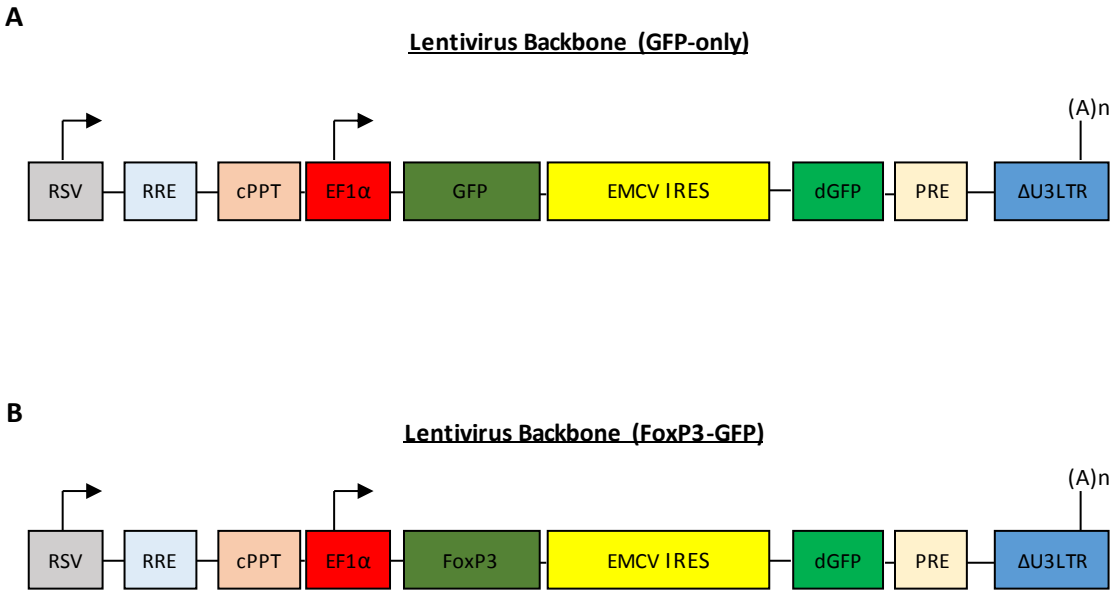


Figure 2.2: The vectors required for the production of the pLVEIG lentivirus.
The pLVEIG lentiviral vectors contain either GFP (A) or full-length FOXP3 (B), driven by the EF1α promoter. An EMCV IRES allows the expression of the GFP reporter downstream. It also includes a Rev-Responsive Elemebt (RRE), Post-transcriptional Regulatory Element, Rous Sarcoma Virus promoter (RSV) and the central Polypurine Tract (cPPT) which enhances the transduction efficiency.

2.7.3. LENTIVIRAL TRANSDUCTION OF MAMMARY EPITHELIAL ORGANOIDS

Human mammary epithelial organoids were thawed as described earlier (section 2.4). Organoids were resuspended in complete Medium 171 containing 8µg/ml polybrene (Sigma-Aldrich; Cat# H9268) and transduced with 5µl of lentiviral particle solution at multiplicity of infection (MOI) of 10 at 37°C, 5% CO_2 incubator for 6 hours. Transduced cells were then centrifuged at 300 x g for 5 minutes, and the pellet was mixed with 160µl of Matrigel and cultured in 8-well chamber slides for 5 days. Transduction efficiency was then assessed qualitatively and quantitatively using confocal microscopy and RT-PCR. To confirm the efficiency, the intensity of GFP as well as the expression of mRNA encoding FOXP3 were measured in FOXP3-GFP-transduced cells and compared with the control cells.

2.8. HISTOLOGY AND IMMUNOHISTOCHEMISTRY

2.8.1. TISSUE EMBEDDING AND SECTIONING

Breast explant tissues were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich; Cat# P6148), for 24 hours at 4°C, washed with 1X PBS overnight, and kept in 70% ethanol solution until tissue processing. Tissue was processed, and paraffin embedded using Excelsior tissue processor (Thermo Fisher Scientific) which involved the following incubation protocol: 1 minute 75% ethanol, 1 minute 90% ethanol, 1 minute 95% ethanol, 3 x 1 minute absolute ethanol, 3 x 1 minute xylene, 3 x 1 minute paraffin wax at 62°C. Blocks and sections were stored at room temperature prior to staining.

2.8.2. HAEMATOXYLIN AND EOSIN STAINING

5µm serial sections were cut using a Microtome (Leica Biosystems, Australia) and four sections per slide were mounted on adhesive glass slides (HD Scientific, NSW, Australia). Sections were dewaxed through three changes of xylene (Merck Millipore, Darmstadt, Germany; Cat# 108298) and treated with 100%, 95%, 70%, and 50% ethanol gradually to rehydrate. The 5th

and 15th slide were then stained with haematoxylin (Sigma-Aldrich; Cat# HHS16) and counterstained with eosin (Sigma-Aldrich; Cat# 318906) before dehydrating and mounting with microscopy Entallen (Proscitech, Australia; Cat# IM022). H&E stained sections were then used to identify the slides containing alveolar epithelium (i.e. clusters of epithelial structures containing alveolar lumens) for immunohistochemical staining.

2.8.3. S100A8 IMMUNOSTAINING ON HUMAN BREAST EXPLANTS

To investigate the effects of estrogen and progesterone on S100A8 expression, paraffin-embedded sections were heated at 60°C for 1 hour before cooling to room temperature for 20 minutes. Sections were then dewaxed twice with xylene for 5 minutes and rehydrated gradually with 100%, 90%, 70% and 50% ethanol, and H_2O. The slides were incubated in citrate buffer (pH=6) for 30min at 95°C in a water bath. The slides were then removed from the water bath and allowed to cool, in the citrate buffer, for 20 minutes. Slides were then washed with envision wash buffer (Dako, USA; Cat# K8007) before incubating with 2µg/ml mouse anti-human S100A8 monoclonal antibody (Thermo Fisher Scientific; Cat# RG2231245) (1:100 dilution in antibody diluent (Dako; Cat# S0809); 1 hour at room temperature). Any unbound primary antibody was removed through three washes in Envision wash buffer and secondary antibody was applied (Envision Flex/HRP (RTU) (Dako; Cat# DM842) for 30 minutes at room temperature. After washing, the sections were incubated with 3,3′-Diaminobenzidine (DAB) (Dako; Cat# K3468) for 10 minutes at room temperature. Sections were then counterstained with hematoxylin and dehydrated with 90% and 100% ethanol (each for 3 minutes) and xylene (2X for 5 minutes), and coverslipped with microscopy Entallen. The negative controls had no application of primary antibody. Stained sections were then visualized using a Nanozoomer digital scanner (Hamamatsu photonics, Japan) and the images were acquired at 40X magnification using the Nanozoomer software for further analysis.

2.9. PROTEIN ANALYSIS

2.9.1. WESTERN BLOT

Protein was extracted from the cells using 200µl of RIPA buffer (Sigma-Aldrich; Cat#R0278) containing 1% β-mercaptoethanol (Sigma-Aldrich; Cat# M6250). Protein samples were quantified using Bradford Protein Assay (Bio-Rad, USA; Cat# 5000002) as according to the manufacturer's instructions and 20µg from each sample was used for western blot analysis. Western blots were performed as per the Bio-Rad protocol for western blotting. Briefly, samples were separated on 12% precast polyacrylamide gel (Bio-Rad; Cat# 4568044) at 200V for 30 minutes. Proteins were then transferred onto a nitrocellulose membrane using Trans-Blot Turbo Transfer Packs (Bio-Rad; Cat# 1704156) in a Trans-Blot Turbo Transfer chamber system (Bio-Rad; Cat# 17001919). The membrane was blocked with 5% skim milk for 1 hour at room temperature prior to incubation with 20ng/ml of primary antibody overnight at 4°C. It was then incubated with 0.5µg/ml of appropriate secondary antibody conjugated to Horse Radish Peroxidase (HRP) for 1 hour at room temperature and washed 5X with TBST solution (containing 50mM Tris, 150mM NaCl, and 0.1% Tween 20). The membrane was incubated with the Clarity Western ECL substrate reagent mix (Bio-Rad; Cat# 170-5060) for 5 minutes to visualise the proteins using ChemiDoc Touch Imaging System (Bio-Rad; Cat# 17001402). To re-probe the membrane, it was stripped in 1X antibody stripping solution for 15 minutes at room temperature as per manual for western blot recycling kit (Alpha Diagnostics, TX USA), blocked with 1X Blocking Buffer and subsequently re-probed with primary antibodies as described above.

2.10. ANIMALS AND SURGERIES

2.10.1. MICE

All animals in this study were approved by the University of Adelaide Animal Ethics Committee (Ethics# M-2015-268) and were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (137). All mice were

maintained under specific pathogen-free conditions with controlled light (12hr light, 12hr dark cycle) and temperature at the Laboratory Animal Services Medical School South facility.

2.10.1.1. FOXP3 HETEROZYGOUS MICE (FOXP3$^{Sf/+}$)

The scurfy mouse genotype developed spontaneously from a multiple recessive stock of seven mutations, at the Oak Ridge National Laboratory in 1949 (138). This mutation is characterised by insertion of two adenosine residues into exon 8, leaving scurfy mice with a truncated Foxp3 protein that lacks the carboxy terminal-forkhead domain. Homozygous scurfy male mice (known as Foxp3$^{Sf/Y}$) develop T cell tolerance leading to an X-linked lymphoproliferative disease, and generally die by 16-25 days of age. Phenotypes associated with scurfy mice include runting, scaly skin on the ears, eyes and tails, squinted eyes, dermal thickening, swelling of the genital papilla, and small testicles retained in the abdominal cavity (91, 92).

In this project, all the experiments used Foxp3$^{Sf/+}$ heterozygous female mice on a C57BL/6J background. Foxp3$^{Sf/+}$ were bred with the wildtype C57BL/6J mice and the breeding pairs produced progeny that were homozygous for a targeted mutation in the Foxp3 gene (Foxp3$^{Sf/Y}$), heterozygous (Foxp3$^{Sf/+}$), or homozygous wild-type (Foxp3$^{+/+}$). Wild-type C57BL/6J mice from Jackson Laboratory (Maine, USA) were used as controls for the heterozygous scurfy mice. Mice were weaned at three weeks and 5mm of their tail tips were cut for genotyping (protocol described in section 2.11.2). Homozygous scurfy male mice were culled as soon as they developed the disease to prevent them from suffering.

2.10.2. BLOOD COLLECTION

Cardiac puncture was used to collect blood (up to 1ml) from mice while they were deeply anaesthetised with 0.4-0.5 ml 2% Avertin (Sigma-Aldrich; Cat# T48402) using a 20 g needle. It was incubated at room temperature for 30 minutes, then centrifuged for 8 minutes at 14,000 rpm. Serum was collected and stored at -80 °C.

2.10.3. ESTROUS CYCLE TRACKING

Vaginal smear analysis was used to determine the estrous cycle stage as described by (139, 140). Daily vaginal smears were conducted between 8:00am–11:00am for at least 28 days. 20µl of sterile PBS was used to flush the vagina; the contents were smeared on a glass slide and covered with a cover slip. The cellular contents were examined using a phase contrast microscope. Each stage of the cycle was determined based on the relative proportion of each cell type (i.e. nucleated epithelial cells, cornified epithelial cells, or leukocytes) (Table 2.1). Representative images of these cell types in the vaginal smears are shown in Figure 2.3.

Table 2.1: Determining the estrous cycle stages by vaginal smears.

Estrous cycle stage	Cytology of the cells in vaginal smears
Proestrus	E or EC
Estrus	EC+ or C+
Metestrus	C++ clumps or C+L+
Diestrus	L++

E= epithelial cells, C= cornified epithelial cells, L= leukocytes, ++ indicates many cells

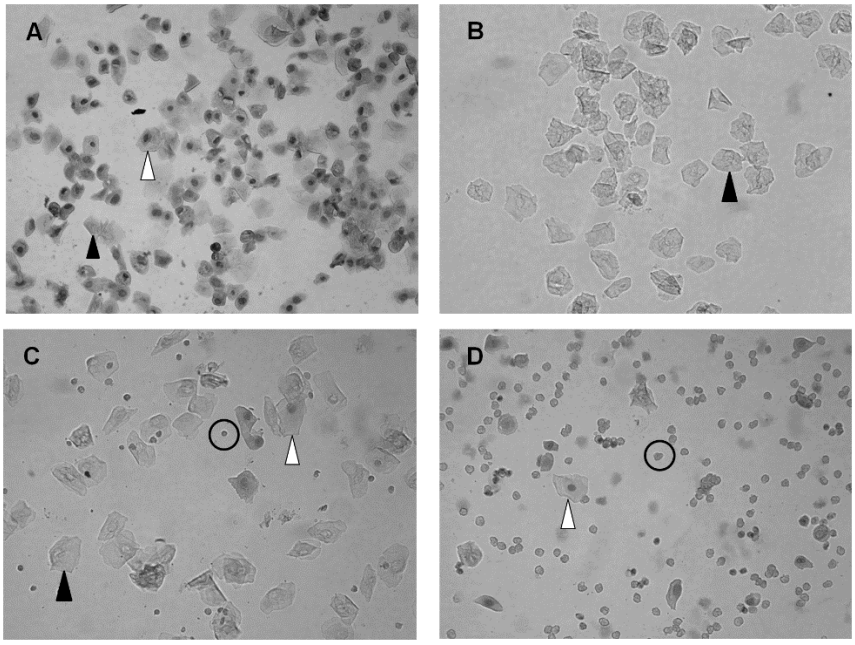


Figure 2.3: Representative images of vaginal cytology at each stage of the estrous cycle. Images are derived from (139).
Three different cell types identified in vaginal smears of mice at each stages of the estrous cycle A) proestrus, B) estrus, C) metestrus, and D) diestrus.

2.10.4. MAMMARY GLAND WHOLE-MOUNT PREPARATION

For mammary gland whole-mount preparation, the 4th left inguinal mammary gland was excised and spread on a glass slide. The slides were fixed for a minimum of 4 hours in Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid). The fixed mammary glands were washed in 70% ethanol for 15 minutes and then rinsed in MQ water for 5 minutes. Tissues were stained with carmine alum [2% carmine (Sigma-Aldrich; Cat# C1022), 5% aluminium potassium (Sigma Aldrich; Cat# 237086) overnight at 4°C]. The slides were washed in 70% ethanol, followed by 95% and 100% ethanol (15 minutes each). Finally, the slides were cleared in xylene for one week and mounted on the slide with a cover slip using DPX (Merck). Mammary whole-mounts were photographed using an Olympus SZ61 stereo microscope (Olympus, Japan) and a SC50 camera (Olympus).

2.10.5. DUCTAL BRANCHING ANALYSIS

The number of branch points per millimetre length of epithelial ducts was counted using Image J software. The three longest ducts from each mammary gland were selected, and the number of branch points on each duct was counted manually (assessor was blinded to mouse genotype). A mean value of three ducts per mammary gland was calculated and expressed as branch points/mm to give a single value for each animal.

The number of TEBs and the ductal length was also quantified in the mammary gland whole-mounts. TEBs projected out from the distal end of the ducts were counted manually across the widest portion of the mammary gland. Only terminal end buds greater than 100μm were counted. The length of ductal epithelial growth in the mammary gland was measured using Image J software, from the nipple to the furthest TEB.

2.11. NUCLEOTIDE ANALYSIS

2.11.1. DNA EXTRACTION:

Mouse tail tips were digested in 250µl digestion buffer containing 17mM tris, 17mM EDTA, 170mM sodium chloride, 0.85% SDS (PH 7.8) with 0.1mg proteinase K (all from Sigma-Aldrich) at 55°C for 4 hours. To precipitate out the proteins and cellular debris, 250µl of 4M ammonium acetate was added to each sample and incubated for 25 minutes at room temperature with regular mixing. Samples were then centrifuged at 12,000 rpm for 10 minutes and the clear supernatant was transferred onto a new tube. 800µl of 100% Ethanol was added to each sample and centrifuged at 12,000 rpm for 8 minutes to pellet the DNA. The pellet was washed with 70% ethanol and air dried on a heat block for 15-20 minutes. Next, 50µl of RNase-free water was added to break down the DNA pellet. DNA concentration was measured using spectrophotometer ND 1000 Nanodrop (Thermo Fisher Scientific). DNA was stored at 20°C prior to genotyping.

2.11.2. GENOTYPING MICE:

TaqMan genotyping assay was used to determine the genotypes of mice. DNA from tail biopsies were analysed for the expression of intact or mutant *Foxp3* gene.

2.11.2.1. PCR PRIMERS AND CONDITIONS:

The primers and conditions used to determine the genotype of mice in respect to FoxP3 mutation were followed based on the method recommended by JAX genotyping Resources (Jackson Laboratory). The mutant (MUT) probe has two adenosine residues inserted into exon 8 in the reading frame. This reading frameshift is predicted to produce a truncated non-functional protein lacking the carboxy-terminal forkhead domain. Table 2.2 specifies the sequence *Foxp3* primer along with wild-type (WT) and MUT probes labelled with specific fluorophore dyes.

Table 2.2: The protocol TaqMan assay used for genotyping mice.

Primer Type	5' Label	Sequence 5' --> 3'	3' Label
Forward	---	GGC TAC AAT GAA ATG ACA AGC	---
Reverse	---	GAC TCC ACT GAC CTG GGA AG	---
WT Probe	VIC	CAA TGG ACA AGA GCT CTT GC	QSY quencher
MUT Probe	6-FAM	CAA TGG ACA AAA GAG CTC TTG C	QSY quencher

2.11.2.2. TAQMAN ASSAY:

To determine the genotype of mice, TaqMan gene expression assays were performed using ViiA7 Real Time PCR system (Applied Biosystem) based on the expression of *Foxp3* variants in the DNA. Reactions were prepared separately for each variant (i.e. FoxP3 WT or MUT probe) in a total volume of 20µl containing 1µl of the probe, 10µl of TaqMan Fast Advanced Master Mix (Applied Biosystem, Cat# 4444557), 7µl of nuclease-free water and 2µl of DNA. A no template control (nuclease-free water) was used to confirm the absence of DNA contamination. Tables 2.3 and 2.4 summarise the reaction set up and PCR conditions for genotyping the mice.

Table 2.3: The reaction set up for genotyping mice using WT and MUT probes.

Reaction Component	Final Concentration	Volume/reaction (µl)
Probe (WT/MUT)	15 µM	1
ddH2O -	---	7
TaqMan Fast Advanced Master Mix	1X	10
DNA	100ng	2
Total	---	20

Table 2.4: PCR conditions for genotyping mice.

Step #	Temperature °C	Time	Note
1	95	3min	---
2	95	5sec	---
3	60	30sec	---
4	---	---	Repeat steps 2-3 for 40 cycles
5	4	---	∞

Genotypes were determined based on the presence or absence of WT or MUT alleles as following: WT DNA only expressed the *Foxp3* WT allele; scurfy DNA only expressed the *Foxp3* MUT allele; and heterozygous mice expressed both WT and MUT alleles in their DNA

(Table 2.5). Examples of amplification plot for three mice with different genotypes are shown in Figure 2.4.

Table 2.5: The summary of End Point Analysis outcomes for each Foxp3 genotype.

Mouse Genotype	WT	MUT
Homozygous Mutants ($Foxp3^{Sf/Y}$)	×	✓
Homozygous Wild-type ($Foxp3^{+/+}$)	✓	×
Heterozygous ($Foxp3^{Sf/+}$)	✓	✓

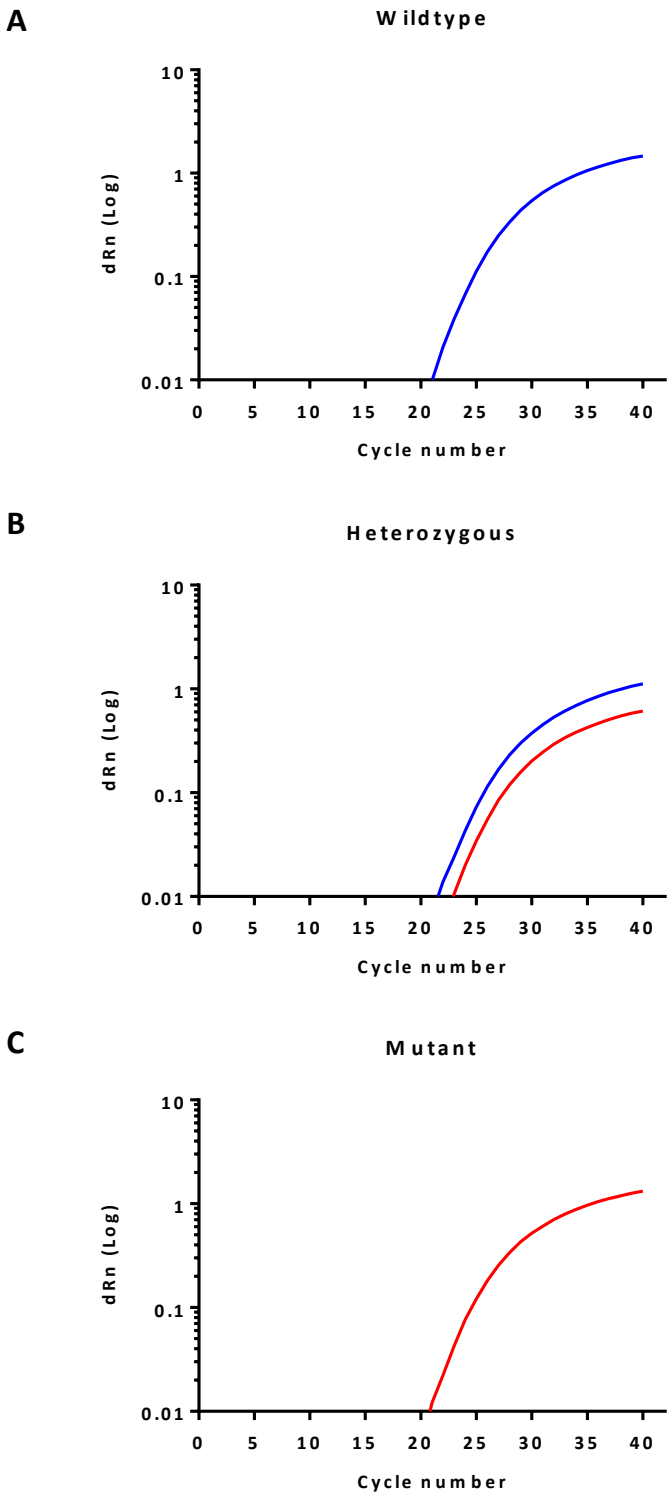


Figure 2.4: Amplification plots for mouse genotyping using TaqMan assay.
Examples of the amplification plots for mice with different genotypes is shown: A) DNA from wildtype homozygous mouse expressing only the wildtype allele of FoxP3, B) DNA from Foxp3$^{Sf/+}$ heterozygous mouse expressing both wildtype and mutant alleles of Foxp3, C) DNA from Foxp3$^{Sf/Y}$ homozygous mouse expressing only the mutant allele of *Foxp3* gene.

2.11.3. TOTAL RNA EXTRACTION

Third pair mammary glands and the lymph nodes (pooled sample of Mesenteric, Lumbar, and Axillary lymph nodes) were collected from all female mice to quantify the expression of mRNA encoding the genes of interest. Tissues were dissected under RNase Free conditions, snap frozen in liquid nitrogen and stored at -80°C until processing. Total RNA was isolated from tissues using 1ml of TRIZOL reagent (Invitrogen, Cat# 15596026) for each sample. Tissues were transferred into tubes containing 0.6g of 1.4mm ceramic beads (QIAGEN, Victoria, Australia, Cat# 13113-325) and were homogenised with the Powerlyzer 24 Bench Top Bead Based Homogenizer (MoBio, USA) (30Hz, 5 minutes). After 5 minutes incubating at room temperature, 200µl of Chloroform (Sigma Aldrich; Cat# 288306-1L) was added to the tubes. Samples were vigorously mixed by manual shaking and centrifuged at 12000 x g for 15 min at 4°C. Approximately 400µl of the top aqueous phase (containing RNA) was transferred into a fresh tube and 500µl of Isopropanol (Sigma-Aldrich; Cat# 563935) was added to the tubes. Samples were centrifuged at 12000 x g for 10 minutes at 4°C. The pellet was washed with 1ml of 75% ethanol and centrifuged at 7500 x g for 5 minutes at 4°C. The pellet was air dried for 10 minutes before being resuspended in 50µl of RNase-free water. Extracted RNA was quantified using spectrophotometer ND 1000 Nanodrop. 10µg RNA from each sample was treated with DNase to remove genomic DNA contamination using DNA-free kit (Life Technologies; Cat# AM1906) according to the manufacturer's instructions. 5µl of 10X DNase I buffer and 1µl of DNase I was added to each sample and incubated for 30 minutes at 37°C. Next, 5µl of DNase Inactivating Reagent was added to each sample and incubated for 2 minutes at room temperature. Samples were then centrifuged at 10,000 x g for 2 minutes at room temperature, and the RNA-containing supernatant was transferred to a clean tube. DNase-treated RNA was quantified using the Nanodrop Spectrophotometer.

To isolate RNA from the organoids and the cell lines, an RNeasy Mini Plus kit (QIAGEN; Cat# 74104) was used according to the manufacturer's instructions, and treated with RNase-free

DNase (QIAGEN; Cat# 79254) to remove any trace of genomic DNA. RNA yield was measured using 2µl of each sample on the Nanodrop Spectrophotometer.

2.11.4. COMPLEMENTARY DNA (CDNA) SYNTHESIS

An iScript cDNA synthesis kit (Bio-Rad; Cat# 1708890) was used to reverse-transcribe messenger RNA into cDNA. All cDNA synthesis reactions were set up using appropriate amounts of RNA, 4µl of 5 x iScript reaction mix (containing dNTPs, MgCl2, and buffer), 1µl of iScript reverse transcriptase and nuclease-free water in a total volume of 20µl. Reaction set up conditions were as follows:

- The amount of RNA used in the reaction set up was 500ng from the organoids, 1µg from the cell lines, and 2µg from mice tissues.
- Samples were incubated on the PTC-100 programmable thermal controller (MJ Research, USA) for 5 minutes at 25°C to anneal primers, 30 minutes at 42°C for reverse transcription, and 5 min at 85°C to terminate the reaction.
- Synthesized cDNA was diluted 1:10 in nuclease-free water (giving a final concentration of 3ng/µl for the organoids, 10ng/µl for the cell lines, and 20ng/µl for mice) in a total volume of 200µl. It was then stored at -20°C freezer until required.

2.11.5. QUANTITATIVE REAL TIME-PCR

2.11.5.1. SYBR GREEN-BASED DETECTION

To assess abundance of cDNA, quantitative real time PCR (qRT-PCR) was performed using a CFX96 Real Time Detection System running CFX Manager 3.0 software (Bio-Rad). Reactions were performed in a total volume of 10µl containing 5µl master-mix of SYBR green (Bio-Rad), 0.4µl of each primer set (i.e. reverse and forward primer), 2.2µl of nuclease-free water and 2µl of diluted cDNA. Reactions were then incubated on the thermocycler, which was set based on the following conditions:

- 3 minutes at 95°C

- 40 amplification cycles at 95°C for 15 seconds,
- 15 seconds at 60°C, and
- 30 seconds at 72°C.

All samples were run in triplicates and a no template control (nuclease-free water) was used for each PCR plate. Quantitative RT-PCR data was analysed by the comparative Ct method (i.e. 2-(ΔΔCT) method) (141), relative to expression of the housekeeping gene. The results were normalized to this gene so that the average of the control samples was 1. All primers were purchased from Geneworks (Adelaide, Australia). The sequence of all primers are provided in Table I in the appendix.

2.11.5.2. SELECTION OF HOUSEKEEPING GENES:

Housekeeping genes are constitutive genes typically involved in basic cellular functions and are expressed in most cells of an organism under normal conditions. However, the expression of some housekeeping genes may vary depending on the experimental conditions. Housekeeping genes that exhibit minimal variation in messenger RNA quantity in response to different hormone treatments provide valuable controls for relative quantification of data (142). Overall, housekeeping genes are expected to be expressed at constant levels in all cells and conditions. This is because these genes are involved in maintaining basic cellular functions that are essential for cell life (143). Three housekeeping genes, *MPRL19, PPIA* and *GAPDH* were assessed to determine a suitable housekeeper gene for these studies. The expression of *GAPDH* and *PPIA* was highly correlated with many of the target genes and it was altered by hormone treatments in the cultures. As a result, *GAPDH* and *PPIA* were not used as the endogenous control gene for normalisation of the qRT-PCR data. Quantitative RT-PCR results showed that the housekeeping gene *MPRL19* has an invariable expression in response to all hormone treatments; thus, it was used as the endogenous control gene.

2.12. STATISTICAL ANALYSIS:

For statistical analysis of RT-PCR data from human mammary epithelial organoids, the "R software" (144) and the lme4 library (145) were used, which enabled us to analyse multiple genes on the same sample. This method of statistical analysis was specifically chosen for this dataset to account for within sample correlations that might lead to an underestimate of the "true" standard deviation, which would increase the risk of type I error.

For statistical analysis of RT-PCR data from human mammary epithelial cell lines and mice, data were assessed using SPSS Statistics Version 24 (IBM Corporation, Armonk, NY, USA). Relative expression of genes was analysed by one-way ANOVA, followed by post-hoc Tukey test to assess the associations between two variables and comparisons between groups. Data are presented as the mean ± SEM (standard error of mean). The difference between groups was considered statistically significant if $p<0.05$. An asterisk (*) identifies a treatment that differs significantly from the control.

CHAPTER THREE

HORMONAL REGULATION OF MAMMARY EPITHELIAL CELLS

3.1. INTRODUCTION

Fluctuations in circulating estrogen and progesterone during the menstrual cycle affect mammary gland morphology. Mammary epithelial cells proliferate in the early luteal phase when the concentration of circulating estrogen and progesterone is high. However, when the concentration of progesterone declines during the late luteal and menstrual phase, mammary epithelial cells undergo apoptosis and the breast tissue reverts to its basic architecture (18). The cumulative effects of fluctuating ovarian hormones on mammary epithelial proliferation, differentiation and apoptosis over successive menstrual cycles are considered a highly significant factor leading to increased risk of breast cancer (124). Hence, it is important to study the precise cellular and hormonal mechanisms that regulate mammary gland development over the course of the menstrual cycle.

The mammary gland is composed of a number of different cell types including epithelial, adipose, and stromal cells, which together form complex interactive networks required for the normal development and function of this tissue (146). Although the majority of breast carcinomas originate from mammary epithelial cells (8), immune cells in the mammary gland stroma have been implicated as paracrine regulators affecting breast cancer susceptibility, development and progression (30). Previous studies by our group have shown that macrophages play important roles in mammary epithelial cell proliferation, phagocytosis, and tissue remodelling (20). In the adult mammary gland, macrophages are in direct contact with the epithelial cells and their abundance fluctuates over the course of the estrous cycle in mice (20, 40). As mammary gland macrophages are physically located in close proximity with the

hormonally responsive mammary gland epithelium, ovarian hormones may indirectly affect the function of macrophages through production of cytokines by mammary epithelial cells (113).

Mammary epithelial cells and immune cells both produce a number of cytokines including TGFB1, TNFA, and IL12, which mediate different aspects of mammary gland development and affect other stromal cells including the phenotype and activity of local macrophage populations (113, 147). The signal transducer and activator of transcription (STAT) family of transcription factors also play a wide range of functions in mammary gland development and cytokine signal transduction, and are associated with T cell lineages (62, 107, 148). In particular, STAT5 and STAT3 are of great interest as they are essential for alveolar development during pregnancy and tissue remodelling during mammary gland involution (64). In this study, we will investigate the expression of some of these cytokines in the mammary epithelium to understand how they are hormonally regulated.

It has been suggested that the cytokine signature of mammary epithelial cells is specific and depends on differentiation status (42). The function of cytokines can be pro-inflammatory, anti-inflammatory, tissue growth promoting, or immunoregulatory. Since breast cancer is an inflammation-linked disease and the tumour microenvironment is enriched with inflammatory components (149), these inflammatory mediators may play essential roles in increasing breast cancer risk.

To confirm the efficacy of estradiol and progesterone in our *in vitro* experiments on human mammary epithelial cells, we analysed the mRNA expression of genes encoding PGR and FKBP51. Estrogen activates the expression of the gene encoding PGR which is followed by the expression of PGR protein (150) and thus *PGR* can be used to assess estradiol responsiveness in cell culture. It has also been observed that estrogen induces expression of PGR in female

reproductive tissues as well as in normal and malignant mammary epithelial cells (151, 152). On the other hand, FKBP51 is expressed abundantly in a number of tissues, and its expression is strongly enhanced by progesterone (153, 154).

The experiments described in this chapter were devised to investigate the hormonal regulation of cytokines in mammary epithelial cells using three different models. Firstly, we used 3D culture of human primary mammary epithelial organoids obtained from human breast of women undergoing surgery. 3D organoids maintain their paracrine signalling relationships between adjacent epithelial cells which are essential for primary cells to retain their estrogen and progesterone responsiveness in culture. This 3D culture protocol was specifically developed by Graham, Mote (126) to investigate the effects of estrogen and progesterone in mammary epithelial cells in culture, and this approach is superior to alternative approaches utilising 2D primary epithelial cell cultures. Secondly, hormone-sensitive human mammary epithelial cell lines, which have more defined hormone receptor profile than primary cells, were used to explore the cytokine expression in the epithelial cells. Lastly, to investigate physiological fluctuations in ovarian hormones in the mammary gland, the cytokine profile during the estrous cycle *in vivo* using adult female virgin mice was assessed. We found there was a large degree of variation in estradiol and progesterone regulation of the expression of mRNA encoding cytokines, depending on the hormone receptor profile of the mammary epithelial cells.

3.2. RESULTS

3.2.1. MESSENGER RNA EXPRESSION OF CYTOKINES IN THE HORMONE-TREATED MAMMARY EPITHELIAL CELL ORGANOIDS

To investigate the effects of estrogen and progesterone on mammary epithelial cell cytokine production, primary human mammary epithelial organoids (n=6) were embedded in Matrigel and treated with combinations of estradiol (10nM) and progesterone (100nM) for 72 hours. Messenger RNA expression of genes *PGR, FKBP51, TGFB1, TNFA, STAT3, STAT5*, and *IL12* were examined from hormone-treated human mammary epithelial organoids. The comparative Ct method (i.e. 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to the mean of expression of housekeeping gene *MPRL19* from the same patient.

The expression of mRNA encoding TGFB1 was significantly attenuated by the combination of estradiol and progesterone treatment (Figure 3.1.C; $p<0.05$). However, there was a large extent of variability in production of all other genes in the primary organoid cultures between different women, and no other significant relationship with hormone treatments was observed (Figure 3.1).

