Cytokine-macrophage regulatory network in mammary gland development and tumourigenesis

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

April 2015

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Abstract

Development and function of the mammary gland involves complex and dynamic interactions between epithelial and stromal cells under the influence of hormones and cytokines. Macrophages are a major component of the mammary gland stroma and they are capable of many roles in mammary gland development; importantly, their functions are tightly regulated by signals within the local cytokine microenvironment. The mammary epithelium secrets a number of cytokines, including transforming growth factor beta 1 (TGFB1) and chemokine ligand 2 (CCL2), that might affect the phenotype and function of adjacent stromal macrophages. Furthermore, alterations in cytokine secretion, and macrophage abundance and phenotype have been observed throughout different stages of normal mammary gland development and in tumourigenesis. A number of studies have demonstrated the significance of TGFB1 and CCL2 in regulating macrophages in many other tissues; however, the importance of the function of this cytokine-macrophage regulatory network in mammary gland development and tumourigenesis is yet to be investigated. The studies described in this thesis aimed to investigate the significance of epithelial cell-derived TGFB1 and CCL2 in regulation of macrophages in mammary gland development and mammary cancer susceptibility in the mouse and human mammary gland.

Utilising a mouse mammary gland transplant model whereby the mammary gland tissue from *Tgfb1* null mutant and wild-type mice were transplanted into TGFB1 replete recipients, we have demonstrated that deficiency in epithelial cell-derived TGFB1 caused a 50% increase of F4/80-positive macrophages invaded into the mammary epithelium, moreover, the number of iNOS-positive ("M1") and CCR7-positive ("M1") macrophages was increased by 78% and 200% respectively in the absence of epithelial cell-derived TGFB1. Similarly, immunohistochemical analysis of human non-neoplastic breast tissue revealed that there was a significant inverse relationship between the abundance of latent TGFB1 protein and the abundance of CD68-positive macrophages. We also observed a significant positive relationship between the abundance of latent TGFB1 and the density of stromal-associated CD206-positive ("M2") macrophages.

Further investigation of the role of TGFB-regulated macrophages in mammary gland development and tumourigenesis was undertaken utilising a transgenic (*Cfms-rtTA* x *TetO-TgfbrII*) mouse model whereby a dominant negative TGFB receptor is activated in macrophages in the presence of doxycycline, which in turn attenuates TGFB signalling in macrophages in these mice. Whole mount and H&E analysis revealed that impaired TGFB signalling in macrophages caused a 15% and 7% increase in the number

of ductal branch points and the percentage of alveolar epithelium respectively in the mammary gland at diestrus. Immunohistochemical analysis using macrophage markers indicated that impaired TGFB signalling in macrophages resulted in a similar alteration in macrophage phenotypes observed in TGFB replete mice transplanted with *Tgfb1-/-* epithelium. There was a 50% increase in abundance of macrophages invaded into the mammary epithelium, and the number of iNOS-positive ("M1") macrophages and CCR7-positive ("M1") stromal macrophages was increased by 110% and 37% respectively. The effect of impaired TGFB signalling in macrophages on mammary gland cancer susceptibility in mice was investigated by challenging the mice with DMBA carcinogen; a significant decrease in mammary tumour incidence and prolonged tumour free survival was observed in mice with impaired TGFB signalling in macrophages compared to controls.

The role of epithelial cell-derived CCL2 in regulation of macrophages in mammary gland development and cancer susceptibility was explored in a transgenic mouse model, *Mmtv-Ccl2,* whereby CCL2 is constitutively expressed by the mammary epithelium under the control of the MMTV promoter. Whole mount and H&E analysis revealed that the number of ductal branch points and the area comprised by alveolar epithelium were increased by 26% and 22% respectively in the presence of abundant epithelial cell-derived CCL2 at proestrus. Immunohistochemical analysis revealed that CCL2 did not affect the proliferation or apoptosis of mammary epithelial cells; however, there was a 40% and 53% increase in macrophage density and collagen deposition respectively around the ductal epithelium of mammary glands of transgenic mice compared to non-transgenic controls. Moreover, quantitative PCR analysis showed that the expression of *Lox* and *Timp3* was increased by 160% and 170% respectively in the mammary glands with constitutive CCL2 expression. In addition, we investigated the effect of constitutive expression of epithelial cell-derived CCL2 on mammary gland cancer susceptibility by challenging the *Mmtv-Ccl2* mice with DMBA carcinogen. A significant increase in mammary gland tumour incidence and reduced tumour latency was seen in mice with overabundant CCL2 expression compared to controls. Non-neoplastic breast tissue exhibited variable expression of CCL2 in the epithelium, with protein abundance ranging from low, to moderate and high. However, immunohistochemical analysis of human non-neoplastic breast tissue did not show a significant correlation between the expression of CCL2 and the abundance of macrophages. Interestingly, it was demonstrated that a significant negative relationship was found between the expression of CCL2 and the abundance of stromal-associated iNOS-positive cells in our human breast tissue.

Together, these studies suggest that epithelial cell-derived TGFB and CCL2 exert effects on mammary gland development and tumourigenesis through regulation of macrophage functions and phenotypes.

This implies that the finely orchestrated cytokine-macrophage regulatory network may be a contributing factor in mammary gland cancer susceptibility. These studies also reveal the possibility of targeting both TGFB and CCL2 signalling as a novel therapeutic approach to breast cancer prevention and/or treatment. However, more research will first be required on the upstream signalling events and underlying mechanisms that affect epithelial cell-derived TGFB and CCL2 macrophage-mediated mammary cancer risk.

Declaration

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Xuan Sun

April 2015

Acknowledgements

I would like to thank my principle supervisor, Associate Professor Wendy Ingman, for the extensive mentoring she has provided during my time in the Breast Biology and Cancer Unit. I am truly grateful to her for such an opportunity, and appreciate all of the support, encouragement and intellectual expertise that she has provided throughout my studies. I would also like to thank my co-supervisor, Professor Sarah Robertson, for the mentoring, support, and technical expertise that I received. I am indebted to you all for all of my future endeavours.

I would like to thank the members of the Breast Biology and Cancer Unit and the Reproductive Immunology Laboratory, who have provided intellectual support and friendship. In particular I would like to thank Dr Danielle Glynn, Ms Leigh Hodson, Dr John Schjenken and Dr David Sharkey for their technical expertise, despite their busy schedules and for the friendship and support they have provided. I would like to thank previous and present Ph D students for their friendship and assistance throughout my studies: Siti, Bihong, Lorreta, Zahied, Noor, Siew, Hanan and Alison. Thank you all.

I am fortunate to receive scholarships from Adelaide Graduate Research Scholarship, Florey Foundation, Breast Biology and Cancer Unit and Robinson Institute for supporting my Ph D journey and opportunity to present my research in conferences. Thank you also to the staff of Discipline of Obstetrics and Gynaecology for excellent resources throughout my studies.

Thank you to my friends Sheechee, Olivia, Cherlynn, Cingel, Witney and Tony for your friendship and support.

Last, but not least, a big thankyou to Mum and Dad for everything that they have done for me and made me who I am today. There's no way that I can thank you enough for your endless love, support and encouragement.

Publications arising from this thesis

1. **Sun X**, Ingman WV. *Cytokine Networks That Mediate Epithelial Cell-Macrophage Crosstalk in the Mammary Gland: Implications for Development and Cancer*. J Mammary Gland Biol Neoplasia. 2014; 19:191–201.

2. **Sun X**, Robertson SA, Ingman WV. *Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland*. Cytokine. 2013; 61(2):377–88.

3. **Sun X**, Robertson SA, Ingman WV. (In preparation) *TGFB-regulated macrophages constrain mammary gland development and promote tumourigenesis.*

4. **Sun X**, Robertson SA, Ingman WV. (In preparation) *The role of epithelial cell-derived CCL2 in regulation of macrophages in mammary gland development and tumourigenesis.*

Abstracts arising from this thesis

2014

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"The role of CCL2 in mammary gland development and cancer"*, Research Centre for Reproductive health (RCRH) conference, Adelaide, Australia. Poster Presentation, November 2014.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*The role of CCL2 in mammary gland development and cancer"*, Society of Reproductive Biology (SRB) Annual Scientific Meeting, Melbourne, Australia, Poster Presentation, August 2014.

2013

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"TGFB1 is a key regulator of mammary gland macrophages"*, Research Centre for Reproductive health (RCRH) conference, Adelaide, Australia. Poster Presentation, November 2013.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "Impaired TGFB signalling in macrophages perturbs mammary gland development", Society of Reproductive Biology (SRB) Annual Scientific Meeting, Sydney, Australia, Oral Presentation, August 2013.

2012

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"Epithelial cell-derived TGFB1 regulates macrophages abundance and phenotypes in the mammary gland"*, Gordon Research Conference, Mammary Gland Biology Conference, Pisa, Italy, Poster Presentation, June 2012.

2011

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"Epithelial cell-derived TGFB1 regulates macrophages abundance and phenotypes in the mammary gland"*, Research Centre for Reproductive health (RCRH) conference, Adelaide, Australia, Poster Presentation, November 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"The effect of epithelial cell-derived TGFB1on macrophage abundance and phenotype in the mammary gland"*, The Queen Elizabeth Hospital Research Day conference, Adelaide, Australia, Oral Presentation, October 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*Epithelial cell-derived TGFB1 regulates macrophages abundance and phenotypes in the mammary gland"*, Faculty of Health Science (FHS) Meeting, Adelaide, Australia, Poster Presentation, August 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"Regulation of mammary gland macrophages by epithelial cell-derived TGFB1"*, Australian Society for Medical Research (ASMR) Scientific Meeting, Adelaide, Australia, Oral Presentation, June 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*Epithelial cell-derived TGFB1 regulates macrophages abundance and phenotypes in the mammary gland"*, Pacific Rim Breast and Prostate Cancer Conference, Tweed Coast, Australia, Poster Presentation, May 2011.

2010

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*Location of active TGFB1 in the mammary gland during different stages of development*", Society of Reproductive Biology (SRB) Annual Scientific Meeting, Sydney, Australia, Oral Presentation, August 2010.

Abbreviations

ArgI	Arginase I
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CCL2	Chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CCR7	C-C chemokine receptor 7
CDs	Cluster of differentiation
Col 1	Collagen 1
COX2	Cyclooxygenase 2
CRP	C-reactive protein
CSF1	Clony stimulating factor 1
CSF1R	Clony stimulating factor 1 receptor
DAB	3,3 diaminobenzadine
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DMBA	7,12-Dimethylbenz (a) anthracene
DNA	Deoxyribonucleic acid
Dox	Doxycylcine
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FBXW7	F-box/WD repeat-containing protein 7
HRP	Horseradish peroxidase
IFNG	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kb	Kilo base
LAP	Latency-associated peptide
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
LTBP	Latent TGFB binding protein
LTGFB1	Latent transforming growth factor 1
MD	Mammographic density

MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MMTV	Mouse mammary tumour virus
MMTV-LTR	Mouse mammary tumour virus long terminal repeat
NO	Nitric oxide
PBS	Phosphate buffered saline
PCNA	Proliferating cellular nuclear antigen
PCR	Polymerase chain reaction
PyMT	Polyoma middle T antigen
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
SEM	Standard error of the mean
SOCSI	Suppressor of cytokine signalling 1
TAM	Tumour-associated macrophages
TGFB1	Transforming growth factor beta 1
TGFBRI	Transforming growth factor beta type I receptor
TGFBRII	Transforming growth factor beta type II receptor
TIMPs	Tissue inhibitors of matrix metalloproteinases
TLR	Toll-like receptor
TNFA	Tumour necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
WAP	Whey acid protein

Chapter 1 Literature Review

Cytokine networks that mediate epithelial cell-macrophage crosstalk in the mammary gland: implications for development and cancer

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Key words: Macrophage, cytokines, TGFB, CSF1, CCL2

Received: 4 May 2014; Accepted: 19 May 2014; Published online: 13 June 2014

J Mammary Gland Biol Neoplasia (2014) 19:191–201

Statement of Authorship

Title of Paper	Cytokine networks that mediate epithelial cell-macrophage crosstalk in the mammary gland: implications for development and cancer
Publication Status	● Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Sun X, Ingman WV. *Cytokine Networks That Mediate Epithelial Cell-Macrophage Crosstalk in the Mammary Gland: Implications for Development and Cancer.* J Mammary Gland Biol Neoplasia. 2014; 19:191–201.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Xuan Sun		
Contribution to the Paper	Developed concepts discussed in the manuscript, and wrote the manuscript. Certification that the statement of contribution is accurate.		
Signature		Date	7/7/15

Name of Co-Author	Wendy Ingman		
Contribution to the Paper	Supervised development of concepts discussed in the manuscript, assisted in manuscript evaluation and editing, and acted as corresponding author. Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.		
Signature		Date	6/7/15

1.1 Abstract

Dynamic interactions between the hormone responsive mammary gland epithelium and surrounding stromal macrophage populations are critical for normal development and function of the mammary gland. Macrophages are highly versatile cells capable of diverse roles in mammary gland development and maintenance of homeostasis, and their function is highly dependent on signals within the local cytokine microenvironment. The mammary epithelium secretes a number of cytokines, including colony stimulating factor 1 (CSF1), transforming growth factor beta 1 (TGFB1), and chemokine ligand 2 (CCL2) that affect the abundance, phenotype and function of macrophages. However, aberrations in these interactions have been found to increase the risk of tumour formation, and utilisation of stromal macrophage support by tumours can increase the invasive and metastatic potential of the cancer. Studies utilising genetically modified mouse models have shed light on the significance of epithelial cell-macrophage crosstalk, and the cytokines that mediate this communication, in mammary gland development and tumourigenesis. This article reviews the current status of our understanding of the roles of epithelial cell-derived cytokines in mammary gland development and cancer, with a focus on the crosstalk between epithelial cells and the local macrophage population.

Key words: Macrophage, cytokines, TGFB, CSF1, CCL2

1.2 Introduction

Development and function of the mammary gland is dependent upon dynamic interactions between the hormone responsive mammary gland epithelium and surrounding stroma. As the epithelium undergoes development to form a network of ducts during puberty, differentiation into alveolar buds during pregnancy, and the involution process that follows weaning of the offspring, local stromal cells, including fibroblasts, adipocytes and immune cells provide essential support through complex interactions (Hovey et al., 2002, Hennighausen and Robinson, 2005, Fata et al., 2001). Macrophages are a major component of mammary gland stroma, and have the capacity to direct a diverse range of biological processes, including cell proliferation, differentiation, phagocytosis, and tissue remodelling under the direction of different cytokines and hormones (Fata et al., 2001, Pollard and Hennighausen, 1994, Sun et al., 2013, Gouon-Evans et al., 2002, Qian et al., 2011, Hodson et al., 2013).

In the mammary gland, a number of cytokines are secreted by the epithelium under particular hormonal conditions or during specific developmental stages, where they affect macrophage phenotype and function. Transforming growth factor beta 1 (TGFB1), colony stimulating factor 1 (CSF1) and chemokine ligand 2 (CCL2) are known as key epithelial cell-secreted cytokines that mediate macrophage recruitment, phagocytosis and activation in the mammary gland (Li et al., 2006, Tsunawaki et al., 1988, Nandan and Reiner, 1997, Soria and Ben-Baruch, 2008, Lin et al., 2002). An abundance of other cytokines, including interleukin 4 (IL4), interferon gamma (IFNG) and tumour necrosis factor alpha (TNFA) are also secreted by the epithelium and are likely to affect local macrophage populations. Significantly, aberrations in expression of cytokines that mediate epithelial cell-macrophage crosstalk increases the risk of tumour formation in some cases, and utilisation of stromal cell support by tumours can also increase the invasive and metastatic potential of the cancer. Here, we review the current understanding of the significance of epithelial cell-macrophage crosstalk in mammary gland development and tumourigenesis, and the cytokines that mediate this communication.

1.3 Macrophages in the mammary gland

Macrophages are highly versatile cells derived from bone marrow haematopoietic stem cells, and capable of diverse roles in development, homeostasis and immunity. Macrophages can undergo either classical “M1” activation or alternative “M2” activation (Mantovani et al., 2002). The “M1” phenotype is characterised by the expression of high levels of pro-inflammatory cytokines and production of nitric oxide and reactive oxygen species, which can suppress tumour development, however, continuing

expression of inflammatory mediators can also drive tumour progression (Wu and Zheng, 2012, Cook and Hagemann, 2013). In contrast, alternatively activated “M2” macrophages are considered to be involved in tissue repair and remodelling, phagocytosis of dying cells, suppression of adaptive immunity and promotion of tumour progression (Sica et al., 2006, Mantovani et al., 2002). The classification of “M1” and “M2” macrophages provides a valuable framework in understanding and predicting macrophage functions in response to different stimuli. However the increasingly recognised phenotypic and functional diversity of macrophages in health and disease suggests that macrophage populations are far more diverse and complex (Cook and Hagemann, 2013). Therefore, not all macrophage phenotypes fit into this simplistic model.

At all stages of postnatal and adult development, macrophages are located in very close proximity to mammary gland epithelial cells. During early postnatal development in mice, macrophages are located in the mammary gland around the rudimentary ductal tree that emanates from the nipple at two weeks of age, and macrophages increase in abundance at 3 weeks age when terminal end buds begin to form (Gouon-Evans et al., 2000, Van Nguyen and Pollard, 2002). During puberty, macrophages are found within the stroma that forms along the neck of the terminal end buds (Gouon-Evans et al., 2000, Ingman et al., 2006). In adult life, macrophages are found to be localised around the mammary epithelium over the course of ovarian cycle, with the highest abundance during diestrus (Chua et al., 2010). During pregnancy and lactation, macrophages are closely associated with the alveolar epithelium (Schwertfeger et al., 2006, Pollard and Hennighausen, 1994). During mammary gland involution, macrophages increase in abundance on day 4 following forced weaning, and are located adjacent to involuting epithelial cells (Stein et al., 2004). The close spatial vicinity between macrophages and the mammary gland epithelium suggests that crosstalk between them is likely to occur. Indeed, macrophages are part of the epithelial stem cell niche in the mammary gland, and macrophage-derived factors are believed to be important for normal stem cell activity (Gyorki et al., 2009). A number of mouse studies have shown that macrophages are essential for mammary gland development during puberty, ovarian cycling, pregnancy, lactation and involution (Table1.1) (Gouon-Evans et al., 2000, Ingman et al., 2006, Pollard and Hennighausen, 1994, Van Nguyen and Pollard, 2002, O'Brien et al., 2012, Chua et al., 2010). Although the cellular and molecular mechanisms of how macrophages interact with epithelial cells to regulate mammary morphogenesis are unclear, macrophages are likely to secrete a variety of cytokines and growth factors that assist hormone-mediated epithelial cell development. For example, the lactogenic effect of prolactin is inhibited by epithelial cell expression of suppressor of cytokine signalling 1 (SOCS1) (Lindeman et al., 2001). This intracellular protein attenuates signalling of not only prolactin, but a number of cytokines including interleukin 6, tumour necrosis factor alpha and

leukaemia inhibitory factor, all of which can be expressed by macrophages under certain conditions, and may interact with epithelial cells to modulate appropriate lobuloalveolar development (Motta et al., 2004, Clarkson et al., 2006) In addition to supporting mammary gland development, macrophages also play a crucial role in cancer progression. Macrophages participate in anti-tumour immune system responses; however, the capacity of macrophages to promote tumorigenesis has also been widely recognised in recent years. Macrophages suppress cancer development as part of innate and adaptive anti-tumour immune responses, through recognition of DNA damaged cells, phagocytosis and antigen presentation to tumour-specific T cells (Gordon, 2007, Gordon and Taylor, 2005, Hume et al., 2002) . On the other hand, macrophages can promote the proliferation and survival of cancer cells, facilitate tumour invasion, increase angiogenesis, and up-regulate the production of pro-tumourigenic factors within the tumour microenvironment, and thus promote tumour progression (Mantovani et al., 2002, Qian and Pollard, 2010). Solid tumours, including breast, lung, ovarian and pancreatic cancers, frequently contain a high abundance of infiltrating macrophages (Martinez et al., 2009, Qian and Pollard, 2010, Wu and Zheng, 2012, Guo et al., 2012), and the presence of tumour-associated macrophages has been found to correlate with poor prognosis in breast cancer (Mahmoud et al., 2012). It is evident that the multiplicity of macrophage populations and phenotypes allows their direction of distinct biological processes, with the balance of pro-tumourigenic or anti-tumourigenic activities likely to be dependent on cytokines derived from the local tissue microenvironment (Wu and Zheng, 2012, Sun et al., 2013).

Table 1.1 Macrophages have diverse and essential roles in mammary gland development

	Role of macrophages in mammary gland development and cancer	*References*
Puberty	Ductal extension, terminal end bud morphology and Collagen fibrillogenesis	Cecchini et al., 1994., Gouon-Evans et al., 2000., Nguyen et al., 2002., Ingman et al., 2006
Ovarian cycle	Epithelial cell proliferation, alveolar budding, clearance of dying cells, tissue remodelling	Chua et al., 2010
Pregnancy	Secondary branching and alveoli development	Pollard and Hennighausen, 1994
Lactation	Transition of gland to lactational state	Pollard and Hennighausen, 1994
Involution	Promote epithelial cell apoptosis and adipocyte repopulation	O'Brien et al., 2012

1.4 CSF1

CSF1 has long been recognised as a critical cytokine that regulates recruitment, survival, proliferation and differentiation of macrophages (Gordon and Taylor, 2005, Wiktor-Jedrzejczak et al., 1990). CSF1 signals via the binding to its cell surface receptor, CSF1 receptor (CSF1R), which is encoded by the *c-fms proto-oncogene* (Hamilton, 1997, Rohrschneider et al., 1997). CSF1 and its receptor are expressed by many different cell types, such as endothelial cells, tissue macrophages, epithelial cells, osteoblasts and trophoblasts (Ryan et al., 2001, Cecchini et al., 1994, Pollard et al., 1987, Byrne et al., 1981). Mammary epithelium has been identified as a major source of CSF1 production affecting local macrophage populations during mammary gland development. In human breast, high abundance of CSF1 mRNA transcripts and protein was detected in normal and non-lactating breast epithelium, with an increased abundance of CSF1 observed in lactating breast (Kacinski et al., 1991, Sapi, 2004, Sapi et al., 1998). Furthermore, transgenic mice expressing a reporter gene under the control of the CSF1 promoter showed strong expression of the reporter in normal and pregnant mammary epithelium which suggests CSF1 is primarily synthesised by mammary epithelium (Ryan et al., 2001). CSF1R is predominantly expressed by macrophages in the mouse mammary gland, indicating that macrophages are the major cell type that responds to CSF1 in this tissue (Gouon-Evans et al., 2000, Gouon-Evans et al., 2002).

1.4.1 CSF1 in mammary gland development

The *Csf1* null mutant mouse, which is almost completely devoid of macrophages, has been used extensively to investigate the role of CSF1-regulated macrophages in mammary gland development. During puberty, *Csf1* null mutant mice exhibit an atrophic and poorly branched ductal structure, with fewer terminal end buds, a lower rate of ductal elongation, and reduced branching morphogenesis compared to CSF1 replete mice (Gouon-Evans et al., 2000, Cecchini et al., 1994). A study utilising multi-photon imaging further analysed mammary glands of pubertal *Csf1* null mutant mice, and demonstrated that CSF1-regulated macrophages promote collagen fibrillogenesis around the terminal end bud, and development of the characteristic club shape of the terminal end bud, likely to be important for normal ductal extension (Ingman et al., 2006). CSF1 regulated macrophages are also required for regulation of mammary gland development during pregnancy and lactation (Pollard and Hennighausen, 1994). Pregnant *Csf1* null mutant mice exhibit reduced branching morphogenesis, with premature alveolar development and milk protein expression (Pollard and Hennighausen, 1994). After parturition, lactation failure is observed in *Csf1* null mutant mice. Milk proteins continue to accumulate in the mammary gland, but the switch to a lactational state does not occur, and the milk is not secreted (Pollard and Hennighausen, 1994).

These descriptive and functional studies have shown that CSF1-regulated macrophages contribute to a number of stages of mammary gland development through regulating epithelial duct morphogenesis and alveolar development. The significance of epithelial cells as a source of CSF1 that directs macrophage activity was demonstrated by rescue of perturbed pubertal mammary gland development in *Csf1* null mutant mice with a mammary epithelial cell-specific transgene driving expression of CSF1 (Van Nguyen and Pollard, 2002, Ingman et al., 2006). This demonstrates that epithelial cell-derived CSF1 can recruit macrophages in the absence of systemic CSF1, to provide critical support for epithelial duct morphogenesis. Thus, a positive paracrine loop is established, where epithelial cells direct macrophage populations to provide functional support for further epithelial cell development.

1.4.2 CSF1 in breast cancer

A number of studies have revealed that abnormal expression of CSF1 and CSF1R is frequently observed in human breast carcinomas, and patients with advanced breast cancer have a 10-fold increase in serum levels of CSF1 compared to normal individuals (Kacinski et al., 1991, Sapi, 2004, Lin et al., 2001, Beuvon et al., 1993). Moreover, a high level of expression of mRNA encoding CSF1 in breast cancer has also been found to correlate with infiltration of tumour-associated macrophages and poor prognosis (Beuvon et al., 1993, Aharinejad et al., 2013). These studies suggest that CSF1 not only regulates macrophage function in mammary gland development, but also plays a significant role in breast cancer. Although the underlying mechanism of how tumour-derived or epithelial cell-derived CSF1 regulates macrophages in breast carcinogenesis is still unclear, several mouse studies have shed light on the significance of CSF1-regulated macrophages in mammary gland tumourigenesis.

The role of CSF1-regulated macrophages in tumour progression and metastasis was demonstrated through crossing *Csf1* null mutant mice with transgenic mice carrying the polyoma middle T antigen (PyMT) oncogene driven by a mammary gland specific mouse mammary tumour virus (MMTV) promoter (Lin et al., 2002). Deficiency in CSF1 resulted in reduced abundance of tumour-infiltrating macrophages. Although growth of the primary tumour was not affected by CSF1 deficiency, the absence of CSF1 resulted in a remarkable reduction in tumour progression and lung metastasis. Furthermore, re-introduction of CSF1 to the mammary epithelium of *Csf1* null mice caused increased infiltration of tumour-associated macrophages and accelerated tumour progression (Lin et al., 2001). Another study used immune-deficient mice with human mammary tumour cell xenografts, demonstrating that administration of small interfering RNAs and antibodies directed against CSF1 could suppress

mammary tumour growth and macrophage infiltration (Aharinejad et al., 2004). Further studies in *Csf1* null mice have demonstrated that crosstalk between tumourigenic epithelial cells and CSF1-regulated macrophages results in a feedback loop that promotes tumour cell motility and metastasis (Wyckoff et al., 2004).

1.5 TGFB1

TGFB1 is one of three mammalian isoforms of TGFB, and was first identified as a factor that promotes the transformation of cultured fibroblasts (Tucker et al., 1984). It is a multifunctional cytokine that controls diverse biological processes, including cell proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis and immune system responses (Lyons et al., 1990, Ingman and Robertson, 2009, Jakowlew, 2006). TGFB receptors are expressed by most cell types in the mammary gland, and thus TGFB1 is able to act on many different cell types, such as epithelial cells, fibroblasts and macrophages (Lyons et al., 1990, Jakowlew, 2006). TGFB1 binds with high affinity to transforming growth factor beta type II receptor (TGFBRII), which transactivates the TGFB type I receptor (TGFBRI). The receptor-ligand binding triggers TGFB signal transduction causing phosphorylation of SMAD protein 2 and 3. Phosphorylated SMAD2 and SMAD3 form a complex with SMAD4, and the SMAD protein complex translocates to the nucleus and activates target gene expression (Nakao et al., 1997, Brown et al., 1999). However, the cellular responses upon TGFB1 signal trasnduction are highly dependent on the cell type that TGFB1 acts upon, the activation state of the cell, and other stimuli present within the cell microenvironment (Jakowlew, 2006, Fleisch et al., 2006, Chong et al., 1999).

TGFB1 is widely secreted by many different cell types as an inactive latent complex (LTGFB1), which consists of the active mature TGFB1 dimer non-covalently bound to a latency-associated peptide (LAP). This large latent complex is also associated with latent TGFB binding protein (LTBP) (Barcellos-Hoff, 1996, Lawrence et al., 1985, Fleisch et al., 2006). A number of studies have shown that a range of extracellular stimuli including proteolysis, heat, acid or alkaline treatment, and irradiation can activate TGFB1 (Lyons et al., 1990, Nunes et al., 1997, Lawrence et al., 1985, Ehrhart et al., 1997, Barcellos-Hoff et al., 1994). Activation of latent TGFB1 is necessary before TGFB1 can exert biological effects (Barcellos-Hoff, 1996, Nunes et al., 1997). TGFB1 associated with its latent complex has a half-life of 90 minutes (Wakefield et al., 1990), whereas active TGFB1 has a very short half-life of approximately 2 minutes. As a result, the bioavailability of TGFB1 to a target cell is highly dependent on the sites and circumstances of its activation (Ewan et al., 2002, Wakefield et al., 1990, Fleisch et al., 2006).

1.5.1 TGFB1 in mammary gland development

The gene encoding TGFB1 is highly expressed by mammary epithelial cells in pubertal and virgin adult mice, lowly expressed during pregnancy-induced alveolar morphogenesis, and is not detected in the lactating gland (Robinson et al., 1991). The expression and activation pattern of TGFB1 in the non-pregnant adult mouse mammary gland also fluctuates over the course of the ovarian cycle (Ewan et al., 2002). Immunofluorescence utilising antibodies specific to latent and active TGFB1 demonstrated that both forms were observed in greatest abundance during the diestrus phase of the cycle, with a linear positive relationship between their expression. During the estrus phase of the cycle however, two distinct populations of epithelial cells were evident, one population was characterised by low abundance of latent TGFB1 and the other population characterised by high abundance of active TGFB1, suggesting differential activation of latent TGFB between the two cycle stages. TGFBR1 is highly expressed by mammary epithelial cells and surrounding stroma cells in adult, pregnant and involuting mammary gland, indicating that both epithelial and stroma cells can respond to TGFB1 (Pollard, 2001, Joseph et al., 1999).

The role of TGFB1 in mammary gland development was first demonstrated by a series of experiments whereby pellets containing TGFB1 were implanted into the pubertal mammary gland. TGFB1 caused cessation of DNA synthesis in the highly proliferative terminal end buds resulting in complete inhibition of ductal growth (Daniel et al., 1989, Robinson et al., 1991, Silberstein et al., 1992). The use of transgenic and null mutant mouse models further explored the role of TGFB1 in mammary gland development. Transgenic mice that constitutively express active TGFB1 in the mammary epithelium under the control of the MMTV promoter caused remarkable retardation of ductal development as well as a significant reduction in mammary epithelial cell proliferation rate. However, alveolar development was not inhibited during pregnancy in these mice and all the transgenic females could feed full litters (Pierce et al., 1993). However, a different transgenic model, whereby the whey acidic protein (WAP) promoter was used to drive overexpression of active TGFB1 in the mammary epithelium during pregnancy and lactation, demonstrated that alveolar development was inhibited during pregnancy (Jhappan et al., 1993). Comparison of the transgene expression level between the two transgenic strains indicated that expression of mRNA encoding TGFB1 was higher with the WAP promoter than with MMTV (Jhappan et al., 1993, Pierce et al., 1993) which may account for the different findings on the effect of TGFB overexpression on alveolar development during pregnancy. These studies support the notion that the primary role of epithelial cell-derived TGFB1 is to inhibit branching morphogenesis and ductal outgrowth in the mammary gland.

Other transgenic mouse approaches to investigate the role of TGFB in the mammary gland have utilised a dominant-negative TGFB receptor which can inhibit all TGFB isoform signalling in the mammary gland (Pollard, 2001, Gorska et al., 1998, Joseph et al., 1999, Crowley et al., 2005). This approach has enabled investigation of the impact of disabled TGFB signalling in a specific cell type on the overall mammary gland phenotype. The expression of such a dominant-negative receptor in the mammary epithelium under control of the MMTV promoter in virgin female mice resulted in mammary epithelial cell hyperplasia and inappropriate alveolar development (Gorska et al., 1998). This finding suggests that TGFB acts directly on epithelial cells to maintain normal mammary gland development. The dominant negative receptor construct has also been expressed in mouse mammary stroma under control of the metallothionein promoter, with the expression of this construct induced by zinc (Joseph et al., 1999). An increase in lateral branching of the ductal epithelium in the mammary gland was observed, suggesting that TGFB1 also acts on stromal cells to inhibit mammary gland development (Joseph et al., 1999). Further reciprocal transplantation experiments confirmed that increased branching morphogenesis was due to the dominant negative receptor activity in the stroma, and the resulting mammary gland phenotype was mediated through TGFB-regulated stromal cells (Crowley et al., 2005). Taken together, these studies suggest that epithelial cell-derived TGFB1 exerts biological effects on both epithelial and stromal cells, and TGFB responsiveness in both cell compartments is necessary for appropriate mammary gland morphogenesis.

The generation of *Tgfb1* null mutant mice has provided further insights into the role of TGFB1 in the mammary gland. A study by Ewan et al investigated mice heterozygous for the *Tgfb1* null mutation, which have only 10% of wild-type TGFB1 abundance, and reported significantly increased ductal invasion and increased epithelial cell proliferation rate during pubertal mammary gland development (Ewan et al., 2002). This study did not address the effect of complete deficiency of TGFB1 on mammary gland development, as mice homozygous for the *Tgfb1* null mutation die at 3 weeks of age due to multifocal inflammation (Shull et al., 1992, Kulkarni et al., 1993). However, by using mice on a severe combined immunodeficient background, *Tgfb1* null mutant mice can develop to adulthood (Diebold et al., 1995). *Tgfb1* null mutant and heterozygous mice on a background of severe combined immunodeficiency exhibit normal mammary gland development during puberty apart from fewer number of terminal end buds in *Tgfb1* null mice (Ingman and Robertson, 2008). The discrepancy between the mammary gland phenotype of *Tgfb1* heterozygous mice reported by Ewan et al and Ingman and Robertson is likely to be caused by the different abundance of TGFB1 in the mice, with 10% and 50% of wildtype TGFB1 abundance respectively (Ewan et al., 2002, McGrath et al., 2009, Ingman and Robertson, 2008). However, accelerated ductal extension was observed in both studies when

epithelium with very low or absent TGFB1 expression was transplanted into wildtype recipients. Apparently normal mammary gland development in *Tgfb1* null mutant mice together with accelerated ductal development in *Tgfb1* null epithelium transplanted into TGFB1 replete mice suggested two opposing roles of TGFB1 in the mammary gland; autocrine inhibition of epithelial cell proliferation and development, and stromal cell or systemic stimulation of development (Ingman and Robertson, 2008).

1.5.2 TGFB1 affects macrophage phenotype and function

Although the identity of the TGFB1-responsive cell type in the mammary stroma that supports pubertal development is not known, TGFB1 is known as a key cytokine that regulates macrophage function in many other tissues. TGFB1 acts as a chemoattractant for immature monocytes during inflammation, however when monocytes differentiate into macrophages, TGFB1 exerts primarily inhibitory effects (Li et al., 2006). TGFB1 down-regulates the expression of macrophage scavenger receptor during macrophage mediated phagocytosis, down-regulates the production of nitric oxide (NO) and the expression of "M1" marker inducible NO synthase (iNOS) in activated macrophages, and inhibits the production of inflammatory cytokines (Tsunawaki et al., 1988, Bottalico et al., 1991, Sherry et al., 1998). These inhibitory effects of TGFB1 on macrophages are likely to play significant roles in resolving inflammation and preventing disease progression (Li et al., 2006). It has been noted that *Csf1* and *Tgfb1* null mutant mice have strikingly similar reproductive phenotypes, including perturbations in mammary gland development and function, which suggests the possibility of a mechanistic link between TGFB1 and CSF1-regulated macrophages in mammary gland development (Ingman and Robertson, 2009).

Direct regulation of macrophage phenotype by epithelial cell-derived TGFB1 has been demonstrated through transplantation studies of *Tgfb1* null and wild-type epithelium into the cleared mammary gland of wild-type recipients (Sun et al., 2013). Deficiency in epithelial cell-derived TGFB1 caused increased macrophage invasion into the epithelium and increased abundance of "M1" iNOS positive macrophages. TGFB signalling promotes expression of genes associated with "M2" macrophages including *ym1*, *mcr2* and *mgl2* and expression of "M2" phenotypic marker Arginase I (Arg1) on macrophages suggesting that TGFB is involved in "M2" macrophage polarization (Gong et al., 2012). These recent findings suggest that expression of TGFB1 by mammary epithelial cells during specific stages of development alters the balance between macrophage phenotypes. However, the effect of TGFB1 on macrophage function in the mammary gland, and how this influences mammary gland morphogenesis is still far from clear.

1.5.3 TGFB1 and breast cancer

The International Breast Cancer Consortium reported a significant association between breast cancer risk and the TGFB1 L10P gene polymorphism (Cox et al., 2007). The TGFB1 L10P gene is linked to increased cellular expression of TGFB1 and elevated circulating TGFB1 (Krippl et al., 2003), suggesting that increased abundance of TGFB1 might increase breast cancer risk. However, the roles of TGFB1 in breast cancer are complex and conflicting, and this cytokine both suppresses tumour development and promotes tumour invasion and metastasis (Jakowlew, 2006). TGFB1 can suppress tumour development through inhibition of cell proliferation, induction of apoptosis, and suppression of growth factor and cytokine production (Yang et al., 2010a, Dumont and Arteaga, 2000). However, sensitivity to these suppressive effects of TGFB1 is frequently lost through mutations or functional inactivation of TGFB1 signalling pathways. At later stages of cancer progression, tumour cells can express large amounts of TGFB1 which appear to have pro-tumourigenic effects (Pardali and Moustakas, 2007, Moustakas et al., 2002). This overexpression of TGFB1 by tumour cells modulates epithelial-mesenchymal transition, impaired immune surveillance and promotes angiogenesis, therefore promoting tumour invasion and metastasis (Pardali and Moustakas, 2007, Yang et al., 2010a).

The utilisation of genetically modified mouse models has been particularly enlightening for the exploration of the roles of TGFB1 in breast cancer. Overexpression of TGFB1 in the mammary epithelium driven by the MMTV promoter resulted in decreased incidence of mammary tumour formation following challenge with the chemical carcinogen 7,12-Dimethylbenz(a) anthracene (DMBA) (Pierce et al., 1995). Similarly, MMTV-Neu transgenic mice with expression of the activated form of TGFB receptor in the mammary epithelium exhibit reduced tumour formation and longer tumour latency (Siegel et al., 2003). On the other hand, transgenic mice carrying a dominant-negative TGFB receptor driven by the MMTV promoter develop mammary tumours spontaneously, and exhibit increased susceptibility to mammary gland tumour following DMBA administration (Gorska et al., 2003, Bottinger et al., 1997). Furthermore, loss of TGFB signalling via conditional deletion of TGFBRII in the mammary epithelium results in shorter tumour latency (Forrester et al., 2005) and promotion of tumour cell survival, tumour heterogeneity and enhanced invasion and metastasis (Bierie et al., 2008, Yang et al., 2008). These studies provide strong evidence that epithelial cell-derived TGFB1 exerts tumour suppressive effects in mammary gland tumourigenesis, possibly due to its autocrine role in inhibiting epithelial cell proliferation and mammary morphogenesis.

However, when tumours attain resistance to the suppressive effects of TGFB, epithelial cell-derived TGFB1 acts on adjacent stromal cells to promote an immune microenvironment conducive to tumour progression. Loss of TGFB signalling in epithelial cells is accompanied by increased expression of the gene encoding inflammatory COX2, and increased immune cell infiltration including macrophages (Bierie et al., 2008). In MMTV-Neu transgenic mice, although expression of a dominant negative TGFB receptor transgene suppressed mammary tumour formation, the later stages of tumorigenesis including tumour invasion and metastasis was significantly promoted (Siegel et al., 2003). Moreover, overexpression of active TGFB1 in the mammary gland of MMTV-Neu transgenic mice resulted in acceleration of tumour invasion and metastasis (Muraoka et al., 2003). Systemic attenuation of TGFB1 through administration of a soluble TGFB1 antagonist resulted in reduced spontaneous metastasis in both MMTV-Neu transgenic mice and in mice injected intravenously with malignant carcinoma cells (Yang et al., 2002). In MMTV-PyMT transgenic mice, ablation of TGFB signalling in the mammary epithelium caused a significant increase in pulmonary metastasis (Forrester et al., 2005, Bierie et al., 2008). The pro-tumourigenic effects of TGFB1 may be a result of a number of autocrine and paracrine interactions within the tumour microenvironment, particularly in mediating stromal cell tumour support, reducing anti-tumour immunity and promoting invasion and angiogenesis (Dumont and Arteaga, 2000, Tan et al., 2009, Bhowmick et al., 2004).

Clearly, TGFB is an essential component of a complex and finely orchestrated signalling pathway, which underpins normal mammary gland development and affects breast cancer progression. The role of epithelial cell-derived TGFB1 in tumourigenesis is multifaceted; it initially acts as a tumour suppressor and subsequently a tumour promoter through modulation of surrounding stroma including immune cells. Although genetically modified mouse studies have shed light on these interactions, caution should be taken in interpretation of these findings. Gene manipulation in mice generally results in complete ablation or non-physiological overexpression of the target gene, whereas altered TGFB signalling in human breast cancer is far less definitive. The roles of TGFB1 are cell specific, context dependent and can be dramatically altered through regulation of TGFB1 activation, TGFB binding proteins, receptor binding and are dependent on the concentration of TGFB1 protein at the cell surface. No doubt the rich literature on the role of TGFB1 as an essential autocrine and paracrine cytokine in the mammary gland will continue to develop as we further explore these epithelial-stromal interactions in human and mouse models.

1.6 CCL2

CCL2, also known as monocyte chemotactic protein 1, is a small pro-inflammatory cytokine and a highly potent chemoattractant for monocytes and macrophages to sites of tissue injury and inflammation (Yadav et al., 2010, Deshmane et al., 2009). CCL2 is produced by various cell types, including epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, monocytes and adipocytes; however, as CCL2 is an "inflammatory cytokine", it is not normally constitutively expressed. Expression of CCL2 can be induced by oxidative stress and activation of the cell by other cytokines and growth factors (Yadav et al., 2010, Cushing et al., 1990, Standiford et al., 1991). The sole receptor for CCL2 is CCR2 which is primarily expressed by leukocytes, particularly monocytes and macrophages. The binding of CCL2 to its receptor on leukocytes results in cell activation and recruitment (Yadav et al., 2010, Deshmane et al., 2009).

The chemoattractant ability of CCL2 was first identified through a series of *in vitro* studies, where purified CCL2 protein was able to induce chemotaxis of various leukocytes, including monocytes, T lymphocytes and natural killer cells (Matsushima et al., 1989, Yoshimura et al., 1989, Carr et al., 1994, Allavena et al., 1994). *In vivo* studies with genetically modified mouse models have further demonstrated the chemoattractant role of CCL2 in regulating macrophage recruitment. These studies have shown that deficiency in CCL2 or CCR2 causes perturbed recruitment of monocytes and macrophages, whereas overexpression of CCL2 results in increased macrophage infiltration (Fuentes et al., 1995, Lu et al., 1998, Rutledge et al., 1995).

The human breast epithelium expresses low but detectable quantities of CCL2 protein (Soria and Ben-Baruch, 2008, Ueno et al., 2000). Although the role of CCL2 in mammary gland morphogenesis has not been studied, this cytokine may be responsible for macrophage recruitment under particular conditions. CCL2 protein increases on day 2 of involution in rats (O'Brien et al., 2010a), and may act as an early chemoattractant for macrophages that infiltrate the involuting mammary gland. Similarly, an increase in serum CCL2 precedes macrophage recruitment to the mammary gland following lipopolysaccharide-induced inflammation during lactation (Glynn et al., 2014). Increased abundance of CCL2 in this context may form part of the immune signalling fingerprint that promotes partial mammary gland involution.

1.6.1 CCL2 in breast cancer

In breast carcinomas, CCL2 can be highly expressed by both the tumour and surrounding stromal cells (Soria and Ben-Baruch, 2008, Ueno et al., 2000). CCL2 is implicated in early relapse of breast cancer and a number of studies have shown that CCL2 expression in breast carcinomas is highly associated with macrophage infiltration (Fujimoto et al., 2009, Arendt et al., 2013, Ueno et al., 2000, Qian et al., 2011). This is likely to be a causal relationship, as administration of a neutralising antibody to CCL2 to mice xenografted with human breast tumour cells resulted in a significant reduction in the number of infiltrated macrophages and reduced tumour growth (Fujimoto et al., 2009).

Population studies support the notion that increased CCL2 expression increases breast cancer risk and metastasis of established tumours. The risk of developing metastasis is significantly increased in breast cancer patients carrying the CCL2-2518 A/G promoter polymorphism compared to A/A homozygotes (Ghilardi et al., 2005). As the A/G genotype causes increased CCL2 production in monocytes after stimulation compared to the A/A genotype (Rovin et al., 1999), this suggests that increased production of CCL2 plays a significant role in promoting cancer metastasis. On the other hand, the CCR2-64I polymorphism, which results in slightly impaired capacity to transduce inflammatory signals, is underrepresented in breast cancer patients (Zafiropoulos et al., 2004, Soria and Ben-Baruch, 2008), and this might provide protection from tumour formation through reduced recruitment of tumour-promoting macrophages.

Recent mouse studies have implicated tumour epithelial cell-derived CCL2 and CCR2-expressing macrophages as a critical factor in metastasis of breast cancer to the bone and lungs. CCL2 administered intravenously increased metastasis of organ-specific metastatic human breast cancer cells to both sites, conversely, blockade of CCL2 with a neutralising antibody largely suppressed metastasis (Lu and Kang, 2009). Increased metastasis was highly associated with increased infiltration of macrophages to the distant site, and tumour epithelial cell-derived CCL2 required engagement with CCR2 on cells of monocytic origin to promote tumour seeding (Lu and Kang, 2009). Extravasation of tumour cells into the lung involves a distinct population of inflammatory monocytes that require expression of CCR2 for migration (Qian et al., 2011). Depletion of tumour cell-derived CCL2 inhibits migration of these monocytes, and as a consequence inhibits metastasis and increases the lifespan of tumour-bearing mice. Non-epithelial cell sources of CCL2 are also significant in regulating cancer metastasis, as macrophage infiltration to the primary tumour and lung metastasis are reduced in *Ccl2*

null mutant mice transplanted with epithelial carcinoma cells into the mammary gland fat pad (Yoshimura et al., 2013).

However, not all studies support the notion that CCL2 promotes metastasis in breast cancer. Indeed, CCL2 may in certain conditions play an inhibitory role. Overexpression of CCL2 in breast cancer cells that are highly metastatic to the bone resulted in reduced metastasis to the lung and bone and prolonged mouse survival (Takahashi et al., 2009). In an orthotopic breast cancer model utilising metastatic breast cancer cells, CCL2 inhibits metastasis through recruitment of tumour-entrained neutrophils (Granot et al., 2011). In addition, null mutation in the gene encoding either CCL2 or CCR2 results in increased lung metastasis in MMTV-PyMT transgenic mice (Li et al., 2013). The role of CCL2 in breast cancer metastasis is likely to be dependent on the specific tumour subtype and other factors within the microenvironment that regulate immune responses to the tumour. Therefore, although CCL2 exhibits an overall pro-tumourigenic role in cancer development and metastasis, it may also play a role in regulating local immune cell populations which may in some cases provide protection against tumorigenesis.

1.7 Other epithelial cell-derived cytokines

Mammary epithelial cells secrete a variety of other cytokines which are likely to affect local macrophage populations and mammary gland morphogenesis. Notably, KIM2 cells, which are a mouse mammary epithelial cell line, express interleukin 12 (IL12), interferon gamma (IFNG) and tumour necrosis factor alpha (TNFA) under undifferentiated conditions, and switch to expression of interleukin 4 (IL4), interleukin 5 (IL5) and interleukin 13 (IL13) when differentiated into luminal epithelial cells, suggesting that mammary epithelial cells secrete a specific cytokine signature dependent on differentiation status. IFNG appears to have no essential role in alveolar development during pregnancy (Lindeman et al., 2001) IL4 and IL13 are key cytokines that promote the “M2” macrophage phenotype, and increase in abundance in the mammary gland during involution (O'Brien et al., 2010a). Null mutation in the genes encoding both IL4 and IL13 results in delayed lobuloalveolar development during pregnancy (Khaled et al., 2007). However the role of these cytokines in mammary gland involution, and whether macrophages participate in IL4/IL13-mediated morphogenesis, has not been investigated.

1.8 Conclusions

There is substantial evidence from genetically modified mouse models suggesting that mammary epithelial cell-derived CSF1, TGFB1 and CCL2 play significant roles in regulating macrophages during mammary gland development (Figure 1.1). Cytokines expressed by mammary epithelial cells become part of a complex microenvironment that includes other stromal cells and extracellular matrix. Together with hormonal cues, this microenvironment directs macrophage function, and is essential for normal mammary gland development. However, cytokine-mediated epithelial cell-macrophage interactions can also act to both affect the risk of breast cancer, and coerce stromal macrophages to support development of established tumours. Cytokine-based therapies for breast cancer treatment are impaired by the fundamental requirements for these proteins in normal tissue homeostasis. Further studies that dissect the specific cell types and cytokine signalling pathways that regulate mammary gland morphogenesis, breast cancer risk, tumour progression and metastasis are essential to develop improved therapeutic approaches to breast cancer prevention and treatment.

1.9 Acknowledgement

X Sun is supported by a University of Adelaide Graduate Research Scholarship and a Florey Medical Research Foundation Postgraduate Cancer Research Top Up Scholarship, W Ingman is an NBCF/THRF Fellow.

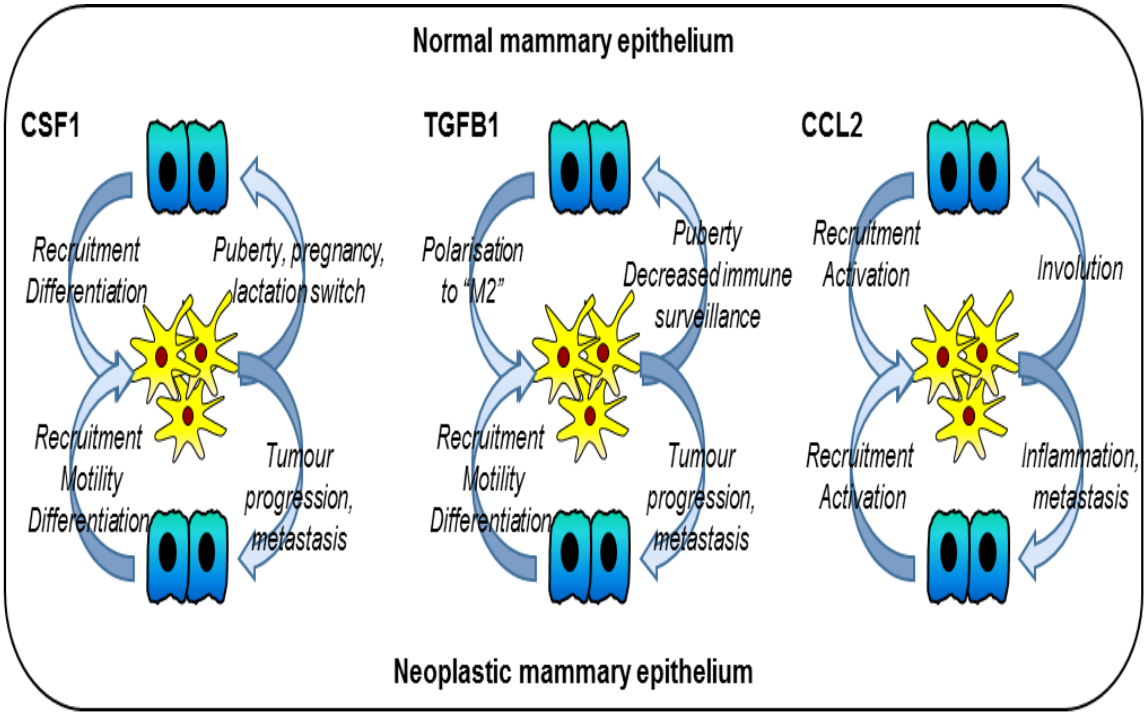

Figure 1.1 Summary of the actions of cytokines known to mediate epithelial cell-macrophage cross-talk in the mammary gland.

Epithelial cell-derived CSF-1, TGFB1 and CCL2 affect macrophage recruitment and activation. Cytokine-regulated macrophages have essential roles in normal mammary gland morphogenesis and affect progression and metastasis of neoplastic breast epithelium.

1.10 Hypotheses and Aims

There is substantial evidence suggesting that the cross-talk between epithelial cell-derived cytokines and macrophages play significant roles in mammary gland development and tumourigenesis. Both epithelial cell-derived TGFB1 and CCL2 have found to be involved extensively in mammary cancer progression, however, the significance and the underlying mechanisms of the cross-talk between macrophages and epithelial cell-derived TGFB1 and CCL2 in the mammary gland is still unclear, and there is little understanding of how cytokine-macrophage networks affects breast cancer susceptibility. The known and proposed roles of key epithelial cell-derived cytokines in regulation of macrophages in mammary gland development and tumourigenesis are summarised in Figure 1.1

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

- Epithelial cell-derived TGFB-macrophage cross-talk promotes mammary gland development and increases mammary tumour susceptibility
- Epithelial cell-derived CCL2 regulates macrophage recruitment and infiltration in the mammary gland, and induces chronic inflammation leading to increased tumour susceptibility

The experiments described in this thesis will address the following aims:

- To investigate the effect of epithelial cell-derived TGFB1 on macrophage phenotype in mice and human mammary gland
- To investigate the role of TGFB-regulated macrophages in mammary gland development and tumorigenesis in mice
- To investigate the role of CCL2 in regulation of macrophages in mammary gland development and tumorigenesis in mice

Chapter 2 Methods and Materials

2.1 Human Non-neoplastic Breast Tissue

Breast tissue was collected from female patients (n=20) undergoing either reduction mammoplasty or mastectomy surgery at The Queen Elizabeth Hospital. Tissue collection was approved by the Human Ethics Committee at the University of Adelaide and The Queen Elizabeth Hospital (TQEH Ethics Approval #2011120). The participants donated healthy breast tissue, a blood sample at the time of surgery and completed a comprehensive medical and personal history survey after surgery. Participants were included if they were a woman aged between 18-75 years of age and capable of giving informed consent. Participants were excluded from the study in the event of pregnancy, current chemotherapy, or high dependence on medical care resulting in inability to give informed consent. Participant's tissue was fixed and paraffin embedded for histological analysis as described in 2.6.1.2.2.

2.2 Animals and Surgeries

2.2.1 Mice

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th ed., 2004). All mice were maintained in specific pathogen-free conditions with controlled light (12hr light, 12 hr dark cycle) and temperature at the Laboratory Animal Services Medical School facility. Food and water were provided ad libitum.

2.2.1.1 *Tgfb1 null mutant, wild-type and Balb/C Prkdc^{scid} mice*

Tgfb1 null mutation was produced by embryonic stem cell targeting by Shull et al. (1992) at the University of Cincinnati College of Medicine, Ohio, USA. In this model, a neomycin resistance gene (*neor*) was inserted into the *Tgfb1* gene at exon 6, and the modified gene was electroporated into D3 embryonic stem cells for selection of neomycin resistance. The embryonic stem cells were electroporated into C57Bl/6 blastocysts and implanted into pseudopregnant females (Shull et al., 1992). The *Tgfb1* null mutant mice (*Tgfb1-/-*) were found to develop a wasting syndrome at around 3 weeks of age characterised by severe inflammation and necrosis of multiple major organs (Shull et al., 1992). *Tgfb1* null mutant mice survive beyond 3 weeks of age when bred onto a lymphocyte-deficient background, afforded by severe combined immunodeficiency (*Prkdc^{scid}* mutation). The experiments described in this thesis used *Tgfb1* null mutant mice on a scid background and on a mixed CF1/129/C3H background. Heterozygous (*Tgfb1+/-*) breeding pairs produced progeny that were

homozygous for a targeted null mutation in the *Tgfb1*gene (*Tgfb1-/-*), heterozygous (*Tgfb1+/-*), or homozygous wild type (*Tgfb1+/+*). All mice in the colony are homozygous for the *Prkdc*^{scid} mutation. At the time of weaning (3 weeks), 5 mm of the tail tip of each mouse was cut for genotyping (protocol described in 2.3.1).

Wild-type Balb/C *Prkdc*^{scid} female mice were obtained from the Waite campus of the University of Adelaide, South Australia as recipients for mammary gland transplant procedure (see section 2.2.3).

2.2.1.2 *Cfms-rtTA, TetO-TgfbrII and TGF-Mac transgenic mice*

The *Cfms-rtTA* transgenic mouse was described by Yan et al. (2006) and imported from Indiana University, USA, Prof Cong Yan's laboratory. In brief, the *Cfms-rtTA* transgenic mouse was generated through pronuclear-microinjection technique where a construct containing a 7.2-kb *cfms* promoter fragment, the *rtTA* cDNA, and the SV40 polyadenylation signaling sequence was microinjected into zygotes of FVB mice, which were then implanted into pseudopregnant foster mothers (Yan et al., 2006). The expression of the reverse tetracycline/doxycycline-responsive transactivator (*rtTA*) is driven by the macrophage specific *Cfms* promoter, so that the *rtTA* expression is restricted to macrophages in the transgenic mouse.

The *TetO-TgfbrII* mice, described by Frugier et al. (2005) were purchased from Jackson Laboratories (Maine, USA). The generation of the *TetO-TgfbrII* transgenic mouse was also achieved by pronuclear-microinjection, in which a construct containing enhanced green fluorescent protein gene (*Egfp*), a bidirectional tetracycline/doxycycline transactivator promoter (*TetO*), and a disrupted *TgfbrII* gene (*ΔTgfbrII*) with a *C-myc* tag was micro-injected into zygotes of FVB mice, which were then implanted into pseudopregnant foster mothers (Frugier et al., 2005). The disrupted *TgfbrII* gene encodes for a dominant negative TGFB receptor which results in disrupted TGFB signaling. The expression of both EGFP and the dominant negative TGFB receptor is under the control of the *TetO* promoter. However, both transgenes are silent in *TetO-TgfbrII* mice since the *TetO* promoter can only drive the expression of transgenes after the activation by a specific tetracycline/doxycycline transactivator.

Cfms-rtTA x *TetO-TgfbrII* (*TGF-Mac*) double transgenic mouse was generated from crossbreeding of *Cfms-rtTA* and *TetO-TgfbrII* single transgenic mice, such that *TGF-Mac* mice carried one allele for each

of the two transgenes. In the *TGF-Mac* double transgenic mouse, the expression of the transgenes can be controlled by addition or removal of doxycycline. In the presence of doxycycline, the rtTA transactivator binds to the *TetO* promoter and subsequently induces the expression of the dominant negative TGFB receptor and EGFP in the *TGF-Mac* double transgenic mouse. The dominant negative TGFB receptor can bind TGFB (all three isoforms) but cannot transduce the signal to activate the downstream TGFB signalling pathway in the cell, which leads to the cells being unresponsive to TGFB. In the absence of doxycycline, the rtTA transactivator does not recognise and bind to the *Teto* promoter so the dominant negative TGFB receptor is not expressed, and hence the normal TGFB receptor can bind TGFB and trigger the TGFB signalling pathway (Figure 2.1).

The experiments described in this thesis used both single transgenic mice as controls for the *TGF-Mac* double transgenic mouse. At the time of weaning (3 weeks), 5 mm of the tail tip of each *TGF-Mac* double transgenic mouse was cut for genotyping (protocol described in 2.3.1).

2.2.1.3 *Mmtv-Ccl2 and FVB mice*

The *Mmtv-Ccl2* transgenic mouse in which the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) constitutively drives the expression of the chemokine (C-C motif) ligand 2 (CCL2) was generated and developed through sub-cloning and pronuclear-microinjection techniques by Ingman et al. (unpublished) at the Albert Einstein College of Medicine (New York, USA). The *Mmtv-Ccl2* expression cassette was constructed by insertion of a 3.2-kb *EcoRI* fragment of the mouse genomic *Ccl2* cDNA from the construct pMMJE+20 (Rutledge et al., 1995) into the *EcoRI* site of the plasmid MMTV-SV40-Bssk which contain regulatory elements of the MMTV-LTR followed by the SV40 poly A site and the ampicillin-resistance gene (*amp*^{r}) (Huang et al., 1981) (Figure 2.2 A). This construct was transformed into DH5-α cells by heat shock and selected for ampicillin resistance. DNA was extracted from the successfully transformed cells and the *Ccl2-Mmtv* expression cassette was released as a *PVUII* fragment of 8 kb in size. This purified *Ccl2-Mmtv* expression cassette was micro-injected into zygotes of FVB mice, which were then implanted into pseudopregnant foster mothers.

Twenty-nine offspring were generated, and three founder lines (*Mmtv-Ccl2* #13, #20 and #29) were identified by PCR screening using a primer pair (forward: 5'-CGT CCA GAA AAC CAC AGT CA -3'; reverse: 5'-CCG CTC GTC ACT TAT CCT TC-3') covering the *Mmtv* promoter sequence which

produced a product size of 196 bp (Figure 2.2 B). All three founders were cross-bred with background strain FVB mice and the genotypes of all progeny were confirmed by PCR. One female pup was selected from each founder line and the expression of *Ccl2* mRNA from different tissues was measured by RT-PCR using a primer pair that spanned both endogenous genomic and cloned *Ccl2* (forward: 5-CCC AAT GAG TAG GCT GGA GA-3'; reverse: 5'-TCT GGA CCC ATT CCT TCT TG-3') and produced product size of 451 bp and 125 bp respectively (Figure 2.2 C). The offspring from founder 29 exhibited highest expression of *Ccl2* in the mammary gland and was chosen for further analysis. A homozygous *Mmtv-Ccl2* transgenic mouse line was successfully established from founder 29 and maintained over 5 generations on a FVB background.

The experiments described in this thesis used FVB mice as control for the *Mmtv-Ccl2 transgenic* mice. At the time of weaning (3 weeks), 5 mm of the tail tip of each *Mmtv-Ccl2* transgenic mouse was cut for genotyping to confirm homozygosity (protocol described in 2.3.1).

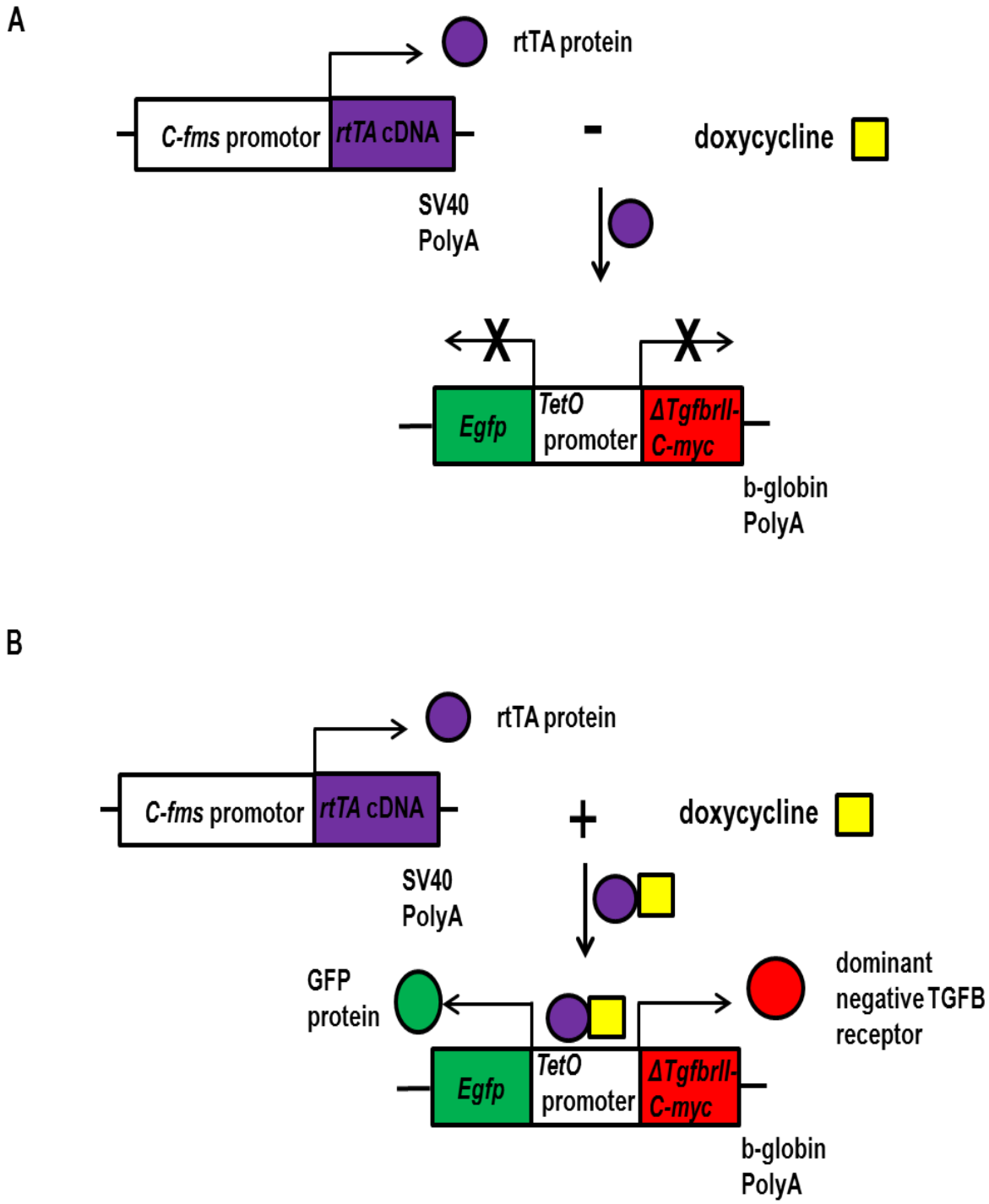

Figure 2.1 Schematic outline of the regulation of transgene expression in *TGF-Mac* double transgenic mice.

In the *TGF-Mac* double transgenic mouse, the expression of the transgenes can be controlled by addition or removal of doxycycline. **A.** In the absence of doxycycline, the reverse tetracycline-controlled transactivator (rtTA) cannot recognise the bidirectional tetracycline/doxycycline transactivator promoter (*TetO*) and thus no expression of both reporter genes, *Egfp* and disrupted *Tgfbrll* (*ΔTgfbrII*). **B**. In the presence of doxycycline, the rtTA transactivator recognises and binds to the *TetO* promoter and subsequently induces the expression of the dominant negative TGFB receptor and EGFP.

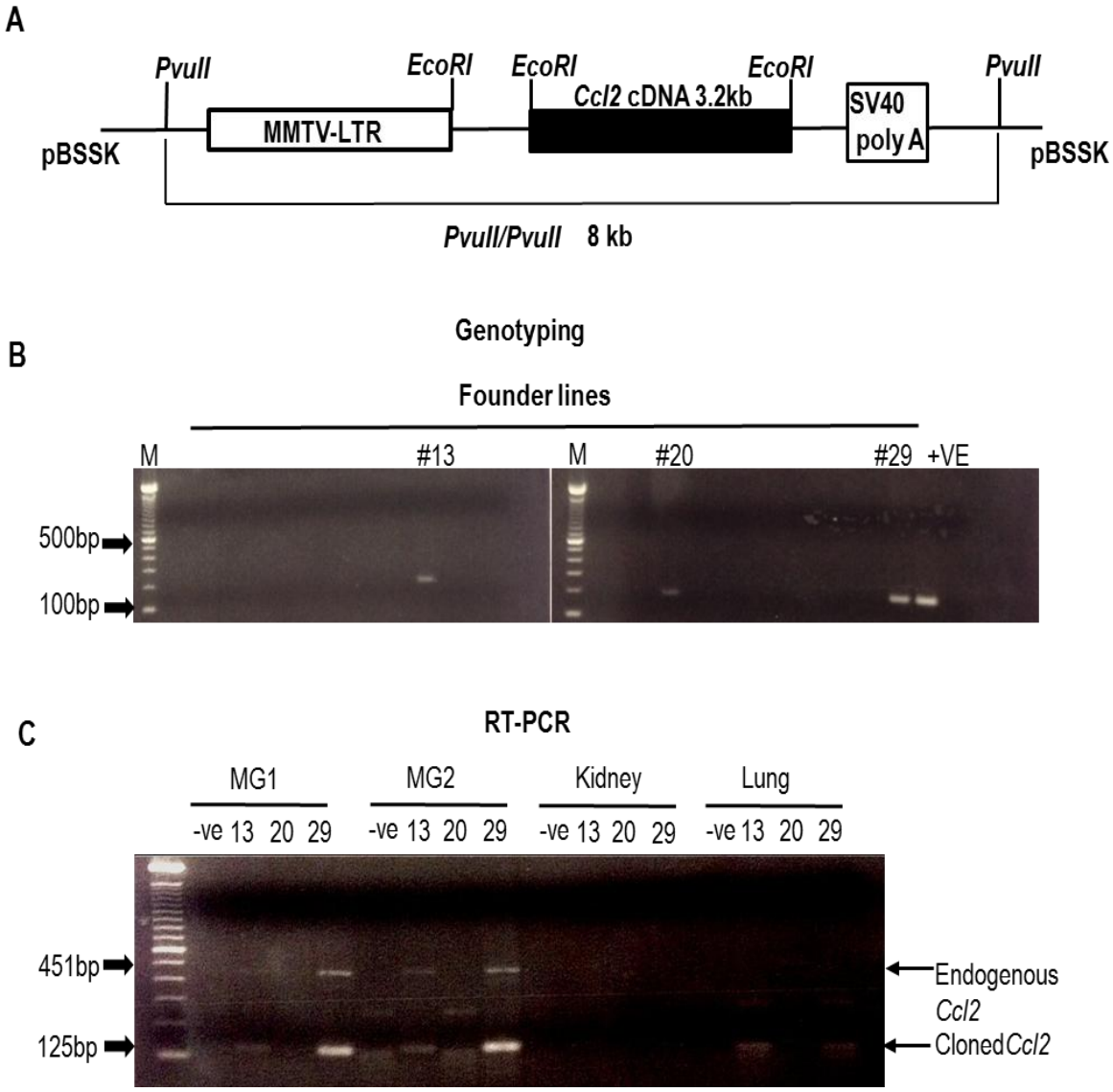

Figure 2.2 Generation of *Mmtv-Ccl2* transgenic mice.