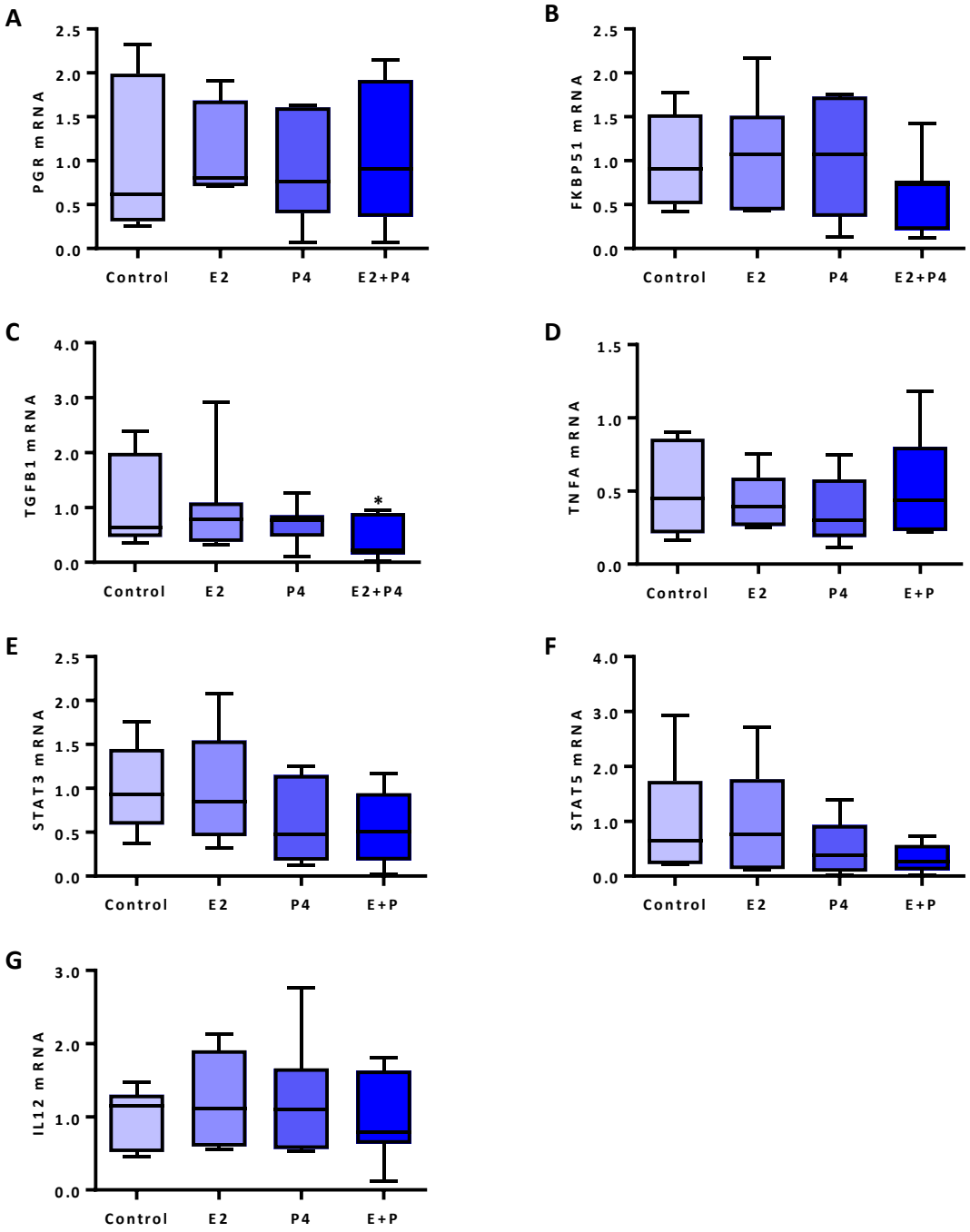


Figure 3.1: Hormonal regulation of mRNA encoding cytokines in 3D organoid cultures.
Box plots represent the mean variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TGFB1*, D) *TNFA* E) *STAT3,* F) *STAT5* and G) *IL12* from Matrigel-embedded human epithelial organoids upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 72 hours (n=6). Data are Mean ± SEM; normalized to the housekeeper gene *MPRL19*.

3.2.2. MESSENGER RNA EXPRESSION OF GENES IN THE HORMONE-TREATED MAMMARY EPITHELIAL CELL LINES:

To assess the efficacy of hormone treatments, and to provide cell line comparisons with the results obtained from the 3D organoid model, human mammary epithelial cell lines were analysed over different time courses. Messenger RNA expression of cytokines *FKBP51, PGR, TGFB1, TNFA, STAT3, STAT5,* and *IL12* were examined from MCF7, T47D, and ZR751 human mammary epithelial cell lines treated with combinations of estradiol and progesterone for i) 24 hours and ii) 72 hours. The comparative Ct method (i.e. 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of housekeeper gene *MPRL19* in each treatment group. The results were normalised so that the average of the control samples was 1.

MCF7 cells are exquisitely responsive to estradiol and less responsive to progesterone, as they contain high affinity specific receptors of estrogen (133, 155). T47D cell lines have naturally high levels of progesterone receptor and are highly responsive to progesterone (133) while being less responsive to estradiol. ZR751 cells respond to both estrogen and progesterone, although these cells mainly express the estrogen receptors (156). Our results show that the maximal *PGR* mRNA expression was observed in MCF7 cells treated with estradiol for 72 hours (Figure 3.5A), while the highest expression of mRNA encoding FKBP51 was in T47D cells treated with progesterone for 72 hours (Figure 3.6B). Also, the expression of both *PGR* and *FKBP51* genes was induced in ZR751 cells treated with estradiol and progesterone respectively, confirming the dual hormone responsiveness of this particular cell line (Figure 3.4 and 3.7). Progesterone treatment was effective in down-regulating expression of its own receptor in all three cell lines. These results support the previous studies that describe the differential effects of estradiol and progesterone on T47D, MCF7, and ZR751 cells, and confirm

that estradiol and progesterone were effective in stimulating epithelial cells, as reported previously.

In MCF7 cells treated with hormones for 24 hours, the expression of mRNA encoding TNFA was increased by 60% upon treatment with estradiol compared to the control-treated cells ($p<0.05$). On the other hand, the expression of mRNA encoding STAT5 and IL12 genes were significantly attenuated by 50% and 30% respectively ($p<0.05$ for both genes) upon treatment with the combination of estradiol and progesterone (Figure 3.2F and 3.2G). Hormone treatment of these cells for 24 hours did not have any significant effect on the mRNA expression of *FKBP51, TGFB1* and *STAT3* (Figure 3.2).

Hormone treatments of T47D cells for 24 hours resulted in more dramatic changes in the expression of genes encoding cytokines. Abundance of mRNA encoding TGFB1 and TNFA was significantly attenuated upon treatment with progesterone or a combination of estradiol and progesterone by more than 60%, compared to estradiol-treated and control-treated cells ($p<0.01$ for both cytokines) (Figure 3.3C and 3.3D). However, the same treatments significantly increased the expression of mRNA encoding STAT5 by approximately 12-fold ($p<0.01$) compared to estradiol-treated and control-treated cells. There was a trend of increased mRNA expression of this gene by approximately 6-fold upon treatment with the combination of estradiol and progesterone; however, the results were not significant (Figure 3.3F). Furthermore, treatment with the combination of hormones significantly increased the expression of mRNA encoding STAT3 by 4-fold, compared to the control-treated cells (Figure 3.3E). There was no significant effect observed in the expression of *IL12* upon hormone treatment in T47D cells (Figure 3.3G).

In ZR751 mammary epithelial cell lines, the combination of estradiol and progesterone treatment for 24 hours significantly increased the expression of mRNA encoding STAT5 by approximately 5-fold, compared to estradiol-treated and control-treated cells ($p<0.01$) (Figure 3.4F). Furthermore, estradiol treatment of these cells significantly increased the expression of mRNA encoding TGFB1 by 50%, compared to all other treatments ($p<0.01$ for P4; $p<0.05$ for control and E2+P4). However, the expression of *STAT3* and *IL12* were not significantly affected by hormone treatments in ZR751 cells (Figure 3.4E and 3.4G).

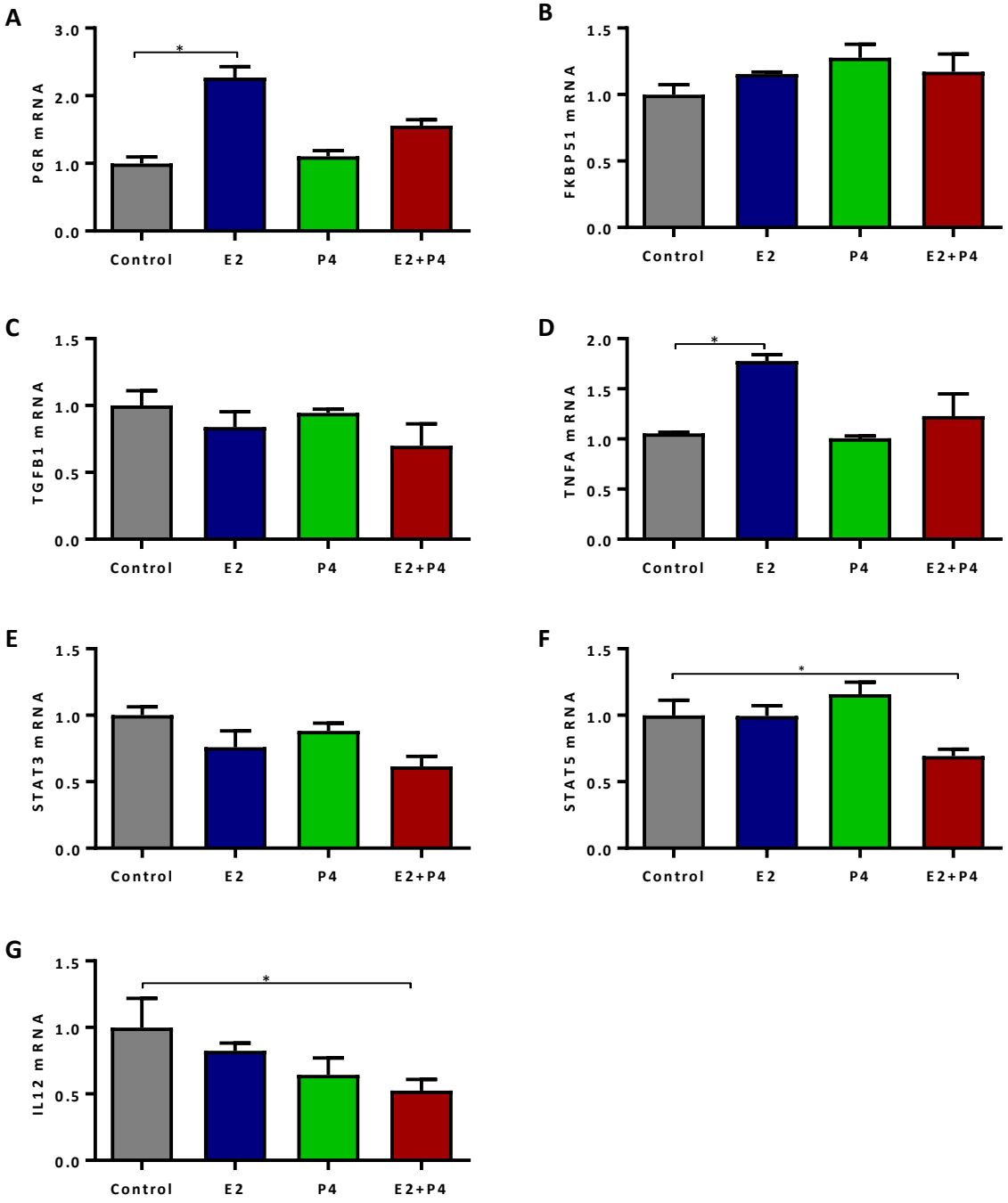


Figure 3.2: Cytokine gene profile in MCF7 mammary epithelial cell lines after 24 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TFGB1*, D) *TNFA*, E) *STAT3,* F) *STAT5,* and G) *IL12* in human mammary epithelial cell lines (MCF7) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

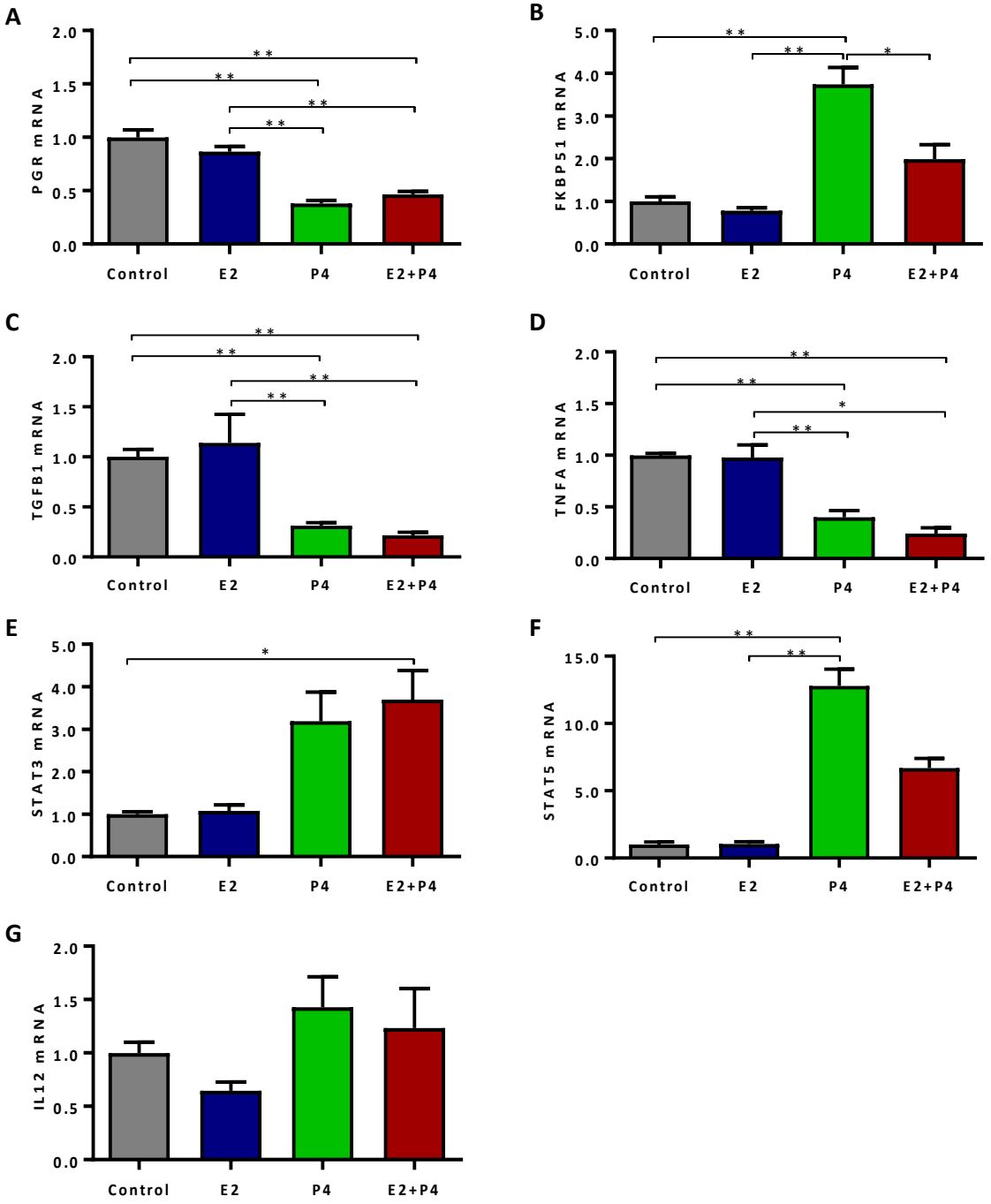


Figure 3.3: Cytokine gene profile in T47D mammary epithelial cell lines after 24 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TFGB1*, D) *TNFA*, E) *STAT3,* F) *STAT5,* and G) *IL12* in human mammary epithelial cell lines (T47D) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

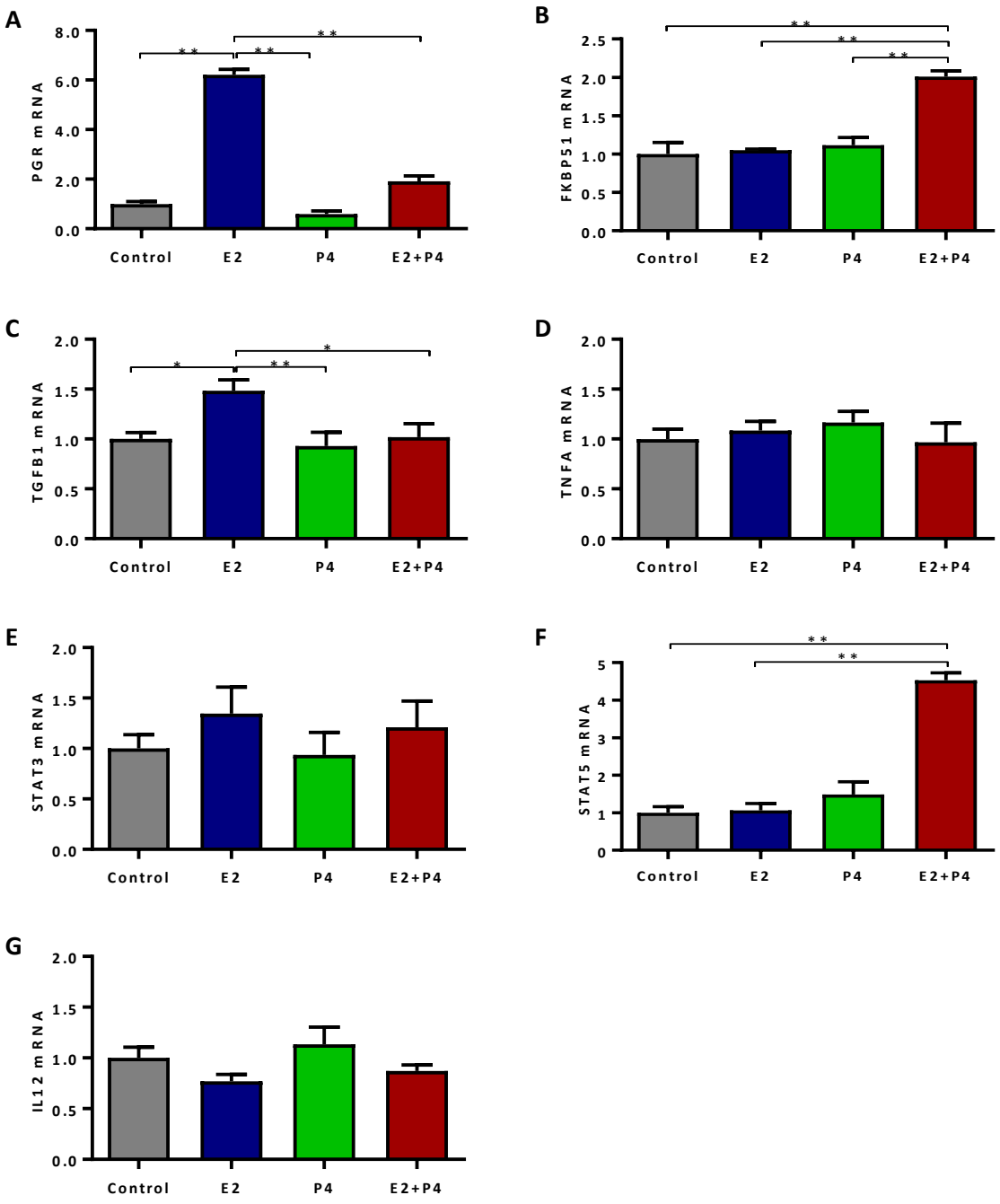


Figure 3.4: Cytokine gene profile in ZR751 mammary epithelial cell lines after 24 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TFGB1*, D) *TNFA*, E) *STAT3,* F) *STAT5,* and G) *IL12* in human mammary epithelial cell lines (ZR751) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

In MCF7 mammary epithelial cell lines treated with hormones for 72 hours, the expression of mRNA encoding TGFB1 was attenuated by approximately 90% in response to progesterone alone or in combination with estradiol, compared to the other treatments ($p<0.01$) (Figure 3.5C). The same hormone treatments dramatically reduced the expression of mRNA encoding STAT3 and STAT5 genes by more than 100-fold compared to the control-treated cells ($p<0.05$) (Figure 3.5E and 3.5F). Expression of mRNA encoding TNFA was significantly downregulated upon progesterone treatment by approximately 60% ($p<0.05$) compared to estradiol-treated and control-treated cells. The mRNA expression of this cytokine was also reduced by 40% when MCF7 cells were treated with the combination of estradiol and progesterone (non-significant) (Figure 3.5D). Hormone treatments tended to reduce the expression of mRNA encoding IL12 in MCF7 cells, however, the results were not statistically significant (Figure 3.5G).

In T47D cells, 72 hours treatment with progesterone resulted in induced expression of mRNA encoding STAT3 and TNFA by approximately 100% ($p<0.05$ for STAT3; and $p<0.01$ for TNFA) (Figure 3.6E and 3.6D). The expression of mRNA encoding STAT5 gene was dramatically upregulated by approximately 35-fold upon progesterone treatment, compared to all other treatments. However, when estradiol and progesterone were combined, the expression of this gene tended to be increased by only 7-fold compared to control-treated cells, however, this finding was not significant (Figure 3.6F). There was also a tendency for estradiol and progesterone to reduce the expression of mRNA encoding IL12 in T7D cells, however, the results were not significant (Figure 3.6G).

In ZR751 cells, the expression of mRNA encoding TGFB1 was significantly upregulated by 100% upon treatment with estradiol compared to progesterone-treated cells ($p<0.05$) (Figure 3.7C). Moreover, there was a tendency for estradiol to reduce the expression of mRNA encoding STAT3 and STAT5 genes in ZR751 cells, however, the results were not significant.

The expression of mRNA encoding TNFA and IL12 was not significantly affected upon treatment with the hormones (Figures 3.7D and 3.7G).

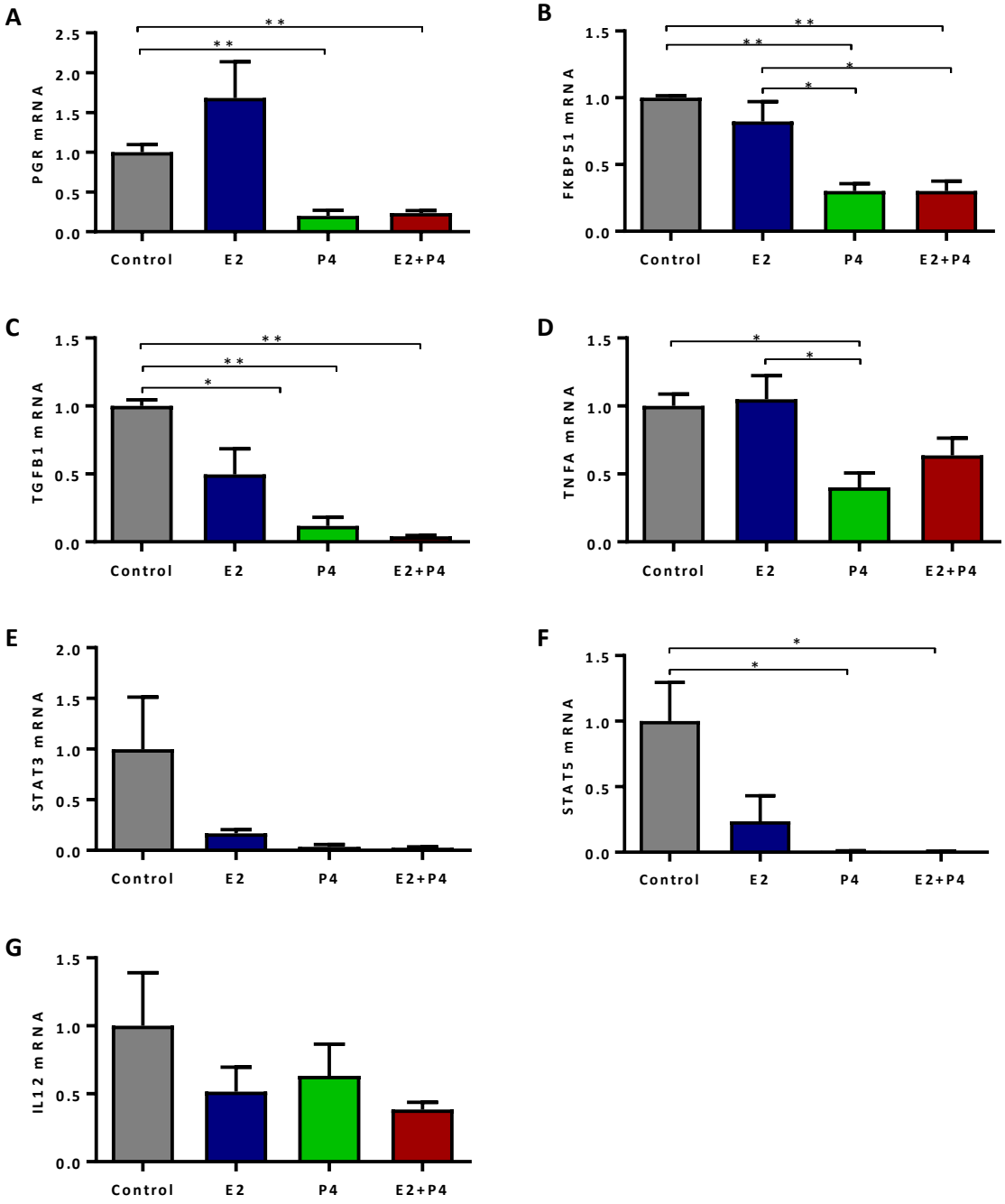


Figure 3.5: Cytokine gene profile in MCF7 mammary epithelial cell lines after 72 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TFGB1*, D) *TNFA*, E) *STAT3,* F) *STAT5,* and G) *IL12* in human mammary epithelial cell lines (MCF7) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 72 hours (n=1 experiment in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

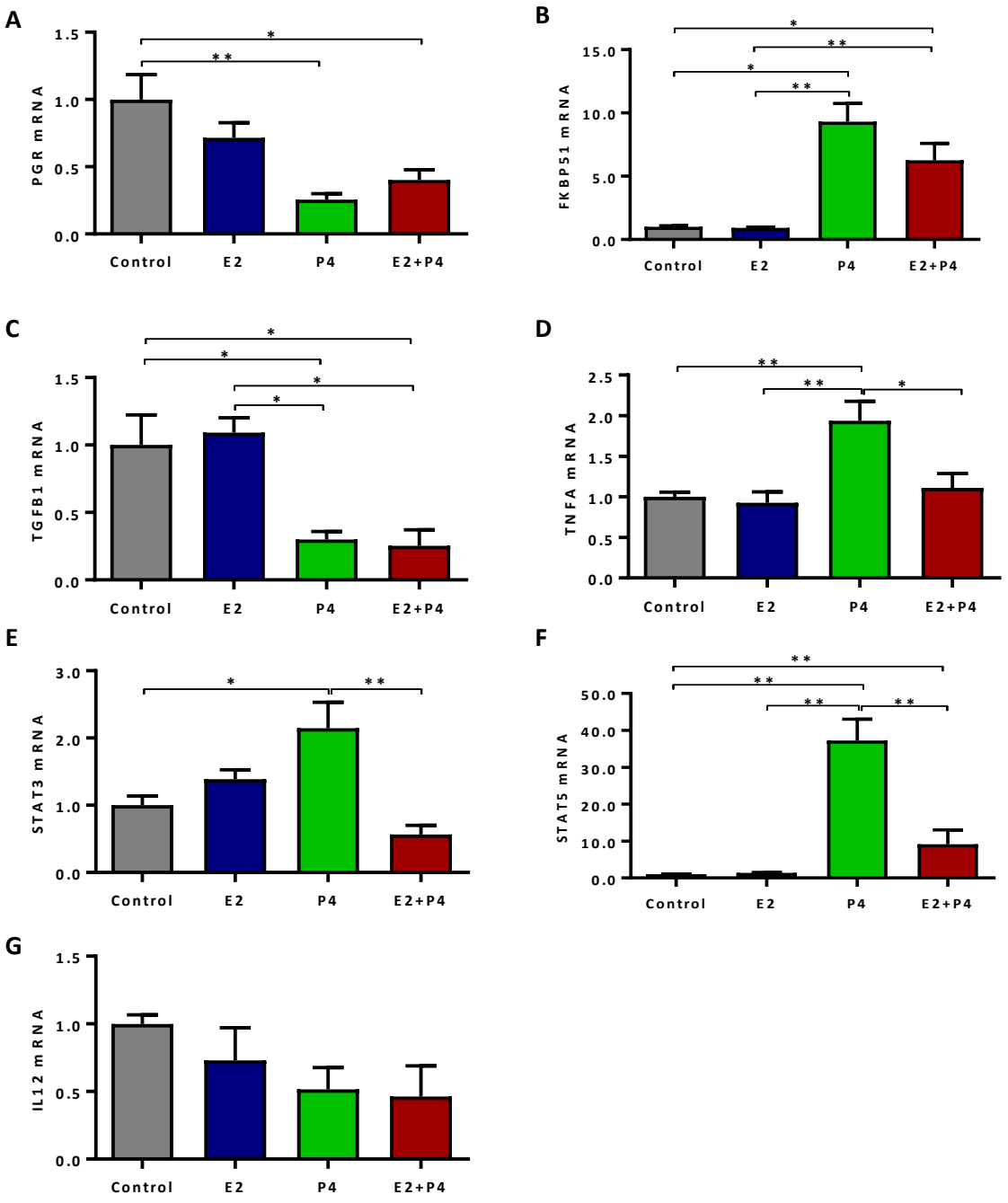


Figure 3.6: Cytokine gene profile in T47D mammary epithelial cell lines after 72 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TFGB1*, D) *TNFA*, E) *STAT3,* F) *STAT5,* and G) *IL12* in human mammary epithelial cell lines (T47D) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 72 hours (n=1 experiment in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, p<0.05; **, p<0.01).

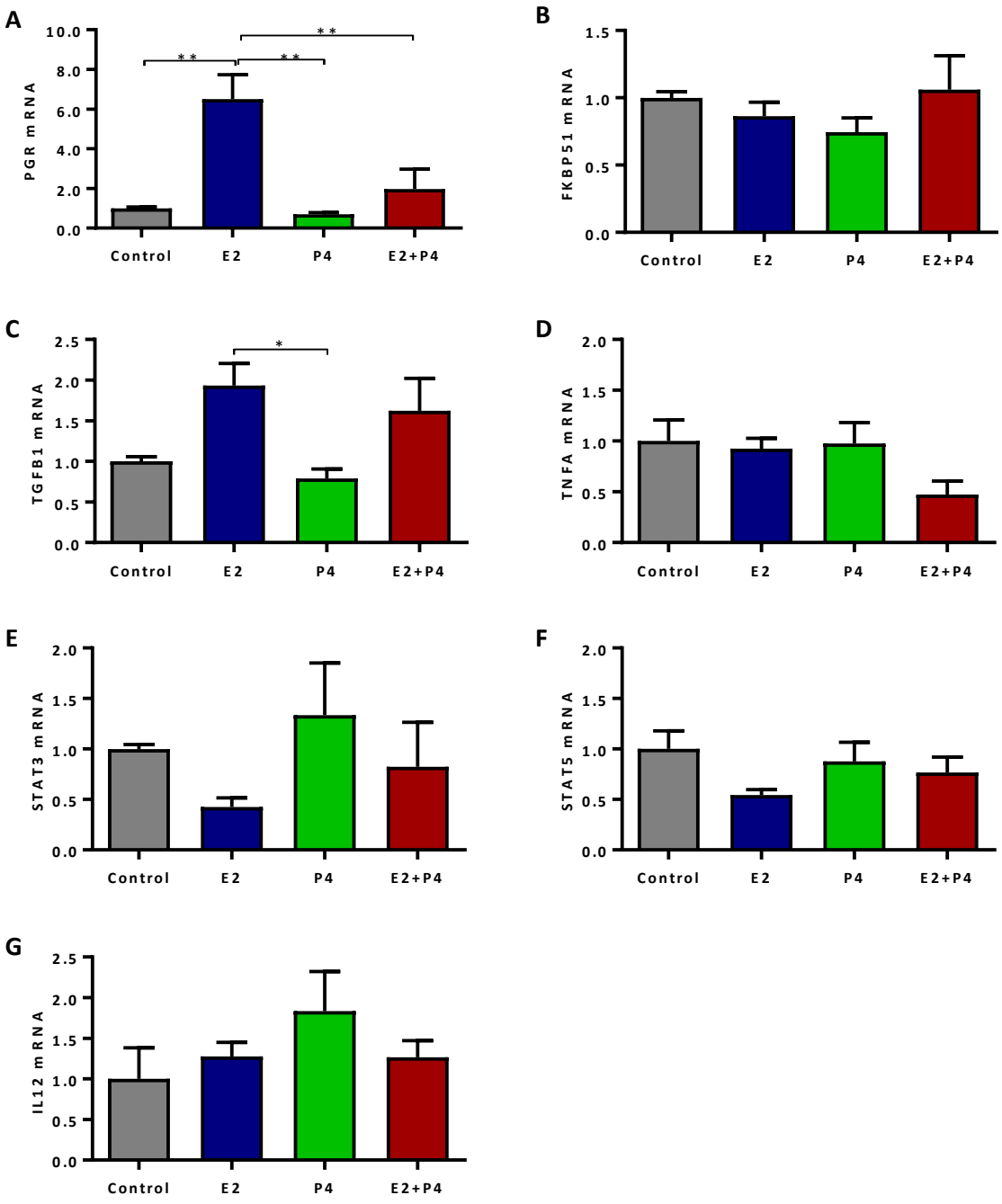


Figure 3.7: Cytokine gene profile in ZR751 mammary epithelial cell lines after 72 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *PGR*, B) *FKBP51*, C) *TFGB1*, D) *TNFA*, E) *STAT3,* F) *STAT5,* and G) *IL12* in human mammary epithelial cell lines (ZR751) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 72 hours (n=1 experiment in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

3.2.3. GENE REGULATIONS IN MICE MAMMARY GLAND DURING THE ESTROUS CYCLE:

To confirm that adult female C57BL6 mice in these experiments were undergoing regular estrous cycles, cycles were tracked for a period of 28 days by histological analysis of vaginal smears from 8 weeks of age (n=37 mice in total). The average cycle length was assessed over 28 days in mice, and a single complete cycle was defined as the first day of estrus through to the first day of the next estrus. Furthermore, to verify that estrous cycling was changing the morphology of the mammary gland in C57BL6 mice, as has been previously described in (19, 20), the 4th pair of the mammary glands from each mouse was collected at each of the four stages of the cycle and whole-mounts were stained with carmine alum. Examples of mammary gland whole-mounts at each phase of the cycle are shown in Figure 3.8.

Vaginal cytology analysis revealed that all mice underwent normal estrous cycling with an average cycle length of 6.0 ± 0.2 days. Ductal branching analysis on mammary gland whole-mounts showed that there were approximately 2.0 ± 0.2 branch points/mm during the estrus phase (n=8). Similarly, mice had 2.0 ± 0.1 branch points/mm in their mammary fat pad during the metestrus phase (n=10). However, the highest number of mammary ductal branch points could be observed at the diestrus phase with 2.3 ± 0.1 branch points/mm (n=10). This was significantly higher (20%) than the branch points quantified at the proestrus phase with 1.8 ± 0.1 branch points/mm (n=9; $p<0.05$) (Figure 3.8). These studies confirmed that the mice in this experimental cohort were undergoing regular ovarian cycling that was affecting mammary gland morphology as previously described (19, 20).