A. The *Mmtv-Ccl2* expression cassette was constructed by insertion of a 3.2-kb *EcoRI* fragment of the mouse genomic *Ccl2* cDNA from the construct pMMJE+20 into the *EcoRI* site of the plasmid MMTV-SV40-Bssk which containing regulatory elements of the MMTV-LTR followed by the SV40 poly A site. **B.** Twenty-nine offspring were generated, and three founder lines (#13, #20 and #29) were identified by PCR screening. **C.** One female offspring was selected from each founder line and the expression of both endogenous genomic and cloned *Ccl2* mRNA from different tissues was measured by RT-PCR with product sizes of 451 bp and 125 bp respectively. The offspring from founder #29 exhibited highest expression of *Ccl2* in the mammary gland and was chosen for further analysis.

2.2.2 General Surgical Procedures

All surgical instruments were sterilised by autoclaving, or submerged in 70% ethanol (ANALAR, Melbourne, Australia) prior to use. For anaesthesia, mice received an intra-peritoneal (i.p.) injection of 15 µl/g body weight of 2% Avertin (tribromoethanol; Sigma-Aldrich, St. Louis, USA), or alternatively, anaesthesia was induced using fluorothane (Veterinary Companies of Australia, Sydney, Australia). Surgical incisions were closed using 9 mm MikRon® wound clips (Becton Dickinson, Sparks, USA), which were removed 2 weeks following surgery. Following anaesthesia, mice were housed on a 37^{o}C heating pad until the following morning. When mice regained consciousness, they were injected subcutaneously with buprenorphine analgesia (Temgesic; Intervet, Schering-Plough, VIC, AU). Buprenorphine was administered at 0.8 µg/10 g of body weight.

2.2.3 Mammary Gland Transplants

Mammary gland transplants were performed using donor mammary gland tissue obtained from 8-week-old *Tgfb1*+/+ and *Tgfb1*-/- female mice as described previously (Ingman and Robertson, 2008). The mice were killed and the 4th pair of mammary glands dissected. Single tissue fragments (1-mm^3) isolated from the donor mammary glands were rinsed and maintained in ice-cold PBS. Under 2% Avertin anesthesia, donor mammary gland tissue was transplanted into the cleared fat pad of recipient 24 day old BalbC *Prkdc*^{scid} female mice. To clear the fat pad, the portion of mammary gland from the nipple to the mammary gland lymph node was dissected and removed. In each recipient, donor tissue fragments were inserted into the cleared 4th pair mammary fat pads, one side was transplanted with *Tgfb1*+/+ mammary tissue from a *Tgfb1*+/+ donor; the contra-lateral side was transplanted with *Tgfb1*-/- mammary tissue. The transplants were performed using *Tgfb1*-/- and *Tgfb1*+/+ donors (n=3 per genotype) to generate a total of 20 recipient mice.

2.2.4 Overiectomy

Ovaries were surgically removed from Balb/C *Prkdc*^{scid} mice at the age of 12 weeks as described previously (Olson and Bruce, 1986). In brief, mice were anaesthetised under fluorothane anaesthesia; the dorsal region was shaved and swabbed with 70% ethanol. A small longitudinal dorsal incision was made, followed by a retroperitoneal incision above the ovarian fat pad. The ovary and associated fat were exteriorised and the oviduct was severed close to the uterotubal junction. The remaining reproductive tract tissue was placed back into the peritoneal cavity and the procedure was repeated on the other side. The skin was closed using wound clips.

2.2.5 Hormone Replacement

Hormone replacement regimen was employed in the ovartiectomised Balb/C *Prkdc*^{scid} mice to standardise the hormonal environment and mimic diestrus. This model has been shown to support mammary gland ductal and alveolar development and mimics the diestrus phase of the ovarian cycle (Aupperlee and Haslam, 2007, Hodson et al., 2013). The mice were allowed to recover for 1 week after ovariectomy. The mice were then injected daily for three days with 17β-estradiol (10 μg/ml in sesame oil) (Sigma-Aldrich) and progesterone (10 mg/ml in sesame oil) (Sigma-Aldrich) administered subcutaneously.

2.2.6 Blood Collection

Blood was collected from the mice by cardiac puncture. The mice were deeply anaesthetised with 0.4-0.5 ml 2% Avertin and up to 1 ml of blood was extracted directly from the heart using a 20 g needle. Blood was incubated at room temperature for 30 mins, then centrifuged for 10 minutes at 2000 rpm and serum was collected and stored at -80 ^{o}C.

2.2.7 Estrous Cycle Tracking

Estrous cycle stage was determined by analysis of vaginal smears as described previously (Allen, 1922). Vaginal smears were conducted between 8:00 – 11:00 h. Sterile phosphate buffered saline (PBS) (25μl) was used to flush the vagina of female mice and the contents smeared on a glass slide and covered with a cover slip. The cellular contents were analysed under a phase contrast microscope to determine the stage of the ovarian cycle (Table 2.1). The estrous cycles of *Cfms-rtTA*, *TetO-TgfbrII*, *TGF-Mac*, *Mmtv-Ccl2* and FVB mice were tracked for at least 28 days.

Table 2.1 Estrous stage determination by vaginal smears

Stage of the estrus cycle	Cytology of cells in vaginal smears	Length of each stage
Proestrus	E or EC	24 hrs
Estrus	EC+ or C+	12 hrs
Metestrus	C++ clumps or C+E L++	36 to 48 hrs
Diestrus	Low number of cells C-- L--	36 hrs

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

2.2.8 Doxycycline Administration

Cfms-rtTA, *TetO-TgfbrII* and *TGF-Mac* transgenic mice received doxycycline (Sigma-Aldrich) in autoclaved drinking water changed twice weekly at a concentration of 2 mg/ml from the age of 4 weeks until culled.

2.2.9 DMBA Administration

Cfms-rtTA, *TetO-TgfbrII*, *TGF-Mac, Mmtv-Ccl2 and* FVB mice received DMBA in sesame oil (1 mg/ml) weekly by oral gavage for 6 weeks from 6 weeks of age. Mice were gently palpated for tumours weekly until positive identification of the first mammary tumour or for up to a year from the last carcinogen treatment. Mice were then killed by cervical dislocation and tumours were dissected for further analysis.

2.3 Nucleotide Analysis

2.3.1 Genotyping Mice

Mouse genotypes were determined by polymerase chain reaction (PCR) using tail DNA. DNA from *Tgfb1* null mutant (*Tgfb1-/-)*, heterozygous (*Tgfb1+/-*), or homozygous wild-type (*Tgfb1+/+*) mice were analysed for the intact *Tgfb1* gene and the mutant *Tgfb1* null gene, DNA from *TGF-Mac* double transgenic mice was analysed for both the *rtTA* and dominant negative TGFB receptor transgene (*Egfp-ΔTgfbrII*) and DNA from *Mmtv-Ccl2* mice was analysed for the *Mmtv* transgene.

2.3.1.1 *DNA Extraction*

DNA extraction was performed using autoclaved reagents and materials. Tails were digested in 350 μl digestion buffer containing 17 mM tris, 17 mM EDTA, 170 mM sodium chloride, 0.85% SDS (PH 7.8) with 0.1 mg proteinase K (all from Sigma-Aldrich) at 55^{o}C for 3-6 hours. 5 μl of each digested tail was added to 95 μl of autoclaved milli-Q (MQ) water in 1.5 ml eppendorf tubes. The tubes were heated to 95^{o}C for 15 minutes to inactivate the proteinase K and stored at 4^{o}C until PCR analysis was performed.

2.3.1.2 *PCR Primers and Conditions*

The primers and conditions used to determine the genotype of the mice in respect to *Tgfb1* mutation (Table 2.2) were designed by Diebold et al. (1995). The primers used to confirm the presence of *rtTA* and *Egfp-ΔTgfbrII* transgenes in *TGF-Mac* mice were designed by Yan et al. (2006) and Frugier et al. (2005) respectively. The primers used to confirm the homozygosity of *Ccl2-Mmtv* mice were designed using Primer Express version 2 software (Life Technologies, Applied Biosystems, CA, USA). All primers were purchased from Geneworks (Adelaide, SA, AU). The sequence, PCR product length of the primers and Genbank accession number are provided in Table 2.2.

Table 2.2 PCR primers for mouse genotyping

Gene	5'-3' Primer sequence	Product size (bp)	Genebank
Tgfb1 intact	5' GAGAAGAACTGCTGTGTGCG	134	L42461
	3' GTGTCCAGGCTCCAAATATAGG		
Tgfb1 neo	5' GAGAAGAACTGCTGTGTGCG	545	L42461&U43611
	3' CTCGTCCTGCAGTTCATTCA		
rtTA	5' TGATTGAAGGGTCCAGACATTC	280	AF290879.1
	3' AGTGTAGGCTGCTCTACACCAAGC		
Egfp-ΔTgfbrII	5' TTCGAGCTCGGTACCCGGGGAT	625	NM_008610.2
	3' CCCGAGACGGTAGCAGTAGAAGA		
Mmtv	5' CGTCCAGAAAACCACAGTCA	195	AF119341.1
	3' CCGCTCGTCACTTATCCTTC		

2.3.1.3 *Polymerase Chain Reaction*

PCR reaction mixtures contained 1x PCR buffer, 2.5 mM $MgCl_2$, and 0.55 U Taq polymerase (all from Fisher Biotec, WA, AUS), 200μM dNTPs (Roche, Germany), 10 μM each primer and 2 μl of extracted DNA in a 25 μl reaction mixture. PCR products were stored at -20°C until analysed. The PCR cycle

conditions to detect the *Tgfb1* genes were one cycle of 94°C for 45 seconds; 35 cycles of 94°C for 45 seconds, 61°C for 45 seconds, 72°C for 45 seconds; and one cycle of 72°C for 3 minutes. The PCR cycle conditions to detect ,*rtTA* ,*Egfp-ΔTgfbrII* and *Mmtv* transgenes were one cycle of 95°C for 2 minutes; 35 cycles of 95°C for 15 seconds, 62°C for 15 seconds, 72°C for 50 seconds; and one cycle of 72°C for 7 minutes. PCR reactions were carried out using either OmniGene TRSM2 thermal cycler (Hybaid Lmited, Middlesex,UK) or GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, CA, USA).

2.3.1.4 *Detection of PCR Products*

The PCR products were separated by size using gel electrophoresis. PCR products of *Tgfb* mutation (Figure 2.3A), *rtTA*, and *EGFP-ΔTgfbrII* (Figure 2.3B) containing 1x loading buffer were run on a 2% agarose gel (Promega, WI, USA) containing Ethidium Bromide (Invitrogen Life Technologies, CA, USA) diluted in TAE for 40 minutes. PCR product of *Mmtv* (Figure 2.3C) containing 1x loading buffer was run on a 2% agarose gel with 0.01% GelRed™(Biotium, Hayward, CA, USA) diluted in TAE for 40 minutes. PUC19 (Geneworks) was used as a size marker. The gel was photographed using either DC120 (Kodak) digital camera for Ethidium Bromide-stained gels or Gel Doc™ EZ Imager (Bio-Rad Laboratories Inc., CA,USA) for GelRed™ under UV light to detect the PCR products.

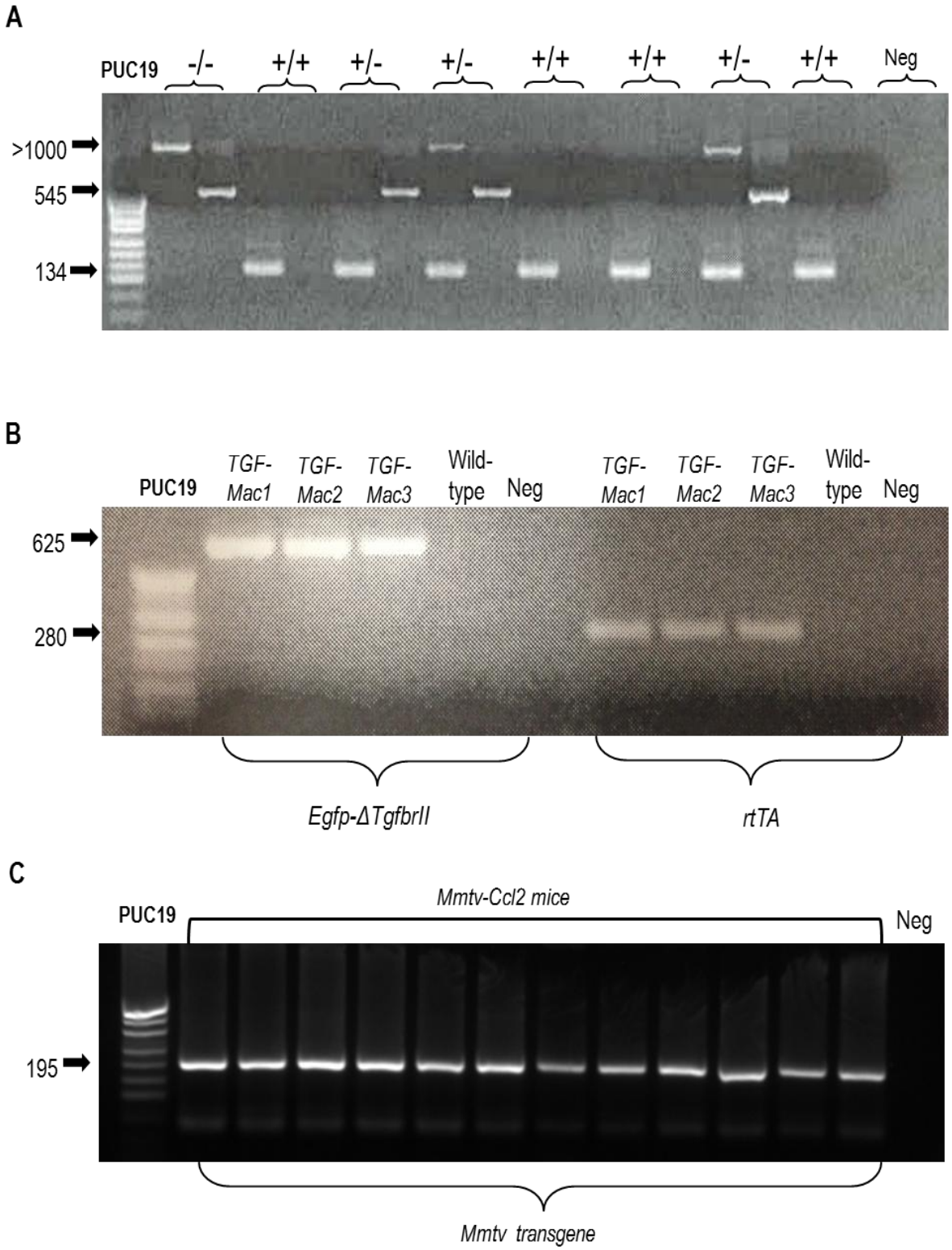

Figure 2.3 Genotyping *Tgfb1* mutation, *rtTA* transgene, *Egfp-ΔTgfbrII* transgene and *Mmtv* transgene by PCR.

A. DNA from each mouse was amplified for *Tgfb intact* (134bp; 1st lane, then alternate lanes) and transgene *Tgfb1 neo* (545bp; 2nd lane, then alternate lanes) to detect wildtype (+/+), heterozygous (+/-) and null (-/-) mice. **B.** DNA from *TGF-Mac* and wild-type mice was amplified for *rtTA* (280bp) and *Egfp-ΔTgfbrII* (625bp) transgenes, only *TGF-Mac* mice showed expression of the transgenes. **C.** DNA from *Mmtv-Ccl2* mice was amplified for *Mmtv* (195bp) transgene to confirm the presence of the transgenic mice.

2.3.2 Quantitative Real-Time PCR

2.3.2.1 RNA Extraction

Spleens were dissected from *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* mice to quantify expression of mRNA encoding the dominant negative TGFB receptor (ΔTGFBRII). Spleen, mammary gland, kidney, ovary and salivary gland were dissected from *Mmtv-Ccl2* and FVB mice to quantify expression of *Ccl2*, *Mmtv*, *Mmp2, Mmp9, Lox, Timp1, Timp2 and Timp3* mRNA expression.

All tissues were dissected under RNase free conditions, and the tissue was snap frozen in liquid nitrogen and stored at -80^{o}C until processing. Total tissue RNA extraction was performed by adding 1 ml of Trizol (Invirtrogen, CA, USA) to tubes containing 0.6 g of 1.4 mm ceramic beads (Geneworks). Tissues were homogenised by Powerlyser (MoBio, CA, USA) (2 x 30s). 200 μl of chloroform (Unilab, Ajax Finechem, Tarepoint, Australia) was added and samples were vigorously mixed by manual shaking. Samples were centrifuged at 12000 g for 15 min at 4^{o}C. Approximately 400 μl of the aqueous RNA phase was removed and placed into a fresh tube. Isopropanol (500 μl) (Sigma-Aldrich) was added and the sample was precipitated overnight at -20^{o}C. The following morning, samples were pelleted at 12000 g for 10 minutes at 4^{o}C. The pellet was washed with ice-cold 75% ethanol and centrifuged at 7500 g for 5 minutes at 4^{o}C. Excess ethanol was removed and the pellet was air dried for 10 minutes before being resuspended in 50 μl of RNase free Milli-Q water. Extracted RNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, USA).

The extracted RNA (10 μg) from each sample was treated with DNase to remove contaminating DNA; using reagents supplied in a TURBO DNA-free kit (Life technologies) according to the manufacturer's instructions. 5 μl of 10 x DNase I buffer and 1 μl of DNase I were added to each sample and incubated for 30 minutes at 37^{o}C. Following this, 5 μl of DNase Inactivating Reagent was incubated with each sample for 2 min at room temperature. Samples were centrifuged at 10000 g for 2 minutes at room temperature, and the RNA-containing supernatant was transferred to a clean tube. DNase treated RNA was quantified again using the Nanodrop Spectrophotometer.

2.3.2.2 *Reverse Transcription and cDNA Generation*

Samples were resuspended at 2 μg in 12 μl of RNase free Milli-Q water prior to incubation with 1 μl of 10 mM dNTP and 1 μl of 125 ng/ul random hexamers (Geneworks) at 65 °C for 5 minutes. Samples were then incubated on ice for 5 minutes. Reverse transcription master mix (4 μl of 5 x strand buffer, 1 μl of 0.1 M DTT and 1 μl of Superscript III enzyme) (Invitrogen) was added to each sample and incubated at 25 °C for 5 minutes and then 50 °C for 60 minutes. The reverse transcription was stopped by incubating the samples at 70 °C for 15 minutes. For quantitative real-time PCR (qRT-PCR), cDNA was diluted to a final concentration of 20 ng/μl and stored at -20 °C.

2.3.2.3 *Primer Design*

2.3.2.3.1 Primers designed by Custom Plus TaqMan® RNA Assay

Primer pairs specific for dominant negative TGFB receptor (*ΔTgfbII*) mRNA expression were designed and custom made by Life Technologies (Custom Plus TaqMan® RNA Assay) and supplied as a single tube that contains primers and probe. These primers and probes were pre-optimised and experimentally tested by Life Technologies and were guaranteed to give reaction efficiency of 100% +/- 10% with certificate of analysis of each assay. A list of target gene, amplicon length, assay ID and Genbank accession number are provided in Table 2.3.

2.3.2.3.2 Primers designed by Primer Express

Primer pairs specific for mRNA encoding *Ccl2* and *Mmtv* were designed using Primer Express version 2 software (Life Technologies). Messenger RNA sequences were downloaded from the Entrez nucleotide database, accessible from the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Primers were designed to meet previously optimised criteria to suit the ABI Prism 7000 Sequence Detection System (Applied Biosystems). All primers were purchased from Geneworks. A list of all sequences for target genes analysed, including product size and Genbank accession number are provided in Table 2.4. Primer specificity was determined by gel electrophoresis (2% agarose gel) to confirm the correct product size and analysis of the dissociation curve and gel electrophoresis was used to exclude the formation of primer dimers or non-specific products.

Table 2.3 Custom made primer probes for TaqMan® qRT-PCR

Gene	Amplicon Length (bp)	Assay ID	Genbank
ΔTgfbrII	79	AJ89J6H	NM_029575.3
Actb	115	Mm00607939_s1	NM_007393

Table 2.4 Primers designed by Primer Express for qRT-PCR

Gene	5'-3' Primer sequence	Product size (bp)	Genebank
Actb	5' GTGTGACGTTGACATCCGTAAAG	151	M12481
	3' CTCAGGAGGAGCAATGATCTTGAT		
Ccl2	5' CCAGCAAGATGATCCCAATGA	101	NM_011333
	3' TCTCTTGAGCTTGGTGACAAAAAC		
Mmtv	5' CGTCCAGAAAACCACAGTCA	195	AF119341.1
	3' CCGCTCGTCACTTATCCTTC		
Mmp2	5' CAAGTTCCCCGGCGATGTC	171	NM_008610.2
	3' TTCTGGTCAAGGTCACCTGTC		
Mmp9	5' CAGACGTGGGTCGATTCCA	103	NM013599
	3' TGTCTCGCGGCAAGTCTTC		
Timp1	5' AGTCCCAGAACCGCAGTGAA	100	NM_001294280.2
	3' AGTACGCCAGGGAACCAAGA		
Timp2	5' GAGCCTGAACCACAGGTACCA	103	NM_011594.3
	3' GTCCATCCAGAGGCACTCATC		
Timp3	5' CTTCTGCAACTCCGACATCGT	162	NM_011595.2
	3' TCAGAGGCTTCCGTGTGAATG		
Lox	5' TGCCAGTGGATTGATATTACAGATGT	125	NM_010728.3
	3' AGCGAATGTCACAGCGTACAA		

2.3.2.3.3 Quantitative Real-Time PCR

Quantitative RT-PCR was performed using an ABI Prism 7000 Sequence Detection System either with a Taqman Universal MasterMix II (Life Technologies) or a 2 x SYBR Green PCR Master Mix (Life Technologies) according to the manufacturer's instructions respectively. Each reaction (20 μl in total) either contained 2.5 μl of cDNA, 1 μl of primer/probe and 16.5 μl of Taqman Universal MasterMix II or 1 μl of cDNA and 0.25 μM 5' and 3' primers and 10 μl of SYBR green. The negative control included in each reaction contained RNase free Milli-Q water subsitituted for cDNA (non-template) control and showed that primers did not produce primer dimers as evidenced by no amplification.

PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60oC. Amplification plots for each sample were obtained and cycle threshold (Ct) limits determined using ABI 7700 software package.

All samples were amplified in duplicate for each primer set. The quantity of cDNA for each sample was normalised to the housekeeper gene *actb* using the ΔΔCt method (Livak and Schmittgen, 2001). Relative change in cDNA quantity was calculated by multiplying the cDNA quantity by a constant such that the average expression of the gene of interest in control tissues equalled an arbitrary number of 100. This enabled the data for all samples set to be expressed relative to the abundance of mRNA for the gene of interest in the control tissue.

2.4 Protein Analysis

Spleen, kidney, liver, ovary, mammary glands, salivary gland and serum were collected from *Mmtv-Ccl2* and FVB mice to quantify the abundance of CCL2 protein.

2.4.1 Protein Extraction

All tissues and serum were collected under sterile conditions, and the tissue was snap frozen in liquid nitrogen and stored at -80°C until processing. Protein was extracted from tissues in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton-X, 2 mM EDTA, 1 mM DTT, PH7.4) containing Complete Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). Tissues were homogenised with the

Powerlyzer® 24 Bench Top Bead Based Homogenizer using 0.6 g of 1.4 mm ceramic beads. Homogenised protein samples were incubated for 10 minutes on ice and then centrifuged at 14,000 g for 10 minutes at 4°C, the supernatant was transferred into a new eppendorf tube for protein concentration quantification.

2.4.2 Pierce™ BCA Protein Assay

Protein concentration of each sample was quantified using the BCA protein assay (Thermo Scientific) following the manufacturer's instructions. The limit of detection was between 20 μg/ml and 2000 μg/ml.

2.4.3 CCL2 ELISA

A CCL2 specific sandwich ELISA (eBioscience, San Diego, USA) was used to quantify mouse CCL2 protein in different tissues, according to the manufacturer's instructions. Briefly, an anti-mouse CCL2 antibody was coated onto a 96 well microtitre plate to capture CCL2 from either recombinant standard or assay samples. A biotinylated anti-mouse CCL2 detection antibody was then used and bound antibody was quantified by the addition of streptavidin-conjugated HRP, followed by the addition of a chromagen substrate. After incubation for 20 min at room temperature, the substrate product was acidified by the addition of 50 μl of 1M HCl, and absorbance at 450 nm (reference wavelength 570 nm) was measured using a Benckmark™ microplate reader (Bio-Rad Laboratories). The concentration of CCL2 within tissues and serum was then calculated from a standard curve (5-parameter logistic curve) using known concentrations of recombinant CCL2. This assay was reported by the manufacturer to have a minimum detection limit of 15 pg/ml, with intra- and inter-assay precision of approximately 5%. The concentration of CCL2 in each sample was then normalised the protein concentration of the tissue and represented as CCL2 (pg)/protein (pg).

2.5 Histology and Immunohistochemistry

2.5.1 Tissue collection, embedding and sectioning

2.5.1.1 *Fresh-Frozen Tissue*

Mammary glands were collected from mice at autopsy. Tissue was embedded in Tissue-Tek OCT compound (Sakura Fintek, Torrance, USA) and stored at -80°C. Tissue was sectioned at 6 μm using a

Leica CM 1850 cryostat (Leica Microsystems, NSW, Australia) and disposable microtome blades (Feather, Osaka, Japan) and SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). Slides were allowed to dry for approximately 1 h before being stained or were stored with desiccant (silica gel; Sigma Aldrich) at -80^{o}C.

2.5.1.2 *Paraffin-Embedded Tissue*

2.5.1.2.1 Mouse Mammary gland

Mammary glands were dissected from mice and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 24 h at 4^{o}C. Tissue was washed in 1 x PBS twice over the following two days, and on the third day, tissue was transferred to a 70% ethanol solution where it remained until tissue processing. Tissue was processed and embedded using the Leica TP1020 Tissue Processor (Leica Microsystems) involving the following dehydration and embedding protocol; 30 minutes 70% ethanol, 30 minutes 85% ethanol, 30 minutes 90% ethanol, 30 minutes 96% ethanol, 2 x 30 minutes absolute ethanol (ANALAR), 2 x 30 minutes 100% Xylene (Ajax Finechem), 2 x 30 minutes paraffin wax (Ajax Finechem) under vacuum conditions. Tissue was moulded into wax blocks and was cut on a Leica Rotary Microtome (Leica Microsystems) onto SuperFrost Plus slides. Slides were dried overnight (O/N) at 37^{o}C. Blocks and sections were stored at room temperature prior to staining.

2.5.1.2.2 Human Breast Tissue

Breast tissue was collected from female participants undergoing either reduction mammoplasty or mastectomy surgery at the Queen Elizabeth Hospital. Breast tissue from each participant was sliced into small fragments and fixed in 4% PFA for 24 h at 4^{o}C and was then washed twice in 1 x PBS over the following 2 days, and on the third day, tissue was transferred to a 70% ethanol solution where it remained until tissue processing. Tissue was processed and embedded using the Leica TP1020 Tissue Processor involving the following dehydration and embedding protocol; 75 minutes 70% ethanol, 75 minutes 85% ethanol, 75 minutes 90% ethanol, 75 minutes 96% ethanol, 2 x 75 minutes absolute ethanol, 2 x 75 minutes 100% Xylene, 2 x 75 minutes paraffin wax under vacuum conditions. Tissue was moulded, sectioned and stored the same as for mouse mammary glands. Blocks and sections were stored at room temperature prior to staining.

2.5.1.3 Mammary Gland Whole Mount Preparation

Mammary glands were dissected from mice and spread on a glass slide. The slides were incubated in Carnoy's fixative (6 parts of 100% ethanol, 3 parts of chloroform and 1 part of acetic acid) for 2 to 4 hours. Fixed mammary glands were then washed in 70% ethanol for 15 minutes, transferred to MQ water for 5 minutes. The tissue was stained in carmine alum [2% carmine (Sigma-Aldrich), 5% aluminium potassium sulphate (Sigma-Aldrich)] overnight. The slides were washed in 70% ethanol for 15 minutes followed by 2 washes in 100% ethanol for 15 minutes each. Finally, the slides were cleared in Safsolv (Ajax Finechem) for 1 week and mounted with a cover slip using DPX (Merck, Darmstadt, Germany).

2.5.2 Immunohistochemistry Protocol

2.5.2.1 Mouse Mammary Gland Tissue

2.5.2.1.1 Fresh-Frozen Tissue

2.5.2.1.1.1 Immunofluorescence to detect TGFB1, F4/80, TROMA-1, MHCII and Collagen 1

Primary antibodies were the polyclonal affinity-purified chicken anti-TGFB1 (R&D Systems, MN, USA), rat anti-F4/80 monoclonal macrophage-specific antibody (Caltag Laboratories, CA, USA), monoclonal anti-TROMA-1 antibody (Developmental Studies Hybridoma Bank, University of Iowa; kindly donated by Dr Carmela Ricciardelli, the University of Adelaide), biotinylated anti-mouse F4/80 antibody (eBioscience), biotinylated anti-mouse MHCII antibody (eBioscience) and polyclonal rabbit anti-mouse collagen-1 antibody (Chemicon, MA, USA) (Table 2.5). Fresh frozen tissue sections were fixed in either 100% acetone (Sigma-Aldrich) or 2% PFA for 15 minutes at 4^{o}C. Slides were then incubated with primary antibodies either diluted in PBS-BSA or PBS-BSA containing 2% goat serum (Sigma-Aldrich) overnight at 4^{o}C in a humidified chamber, except polyclonal rabbit anti-mouse collagen-1 was incubated for 60 minutes at room temperature. TGFB1 and F4/80 was detected by applying anti-chicken IgY FITC conjugated secondary antibody (1:200 dilution; Sigma-Aldrich) and Alexa Fluor® 594 rabbit-anti rat IgG (1:200 dilution; Invitrogen) respectively for 60 minutes at room temperature. TROMA-1, biotinylated F4/80 and biotinylated MHCII was detected by incubating with Alexa Fluor® 594 rabbit-anti rat IgG (1:200 dilution) and strepavidin FITC (1:200 dilution; eBioscience) respectively for 60 minutes at room temperature. Collagen-1 was detected using Alexa Fluor® 594 goat-anti rabbit IgG (1:800 dilution;). Slides stained with secondary antibodies only or with isotype matched antibody was included as negative controls. All sections were mounted in fluorescent mounting medium (Dako, Glostrup,

Denmark) with 4', 6-diamidino-2-phenylindol (DAPI) (Sigma, St Louis, USA) and were stored at 4°C in the dark until image capture.

2.5.2.1.1.2 Immunohistochemistry to detect iNOS

Fresh frozen tissue sections were fixed in cold 100% acetone for 15 minutes at 4°C. Slides were then washed in PBS (3 x 5 minutes) and incubated with 1% hydrogen peroxide (Thermo Fisher Scientific, Vic, AU) in PBS for 10 minutes before the slides were blocked in PBS containing 10% goat serum and 2% BSA for 40 minutes at room temperature. All incubations were performed in a humidified chamber. Polyclonal rabbit anti-iNOS/iNOS type 2 (BD Pharmingen, BD Biosciences, San Diego, USA) was applied to sections diluted in PBS-BSA with 2% goat serum, and the slides incubated at 4°C overnight (Table 2.5). The following morning, a HRP-conjugated secondary antibody (goat anti-rabbit HRP; Dako) was diluted 1:400 in PBS-BSA containing 2% goat serum and incubated for 40 minutes at room temperature. The detection of bound antibody was performed using 3,3 diaminobenzadine (DAB; Dako) according to the manufacturer's instructions. Slides stained with secondary antibodies only or with isotype matched antibody was included as negative controls. Tissue sections were counterstained with haematoxylin (Sigma-Aldrich) for 30 seconds, before being dehydrated in 95% (5 minutes) and absolute ethanol (5 minutes), and cleared in 2 changes of Safsolv for 5 minutes. Slides where left to dry O/N before coverslips were mounted using DPX mountant.

Table 2.5 Primary antibodies used for fresh-frozen mouse mammary gland tissue

Antigen	Fixation	Reactivity	Manufacturer	Isotype	Clone	Concentration/ Dilution
TGFB1	2% PFA	Mouse/ Human	R&D systems	Chicken IgY	240-B	5 μg/ml (1:40 overnight)
F4/80	2% PFA	Mouse	Caltag Laboratories	Rat IgG2a	BM8	10 μg/ml (1:100 overnight)
TROMA1	2% PFA	Mouse	Developmental Studies Hybridoma Bank	Rat IgG2a	SP2/0	4.96 μg/ml (1:50 overnight)
Biotinylated F4/80	2% PFA	Mouse	eBioscience	Rat IgG2a	BM8	10 μg/ml (1:50 overnight)
Biotinylated MHCII	2% PFA	Mouse	eBioscience	Rat IgG2a	M5/114.1 5.2	10 μg/ml (1:50 overnight)
Collagen 1	100% acetone	Mouse	Chemicon	Rabbit IgG	N/A	2.5 μg/ml (1:400 for an hour)
iNOS	100% acetone	Mouse/ Human	BD Biosciences	Rabbit IgG	N/A	0.83 μg/ml (1:300 overnight)

N/A: Not applicable

2.5.2.1.2 Paraffin-Embedded Tissue

2.5.2.1.2.1 Immunohistochemistry to detect F4/80, CCR7 and CCL2

Primary antibodies were rat anti- mouse F4/80 monoclonal macrophage-specific antibody (Caltag Laboratories), rat anti-mouse CCR7 monoclonal antibody (Clone 4B12; kindly donated by Dr Marina Kochetkova, the University of Adelaide, SA, AU) and polyclonal rabbit anti-mouse CCL2 (Santa Cruz Biotechnology, CA, USA) (Table 2.6). Paraffin-embedded mammary gland tissue sections were dewaxed in Safsolv and rehydrated through graduated dilutions of ethanol for 3 minutes each (2 x 100%, 1 x 90% ,1 x 70% and 1 x 50% ethanol) followed by 1 x 2 minutes in MQ water. No antigen retrieval was required for detection of F4/80 and CCR7. Whereas for detection of CCL2, slides were placed in 10mM sodium citrate buffer [0.00378 g/ml citric acid (Sigma-Aldrich) and 0.0024 g/ml sodium citrate (Sigma-Aldrich) in MQ water, Ph=6] and brought to 90^{o}C in a water bath for 20 minutes for antigen retrieval and

washed 2 times in PBS for 3 minutes each. Endogenous peroxidase activity was quenched by incubating slides with freshly prepared 30% hydrogen peroxide solution in methanol (Sigma-Aldrich; solution contained 10 ml hydrogen peroxide, 100 ml methanol, 90 ml water) for 15 minutes. Slides were blocked with 15% normal rabbit serum in PBS for 30 minutes at 37^{o}C before incubation with rat anti-mouse F4/80 monoclonal macrophage-specific antibody, with 10% normal rabbit serum in PBS for 30 minutes at room temperature before incubation with rat anti-mouse CCR7 monoclonal antibody or with 10% goat serum in PBS for 30 minutes at room temperature before incubation with polyclonal rabbit anti-mouse overnight at 4^{o}C respectively in a humidified chamber. Sections were then incubated with biotinylated rabbit anti-rat IgG (1:100 dilution; Vector Laboratories, Burlingame, USA) for 40 minutes at room temperature followed by ABC Elite kit (Vector Laboratories) for 30 minutes, incubated with rabbit anti-rat HRP (1:200 dilution; Dako) for 60 minutes at room temperature or incubated with goat anti-rabbit HRP (1:200 dilution; Dako) for 60 minutes at room temperature respectively. The detection of bound antibody was performed using DAB according to the manufacturer's instructions. Tissue sections were counterstained with haematoxylin for 30 seconds, before being rehydrated through a graduated increase in ethanol concentration (the reverse of the dehydration protocol) and cleared in two changes of Safsolv for 5 minutes. Slides stained with secondary antibodies only or with isotype matched antibody was included as negative controls. The slides were dried O/N and then mounted with coverslips using DPX mountant.

2.5.2.1.2.2 Haematoxylin and eosin stain

Tissue sections were counterstained with haematoxylin haematoxylin for 30 seconds and then stained in eosin (Sigma-Aldrich) for 10 seconds prior to dehydration, clearing and mounting, as described above.

2.5.2.1.2.3 Masson's trichrome stain

This protocol was used for detection of collagen fibres in paraffin-embedded mouse mammary gland tissue (all reagents were from Sigma-Aldrich). Tissue were dewaxed and rehydrated as described above. Slides were placed in Bouin's fluid overnight at room temperature. The following day, slides were placed in Weigert's Haematoxylin working solution (50% A and 50% B) for 10 minutes at room temperature and immersed in 80% ethanol before washed with MQ water. Slides were placed in Biebrich Scarlet solution for 2 minutes and immersed in 5% Phosphottungstic acid for 15 minutes followed by immersing in Light green solution for 10 minutes. Slides were immersed in 1% glacial acetic acid for 5 minutes prior to dehydration, clearing and mounting, as described above.

2.5.2.1.2.4 TUNEL stain

The detection of apoptotic cells by TUNEL (terminal transferase-mediated dUTP nice end labelling) staining in paraffin-embedded mouse mammary gland tissue was conducted by using the In Situ Cell Death Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. In brief, tissue were dewaxed and rehydrated as previously described. Tissue sections were treated with proteinase K (20 μg/ml; Sigma-Aldrich) in PBS for 10 minutes at 37°C followed by incubation with enzyme and label solution mix in PBS (10% enzyme solution, 70% label solution and 20% PBS; Roche Diagnostics, Germany) for 60 minutes at 37°C. All sections were mounted in fluorescent mounting medium with DAPI and were stored at 4°C in the dark until image capture.

2.5.2.1.2.5 Brdu incorporation

One hour prior to sacrifice, mice received an i.p. injection of 100 μl of 10 mg/ml BrdU (Sigma). To identify proliferating cells in paraffin embedded mouse mammary gland tissue, the bromodeoxyuridine (BrdU) In-Situ Detection Kit (BD Pharmingen, BD Biosciences), containing a biotinylated anti-BrdU antibody (Table 2.6), was used according to the manufacturer's instructions.

Table 2.6 Primary antibodies used for paraffin embedded mouse mammary gland tissue

Antigen	Antigen Retrieval	Reactivity	Manufacturer	Isotype	Clone	Concentration/ Dilution
F4/80	None	Mouse	Caltag Laboratories	Rat IgG2a	BM8	10 μg/ml (1:50) overnight
CCR7	None	Mouse	Abnova	Rat IgG2a	4B12	0.5 μg/ml (1:200 overnight)
CCL2	Citrate Buffer PH=6	Mouse	SantaCruz Biotechnology	Rabbit IgG2	N/A	4 μg/ml (1:50 overnight)
BrdU	As provided in the kit	Synthetic nucleotide	BD Biosciences	Mouse Monoclonal (Not disclosed	N/A	Not disclosed

N/A: Not applicable

2.5.2.1.3 Image Capture

Dual immunofluorescence images of TGFB1/F4/80, TROMA1/F480 and TROMA1/MHCII were captured and collected by a Leica SP5 spectral scanning confocal microscope (Adelaide Microscopy, The University of Adelaide, SA, AU) at 63 X magnification using the AMIRA and analySIS softwares. Fluorescence images of collagen-1 and TUNEL-positive apoptotic epithelial cells were captured and collected using FV10i Confocal Microscope (Olympus, USA) with laser-power and photomultiplier settings were kept constant for all experiments and images were captured at 60 x magnification. Non-fluorescent stained tissue sections were captured as a digital image using a Nanozoomer 1.0 (Hamamatsu, Shizouka, Japan) at a zoom equivalent to 40 x objective lens. Whole mount images of mammary glands were captured by MZ16 FA-Stereo microscope (Leica, The University of Adelaide, SA, AU).

2.5.2.1.4 Histology and Immunohistochemistry Quantification

All quantification analysis was performed by an assessor blinded to mouse genotype. To determine the extent of ductal branching morphogenesis in whole-mounted mammary glands, the three longest ducts from each mammary gland were selected and the number of branch points on each duct was counted manually and a mean value of 3 ducts per mammary gland were calculated and expressed as branch points/ mm.

To quantify the extent of alveolar development in H&E stained sections, epithelium was categorized as ductal (single epithelium layer) or alveolar (clusters of epithelial structures containing alveolar lumens) and the numbers of ductal and alveolar epithelial structures were counted manually. The total number of alveolar buds was expressed as percentage of total epithelial structures (ductal plus alveolar).

To determine the ratio of stroma and epithelium area within the mammary gland in H&E stained sections, five ductal epithelium and alveolar epithelium were randomly chosen for quantification. The area of stroma and epithelium was measured and represented as stroma area (mm^2)/epithelium area (mm^2). To determine the number of epithelial cells within the ductal epithelium, the number of haematoxylin-positive nuclei was counted.

To determine the deposition of collagen around mammary epithelium, the amount of fibre stain (green) around five randomly chosen ductal epithelium and alveolar epithelium was quantified. To determine the number of proliferating epithelial cells and apoptotic epithelial cells, the number of BrdU-positive cells and TUNEL-positive cells within the epithelium was counted respectively. The number of F4/80, CCR7 and iNOS positive cells in the mammary gland was counted and only positive cells with visible haematoxylin stained nuclei were included. For F4/80 staining, F4/80 positive macrophages were distinguished from F4/80 stained eosinophils on the basis of nuclear morphology. All results were expressed as positive cells/mm^2 or % positive cells. The mean density of positive cells within the five ductal and the five alveolar stroma was calculated, and grouped by mouse genotype.

The mean fluorescence intensity (mean grey-scale value) of collagen-1 was determined and analysed using ImageJ software and a mean value of five randomly chosen epithelial ducts was calculated.

2.5.2.2 *Human Breast Tissue*

2.5.2.2.1 Immunofluorescence to detect latent TGFB1

Paraffin embedded human breast tissue sections were placed on hotplate at 60^{o}C for 60 minutes before being dewaxed in two 5 minutes washes in Safsolv and passed through 100%, 95%, 80% and 70% ethanol gradually for rehydration. Slides were placed in 10 mM sodium citrate buffer [0.00378 g/ml citric acid and 0.0024 g/ml sodium citrate in MQ water, Ph=6] and brought to 90^{o}C in a water bath for 20 minutes and washed 2 times in PBS for 3 minutes each. The sections were incubated with anti-human LAP antibody (R&D systems) overnight at 4^{o}C (Table 2.7). The sections were washed again as before, coated in PBS/BSA/FCS and incubated with biotinylated rabbit anti-goat IgG (1:200 dilution; Millipore, MA, USA) at room temperature for 60 minutes followed by incubation with streptavidin Alexa Fluor® 594 (1:200 dilution; Invitrogen) for 60 minutes in the dark at room temperature. All sections were coverslipped in fluorescent mounting medium with DAPI and stored at 4^{o}C in the dark until image capture. Negative control with no application of primary antibody was included for each patient.

2.5.2.2.2 Detection of CD68, CD206 and CCL2 with Dako EnVision™ system

Paraffin embedded human breast tissue sections were placed on a hotplate at 60^{o}C for 60 minutes before dewaxed in two 5 minutes washes in Safsolv and passed through 100%, 95%, 80% and 70%

ethanol gradually for rehydration as described previously. All primary antibodies were diluted in EnVision™ antibody diluent (Dako). Before sections were incubated with mouse anti-human CD68 antibody (Dako) or mouse anti-human CD206 antibody (Dako) for 30 minutes at room temperature, sections were placed in Dako EnVision™ high Ph antigen retrieval solution (Dako) and brought to 90^{o}C in a water bath for 20 minutes followed by Envision™ wash buffer (Dako) for 5 minutes and incubated with endogenous peroxidase block (Dako) for 10 minutes at room temperature (Table 2.7). Before sections were incubated with mouse anti-human CCL2 antibody (R&D systems) for 30 minutes at room temperature, sections were placed in Dako EnVision™ low Ph antigen retrieval solution (Dako) and brought to 90^{o}C in a water bath for 20 minutes followed by Envision™ wash buffer wash and endogenous peroxidase block as described above (Table 2.7). After primary antibody incubation, sections were incubated with Dako EnVision™ HRP (ready-to-use, Dako) for 30 minutes at room temperature and washed in Envision™ wash buffer for 5 minutes. The detection of bound antibody was performed using according to the manufacturer's instructions. Tissue sections were counterstained with haematoxylin prior to dehydration, clearing and mounting, as described above. SIides stained with secondary antibodies only or with isotype matched antibody were included as negative controls.

2.5.2.2.3 Detection of iNOS and CCR7 with Citrate Buffer Antigen Retrieval

Paraffin embedded human breast tissue sections were heated, dewaxed and rehydrated as described previously. Slides were placed in 10 mM sodium citrate buffer [0.00378 g/ml citric acid and 0.0024 g/ml sodium citrate in MQ water, Ph=6] and brought to 90^{o}C in a water bath for 20 minutes for antigen retrieval and washed 2 times in PBS for 3 minutes each as described previously. Endogenous peroxidase activity was quenched by incubating slides with freshly prepared 30% hydrogen peroxide solution in methanol (solution contained 10 ml hydrogen peroxide, 100 ml methanol, 90 ml water) for 15 minutes. Slides were blocked with 10% goat serum in PBS for 30 minutes at room temperature before incubation with rabbit anti-mouse iNOS antibody (Santa Cruz Biotechnology) or rabbit monoclonal anti-CCR7 antibody (Abcam, Cambridge, UK) overnight at 4^{o}C respectively in a humidified chamber (Table 2.7). Sections were then either incubated with biotinylated goat anti-rabbit IgG (1:500; Dako) for 40 minutes at room temperature followed by ABC Elite kit for 30 minutes; or incubated with goat anti-rabbit HRP (1:200 dilution; Dako) for 60 minutes at room temperature. The detection of bound antibody was performed using DAB according to the manufacturer's instructions. Tissue sections were counterstained with haematoxylin prior to dehydration, clearing and mounting, as described above. Slides stained with secondary antibodies only or with isotype matched antibody was included as negative controls.

Table 2.7 Primary antibodies used for paraffin-embedded human breast tissue

Antigen	Antigen Retrieval	Reactivity	Manufacturer	Isotype	Clone	Concentration/ Dilution
Latent TGFB1 (LAP)	Citrate Buffer Ph=6	Human	R&D systems	goat IgG	N/A	20 μg/ml (1:50 overnight)
CD68	Dako EnVision™ high Ph antigen retrieval solution	Human	Dako	Mouse IgG	PG-M1	4μg/ml (1:50 30 minutes)
CD206	Dako EnVision™ high Ph antigen retrieval solution	Human	R&D systems	Mouse IgG	685645	4 μg/ml (1:50 30 minutes)
CCL2	Dako EnVision™ low Ph antigen retrieval solution	Human	R&D systems	Mouse IgG2b	23002	2.5 μg/ml (1:50 30 minutes)
iNOS	Citrate Buffer Ph=6	Mouse/ Human	SantaCruz Biotechnology	Rabbit IgG	N/A	0.25 μg/ml (1:800 overnight)
CCR7	Citrate Buffer Ph=6	Human	Abcam	Rabbit IgG	Y59	20 μg/ml (1:2000 overnight)

N/A: Not applicable

2.5.2.2.4 Image Capture

Fluorescence images of LAP were captured and collected using FV10i Confocal Microscope with laser-power and photomultiplier settings were kept constant for all experiments and images were captured at 60 x magnification. Non-fluorescently stained tissue sections were captured as a digital image using a Nanozoomer 1.0 at a zoom equivalent to 40 x objective lens.

2.5.2.2.5 Histology and Immunohistochemistry Quantification

All quantification analysis was performed blinded. Three epithelium clusters were randomly selected from each section for quantification. For each epithelial cluster, the total area of cluster (mm^2), the area

of epithelium (mm^2) and the area of stroma (mm^2) was measured and calculated. The number of CD68, CD206 and iNOS positive cells in each cluster was counted manually and only positive cells with visible haematoxylin stained nuclei were included. All results were expressed as positive cells/mm^2. The mean density of positive cells within the cluster's epithelium and stroma from each patient was calculated.

To determine the percent of CCR7 positive cells within each cluster, staining was quantified using the video image analysis software, Video Pro (Leading Edge Software, Adelaide, AU). The area of positive staining was calculated as the percent positive staining (DAB) of the total stained (haematoxylin + DAB) area, expressed as a percentage. To determine the intensity of CCL2 positive staining within the epithelium of each cluster, staining was quantified using the AnalySIS LS Professional (The University of Adelaide, SA, AU).The area of positive staining was calculated as the percent positive staining (DAB) of the total stained (haematoxylin + DAB) area within the epithelium, expressed as a percentage.

The mean fluorescent intensity of LAP within three randomly selected epithelium clusters from each section was determined and analysed by using ImageJ software and a mean value of 3 clusters per patient was calculated.

2.6 Statistical Analysis

Data were assessed for normal distribution with a Shapiro-Wilk normality test using GraphPad Prism 5 (GraphPad software Inc, San Diego, USA) or SPSS Statistics Version 17.0 (IBM Corporation, Armonk, NY, USA). Normally distributed data were analysed using an unpaired t-test. Not normally distributed data were analysed using a Mann-Whitney U test. Data are presented as the mean ± SEM (standard error of mean). Linear regression analysis was conducted using SPSS Statistics Version 17.0 (IBM Corporation, Armonk, NY, USA) if analysis of relationships between variables were required. Kaplan-Meier survival curves were generated using SPSS Statistics Version 17.0 to analyse survival function and LogRank test was used to compare different Kaplan–Meier curves between groups. The difference between groups was considered statistically significant if $p < 0.05$. In some cases different superscripts (a, b and c) were used to indicate statistical significance between group. In other cases an asterisk (*) identifies a treatment or genotype that differs significantly from the control.

Chapter 3 Regulation of macrophage phenotype in the mammary gland by epithelial cell-derived TGFB1

Statement of Authorship

Title of Paper	Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland
Publication Status	◉ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Sun X, Robertson SA, Ingman WV. Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland. Cytokine. 2013; 61(2):377–88

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Performed analysis on all samples, interpreted data, wrote manuscript Certification that the statement of contribution is accurate.
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Signature	Date: 6/7/15

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Contribution to the Paper	Helped to evaluate and edit the manuscript. Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis
Signature	Date: 8/07/15

3.1 Introduction

Transforming growth factor beta1 (TGFB1) is a multifunctional cytokine that controls many aspects of cellular function, including proliferation, differentiation, migration, apoptosis, and the immune response (Ingman and Robertson, 2002, Lyons and Moses, 1990, Jakowlew, 2006). A strong association between breast cancer risk and the TGFB1 L10P gene polymorphism has been reported by the International Breast Cancer Consortium (Cox et al., 2007). The TGFB1 L10P gene is linked to both increased cellular expression of TGFB1 and elevated circulating TGFB1 (Cox et al., 2007, Dunning et al., 2003), suggesting that TGFB1 expression is an important determinant of breast cancer risk.

TGFB1 has both stimulatory and inhibitory roles in regulating tissue homeostasis, development, remodelling, and cancer progression (Pardali and Moustakas, 2007, Jakowlew, 2006). TGFB1 acts as a tumour suppressor in the early phase of cancer development, and promotes invasion and metastasis during the later stages of cancer progression (Pardali and Moustakas, 2007). TGFB1 can suppress tumour development through inhibition of cell proliferation, induction of apoptosis, and suppression of growth factor, cytokine and chemokine production (Yang et al., 2010a, Dumont and Arteaga, 2000). However, as tumours progress, resistance to TGFB1 is acquired through mutations or inhibition of TGFB1 signalling pathways. At this time, tumour cells begin to secrete large quantities of TGFB1 which appears to further promote tumour progression (Moustakas et al., 2002, Pardali and Moustakas, 2007). This surge in TGFB1 production by tumour cells mediates epithelial-mesenchymal transition, increased angiogenesis and impairs immune surveillance, thereby promoting tumour invasion and metastasis (Dumont and Arteaga, 2000, Pardali and Moustakas, 2007, Yang et al., 2010a, Moustakas et al., 2002).

Latent TGFB1 is highly expressed by mammary epithelium during puberty, in cycling virgin mice, and during pregnancy (Robinson et al., 1991, Ewan et al., 2002, Daniel et al., 1989). The highest expression of both latent and active TGFB1 is observed at the diestrus phase of the ovarian cycle (Ewan et al., 2002). TGFB1 has a largely inhibitory role in regulating mammary gland epithelial cell proliferation and ductal development (Jhappan et al., 1993, Pierce et al., 1993, Gorska et al., 1998, Bierie et al., 2009, Ewan et al., 2002, Ingman and Robertson, 2008). However, as a multi-functional cytokine, it is highly likely that TGFB1 in the mammary gland acts on other cell types within the stroma. These paracrine effects are yet to be investigated, and may contribute to the increased breast cancer risk associated with increased expression of TGFB1.

Key target cells for TGFB1 action are macrophages, which are bone marrow-derived cells present in the mammary gland stroma that regulate epithelial cell function in normal development. In estrous cycling adult mice, macrophages promote epithelial cell proliferation, alveolar development, phagocytosis dying epithelium and tissue remodelling (Chua et al., 2010). In addition to their function in regulation of epithelial cell turnover during the ovarian cycle, colony-stimulating factor1 (CSF1)-regulated macrophages contribute to development during puberty (Gouon-Evans et al., 2000, Ingman et al., 2006), pregnancy (Pollard and Hennighausen, 1994) and the switch to a lactational state (Pollard and Hennighausen, 1994). Macrophages have also been shown to support stem cell activity in the mammary gland (Gyorki et al., 2009). In breast cancer, macrophages play multiple roles in metastasis, and promote growth and survival of tumour cells, angiogenesis and cell invasion (Pages et al., 2010, Yang et al., 2010a).

Macrophages are considered as key players in immune system responses that protect against tumourigenesis through their actions as professional antigen-presenting cells involved in phagocytosis of tumour cells and processing and presentation of tumour antigens (Pollard, 2008). Macrophages are also implicated in promotion of tumourigenesis in many tissues, particularly tumours of epithelial cell origin in the breast, ovary and endometrium (Lin et al., 2001, Kacinski, 1995). Anti- and pro-tumorigenic properties of macrophages have been generally assigned as “M1” and “M2” macrophage functions respectively (Mantovani et al., 2002). In broad terms, M1 macrophages are mainly engaged in antigen presentation and immune surveillance, while M2 macrophages participate in tissue development, wound healing and repair (Gordon and Taylor, 2005, Mantovani et al., 2002, O'Brien et al., 2010b, Stout et al., 2009). Although this paradigm is likely to be a simplification of the physiological situation (Mosser and Zhang, 2008), it is clear that the diversity of macrophage phenotypes permits their direction of distinct biological processes, with the balance of tumour promoting or tumour inhibiting effects being dependent on signals emanating from the local tissue microenvironment.

Tgfb1 and *Csf1* null mutant mice have strikingly similar reproductive phenotypes, including perturbation in mammary gland development and function (Ingman and Robertson, 2009), suggesting the possibility of a mechanistic link between TGFB1 and macrophages in the mammary gland. Furthermore, in established mammary gland tumours, TGFB signalling in the epithelium regulates recruitment of macrophages and expression of inflammatory markers (Bierie et al., 2008). The experiments described in this chapter were devised to investigate cross-talk between epithelial cell-derived TGFB1 and

macrophages, to better understand the role of TGFB1 in regulation of function in of both the mouse and human mammary gland. A mouse mammary gland transplant mouse model was established where the mammary gland tissue from *Tgfb1* null mutant and wild-type mice were transplanted into TGFB1 replete recipients, and the role of epithelial cell-derived TGFB1 in regulation of macrophage functions in mice was investigated. The correlation between TGFB1 expression and macrophage phenotype in human breast was also examined.

3.2 Co-localisation of active TGFB1 and macrophages in mammary gland

TGFB1 is secreted as an inactive latent complex (LTGFB1) consisting of the active mature TGFB1 dimer non-covalently bound to a latency associated peptide (LAP) (Barcellos-Hoff, 1996, Lawrence et al., 1985, Fleisch et al., 2006). This large complex is associated with latent TGFB binding protein (LTBP) (Lawrence et al., 1985). Active TGFB1 has a half-life of 2 minutes, whereas TGFB1 associated with its latent complex is substantially more stable, with a half-life of 90 minutes (Wakefield et al., 1990). Activation of latent TGFB1 through heat, altered Ph or proteolysis (Barcellos-Hoff, 1996, Lawrence et al., 1985, Lyons et al., 1990, Ehrhart et al., 1997) is necessary before TGFB1 can exert biological effects (Barcellos-Hoff, 1996). Therefore, activation of TGFB1 from the latent form is a key regulatory event that controls its biological availability (Ewan et al., 2002, Wakefield et al., 1990, Fleisch et al., 2006).

In order for TGFB1 to regulate macrophage function, active TGFB1 must be localised in the immediate vicinity of macrophages within the stroma surrounding mammary epithelial cells. To examine the spatial relationship between active TGFB1 and macrophages in the mammary gland, dual-label immunofluorescence was performed on frozen sections from wild-type FVB mice at diestrus. Active TGFB1 was observed throughout the mammary gland epithelium and stroma (Figure 3.1A, 3.1C, 3.1D and 3.1F), as previously described (Ewan et al., 2002). The sections were also counterstained with DAPI (Figure 3.1E). Macrophages were localised immediately subjacent to the mammary epithelium within the stroma and were in close proximity to active TGFB1 (Figure 3.1B, 3.1C, 3.1D and 3.1F). Positive staining with anti-active TGFB1 antibody was not observed in *Tgfb1* null mutant mouse mammary tissue (Figure 3.1G and 3.1J), or in sections stained with isotype matched negative control antibodies (Figure 3.1H and 3.1K). No positive staining of active TGFB1 was observed in TGFB1 replete mammary gland transplanted with *Tgfb1-/-* epithelium (Figure 3.1I and 3.1L), confirming previous reports that the epithelium is the primary source of TGFB1 production in the mammary gland (Ewan et al., 2002, Daniel et al., 1989, Robinson et al., 1991).

3.3 Dual-label immunofluorescence of mammary epithelial cells and macrophages in the mammary gland

To investigate whether mammary epithelial cells express macrophage markers, which would confound interpretation of our results, dual-label immunofluorescence was performed on frozen sections from adult FVB mice using the luminal epithelial cell marker TROMA-1 (cytokeratin 8) and macrophage markers F4/80 and MHCII. F4/80-positive macrophages and MHCII-positive macrophages were mainly located within the mammary gland stroma (Figure 3.2A and 3.2B respectively), whereas mammary epithelial cells expressing cytokeratin 8 were clearly a distinct population (Figure 3.2D and 3.2E). Neither macrophage marker F4/80 nor MHCII was expressed by TROMA-1-positive epithelial cells; however F4/80-positive macrophages and MHCII-positive macrophages were in close physical contact with the mammary epithelium (Figure 3.2G, 3.2H, 3.2J and 3.2K). Positive staining was not observed in mammary gland tissue stained with isotype matched negative control antibodies (Figure 3.2C, 3.2F, 3.2I and 3.2L).

3.4 Effect of epithelial cell-derived TGFB1 on macrophage abundance and phenotype in the mammary gland at diestrus

To investigate the effect of epithelial cell-derived TGFB1 on macrophage function in mouse mammary gland, a mammary gland transplant model was established (as described in Chapter 2.2.3). In each recipient mouse, one side of the mammary epithelium was TGFB1 deficient and the contra-lateral side of the mammary epithelium was TGFB1 replete, however, the stroma of each side of transplanted mammary epithelium was TGFB1 replete.

Expression of TGFB1 by mammary gland epithelium is highest at diestrus (Ewan et al., 2002), and therefore this stage was selected to analyse the effect of epithelial cell-derived TGFB1 on macrophage function. However, between mouse variation in progesterone secretion at diestrus could confound these experiments, as progesterone also regulates macrophage phenotype and function (Chua et al., 2010). In order to standardise the hormonal environment and mimic diestrus, a model employing hormone replacement in ovariectomised mice was utilised (as described in Chapter 2.2.4 and 2.2.5). This model has been shown to promote mammary gland ductal and alveolar development similar to that observed at the diestrus phase of the ovarian cycle (Hodson et al., 2013, Chua et al., 2010).

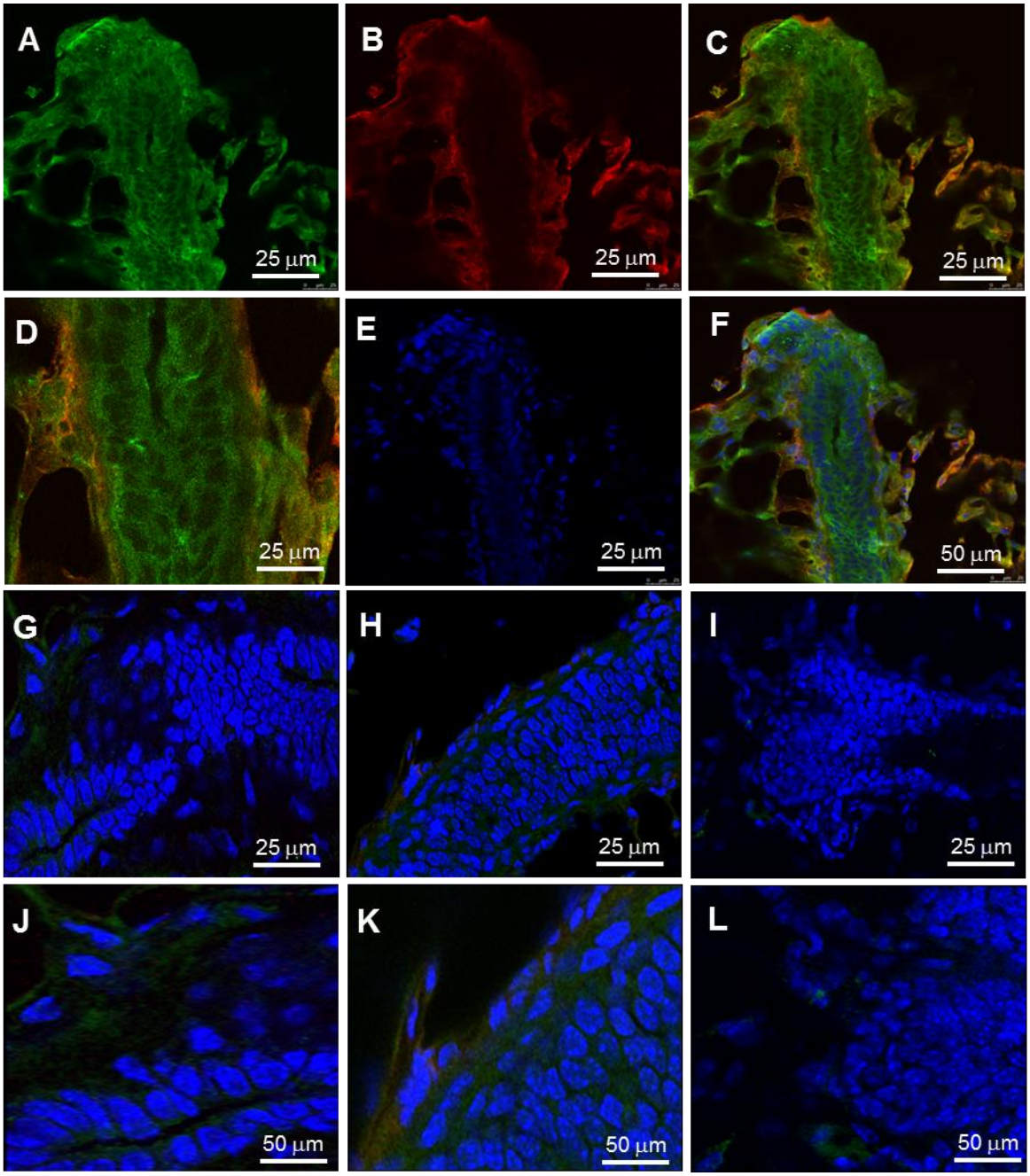

Figure 3.1 The co-localisation of active TGFB1 and macrophages in the mammary gland at diestrus.

Frozen mammary gland tissue sections from adult diestrus FVB mice were stained simultaneously with antibody reactive with active TGFB1 and F4/80 antibody to detect macrophages. Active TGFB1 (green) (A) and macrophages (red) (B) were visualised simultaneously under a confocal microscope (C: merged picture of A and B; D: zoomed in version of C) .The sections were also counterstained with DAPI (E; F: merge picture of C and E). The application of anti-active TGFB1 antibody on mammary gland tissue from an adult Tgfb1 null mutant mouse (G; J: zoomed in version of G), the application of secondary antibodies only on mammary gland tissue from a diestrus FVB mouse (H; K: zoomed in version of H) and the application of anti-active TGFB1antibody on transplanted Tgfb1-/- tissue (I; L: zoomed in version of I) were used as negative controls.

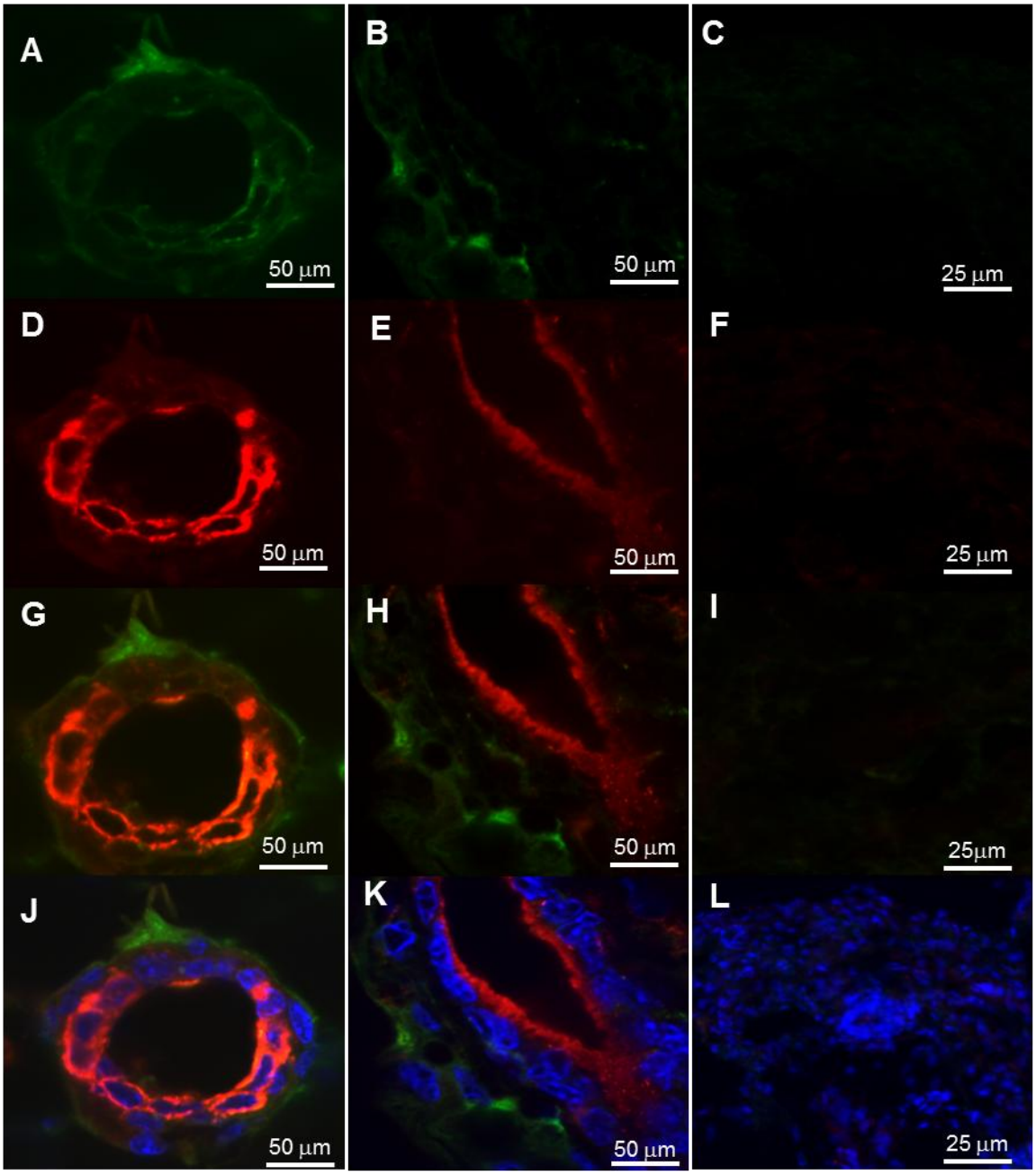

Figure 3.2 The dual-label immunofluorescence of mammary epithelial cells and F4/80-postive/MHCII-positive macrophages in the mammary gland.