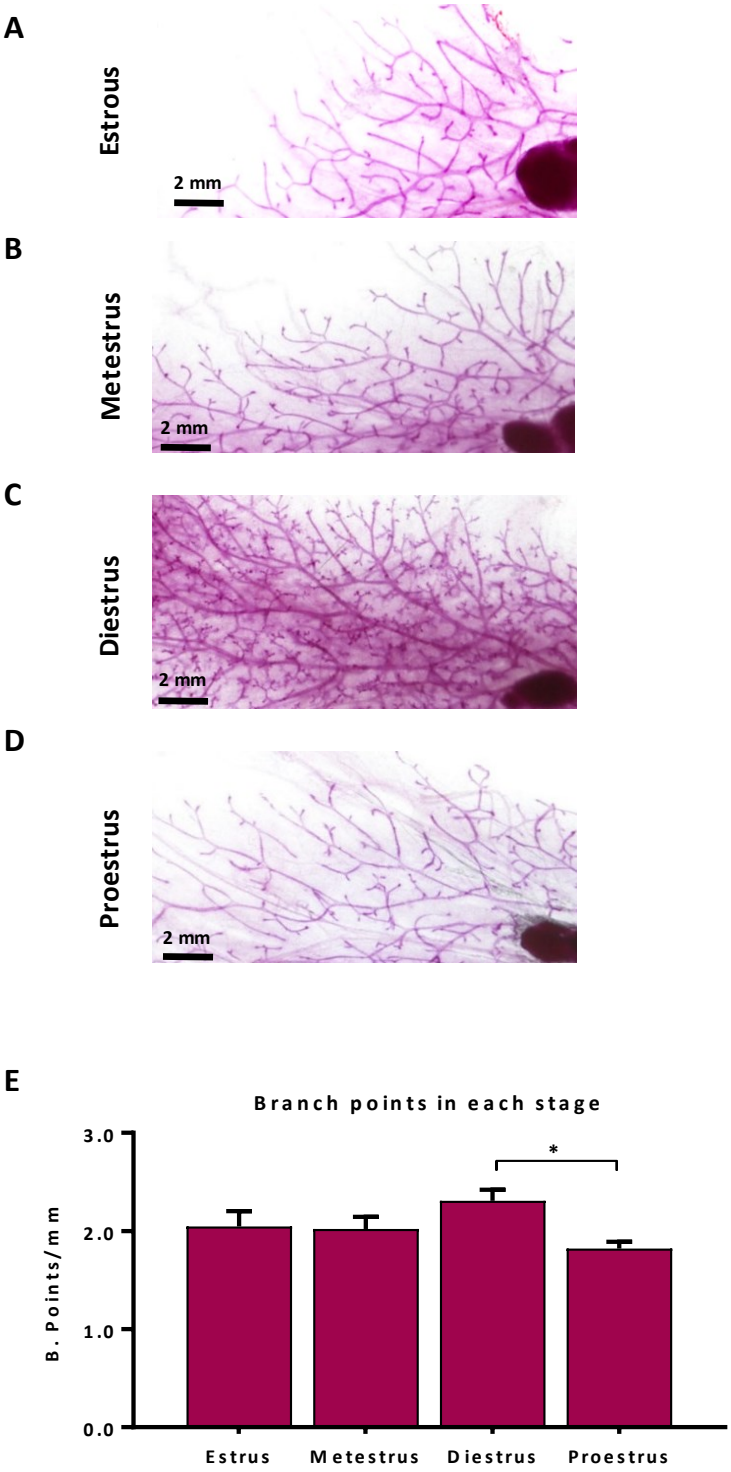


Figure 3.8: Ductal branch point analysis in virgin adult mice during the estrous cycle.
Mammary gland whole-mounts of virgin adult C57BL6 mice were stained with carmine alum at estrus (n=8), metestrus (n=10), diestrus (n=10) and proestrus (n=9). A-D) Representative images of mammary gland whole-mounts during all stages of the estrous cycle. E) The number of branch points per millimetre was calculated. Data are mean + SEM and were analysed by one-way ANOVA followed by post-hoc Tukey test (*, $p<0.05$).

To investigate the expression of mRNA encoding cytokines in mice during the estrous cycle, third pair mammary glands were collected at each of the four stages of the cycle from female adult mice. The comparative Ct method (i.e., 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of housekeeper gene *Rpl13a* in each mouse. The results were normalised to the average expression of each gene in the estrus phase so that the average of mRNA expression at this stage is 1 (n=8-10 per stage).

RT-PCR results showed that there was a high degree of variability in the expression of mRNA encoding all genes during the estrous cycle and none of the results were significant (Figure 3.9).

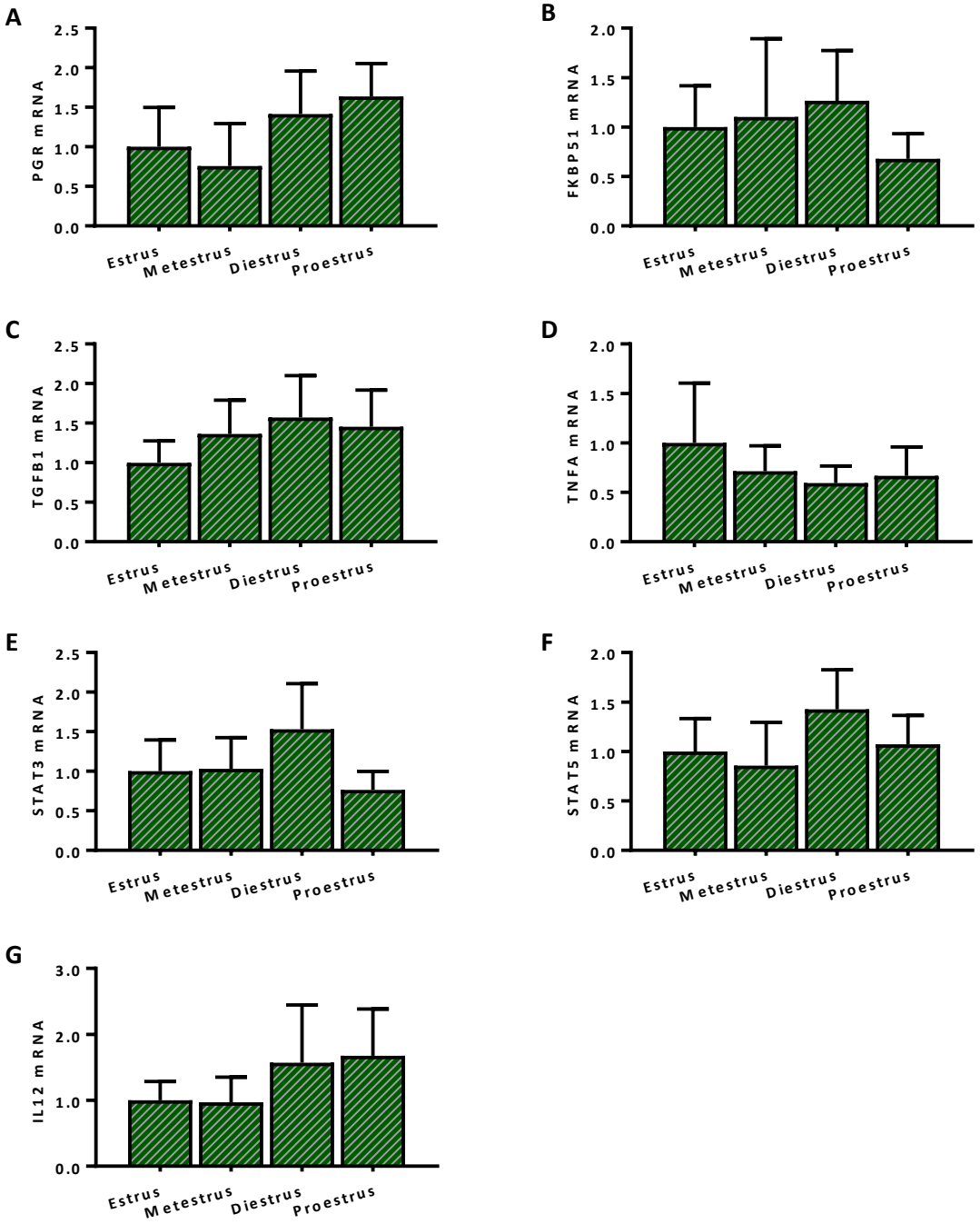


Figure 3.9: Cytokine gene profile in C57BL/6 mice's mammary glands at different stages of the estrous cycle.

RNA was extracted from the third mammary gland of female adult mice. Using RT-PCR, the messenger RNA expression of *Pgr, Fkbp51, Tgfb1, Tnfa, Stat3, Stat5*, and *Il12* was analysed in the mammary gland at all stages of the estrous cycle. The average expression of these genes in mice was normalized to the average expression in estrus stage so that the average of mRNA expression in this stage is 1 (n=8-10 per stage). Data are mean + SEM, and were analysed using a one-way ANOVA.

3.3. DISCUSSION

The mechanisms by which estrogen and progesterone act *in vivo* are difficult to disentangle due to the complex interactions of endocrine hormones at the cellular, tissue and organismal levels (16). Moreover, estrogen and progesterone do not necessarily act directly on the target cells that express their receptors, but often they exert their effects on the mammary epithelium indirectly via paracrine signalling and stromal-epithelial interactions. Therefore, a variety of experimental approaches including cell lines, primary organoids and mouse models were used in this study to investigate the effects of ovarian hormones on cytokine expression by human mammary epithelial cells. The experiments described in this chapter have clearly demonstrated that estradiol and progesterone play a significant role in regulating the expression of transcription factors and inflammatory cytokines in mammary epithelial cells.

3.3.1. HORMONAL REGULATION OF TGFB1 EXPRESSION IN THE MAMMARY GLAND

Expression of *TGFB1* in human mammary epithelial cell lines suggests that progesterone alone or combined with estradiol has inhibitory effects on *TGFB1* production by mammary epithelial cells. Similarly, mRNA expression of this cytokine was significantly attenuated in primary human mammary epithelial organoids treated with the combination of estradiol and progesterone. In the mouse mammary gland, *Tgfb1* mRNA expression was variable. However, there is a tendency for this gene to be lower during estrus compared to other phases of the cycle. According to the literature, the highest expression of latent Tgfb1 is observed at the diestrus phase of the ovarian cycle, when circulating progesterone peaks (157). This is associated with the increased proliferation and apoptosis rate in response to high levels of progesterone during the diestrus phase compared to estrus (19). Conversely, Ewan, Shyamala (157) observed that overabundance of Tgfb1 in the mammary gland reduced proliferation of terminal end bud epithelium and eventually caused regression of these structures. Furthermore, reduced

expression of this cytokine resulted in induced epithelial cell proliferation and ductal extension in Tgfb1 knockout mouse models (157).

Epithelial cell-derived Tgfb1 exerts strong inhibitory effects on mammary epithelial cell proliferation, demonstrated in a mammary transplant mouse model, where Tgfb1 deficient epithelium was transplanted in a Tgfb1 replete host, resulting in increased proliferation of mammary epithelial cells in the mammary gland (113). Previous studies highlight that TGFB1 plays important roles in mediating the complex processes involved in mammary gland development (158, 159). Robinson, Silberstein (160) have shown that mammary epithelial cells express the gene encoding Tgfb1 at high levels in pubertal and virgin adult mice, whereas it is lowly expressed during pregnancy-induced alveolar development, with diminished expression in the lactating gland. Therefore, the reduction in *TGFB1* mRNA expression in the progesterone-stimulated breast cancer cell lines in our studies may suggest the removal of an inhibitory signal, and enabling proliferation of mammary epithelial cells, which occurs during the luteal phase of cycle.

Sun, Robertson (113) reported that epithelial cell-derived Tgfb1 acts through macrophages and other stromal cells during the ovarian cycle and inhibits macrophage activation and immune surveillance by suppressing Inducible Nitric Oxide Synthase (iNOS) expression. In turn, these stromal actions of epithelial cell-derived TGFB1 might exert feedback effects on the epithelium, and affect further expression of TGFB1. In addition, basal TGFB1 expression is known to be affected by polymorphism in the *TGFB1* gene. The L10P gene polymorphism in particular causes elevated TGFB1 at the cellular level and in serum (161). This polymorphism is also associated with a significant increase in breast cancer risk in a large international consortium study (161).

Overall, the roles of TGFB1 in mammary gland development and tumorigenesis are critical. TGFB1 suppresses development of breast tumours by inhibiting cell proliferation, inhibiting production of growth factors, cytokines and chemokines or by inducing apoptosis. However, high expression of this cytokine can promote tumour invasion and metastasis by impairing immune surveillance and increasing angiogenesis (162). Also, elevated TGFB1 expression has been found in many breast cancer patients and strong correlations between TGFB1 polymorphism and breast cancer risk are reported, as highlighted above. Our findings in this experiment suggest that *TGFB1* expression in the mammary epithelial cells is inhibited by progesterone. It appears that expression of this cytokine varies widely between women and fluctuates according to altered ovarian hormone signalling throughout the different phases of the ovarian cycle. However, the exact mechanisms through which the ovarian hormones affect TGFB1 expression are still unclear. Further research is required to investigate the roles of this cytokine in the mammary gland during the menstrual cycle and its link to breast cancer development.

3.3.2. SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT 3 AND STAT5):

Our results derived from the cell lines and mouse mammary gland suggest that *STAT3* is positively regulated by progesterone. Messenger RNA expression of *STAT3* was significantly increased in progesterone-responsive T47D and ZR751 cells upon treatment with progesterone. There was also a tendency for the expression of this gene to be increased during the progesterone-dominant diestrus phase in mice. On the contrary, hormone treatments of the estrogen-responsive MCF7 cells for 72 hours resulted in a dramatic reduction of *STAT3*. Previous studies have demonstrated that induced expression of STAT3 can be a death signal for activating mammary epithelial cell apoptosis and returning the mammary gland to its basic structure. This might affect the function and phenotype of immune cells such as macrophages

and mast cells since STAT3 regulates the influx of these cells during involution and drives a polarisation of macrophages towards an alternatively activated (M2) phenotype (163). On the other hand, a lack of STAT3 in the mammary epithelium is associated with a significant increase in the abundance of iNOS, suggesting the existence of classically activated macrophages (163). In addition, STAT3 regulates the expression of genes associated with innate immunity during involution and mediates tissue remodelling by balancing the inflammatory and anti-inflammatory signalling (62). Thus, it is likely that dysregulation of STAT3 by ovarian hormones influences the function of immune cells and promotes an inflammatory environment in the mammary gland which has pro-tumorigenic potential (164).

Similar results were obtained for *STAT5* upon treatment with estradiol and progesterone in the cell lines. However, transcriptional changes observed in *STAT5* seem to be more significant than the changes observed in *STAT3*, suggesting that hormone signalling strongly activates this gene in the mammary gland. It is worth pointing out that despite their high homology, STAT3 and STAT5 have opposing roles in mammary gland development as well as in breast cancer (165). Overexpression of Stat5 in the mouse mammary gland resulted in induced mammary epithelial proliferation and differentiation and delayed involution by preventing Stat3 activation, and also contributed to mammary tumorigenesis (166). In breast cancer, these transcription factors regulate a subset of target genes (such as BCL6) in an opposite fashion such that the inhibitory effects of STAT5 on gene expression is dominant over the stimulatory effects of STAT3 (63). Given that STAT5 is essential for mammary epithelial cell survival and maintenance (167), it is likely that dysregulation of this gene by ovarian hormones may alter cell proliferation and differentiation rate, and increase the risk of DNA damage in the mammary epithelium. Although the roles of STAT3 and STAT5 in mammary gland development and tumorigenesis are known, the importance of these transcription factors in the mammary gland during the menstrual cycle is not well-understood. Given the roles they play in breast cancer,

their impaired activity during the menstrual cycle could represent a genuine risk factor for this disease.

3.3.3. HORMONE REGULATION OF PRO-INFLAMMATORY CYTOKINES IN THE MAMMARY GLAND:

Our mouse studies show that the mRNA expression of *Tnfa* was highly variable; however, there is a trend for this cytokine to be induced during the estrus phase of the cycle. This is in parallel with the observations by Dasari, Sharkey (111) who showed using multiplex assay that the protein concentration of Tnfa is increased in the mammary gland during the estrus compared with other phases. These observations suggest that estradiol has stimulatory effects on Tnfa expression in the mammary gland. This is supported by our findings in MCF7 cells treated with estradiol (for 24 hours) which resulted in a 50% increase in *TNFA* mRNA expression. TNFA can either induce tumour necrosis and apoptosis or promote tumour development (168). Stimulation of T47D cells with TNFA in the presence of estradiol resulted in an enhanced breast cancer proliferation *in vitro* (169). However, our results along with previous observations by Dasari, Sharkey (111) show that progesterone has significant inhibitory effects on the expression of *TNFA*. Thus, an increase in the expression of this cytokine during the estrus phase may promote a pro-inflammatory and tumorigenic environment in the mammary gland, which may get dampened by progesterone at other phases of the cycle.

On the other hand, the expression of *IL12* was not noticeably affected by hormone treatments. It is important to note that pro-inflammatory cytokines are not constitutively expressed by normal cells in the tissue. Indeed, they require inflammatory insults, such as infection or necrosis, in order to be highly expressed and promote inflammatory responses (170). The variability in regulation of the cytokines reported in the literature and in our study, suggest that other stromal components rather than epithelial cells might affect their expression in the

mammary gland. As mentioned earlier, many different cell types such as adipocytes, fibroblasts, macrophages and mast cells can produce these cytokines. Therefore, to fully understand the mechanisms involved in the hormonally regulated cytokine production in the mammary gland, the cell sources for each cytokine should be identified.

3.3.4. LIMITATIONS:

Each model used in this study possesses limitations which might have affected the results. Analysis of RT-PCR results obtained from primary organoids and mouse mammary glands demonstrates enormous variability in mRNA expression between different samples, which makes it very difficult to derive any conclusion from the data. However, this was not surprising, as inter-individual variability is expected due to many genetic and potentially epigenetic factors. This heterogeneity truly represents the nature of primary tissues. Another important factor that might explain this variability is that the organoids growing in this model recapitulate estrogen or progesterone receptor expression observed *in vivo*. Between 10 and 25% of epithelial cells from human breast tissue express estrogen or progesterone receptors *in vivo* (126). Hence, only a low proportion of cells respond to estrogen and progesterone; demonstrating that hormone regulation in some samples of primary mammary epithelium can be quite challenging to detect. To validate the hormone responsiveness of our models, we analysed the expression of mRNA encoding the estrogen-responsive gene encoding PGR and the progesterone-responsive gene encoding FKBP51, however, the results were highly variable amongst the samples. Future studies could investigate whether higher concentrations of hormones for the organoid cultures, and pre-treatment of these cells with estradiol to induce progesterone receptor expression, could result in a less variable response. It is also noteworthy that freezing the primary mammary epithelial organoids prior to culture might have affected their responsiveness to estrogen and progesterone. Future studies should investigate this.

Furthermore, studies in the cell lines revealed that the time course of hormone treatments can significantly affect the gene expression. Although all cell lines showed great responsiveness to estradiol and progesterone, the extent of hormonal response varied in some cells depending on the treatment time course. For instance, progesterone treatment of T47D cells for 24 hours increased the expression of mRNA encoding FKBP51 up to 4-fold, while 72 hours treatment of these cells resulted in a 10-fold increased expression of this gene. This suggests that longer exposure to hormones would activate a higher number of receptors, leading to a larger effect on gene expression, possibly through an indirect pathway. A similar effect could be observed in the mRNA expression of our genes of interest. For instance, hormone treatments of MCF7 cell lines for 24 hours did not have any effect on the expression of mRNA encoding STAT3 and STAT5; however, 72 hours treatment of these cells with estradiol and progesterone resulted in a significant reduction in the expression of these genes. Such effects could also be due to the half-life of the specific mRNA, which is an important factor in determining how long it takes to detect a change in the mRNA level of a specific gene (171). Nonetheless, the results obtained from these experiments suggest that treating cells with hormones at different time points produces different results, most of which can depend on the hormone receptor profile of the cells.

3.4. CONCLUSION

Using three different cell culture settings, we showed that hormone regulation of cytokines in the mammary epithelium is dependent on the hormone receptor responsiveness of the cells, as well as the exposure time to the ovarian hormones. However, there are inconsistent results from different mouse and cell line studies, and primary organoid cultures exhibit too much variability to significantly confirm hormonal regulation. On the other hand, most of the cytokines have paradoxical activities in regulation of mammary gland development and breast tumorigenesis. Clearly, hormonal regulation of immune-associated cytokines is complex and it is likely that

the expression of these cytokines is affected by a number of factors such as infection, genetics, and interactions with immune cells and other components of the microenvironment. Therefore, further experiments are required to understand the precise roles and effects of these cytokines in mammary gland development and breast cancer risk. Studies in the following chapters will investigate the role of other important transcription factors in regulating cytokine expression in mammary epithelial cells.

CHAPTER FOUR

THE ROLE OF ELF5 IN HORMONE REGULATED CYTOKINE EXPRESSION BY MAMMARY EPITHELIAL CELLS

4.1. INTRODUCTION

The previous chapter demonstrated that estradiol and progesterone regulate the mRNA expression of specific transcription factors and inflammatory cytokines in mammary epithelial cells. However, the precise molecular mechanisms through which these hormones regulate cytokine expression in epithelial cells is not well-defined. To address this, we focused on an important epithelial cell-specific transcription factor, and analysed its role in hormone regulated cytokine secretion by mammary epithelial cells.

E74-like factor 5 (ELF5, also known as ESE-2) is an epithelial cell-specific member of the large family of Ets transcription factors, found in the lung, kidney, placenta, and most prominently in mammary glands (75, 76). ELF5 plays roles in mammary epithelial cell proliferation and differentiation (77). It specifies the mammary secretory cell lineage and is mainly expressed by the luminal progenitor cells in the mammary gland (78). Studies in Elf5 null mutant mouse models demonstrate that these animals are either unable to lactate due to failed alveolar development, or exhibit impaired functional secretory units due to improper differentiation of alveoli. This indicates that ELF5 is a crucial transcriptional mediator required for structural and functional morphogenesis of lobuloalveoli (77).

The expression of ELF5 is altered in various cancers (172) and implicated in breast cancer as a critical regulator of tumour subtypes. ELF5 is involved in mammary tumour metastasis in the luminal A breast cancer subtype, by recruiting particular cells of the innate immune system such as myeloid-derived suppressor cells (82). Furthermore, high expression of ELF5 correlates with more aggressive basal cancers and resistance to anti-estrogen cancer therapies (84). ELF5 is a direct transcriptional target of the progesterone receptor, and its expression is increased by progestin treatment *in vivo* in mice and *in vitro* in T47D human breast cancer cell lines (85). ELF5 can be considered an essential mediator of progesterone-regulated mammary epithelial cell proliferation and differentiation. It might affect cancer susceptibility through the production of cytokines that affect interactions between the mammary epithelium and surrounding immune cell populations. Little is known of the role of this transcription factor in directing epithelial cell-specific cytokine secretion and its association with immune cells. However, using chromatin immunoprecipitation sequencing (ChIP-Seq), Ormandy and his colleagues found that ELF5 binding sites are located near the promoter region of specific immune-related cytokines such as CXCL12, S100A8, and S100A9, suggesting these to be key targets of ELF5 function in the mammary gland and in breast cancer (Unpublished observations).

CXCL12 is a homeostatic chemokine for lymphocytes and monocytes and has roles in breast cancer progression (117, 118). It is primarily expressed in hormone receptor-positive luminal cells (119), and its expression is strongly upregulated in estrogen-treated MCF7 breast cancer cells (120). The calcium-binding proteins S100A8 and S100A9, expressed by neutrophils, macrophages, and activated monocytes (121), are damage-associated molecular pattern (DAMP) molecules that are overexpressed in inflammation and breast cancer suggesting that they might have a potential role in inflammation-associated tumorigenesis (123). Also, their expression is associated with estrogen receptor loss in breast cancer cell lines (122).

Nonetheless, the molecular mechanisms through which these cytokines are involved in breast cancer is not well understood. Considering the role of hormone-regulated ELF5 in breast cancer, it is possible that it regulates the expression of immune-related cytokines to either promote immune tolerance or dampen immune surveillance in the breast, subsequently increasing the risk of cancer. The experiments described in this chapter were devised to investigate the role of ELF5 transcription factor in regulating immune cell-associated cytokines by mammary epithelial cells, to better understand the role of ELF5 in the mammary gland.

4.2. RESULTS:

4.2.1. MESSENGER RNA EXPRESSION OF CYTOKINES IN HORMONE-TREATED ORGANOIDS:

To investigate the effects of estrogen and progesterone on mammary epithelial cell cytokine production, primary human mammary epithelial organoids (n=6) were embedded in Matrigel and treated with combinations of estradiol (10nM) and progesterone (100nM) for 72 hours. The abundance of mRNA encoding cytokines ELF5, CXCL12, S100A8, and S100A9 were examined from hormone-treated human epithelial organoids. The comparative Ct method (i.e. 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to the mean of expression of housekeeping genes *MPRL19* from the same sample.

The expression of all cytokines was highly variable amongst patients with no trend, and no statistically significant result was obtained (Figure 4.1).

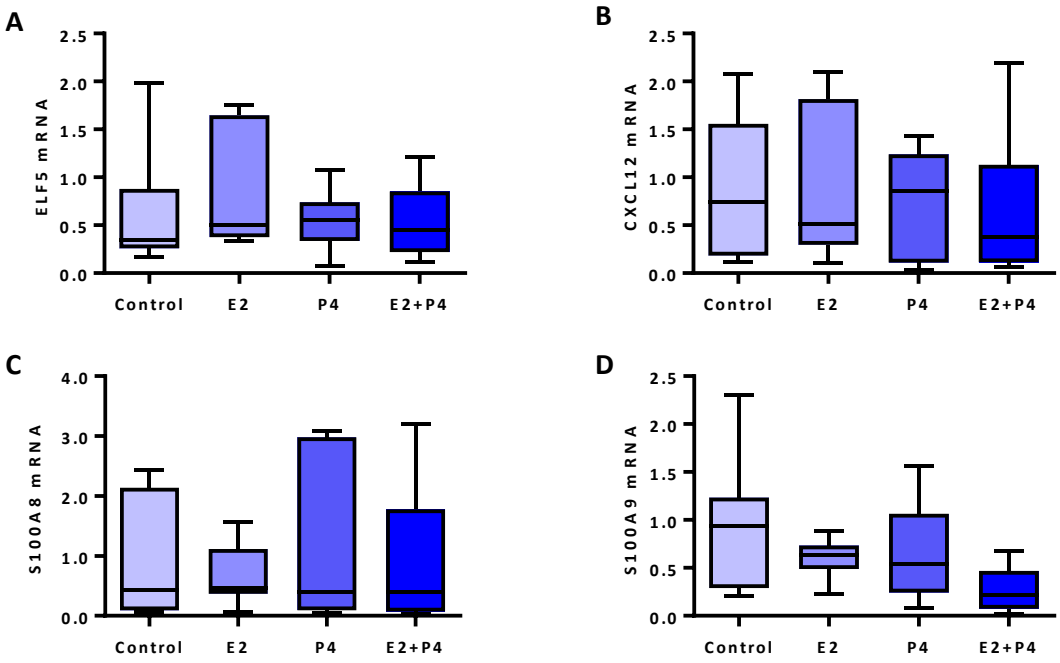


Figure 4.1: Hormonal regulation of mRNA encoding cytokines in 3D organoid cultures.
Box plots represent the mean variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* from Matrigel-embedded human epithelial organoids upon treatment with combinations of estradiol (E2) and progesterone (P4) for 72 hours (n=6). Data are Mean ± SEM; normalized to the housekeeper gene *MPRL19*.

4.2.2. MESSENGER RNA EXPRESSION OF CYTOKINES IN HUMAN MAMMARY EPITHELIAL CANCER CELL LINES:

To investigate the effects of estrogen and progesterone on cytokine production by mammary epithelial cells, human mammary epithelial cancer cell lines cultured at optimal density were treated with estradiol (10nM) and progesterone (100nM). Messenger RNA encoding cytokines ELF5, CXCL12, S100A8, and S100A9 was quantified in MCF7, T47D, and ZR751 human mammary epithelial cell lines treated with various combinations of estradiol and progesterone for i) 24 hours and ii) 72 hours. The comparative Ct method (i.e., 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of housekeeper gene *MPRL19* in each sample. The results were normalised to this gene so that the average of the control samples was 1.

In MCF7 cell lines treated with hormones for 24 hours, progesterone increased abundance of *ELF5* mRNA by 50% ($p<0.05$) compared to ethanol-treated control cells (Figure 4.2A). However, the same effect was not observed when both estradiol and progesterone were combined as a treatment. Although the expression of all other cytokines was variable, there was a trend for *CXCL12* to be upregulated by about 50% upon treatment with either estradiol or a combination of hormones (non-significant) (Figure 4.2B).

In T47D cells, 24 hours treatment with estradiol or a combination of hormones had inhibitory effects on the expression of mRNA encoding ELF5 which was downregulated by approximately 50% compared with the controls ($p<0.01$) (Figure 4.3A). Moreover, progesterone alone or combined with estradiol significantly attenuated *CXCL12* mRNA by more than 90% ($p<0.01$) compared to estradiol-treated and control cells (Figure 4.3B). The expression of mRNA encoding S100A8 was significantly increased by 50% upon treatment with progesterone, compared with estradiol-treated cells ($p<0.05$). However, no changes were

observed in the expression of mRNA encoding S100A9 in cells treated with estradiol and progesterone either alone or in combination (Figure 4.3D).

In ZR751 cell lines, progesterone treatment for 24 hours resulted in a significant increase (by approximately 50%) in the expression of mRNA encoding ELF5 and S100A9, compared with estradiol-treated and control cells ($p<0.05$ for both genes) (Figure 4.4A and 4.4D). Treatment of these cells with estradiol dramatically elevated the expression of mRNA encoding CXCL12 by 35-fold ($p<0.01$), an effect which was dampened by approximately 10-fold when progesterone and estradiol were combined ($p<0.01$) (Figure 4.4B).

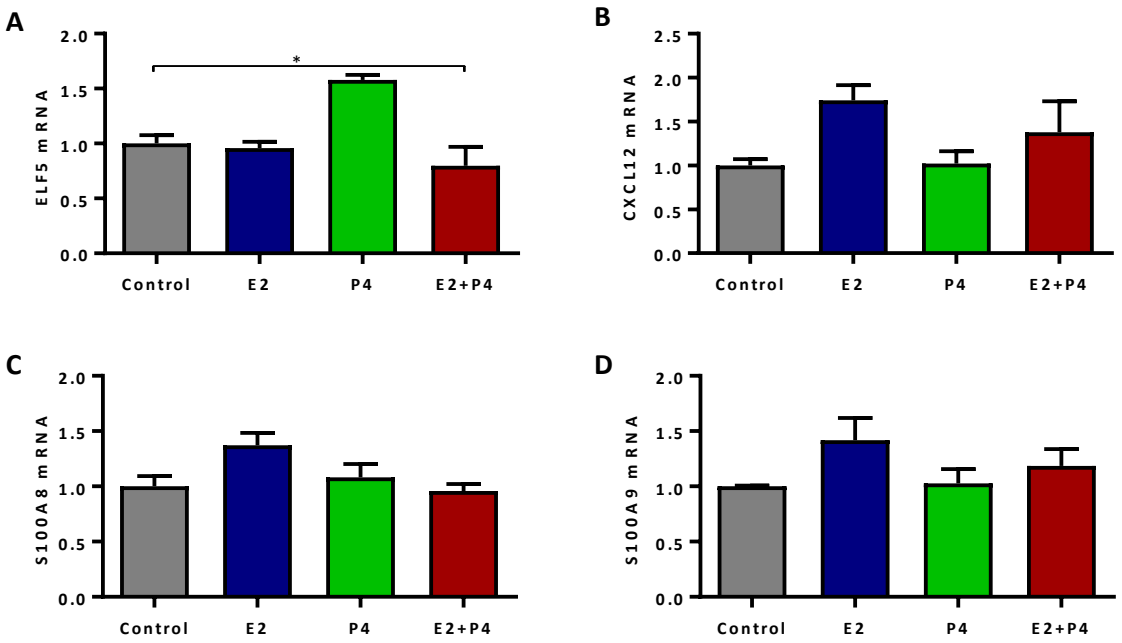


Figure 4.2: Cytokine gene profile in MCF7 mammary epithelial cell lines after 24 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (MCF7) upon treatment with combinations of estradiol (E2) and progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

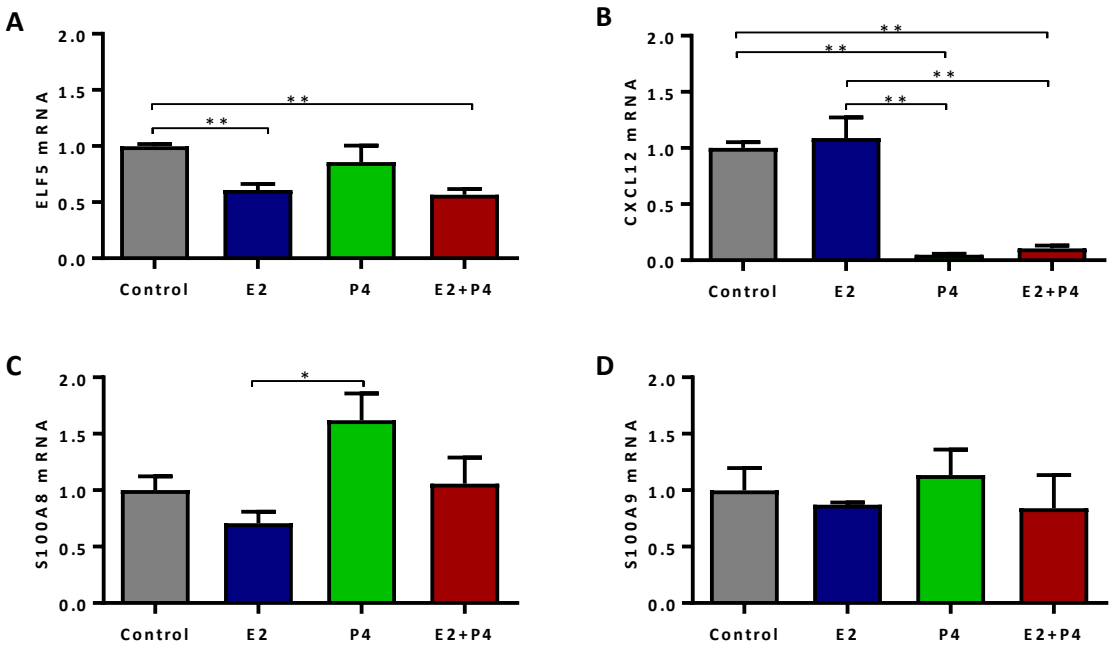


Figure 4.3: Cytokine gene profile in T47D mammary epithelial cell lines after 24 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (T47D) upon treatment with combinations of estradiol (E2) and progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

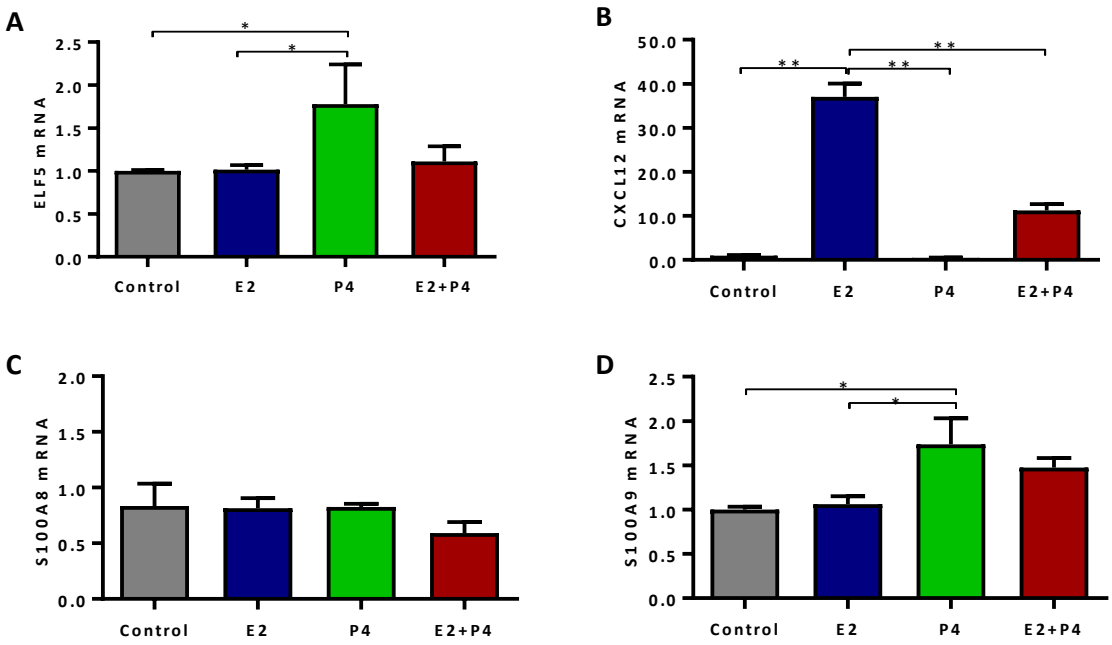


Figure 4.4: Cytokine gene profile in ZR751 mammary epithelial cell lines after 24 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (ZR751) upon treatment with combinations of estradiol (E2) and progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

Although it was statistically non-significant, the expression of mRNA encoding ELF5 tended to be dampened by about 50% upon hormone treatments of MCF7 cell lines (Figure 4.5A). Progesterone treatment alone and in combination with estradiol tended to reduce *CXCL12* mRNA expression by more than 80% in these cells (non-significant results). The expression of mRNA encoding S100A8 was significantly upregulated by 6-fold upon treatment with the combination of estradiol and progesterone compared to all other cells ($p<0.01$) (Figure 4.5C). Estradiol treatment induced the expression of mRNA encoding S100A9 by approximately 3-fold compared to all other treatments ($p<0.01$) (Figure 4.5D).