Frozen mammary gland tissue sections from adult FVB mice were stained subsequently with antibody reactive with mammary luminal epithelial cells and F4/80 or MHCII antibody to detect macrophage. F4/80-positive macrophages (green) (A) or MHCII-positive macrophages (green) (B) and mammary luminal epithelial cells (red) (D and E) were visualised simultaneously under a confocal microscope (G: merge picture of A and D; H: merge picture of B and E). The application of secondary antibodies only on the section from the same FVB mouse (C: only strepavidin FITC was applied; F: only Alexa Fluor 594 was applied) was used as negative control (I: merge picture of C and F). All the sections were counterstained with DAPI (J: merge picture of G and DAPI; K: merge picture of H and DAPI; L; merge picture of I and DAPI).

To evaluate the role of epithelial cell-derived TGFB1 in regulation of macrophages, immunohistochemistry was performed to detect F4/80, CCR7 and iNOS positive cells from mammary gland transplanted with *Tgfb1*+/+ or *Tgfb1*-/- epithelium.

3.4.1 F4/80

F4/80 is expressed at high levels on the cell membrane of macrophages, and is a well-known macrophage differentiation marker (Austyn and Gordon, 1981). F4/80-positive macrophages were observed in close proximity to ductal and alveolar epithelium in mammary glands transplanted with *Tgfb1*+/+ (Figure 3.3A and 3.3D respectively) and *Tgfb1*-/- epithelium (Figure 3.3B and 3.3E respectively). No positive staining was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.3C, 3.3F). In both genotypes and epithelial cell subpopulations, the F4/80-positive macrophages were localised both within the stroma surrounding the epithelium, as well as in between epithelial cells. There was no significant difference in macrophage density within the stroma between *Tgfb1*+/+ and *Tgfb1*-/- epithelium (Figure 3.3G, 3.3H). However, there was a 48% increase in the number of macrophages within *Tgfb1*-/- ductal epithelium (1304 ± 142 macrophage density/mm^2; p=0.026; unpaired t-test) compared to *Tgfb1*+/+ ductal epithelium (884 ± 125 macrophage density/mm^2) (Figure 3.3G, 3.3H). Additionally there was a 50% increase in the number of macrophages within *Tgfb1*-/- alveolar epithelium (1327 ± 154 macrophage density/mm^2; p=0.028; unpaired t-test) compared to *Tgfb1*+/+ alveolar epithelium (882 ± 54 macrophage density/mm^2) (Figure 3.3G, 3.3H).

3.4.2 iNOS

iNOS is highly expressed by activated cytotoxic macrophages involved in antigen presentation and immune surveillance (O'Brien et al., 2010b) and is considered a marker for “M1” type macrophages (Mantovani et al., 2002). iNOS expression was observed in both the mammary gland epithelium and stromal compartment. Previous studies report that iNOS is synthesized by most of the epithelial cells in normal breast tissue as well as by macrophages (Bulut et al., 2005, Tschugguel et al., 1999). Therefore, it is not possible to distinguish between iNOS-positive macrophages and iNOS-positive epithelial cells within the epithelium, and hence only iNOS-positive macrophages within the stroma surrounding ductal and alveolar epithelium in mammary gland transplanted with *Tgfb1*+/+ (Figure 3.4A and 3.4D respectively) and *Tgfb1*-/- (Figure 3.4B and 3.4E respectively) could be quantified. No positive staining

was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.4C, 3.4F). There was a 54% increase in the number of iNOS-positive macrophages within the ductal stroma of *Tgfb1-/-* epithelium (174 ± 16 iNOS-positive cells/mm^2; p=0.014; unpaired t-test) compared to that of *Tgfb1+/+* epithelium (113 ± 7 iNOS-positive cells/mm^2) (Figure 3.4G). There was a 78% increase in the number of iNOS-positive macrophages within the alveolar stroma associated with *Tgfb1-/-* epithelium (208 ± 14 iNOS-positive cells/mm^2; p=0.005; unpaired t-test) compared to alveolar stroma of *Tgfb1+/+* epithelium (117 ± 8 iNOS-positive cells/mm^2) (Figure 3.4G).

3.4.3 CCR7

CCR7 is a chemokine receptor involved in lymphocyte homing (Cunningham et al., 2010) that is highly expressed by breast cancer cells and promotes metastasis (Muller et al., 2001, Andre et al., 2006). CCR7 is also a phenotypic marker of "M1" macrophages and is uniquely expressed on adipose tissue macrophages, which promote inflammation in obese mice (Zeyda et al., 2010, Mantovani et al., 2002). CCR7-positive cells were located in the stroma surrounding ductal and alveolar epithelium of mammary glands transplanted with *Tgfb1+/+* (Figure 3.5A and 3.5D respectively) and *Tgfb1-/-* epithelium (Figure 3.5B and 3.5E respectively). No positive staining was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.5C, 3.5F). There was no significant difference in the number of CCR7-positive cells within the ductal stroma associated with *Tgfb1+/+* epithelium (236 ± 72 CCR7-positive cells/mm^2) compared with *Tgfb1-/-* epithelium (378 ± 66 CCR7-positive cells/mm^2; p=0.17, unpaired t-test) (Figure 3.5G). However, there was a 2-fold increase in the number of CCR7-positive macrophages within the alveolar stroma of *Tgfb1-/-* epithelium (563 ± 80 CCR7-positive cells/mm^2) compared with *Tgfb1+/+* epithelium (256 ± 89 CCR7-positive cells/mm^2; p=0.026; unpaired t-test) (Figure 3.5G).

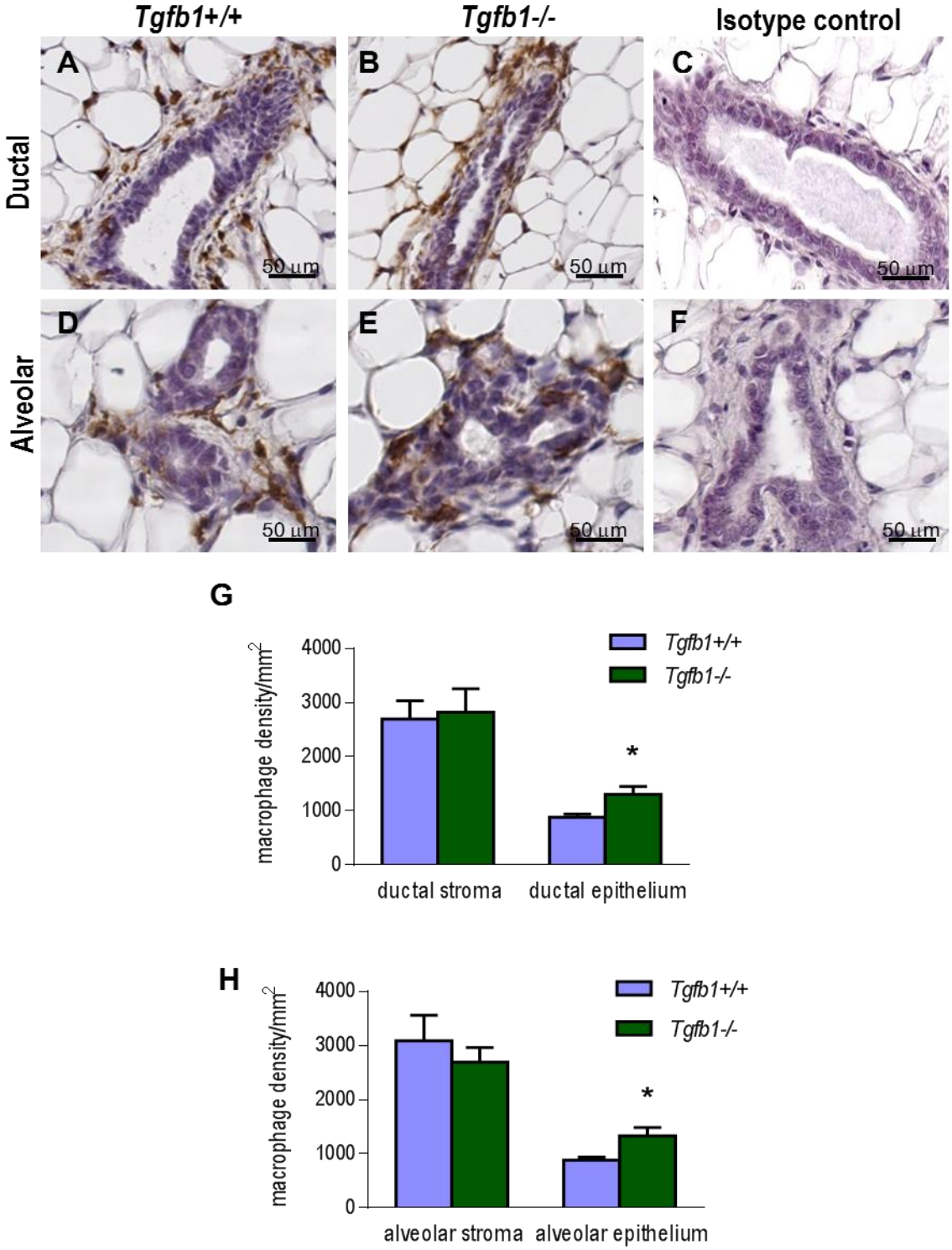

Figure 3.3 The effect of epithelial cell-derived TGFB1 on macrophage abundance and location within and around mammary epithelium.

Paraffin sections of mammary gland tissue transplanted from *Tgfb1*+/+ mice (n=6) and *Tgfb1*-/- mice (n=7) were stained with macrophage-specific F4/80 antibody to detect macrophages within ductal epithelium and stroma (A and B) and within alveolar epithelium and stroma (D and E). Brown staining are macropahages. Isotype matched irrelevant antibody negative controls (C and F are ductal and alveolar epithelium respectively) were included. The number of F4/80-positive cells within ductal epithelium and stroma, and within alveolar epithelium and stroma were calculated and expressed as macrophage density/mm^2 (G and H). Data are presented as mean ± SEM with statistical analysis using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

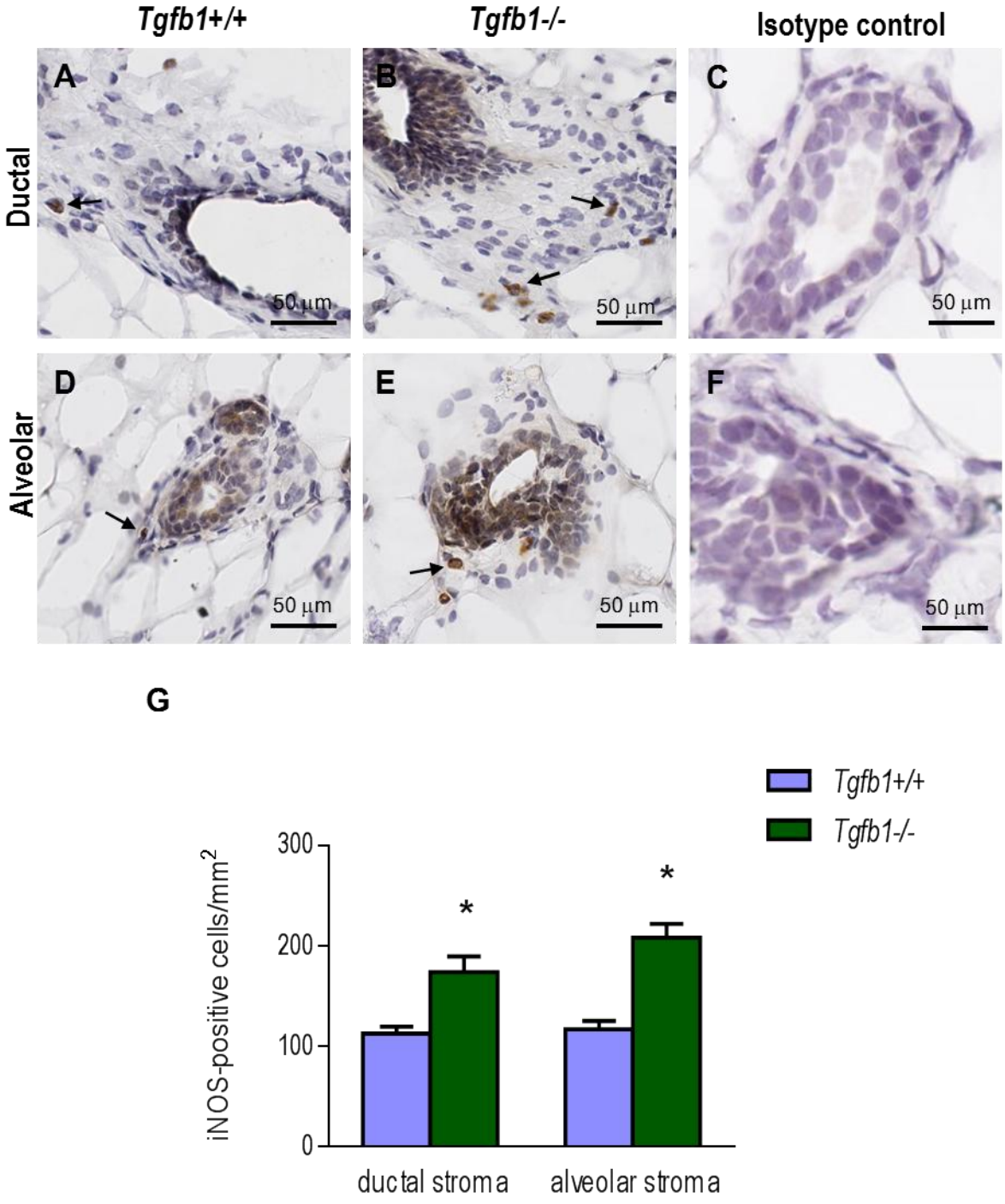

Figure 3.4 The effect of epithelial cell-derived TGFB1 on abundance and location of iNOS-positive cells within mammary epithelium stroma.

Frozen sections of mammary gland tissue transplanted from *Tgfb1+/+* mice (n=5) and *Tgfb1-/-* mice (n=8) were stained with anti-iNOS antibody to detect iNOS-positive macrophages (arrows) within mammary ductal epithelium stroma (A and B) and alveolar epithelium stroma (D and E). Isotype matched irrelevant antibody negative controls (C and F are ductal and alveolar epithelium respectively) were included. The number of iNOS-positive cells within mammary epithelium stroma were calculated and expressed as iNOS-positive cells/mm^2 (G) Data are presented as mean ± SEM with statistical analysis using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

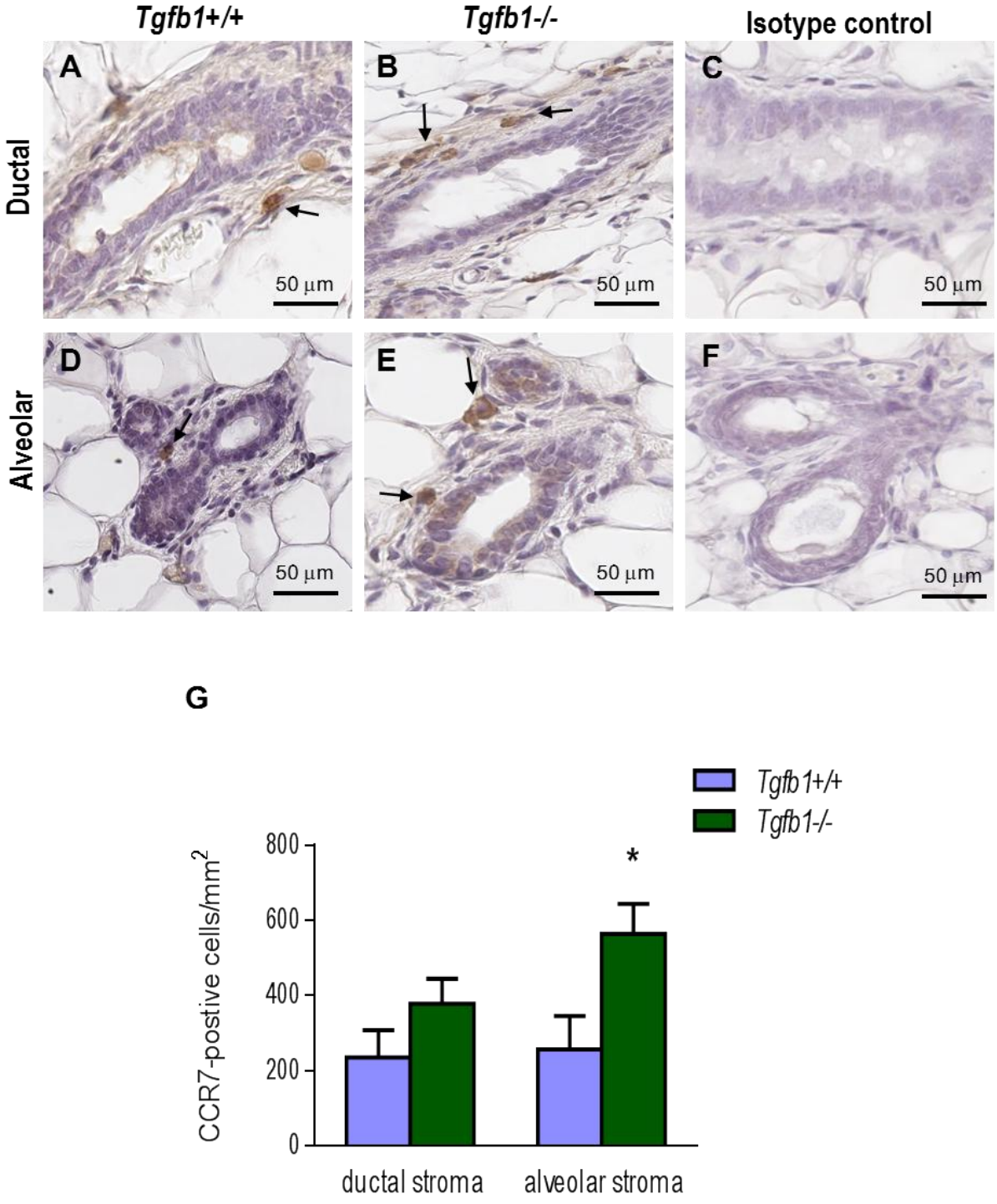

Figure 3.5 The effect of epithelial cell-derived TGFB1 on abundance and location of CCR7-positive cells within mammary epithelium stroma.

Paraffin sections of mammary gland tissue transplanted from *Tgfb1*+/+ mice (n=6) and *Tgfb1*-/- mice (n=7) were stained with anti-CCR7 antibody to detect CCR7-positive macrophages (arrows) within mammary ductal epithelium stroma (A and B) and alveolar epithelium stroma (D and E.) Isotype matched irrelevant antibody negative controls were included (C and F are ductal and alveolar epithelium respectively) were included. The number of CCR7-positive cells within mammary epithelium stroma were calculated and expressed as CCR7-positive cells/mm^2 (G). Data are presented as mean ± SEM with statistical analysis using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

3.5 Relationship between epithelial cell-derived TGFB1 and macrophage phenotype in human non-neoplastic breast tissue

After demonstrating that epithelial cell-derived TGFB1 regulates macrophage function and phenotype in the mouse mammary gland, a series of experiments were conducted to investigate whether TGFB1 plays a similar role in regulating macrophages in the human breast. Polymorphisms in the gene encoding transforming growth factor beta 1 (TGFB1) are known to affect breast cancer risk (Cox et al., 2007), however, the expression pattern of TGFB1 by human mammary epithelium is yet to be investigated. To evaluate the role of epithelial cell-derived TGFB1 in regulation of macrophages in human non-neoplastic breast tissue obtained from women who have undergone breast surgery, immunohistological analysis was performed to detect TGFB1, CD68, CD206, iNOS and CCR7 expression, and correlations between abundance of TGFB1 and different phenotypes were evaluated.

3.5.1 Relationship between TGFB1 expression and CD68+ cell abundance

Latent TGFB1 was observed throughout the non-neoplastic human breast epithelium (Figure 3.6 A, B, G and H), suggesting that TGFB1 is predominantly expressed by the epithelium in the human breast; however, the level of TGFB1 expression was varied among participants. The sections were also counterstained with DAPI (Figure 3.6 D, E and F). No positive staining of latent TGFB1 was observed in tissue stained with secondary antibody only (Figure 3.6 C and I).

CD68 is a well-known macrophage marker which is highly expressed by human monocytes and tissue macrophages (Holness and Simmons, 1993). CD68-positive cells were found to be located within three different locations; inter-epithelial CD68-positive macrophages were macrophages that located between adjacent epithelial cells, epithelial-associated CD68-positive macrophages were macrophages that aligned along the epithelium but between epithelial cells and stromal CD68-positive macrophages were macrophages within the stroma (Figure 3.7 A). No positive staining was observed in mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.7 B).

There was no significant statistical correlation between latent TGFB1 expression and the abundance of epithelial-associated CD68-positive macrophages (Figure 3.8 A; R^2= 0.1963, p=0.057). However, there was a significant negative relationship between the expression of latent TGFB1 and the abundance of

inter-epithelial-associated CD68-positive macrophages (Figure 3.8 B; R^2= 0.2555, p=0.027) and there was a significant negative relationship between the level of latent TGFB1 expression and the abundance of stromal-associated CD68-positive macrophages (Figure 3.8 C; R^2= 0.3289, p=0.01).

3.5.2 Relationship between TGFB1 expression and CD206+ cell abundance

CD206 is a macrophage mannose receptor, and it is widely accepted as a "M2" macrophage marker. CD206 is found to participate in phagocytosis of bacteria and fungi, antigen presentation and resolution of inflammation (Ezekowitz and Gordon, 1984, Stein et al., 1992, Gazi and Martinez-Pomares, 2009). CD206-positive cells were found to be located within three different locations; inter-epithelial CD206-positive cells were cells that located between adjacent epithelial cells, epithelial-associated CD206-positive cells were cells that aligned along the epithelium but between epithelial cells and stromal CD206-positive cells were cells within the stroma (Figure 3.9 A). No positive staining was observed in mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.9 B).

There was no significant statistical correlation between latent TGFB1 expression and the abundance of epithelial-associated or inter-epithelial-associated CD206-positive cells (Figure 3.10 A; R^2= 0.0019, p=0.864; Figure 3.10 B; R^2= 0.1303, p=0.14). However, there was a significant positive relationship between the expression of latent TGFB1 and the abundance of stromal-associated CD207-positive cells (Figure 3.10 C; R^2= 0.327, p=0.013).

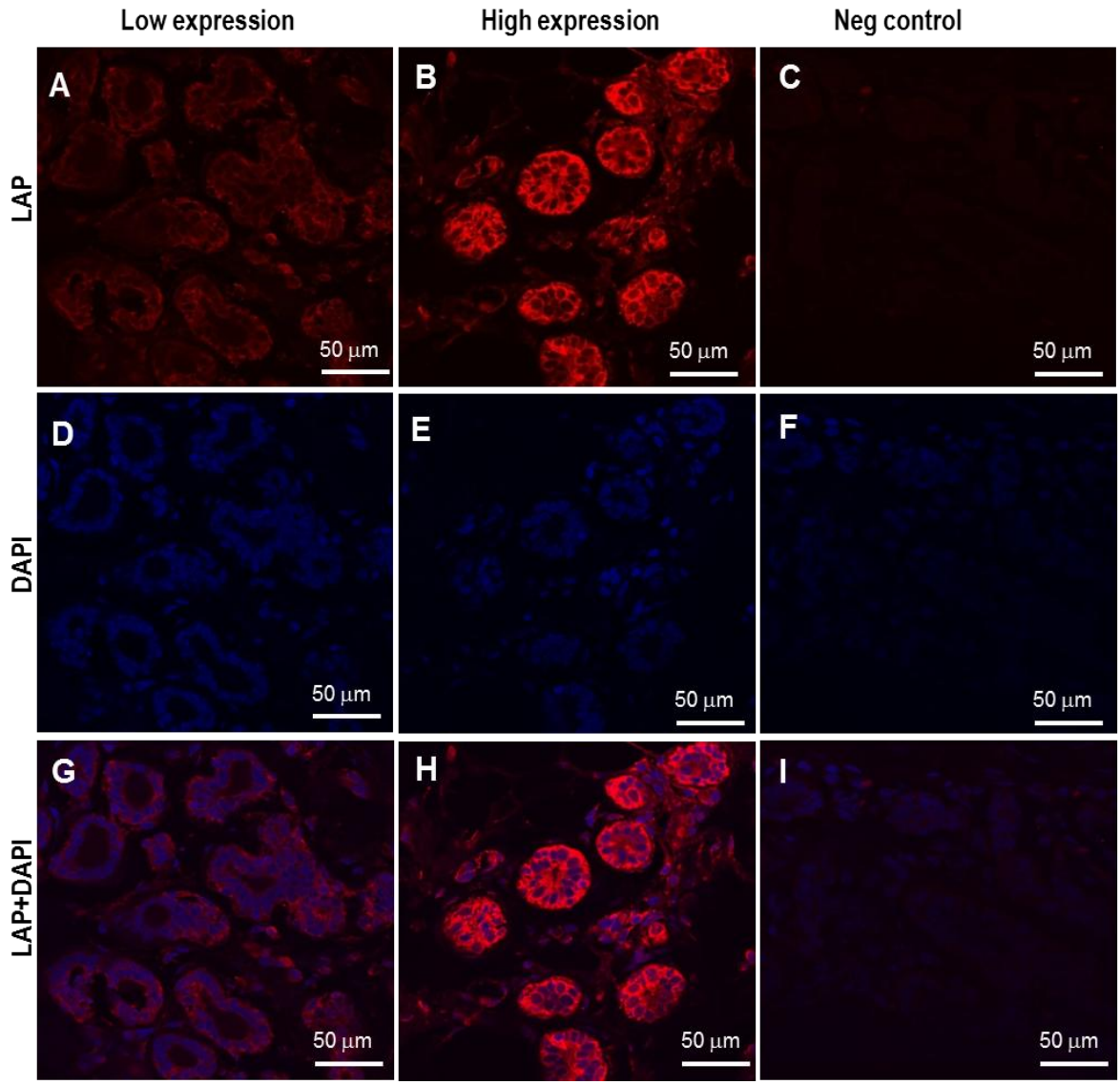

Figure 3.6 Detection of latent TGFB1 in human non-neoplastic breast tissue.

Anti-latent TGFB was used to detect TGFB1 expression in non-neoplastic human breast tissue (n=19) (A and B). The sections were also counterstained with DAPI (D, E and F). Latent TGFB1 and DAPI were visualised simultaneously under a confocal microscope (G: merged picture of A and D; H: merged picture of B and E). Immunostainings of A, D and G represented breast tissue with low level expression of TGFB1, whereas immunostainings of B, E and H represented breast tissue with high level expression of TGFB1.The application of secondary antibodies only on human breast tissue (C; I: merged picture of C and F) was used as negative control.

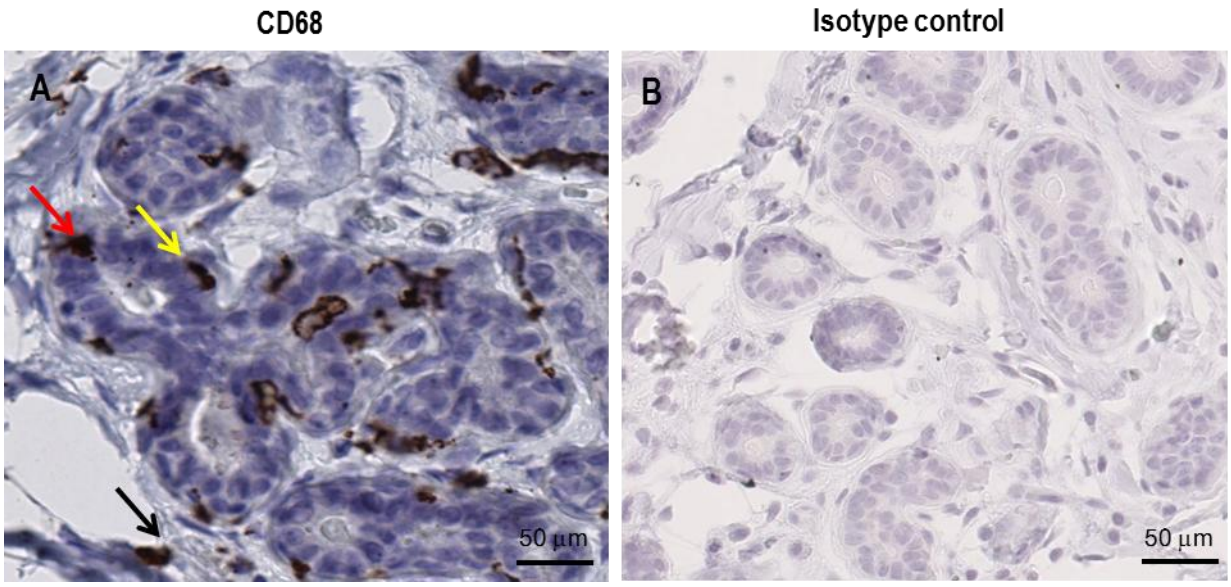

Figure 3.7 Detection of the abundance of CD68-positive macrophages in human non-neoplastic breast tissue.

Anti-CD68 macrophage antibody was used to detect the density of CD68-positive macrophage (A) in non-neoplastic human breast tissue (n=19). Macrophages were found within the epithelium (red arrow), aligned along the epithelium (yellow arrow) and within the stroma (black arrow). Isotype matched irrelevant antibody negative control (B) was included.

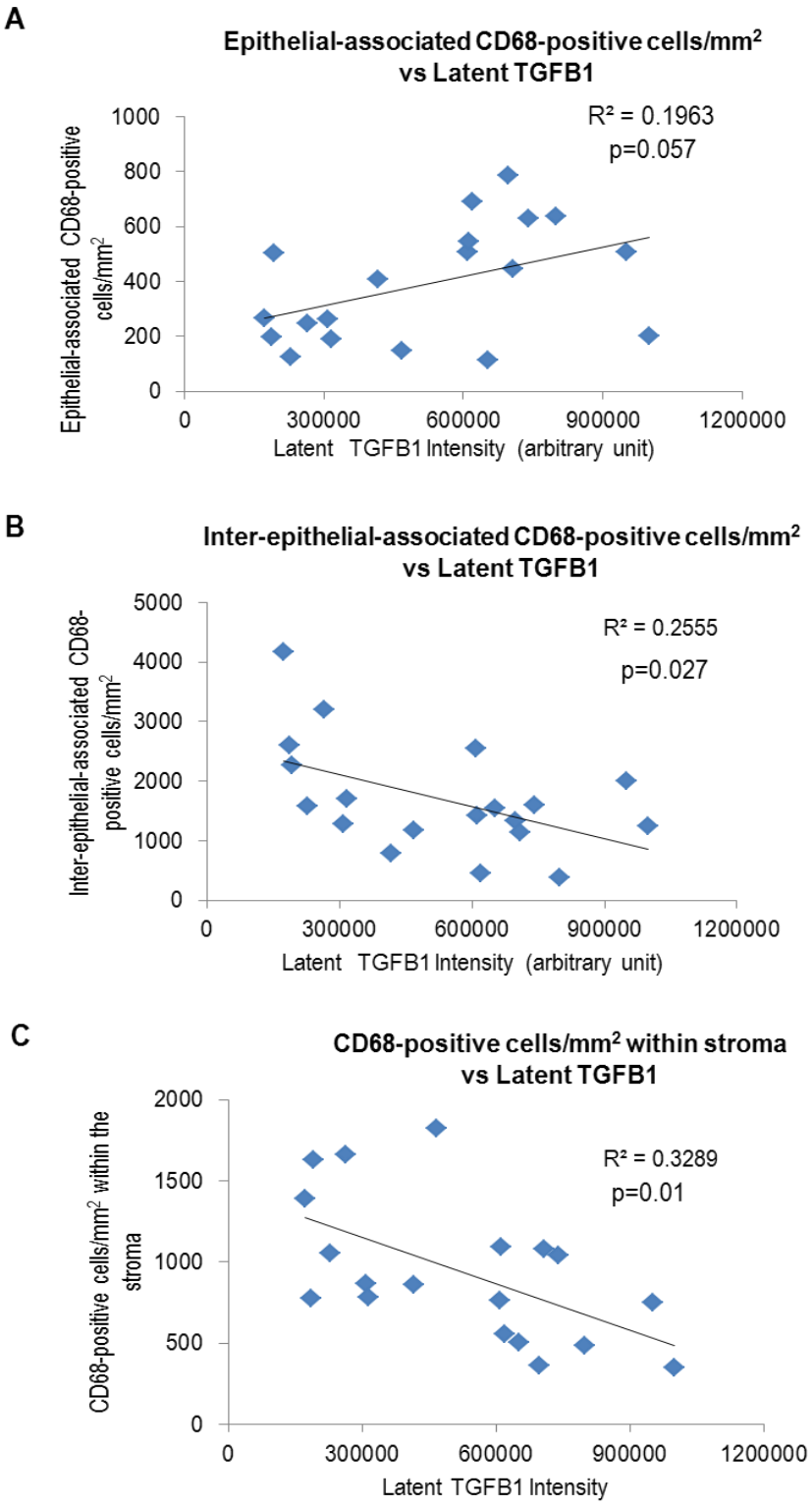

Figure 3.8 Relationship between the expression of latent TGFB1 and the abundance of CD68-positive macrophages.