The most dramatic and consistent results were obtained from T47D cells treated with hormones for 72 hours. Progesterone alone and in combination with estradiol increased the expression of mRNA encoding ELF5 by approximately 200% and 100%, respectively ($p<0.01$) (Figure 4.6A). The mRNA expression of *CXCL12* was significantly attenuated in the cells treated with either progesterone alone (by approximately 80%; $p<0.01$) or the combination of estradiol and progesterone (by approximately 90%; $p<0.01$), compared to estradiol-treated and control-treated cells (Figure 4.6B). Also, progesterone treatment significantly increased the mRNA expression of genes encoding S100A8 and S100A9 by 30- and 15-fold, compared to estradiol-treated and control-treated cells ($p<0.01$). These effects were dampened by 60% when estradiol and progesterone were combined ($p<0.01$) (Figure 4.6C and 4.6D).

72 hours treatment of ZR751 cells with estradiol resulted in a significant upregulation of *CXCL12* cytokine (by approximately 20-fold), compared to progesterone-treated and control-treated cells ($p<0.01$). Also, the combination of hormones tended to increase *CXCL12* mRNA expression by approximately 10-fold; however, this result was not statistically significant (Figure 4.7B). None of the other results obtained from hormone-treated ZR751 cells were statistically significant. However, progesterone tended to decrease the expression of mRNA

encoding ELF5 by approximately 50% compared to control cells (Figure 4.7A). The expression of *S100A8* and *S100A9* was not affected by hormone treatments (Figure 4.7C and 4.7D).

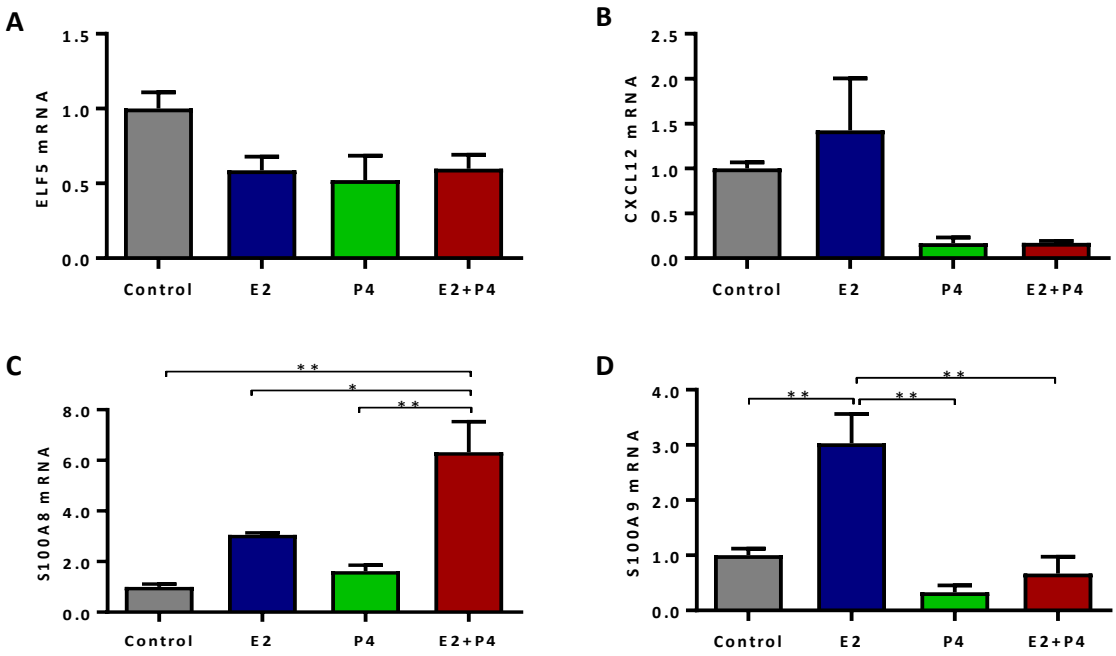


Figure 4.5: Cytokine gene profile in MCF7 mammary epithelial cell lines after 72 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (MCF7) upon treatment with combinations of estradiol (E2) and progesterone (P4) for 72 hours (n=1 experiment in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

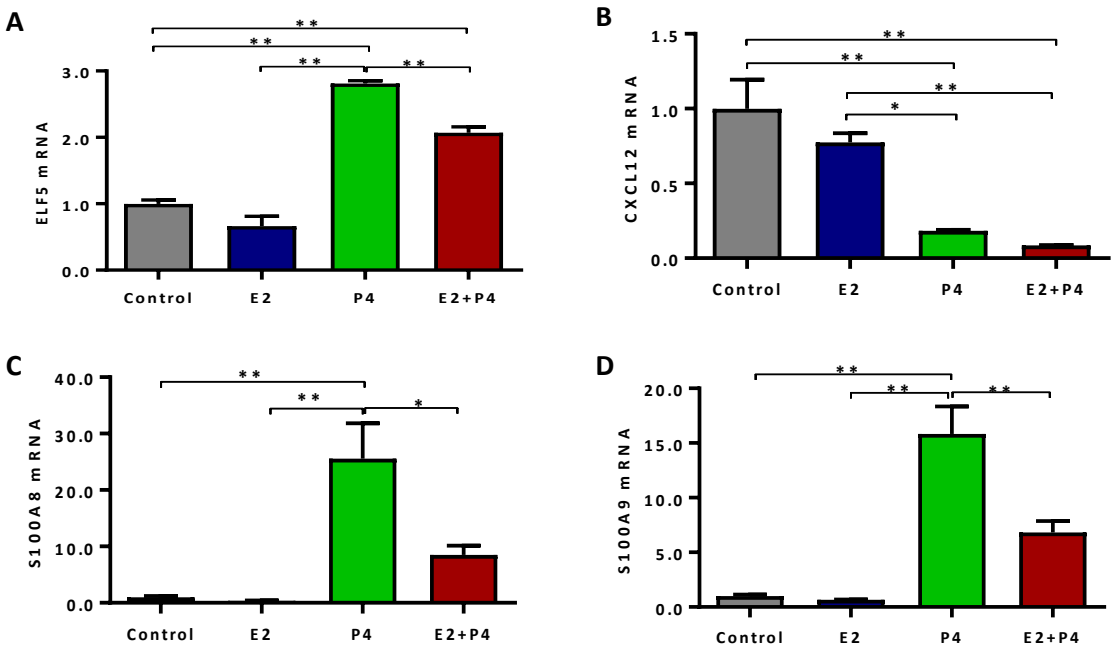


Figure 4.6: Cytokine gene profile in T47D mammary epithelial cell lines after 72 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (T47D) upon treatment with combinations of estradiol (E2) and progesterone (P4) for 72 hours (n=1 experiment in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

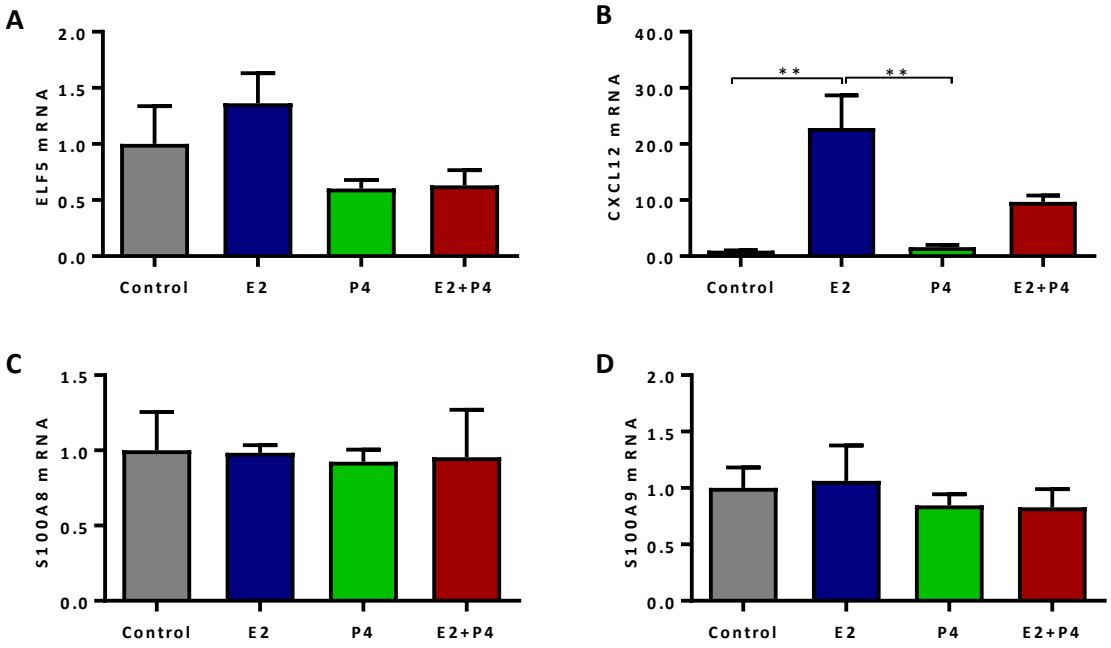


Figure 4.7: Cytokine gene profile in ZR751 mammary epithelial cell lines after 72 hours treatment with hormones.

Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (ZR751) upon treatment with combinations of estradiol (E2) and progesterone (P4) for 72 hours (n=1 experiment in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

4.2.3. MESSENGER RNA EXPRESSION OF CYTOKINES IN THE MOUSE MAMMARY GLAND DURING THE ESTROUS CYCLE:

To investigate the effects of ovarian hormones on cytokine expression during the estrous cycle in the mammary gland, third pair mammary glands from wildtype C57BL/6 mice were collected at each of the four stages of the cycle. Messenger RNA expression of cytokines *Elf5, Cxcl12, S100a8,* and *S100a9* were examined from the mammary glands at each stage of the estrous cycle. The comparative Ct method (i.e. 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to the mean of expression of housekeeping gene *Rpl13a* from the same sample.

Although none of the results were statistically significant, the expression of all four cytokines tended to change over the course of the estrous cycle. The expression of mRNA encoding Elf5 was increased by 100% during metestrus and diestrus phases of the cycle compared to the estrus phase, while it dropped to baseline during proestrus. *Cxcl12* expression was increased during metestrus by about 70%; however, it decreased gradually towards the proestrus phase. The expression of mRNA encoding S100a8 and S100a9 were also upregulated during diestrus (by 100 % and 60% respectively) compared to the estrus phase of the cycle (p=0.088 for S100a8 and p=0.334 for S100a9) (Figure 4.8).

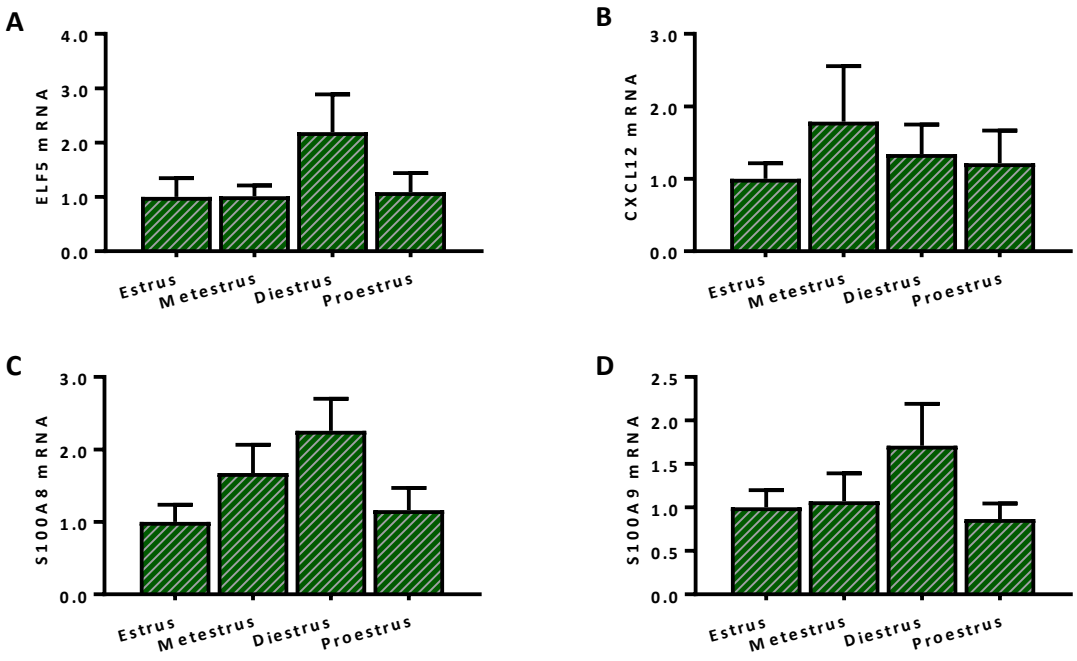


Figure 4.8: Cytokine gene profile in C57BL/6 mice's mammary glands at different stage of the estrous cycle.

RNA was extracted from the third mammary gland of wildtype mice. Using RT-PCR, the messenger RNA expression of *Elf5, Cxcl12, S100a8* and *S100a9* was analysed in the mammary gland at all stages of the estrous cycle. The average expression of these genes in mice was normalized to the average expression in estrus stage so that the average of mRNA expression in this stage is 1 (n=8-10 per stage). Data are mean + SEM, and were analysed using a one-way ANOVA, followed by Tukey post-hoc test.

4.2.4. THE EFFECT OF PROGESTERONE ON CYTOKINE EXPRESSION IN T47D CELLS:

The most dramatic changes in the expression of mRNA encoding cytokines were observed in the T47D cell line treated with progesterone for 72 hours. Hence, further studies were conducted using T47D cells in such culture condition. To confirm the efficacy and reproducibility of results obtained from the previous section, T47D cells were treated with progesterone alone (100nM) for 72 hours. Messenger RNA expression of genes encoding ELF5, CXCL12, S100A8, and S100A9 was examined using RT-PCR. The comparative Ct method (i.e., 2-($\Delta\Delta$CT) method) was used to analyse quantitative real-time PCR data, relative to expression of housekeeper gene *MPRL19* in each treatment group. The results were normalised to this gene so that the average of the control samples was 1.

There are dramatic changes in the expression of all cytokines of interest in T47D cells treated with progesterone. As it can be seen from Figure 4.9, *ELF5* mRNA expression was induced significantly by 150% compared to the control cells ($p<0.05$). This was followed by a significant rise in the expression of mRNA encoding S100A8 and S100A9 (20- and 13-fold, respectively; $p<0.05$)). In addition, the expression of CXCL12 was reduced by more than 80% ($p<0.01$) upon treatment with progesterone compared to the control.

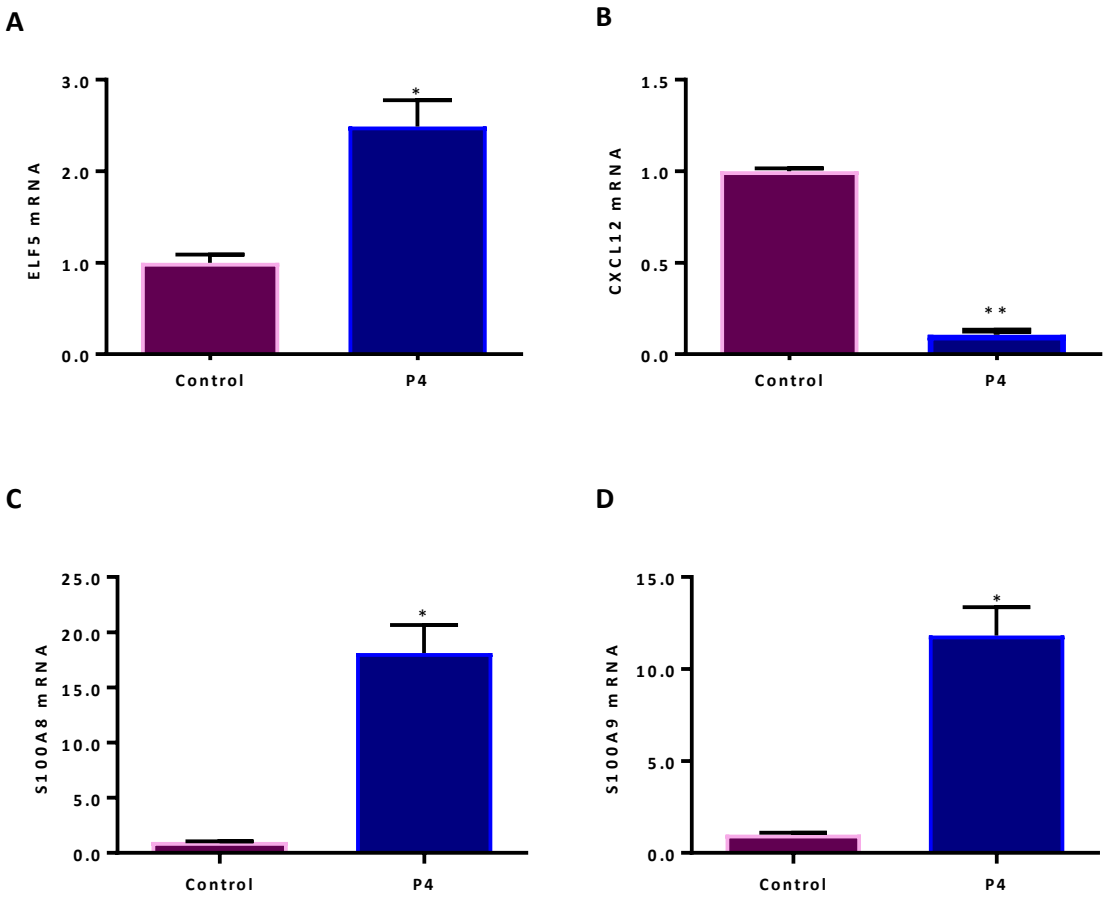


Figure 4.9: Cytokine gene profile in T47D mammary epithelial cell lines after 72 hours treatment with progesterone.
Graphs represent the variation in the expression of genes A) *ELF5*, B) *CXCL12*, C) *S100A8*, and D) *S100A9* in human mammary epithelial cell lines (T47D) upon treatment with progesterone (P4) for 72 hours (n=6 experiment, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

4.2.5. SIRNA TRANSFECTION OF T47D CELL LINE:

To investigate the role of *ELF5* in the hormonal regulation of cytokine production by mammary epithelial cells, the human mammary epithelial cell line T47D was transfected with SMARTpool human *ELF5* siRNA and/or non-targeting siRNA oligos for 24 hours. Cells were then treated with progesterone for 72 hours post-transfection. Messenger RNA expression of genes ELF5, CXCL12, S100A8 and S100A9 was examined from the transfected cells. The comparative Ct method (i.e. 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of *ELF5* in the cells transfected with the non-targeting siRNA.

ELF5 gene was silenced at the mRNA level with more than 80% efficiency ($p<0.05$) compared to the cells which were transfected with non-targeted siRNA oligos (Figure 4.10A). All cytokines were hormonally regulated and followed a similar trend as it was observed in Figure 4.9. However, when *ELF5* was knocked down, the expression of mRNA encoding *S100A8* and *S100A9* genes were increased by about 100% in the cells treated with progesterone ($p<0.05$ for S100A9). On the other hand, *CXCL12* mRNA was attenuated by about 50% ($p<0.05$) in the progesterone-treated cells lacking *ELF5* compared to the cells which had functional *ELF5*. Moreover, silencing this transcription factor did not affect the expression of any of the cytokines in the hormone-free control-treated cells (Figure 4.10).

ELF5 knockdown efficiency was also assessed at the protein level using Western Blot. Protein was extracted from transfected cells, and ELF5 was detected in the samples using monoclonal ELF5 antibody. Consistent with the results obtained from RT-PCR, the protein expression of ELF5 increased with progesterone treatment (approximately 150% compared to the control), particularly in the cells transfected with non-targeting siRNA. Although ELF5 protein was not reduced in control-treated cells, it was attenuated in progesterone-treated cells. (Figure 4.11).

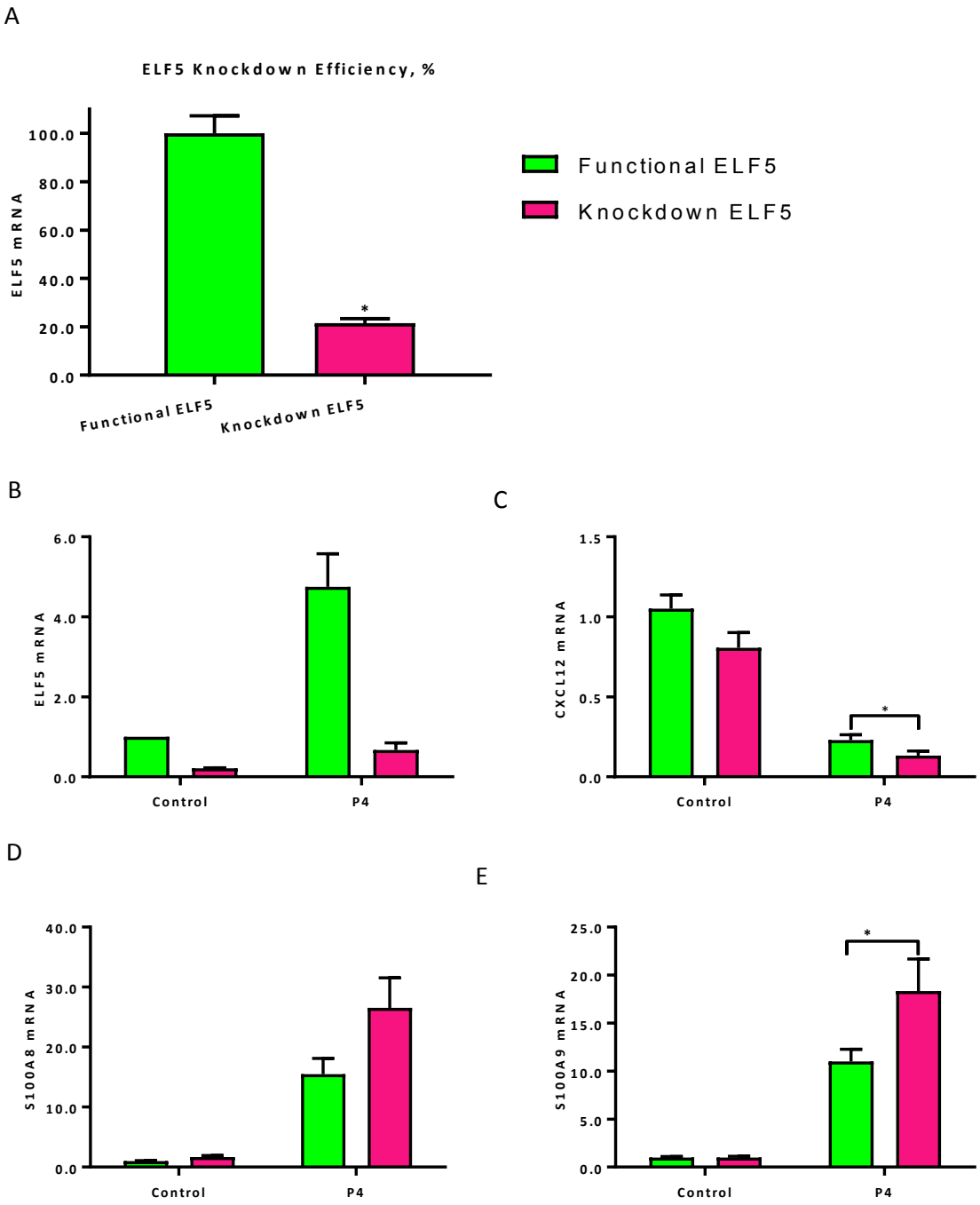
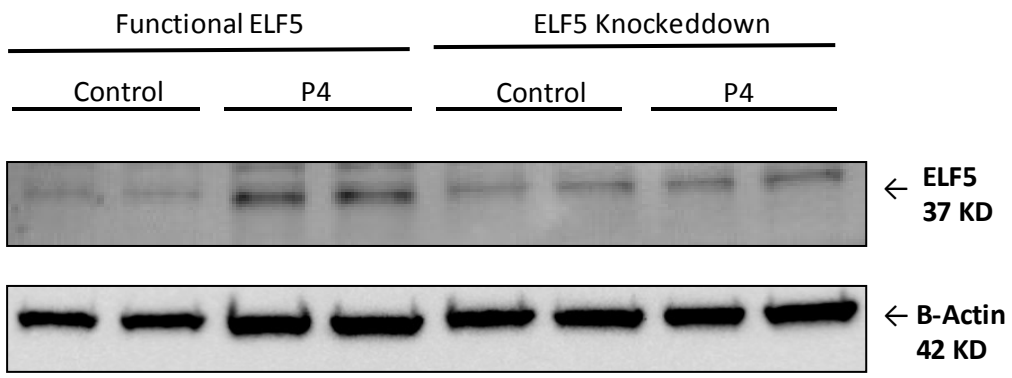


Figure 4.10: Progesterone regulation of cytokines in T47D cell line transfected with ELF5 siRNA and non-targeting siRNA.

A) *ELF5* mRNA knockdown efficiency in T47D cells was assessed using RT-PCR. Graphs represent the variation in the expression of genes B) *ELF5*, C) *CXCL12,* D) *S100A8*, and E) *S100A9* in ELF5-transfected human mammary epithelial cell lines (T47D) upon treatment with progesterone (P4) for 72 hours (n=4 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples in non-targeting transfected cells was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, p<0.05; **, p<0.01).

A.



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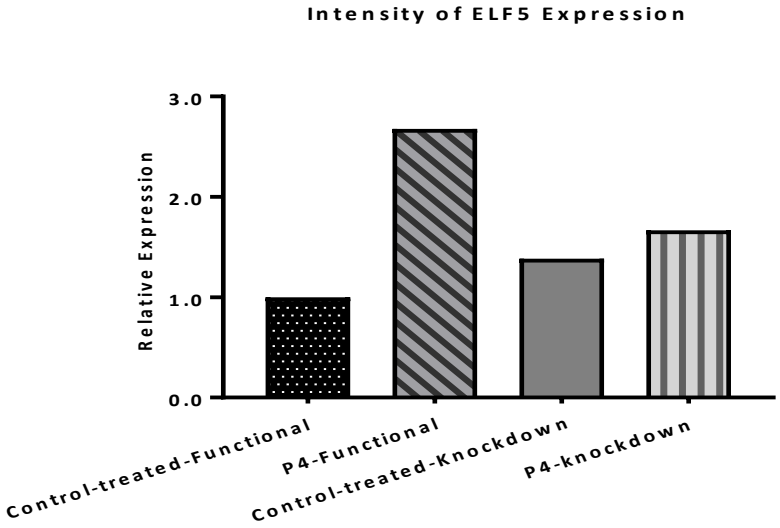

Figure 4.11: Western blot for progesterone regulation of ELF5 in T47D cell line transfected with ELF5 siRNA and non-targeting siRNA.

T47D cells were transfected with either ELF5 siRNA or non-targeting siRNA oligos for 24 hours and treated with either progesterone (100nM) or ethanol control for 24 hours post transfection. The comparative Ct method (i.e. 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of ELF5 in the cells transfected with the non-targeting siRNA. A) Whole cell lysate extracted from T47D cell line was subjected to western blotting with a monoclonal ELF5 antibody. A beta actin monoclonal antibody was used as a loading control. B) Bar graph represents the expression of ELF5 protein in the cells transfected with ELF5 siRNA, relative to the expression of control-treated cells transfected with non-targeting siRNA oligos.

4.2.6. PROTEIN EXPRESSION OF S100A8 IN HORMONE-TREATED HUMAN BREAST EXPLANTS:

To investigate the effects of estrogen and progesterone on protein expression of S100A8, human breast tissues were dissected into small pieces and cultured on dental sponges as explants (n=8). Explant cultures were then treated with a combination of hormones for 3 days, sectioned, fixed and paraffin embedded. The paraffin-embedded sections were incubated with mouse anti-human S100A8 monoclonal antibody overnight, stained with streptavidin/Alexa Fluor 594, and counterstained with DAPI.

The intensity of S100A8 expression (brown regions) in the mammary gland was scored in three random clusters of epithelial alveolar buds in each section (Figure 4.12). The scoring system was based on the intensity of positive stained cells in each section compared to the negative controls in a blinded analysis. Indeed, positive cells were analysed in two ways: epithelial cells with visible haematoxylin stained nuclei were included in the ‘epithelial’ analysis, while stromal regions with positive staining were scored and categorized into ‘stromal’ analysis.

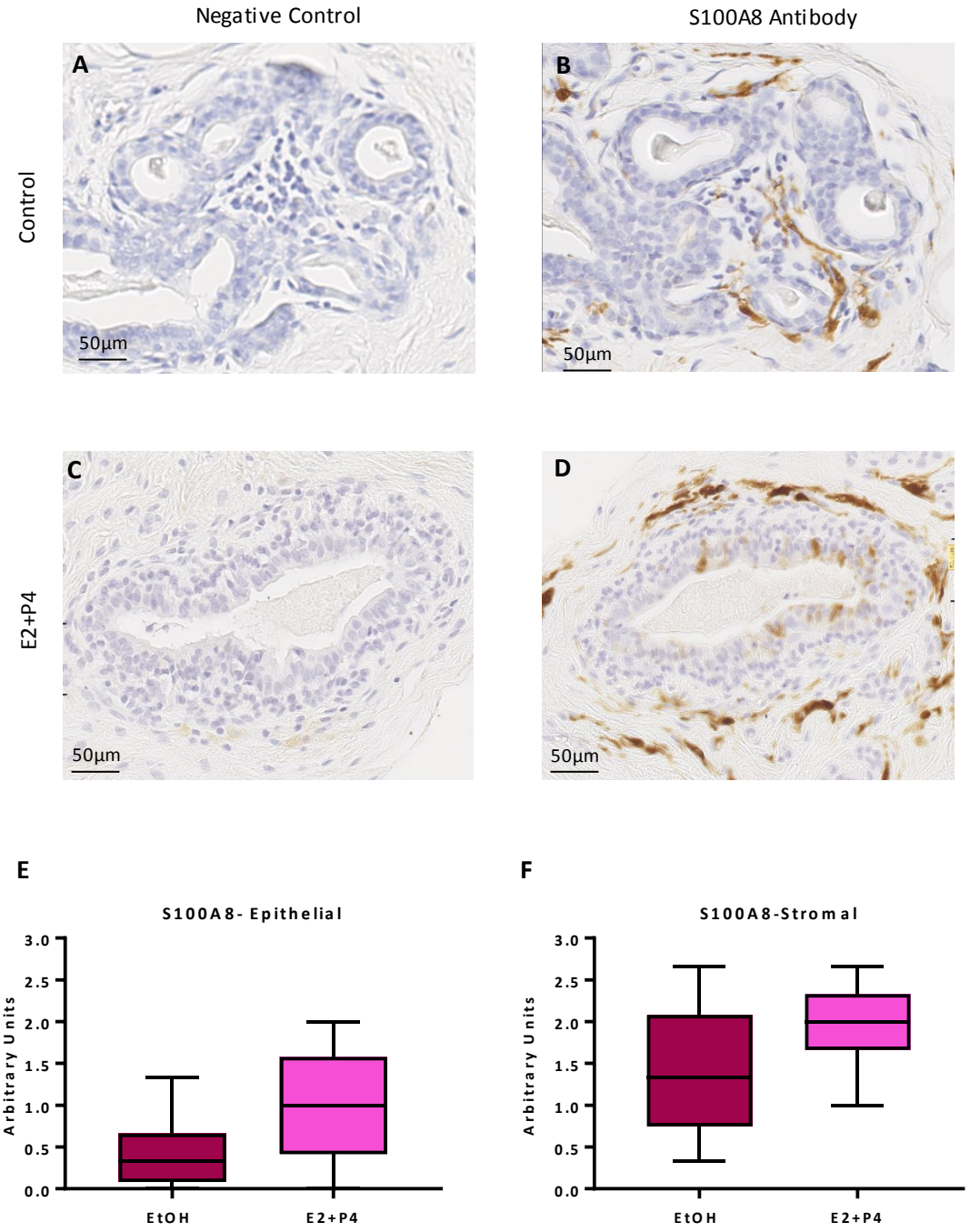

Figure 4.12: Hormonal regulation of S100A8 in human mammary epithelial explant models (n=8). Mammary epithelial explant cultures were treated with ethanol or a combination of estradiol and progesterone and the tissue sections were stained with S100A8 monoclonal antibody. Representative images of human breast explants stained with S100A8 antibody are shown: A) Negative control for control-treated breast tissue explant, B) control-treated explant stained with S100A8 monoclonal antibody, C) Negative control for E2+P4 treated explant, D) E2+P4 treated explant stained with S100A8 antibody. Box plots represent the mean score of S100A8 expression from human *ex vivo* cultures upon treatment with combination of hormones and/or ethanol: E) S100A8 expression in the mammary epithelial cells, F) S100A8 expression in the mammary stromal regions. Data are mean ± SEM and were statistically analysed using an unpaired t test.

4.3. DISCUSSION:

From the results there are two main observations. First, *ELF5* and its putative downstream cytokines were hormonally regulated in mammary epithelial cells. Second, silencing *ELF5* at the mRNA level resulted in induction of *S100A8* and *S100A9*, and a reduction in the mRNA expression of *CXCL12* in progesterone-stimulated cells. These results suggest that *ELF5* reduces, but does not eliminate the pro-inflammatory effects of progesterone on these cytokines. Indeed, these expression patterns could be related to activation of specific signalling pathways governed by ELF5 transcription factor.