TGFB1 expression by breast epithelium was not significantly associated with CD68-positive macrophage density around the epithelium (A), however, it was significantly negatively associated with CD68-positive macrophage density within the epithelium (B) and significantly negatively associated with CD68-positive macrophage density within the stroma (C). Linear regression analysis was conducted using SPSS Statistics Version 17.0, Significant differences were inferred when p values were ≤0.05, n=19.

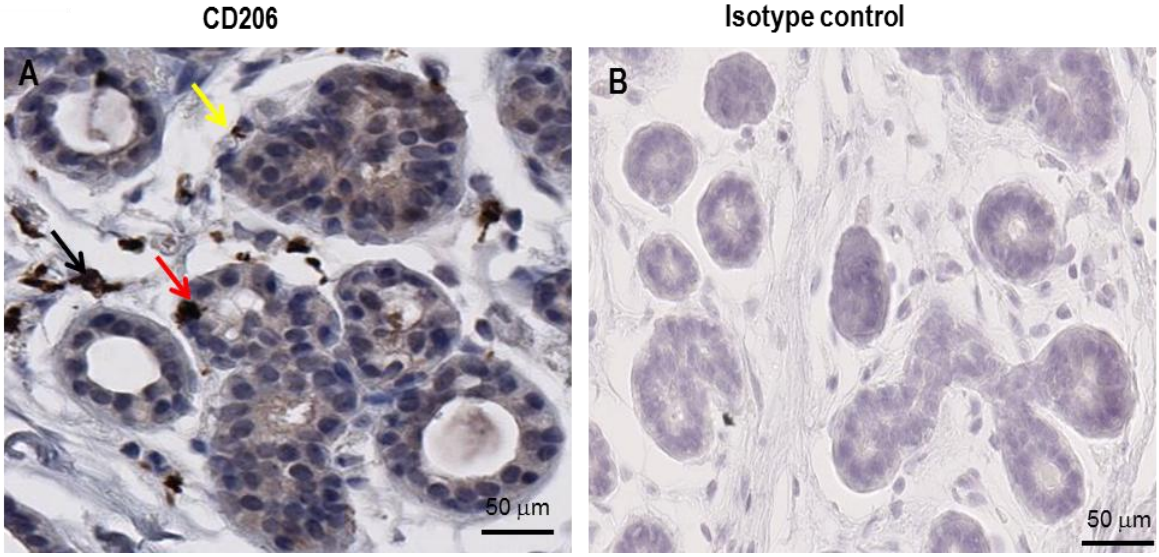

Figure 3.9 Detection of the abundance of CD206-positive cells in human non-neoplastic breast tissue.

Anti-CD206 antibody was used to detect CD206-positive cell density (A) in non-neoplastic human breast tissue (n=19). CD206-positive cells were found within the epithelium (red arrow), aligned along the epithelium (yellow arrow) and within the stroma (black arrow). Isotype matched irrelevant antibody negative control (B) was included.

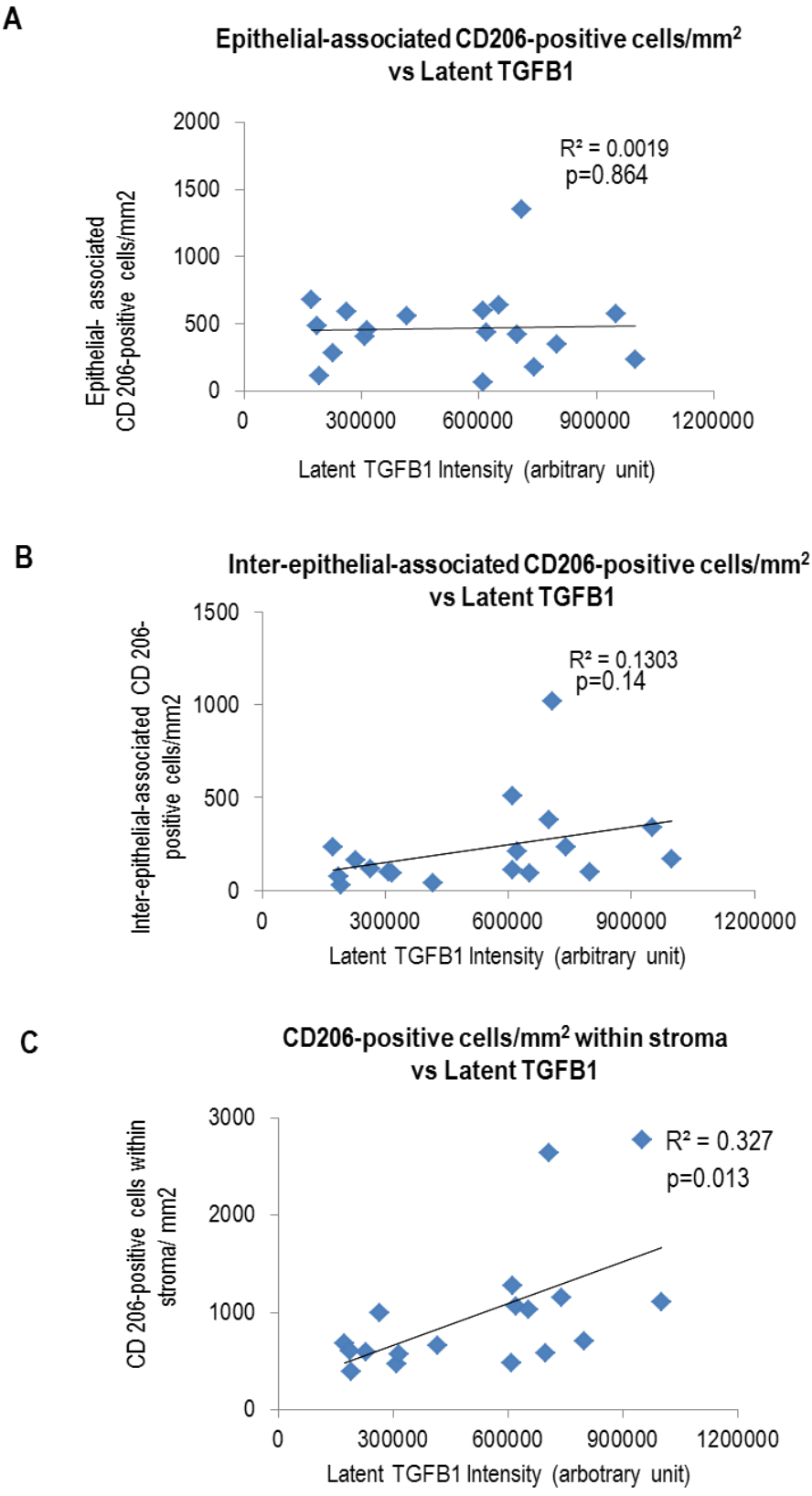

Figure 3.10 Relationship between the expression of latent TGFB1 and the abundance of CD206-positive cells.

TGFB1 expression by breast epithelium was not significantly associated with CD206-positive cell density around the epithelium (A) or within the epithelium (B), however, it was significantly negatively associated with CD206-positive cell density within the stroma (C). Linear regression analysis was conducted using SPSS Statistics Version 17.0, Significant differences were inferred when p values were ≤0.05, n=19.

3.5.3 Relationship between TGFB1 expression and iNOS abundance

iNOS-positive cells were mainly located within the stroma of breast mammary epithelium (Figure 3.11 A). No positive staining was observed in mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.11 B). There was no significant statistical correlation between the expression of latent TGFB1 and the abundance of stromal-associated iNOS-positive cells (Figure 3.11 C; R^2= 0.0218, p=0.55).

3.5.4 Relationship between TGFB expression and CCR7 abundance

CCR7-positive cells were mainly located around the mammary epithelium (Figure 3.12 A). No positive staining was observed in mammary epithelium stained with isotype matched irrelevant antibody (Figure 3.12 B). There was no significant statistical correlation between the expression of latent TGFB1 and the percentage of CCR7-positive cells (Figure 3.12 C; R^2= 0.1291, p=0.156).

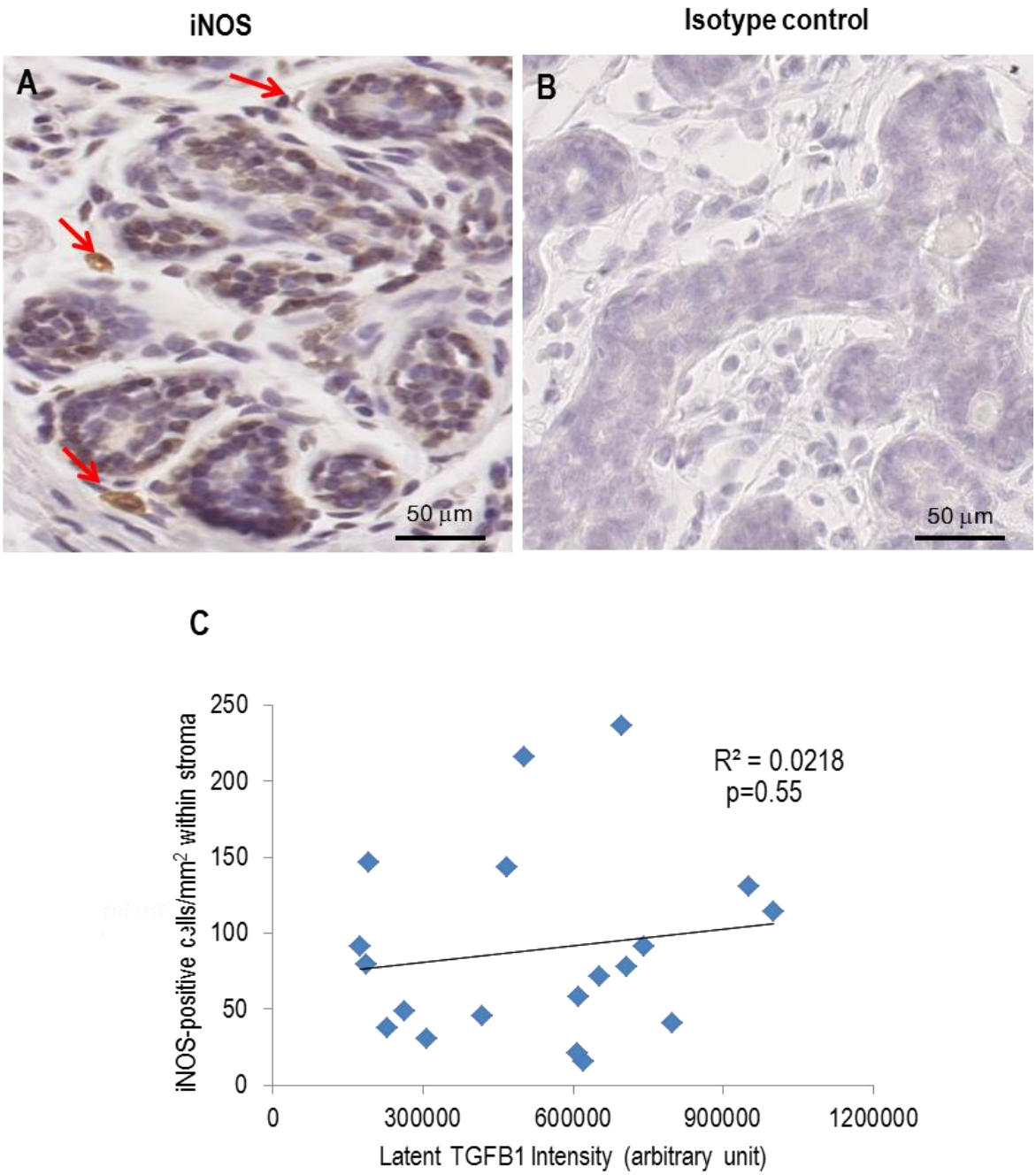

Figure 3.11 Relationship between the expression of latent TGFB1 and the abundance of iNOS-positive cells.

Anti-iNOS antibody was used to detect iNOS-positive cell density (A) in non-neoplastic human breast tissue. Isotype matched irrelevant antibody negative control (B) was included. TGFB1 expression by breast epithelium was not significantly associated with iNOS-positive cell density within the stroma (C). Linear regression analysis was conducted using SPSS Statistics Version 17.0, Significant differences were inferred when p values were ≤0.05, n=19.

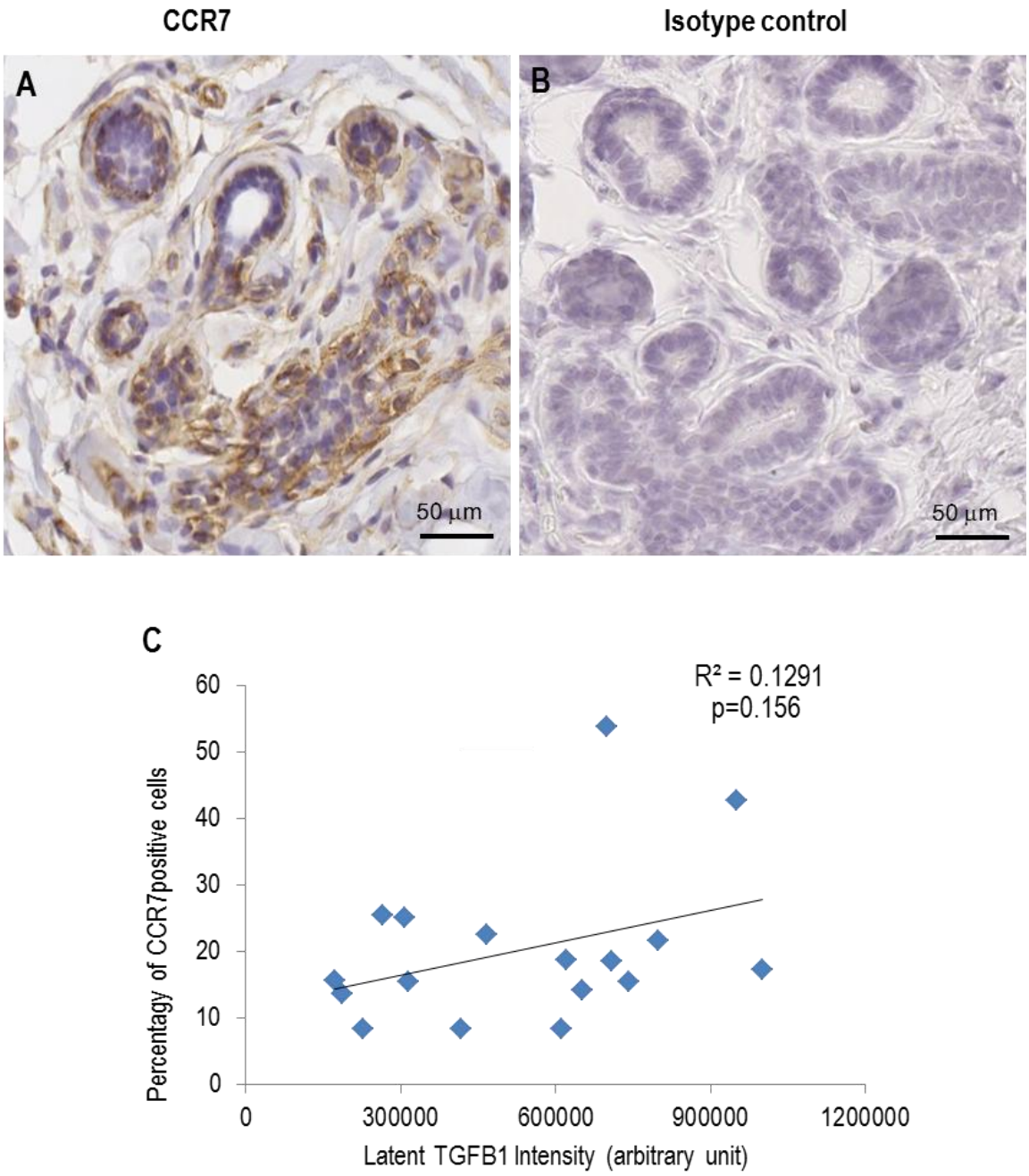

Figure 3.12 Relationship between the expression of latent TGFB1 and the abundance of CCR7-positive cells.

Anti-CCR7 antibody was used to detect CCR7-positive cell density (A) in non-neoplastic human breast tissue. Isotype matched irrelevant antibody negative control (B) was included. TGFB1 expression by breast epithelium was not significantly associated with the percentage of CCR7-positive cell density around the epithelium (C) Linear regression analysis was conducted using SPSS Statistics Version 17.0, Significant differences were inferred when p values were ≤0.05, n=19.

3.6 Discussion

Studies utilising a variety of genetically modified mouse models have shown both macrophages and TGFB1 play essential roles in mediating complex developmental processes in the mammary gland (Pierce et al., 1993, Gorska et al., 1998, Ingman and Robertson, 2008, Gouon-Evans et al., 2000, Pollard and Hennighausen, 1994, Van Nguyen and Pollard, 2002, Cecchini et al., 1994). Although macrophages are considered key target cells for TGFB1 action based on studies in other tissues, the cross-talk between epithelial cell-derived TGFB1 and macrophages in the mammary gland has not previously been investigated. The experiments described in this chapter have clearly demonstrated that epithelial cell-derived TGFB1 plays a significant role in regulation of local macrophage abundance and phenotype in both mouse and human mammary gland.

3.6.1 Co-localisation of TGFB1 and macrophages in the mammary gland

TGFB1 is produced in a latent form and must be activated to exert a biological effect (Barcellos-Hoff, 1996, Nunes et al., 1997). Active TGFB1 has a short half-life and its site of action is therefore likely to be limited to the immediate vicinity (Nunes et al., 1997). In the mammary gland, *Tgfb1* mRNA is mainly expressed by mammary epithelial cells (Daniel et al., 1989). Previous studies have shown macrophages have close spatial association with the mammary epithelium and they are highly abundant in cycling non-pregnant gland during diestrus (Cecchini et al., 1994, Chua et al., 2010). Dual-staining immunofluorescence confirms the co-localisation of epithelial cell-derived TGFB1 and macrophages at diestrus, implying that TGFB1 would be available to target macrophages and other stromal cells in the non-pregnant mammary gland.

3.6.2 Epithelial cell-derived TGFB1 regulates macrophages in the mouse mammary gland

Increased penetration of macrophages into both the ductal and alveolar epithelium was observed in mammary glands deficient in epithelial cell-derived TGFB1. In the naturally cycling mammary gland, macrophages invade into the epithelium particularly at proestrus, where they assist in the breakdown of alveolar buds through phagocytosis of apoptotic cells and tissue remodelling (Chua et al., 2010). We have previously reported that deficiency in TGFB1 resulted in increased apoptosis in the mammary gland epithelium (Sun et al., 2013), and macrophages may be recruited to the epithelium to phagocytose these dying cells. Increased invasion of macrophages may be the consequence of signals emanating from the increased number of apoptotic epithelial cells when TGFB1 is absent. Alternatively,

increased macrophage invasion might be due to alterations in the chemoattractant signals produced by TGFB1 deficient epithelium or in the composition of extracellular matrix between the epithelium and stroma.

The abundance of stromal cells expressing iNOS, and CCR7 was increased by deficiency in epithelial cell-derived TGFB1. TGFB1 acts to down-regulate the expression of iNOS in activated macrophages and thus resolves inflammation by deactivating macrophages (Tsunawaki et al., 1988, Vodovotz et al., 1993). This increased abundance of cytotoxic macrophages when TGFB1 is deficient suggests epithelial cell-derived TGFB1 might play a role in inhibiting macrophage activation and immune surveillance. Deficiency in epithelial cell-derived TGFB1 also caused an increased abundance of CCR7-positive macrophages within the alveolar stroma, implying that TGFB1 suppresses immune-associated activities of CCR7-postive macrophages in the mammary gland during diestrus.

Together, these findings suggest that epithelial cell-derived TGFB1 restrains the immune regulatory activities of macrophages and inhibits invasion of macrophages into the epithelium. Through suppression of iNOS and CCR7, high TGFB1 expression would dampen inflammatory "M1" macrophage activity, which in turn would be expected to impair tumour immune surveillance in the mammary gland.

3.6.3 Relationship between epithelial cell-derived TGFB1 and macrophage location and phenotypes in the human breast

Similar to what was observed in the mouse mammary gland, TGFB1 is widely expressed by human non-neoplastic breast epithelium; however, the expression level was variable among individuals. Macrophages are mainly located around the epithelium, within the epithelium and the stroma, and therefore macrophages are in close proximity to TGFB1. This suggests epithelial cell-derived TGFB1 is likely to exert regulatory actions on macrophages in the human breast, to an extent that varies between individuals.

There was a trend, although not statistically significant (p=0.057), towards a possible association between expression of epithelial cell-derived TGFB1 and abundance of epithelial cell-associated macrophages. However, a significant negative relationship was found between the abundance of TGFB1 and the abundance of inter-epithelial cell-associated and stromal-associated macrophages, implying that high TGFB1 expression leads to lesser macrophages invaded into the breast epithelium and fewer macrophages within the stroma. This finding is consistent with what we have demonstrated in mice, in which the presence of abundant TGFB1 prevents macrophage invasion into the mammary epithelium.

The abundance of stroma-associated CD206-positive macrophages was increased with the presence of increased abundance of epithelial cell-derived TGFB1. Our finding is consistent with a recent study which has demonstrated TGFB signalling promotes expression of genes associated with “M2” macrophages including *ym1*, *mcr2* and *mgl2* and expression of “M2” phenotypic marker Arginase I (Arg1) on macrophages (Gong et al., 2012). This increased abundance of “M2” macrophages when TGFB1 is abundant suggests epithelial cell-derived TGFB1 might play a role in promoting “M2” macrophage polarization. Studies have shown that TGFB1 may promote tumour invasion and metastasis at the later stage of cancer progression (Jakowlew, 2006, Moustakas et al., 2002, Pardali and Moustakas, 2007). Tumour-associated macrophages (TAM) which involved in tumour progression and metastasis exhibit abundant level of CD206 expression and up-regulated “M2” macrophage phenotypes (Luo et al., 2006). These findings together suggest that the exacerbation of the pro-tumourigenic properties of macrophages in cancer may be mediated through increased expression of TGFB1, and TGFB1 might be involved in promoting cancer progression through up-regulation of “M2” macrophage functions.

The abundance of iNOS-positive macrophages and CCR7-positive macrophages was not significantly associated with TGFB1 expression by the epithelium in human breast, and this finding is not consistent with what we found in mice. This difference may in part be due to the high variations among human breast tissue samples. In our mouse model, the factors that might induce variations in the study such as age, genetic background and hormone environment were kept the same and standardised, however, these factors were highly variable in human breast samples, which could confound and limit our interpretation of the data. A larger sample size with participants at a certain age with relatively similar hormone profiles is necessary to further explore the relationship between TGFB1 and macrophages in the human breast.

Together, these findings suggest that epithelial cell-derived TGFB1 plays a similar role in the human breast as it does in the mouse mammary gland, that is, inhibits macrophage invasion into the epithelium and stroma. Furthermore, through promotion of CD206, high TGFB1 expression would up-regulate anti-inflammatory and pro-tumourigenic “M2” macrophage activity in the breast, which in turn may skew the immune environment in favour of tolerance and tumourigenesis.

3.7 Conclusion

These experiments indicate that resident tissue macrophages are key targets of epithelial cell-derived TGFB1 expression in both the mouse and human mammary gland and suggest that TGFB1-macrophage cross-talk may be a pivotal pathway in increased breast cancer risk in women carrying the TGFB1 L10P polymorphism. Studies in the following chapter will attempt to describe the significance of macrophages as intermediary cells through which TGFB1 affects tumourigenesis in the mammary gland.

Chapter 4 The role of TGFB-regulated macrophages in mammary gland development and cancer

4.1 Introduction

The previous chapter demonstrated that epithelial cell-derived TGFB1 modulates macrophage abundance location and phenotype in both mouse and human breast tissue. A mammary gland transplant mouse model was established to study the significance of epithelial cell-derived TGFB1 in regulating macrophages in mice, and the results implied a possible role of TGFB1 in skewing the immune environment in favour of tolerance and tumourigenesis. However, both *Tgfb1* null mutant mice and recipient mice are on a genetic background of severe combined immunodeficiency and therefore tumourigenesis in these mice cannot be studied in the context of a fully functioned immune system. Hence, a new model was needed to further investigate the full spectrum of roles of TGFB-regulated macrophages in mammary gland development and tumourigenesis.

In order to overcome these limitations, a new transgenic mouse model was developed whereby TGFB signaling could be switched off specifically in macrophages. TGFB1 is the main TGFB isoform present in the adult mouse mammary gland (Ewan et al., 2002). TGFB receptors are expressed by most cell types in the mammary gland, and thus TGFB1 is able to act on many different cell types, such as epithelial cells, fibroblasts and macrophages (Lyons et al., 1990, Jakowlew, 2006). In the previous chapter we have shown that macrophages are a key target cell type of TGFB1 in the mammary gland, and hence the binding between TGFB and its receptor on macrophages must be crucial for TGFB signal transduction and activation of gene expression in the mammary gland. In the experiments described in this chapter, we developed a double transgenic mouse model *TGF-Mac* whereby a dominant negative TGFB receptor is activated in macrophages in the presence of doxycycline, which in turn attenuating the TGFB signalling pathway in macrophages (described in section 2.2.1.2). The generation of this model enables us to investigate the effect of impaired TGFB signalling in macrophages on mammary gland development and mammary cancer susceptibility.

4.2 Expression of dominant negative TGFB receptor in *TGF-Mac* mice

To determine whether the dominant negative TGFB receptor transgene is specifically expressed in double transgenic *TGF-Mac* mice, *ΔTgfbrII* mRNA was quantified in the spleens of doxycycline-treated *Cfms-rtTA* single transgenic control, doxycycline-treated *TetO-TgfbrII* single transgenic control, non-doxycycline treated *TGF-Mac* mice (*TGF-Mac* no dox) and doxycycline-treated *TGF-Mac* mice (*TGF-Mac*+dox) by RT-PCR (Figure 4.1). Doxycycline-treated mice were treated with doxycycline from age of

4-week old for a period of 6 weeks to 8 weeks. Tissue collection and RT-PCR was performed as described in section 2.3.2 and expression of *ΔTgfbrII* was normalised to the house-keeping gene *Actb*. The relative quantity of PCR product was multiplied by a constant to give an average for the control tissue sample (doxycycline-treated *Cfms-rtTA* controls) the arbitrary value of 1. The expression of *ΔTgfbrII* mRNA was significantly higher in doxycycline-treated *TGF-Mac* mice compared to doxycycline-treated *Cfms-rtTA* controls, doxycycline-treated *TetO-TgfbrII* controls and non-doxycycline treated *TGF-Mac* mice (11-fold increased vs. doxycycline-treated *Cfms-rtTA* controls, p=0.045; 9.4-fold increased vs. doxycycline-treated *TetO-TgfbrII* controls, p=0.039; 6.7-fold increased vs. non-doxycycline treated *TGF-Mac* mice, p=0.048; unpaired t-test) (Figure 4.1). There were no significant differences in the expression of *ΔTgfbrII* mRNA between doxycycline-treated *Cfms-rtTA*, doxycycline-treated *TetO-TgfbrII* controls or non-doxycycline treated *TGF-Mac* mice (unpaired t-test) (Figure 4.1). This result confirmed that the dominant negative TGFB receptor transgene is specifically expressed by *TGF-Mac* mice in the presence of doxycycline, and not in non-treated *TGF-Mac* mice, or in single transgenic controls.

Further validation of the specificity of impaired TGFB signalling in macrophages was attempted through a series of *in vitro* experiments; however, the administration of doxycycline caused interfered with our *in vitro* studies. *In vitro* culture with doxycycline dramatically altered macrophages responses to TGFB at both the mRNA and protein level of a number of different TGFB-responsive genes in wild-type and single transgenic control mice, such that we were unable to assess TGFB-responsiveness *in vitro*. The expression of the other inbuilt reported gene *Egfp* was explored through flow cytometry in doxycycline-treated *TGF-Mac* mice to further examine the expression of the transgenes in macrophages, however, the expression of the EGFP protein was unstable and inconsistent between mice (possibly due to the influence of doxycycline), as a result an alternative approach was needed to further assess the effect of impaired TGFB signalling in macrophages in these mice. Hence, we determined to further validate this model through *in vivo* experiments. Both *Cfms-rtTA* and *TetO-TgfbrII* single transgenic mice were used as controls for double transgenic *TGF-Mac* mice. All three groups of mice used in the *in vivo* studies were treated with doxycycline from age of 4-week old for a period of 8 weeks to ensure that impaired TGFB signalling in macrophages is the only variable factor among the three groups of mice.

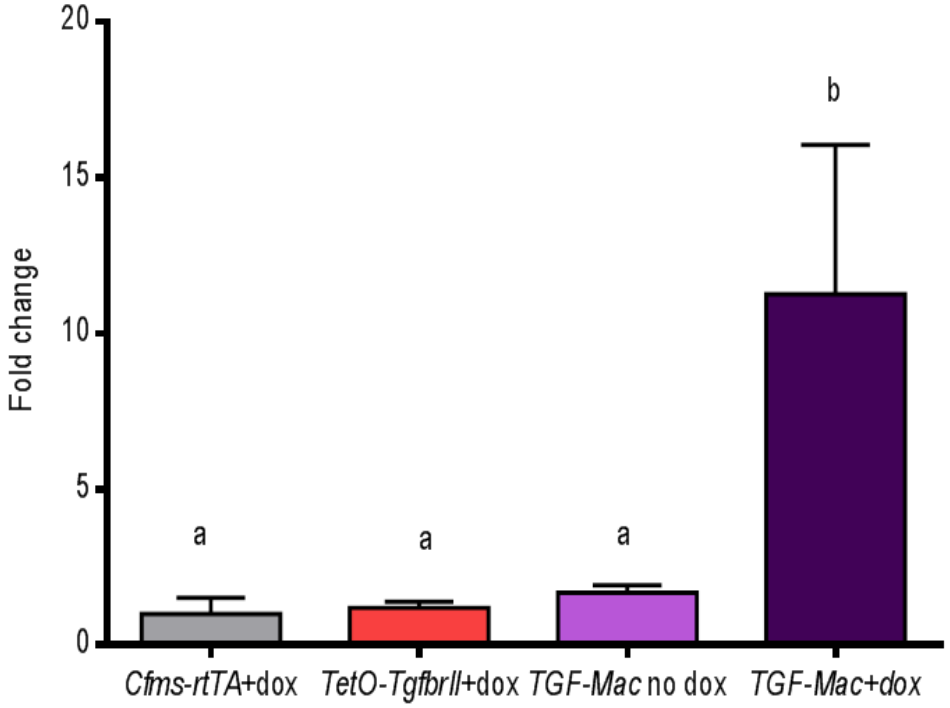

Figure 4.1 Expression of *ΔTgfbrII* mRNA in doxycycline-treated *Cfms-rtTA* mice, doxycycline-treated *TetO-TgfbrII* mice, non-doxycycline treated *TGF-Mac* mice and doxycycline-treated *TGF-Mac* mice measured by RT-PCR.

Spleens from each group of mice (n=12-13) were dissected and frozen in liquid nitrogen. *ΔTgfbrII* mRNA expression was measured by RT-PCR. Amount of mRNA was normalised to *Actb* expression, and is given in arbitrary units where the average of the control is 1. Data are presented as mean + SEM with statistical analysis using an unpaired t-test, a and b are significantly different, whereas ab is not different to a or b ($p<0.05$).

4.3 Effect of impaired TGFB signalling in macrophages on estrous cyclicity

To investigate the effect of impaired TGFB signalling in macrophages on estrous cyclicity, cycles were tracked for a period of 28 days by histological analysis of vaginal smears in adult females for doxycycline-treated *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* mice. The estrous cycle length or the number of cycles within 28 days was unaffected by mouse genotype (Table 4.1). There was no significant difference in the percentage of time spent in each stage of the estr0us cycle among the three groups (Figure 4.2)

Table 4.1 The average length of estrous cycle and the number of estrous cycles within 28 days in adult doxycycline treated *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* mice

	Cfms-rtTA	*TetO-TgfbrII*	*TGF-Mac*
Cycle Length (days)	5.04±0.07	4.93±0.11	4.84±0.10
Cycles (number)	4.8± 0.13	4.63±-0.11	4.75±-0.25

Estrous cycles were tracked for 28 days (females were approximately 8 weeks of age) by histological analysis of vaginal smears. Estrus was defined as when >90% of cells in vaginal smear were cornified epithelial cells. A single complete cycle was defined as the first day of estrus through to the first day of the next estrus (n=8-12 per group). Data are presented as mean ± SEM, and were analysed by an unpaired t-test.

4.4 Effect of impaired TGFB signalling in macrophages on mammary gland development at diestrus

Previous studies have shown that both TGFB1 (Ewan et al., 2002) and macrophages (Chua et al., 2010) are most abundant in the mammary gland at diestrus, therefore this stage of cycle was chosen to examine the role of TGFB-regulated macrophages on mammary gland development. Three month old virgin doxycycline-treated *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* females were dissected at diestrus and one side of the inguinal pair of mammary glands was stained as a whole mount with carmine alum, and the other side of the mammary gland was paraffin-embedded, sectioned and stained with haematoxylin and eosin (H&E).

The number of ductal branch points was increased by 15% in the mammary glands of doxycycline-treated *TGF-Mac* mice (Figure 4.3 C; 1.59 $\pm$ 0.075 branch points/mm; p=0.04; unpaired t-test)

compared to doxycycline-treated *Cfms-rtTA* control mice (Figure 4.3 A; 1.39±0.053 branch points/mm) (Figure 4.3 D). However, no significant difference was found in the number of branch points in the mammary glands of doxycycline-treated *TetO-TgfbrII* (Figure 4.3 B; 1.49±0.046 branch points/mm) when compared with that of doxycycline-treated *Cfms-rtTA* control mice (p=0.14; unpaired t-test) or with that of *TGF-Mac* mice (p=0.34; unpaired t-test) (Figure 4.3 D).