4.3.1. HORMONE REGULATION OF ELF5 AND ITS DOWNSTREAM CYTOKINES

Our results show that estradiol and progesterone regulate the expression of mRNA encoding ELF5 and its downstream cytokines in mammary epithelial cells. S100A8 and S100A9 act as pro-inflammatory danger signals and are released to the extracellular microenvironment in response to inflammation and cell damage (173). Acting as DAMPs, they bind to cell surface receptors such as receptor of advanced glycation end product (RAGE) and Toll-like receptors (TLRs), as part of the innate immune signalling that recognizes danger signals derived from pathogens, cellular stress, or damaged cells, and subsequently mounts intracellular immune responses (174, 175). S100A8 and S100A9 also serve as potential markers of tumour invasion and metastasis, and their expression is reported to be correlated with tumour progression and a poor outcome in breast cancer patients (176). Therefore, it is likely that upregulation of S100A8 and S100A9 in the mammary gland induces myeloid cell recruitment and exerts inflammatory responses which can assist in inflammation-associated tumorigenesis.

The dramatic changes in the mRNA expression of *CXCL12* in the cell lines suggest that estradiol strongly activates *CXCL12*, whereas progesterone has inhibitory effects on this

chemokine. This is also in line with our mouse studies which illustrated that *Cxcl12* mRNA expression is higher during the metestrus phase compared to all other stages of the estrous cycle. CXCL12 binds to its receptor and activates a wide array of signal transduction pathways which mediate the migration and survival of monocytes and lymphocytes. Indeed, neutrophils and macrophages express CXCR4 on their surface which allows them to migrate towards CXCL12 present at the site of inflammation. Moreover, CXCL12 signalling can induce the differentiation of monocytes into proangiogenic, immunosuppressive macrophages in the tumour microenvironment (177). CXCL12 acts as a direct target for ERA, and estradiol induces its secretion in MCF7 cancer cells (120), however, its association with progesterone in the mammary epithelium is not well-understood. In 2011, Okada, Okamoto (178) reported that progestins attenuate estradiol-induced CXCL12 production *in vitro* in hormone-responsive human endometrial stromal cells. Our results agree with this, showing that progesterone has inhibitory effects on the expression of *CXCL12* in malignant breast epithelial cells. Although downregulation of this chemokine in dermal wound tissues has previously been observed in response to inflammation (179), it is still not clear how progesterone affects its function in the mammary gland.

Collectively, from these results, it can be suggested that progesterone produces an inflammatory signal in the breast epithelium which is associated with an upregulation of *S100A8* and *S100A9* and a reduction in the expression of *CXCL12*. These cytokines play roles in the recruitment of inflammatory and resident immune cells; hence, alterations in their expression due to hormonal fluctuations may influence the phenotype and function of immune cells in the mammary gland microenvironment. It would be of great interest to understand the precise cellular mechanisms and signalling pathways that govern these hormonal effects in the mammary gland. In this context, it was hypothesised that transcription factor ELF5 has a role in directing the expression of these cytokines.

4.3.2. THE ROLE OF ELF5 IN HORMONE-REGULATED CYTOKINE EXPRESSION:

Different transcription factors intrinsic to the mammary epithelium have been identified, and often involved in both normal mammary gland development and in development of breast cancer; however, their target genes are still not well-defined (51). An important candidate implicated in mammary gland development and breast cancer risk is ELF5. A few studies have already investigated the associations of ELF5 and the ovarian hormones in the mammary gland. Using mammary epithelial cell lines, the effect of ELF5 expression on 289 estrogen-induced genes was analysed and it was noted that the predominant effect of ELF5 expression is to suppress estrogen-induced gene expression (75). Moreover, Hilton, Kalyuga (85) showed that ELF5 plays a role in progestin-mediated regulation processes such as cell cycle control, adhesion and cell signalling. However, there is still limited information about ELF5 might impact on the immune microenvironment through cytokine expression by mammary epithelial cells. In our study, T47D cell lines were used to investigate the role of ELF5 in mediating the hormonally regulated cytokine expression by mammary epithelial cells *in vitro*. Silencing *ELF5* gene at the mRNA level resulted in induction of *S100A8* and *S100A9* by 100%, and a 50% reduction in the mRNA expression of *CXCL12* in progesterone-stimulated cells. This suggests that *ELF5* reduces, but does not eliminate the pro-inflammatory effects of progesterone on these cytokines. These expression patterns could be related to activation of specific signalling pathways governed by ELF5 transcription factor.

Although the importance of ELF5 in regulating normal mammary gland development and function is well established, there is much debate on its role in breast tumorigenesis. A few studies suggest that this transcription factor contributes to cancer development and metastasis (180, 181), while others consider ELF5 as a tumour suppressor gene (79). In 2015, Gallego-

Ortega, Ledger (82) noted that ELF5 overexpression promotes the recruitment of myeloid derived suppressor cells (MDSCs) in luminal breast cancer and drives metastasis into the lungs in mice. In contrast, Chakrabarti, Hwang (81) reported that silencing ELF5 in polyoma middle T antigen (PyMT) mouse mammary glands and T47D cancer cells induce epithelial to mesenchymal transition (EMT) and metastasis (81). MDSCs are a group of myeloid cells including myeloid progenitors, immature granulocytes, dendritic cells, and macrophages which have critical roles in suppression of immune responses and induction of tolerance in cancer (182). Although we did not investigate the role and abundance of these cells, our results suggest that *ELF5* negatively regulates the expression of *S100A8* and *S100A9* which have been implicated in MDSC's function. Upregulation of S100A8 and S100A9 impairs differentiation of mature myeloid cells such as macrophages and dendritic cells and induces accumulation of MDSCs (183). It has also been reported that pro-inflammatory cytokines such as IL6 and IL1 beta can induce MDSCs and block adaptive immunity which may promote tumour progression (184, 185).

To investigate the abundance of ELF5 at the protein level, western blot analysis was performed on T47D cells treated with progesterone. ELF5 expression was induced in progesterone-treated cells, similar to the results obtained at the mRNA level. The results also suggest that ELF5 was knocked down at the protein level, however the protein was stable enough that it was still observed in control-treated cells. It is when the cells attempt to increase the expression of ELF5 that the knockdown effect becomes apparent. There is not much information available about the protein stability of ELF5. However, it has been suggested that post-transcriptional mechanisms such as protein stability impact the cellular abundance and activity of Ets factors (181). Possibly, ELF5 protein has high stability and a long half-life and it does not require constant translation from the mRNA; hence the knockdown efficiency using RT-PCR is not reflected in western blot analysis. Using short hairpin RNA (ShRNA) knockdown via lentiviral

vectors could overcome this issue as it constantly suppresses the mRNA and allows for longer period selection.

It is likely that ELF5 acts a modulator of the inflammatory response which dampens the pro-inflammatory effects of immune cytokines such as *S100A8*, *S100A9* and *CXCL12*. However, these effects do not reflect such cytokines as direct transcriptional targets of ELF5 function. The role of ELF5 in the mammary gland remains controversial. What accounts for the different effects of ELF5 in different cell culture environments and how it regulates the immune cytokine network in the breast is still not clear. However, there might be an optimal level of ELF5 required in the mammary gland to direct immune responses via cytokine regulation. Thus, dysregulation of this transcription factor during the menstrual cycle might be associated with mechanisms leading to tumour initiation and progress.

4.4. CONCLUSION:

A large number of cytokines may contribute to increased breast cancer risk when regulated by hormones or other cellular events. In particular, pro-inflammatory cytokine networks can affect cell survival, proliferation, differentiation, and mutation, as well as epithelial-stromal crosstalk in the breast. Our results suggest that the activation and infiltration of inflammatory macrophages to the mammary gland at the diestrus phase of the mouse ovarian cycle reported by others (20, 40) may be the result of progesterone-induced pro-inflammatory cytokine production by mammary epithelial cells, a process that might be dampened by ELF5 transcription factor. In other words, ELF5 appears to function as a gatekeeper to limit the inflammatory functions of progesterone during the high progesterone phase of the ovarian cycle. Therefore, a reduction of ELF5 in the mammary gland might increase inflammation and lead to an increased breast cancer susceptibility. Future experiments will be needed to further

elucidate the potential mechanisms of ELF5 in mediating cytokine production in the mammary gland microenvironment.

CHAPTER FIVE

THE ROLE OF FOXP3 IN MAMMARY GLAND DEVELOPMENT

5.1. INTRODUCTION

FOXP3 is a member of forkhead/winged family of transcription factors and functions as an essential regulator of CD4+CD25+ Treg development (88). Thymic T cells express FOXP3 and become Tregs to prevent inappropriate immune responses (47, 48). The lack of a functional *FOXP3* gene in lymphoid tissues leads to a deficiency in the population of CD4+ CD25+ Tregs which causes a severe autoimmune lesion in males, manifesting as an X-recessive autoimmune disease called IPEX (89). An analogous disease develops in male mice lacking functional *Foxp3* gene, known as "scurfy" mice, characterised by scaly and ruffled skin, reddened eyes, and enlarged spleen and lymph nodes (91, 92).

FOXP3 is predominantly expressed in the thymus and the spleen, from where Tregs are derived. However, a few reports have demonstrated its expression in epithelial cells of specific tissues such as the breast, lung, and prostate (93-95). FOXP3 expression in the epithelium suggests that it may play a broad function outside of T cells where it can bind and regulate several target genes (93). Although the epithelial cell-specific function of FOXP3 has not been well-investigated, some studies have suggested that FOXP3 acts as a tumour suppressor gene in breast cancer. As a transcription factor, FOXP3 can bind to approximately 700 genes (97) and act as either a transcriptional repressor (e.g. by directly repressing certain oncogenes (95, 96, 98)), or a transcriptional activator (e.g. by maintaining the expression of the p21 tumour suppressor) in the mammary epithelium (99).

In 2007, Zuo, Wang (94) analysed the expression of FOXP3 in normal and cancerous tissues and noted its expression to be present in only 20% of human breast cancer samples (mostly the HER2− or ER+ phenotype), whereas 80% of normal breast tissues expressed this protein. These researchers also found that Foxp3 heterozygous female mice develop mammary carcinomas spontaneously at a high rate as they age (94). In addition, when Foxp3 heterozygous female mice were challenged with the chemical carcinogen DMBA in conjunction with progesterone, a significant increase in the susceptibility to mammary cancer development was observed (94). Further studies in human breast cancer samples showed a high rate of FOXP3 somatic mutations in breast tumours (100, 101), suggesting that FOXP3 defects play a role in breast cancer susceptibility.

In the study by Zuo, Wang (94), 40% of aged Foxp3 heterozygous mice with breast cancer developed lung metastasis, suggesting that Foxp3 is involved in metastatic mechanisms (94, 103). Also, using human and mouse breast cancer cells, Zhang, Zhang (103) found that FOXP3 regulates the transcriptional activity of mircroRNA (miR)-200s, which are promising biomarkers of breast tumour progression and metastasis. On the other hand, transcription factor ZEB1 which promotes the EMT in various human tumours (104, 105), has been shown to inhibit the transcription of miR-200 clusters (105, 106). Since FOXP3 and ZEB1 are linked together through the regulation of miR-200, we hypothesise that regulation of *FOXP3* by ovarian hormones could influence the expression of *ZEB1* in the mammary epithelial cells.

The biological function and regulation of FOXP3 in the mammary gland is not well understood. It has been shown, however, that ovarian hormones regulate the abundance of peripheral Tregs (53, 186) which suggests they may also regulate FOXP3 expression in the mammary gland. *In vitro* and *in vivo* studies in mouse models showed that estrogen at physiological doses expands the number of CD4+CD25+ Tregs in different lymphoid tissues in estradiol-treated

ovariectomised mice and that it induces the expression of the *Foxp3* gene (102). Moreover, progesterone at high levels acts as a potent inducer of Th2 type cytokines produced by T cells (187). The experiments described in this chapter were devised to investigate the effects of Foxp3 heterozygosity on mammary gland development during puberty and the estrous cycle in mice. To investigate hormonal regulation of *FOXP3* in the human breast, mammary epithelial organoids and cell lines were cultured with combinations of estradiol and progesterone. In addition, the effect of overexpression of FOXP3 on cytokine production by human mammary epithelial cells was investigated.

5.2. RESULTS

5.2.1. THE EFFECTS OF FOXP3 HETEROZYGOSITY ON MAMMARY GLAND MORPHOGENESIS AT PUBERTY

To investigate the effects of Foxp3 heterozygosity on mammary gland morphogenesis at puberty, the 4th pair of mammary glands from 6 week-old Foxp3 heterozygous (n=12) and C57BL6 wildtype (n=9) control females were dissected and whole-mounts were stained with carmine alum. The number of branch points per millimetre length of epithelial ducts, the number of TEBs, and the length of ductal epithelial growth were measured using Image J software.

Puberty is a crucial stage of ductal morphogenesis whereby any changes at this period may have critical implications for later patterns of mammary gland development (188). Ductal branch point analysis on mammary gland whole-mounts showed that there was no difference in mammary ductal branching morphogenesis between Foxp3 heterozygous and wildtype control mice. At six weeks of age, both groups of mice exhibited similar number of branches (i.e., 1.6 ± 0.2 branch points per millimetre for Foxp3 heterozygous vs. 1.6 ± 0.1 branch points per millimetre for wildtype mice) (Figure 5.2).

Blinded analysis of the whole-mounts showed that the number of TEBs is also very similar between the two groups of mice. Foxp3 heterozygous females had approximately 9.0 ± 0.7 TEBs, while the wildtype mice had 9.2 ± 1.3 TEBs in their mammary fat pad (Figure 5.3A). Altered development of TEBs can have lasting consequences on mammary gland development (189). However, TEBs in the mammary tissues from Foxp3 heterozygous and wildtype mice appeared to be of normal structure and size.

Ductal growth in the wildtype mice was 27.4 ± 0.6 millimetre, while the average of ductal growth in the heterozygous animals was 24.5 ± 1.3 millimetre (non-significant results), and

there was a high degree of variability within each group of mice (Figure 5.3B). Epithelial ducts in the heterozygous mice elongated normally into the mammary fat pad, similar to that of wildtype controls.

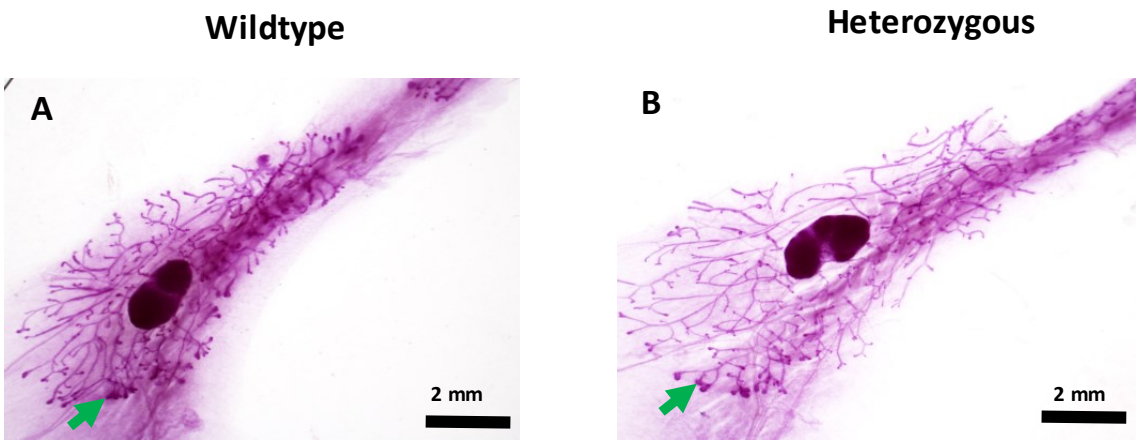

Figure 5.1: Representative images of mammary gland whole-mounts during puberty.
Mammary gland whole-mounts of Foxp3 heterozygous (n=12) and wildtype control (n=9) mice were stained with carmine alum at six weeks of age. The images are representative of mammary glands from A) wildtype and B) Foxp3 heterozygous female mice. Green arrows represent terminal end buds in each mammary gland.

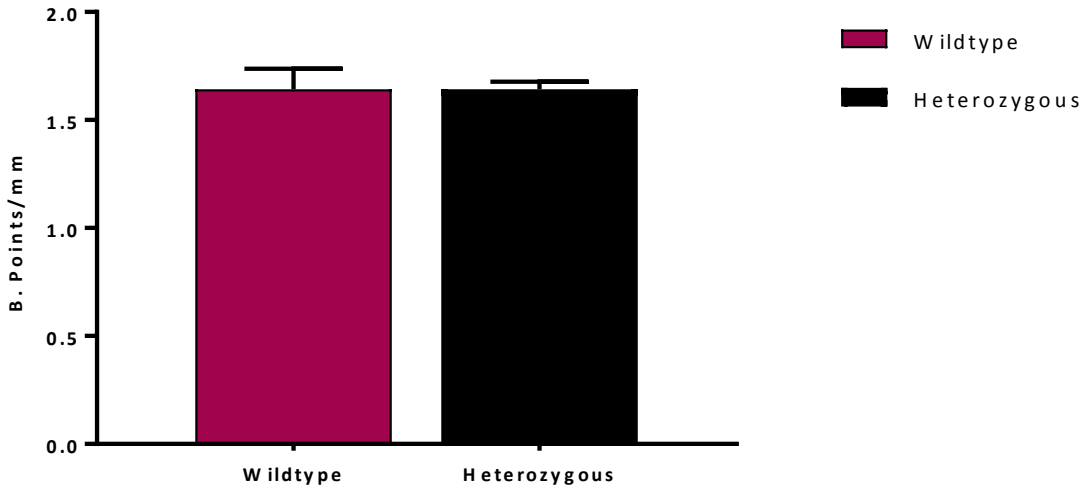

Figure 5.2: The effect of Foxp3 heterozygosity on mammary gland morphogenesis during puberty.
Mammary gland whole-mounts of Foxp3 heterozygous (n=12) and wildtype control (n=9) were stained with carmine alum at six weeks of age. The number of branch points per millimetre was calculated. Data are presented as mean ± SEM and were analysed by an unpaired t-test.

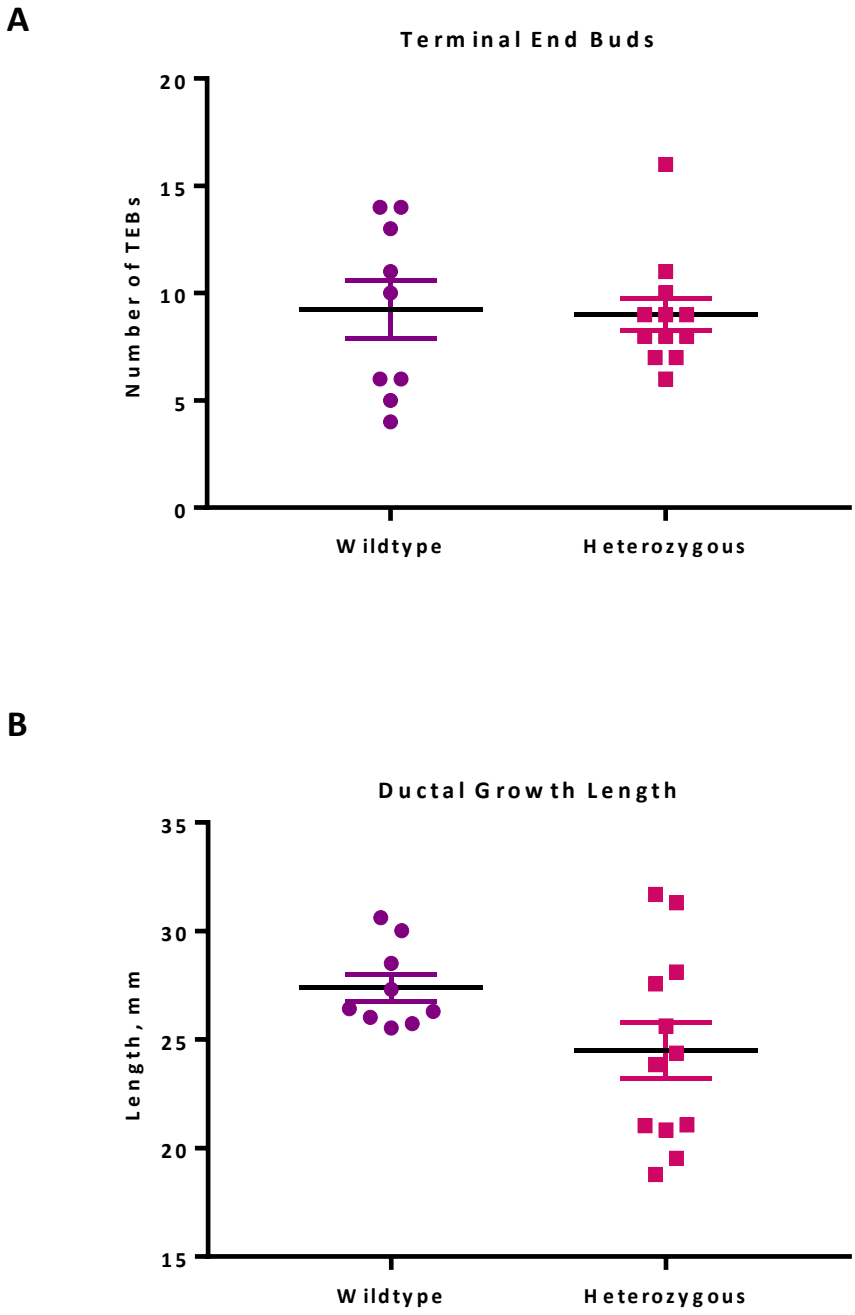

Figure 5.3: The effect of Foxp3 heterozygosity on pubertal mammary gland development.
Mammary gland whole-mounts of Foxp3 heterozygous (n=12) and wildtype control (n=9) were stained with carmine alum at six weeks of age. A) The number of terminal end buds in each mammary gland was counted. Terminal end buds, defined as club-like structures projected out from the distal end of the ducts, were counted in mammary glands. B) Ductal epithelial length was measured from both groups of mice, expressed in millimetre. Data are presented as mean ± SEM and were analysed by an unpaired t-test.

5.2.2. THE EFFECT OF FOXP3 HETEROZYGOSITY ON ESTROUS CYCLING

To investigate the effect of Foxp3 heterozygosity on the regularity of the estrous cycle in mice, cycles were tracked for 28 days by histological analysis of vaginal smears in eight-week old adult virgin females (n=8-10 mice per group). The average cycle length and number were analysed over 28 days in Foxp3 heterozygous and wildtype mice, while a single complete cycle was defined as the day of estrus through to the day of the next estrus. Also, the percentage of the time spent in each of the four stages of the estrous cycle was analysed between the two groups. Data are presented as mean ± SEM and were analysed by an unpaired t-test.

Estrous cycle length was not affected by mouse genotype as both groups had a similar cycle length of approximately six days on average (Figure 5.4). Both groups of mice had similar number of cycles (Table 5.1). Moreover, there was no significant difference in the percentage of the time spent in each stage of the estrous cycle between the two groups. However, Foxp3 heterozygous mice spent slightly more time in metestrus phase (32%) compared to the wildtype mice which were in that phase for about 27% of the time. Both groups of mice spent more time in the metestrus and diestrus phases of the cycle (30% on average) and less time in proestrus (around 17% of the time) compared to the estrus phase (Figure 5.5).

Table 5.1. The average length and the number of estrous cycles in Foxp3 heterozygous and wildtype mice.

	Wildtype	Foxp3 Heterozygous
Cycle Length (Days)	6.0 ± 0.2	6.0 ± 0.3
Cycles (number)	4.1 ± 0.1	3.9 ± 0.2

The average cycle length and number were analysed over 28 days in Foxp3 heterozygous and wildtype mice, while a single complete cycle was defined as the day of estrus through to the day of the next estrus.

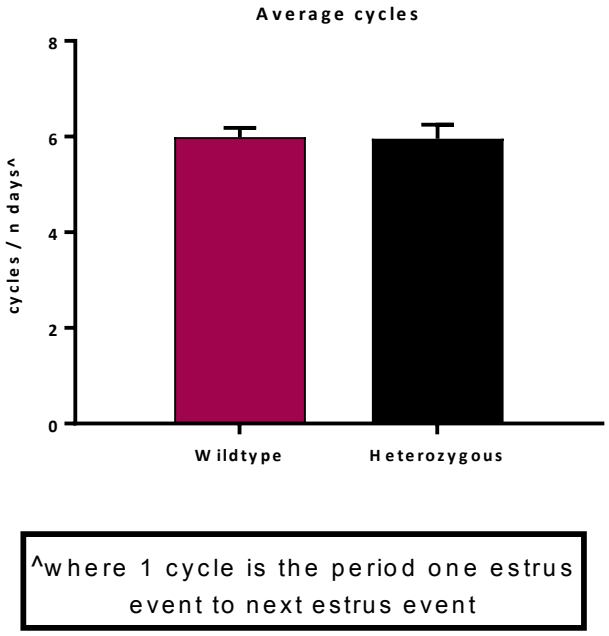

Figure 5.4: The effect of Foxp3 heterozygosity on estrous cycling.
Eight weeks old female Foxp3 heterozygous (n=38) and C57BL6 wildtype mice (n=37) were tracked over the course of estrous cycles for 28 days through histological analysis of vaginal smears. A single complete cycle was defined as the first day of estrus through to the first day of the next estrus (n=8-10 per group). Data are presented as mean ± SEM and were analysed by an unpaired t-test.

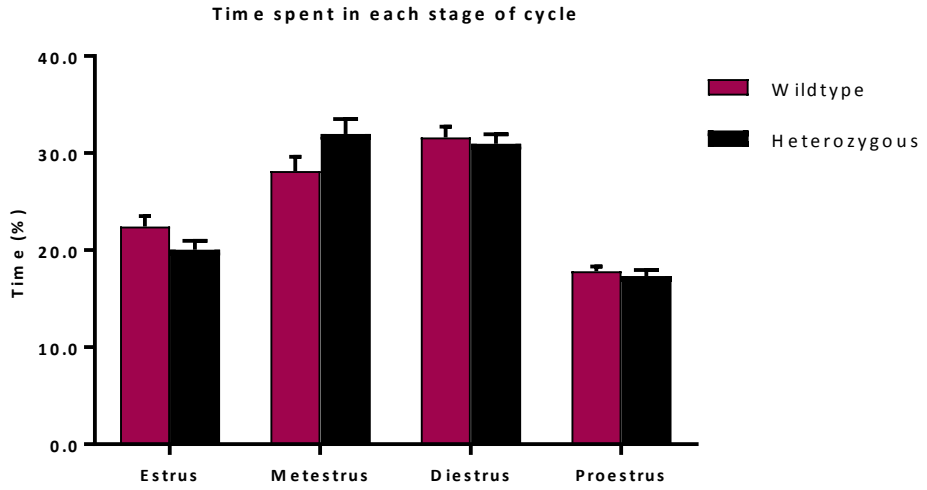

Figure 5.5: Percentage of time spent in each stage of the estrous cycle in Foxp3 heterozygous and C57BL6 wildtype mice.
Daily vaginal smears were analysed by phase contrast microscopy in female mice (n=8-10 per group) over 28 days. The percent of viable epithelial cells, cornified epithelial cells, and leukocytes in the smears distinguished mice in proestrus, estrus, metestrus, and diestrus. Data are mean + SEM and were analysed by an unpaired t-test.

5.2.3. THE EFFECTS OF FOXP3 HETEROZYGOSITY ON MAMMARY GLAND MORPHOGENESIS DURING THE OVARIAN CYCLE

To investigate the effect of Foxp3 heterozygosity on mammary gland morphogenesis over the course of the ovarian cycle, one side of the fourth inguinal pair of mammary glands from Foxp3 heterozygous and wildtype control females were collected at each of the four stages of the cycle and whole-mounts were stained with carmine alum. Representative images of mammary gland whole-mounts at each stage are shown in Figure 5.6. The number of branch points per millimetre length of epithelial ducts was counted using Image J software.

Ductal branching analysis of mammary gland whole-mounts showed that mammary ductal branching morphogenesis is similar between the Foxp3 heterozygous and wildtype control mice. During the estrus phase, Foxp3 heterozygous mice had 1.9 ± 0.1 branch points/mm (n=10) compared to the wildtype mice which had 2.0 ± 0.1 branch points/mm (n=8). Similar results were obtained at metestrus whereby Foxp3 heterozygous mice had 1.9 ± 0.1 branch points/mm (n=9), compared to the controls which had 2.0 ± 0.1 branch points/mm (n=10). Both groups of mice had the highest number of their mammary ductal branch points at the diestrus phase (2.3 ± 0.2 branch points/mm vs. 2.3 ± 0.1 branch points/mm; n=10). The lowest number of ductal branch points were observed during the proestrus phase regardless of genotype. However, Foxp3 heterozygous mice had a comparable number of ductal branch points at this stage, compared to the wildtype mice (1.7 ± 0.1 branch points/mm vs 1.8 ± 0.1 branch points/mm, n=9) (Figure 5.7).

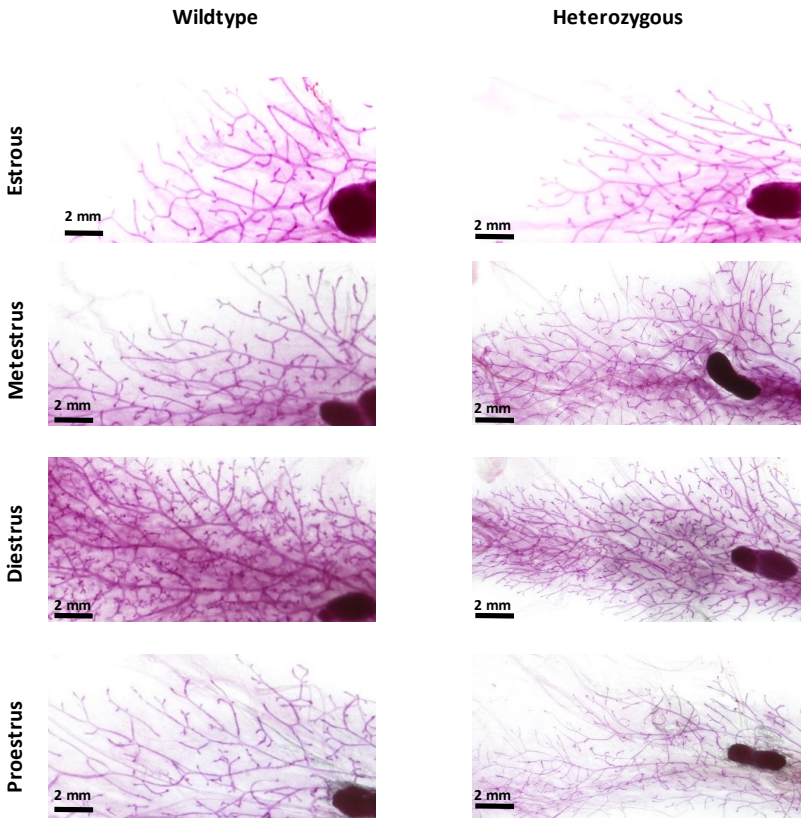

Figure 5.6: Representative images of mammary gland whole-mounts during the estrous cycle for Foxp3 heterozygous and wildtype mice.
The fourth inguinal mammary gland from Foxp3 heterozygous and wildtype mice, stained with carmine alum as whole-mounts at estrus (n=18), metestrus (n=19), diestrus (n=20) and proestrus (n=18). The images are representative of mammary glands from wildtype and Foxp3 heterozygous female mice at each of the four stages of the cycle.

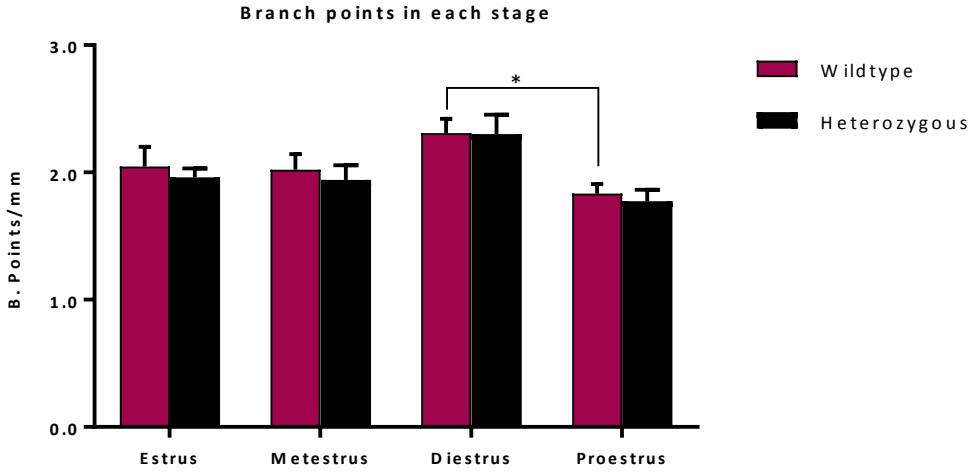

Figure 5.7: The effect of Foxp3 heterozygosity on morphology and branching of mammary glands in virgin adult mice during the estrous cycle.
Mammary gland whole-mounts of Foxp3 heterozygous and wildtype control were stained with carmine alum at estrus (n=18), metestrus (n=19), diestrus (n=20) and proestrus (n=18). The number of branch points per millimetre was calculated. Data are mean + SEM and were analysed by two-way ANOVA.

5.2.4. MESSENGER RNA EXPRESSION OF FOXP3 IN THE MAMMARY GLAND DURING THE ESTROUS CYCLE

Analysis of *Foxp3* utilises a different approach to measure its expression and this requires special reagents for real-time PCR. Due to the high cost and the low availability of reagents, the mRNA expression of *Foxp3* was only assessed at the estrus and diestrus phases of the cycle from third pair mammary glands in Foxp3 heterozygous and wildtype mice. To compare abundance of *Foxp3* in the mammary gland to tissues with known high expression of *Foxp3*, pooled samples of mesenteric, lumbar and axillary lymph nodes (from each mouse) were also assessed as Tregs are largely found in the draining lymph nodes (190). The comparative Ct method (i.e., 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of housekeeper gene *Rpl13a* in each mouse. The results were normalised to the average expression of *Foxp3* in the estrus phase so that the average of mRNA expression in this stage is 1 (n=8-10 per stage).

Although there was high variability within groups and no significant differences, the abundance of *Foxp3* mRNA tended to be lower in the mammary gland during the diestrus compared to the estrus phase of the cycle. Moreover, the expression of *Foxp3* mRNA in the lymph nodes tended to be higher during the diestrus phase compared to the estrus (non-significant results). There was no significant difference between Foxp3 heterozygous and wildtype mice in regards to mRNA expression of *Foxp3* in the mammary glands and lymph nodes (Figure 5.8). This demonstrates a variable pattern in the expression of mammary gland-specific *Foxp3* and Tregs in the lymph nodes.

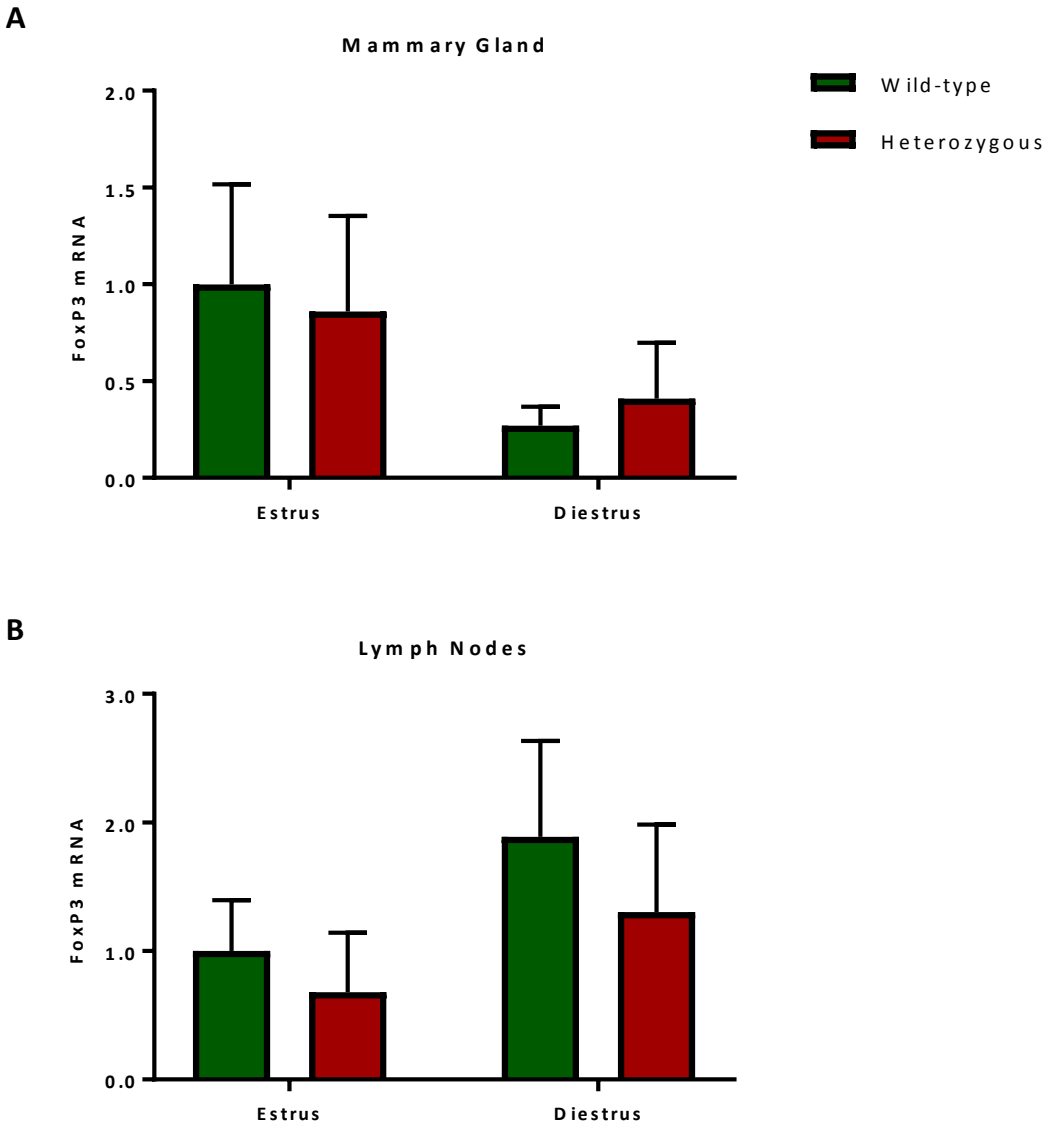

Figure 5.8: mRNA expression of Foxp3 in the mammary gland (A) and lymph nodes (B) of Foxp3 heterozygous and wildtype mice at estrus and diestrus stages of the cycle.
RNA was extracted from the third mammary glands and lymph nodes at estrus and diestrus phases of the cycle. Using RT-PCR, the messenger RNA expression of Foxp3 was analysed in the mammary gland. The comparative Ct method (i.e., 2-(ΔΔCT) method) was used to analyse the data, relative to expression of housekeeper gene RPL13a in each mouse. The results were normalised to the average expression of Foxp3 in the estrus stage so that the average of mRNA expression in this stage is 1 (n=8-10 per stage).

5.2.5. MESSENGER RNA EXPRESSION OF CYTOKINES IN HORMONE-TREATED HUMAN MAMMARY EPITHELIAL ORGANOIDS

To investigate the effects of estradiol and progesterone on regulating the expression of *FOXP3* and *ZEB1* in mammary epithelial cells, primary human mammary epithelial organoids (n=6) were embedded in Matrigel and treated with various combinations of estradiol and progesterone for 72 hours. Messenger RNA expression of *FOXP3* and *ZEB1* were examined from hormone-treated human epithelial organoids. The comparative Ct method (i.e., 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to the mean of expression of housekeeping genes *MPRL19* from the same patient.

The expression of both genes was highly variable amongst patients with no trend, and no significant result was obtained (Figure 5.9).

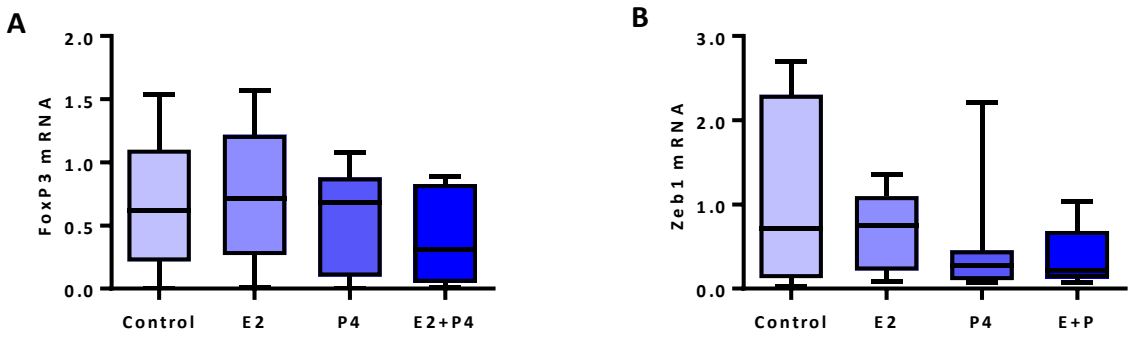

Figure 5.9: Hormonal regulation of mRNA encoding FOXP3 and ZEB1 in 3D organoid cultures.
Box plots represent the mean variation in the expression of genes A) *FOXP3* and B) *ZEB1* from Matrigel-embedded human epithelial organoids upon treatment with combinations of estradiol (E2) and progesterone (P4). Data are Mean ± SEM (n=6); normalized to the housekeeper gene *MPRL19*.

5.2.6. MESSENGER RNA EXPRESSION OF CYTOKINES IN HUMAN MAMMARY EPITHELIAL CANCER CELL LINES

To investigate the effects of estradiol and progesterone on *FOXP3* production by mammary epithelial cells, human mammary epithelial cell lines were treated with estradiol and progesterone over different time courses. Messenger RNA expression of *FOXP3* and *ZEB1* genes were examined from MCF7, T47D, and ZR751 human mammary epithelial cell lines treated with combinations of estradiol and progesterone for i) 24 hours and ii) 72 hours. The comparative Ct method (i.e., 2-(ΔΔCT) method) was used to analyse quantitative real-time PCR data, relative to expression of housekeeper gene *MPRL19* in each treatment group. The results were normalised to this gene so that the average of the control samples was 1.

In MCF7 cells treated with hormones for 24 hours, the mRNA expression of the genes encoding FOXP3 and ZEB1 was not significantly regulated by hormones. However, there was a tendency for the combination of estradiol and progesterone to downregulate *FOXP3* expression by approximately 40% compared to control-treated cells. Moreover, the expression of mRNA encoding ZEB1 was slightly increased upon treatment with either estradiol or progesterone (Figure 5.10A and 5.10B).

In T47D cells, treatment with progesterone significantly downregulated the expression of mRNA encoding FOXP3 by approximately 40% compared to the controls ($p<0.01$), while the combination of estradiol and progesterone decreased *FOXP3* expression by the same extent compared to estradiol-treated cells ($p<0.05$). Although non-significant, estradiol and progesterone treatment alone seemed to attenuate the expression of mRNA encoding FOXP3 gene (Figure 5.10C). On the other hand, the combination of hormone treatment significantly increased the expression of mRNA encoding ZEB1 gene by 4-fold compared to the control-treated cells ($p<0.05$). Interestingly, there was a tendency for either of the hormones to

upregulate the expression of mRNA encoding ZEB1 gene in these cells (non-significant results) (Figure 5.10D).

Hormone treatment of ZR751 mammary epithelial cells for 24 hours had no significant effect on the expression of mRNA encoding FOXP3 and ZEB1 genes. However, there was a tendency for estradiol to increase the expression of these genes in ZR751 cells (Figure 5.10E and 5.10F).

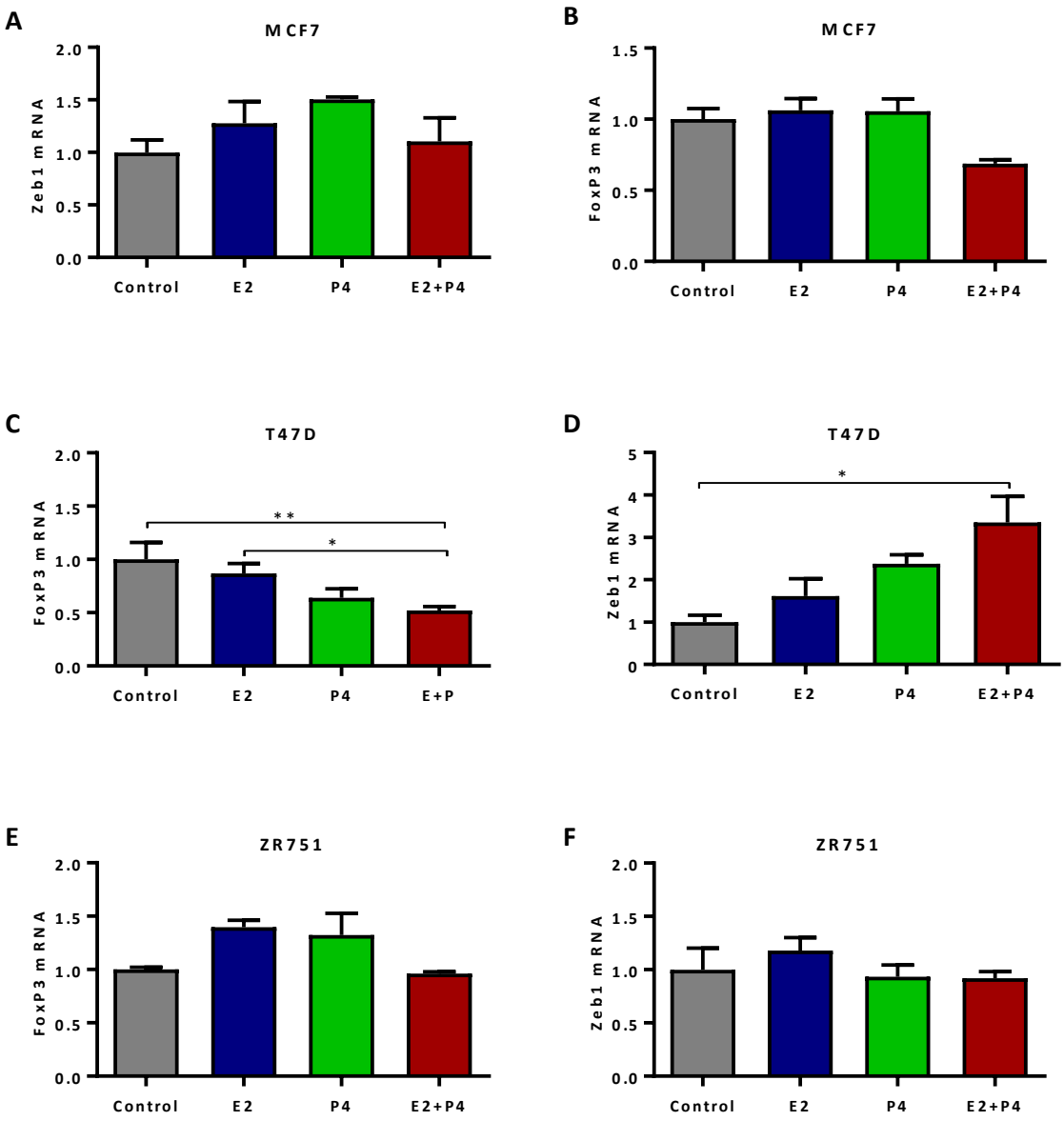

Figure 5.10: Messenger RNA expression of *FOXP3* and *ZEB1* in human mammary epithelial cell lines (MCF7, T47D, and ZR751) after 24 hours hormone treatment.
Graphs represent the variation in the expression of genes *FOXP3* and *ZEB1* in human mammary epithelial cell lines (MCF7, T47D, and ZR751) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 24 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene *MPRL19* so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

In MCF7 cells treated with hormones for 72 hours, progesterone had significant suppressive effects on the expression of mRNA encoding FOXP3 and ZEB1. Treatment with progesterone alone significantly downregulated the mRNA expression of FOXP3 and ZEB1 by more than 80% and 50% respectively ($p<0.05$). Similar effects were observed when estradiol was combined with progesterone; the expression of mRNA encoding FOXP3 and ZEB1 was significantly attenuated by approximately 80% and 90% respectively, compared to the control-treated cells ($p<0.05$ for *FOXP3* and $p<0.01$ for *ZEB1*) (Figure 5.11A and 5.11B).

The expression of mRNA encoding FOXP3 in T47D cells treated with progesterone was significantly upregulated compared to the cells treated with vehicle, estradiol or a combination of hormones ($p<0.01$ for all groups). Similar results were obtained for *ZEB1* mRNA expression which was approximately 4-fold higher in progesterone-treated cells, compared to the cells treated with either ethanol or estradiol ($p<0.05$) (Figure 5.11C and 5.11D).

The expression of mRNA encoding FOXP3 in ZR751 cells was not altered by hormone treatment. There was a tendency for estradiol alone to increase the expression of *ZEB1* compared to the control-treated cells (non-significant results) (Figure 5.11E and 5.11F).

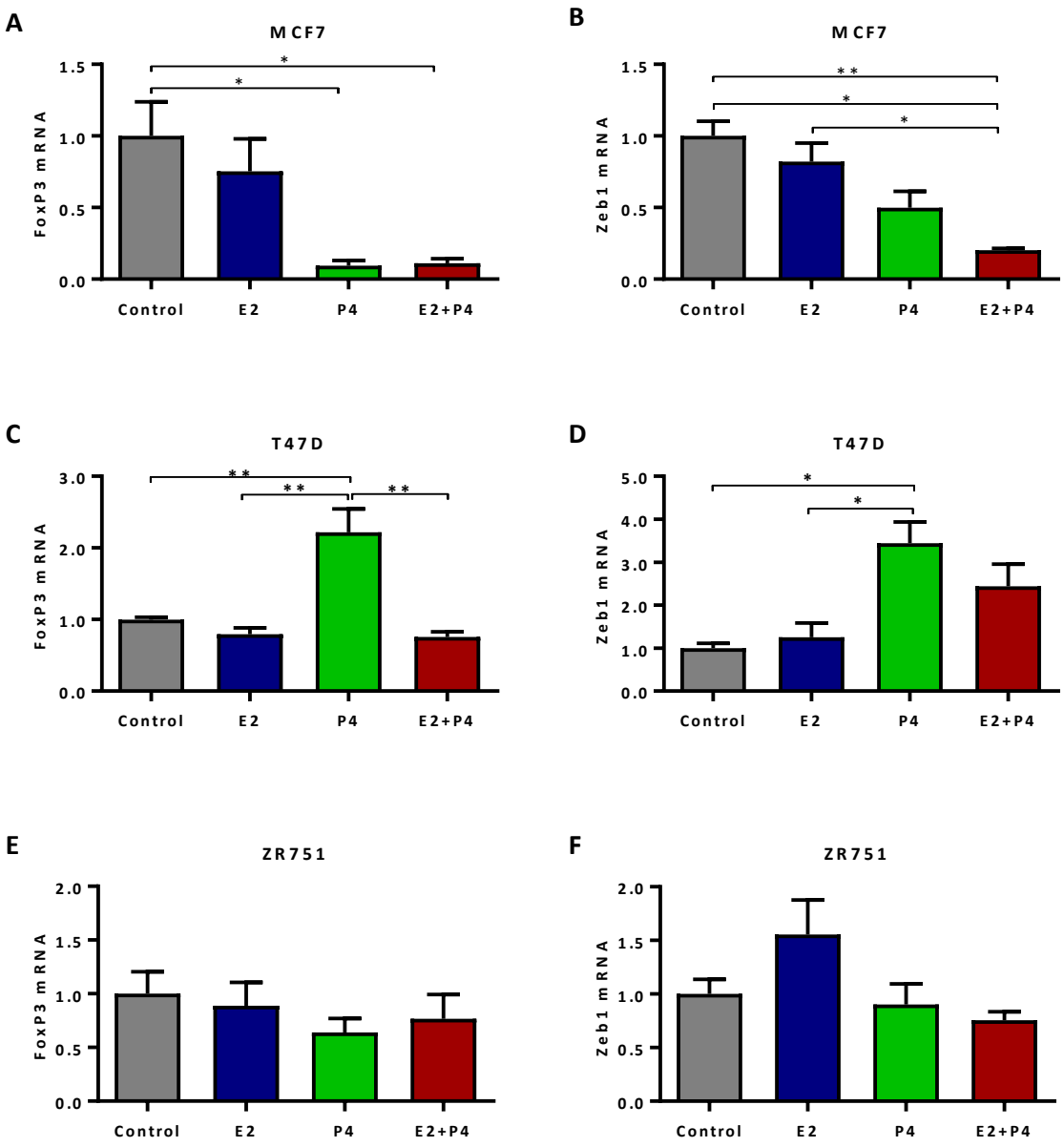

Figure 5.11: Messenger RNA expression of *FOXP3* and *ZEB1* in human mammary epithelial cell lines (MCF7, T47D, and ZR751) after 72 hours hormone treatment.

Graphs represent the variation in the expression of genes *FOXP3* and *ZEB1* in human mammary epithelial cell lines (MCF7, T47D, and ZR751) upon treatment with combinations of estradiol (E2) and/or progesterone (P4) for 72 hours (n=3 experiments, each in triplicate). Results were normalised to the housekeeper gene MPRL19 so that the average of the control samples was 1. Data are mean + SEM and were analysed by one-way ANOVA, followed by post-hoc Tukey test (*, $p<0.05$; **, $p<0.01$).

5.2.7. FOXP3 OVEREXPRESSION IN HUMAN MAMMARY EPITHELIAL ORGANOIDS

Lentiviral vectors are efficient tools to introduce the gene of interest into dividing and non-dividing cells. To investigate the consequences of FOXP3 overexpression in human normal breast epithelial cells, lentiviral vectors were used to induce stable, long-lasting FOXP3 expression. In order to obtain sufficient number of cells to conduct the experiment, samples of human mammary epithelial organoids were pooled from 10 patients and were transduced with lentiviral vectors either carrying FOXP3-GFP or GFP alone. Un-transduced cells were also used as control. Cells were embedded in Growth Factor Reduced Matrigel and cultured for five days to allow organoids to stabilise and grow, before RNA extraction. This experiment was conducted once in duplicate due to the limited number of primary mammary epithelial organoids. The transduction efficiency was assessed using confocal microscopy and RT-PCR.

Fluorescence intensity was determined qualitatively relative to the un-transfected control cells (No LV). The green fluorescence was clearly visible by fluorescence microscopy in the GFP and FOXP3-GFP transduced cells, compared to un-transduced control cells (Figure 5.12A-C). *FOXP3* mRNA expression was increased (up to 10-fold) in cells transduced with FOXP3-GFP lentiviral vectors compared with control cells (Figure 5.12F). When GFP transfection was assessed quantitatively using RT-PCR, the expression of mRNA encoding GFP was much higher (up to 150-fold) than that of FOXP3-GFP-transduced cells (Figure 5.12E). However, these data could not be statistically analysed due to the low sample size. Following stable FOXP3 overexpression in human mammary epithelial organoids, an apparent increase was observed in the expression of *ZEB1* mRNA (approximately 100%), compared to GFP-transduced and un-transduced cells (Figure 5.13A). The expression of *HER2* and *CXCL12* was reduced by approximately 80% in both groups of lentiviral-transduced cells compared to un-transduced cells (Figure 5.13B-C). No overt changes were observed in the expression of mRNA encoding TGFB1 and the housekeeper gene, PPIA.

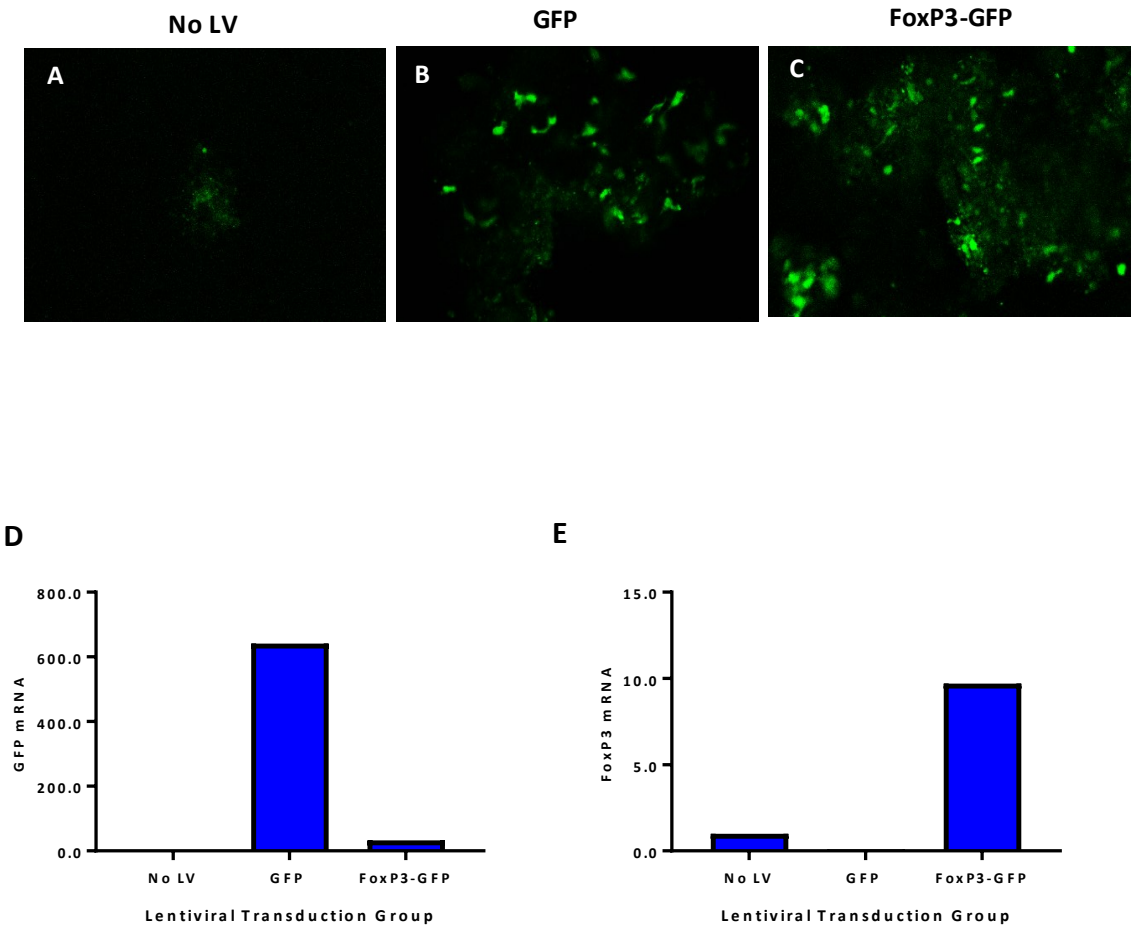

Figure 5.12: Lentiviral transduction of human mammary epithelial organoids.
Pooled samples of human mammary epithelial organoids were transduced with lentiviral vectors expressing FOXP3 and GFP. Control groups include a no lentiviral treatment (No LV) and lentiviral vectors which express GFP alone. Representative images are shown for GFP detection in the cells: A) un-transduced B) GFP transduced, and C) FOXP3-GFP transduced. RNA was extracted from the cells and mRNA encoding GFP and FOXP3 were measured using RT-PCR (D-E). Data shown are the mean of duplicate values (n=1 experiment in duplicate), normalized to the housekeeper gene *MPRL19*. No statistical analysis was conducted.

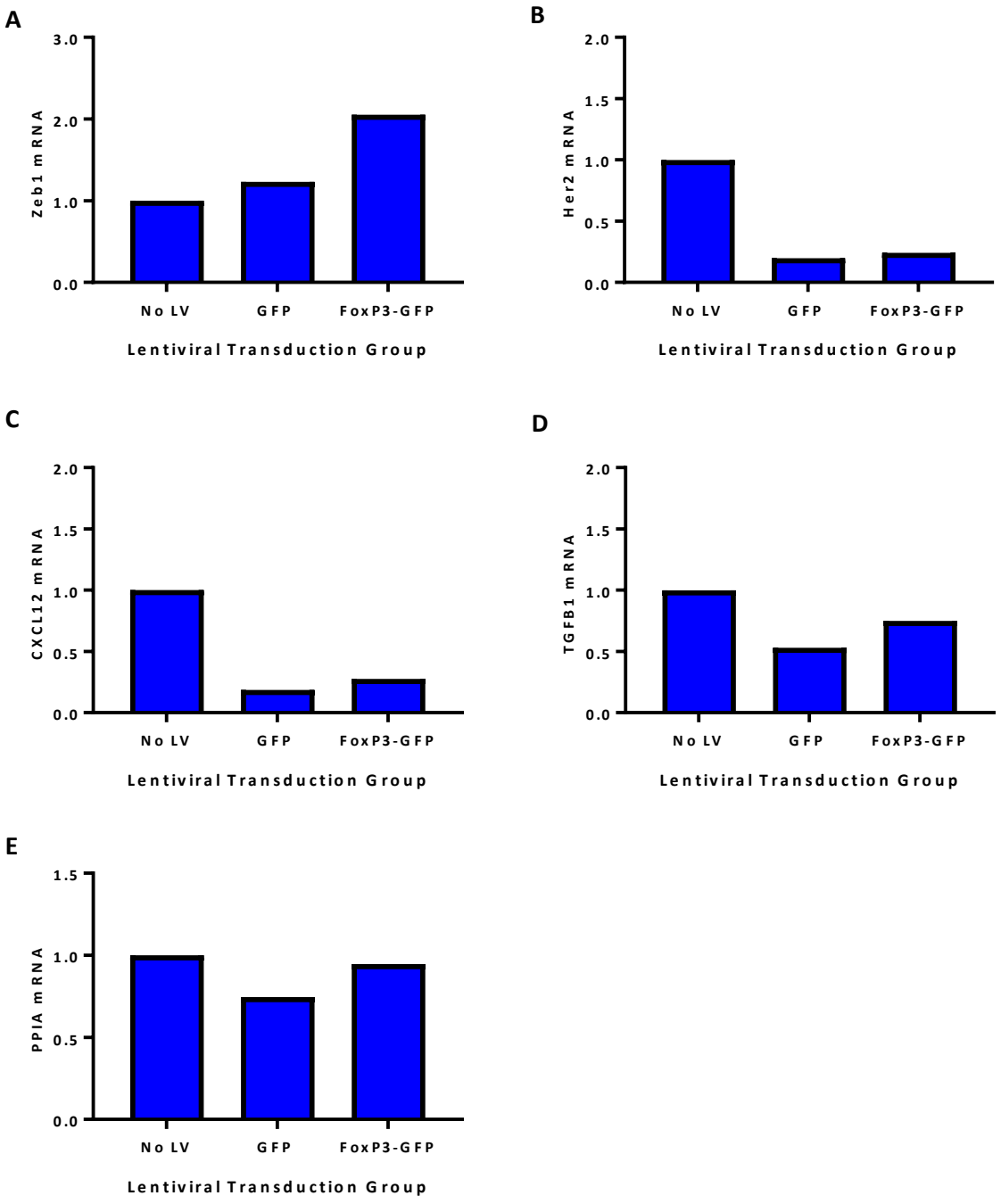

Figure 5.13: Lentiviral transduction of human mammary epithelial organoids.
Pooled samples of human mammary epithelial organoids were transduced with lentiviral vectors expressing FOXP3 and GFP. Control groups include a no lentiviral treatment (No LV) and lentiviral vectors which express GFP only. Graphs represent the variation in the expression of genes *ZEB1, HER2*, *CXCL12, TGFB1,* and *PPIA* in human mammary epithelial organoids transduced with different lentiviral transduction group. Data shown are the mean of duplicate values (n=1 experiment in duplicate), normalized to the housekeeper gene *MPRL19*. No statistical analysis was conducted.

5.3. DISCUSSION

Unlike most tumour suppressor genes which are autosomal, FOXP3 is X-linked and is subject to X chromosome inactivation, meaning that it can be inactivated by a single genetic or epigenetic hit (87). Although Foxp3 heterozygous mice appear healthy in the first year of life, there is little known about their mammary gland development and function, and how they develop cancer when they are older. Studies have suggested that mammary gland development may play an essential role in determining future breast cancer risk (188, 191). We hypothesized that Foxp3 heterozygosity might impair mammary gland development and affects its architecture, a process which may ultimately increase the risk of cancer. However, our results suggest that Foxp3 heterozygosity has no effects on mammary gland development during puberty and over the course of the estrous cycle. Despite these observations, epithelial-specific *FOXP3* and its potential downstream cytokine, *ZEB1*, seemed to be regulated by estradiol and progesterone in mammary epithelial cancer cell lines.

5.3.1. FOXP3 HETEROZYGOSITY DOES NOT AFFECT MAMMARY GLAND DEVELOPMENT IN MICE

Our results indicate that Foxp3 heterozygous mice exhibit overtly normal mammary gland ductal architecture, suggesting that Foxp3 heterozygosity has no effects on the mammary gland development during puberty and adulthood. The analysis of the estrous cycle regularity in adult virgin cycling mice showed that Foxp3 heterozygous mice undergo regular estrous cycling, indistinguishable from the wildtype controls, suggesting that the ovarian hormones are normally circulated in these mice. The normal phenotypes of mice observed in our study suggest that half-dose expression of Foxp3 is sufficient for its normal function, and does not impair mammary gland development or the estrous cycle regularity.

Our results suggest that Foxp3 heterozygosity does not impair mammary gland morphogenesis or function in young adult mice, suggesting that other factors might be involved in making these

mice susceptible to cancer development. One possibility for the rise of breast cancer risk in aged Foxp3 heterozygous mice could be due to indirect effects of immune cells in the mammary gland, which has not yet been investigated. It is also noteworthy that the mice used in the study by Zuo, Wang (94) were in BALB/C genetic background whereas in our study we used Foxp3 heterozygous mice on the C57BL6 background. Indeed, different strains of mice can show variable side branching or alveologensis in the mammary gland (192). It would be informative to examine these phenotypes in Foxp3 heterozygous mice on a BALB/C background to observe any defects in the mammary epithelial ductal branching.

Loss of the wildtype Foxp3 allele has been suggested to be exclusive to cancer, since skewed X-inactivation was only observed in tumour cells, but not the adjacent healthy breast tissue in Foxp3 heterozygous mice (94). Whether or not FOXP3 plays a role in mammary gland development in humans remains speculative. The pattern of X chromosome inactivation was analysed in the peripheral blood of a woman heterozygous for FOXP3 mutation, and it was found that there was no significant imbalance between T cells expressing the mutated FOXP3 allele and normal T cells (90). Although human female carriers of FOXP3 mutation are completely healthy and have normal immunological parameters including lymphocyte subpopulations (90), it is possible that genetic alterations of FOXP3 work in concert with additional genetic hits to increase breast cancer risk. This X-linked gene may be inactivated by various events such as mutations, loss of heterozygosity, bi-allelic methylation, and skewed X-inactivation in breast cancer (193). The mechanisms through which FOXP3 may get altered in the normal breast is not clear; however, the ovarian hormones might be involved. It should also be noted that there are few differences between human and mouse Foxp3, one of which is the presence of three isoforms in humans, while there is only a single Foxp3 isoform present in mice (92). The longest form resembles the murine Foxp3, while the other two isoforms are unique to humans (194). The PCR primer sets used in our experiments amplify all isoforms;

however, it would be intriguing to investigate the mammary epithelial-specific expression of different FOXP3 isoforms in the human breast.

5.3.2. HORMONE REGULATION OF FOXP3 IN MAMMARY EPITHELIAL CELLS AND ITS EFFECT ON ZEB1

Nuclear expression of FOXP3 is much lower in cancer cells than in normal breast epithelia (94). Its expression is abnormal and variable in different cancer cell lines, with the highest expression in MCF7 cells (94, 195). This is in parallel with our observations which also showed that the mRNA expression of *FOXP3* is variably regulated by estradiol and progesterone, depending on the cancer cell type or the treatment time course.

Considering the role of FOXP3 as a tumour suppressor gene, it is likely that downregulation of this gene by hormones may increase the expression of oncogenes such as HER2 or SKP2 (94, 95) and contribute to breast cancer development. This is supported by a recent study in which lower expression of the nuclear FOXP3 protein in primary tumour samples was significantly correlated with tumour clinical stage and lymph node metastasis (196). On the other hand, upregulation of this gene can be an ideal effect considering the role of FOXP3 as an inducer of transcriptional activity of tumour suppressor genes in both normal and malignant breast epithelial cells (99). Peripheral FOXP3 has been shown to regulate the expression of several DNA damage-associated genes, suggesting that it may suppress tumour cell growth by either preventing the repair of DNA damaged cells or promoting their apoptosis (197). If the epithelial-specific FOXP3 functions in the same manner, it may inhibit the proliferation of DNA-damaged cells in the breast during the menstrual cycle. Several studies have reported the higher expression of FOXP3 to be a prognostic factor for breast cancer (198, 199), although a few have suggested the opposite role (200, 201). High expression of the FOXP3 protein in breast tumours is correlated with the abundance of Tregs, suggesting that a higher immune

tolerance occurs in mammary gland tumours. This could affect aggressive tumour growth, leading to the shutdown of any effective anti-tumour immune response.

Our RT-PCR results revealed that the correlation of *ZEB1* and *FOXP*3 mRNA expression is dependent on the properties of breast cancer cell lines as well as the hormone treatment time course. For instance, both *FOXP3* and *ZEB1* mRNA expression was induced by progesterone in T47D cells, whereas these genes were downregulated with estradiol treatment in estrogen-responsive MCF7 cells. It is likely that hormone treatments might trigger regulatory mechanisms between *FOXP3* and *ZEB1* in the mammary gland, which subsequently aids in the transformation of mammary epithelial cells. Whether or not FOXP3 regulates EMT via mechanisms involving ZEB1 requires further investigations using EMT assays and more detailed cell culture models.

It seems that *FOXP3* mRNA expression in the mammary epithelium is variable in different breast cancer cell lines. However, due to the conflicting clinical results in the literature, as well as in our experiments, the significance of mammary epithelial cell-specific *FOXP3* expression, and its regulation by ovarian hormones remain unclear. In the immune system, FOXP3 expression is essential to modulate immune surveillance through Tregs (194). Therefore, hormonal alterations of this gene might affect the immune surveillance in the breast. The results obtained in this study might be just the 'tip of the iceberg' of insights into the crosstalk between the ovarian hormones and Foxp3 expression in the human breast and further research is needed.

5.3.3. FOXP3 OVEREXPRESSION IN HUMAN MAMMARY EPITHELIAL ORGANOIDS:

A recent study showed that overexpression of FOXP3 in breast cancer cell lines has an inhibitory effect on cancer cell adhesion and invasion (196). In another study using MDA-MB-231 breast epithelial cell lines, FOXP3 overexpression was associated with a significant

reduction in CXCR4 and HER2, whereas silencing FOXP3 had the opposite effect (98). These researchers suggest that FOXP3 regulates CXCR4 expression and that a loss of FOXP3 by invasive breast tumours might promote CXCL12-regulated metastasis (98).