The effect of impaired TGFB signalling in macrophages on alveolar development was determined by analysis of H&E stained paraffin sections from mammary glands of doxycycline-treated *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* females at diestrus. Two distinct types of ducts were observed in the mammary glands of three groups of mice: ductal epithelium with a single epithelial cell layer (Figure 4.4 A, C and E) and alveolar epithelium (Figure 4.4 B, D and F) identified as clusters of epithelial cells containing an alveolar lumen (Chua et al., 2010, Fata et al., 2001). There was no significant difference in the percentage of alveolar epithelium in the mammary glands from *Cfms-rtTA* mice compared to that of *TetO-TgfbrII* mice at diestrus (24.9%±1.2 vs. 23.2%±0.84; p=0.25; unpaired t-test; Figure 4.4 G). However, the percentage of alveolar epithelium was significantly higher in the mammary glands from *TGF-Mac* mice compared to that of *Cfms-rtTA* and *TetO-TgfbrII* mice (6% increased vs. *Cfms-rtTA* controls, p=0.011; 7% increased vs. *TetO-TgfbrII* controls, p=0.002; unpaired t-test; Figure 4.4 G).

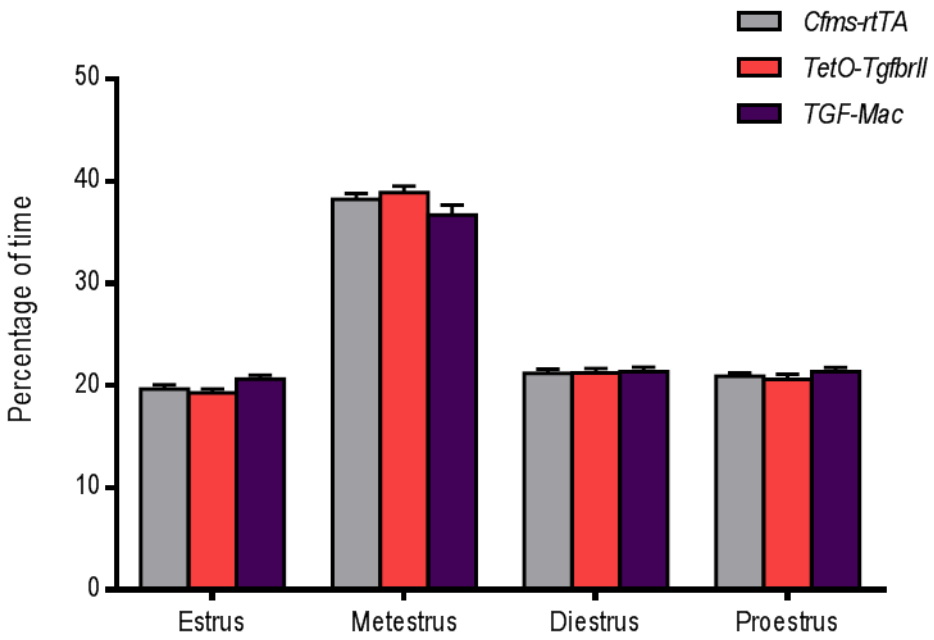

Figure 4.2 Percentage of time spent in each stage of estrous cycle in doxycycline-treated *C-fms-rtTA*, *TetO-Tgfbrll* and *TGF-Mac* adult mice.

Daily vaginal smears were analysed by phase contrast microscopy in female mice (n=8-12 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.

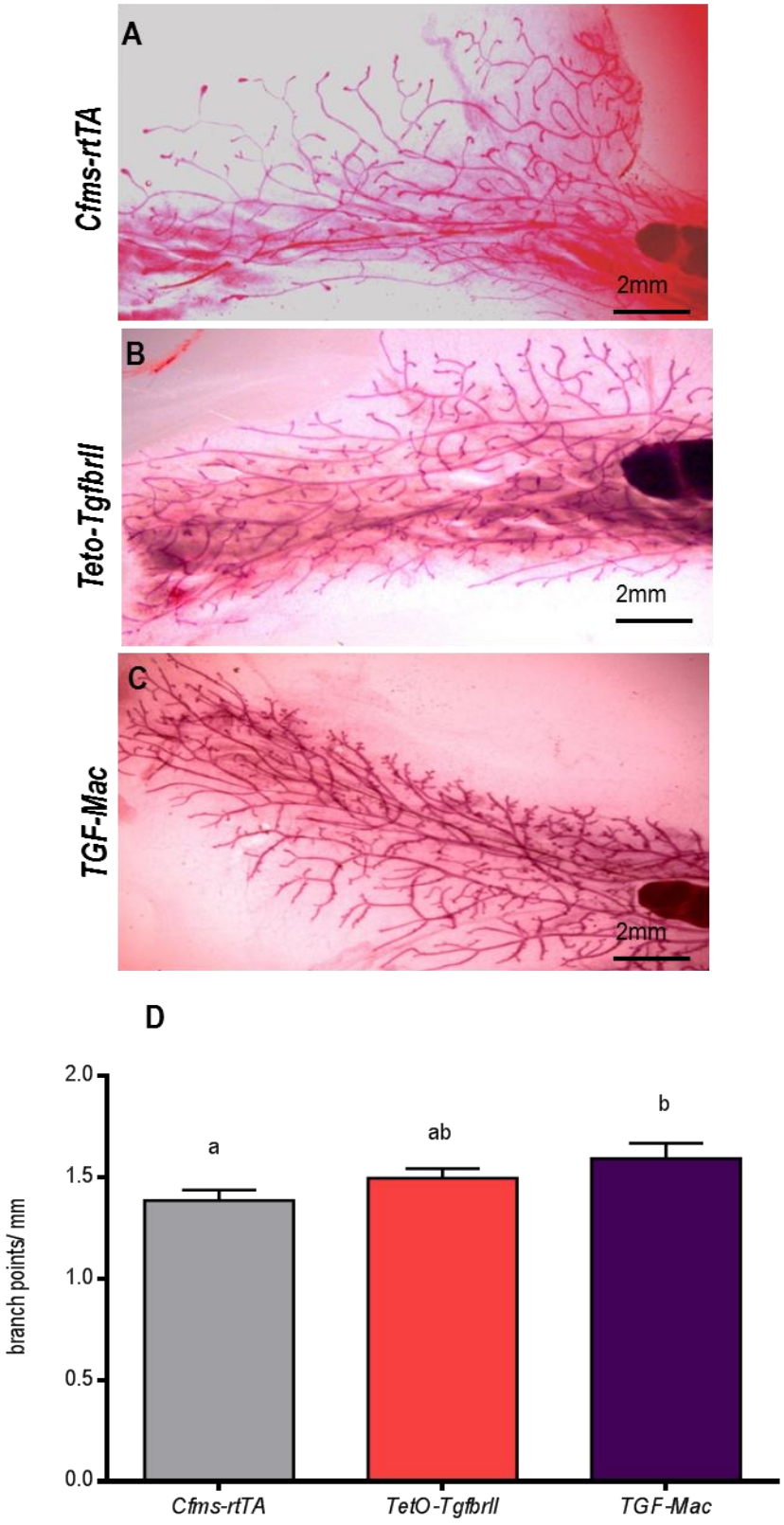

Figure 4.3 The effect of impaired TGFB signalling in macrophages on mammary gland morphogenesis at diestrus.

Mammary gland wholemounts of doxycycline-treated *Cfms-rtTA* (n=10; A), *TetO-Tgfbrll* (n=8; B) and *TGF-Mac* (n=12; C) mice were stained with carmine alum at diestrus. The number of branch points/mm was calculated (D). Data are presented as mean + SEM with statistical analysis using an unpaired t-test, a and b are significantly different, whereas ab is not different to a or b ($p<0.05$).

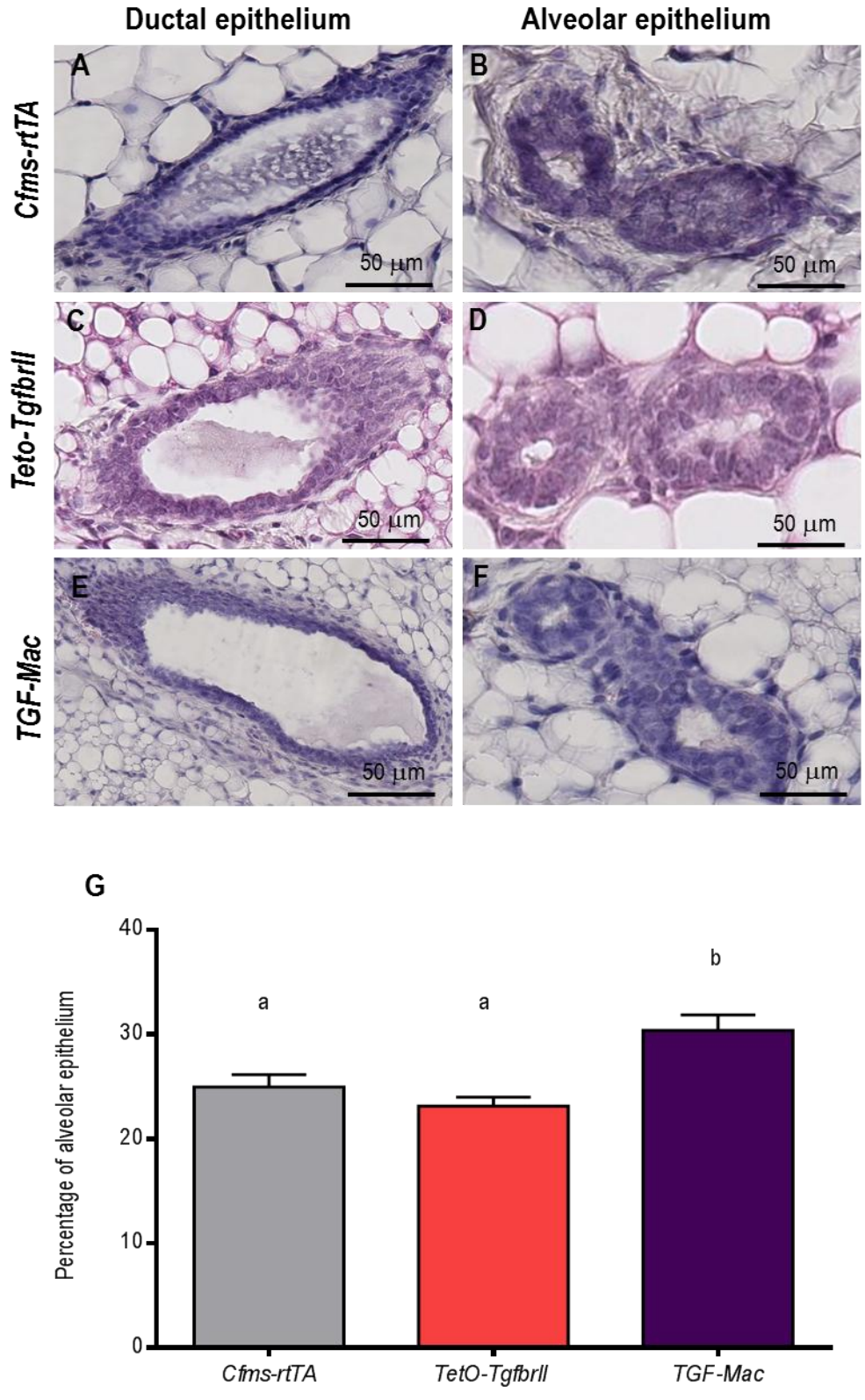

Figure 4.4 The effect of impaired TGFB signalling in macrophages on mammary gland alveolar development at diestrus.

Paraffin sections of mammary gland tissue from doxycycline-treated *Cfms-rtTA* (n=10; A and B), *TetO-Tgfbrll* (n=8; C and D) and *TGF-Mac* (n=12; E and F) mice were stained with haematoxylin and eosin. The percentage of alveolar epithelium was calculated (G). Data are presented as mean + SEM with statistical analysis using an unpaired t-test, a and b are significantly different, whereas ab is not different to a or b ($p<0.05$).

4.5 Effect of impaired TGFB signalling in macrophages on macrophage abundance and phenotype in the mammary gland at diestrus

To evaluate the effect of impaired TGFB signalling in macrophages on macrophage abundance and phenotype in the mammary gland at diestrus, immunohistochemistry was performed to detect F4/80, CCR7 and iNOS positive cells in mammary glands of doxycycline-treated *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* mice.

As differences were observed in macrophage population around ductal versus alveolar epithelium (Chapter 3), these two epithelial cell subsets were again used to dissect different macrophage populations. Ductal epithelium was defined as a single epithelium layer and alveolar epithelium was consisted of clusters of epithelial structures containing alveolar lumens. F4/80-positive macrophages were observed in close proximity to ductal and alveolar epithelium in mammary glands of doxycycline-treated *Cfms-rtTA* (Figure 4.5 A and 4.5 B respectively), *TetO-TgfbrII* (Figure 4.5 C and 4.5 D respectively) and *TGF-Mac* (Figure 4.5 E and 4.5 F respectively) mice. No positive staining was observed in the mammary gland stained with isotype matched irrelevant antibody. The F4/80-positive macrophages were localised both within the stroma surrounding the epithelium, as well as in between epithelial cells. There was no significant difference in macrophage density within the ductal stroma among the three genotypes (Figure 4.5 G). However, there was a 46% and 68% increase in the number of macrophages within *TGF-Mac* ductal epithelium (1312 $\pm$ 150 macrophage density/mm^2; p=0.03 and p=0.01 respectively; unpaired t-test) compared to that of *Cfms-rtTA* ductal epithelium (896 $\pm$ 83 macrophage density/mm^2) and *TetO-TgfbrII* ductal epithelium (780 $\pm$ 51 macrophage density/mm^2) respectively (Figure 4.5 G). No significant difference was found in the number of macrophages within ductal epithelium between the two control groups (p=0.28; unpaired t-test; Figure 4.5 G). There was a 51% and 92% increase in the number of macrophages within *TGF-Mac* alveolar epithelium (1559 $\pm$ 167 macrophage density/mm^2; p=0.015 and p=0.003 respectively; unpaired t-test) compared to that of *Cfms-rtTA* alevolar epithelium (1031 $\pm$ 83 macrophage density/mm^2) and *TetO-TgfbrII* alveolar epithelium (810 $\pm$ 99 macrophage density/mm^2) respectively (Figure 4.5 H). No significant difference was found in the number of macrophages within alveolar epithelium between the two control groups (p=0.1; unpaired t-test; Figure 4.5 H). Additionally there was a 33% and 37% increase in the number of macrophages within *TGF-Mac* alveolar stroma (1809 $\pm$ 115 macrophage density/mm^2; p=0.004 and p=0.013 respectively; unpaired t-test) compared to that of *Cfms-rtTA* alveolar stroma (1358 $\pm$ 68 macrophage density/mm^2) and *TetO-TgfbrII* alveolar stroma (1314 $\pm$ 138 macrophage density/mm^2) respectively

(Figure 4.5 H). No significant difference was found in the number of macrophages within alveolar stroma between the two control groups (p=0.8; unpaired t-test; Figure 4.5 H).

iNOS expression was observed in both the mammary gland epithelium and stromal compartment. However, as described in Chapter 3, only iNOS-positive macrophages within the stroma surrounding ductal and alveolar epithelium in mammary glands of doxycycline-treated *Cfms-rtTA* (Figure 4.6 A and 4.6 B respectively), *TetO-TgfbrII* (Figure 4.6 C and 4.6 D respectively) and *TGF-Mac* (Figure 4.6 E and 4.6 F respectively) mice was quantified. No positive staining was observed in the mammary gland stained with isotype matched irrelevant antibody. There was a 1.8-fold and 1.1-fold increase in the number of iNOS-positive macrophages within *TGF-Mac* ductal stroma (177 ± 24 iNOS-positive cells/mm^2; p<0.001 and p=0.004 respectively; unpaired t-test) compared to that of *Cfms-rtTA* ductal stroma (62 ± 13 iNOS-positive cells/mm^2) and *TetO-TgfbrII* ductal stroma (82 ± 7 iNOS-positive cells/mm^2) respectively (Figure 4.6 G). No significant difference was found in the number of iNOS-positive macrophages within ductal stroma between the two control groups (p=0.24; unpaired t-test; Figure 4.6 G). In addition, there was a 1.8-fold and 0.8-fold increase in the number of iNOS-positive macrophages within *TGF-Mac* alveolar stroma (210±33 iNOS-positive cells/mm^2; p=0.002 and p=0.03 respectively; unpaired t-test) compared to that of *Cfms-rtTA* alveolar stroma (76±18 iNOS-positive cells/mm^2) and *TetO-TgfbrII* alveolar stroma (117±15 iNOS-positive cells/mm^2) respectively (Figure 4.6 G). No significant difference was found in the number of iNOS-positive macrophages within alveolar stroma between the two control groups (p=0.11; unpaired t-test; Figure 4.6 G).

CCR7-positive cells were located in the stroma surrounding ductal and alveolar epithelium in mammary glands of doxycycline-treated *Cfms-rtTA* (Figure 4.7 A and 4.7 B respectively), *TetO-TgfbrII* (Figure 4.7 C and 4.7 D respectively) and *TGF-Mac* (Figure 4.7 E and 4.7 F respectively) mice. No positive staining was observed in the mammary gland stained with isotype matched irrelevant antibody. There was a 47% and 74% increase in the number of CCR7-positive cells within *TGF-Mac* ductal stroma (807±41 CCR7-positive cells/mm^2; p<0.001 and p<0.001 respectively; unpaired t-test) compared to that of *Cfms-rtTA* ductal stroma (549±38 CCR7-positive cells/mm^2) and *TetO-TgfbrII* ductal stroma (463±29 CCR7-positive cells/mm^2) respectively (Figure 4.7 G). No significant difference was found in the number of CCR7-positive macrophages within ductal stroma between the two control groups (p=0.1; unpaired t-test; Figure 4.7 G). Additionally there was a 37% and 63% increase in the number of CCR7-positive cells within *TGF-Mac* alveolar stroma (899 ± 67 CCR7-positive cells/mm^2; p=0.02 and p<0.001

respectively; unpaired t-test) compared to that of *Cfms-rtTA* alveolar stroma (655±65 CCR7-positive cells/mm^2) and *TetO-TgfbrII* alveolar stroma (550±33 CCR7-positive cells/mm^2) respectively (Figure 4.7 G). No significant difference was found in the number of CCR7-positive macrophages within alveolar stroma between the two control groups (p=0.18; unpaired t-test; Figure 4.7 G).

4.6 Effect of impaired TGFB signalling in macrophages on mammary gland cancer susceptibility in mice

To investigate the effect of impaired TGFB signalling in macrophages on mammary gland cancer susceptibility, doxycycline-treated *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* mice were challenged with the chemical carcinogen DMBA. Mice were treated with DMBA for 6 weeks from the age of 6 weeks and monitored weekly for mammary gland tumours by palpation. A Kaplan-Meier survival plot showing the percentage of tumour-free mice as a function of post-DMBA treatment in weeks for *Cfms-rtTA*, *TetO-TgfbrII* and *TGF-Mac* mice was generated (Figure 4.8). Tumour latency was determined as the time to first appearance of palpable mammary tumour. There was a significant reduction in the incidence of mammary tumours in DMBA-treated *TGF-Mac* mice [14 out of 20 *TGF-Mac* mice developed mammary gland tumour (70%); p=0.025 and p=0.015 respectively; Log Rank] compared to that of *Cfms-rtTA* mice [18 out of 20 *Cfms-rtTA* mice developed mammary gland tumour (90%)] and *TetO-TgfbrII* mice [16 out of 19 *TetO-TgfbrII* mice developed mammary gland tumour (85%)] respectively (Figure 4.8). No significant difference was found in the percentage of mammary tumour incidence or mammary tumour latency between the two control groups (p=0.2; Log Rank; Figure 4.8).

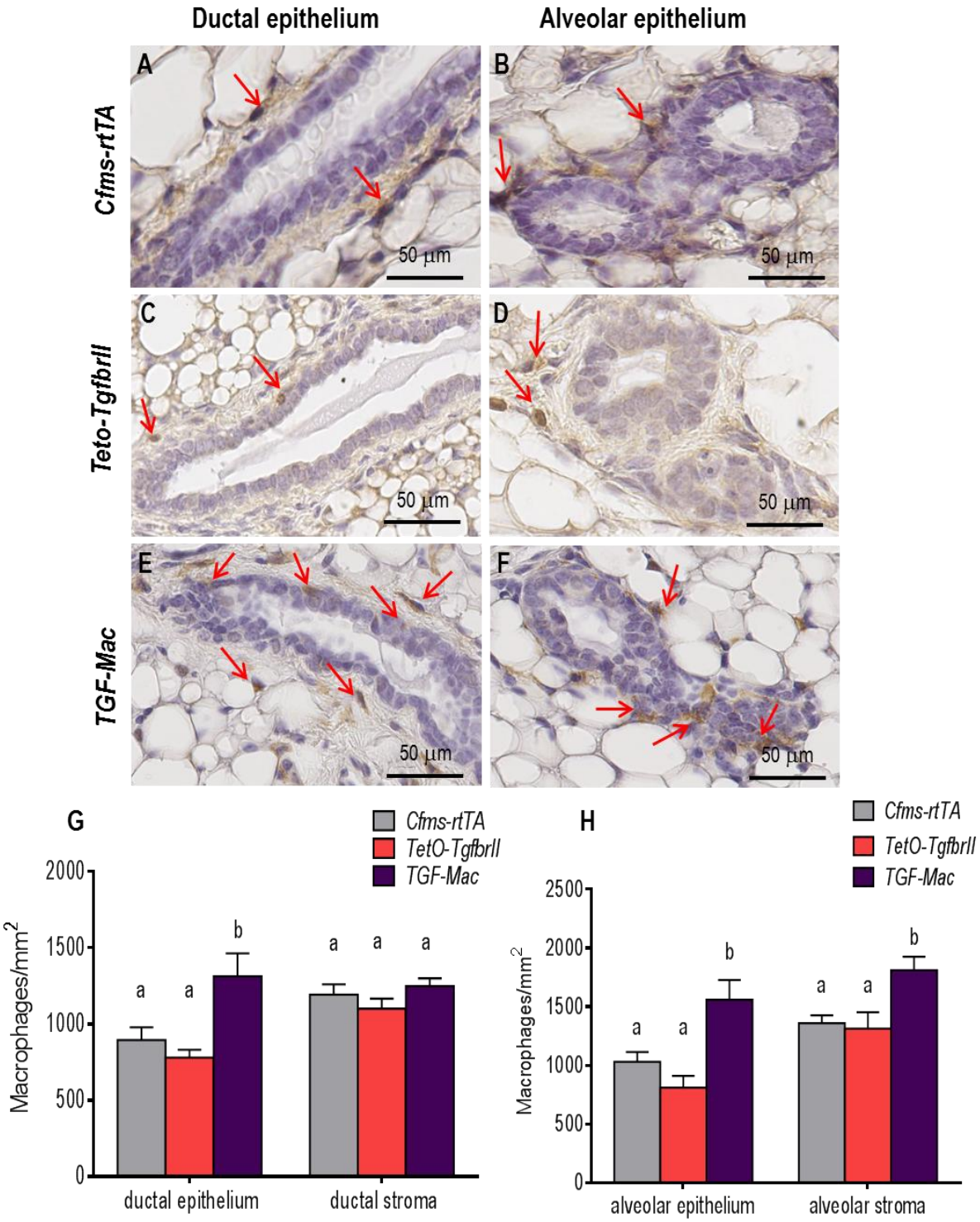

Figure 4.5 The effect of impaired TGFB signalling in macrophages on macrophage abundance and location within and around mammary epithelium.

Paraffin sections of mammary gland tissue from doxycycline-treated *Cfms-rtTA* (n=10; A and B), *TetO-Tgfbrll* (n=8; C and D) and *TGF-Mac* (n=12; E and F) mice were stained with macrophage-specific F4/80 antibody to detect macrophages within ductal epithelium and stroma (A, C and E) and within alveolar epithelium and stroma (B, D and F). Brown-stained macrophages are indicated by red arrows. The number of F4/80-positive cells within ductal epithelium and stroma, and within alveolar epithelium and stroma were calculated and expressed as macrophage density/mm^2 (G and H). Data are presented as mean + SEM with statistical analysis using an unpaired t-test, a and b are significantly different, whereas ab is not different to a or b ($p<0.05$).

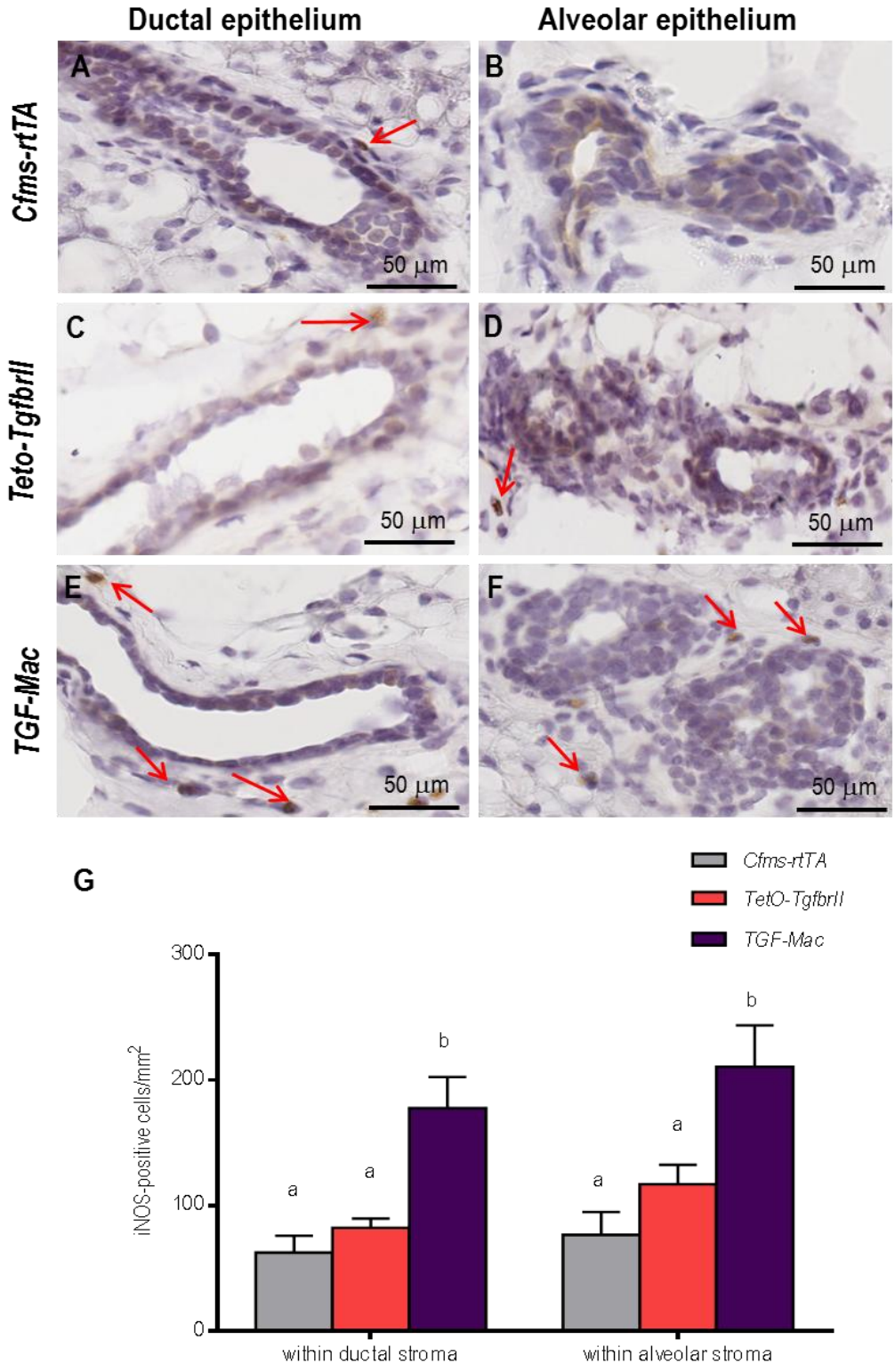

Figure 4.6 The effect of impaired TGFB signalling in macrophages on abundance and location of iNOS-positive cells within mammary epithelium stroma.

Frozen sections of mammary gland tissue from doxycycline-treated *Cfms-rtTA* (n=10; A and B), *TetO-Tgfbrll* (n=8; C and D) and *TGF-Mac* (n=12; E and F) mice were stained with anti-iNOS antibody to detect iNOS-positive cells within ductal (A, C and E) and alveolar stroma (B, D and F). Brown-stained iNOS-positive cells are indicated by red arrows. The number of iNOS-positive cells within ductal and alveolar stroma were calculated and expressed as iNOS-positive /mm^2 (G). Data are presented as mean + SEM with statistical analysis using an unpaired t-test, a and b are significantly different, whereas ab is not different to a or b ($p<0.05$).

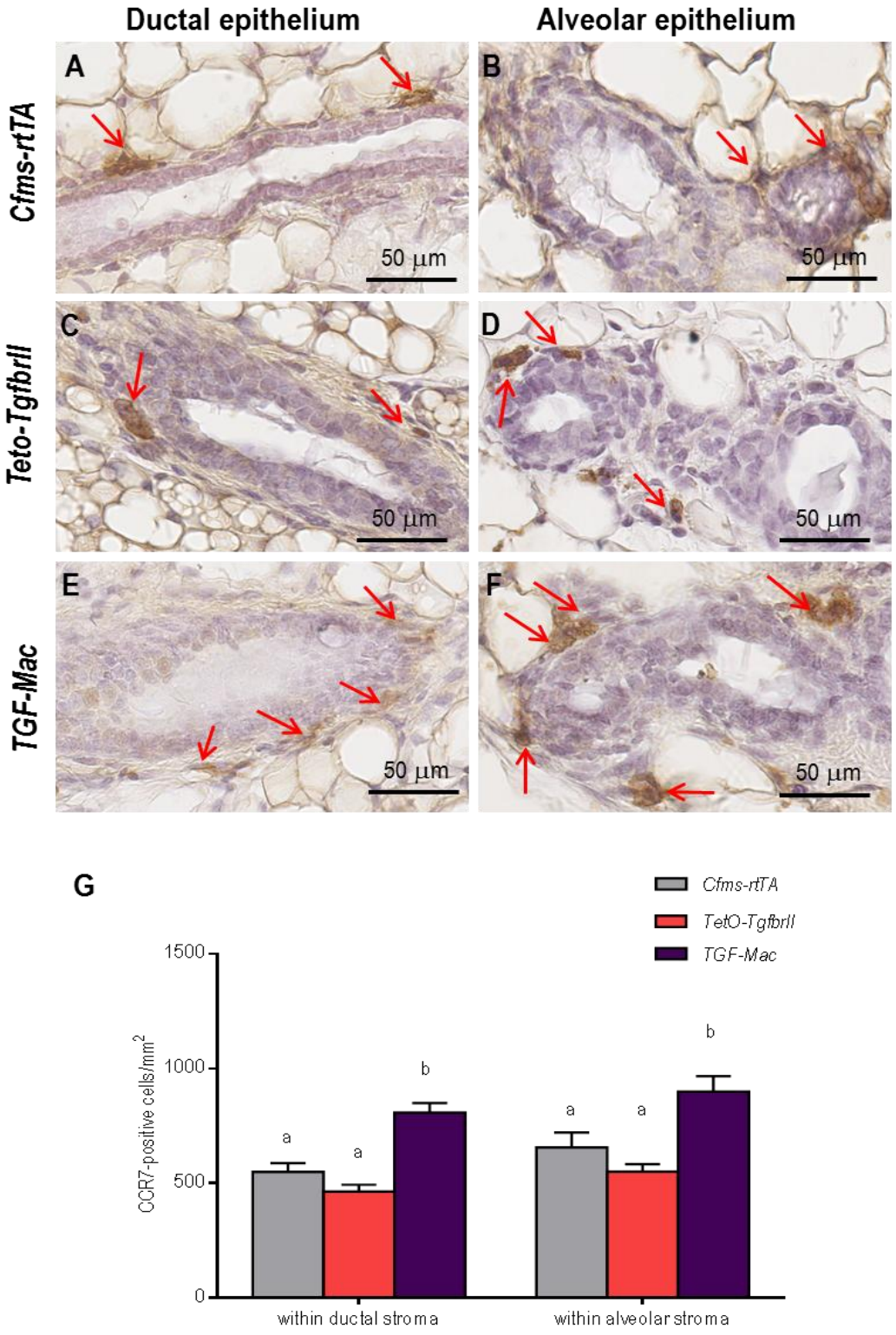

Figure 4.7 The effect of impaired TGFB signalling in macrophages on abundance and location of CCR7-positive cells within mammary epithelium stroma.

Paraffin sections of mammary gland tissue from doxycycline-treated *Cfms-rtTA* (n=10; A and B), *TetO-Tgfbrll* (n=8; C and D) and *TGF-Mac* (n=12; E and F) mice were stained with anti-CCR7 antibody to detect CCR7-positive cells within ductal (A, C and E) and alveolar stroma (B, D and F). Brown-stained CCR7-positive cells are indicated by red arrows. The number of CCR7-positive cells within ductal and alveolar stroma were calculated and expressed as CCR7-positive /mm^2 (G). Data are presented as mean + SEM with statistical analysis using an unpaired t-test, a and b are significantly different, whereas ab is not different to a or b ($p<0.05$).