In our experiment, both groups of cells were infected with a similar multiplicity of infection (MOI) and expressed the reporter GFP at the gene and protein level. The lower expression of GFP in FOXP3-GFP-transduced cells is probably due to the presence of a second gene of interest (i.e., FOXP3), rather than GFP alone (202). In FOXP3-GFP lentiviral vectors, full-length FOXP3 is driven by the EF1-alpha promoter, while an encephalomyocarditis virus (EMCV) Internal Ribosome Entry Site (IRES), located downstream of FOXP3 gene, enables the expression of the GFP reporter. However, GFP lentiviral constructs contain an additional GFP gene in place of FOXP3. Therefore, co-expressing two different transgenes linked by an IRES element in a single bicistronic lentiviral transcript might have affected the low expression of the second gene (i.e. GFP) in FOXP3-GFP-transduced cells. Different mechanisms may contribute to the capacity of an IRES to precisely translate the second gene (downstream of the IRES), however, in most cases, only the first gene is strongly expressed in a bicistronic vector (202, 203). Notwithstanding, the expression of GFP reporter in both groups of cells is clearly higher than that of un-transduced cells, confirming that transduction of the organoids was efficient in this experiment.

Following stable FOXP3 overexpression in human mammary epithelial organoids, an apparent increase was observed in the expression of *ZEB1* mRNA, suggesting that *ZEB1* is a downstream target of *FOXP3*. To an extent, this effect is consistent with our observations in progesterone-treated T47D and MCF7 mammary epithelial cell lines whereby regulation of *FOXP3* was associated with an increase or decrease of *ZEB1* in these cell lines, respectively. Also, consistent with previous findings in the literature, the mRNA expression of *HER2* and *CXCL12* genes was downregulated following an increase in *FOXP3* levels. Unexpectedly, however, a similar effect

was observed in the cells transduced with GFP alone, suggesting that such impact in FOXP3-GFP-transduced organoids is not solely due to FOXP3 overexpression. The reason for this is not apparent; however, it might be either due to technical errors in the assays or the effects of lentiviral vectors interfering with the expression of these genes. Nonetheless, the mRNA expression of the housekeeper gene *PPIA* was constant across the groups. This suggests that the sample variations could have occurred due to other experimental processes such as the cell culture or lentiviral transduction.

It must also be noted that due to the limited number of cells, due to limitation in access to primary mammary epithelial cells from patients, we pooled the organoids from 10 individuals before lentiviral transduction. For the same reason, there were insufficient number of cells required to purify and sort the cells based on their GFP expression which is a marker that the individual cell has been transduced. Hence, the obtained results are from a mixture of transduced and non-transduced cells, making the RT-PCR data less reliable. However, this is the first study that has used such novel model of primary human mammary epithelial organoids to analyse the role of *FOXP3* in the breast. Manipulating the genetic profile of human mammary epithelial organoids using lentiviral vectors serves as a promising resource in experimental research on the breast epithelium. Using a physiologically relevant model system of genetically-engineered primary cells can surpass conventional cell line and transgenic mouse models regarding cost, speed, and reliability. One approach to be taken in future is to use microarray technology or chromatin immunoprecipitation (ChIP) to identify more downstream target genes of FOXP3 in the breast epithelia. Also, it would be reasonable to silence FOXP3 gene in the organoids and analyse its effects on cytokine production by normal mammary epithelial organoids.

5.4. CONCLUSION

The data presented in this chapter revealed that Foxp3 heterozygosity does not affect mammary gland development during puberty and young adulthood in mice. Although *FOXP3* is hormonally regulated in human mammary epithelial cells *in vitro*, its heterozygosity does not affect the estrous cycle regularity in mice. It is likely that subtle effects of Foxp3 heterozygosity on the immune cell compartment and possibly directly on mammary epithelial cells are involved in causing aged Foxp3 heterozygous female mice susceptible to mammary cancer development over a long period of time. Foxp3 expression confers suppressor functions on peripheral CD4+CD25+ Tregs (88). This is required to suppress the function of autoreactive T cells and inhibit the development of autoimmunity. Therefore, it is possible that a defect in this gene in the mammary epithelium may deregulate the immune homeostasis, leading to immune tolerance and increases tumour development. Overall, further investigations are required to dissect the underlying mechanisms of how Foxp3 heterozygosity increases breast cancer risk in mice and what the normal function of this transcription factor is in the mammary gland epithelium.

CHAPTER SIX

GENERAL DISCUSSION

6.1. INTRODUCTION

It is widely accepted that mammary gland development is dependent on the dynamic interactions between hormonally-responsive mammary epithelium and the immune microenvironment in the stroma; these interactions are mediated through activation and function of a variety of transcription factors and cytokines. However, it is not clear how fluctuations of estrogen and progesterone affect the immune cytokine expression in the mammary gland. We attempted to address this question using a variety of different approaches to investigate hormonal regulation of cytokine expression by mammary epithelial cells. The approaches that were employed were three-dimensional human mammary epithelial organoids, cell lines, and mouse mammary glands. Functional three-dimensional primary organoids provide a greater understanding of normal breast structure and development and recapitulate morphogenetic events regulated by ovarian hormones in the breast (128). Human mammary epithelial cell lines have a well-defined hormone receptor profile, and mouse models were used to track the molecular changes in the mammary gland associated with the natural fluctuations in hormones across the estrous cycle.

The general hypothesis of this thesis was that estrogen and progesterone regulate the expression of certain immune-related transcription factors and cytokines in the breast epithelial cells. Our results showed that transcription factors *ELF5* and *FOXP3*, as well as their downstream cytokines, were variably regulated by estradiol and progesterone depending on the hormone receptor profile of the mammary epithelial cells. It was also hypothesized that Foxp3 heterozygosity perturbs mammary gland development in mice; a theory which was negated.

The studies outlined in this thesis are the first to investigate the significance of hormone regulation of these immune-related cytokines in primary mammary epithelial cells. In this chapter, the overall effects of estradiol and progesterone on cytokine expression by mammary epithelial cells are discussed. The current knowledge on the cellular and molecular changes in the human breast during the menstrual cycle is also reviewed. Further, the limitations of these studies to investigate how menstrual cycle-associated hormones affect mammary epithelial cell function are outlined. Finally, opportunities for future research to improve our understanding of the hormone-regulated cytokine networks are highlighted.

6.2. CURRENT KNOWLEDGE ON THE CELLULAR AND MOLECULAR CHANGES IN THE BREAST DURING THE MENSTRUAL CYCLE

Much of what is known about the cellular and molecular interactions directing mammary gland morphogenesis during the ovarian cycle comes from studies involving animals (20, 40, 204, 205). Using mouse models, Chua, Hodson (20) observed that the abundance and phenotype of macrophages fluctuates over the course of the estrous cycle. Following this study, Hodson, Chua (40) reported differential percentage of murine macrophages that express cell surface proteins NKG2D, CD204, and MHCII during different stages of the ovarian cycle, regulated by progesterone and estrogen. NKG2D which was increased during metestrus and diestrus, is a receptor that recognises antigens expressed on the surface of DNA-damaged cells. When activated, it induces production of pro-inflammatory cytokines, causing inflammation at the site of injury. The mammary gland is most resistant to tumour initiation during the development phase of the ovarian cycle (206), which may be the result of heightened immune surveillance mediated by NKG2D-expressing macrophages. Additionally, the percentage of macrophages expressing MHCII and CD204 phenotypes were found to be highest during proestrus which is consistent with the established roles of MHCII and CD204 in the recognition and phagocytosis of apoptotic cells (40). Based on these findings, it is possible that similar phenotypes of

macrophages exist in the human breast which fluctuate with the ovarian hormones and affect immune surveillance in this tissue.

Few studies have investigated the gene expression profile in the human breast during the menstrual cycle. In 1994, Sabourin, Martin (207) analysed the expression of B-cell lymphoma 2 (BCL-2) protein in 50 samples of normal breast tissue and found that its expression in lobular epithelial cells increases during the follicular phase and decreases sharply at the end of luteal phase. BCL-2 prevents apoptotic cell death and plays a critical role in determining the life or death of a cell (208). As noted by Ferguson and Anderson (18), mammary epithelial apoptosis in normal mammary gland increases at the end of the luteal phase when the circulating concentration of estrogen and progesterone decline. Hence, hormonal fluctuations of BCL-2 during the menstrual cycle is part of a normal process in the mammary gland. However, dysregulation of this protein might lead to overabundance of mammary epithelial cells, which can be a trigger for hormone-dependent tumorigenesis in the breast (207). More recently, Pardo, Lillemoe (209) analysed the DNA expression in premenopausal breast epithelium using next-generation whole transcriptome sequencing, and found a significant difference in the expression of 255 genes between the two phases of the cycle. The majority of these genes were upregulated during the luteal phase, and play roles in the cell cycle events such as DNA replication, DNA damage response and mitosis. Interestingly, most of these genes were overexpressed in breast cancer, suggesting that they play a role in tumorigenesis via increasing the mitotic rate in the breast (209).

Other studies revealed that the molecular profile of ECM in healthy human mammary gland undergoes cyclical changes during the menstrual cycle *in vivo* (210, 211). In particular, the expression of proteoglycans syndecan-1, syndecan-4, and decorin was found to be reduced during the luteal phase (211). Since polymorphisms of these genes are associated with breast cancer susceptibility (212, 213), it is likely that their modulation by hormones during the

menstrual cycle deregulate the behaviour of stromal cells and trigger tumorigenesis in the mammary gland microenvironment. Despite all these attempts, no study has investigated the function and phenotypes of immune cells such as macrophages and T cells in the breast over the course of menstrual cycle. There is a pressing need for more research on the cellular cell signalling in healthy breast tissue under normal physiologic settings. However, several considerable challenges have been identified which make it difficult to address these questions.

A major challenge in studies associated with hormone regulation of the mammary gland during the menstrual cycle is to determine the exact phase and duration of menstrual cycling in women prior to breast surgery. Currently, conventional methods such as counting the days from the onset of bleeding, basal body temperature charts, and measuring serum concentrations of estrogen and progesterone are used to estimate the different phases of the menstrual cycle. Also, technology has offered apps which allow women to track their menstrual cycle using their smart phones. However, using a device which can measure the concurrent concentrations of estrogen and progesterone on the days of operation and track the changes affecting the breast overall architecture, could be of great benefit. On the other hand, the abundance of hormone receptors fluctuates during the menstrual cycle (214), making it more difficult to study hormonal effects. Besides, different women with heterogeneous genetic background may respond differently to the ovarian hormones.

Clearly, a larger sample size with participants encompassing a more defined menstrual status is required to adequately explore the effects of these ovarian hormones on the mammary gland microenvironment *in situ*. Using *in vitro* techniques of primary mammary culture would also overcome some of the difficulties associated with the study of hormonal changes in the breast. For instance, isolating cells from a human breast sample and treating the cells with different hormone combinations would recapitulate hormone environment in the breast, and also enables us to perform paired sample statistical analysis in the same patient.

6.3. HORMONAL REGULATION OF IMMUNE-RELATED CYTOKINES IN THE MAMMARY GLAND

Ovarian hormones are likely to have direct and indirect effects on cytokine production by immune cells. For example, progesterone directly promotes the expression of pro-inflammatory cytokines such as TNFA, chemokine (C-C motif) ligand 2 (CCL2), and interleukin 1 beta (IL1B). On the other hand, estrogen mediates anti-inflammatory effects by inhibiting the expression of TNFA, IL1B, and IL6 in human osteosarcoma cell line, through both of its receptors, ERA and ERB (215). Therefore, inhibition of hormone receptors can account for a potential mechanism for deactivating the cytokines and inhibiting their effects. However, indirect effects are also likely to occur, through interactions with mammary epithelial cells and the immune microenvironment of the mammary gland. As described before, mammary epithelial cells secrete an array of cytokines, many of which are regulated by ovarian hormones and are expected to affect local immune cell populations. A similar cytokine network exists in the uterus whereby hormonally responsive uterine epithelial cells direct macrophage abundance and function through the expression of specific cytokines (216). The findings in this thesis suggest that depending on the presence of hormone receptors and downstream signalling pathways that affect mammary epithelial cell function, estrogen and progesterone can activate or dampen immune-regulatory cytokine networks in the breast.

Figure 6.1 provides a heat map summary of the abundance of mRNA encoding cytokines across three different mammary epithelial cancer cell lines, upon treatment with estradiol and progesterone. The results obtained from mammary epithelial organoids, mouse mammary glands, and mammary epithelial cancer cell lines suggest that hormone-regulated cytokine expression can differ remarkably across the different models. In particular, these hormones tend to have inhibitory effects on the cytokines produced by normal mammary epithelial organoids, while having stimulatory effects on the same cytokines in mouse mammary gland and malignant mammary epithelial cell lines. A similar observation, that hormones affect cytokine

expression differently depending on the exposure time, could be noted when cell lines were hormone-treated over different time courses. For example, progesterone treatment of MCF7 cells for 24 hours tended to stimulate the expression of inflammatory cytokines, whereas 72 hours treatment had inhibitory effects, suggesting that short exposure to hormones can lead to inflammation, whereas prolonged exposure can dampen the inflammatory response. Furthermore, MCF7 and T47D cell lines tend to respond to estradiol and progesterone by altering the expressions of mRNA encoding cytokines more so than ZR751 cells, making them useful tools for understanding the effects of these hormones on mammary gland development.

It is likely that ovarian hormones over the menstrual cycle maintain a balance between the pro and anti-inflammatory cytokine signals using a transcriptional regulatory feedback system to mediate the dynamic and complex changes in the breast. Although we could not precisely determine the immune signalling pathways in the breast, our studies suggest that the epithelial cell-specific transcription factor *ELF5* might play a role in mediating the cytokine production in breast epithelia. It might act as a modulator of inflammatory signals in the breast by dampening the effects of immune cytokines such as *S100A8, S100A9* and *CXCL12*. As mentioned earlier, dysregulation of many of these cytokines is found in breast cancer and may contribute to increased risk of tumorigenesis. Therefore, an imbalance in hormone fluctuations that can be caused by an unbalanced lifestyle, chronic stress, and exposure to synthetic oral contraceptives, might influence the function of transcription factors in the mammary gland, leading to dysregulation of immune cytokines and subsequently dampening the immune surveillance in this tissue. Throughout reproductive years, impaired immune surveillance along with increased proliferation of mammary epithelial cells mediated by estrogen and progesterone, might cause genomic instability, increase the likelihood of mutations in the breast and lead to increased risk of breast cancer in women.

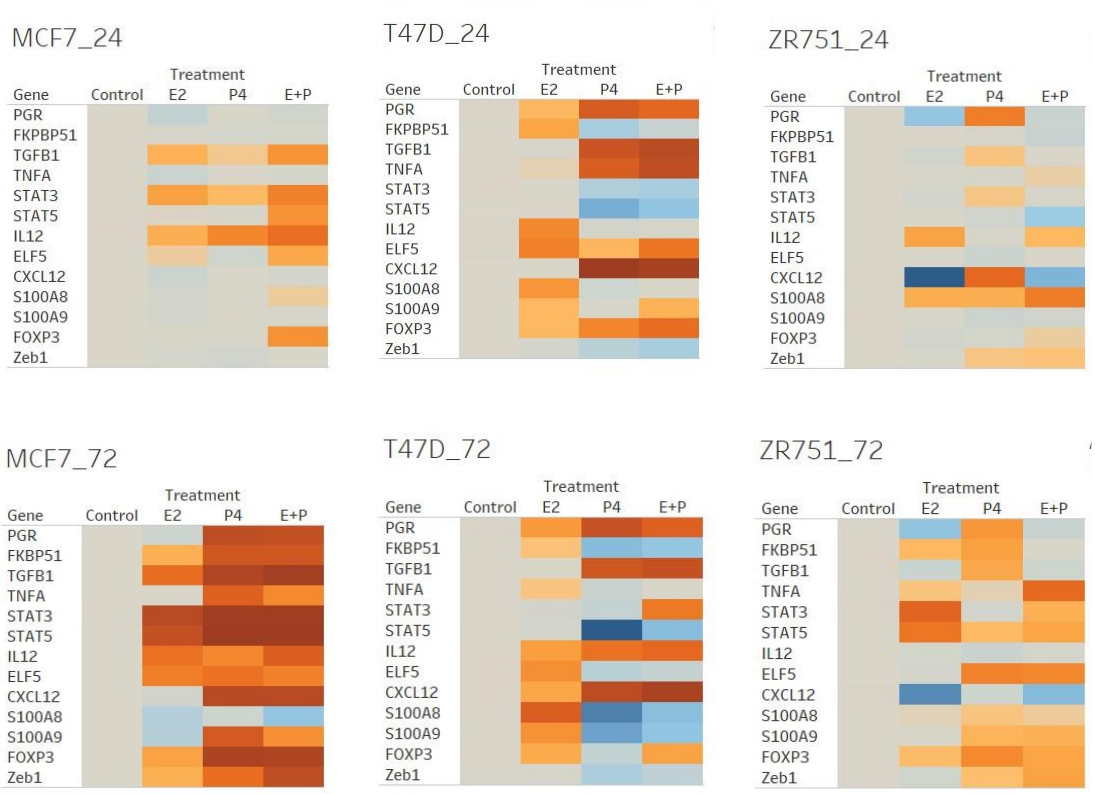

Figure 6.1: Heat-map profiles of mRNA expression of cytokines in mammary epithelial cancer cell lines upon treatments with estradiol and progesterone.

The image represents the scale of mRNA expression of cytokines in different mammary epithelial cancer cell lines. Orange indicates downregulation of mRNA encoding each cytokine, blue indicates upregulation and no change is indicated in grey.

6.4. LIMITATIONS OF THIS STUDY:

There were some weaknesses and limitations to this study. Firstly, we used heterogeneous population of cells including primary normal human cells, cancerous cell lines, and mouse mammary glands which are physiologically different from each other and none of which are an ideal system to represent the events occurring *in vivo* in humans. Moreover, we largely investigated gene expression in our studies rather than protein expression. Gene expression may not be identical to the expression of the corresponding protein, which might be affected by different post-transcriptional modifications, leading to mistaken conclusions on the more physiologically relevant protein secretions. This was evident in our studies involving genetic knockdown of *ELF5* in T47D cells whereby the expression of *ELF5* was efficiently silenced at the mRNA level, but not at the protein level. Also, most of these studies were performed in isolated *in vitro* cell culture settings and in the absence of local microenvironment co-factors including those expressed by immune cells, and physiological concentration of estradiol and progesterone. It is likely that estrogen and progesterone trigger the mechanisms through which immune cells are recruited to the mammary epithelial cells. This was evident in our *ex vivo* studies which showed that hormone treatment of human breast tissue explants result in a higher expression of S100A8 protein on stromal macrophages. Nevertheless, it is important to focus on such models in order to answer the main question regarding hormonal effects on immune cell function in the mammary gland.

Primary organoids are usually impure and contaminated with stromal and non-epithelial cells (217). Although the protocol was designed to isolate the epithelial cells from human breast tissues, the efficiency and the purity of samples could not be determined due to the limited number of cells. It is likely that these organoid samples consist of not only epithelial and myoepithelial cells, but stromal components such as fibroblasts, macrophages and adipocytes which might have contributed to the effects. To tackle this issue, immunohistochemistry

techniques and mammary epithelial cell-specific markers could be used to precisely study on the purity of human mammary epithelial organoids.

Although cell lines are usually highly proliferative and easy to culture, there are a number of limitations which make them a less favourable option in research. Cell lines have lost the true characteristics of the original tissue from which they were derived. Moreover, serial subculture of these cells can cause genotypic and phenotypic variations over an extended period of time (218). The cell lines used in this experiment are from the same aggregate morphological class, however, they originate from different patients, have unique biologic features, and express variable levels of hormone receptors; thus show different gene expression patterns upon hormone treatments. It should also be noted that although these cell lines were purchased from ATCC, their identity was not confirmed by sequencing. Therefore, misidentification of these cell lines could be another reason for the variable results reported in this project.

Another limitation associated with this study is that RNA was extracted from whole mammary glands, and expression of genes encoding specific cytokines may not solely be specific to mammary epithelial cells, and other cell types might be involved in producing such results. Using laser capture microdissection could help us address this issue by isolating the mammary epithelial cells from the tissues and analysing gene expression specifically in mammary epithelial cells. It is also important to note that the serum levels of hormones was not measured in these mice; this makes the results less reliable as the exact cycle stage determined by vaginal cytology was not confirmed with circulating concentration of estradiol and progesterone.

Given more time for these studies, the sample sizes for primary tissue studies would be increased. With the high degree of variation observed in primary cell culture, it is estimated that a sample size of 25 breast tissues is needed for a 95% power result and 5% significance level. It would also be beneficial to conduct more in depth studies on the time frame of hormone

stimulation and the reproducibility of results of organoids, mouse mammary glands and explant cultures. The small amounts of primary tissue we obtained limited our ability to robustly test timing of hormone treatments, and the reproducibility of results obtained from the same patient is currently unknown. However, a larger study should certainly address these questions. We are currently collecting normal and cancerous breast tissue biopsies during different stages of the menstrual cycle within individual patients. This would be a valuable resource as it allows us to analyse hormonal changes as well as gene expression patterns in malignant breast tissues and compare them to those from healthy individuals across the menstrual cycle.

6.5. FUTURE RESEARCH DIRECTIONS

The experiments reported herein demonstrated the complexity of immune cytokine networking in the mammary epithelia which is regulated by estradiol and progesterone. However, the precise molecular mechanisms affecting breast cancer risk in the mammary gland during the menstrual cycle are still far from clear. There are several aspects of the immune-cytokine regulatory networks in the breast that need to be explored. For example, the specific mechanisms through which estrogen and progesterone modulate the phenotype and function of immune cells such as macrophages and T cells in the breast should be investigated. Moreover, transgenic knockout mouse models can be used to investigate the correlation between ovarian hormones and specific cytokines in the mammary gland and their influence on tumour initiation. It would also be interesting to use human breast xenograft mouse models to analyse the effects of exogenous hormones on regulation of molecular networks in the mammary gland. On the other hand, lentiviral transduction techniques can be utilised to manipulate human mammary epithelial cells and investigate the effects of transcription factors and cytokines in the breast. However, it would be more beneficial to use high content screening technology to determine entire pathways rather than individual factors mediating the relationship between cyclic hormone-induced changes in mammary epithelium and tumour stroma.

Furthermore, the effects of Foxp3 heterozygosity on mammary gland morphogenesis will be analysed using aged mice. This is a critical step to understand the cellular changes in Foxp3 heterozygous mice's mammary gland since these animals are prone to breast cancer development when they age. It is also part of our future plan to study other immune-associated gene candidates which have the potential to mediate hormonally-regulated cytokine production in the mammary gland. Research in our lab has discovered an important role for the complement protein C1q in the establishment of breast cancers. C1q plays an essential role in promoting macrophage-mediated clearance of dying mammary epithelial cells during the regression phase of the ovarian cycle. Mouse models lacking C1q protein are far less susceptible to mammary cancers than normal mice, a finding that opens the door for therapeutic intervention. We are planning to produce monoclonal antibodies capable of neutralising C1q activity and test the therapeutic capacity of these antibodies in a mouse model of mammary cancer. A therapeutic antibody that neutralises C1q activity has the potential to prevent breast cancers from becoming established.

Overall, the information from this study can benefit the ongoing research regarding the development of new approaches to prevent breast cancer in premenopausal women. The susceptibility of the human breast to cancer-initiating factors may be altered during specific stages of the menstrual cycle (13). Thus, avoidance of exposure to initiating factors such as alcohol and radiation such as that in imaging X-rays at specific stages of the cycle can help reduce the risk of cancer development. A similar concept could be applied in designing more effective screening and treatment programs. For instance, knowing the best time to get a mammogram during the menstrual cycle could help increase the accuracy of the results, as shown by a recent study suggesting that the sensitivity of mammography in the detection of breast cancer increases when women undergo screening during the first week of their menstrual cycle (219). Furthermore, gene expression profiling tests are commonly used to diagnose breast cancer subtype, and help guide treatment decisions (220-222). However, these tests have been

developed and validated predominantly in postmenopausal women, and it remains unclear whether these tests are appropriate for use in premenopausal women (222). It is possible that breast cancer gene expression and the treatment pathways which stem from its measurement, could fundamentally depend on a woman's menstrual cycle stage at the time of tissue collection. In addition, these studies can help us identify key mechanisms in mammary gland development during menstrual years, and how these factors may increase cancer risk later in life.

6.6. CONCLUSION

Estrogen and progesterone regulate the expression of specific immune cytokines in mammary epithelial cells, however, the response is highly variable and dependent on a number of factors including where the cells originated from (for example whether they are primary cells from an individual patient or a cell line), hormone receptor profile, and the time of exposure to the hormones. The different expression profiles of cytokines have provided us a framework for understanding the complexity of hormone regulatory cytokine signalling in the mammary gland. A complex interplay of transcription factors including ELF5 and FOXP3 might mediate cytokine expression in mammary epithelium. Figure 6.2 represents a schematic of the changes in the cellular components of the mammary gland during menstrual cycling.

Overall, fluctuations in the ovarian hormones estrogen and progesterone, across the course of menstrual cycle, might create a pro-tumorigenic environment in the mammary gland that causes the mammary gland to be more susceptible to cancer. Although the biological mechanisms underlying this pro-tumorigenic microenvironment is still unclear, it may involve dampened immune surveillance and/or tumorigenesis driven by the inflammation associated with altered cytokine signalling. Also, inflammatory factors may induce DNA damage, leading to genetic instability and proliferation of mutated cells (223). Therefore, understanding the cellular and molecular mechanisms that govern mammary epithelial cell cytokine expression can help in identifying potential new treatments for breast cancer.

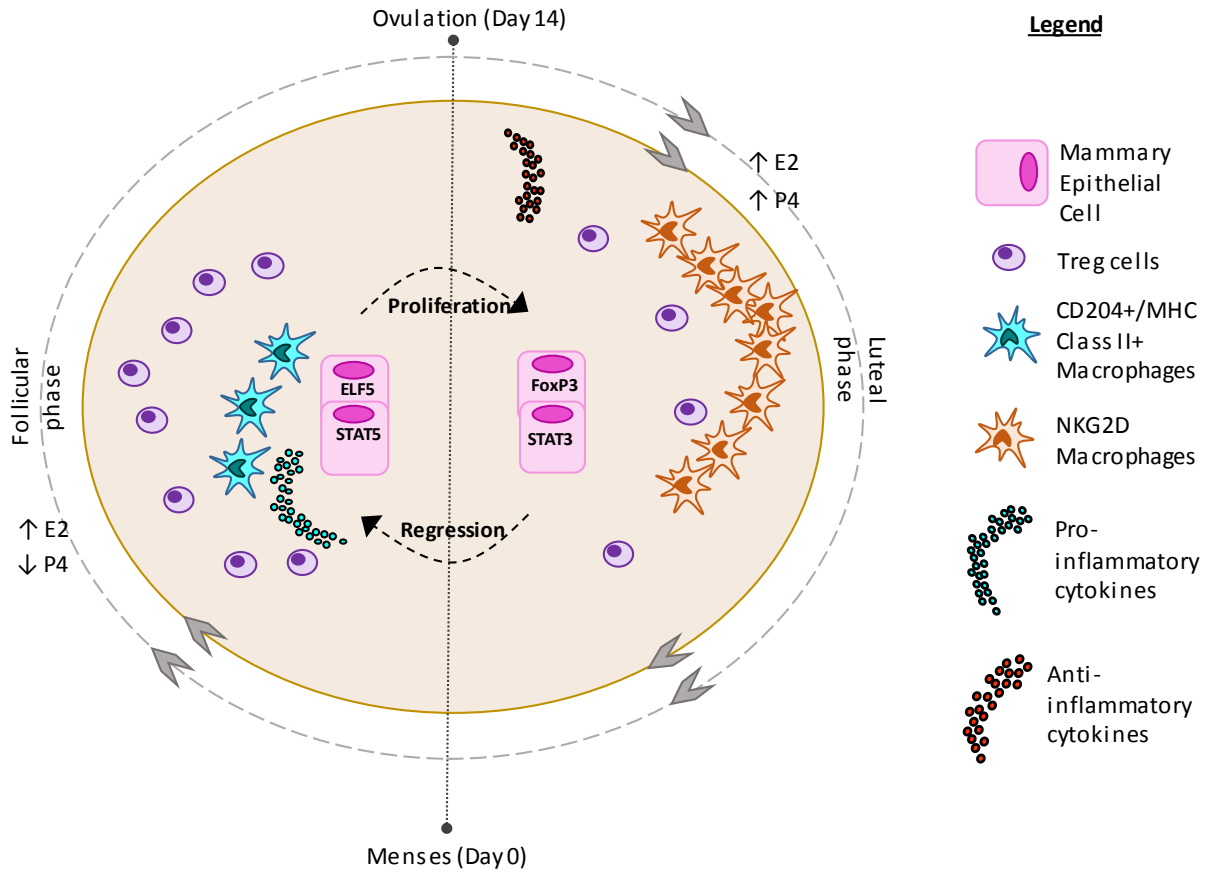

Figure 6.2: Schematic of the changes in the cellular components of the mammary gland during menstrual cycling.
The follicular phase of the menstrual cycle is characterised by high serum levels of estrogen, lower number of mammary epithelial cells, and higher number of Tregs and anti-inflammatory cytokines compared to the luteal phase. In contrast, the luteal phase is characterised by high serum levels of progesterone and increased numbers of mammary epithelial cells as well as increased abundance of macrophages compared to the follicular phase. The phenotype and function of macrophages in the mammary gland also changes throughout the cycle.

APPENDICES

APPENDIX A:

Atashgaran, V., et al. (2016). "Dissecting the Biology of Menstrual Cycle-Associated Breast Cancer Risk." Frontiers in Oncology 6: 267.

frontiers in Oncology

REVIEW
published: 26 December 2016
doi: 10.3389/fonc.2016.00267

Dissecting the Biology of Menstrual Cycle-Associated Breast Cancer Risk

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Fluctuations in circulating estrogen and progesterone across the menstrual cycle lead to increased breast cancer susceptibility in women; however, the biological basis for this increased risk is not well understood. Estrogen and progesterone have important roles in normal mammary gland development, where they direct dynamic interactions among the hormonally regulated mammary epithelial, stromal, and immune cell compartments. The continuous fluctuations of estrogen and progesterone over a woman's reproductive lifetime affect the turnover of mammary epithelium, stem cells, and the extracellular matrix, as well as regulate the phenotype and function of mammary stromal and immune cells, including macrophages and regulatory T cells. Collectively, these events may result in genome instability, increase the chance of random genetic mutations, dampen immune surveillance, and promote tolerance in the mammary gland, and thereby increase the risk of breast cancer initiation. This article reviews the current status of our understanding of the molecular and the cellular changes that occur in the mammary gland across the menstrual cycle and how continuous menstrual cycling may increase breast cancer susceptibility in women.

Keywords: estrogen, progesterone, microenvironment, cytokine, menstrual cycle

OPEN ACCESS

Edited by:
Gilberto Schwartsmann, Universidade Federal do Rio Grande do Sul, Brazil

Reviewed by:
Tamara Louise Kalir, Icahn School of Medicine at Mount Sinai, USA
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Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *31 August 2016*
Accepted: *14 December 2016*
Published: *26 December 2016*

Citation:
Atashgaran V, Wrin J, Barry SC, Dasari P and Ingman WV (2016) Dissecting the Biology of Menstrual Cycle-Associated Breast Cancer Risk. Front. Oncol. 6:267. doi: 10.3389/fonc.2016.00267

INTRODUCTION

The mammary gland is an essential reproductive organ, present in females of all mammalian species, which produces milk for both nourishment and immunological protection of newborns. It is a unique organ, as the vast majority of mammary gland development occurs postnatally, during puberty, pregnancy, and the postpartum period (1). Mammary gland development is highly dependent on the actions of hormones, including estrogen and progesterone, and these endocrine factors act locally within the tissue through complex interactions with growth factors and cytokines in the mammary microenvironment (2, 3). While there has been much interest in the cellular and molecular interactions directing mammary gland development during pregnancy and postpartum involution, surprisingly little is understood of the biological mechanisms that promote development during the menstrual cycle.

A number of risk factors are associated with breast cancer, including family history of breast cancer, increasing age, and high breast density (4). There are also a number of significant risk factors associated with a woman's reproductive history, one of which is increased number of years

of menstrual cycling. A large meta-analysis demonstrated that the period of time between onset and cessation of menstrual cycling strongly correlates with increased breast cancer risk in women (5). For each year younger a girl commences menstrual cycling, there is a 5% increase in lifetime risk of breast cancer. Similarly, for each year older at the time of menopause, there is a 3.5% increased breast cancer risk (5). Studies on naturally postmenopausal women also showed increased breast cancer risk in those who had experienced greater than 490 menstrual cycles in their lifetime as compared to those with fewer or irregular cycles (6). This indicates that fluctuations in ovarian hormones associated with menstrual cycling affect breast cancer susceptibility. However, the biological basis for the link between fluctuations in ovarian hormones and increased breast cancer risk is not well understood.

The mammary gland consists of a number of different cell lineages including epithelial, hematopoietic, endothelial, and stromal (7). Epithelial cells are organized in a hierarchical manner of mammary stem cells (MaSCs), mammary progenitor cells, and committed mammary epithelial cells, including luminal and myoepithelial cells. The majority of human carcinomas originate from mammary epithelial cells (8). Immune cells, extracellular matrix (ECM), fibroblasts, and endothelial cells are all abundant in the stroma of the mammary gland (9), and their roles are mediated *via* a complex network of intracellular and extracellular signaling pathways. It is widely accepted that mutations in mammary epithelial cells are the initial drivers of tumorigenesis. However, what is increasingly appreciated are the cell-to-cell interactions between epithelia and the surrounding stroma that affect DNA mutation rate, survival of DNA-mutated cells, and development of malignancy in the breast. Fluctuations in estrogen and progesterone across the menstrual cycle affect the abundance and function of mammary epithelial cells, stromal immune cells, and the ECM, and these changes are likely to be associated with biological mechanisms that cause increased breast cancer risk associated with menstrual cycling. The focus of this review is to collate current knowledge of the molecular and cellular events that occur during hormone-mediated menstrual cycling that affect epithelial and stromal cells in the mammary gland and how these contribute to increased breast cancer risk in women.

BIOLOGICAL CHANGES IN THE BREAST DURING THE MENSTRUAL CYCLE

The phases of the menstrual cycle in women are regulated by fluctuations in the pituitary gland hormones, follicle-stimulating hormone and luteinizing hormone, and the ovarian hormones, estrogen and progesterone (10). Hormonal fluctuations are cyclical in nature and occur continuously, such that each cycle merges into the next. There is also variability in cycle length (22–36 days) between different women (11). The majority of the literature on menstrual cycle-associated changes in women is consistent with mouse literature and suggests that the main proliferative phase is the mid-luteal phase of the menstrual cycle, during which time circulating progesterone and estrogen are both high and epithelial alveolar buds begin to form. The high level of mitotic activity in this phase suggests progesterone is associated with a proliferative action (12). Conversely, the late luteal phase or the menstruation phase could be considered as the regression phase of mammary gland epithelia (13). During this time, the newly formed alveolar buds undergo apoptosis and tissue remodeling occurs such that the mammary gland returns to its basic architecture ready for another menstrual cycle (1, 14).

Progesterone appears to be a key hormone in regulation of mammary gland development and regression during the cycle. A number of animal studies have demonstrated a positive correlation between the percentage of alveolar epithelial buds in the mammary gland and the concentration of serum progesterone during the ovarian cycle (15–17). Indeed, the highest percentage of alveolar epithelia were observed during the diestrus phase (mouse equivalent of luteal phase), where the concentration of serum progesterone is maximal. Although estrogen exerts proliferative effects on mammary epithelial ducts directly *via* estrogen receptors (ERs) (18), it also upregulates the expression of the progesterone receptor (PR) during the luteal phase of the cycle (19). Importantly, progesterone withdrawal is also a critical regulator of mammary gland function. Newly developed alveolar buds require continuous progesterone signaling and undergo apoptosis and tissue remodeling which returns the mammary gland to a more basic architecture when progesterone falls (17).

These cycles of hormone-driven development and regression are likely to have a significant impact on breast cancer risk, even when breast cancer arises after menopause. Nielsen et al. (20) noted that clinically occult *in situ* breast carcinomas and atypical lesions are frequent in young and middle-aged women; they may remain in the non-invasive phase for 15–20 years before they develop into invasive breast cancers. In other words, hormone-regulated cellular events that occur during premenopausal years may induce persistent changes in the developmental fate of mammary epithelial cells. Alterations in signal transduction pathways, growth factors, and cell cycle regulators (21) associated with these clinically occult cancers would increase the lifetime risk of breast cancer.

DIRECT EFFECTS OF OVARIAN HORMONES ON TUMORIGENESIS

The roles of estrogen and progesterone in mammary gland development have been investigated using mouse models, gene expression analysis, and normal human breast tissues. Animal studies demonstrate the critical role of estrogen in mammary tumorigenesis, as cancer initiation and development can be significantly reduced using anti-estrogenic drugs or by performing oophorectomy (22–24). This is supported in human studies wherein early ablation of the ovaries results in regression of disseminated breast cancer (25). Furthermore, use of exogenous estrogen and progesterone analogs, such as hormone replacement therapy or hormonal oral contraceptives are known to increase breast cancer risk in women (26). The primary mechanism through which exogenous and endogenous hormones are

implicated in carcinogenesis is through promotion of mammary epithelial cell proliferation, which increases the chance of random genetic errors (26).

During the menstrual cycle, the highest proliferative rate of mammary epithelial cells occurs in the mid-luteal phase, as shown in premenopausal women who had highest cellular expression levels of proliferative marker Ki67 in the luteal phase versus highest expression of quiescent marker p27 in the follicular phase (27). A number of studies suggest that the mammary gland is more susceptible to carcinogenesis when there is higher proliferative activity in mammary epithelial cells (24, 28). The higher Ki67+ and the lower p27+ cell frequencies were positively associated with higher breast cancer risk among premenopausal women (27). Higher proliferative activity increases the chance of random mutations or DNA lesions (29). If the DNA-damaged cell is not repaired immediately, it will be used as a template for DNA synthesis in the next proliferative phase of the menstrual cycle, which would lead to the accumulation of faulty cells in the mammary epithelia. Polymorphisms in DNA repair genes such as BRCA2 and XRCC1, which maintain the integrity of the genome, can account for this genetic instability and the inability to repair DNA-damaged cells (30, 31). This genomic instability is favorable for premalignant cells to gain the faulty genotypes that enable tumor progression (32).

Although the role of progesterone in breast cancer etiology is controversial, it has been hypothesized that progesterone is the main driver of breast cancer risk during the menstrual cycle (33). More recently, Brisken et al. (34) proposed that repeated activation of PR signaling during the luteal phase may promote tumorigenesis in the breast. On the other hand, a recent study suggested the anti-mitogenic effects of progesterone, by inhibiting the estrogen-mediated growth of ER-positive tumors in human breast explants and cell lines (35). Overall, it seems that exposure to ovarian hormones affects cell signaling pathways and mammary progenitor cell fate. This leads to higher mitotic activity, which in turn may result in increased risk of genome instability and random genetic errors in DNA replication.

IMPACT OF MENSTRUAL CYCLING ON MaSCs

Mammary stem cells reside within the breast tissue and support mammary gland morphogenesis during different stages of development, such as during pregnancy. These cells are capable of self-renewal divisions as well as generating various lineages of mammary epithelial cells (36, 37). With the recent advances in stem cell biology and the technical frameworks for identification of these cells, the concept that cancers originate from stem cells and that MaSCs are the targets for transformation has become a hot topic in understanding breast cancer risk.

It should be noted that only a small percentage of normal mammary epithelial cells express ER and PR, and MaSCs lack these hormone receptors. Nevertheless, they are highly responsive to and are regulated by estrogen and progesterone *via* paracrine signaling from luminal cells involving receptor activator of nuclear factor-κB ligand (RANKL), WNT, CXCL12, and amphiregulin (38–41). Investigating the phenotypes of MaSCs in the mammary gland, particularly during the menstrual cycle, can be very challenging due to their rarity and the absence of specific markers for identification of these cells (42). Although there is evidence suggesting that ovarian hormones regulate the fate of MaSCs (7), research on the link between estrogen and progesterone and these multi-potent cells during menstrual cycling is limited.

In 2009, Graham et al. (43) reported that progesterone increases proliferation of normal human mammary epithelial cells by activating DNA replication mechanisms and increasing the number of bipotent progenitor cells. However, recent studies by Lombardi et al. (44) implicate another hormone in this process. Progesterone induces normal mammary epithelial cells to secrete pituitary hormone and growth hormone, and subsequently growth hormone increases proliferation of stem and progenitor cells in the mammary gland (44). The highest levels of serum growth hormone occurs in the luteal phase, correlating with the high progesterone levels. Joshi et al. (7) observed that the abundance of MaSCs significantly increases during the diestrus phase in cycling mice as well as in ovariectomized mice treated with the combination of estradiol and progesterone.

It is speculated that the high levels of growth hormone and progesterone during the luteal phase expand the numbers and proliferation rates of undifferentiated stem cells (44). These hormones may alter the phenotypes of mammary progenitor cells and increase the likelihood of transformation of undifferentiated cells into malignancy (7). Moreover, progesterone affects both symmetric and asymmetric cell division of MaSCs by increasing the population of basal and mature mammary epithelial cells (7). An imbalance between asymmetric and symmetric stem cell divisions can occur when there is deregulation in progesterone-regulated self-renewal pathways, such as WNT and RANKL (45, 46). It may be that repetitive menstrual cycling expands the number of undifferentiated MaSCs which are more prone to oncogenic hits (39, 44), leading to an increased risk of breast cancer.

CHANGES IN THE IMMUNE MICROENVIRONMENT DURING THE MENSTRUAL CYCLE

Mammary gland development and function depends on dynamic interactions between hormonally responsive mammary epithelia and the immune microenvironment. Immune cells are closely associated with mammary epithelial cells (47) and contribute to a number of stages of mammary gland development. Macrophages affect development and regression of the mammary gland over the course of the cycle, and these alternating roles of macrophages may affect menstrual cycle-associated breast cancer risk, particularly during the process of mammary gland regression. Another type of immune cell that may affect cancer risk in the mammary gland during the menstrual cycle is regulatory T cells (Tregs). Although immune cells are known to have an active role in the development and function of the mammary gland, it is still not

clear whether these cells affect menstrual cycle-associated breast cancer risk.

If a DNA mutation occurs, there is still a high chance that the immune system will recognize and eliminate the premalignant cell. Failure of the immune system to eliminate transformed cells throughout life can lead to cancer development. Thus, immune surveillance is a critical aspect to protect against tumorigenesis and evasion of the immune response against transformed cells is a hallmark of cancer (48). Studies on breast tumor microenvironment demonstrate that Tregs, macrophages, and other immune cells have critical roles in the immune evasion abilities of the tumor in the breast (49). The abundance and function of these cells change over the course of the menstrual cycle, potentially opening a window of breast cancer risk at specific stages of the cycle.

Fluctuations of estrogen and progesterone during the ovarian cycle influence the abundance, phenotype, and function of local macrophages in the mammary gland. Macrophages promote the proliferation of epithelial cells and formation of alveolar buds when circulating estrogen and progesterone concentrations are high and promote alveolar bud regression and tissue remodeling as circulating progesterone concentration declines (16). These processes of development and regression are associated with altered macrophage phenotype, which may affect the immune microenvironment in the mammary gland (17). The impact of hormone-regulated macrophages on breast cancer risk is not known, but may affect protection against persistence of DNA-mutated cells and tolerance to transformed cells, particularly during mammary gland regression, discussed in the next section.

Abundance of Tregs in the human blood correlates with serum concentration of estrogen; it increases during the follicular phase and decreases during the luteal phase (50). Prieto and Rosenstein (51) reported that exogenous estradiol promotes proliferation of T cell receptor-activated Tregs isolated from the blood of healthy individuals and enhances their suppressive function *in vitro*. On the other hand, progesterone regulates differentiation of naïve T cells into immune suppressive Foxp3+ Tregs in fetal cord blood and promotes immune tolerance (52). It is noteworthy that Tregs must be activated before ovarian hormones can enhance their suppressive functions (50, 51). Stimuli that activate Tregs during the menstrual cycle are not known; however, infections or altered cell signaling pathways may play a role.

Both mammary epithelial cells and immune cells secrete cytokines and chemokines, which act as intercellular mediators in the generation of immune responses. Induction of mammary epithelial differentiation is accompanied by a switch from production of Th1 cytokines (such as TNFA, IFNG, and IL12) to Th2 cytokines (such as IL13, IL10, and IL4) by mammary epithelial cells (53). Interestingly, progesterone has been shown to regulate Th1/Th2 phenotypes in the mammary gland (54) and is a potent inducer of Th2 cytokines during pregnancy (55). Th1 cytokines are more effective in producing antitumor immunity and tumor rejection, whereas Th2 cytokines are mostly produced by tumors; they induce alternatively activated macrophages and are involved in increasing humoral protumorigenic responses (56, 57).

On the other hand, estradiol is shown to induce proinflammatory cytokine profile during the estrus phase in mice, an effect that was strongly mitigated by progesterone during other phases of the cycle (58). This inflammatory milieu may lead to tumor development and cancer progression. Thus, it seems that fluctuations of estrogen and progesterone can direct the cytokine profile of immune cells in the mammary gland. Considering the immunosuppressive roles of the immune cells in the tumor microenvironment, it is possible that these cells may weaken the mammary gland's capability for immune detection at certain stages of the menstrual cycle and potentially affect risk of tumorigenesis in the breast.

INCREASED CANCER SUSCEPTIBILITY DURING MAMMARY GLAND REGRESSION

The ductal and alveolar epithelial structures that form in the breast during diestrus in anticipation of pregnancy become unnecessary when the cycle progresses. These cells must be removed as the breast is remodeled during proestrus to a more basic architecture. The onset of apoptosis in mammary epithelium that occurs at the end of the luteal phase is tied to declining levels of progesterone (59). A number of animal studies have compared the sensitivity of the mammary gland to chemical carcinogens such as 9,10-dimethyl-1,2-benzanthracene (DMBA) or *N*-methyl-*N*-nitrosorea (NMU) between different phases of the ovarian cycle. Although there are some conflicting results in the literature, the majority reported that young rats exposed to chemical carcinogens at proestrus had a higher rate of mammary tumor incidence (28, 60, 61). This was accompanied by increased number of tumors, as well as shorter tumor latency than those rats injected during the metestrus or estrus phase. Proestrus is the phase in which circulating estrogen is high, and circulating progesterone is declining. This suggests that the phase of the cycle associated with mammary gland regression may be more susceptible to the initiating factors that lead to cancer than other phases of the cycle.

Macrophages are central players in the immunologically silent removal of apoptotic cells. Hodson et al. (17) reported differential percentage of murine macrophages that express cell-surface proteins NKG2D, CD204, and MHCII during different stages of the ovarian cycle, regulated by progesterone and estrogen. Macrophages present during epithelial proliferation and alveolar development during diestrus display a greater predominance of an immune surveillance phenotype, characterized by the expression of the NGK2D marker, associated with the recognition of DNA-damaged cells (17). At this stage, they are able to remove epithelial cells that have experienced replication errors. Toll-like receptors, which recognize danger-associated molecular patterns, can also participate in this activity. At proestrus, progesterone levels have declined, and this is associated with decreased NKG2D and an increase in the expression of antigen presentation receptor MHCII and scavenger receptor CD204 on macrophages, which have roles in phagocytosis and antigen presentation of dying epithelial cells (17, 62). Hence, it is suggested that fluctuations

of macrophage phenotypes over the course of the ovarian cycle may regulate their capability to recognize DNA-damaged cells, phagocytose, and present antigen to generate adaptive immune responses, which may subsequently affect tumor incidence in the mammary gland (17).

Previously, it was thought that macrophages only protect the tissues from cancer, by phagocytizing the apoptotic cell debris or presenting tumor-associated antigens to T cells. However, it is now clear that these cells are also involved in breast tumorigenesis, progression, and metastasis, depending on their functional phenotype (63, 64). Macrophages are highly plastic cells that are capable of both anti-tumorigenic and pro-tumorigenic functions (56). Macrophage phenotypes and functions are heterogeneous, complex in human pathologies, and are activated by various stimuli (65). It is possible that hormonal regulations of these immune cells during menstrual cycling direct them toward a pro-tumorigenic state in which they can assist in the growth of potential tumors in the tissue.

Concomitant with the drop in progesterone is the increase in the expression and activation of TGFB1. TGFB1 has pleiotropic effects in the environment of the involuting mammary gland, first by further retarding epithelial cell proliferation and second by inducing epithelial apoptosis and causing a shift to alternative differentiation in the macrophages infiltrating the breast (62, 66). TGFB1 signaling triggers apoptosis through members of the Bcl family, leading to activation of caspase 3 with eventual nuclear condensation and DNA fragmentation (67). This process is accompanied by the release of damage-associated molecular patterns (DAMPs) or "find me" signals for immune phagocytes. Secreted DAMPs include ATP and lysophosphatidylcholine (68). In addition to DAMPs, apoptotic cells express the membrane markers phosphatidylserine (PS) and calreticulin (CRT). PS is normally found in the cell membrane on the cytoplasmic side and extracellular exposure is an early event in apoptosis. Calreticulin is found in the endoplasmic reticulum where it functions in protein folding and calcium retention in the endoplasmic reticulum (69). Apoptosis dysregulates calcium localization, leading to the release of CRT from the endoplasmic reticulum and its eventual exposure on the membrane surface (70). Membrane localization of these two molecules act as a signal of abnormal processes within the host cell and are also important in the eventual clearance of the cell by phagocytes (71, 72).

As apoptosis progresses, membrane blebbing leads to the release of exosomes. The membrane integrity deteriorates and if the cell is not cleared it will eventually become necrotic. In necrosis, the cell membrane ruptures and allows the release of pro-inflammatory cytoplasmic contents, such as Il-1 alpha and HMGB1 (73). Necrosis in breast tissue is not a desirable outcome, especially since this tissue will be exposed to hormone cycling repeatedly with attendant proliferation/apoptosis during the full extent of a woman's reproductive life. Macrophages and other epithelial cells remove apoptotic cells from the breast before they necrose and provoke harmful inflammation. Necrosis attracts a variety of immune cells that interact to produce a vigorous response that increases the possibility of autoimmunity and carcinogenic DNA damage (74).

There is a multiplicity of mechanisms for the removal of apoptotic cells, both in terms of the target and the phagocyte. This allows for an enhanced flexibility in the host immune system and a greater likelihood an apoptotic event will be cleared and not allowed to necrose. And the key component discussed above involves PS only and does not take into account other mechanisms involving lectins, thrombospondin, or ICAM-3 (68). However, the dynamic and changing immune requirements in the breast throughout the menstrual cycle may result in mutated cells persisting from one cycle into the next, increasing the chance of tolerance of pre-cancerous cells and accumulation of further mutations that ultimately result in increased risk of cancer.

OTHER POTENTIAL CANCER PATHWAYS

The RANKL belongs to the tumor necrosis factor superfamily and acts as a paracrine modulator of progesterone action in the adult mouse mammary gland (75). RANKL also plays critical roles in progesterone-induced expansion of MaSCs and is implicated in increased breast cancer risk associated with high exposure to this hormone (76). The mRNA and protein expression of RANKL in mammary epithelium is upregulated during the luteal phase in normal breast tissues from women at standard risk of breast cancer as well as in malignant breast tissue, suggesting a role for RANKL in breast cancer initiation (40, 77). Moreover, Brisken (78) hypothesized, based on mouse model studies that repeated activation of RANKL by progesterone during the luteal phase promotes breast carcinogenesis. In short, RANKL is considered as a potential target in breast cancer treatment and prevention in premenopausal women (79).

Using next-generation whole transcriptome sequencing on 20 samples of normal human breast epithelium, Pardo et al. (40) examined the effects of hormonal fluctuations during menstrual cycle on gene expression. There were significant differences in the expression of 255 genes between the two phases of the menstrual cycle, most of which had higher expression in the luteal phase compared to the follicular phase. Genes elevated during the luteal phase include FOXM1, MYC, BCRA1, and WNT4, and are mainly involved in the cell cycle events, such as DNA replication, DNA damage response, and mitosis. Interestingly, steroid 5 alpha reductase 1 (SRD5A1) gene, which has a role in catalyzing the conversion of progesterone to 5 alpha-pregnenes mitogens *in situ* (80), was highly expressed during the luteal phase. This finding suggests that the fate of progesterone metabolism is affected during the menstrual cycle. Most of the cell cycle genes, which had higher expression in the luteal phase in this study are overexpressed in breast cancer samples (40). Hence, it is likely that the rise of progesterone during the luteal phase drives mitosis, which may increase the likelihood of genome instability and mutations in the breast and subsequently increase the risk of tumorigenesis.

In 1992, Ferguson and his colleagues noted that molecular profile of ECM in the human breast changes during the menstrual cycle *in vivo* (81). Alterations in the molecular profile of ECM would alter cell signaling and deregulate the behavior of stromal cells, which may lead to generation of a tumorigenic

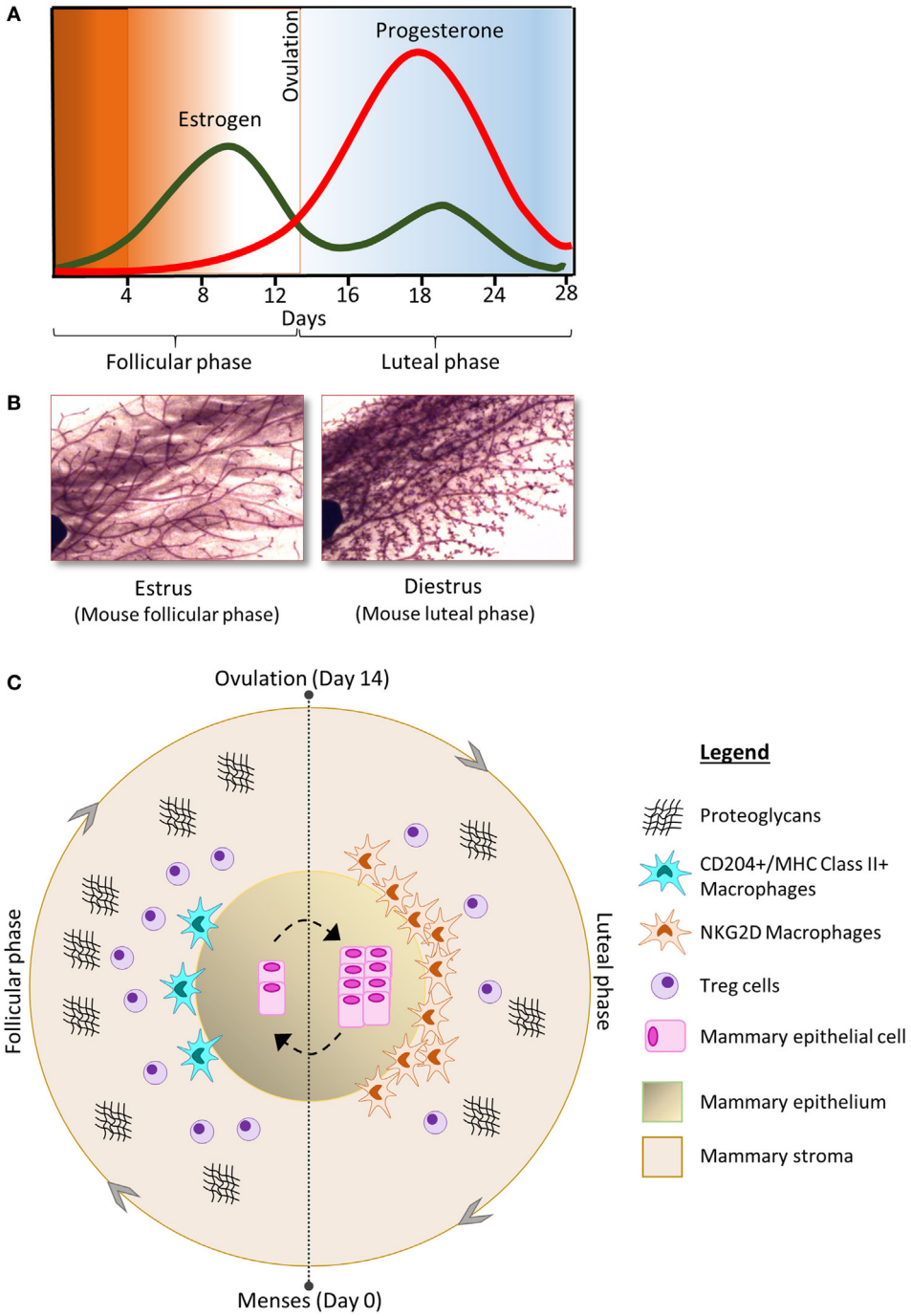

FIGURE 1 | **(A)** The concentration of estrogen (green) and progesterone (red) fluctuates during the phases of the menstrual cycle and is associated with morphological changes in the cellular components of the mammary gland; shown here in carmine alum-stained mouse mammary gland whole-mount preparations **(B)** [adapted from Ref. (17) with permission]. **(C)** The follicular phase of the menstrual cycle is characterized by increase in the number of Tregs and proteoglycans compared to the luteal phase. In contrast, the luteal phase is characterized by increased numbers of mammary epithelial cells as well as increased abundance of macrophages compared to the follicular phase. The phenotype of macrophages in the mammary gland also changes throughout the cycle.

microenvironment (82). More recently, it was observed that gene expression of proteoglycans syndecan-1, syndecan-4, and decorin was reduced during the luteal phase in healthy breast tissues of parous women (83). Single nucleotide polymorphisms in syndeacan-1 are associated with breast cancer susceptibility (84, 85). The expression of both Syndeacan-1 and 4 is significantly correlated with human carcinoma cell proliferation (86). Reduced expression of decorin has also been observed in breast cancer

tissues compared with normal tissues (87). It is suggested that lower expression of decorin weakens the ECM and is correlated with rapid progression, higher recurrence, and poor survival rate in breast cancer patients (87, 88). Proteoglycans regulate the activity of extracellular regulatory proteins in the ECM and interact with growth factors, cytokines, and chemokines (89). The direct effects of exogenous hormones on proteoglycans are not well studied. However, considering the role of proteoglycans in breast carcinomas, it is likely that hormonal regulation of these ECM components during the menstrual cycle affects cell signaling and triggers cancer pathways in the mammary gland microenvironment.

In situ microdialysis on normal human breast tissues revealed that the extracellular levels of vascular endothelial growth factor (VEGF), which is a potent stimulatory factor in angiogenesis, doubled during the luteal phase (90). Angiogenesis and high levels of growth factors are important factors for transformation of normal cells into malignancy (91, 92). Moreover, VEGF mRNA expression increases in breast cancer and is induced with estrogen and progestins in human breast cancer cell lines (93, 94). The higher levels of VEGF during the luteal phase suggest that there is a proangiogenic microenvironment at this time. This reflects the normal capacity of hormone-induced mammary gland to stimulate vascular growth. However, as breast cancer is an angiogenic-dependent disease (94), this proangiogenic profile might provide the essential fuel (i.e., blood supply) for the growth of potential tumor cells.

NEW DIRECTIONS FOR BREAST CANCER DIAGNOSIS AND PREVENTION IN PREMENOPAUSAL WOMEN

Understanding the biological changes that occur over the course of the menstrual cycle could lead to the development of new approaches to prevent breast cancer in premenopausal women. Studies in rodent species, discussed above, suggest that susceptibility to initiating factors that lead to cancer might be elevated during specific stages of the cycle. Alcohol consumption increases breast cancer risk, potentially through increasing circulating estrogen and enhancing estrogen responsiveness, as well as increasing production of reactive oxygen species leading to DNA damage (95, 96). Another risk factor for breast cancer is exposure to radiation. Medical imaging techniques that employ low-dose ionizing radiation, such as computed tomography, x-rays, molecular breast imaging, and mammography, can affect cancer risk (97, 98). As the susceptibility of the breast to these carcinogenic exposures might be altered by menstrual cycle stage in premenopausal women, avoidance of exposure to alcohol and ionizing radiation at specific stages of the cycle has the potential to reduce breast cancer risk.

A better understanding of how immune cell abundance and function fluctuates across the menstrual cycle may provide us with improved potential to harness the immune system to treat and prevent breast cancer. Immunotherapy and immunoprevention of cancer can involve immunization with a vaccine, passive transfer of tumor-specific antibodies, or adoptive transfer of immune cells that kill tumor cells. Changes in the types of macrophages in the breast, the cytokine microenvironment, and the phenotype of tumor-infiltrating lymphocytes across the cycle could affect the efficacy of both adaptive and humoral immune responses that recognize and eliminate tumorigenic cells (99, 100). Further studies on the effect of hormonal fluctuations on immune function could help us address such questions as how to improve immune surveillance and break the immunological tolerance induced during specific stages of the menstrual cycle.

In addition to potential for breast cancer prevention and treatment, research on the effect of menstrual cycle stage on gene expression in the breast is critical in improving the utility of PCR-based diagnostic and prognostic tests for breast cancer. The relative expression of panels of genes, employed in tests such as Oncotype DX and Prosigna, classifies tumor subtype and predicts risk of disease recurrence, in order to guide treatment decision-making (101, 102). However, such tests were developed and validated largely in postmenopausal women, and the utility of these tests in premenopausal women might be affected by fluctuating estrogen and progesterone at different stages of the menstrual cycle (103). Overall, a clearer understanding of the molecular, cellular, and immunological changes that occur in the breast during the menstrual cycle could be fundamental for improving personalized and preventive programs in breast cancer.

CONCLUSION

Women who undergo early menarche and/or late menopause experience higher exposure to estrogen and progesterone, and a higher number of cyclical fluctuations of these hormone during their life time. Together with the direct effects of estrogen and progesterone on cancer initiation, there are a series of coordinated events during the menstrual cycle which are regulated by these ovarian hormones which have been implicated in increased breast cancer risk. **Figure 1** represents a summary of the changes in the cellular components of the mammary gland during menstrual cycling. The circle of proliferation and regression that occurs every month with each menstrual cycle affects the fate of MaSCs, which might increase the chance of random genetic errors and tumor initiation. An imbalance between mammary epithelial cell proliferation and apoptosis may provide the ideal conditions for the growth of potential tumor cells. In addition, deregulation of stromal components, such as ECM, macrophages, and Tregs, may alter the gene signaling pathways and tumor suppressor genes in the human breast, which might become persistent in some women. In addition, hormone-regulated immune cells can influence a microenvironment (e.g., by secreting cytokines and chemokines) in which immune surveillance is dampened and the breast is at increased risk of oncogenic initiation. Over time, the recurrent rise and fall in circulating estrogen and progesterone would provide the conditions for altering cell fate, increasing the risk of genome instability, and random mutations. It is a significant challenge to dissect mechanisms of menstrual cycle-associated breast cancer risk; however, the benefit will be that of a greater

understanding of breast cancer susceptibility in women and the potential for discovery of new cancer biomarkers, indicators of prognosis, and therapeutic strategies to treat and prevent breast cancer.

AUTHOR CONTRIBUTIONS

All the authors contributed intellectually to the content and writing of the manuscript.

FUNDING

The authors would like to thank the Hospital Research Foundation and the National Breast Cancer Foundation for funding our research. WI is a THRF Associate Professor and National Breast Cancer Foundation Early Career Fellow. PD is supported by an Australian Breast Cancer Research Postdoctoral Fellowship. VA is supported by University of Adelaide Graduate Research Scholarship. JW is supported by Australian Postgraduate Award.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX B:

Table I: PCR primers used to quantify cytokine mRNA expression in human breast organoids, human breast epithelial cell lines, and mouse mammary glands.

Gene	Primer Sequence
FoxP3 (Human)	5'GGCACTCCTCCAGGACAG 3'GCTGATCATGGCTGGGCTCT
FoxP3 (Mouse)	5'TACTTCAAGTTCCACAACATGCGACC 3'CGCACAAAGCACTTGTGCAGACTCAG
TGFB1 (Human)	5'AAATTGAGGGCTTTCGCCTTA 3'TGAACCCGTTGATGTCCACTT
TGFB1 (Mouse)	5'GAGAAGAACTGCTGTGTGCG 3'GTGTCCAGGCTCCAAATATAGG
ELF5 (Human)	5'ATG AAAAGTTGAGCAGAGCCCTGAG 3'TGCCACCCGTGTGCATTT
ELF5 (Mouse)	5'GTGGCATCAAGAGTCAAGACTGTC 3'CTCAGCTTCTCGTACGTCATCCTG
MPRl19 (Human)	5'TGCCAGTGGAAAAATCAGCCA 3'CAAAGCAAATCTCGACACCTTG
RPL13a (Mouse)	5'GAGGTCGGGTGGAAGTACCA 5'TGCATCTTGGCCTTTTCCTT
FKBP51 (Human)	5'GGGTCACTAATGAAAAAGGAACAGA 3'CTCTTCCCTCCTTGGCGTG
FKBP51 (Mouse)	5'GTACAACAAAGCCGTGGAGTG 3'GCCCTGTTCTGAGGATTGACT
TNFA (Human)	5'GCCCGACTATCTCGACTTTGC 3'GGAGGCGTTTGGGAAGGTT
TNFA (Mouse)	5'GGCAGGTTCTGTCCCTTTCAC 3'TTCTGTGCTCATGGTGTCTTTTCT
STAT5 (Human)	5'TTACTGAAGATCAAGCTGGGG 3'TCATTGTACAGAATGTGCCGG
STAT5 (Mouse)	5'CGCTGGACTCCATGCTTCTC 3'GACGTGGGCTCCTTACACTGA
STAT3 (Human)	5'GAGGACTGAGCATCGAGCA 3'CATGTGATCTGACACCCTGAA
STAT3 (Mouse)	5'GCACCTTGGATTGAGAGTCA 3'CCCAAGAGATTATGAAACACCA
S100A8 (Human)	5'ATCAGGAAAAAGGGTGCAGACGTC 3'TTTTTGTGGGCTGCCACG
S100A8 (Mouse)	5'AGTGTCCTCAGTTTGTGCAG 3'ACTCCTTGTGGCTGTCTTTG
S100A9 (Human)	5'GTGCGAAAAGATCTGCAAAATTT 3'GGTCCTCCATGATGTGTTCTATGA
S100A9 (Mouse)	5'GTTGATCTTTGCCTGTCATGAG 3'AGCCATTCCCTTTAGACTTGG
ZEB1 (Human)	5' TTCCGAGCGGCCAAGTC 3' TGGGAGATACCAAACCAACTG
ZEB1 (Mouse)	5'GCACCTGAAGAGGACCAGAG 3'GCCATCTGGTGTTCCATTTT
IL12 (Human)	5'CCTTGCACTTCTGAAGAGATTGA 3'ACAGGGCCATCATAAAAGAGGT

IL12 (Mouse)	5’AAGCTCTGCATCCTGCTTCAC 3’GATAGCCCATCACCCTGTTGA
CXCL12 (Human)	5’ACCAGTCAGCCTGAGCTACC 3’GCTCTGGCGATGTGGCTCTC
CXCL12 (Mouse)	5’CAGAGCCAACGTCAAGCA 3’AGGTACTCTTGGATCCAC
HER2 (Human)	5’ CTGAACTGGTGTATGCAGATTGC 3’ TTCCGAGCGGCCAAGTC
EGFP (Human)	5’AAGCTGACCCTGAAGTTCATCTGC 3’CTTGTAGTTGCCGTCGTCCTTGAA
PGR (Human)	5’TGCCTTACCATGTGGCAGATCCC 3’ACTGGGTTTGACTTCGTAGCCCT
PGR (Mouse)	5’CTCCGGGACCGAACAGAGT 3’ACAACAACCCTTTGGTAGCAG

APPENDIX C:

Table II: Primary antibodies used in Western Blots (WB) and Immunohistochemistry (IHC).

Antigen	Isotype	Conjugate	Species Reactivity	Application	Manufacturer	Catalogue number
ELF5	Mouse	Unconjugated	Human	WB/IF	SANTA CRUZ BIOTECHNOLOGY	sc-376737
S100A8	Mouse IgG	Unconjugated	Human, Mouse, Rat	IHC	ThermoFisher	MA191321
Beta Actin	Rabbit IgG	Biotin	Human, Mouse	WB/IF	Abcam	ab222760

APPENDIX D:

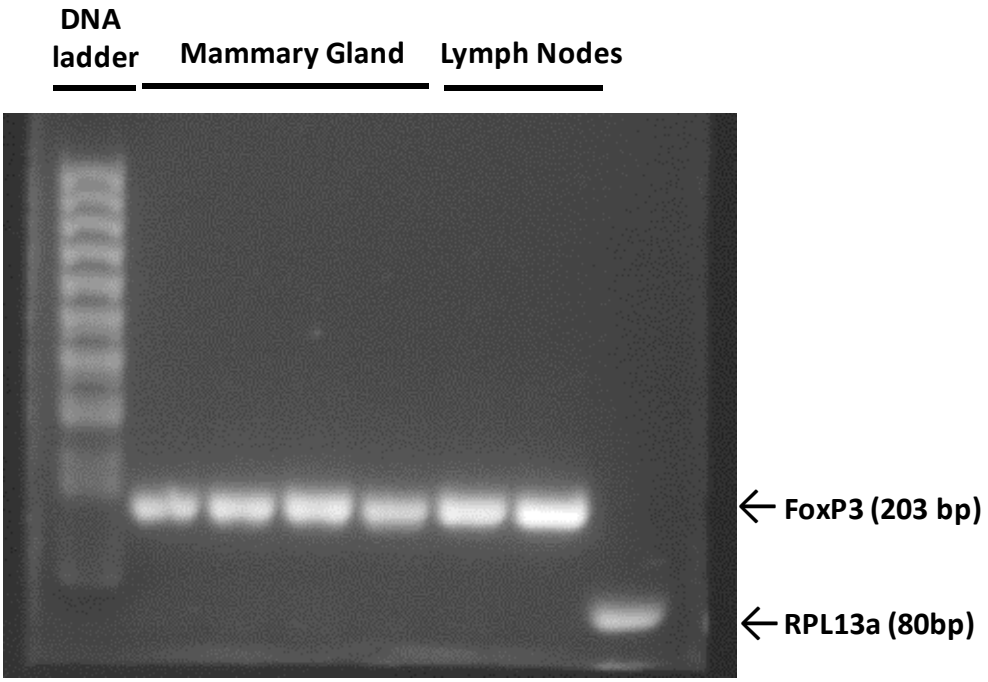

Figure I: Analysis of FoxP3 primer specificity in mice cDNA samples.
5µl of each PCR product was electrophoresed on 1.5% agarose gel in 1X TAE buffer following 40 PCR cycles. Lane 1: DNA ladder size marker (1kb plus), lane 2&3: FoxP3 amplified in WT mouse mammary gland, lanes 4&5: FoxP3 amplified in FoxP3 heterozygous mouse's mammary gland, lane 6: FoxP3 amplified in WT mouse lymph nodes, lane 7: FoxP3 amplified in FoxP3 heterozygous mouse lymph nodes, lane 8: Housekeeper gene RPL13a amplified in WT mouse mammary gland.

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