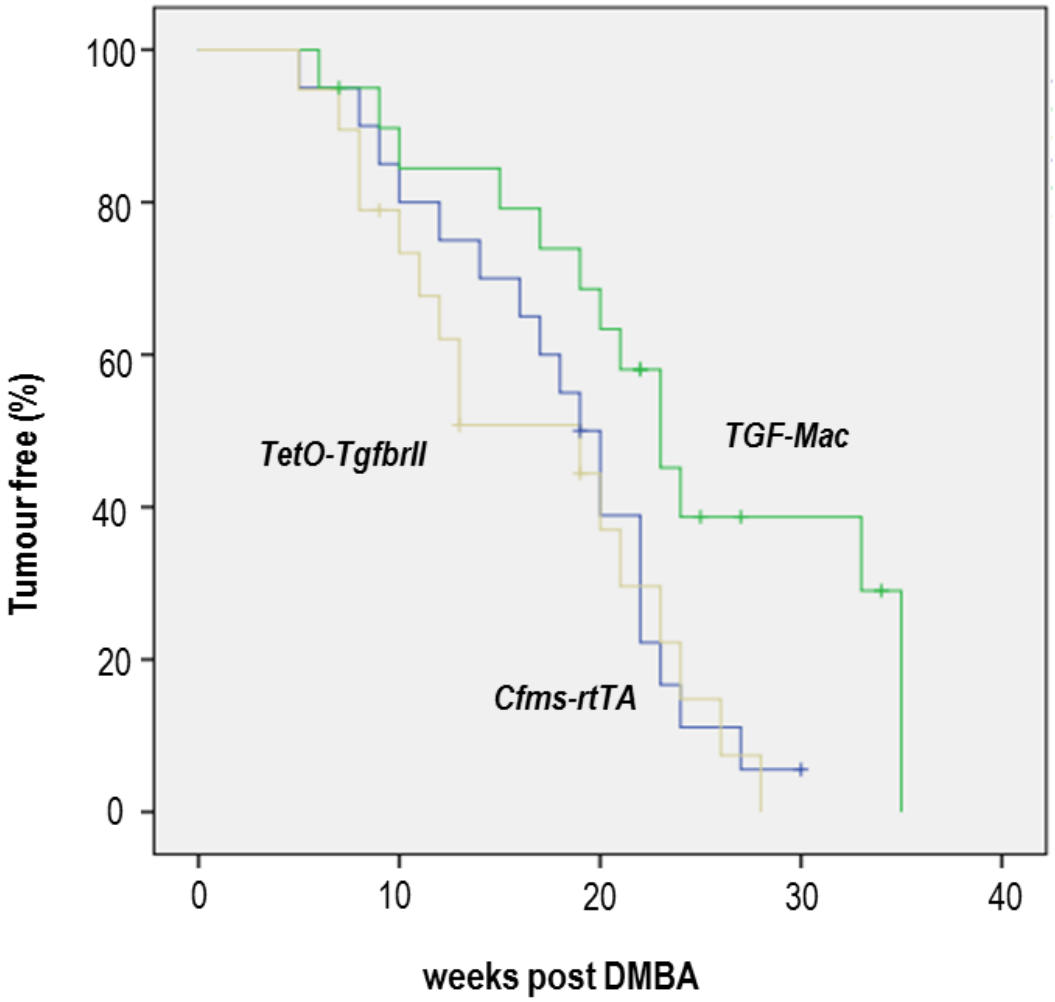

Figure 4.8 The effect of impaired TGFB signalling in macrophages on mammary gland cancer susceptibility in mice.

A Kaplan-Meier survival plot showing the percentage of tumour-free mice as a function of post-DMBA treatment in weeks for doxycycline-treated *Cfms-rtTA* (blue line), *TetO-Tgfbrll* (yellow line) and *TGF-Mac* (green line) mice was generated. Tumour latency was determined as the time to first appearance of palpable mammary tumour. The censored-mice (mice were killed because of other tumours or sickness) were represented by a vertical line on the plot. n=20 per group, statistical analysis was conducted using SPSS Statistics Version 17.0, Log Rank, p=0.025 (*Cfms-rtTA* vs. *TGF-Mac*) and p=0.015 (*TetO-Tgfbrll* vs. *TGF-Mac*).

4.7 Discussion

This study was undertaken to investigate the role of TGFB-regulated macrophages in mammary gland development and tumourigenesis. In the previous chapter we have demonstrated that epithelial cell-derived TGFB1 regulates the function and phenotype of local macrophages in both the human and mouse mammary gland. By using a double transgenic model whereby TGFB signalling is inhibited specifically in the macrophage population, the experiments presented in this chapter have further revealed the significance of TGFB1 as a key regulator of mammary gland macrophages. We report that TGFB-regulated macrophages have multiple roles in mammary gland development and cancer susceptibility.

4.7.1 TGFB-regulated macrophages inhibit mammary epithelial alveolar development at diestrus

Increased ductal branch points and alveolar bud development were observed in mice with impaired TGFB signaling in macrophages at diestrus. Although the cycling non-pregnant gland is relatively quiescent and has been described as being in a "resting state" (Fata et al., 2001), active branching and alveolar budding have been observed over the course of the estrous cycle in mice and the menstrual cycle in humans under the regulation of ovarian hormones and different cytokines (Butler et al., 2000, Fata et al., 2001, Navarrete et al., 2005, Chua et al., 2010, Hodson et al., 2013). TGFB is one of the key signalling pathways that regulates mammary gland development (Chapter 1) (Daniel et al., 2001, Daniel et al., 1989, Silberstein et al., 1992). Many studies have demonstrated that TGFB signalling could inhibit mammary gland development in an autocrine manner (Robinson et al., 1991, Daniel et al., 1989, Pierce et al., 1993), whereby diminished synthesis or signalling capacity of TGFB1 results in increased ductal elongation. Furthermore, attenuation of TGFB signaling by expression of a dominant negative receptor on mammary gland epithelial cells resulted in mammary epithelial cell hyperplasia together with improper alveolar development and differentiation in mice (Gorska et al., 1998). However, transplantation studies have revealed that TGFB1 has both autocrine and paracrine effects on mammary gland development as mice with deficiency in epithelial cell-derived TGFB1 exhibited an overall normal mammary gland development despite of increased epithelial cell turnover rate (Sun et al., 2013) and accelerated ductal expansion (Ingman and Robertson, 2008). These findings could provide an explanation for the observations of increased mammary gland development in mice with impaired TGFB signalling in macrophages at diestrus, which is also implying that besides the previously described autocrine inhibitory effects of TGFB, there are also paracrine effects where epithelial cell development is regulated by TGFB-regulated macrophages in the cycling non-pregnant gland.

In addition to the increased epithelial cell development, there was increased macrophage invasion into the mammary epithelium in mice with impaired TGFB signalling in macrophages. Macrophages have diverse and essential roles in mammary gland development (Chapter 1) (Cecchini et al., 1994, Gouon-Evans et al., 2002, Pollard and Hennighausen, 1994). A recent study has shown that when macrophages were acutely depleted in the mammary gland, macrophages were found to have dual roles during the estrous cycle, one population of macrophages are involved in promoting alveolar development, and the other population are involved in tissue remodeling (Chua et al., 2010). This finding together with the observations of increased ductal development and macrophage invasion in the mammary gland when TGFB signalling is attenuated in macrophages, suggesting that TGFB could be exerting paracrine effects on mammary gland ductal development via regulating macrophage functions. The increased macrophage invasion could be responsible for the increased epithelial cell development in mice with impaired TGFB signalling in macrophages, and TGFB-regulated macrophages may be playing an essential role in maintaining normal ductal development in the mammary gland. Moreover, TGFB signalling could be one of the key signalling pathways that involves in maintaining the balance of different populations of macrophages in the cycling non-pregnant mammary gland, and hence enables the mammary gland undergoes appropriate development and regression over the course of every ovarian cycle.

However, it should be noted that only a small yet significant increase of alveolar development was observed in mammary glands with impaired TGFB signalling in macrophages, implying that the role of TGFB-regulated macrophages in mammary gland development could be perhaps compensated by other factors or signalling pathways and hence sustaining an overall normal mammary gland morphogenesis in these mice. Further investigations are needed to dissect the underlying mechanisms of how TGFB regulates mammary gland ductal development via macrophages.

4.7.2 TGFB signalling to macrophages regulates macrophage invasion and inhibits "M1" macrophage activity

As described in Chapter 1 and Chapter 3, macrophages are important immune cells and capable of roles in immunity, development, tissue homeostasis and cancers (Reed and Schwertfeger, 2010, Schwertfeger et al., 2006). In response to various signals, macrophages might undergo classical "M1" activation or alternative "M2" activation (Mantovani et al., 2002). The "M1" phenotype is characterised by the expression of pro-inflammatory cytokines, high production of reactive oxygen and nitrogen

intermediates which can promote tumour suppression. In contrast, “M2” macrophages are considered to be involved in tissue remodelling and promoting tumour progression (Mantovani et al., 2002, Gordon, 2007). For macrophages to fulfil multiple functions during development and cancer progression, they are likely to function under the direction of different cytokines and hormones.

TGFB1 is known as a key cytokine that regulates macrophage function in many tissues. The experiments presented in Chapter 3 have demonstrated that epithelial cell derived-TGFB1 prevents macrophage invasion into mammary epithelium and suppresses the activities of “M1” macrophages. Similar to what we observed previously, increased penetration of macrophages into both the ductal and alveolar epithelium was observed in mammary glands with impaired TGFB signalling in macrophages, suggesting that TGFB signalling to macrophages plays a significant role in regulating macrophage abundance and invasion. In addition, impaired TGFB signalling in macrophages caused increased abundance of “M1” iNOS-positive and CCR7-positive macrophages within the mammary gland stroma, which is also consistent with our previous findings in Chapter 3 as increased density of “M1” macrophages in the mammary gland was found in the absence of epithelial cell-derived TGFB1, implying a role for TGFB signalling in down-regulating the activities of “M1” macrophages. These findings are supported by previous studies which have demonstrated that TGFB1 inhibits the expression of macrophage scavenger receptor during macrophage mediated phagocytosis and down-regulates the expression of “M1” marker iNOS in activated macrophages and thus resolving inflammation and preventing disease progression (Tsunawaki et al., 1988, Bottalico et al., 1991, Sherry et al., 1998, Li et al., 2006). Taken together, this evidence strongly suggests the role of TGFB signalling to macrophages in the mammary gland appears to be two-fold: (1) inhibition of macrophage invasion into the epithelium and (2) down-regulation of “M1” macrophages.

4.7.3 Impaired TGFB signalling in macrophages reduces mammary cancer susceptibility in mice

Studies have shown that TGFB1 has both stimulatory and inhibitory roles in cancer progression; it acts as a tumour suppressor at the early stage of cancer development and promotes invasion and metastasis at the later stage of cancer progression (Jakowlew, 2006, Yang et al., 2010a, Dumont and Arteaga, 2000). The International Breast Cancer Consortium reported a significant association between breast cancer risk and the TGFB1 L10P gene polymorphism (Cox et al., 2007), in which the presence of TGFB1 L10P gene is linked to increased cellular expression of TGFB1 and elevated circulating TGFB1 (Krippl et al., 2003), suggesting that increased abundance of TGFB1 is associated with increased

susceptibility to breast cancer. This finding is consistent with our observations as a significant reduction in incidence of mammary gland tumour formation, and longer tumour latency, was observed in mice with impaired TGFB signalling in macrophages challenged with DMBA carcinogen, indicating that TGFB-regulated macrophages might promote mammary cancer susceptibility in mice.

Macrophages play multiple roles in tumour development and progression; they suppress cancer development as part of innate and adaptive anti-tumour immune responses and promote tumourigenesis via promoting growth and survival of tumour cells, angiogenesis and cell invasion (Mantovani et al., 2002, Qian and Pollard, 2010). Studies have shown that a high abundance of infiltrating macrophages is often found in solid tumours (Martinez et al., 2009, Qian and Pollard, 2010, Wu and Zheng, 2012, Guo et al., 2012) and the presence of "M2" tumour-associated macrophages (TAM) has been found to correlate with poor prognosis in breast cancer (Mahmoud et al., 2012), suggesting macrophage infiltration and phenotype is associated with tumour progression. However, the balance of pro-tumourigenic or anti-tumourigenic activities of macrophages is dependent on cytokines signalling derived from the local tissue microenvironment, including TGFB signalling (Wu and Zheng, 2012, Sun et al., 2013). Although increased macrophages invasion and abundance was observed in the mammary glands with impaired TGFB signalling in macrophages, the progression of mammary gland cancer upon DMBA challenge in these mice was reduced, implying that TGFB signalling is essential in modulating macrophage abundance in mammary gland tumourigenesis, and impaired TGFB signalling to macrophages could alter the phenotypes and functions of macrophages and thus affect cancer susceptibility. Further, the reduction of mammary gland tumour incidences in mice with impaired TGFB signalling in macrophages could be due to the increased abundance of "M1" macrophages in these mice, as "M1" macrophages are mainly involved in tumour suppression, and hence it is highly likely that TGFB signalling to macrophages promotes mammary gland tumourigenesis through inhibition of the activities of "M1" macrophages. These findings provide compelling evidence that aberrant TGFB signalling to macrophages may impair immune system protection from tumour formation through regulation of macrophage abundance and phenotype, in particular, via suppression of "M1" macrophage function and immunosurveillance, leading to increased risk of breast cancer.

4.7.4 Limitations and future directions

The generation and development of the double transgenic mouse model *TGF-Mac*, whereby attenuation of TGFB signalling in macrophages was achieved by the administration of doxycycline has enabled us

to further investigate the role of TGFB-regulated macrophages in mammary gland development and mammary cancer susceptibility. The limitation of this model is that we were unable to demonstrate that TGFB signalling is specifically switched off in macrophages in the double transgenic *TGF-Mac* mice through *in vitro* experiments. However, the significance of the *in vivo* findings in this chapter that show similar macrophage phenotypes to results in Chapter 3 has added strength to this model. Further experiments are required to demonstrate that TGFB signalling is inhibited specifically in macrophages and therefore further validate this model; this could be potentially achieved by determining the ability of phagocytosis of macrophages primed with TGFB1 in *TGF-Mac* and control mice through phagocytosis assay.

4.8 Conclusion

In summary, the findings we reported in this chapter further demonstrate the significance of TGFB in regulating macrophages in mammary gland development and cancer. We propose that TGFB signalling to macrophages promotes mammary gland tumourigenesis in mice through (1) regulation of macrophage invasion and abundance, and (2) suppression of immune regulatory activities of "M1" macrophages. Future studies should focus on examining how TGFB signalling to macrophages mediates tumour metastasis in the mammary gland, and develop improved therapeutic approaches to breast cancer prevention and treatment through targeting TGFB signalling to macrophages.

Chapter 5 Regulation of macrophages, mammary gland development and cancer by epithelial cell-derived CCL2

5.1 Introduction

Macrophages are highly versatile cells capable of many roles in mammary gland development and cancer (Chapter 1). The plasticity of macrophages allows them to respond to different signals, including cytokines, which they receive from their local microenvironment. In addition to TGFB1, which has been demonstrated as a key cytokine that regulates macrophages in the mammary gland in previous chapters, CCL2 is also known as an important regulator of macrophages.

CCL2 is a pro-inflammatory chemotactic cytokine and is a highly potent chemoattractant for monocytes and macrophages during inflammation (Yadav et al., 2010, Deshmane et al., 2009). Due to its proinflammatory nature, CCL2 is not normally constitutively expressed, however, the expression of CCL2 can be induced by various conditions including oxidative stress and activation of the cell by other cytokines and growth factors (Yadav et al., 2010, Cushing et al., 1990, Standiford et al., 1991). CCR2 is the primary receptor for CCL2. CCR2 is mainly expressed by leukocytes, particularly monocytes and macrophages, suggesting that macrophages are a key target cell type for CCL2.

There is substantial evidence demonstrates that CCL2 can be highly expressed by both the tumour and surrounding stromal cells in breast carcinomas (Soria and Ben-Baruch, 2008, Ueno et al., 2000). A number of studies have shown that CCL2 expression in breast carcinomas is highly associated with macrophage infiltration, and its expression is correlated with poor prognosis in breast cancer patients (Fujimoto et al., 2009, Arendt et al., 2013, Ueno et al., 2000, Qian et al., 2011). Recent mouse studies have implicated tumour epithelial cell-derived CCL2 and CCR2-expressing macrophages as a critical factor in metastasis of breast cancer to the bone and lungs (Lu and Kang, 2009). These studies suggest that high expression of epithelial cell-derived CCL2 in carcinomas might promote tumour invasion and metastasis through increased infiltration of macrophages into tumours.

Despite many studies addressing the role of CCL2 in breast cancer progression (Ueno et al., 2000, Fujimoto et al., 2009), its role in breast biology is still unclear. Studies have shown that the human breast epithelium expresses low but detectable quantities of CCL2 protein (Soria and Ben-Baruch, 2008, Ueno et al., 2000). The role of CCL2 in mammary gland morphogenesis has not been studied; however, this cytokine may be responsible for macrophage recruitment under specific conditions. A recent study

has demonstrated that CCL2 protein increases on day 2 of involution in rats (O'Brien et al., 2010a), and may act as an early chemoattractant for macrophages that infiltrate the involuting mammary gland.

In the experiments described in this chapter, we developed a transgenic mouse model *Mmtv-Ccl2*, whereby CCL2 is constitutively expressed by the mammary epithelium to investigate the role of epithelial cell-derived CCL2 in regulation of macrophages in mammary gland development and cancer susceptibility. The correlation between CCL2 expression and macrophage phenotype in human breast was also examined.

5.2 Validation of *Mmtv-Ccl2* transgenic mouse model

CCL2 is not constitutively expressed by the mammary epithelium in the normal mammary gland, and hence it is difficult to study the roles of CCL2 in regulation of macrophages in mammary gland morphogenesis and tumourigenesis. However, this could be overcome by generating a mouse model in which CCL2 is constitutively expressed by the mammary epithelium under the control of a mammary epithelium specific MMTV promoter (section 2.2.1.3, Chapter 2). The generation of *Mmtv-Ccl2* transgenic mouse model enabled us to investigate the significance of epithelial cell-derived CCL2 in mammary gland development and cancer progression. In order to confirm the expression level of CCL2 in *Mmtv-Ccl2* mice, RT-PCR, immunohistochemistry and ELISA was performed to validate this mouse model.

5.2.1 Expression of *Mmtv* and *Ccl2* mRNA in *Mmtv-Ccl2* mice

To determine whether *Mmtv* and *Ccl2* is over-expressed in the mammary gland of *Mmtv-Ccl2* mice, the abundance of *Mmtv* and *Ccl2* mRNA was measured and detected in spleen (Sp), kidney (Kid), ovary (Ov), liver (Liv), salivary gland (SG) and mammary gland (MG) of both *Mmtv-Ccl2* and control mice by RT-PCR (Figure 5.1). Tissue collection and RT-PCR was performed as described in section 2.3.2. Sequence specific primers for *Mmtv* and *Ccl2* were used to identify changes in the resultant cDNA between the two groups. Expression of each of these genes was normalised to the house-keeping gene *Actb*. The amount of PCR product amplified by specific primers was multiplied by a constant to give an average for the control tissue sample (control mice) the arbitrary value of 1. *Mmtv* mRNA levels were 15-fold ($p=0.006$, unpaired t-test) and 125-fold ($p<0.001$, unpaired t-test) higher in the mammary glands and salivary glands of *Mmtv-Ccl2* mice compared to that of controls respectively (Figure 5.1A). No mRNA encoding *Mmtv* was detected in the spleens, kidneys, ovaries and livers of both groups of mice

(Figure 5.1A). *Ccl2* mRNA was detected in all the tissue from both groups of mice. *Ccl2* mRNA levels were 80-fold ($p<0.001$, unpaired t-test), 5-fold ($p=0.005$, unpaired t-test), 4609-fold ($p<0.001$, unpaired t-test), 218-fold ($p=0.02$, unpaired t-test) and 4-fold ($p=0.01$, unpaired t-test) higher in the spleens, kidneys, salivary glands, mammary glands and livers of *Mmtv-Ccl2* mice compared to that of controls respectively (Figure 5.1B). There was no significant difference in the expression of *Ccl2* mRNA in the ovaries ($p=0.054$, unpaired t-test) between the two groups (Figure 5.1B).

5.2.2 Constitutive expression of CCL2 protein in *Mmtv-Ccl2* mice

To further validate this model, the level of CCL2 protein expression was examined by immunohistochemistry and ELISA. CCL2 positive staining was observed in the mammary epithelium of both control (Figure 5.2 A) and *Mmtv-Ccl2* (Figure 5.2 B) mice. There was a significant increased abundance of CCL2 in the mammary epithelium of *Mmtv-Ccl2* mice compared to that with control mice. No positive staining was observed in the mammary epithelium stained with secondary antibody only (Figure 5.2 C). To determine whether CCL2 is specifically overly-expressed in the mammary gland of *Mmtv-Ccl2* mice, the level of CCL2 protein was measured and detected in spleen (Sp), kidney (Kid), ovary (Ov), liver (Liv), salivary gland (SG),mammary gland (MG) and serum of both *Mmtv-Ccl2* and non-transgenic control mice by mouse CCL2 ELISA (Figure 5.2 D). Tissue collection and ELISA was performed as described in section 2.4. Expression of CCL2 protein from each tissue sample was normalised to its corresponding protein concentration, and the level CCL2 protein expression was represented as CCL2 (pg)/ protein (pg). CCL2 protein expression level was 3-fold ($p<0.001$, unpaired t-test), 1.7-fold ($p<0.001$, unpaired t-test), 90000-fold ($p<0.001$, unpaired t-test), 530-fold ($p=0.003$, unpaired t-test) and 2.5-fold ($p=0.003$, unpaired t-test) higher in the spleens, livers, salivary glands, mammary glands and serums of *Mmtv-Ccl2* mice compared to that with controls respectively (Figure 5.2 D). There was no significant difference in the expression of CCL2 protein in the kidneys ($p=0.06$, unpaired t-test) and ovaries ($p=0.98$, unpaired t-test) between the two groups (Figure 5.2 D). Although elevated levels of *Ccl2* mRNA and protein was observed in other tissues of *Mmtv-Ccl2* mice, this could be due to the high level of CCL2 expression in the mammary glands and salivary glands, causing increasing CCL2 in other tissues of *Mmtv-Ccl2* mice. This finding was consistent with previous reports using CCL2 over-expression cassettes in other tissues (Rollins et al., 1988, Rutledge et al., 1995, Kanda et al., 2006).

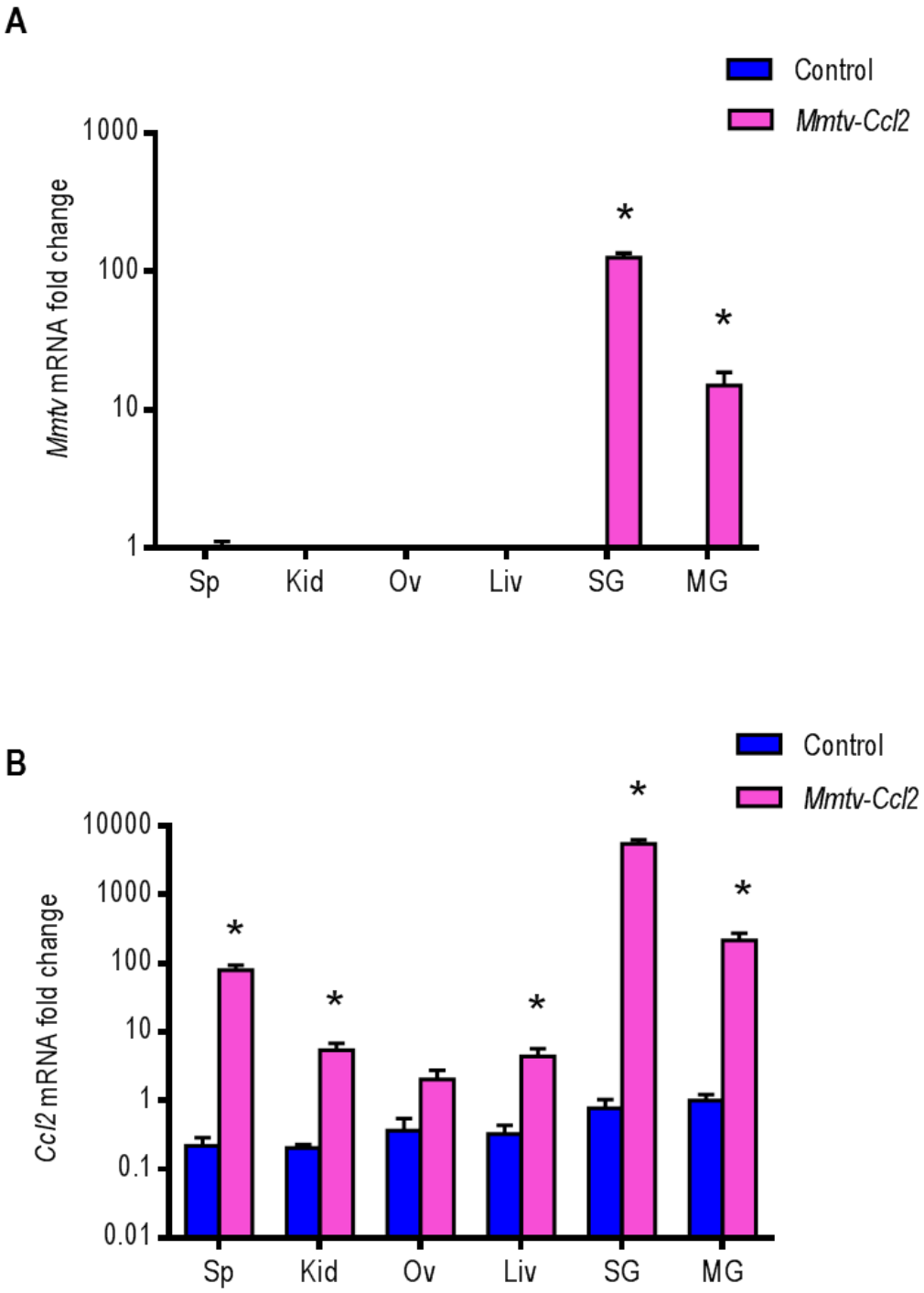

Figure 5.1 Expression of *Mmtv* and *Ccl2* mRNA in *Mmtv-Ccl2* and control mice measured by RT-PCR.

Spleen (Sp), kidney (Kid), ovary (Ov), liver (Liv), salivary gland (SG) and mammary gland (MG) from both groups of mice (n=5) were dissected and frozen in liquid nitrogen. *Mmtv* mRNA (A) and *Ccl2* mRNA (B) was extracted and measured by RT-PCR. Amount of mRNA was normalised to *Actb* expression, and is given in arbitrary units where the average of the control is 1. Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

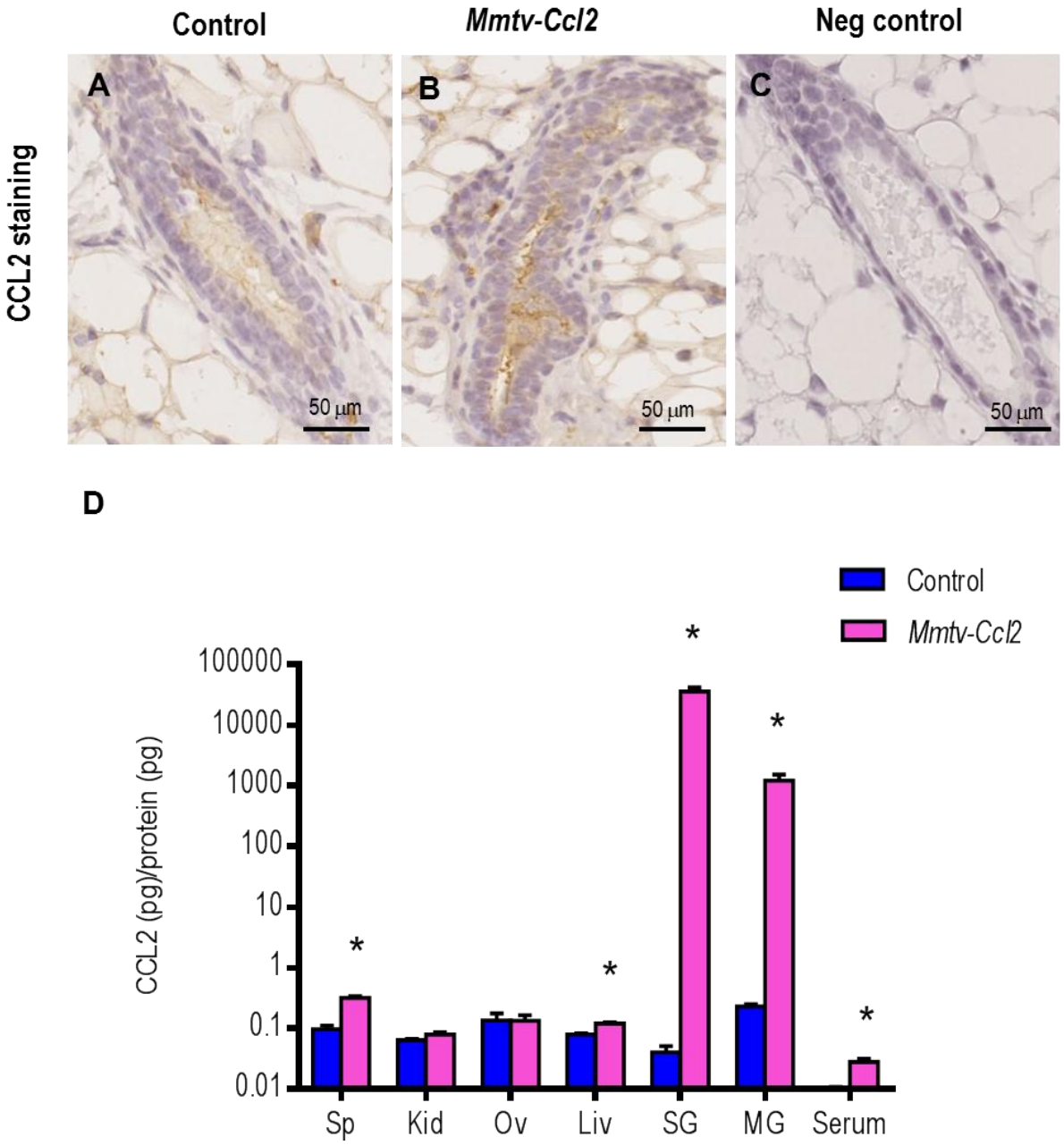

Figure 5.2 Expression of CCL2 protein in *Mmtv-Ccl2* and control mice measured by immunohistochemistry and ELISA.

Paraffin-embedded mammary gland tissue from control (A) and *Mmtv-Ccl2* (B) mice were stained with anti-CCL2 antibody to detect the expression of CCL2 protein within mammary epithelium. Negative control with secondary antibody application only was included (C). Spleen (Sp), kidney (Kid), ovary (Ov), liver (Liv), salivary gland (SG), mammary gland (MG) and serum from both groups of mice (n=5) were collected and the level of CCL2 protein expression was measured by ELISA (D). Expression of CCL2 protein from each tissue sample was normalised to its corresponding protein concentration, the level CCL2 protein expression was represented as CCL2 (pg)/ protein (pg). Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

5.3 Effect of constitutive expression of CCL2 on estrous cyclicity

To investigate the effect of constitutive expression of CCL2 on estrous cyclicity, cycles were tracked for a period of 28 days by histological analysis of vaginal smears in adult females for both control and *Mmtv-Ccl2* mice. The estrous cycle length and the number of cycles within 28 days was unaffected by mouse genotype (Table 5.1). There was no significant difference in the percentage of time spent in each stage of the estrous cycle between the two groups (Figure 5.3).

Table 5.1 The average length of estrous cycle and the number of estrous cycles with 28 days in adult control and *Mmtv-Ccl2* mice

	Control	*Mmtv-Ccl2*
Cycle Length (days)	5.35±0.08	5.40±0.07
Cycles (number)	4.27±0.10	4.26±0.10

Estrous cycles were tracked for 28 days (females were approximately 8 weeks of age) by histological analysis of vaginal smears. Estrus was defined as when >90% of cells in vaginal smear were cornified epithelial cells. A single complete cycle was defined as the first day of estrus through to the first day of the next estrus (n=29 per group). Data are presented as mean ± SEM**,** and were analysed by an unpaired t-test.

5.4 Effect of constitutive expression of epithelial cell-derived CCL2 on mammary gland development during ovarian cycle

To investigate the effect of constitutive expression of epithelial cell-derived CCL2 on mammary gland development over the course of the ovarian cycle, one side of the inguinal pair of mammary glands from 3 month old virgin control and *Mmtv-Ccl2* females was dissected and stained as whole mount with carmine alum and the other side of the mammary gland was paraffin-embedded, sectioned and stained with H&E. Mammary glands were collected from mice at each of the four stages of the ovarian cycle.

Similar mammary gland branching morphogenesis was observed in carmine alum-stained mammary gland wholemounts of control and *Mmtv-Ccl2* mice at estrus (Figure 5.4 A, B; 1.21 ± 0.06 branch points/mm vs. 1.43 ± 0.09 branch points/mm; p=0.07; unpaired t-test; Figure 5.4 I), metestrus (Figure 5.4 C, D; 1.45 ± 0.1 branch points/mm vs. 1.81 ± 0.15 branch points/mm; p=0.07; unpaired t-test; Figure 5.4 I) and diestrus (Figure 5.4 E, F; 1.93 ± 0.14 branch points/mm vs. 2.00 ± 0.19 branch

points/mm; p=0.7; unpaired t-test; Figure 5.4 I). However, at proestrus (Figure 5.4 G, H), the number of ductal branch points was increased by 26% in the mammary glands of *Mmtv-Ccl2* mice (1.48±0.09 branch points/mm; p=0.036; unpaired t-test) compared to control mice (1.17±0.09 branch points/mm) (Figure 5.4 I).

The effect of epithelial cell-derived CCL2 on alveolar development was determined by analysis of H&E stained paraffin sections from mammary glands of control and *Mmtv-Ccl2* females at different stages of the ovarian cycle. Two distinct types of ducts were observed in the mammary glands of both groups of mice: ductal epithelium (Figure 5.5 A and B) and alveolar epithelium (Figure 5.5 C and D). There was no significant difference in the percentage of alveolar epithelium in the H&E stained control mammary epithelium compared to that of *Mmtv-Ccl2* mammary epithelium at estrus (20.3%±2.1 vs. 30.6%±6.3; p=0.17; unpaired t-test; Figure 5.5 E), metestrus (19.7%±2.8 vs. 34.3%±5.9; p=0.06; unpaired t-test; Figure 5.5 E) and diestrus (40.0%±7.7 vs. 55.9%±8.7; p=0.18; unpaired t-test; Figure 5.5 E). However, at proestrus, the percentage of alveolar epithelium was increased by 22% in the mammary glands of *Mmtv-Ccl2* mice (45.8%± 5.4; p=0.004; unpaired t-test) compared to control mice (23.8%± 2.1) (Figure 5.5 E).

These results indicated that constitutive expression of epithelial cell-derived CCL2 could have a significant effect on mammary gland morphogenesis and ductal development particularly at the proestrus stage of the ovarian cycle. To further examine the effect of constitutive expression of epithelial cell-derived CCL2 on mammary epithelium development at proestrus, the ratio of stroma area/epithelium area (Figure 5.5 F) and the density of epithelial cells (represented as epithelial cells/mm^2; Figure 5.5 G) within ductal epithelium was quantified. There was a 2-fold increase in the ratio of stroma/epithelium within/around *Mmtv-Ccl2* ductal epithelium (1.94±0.35; p=0.03; unpaired t-test) compared to that of control (1.03±0.08) (Figure 5.5 F). However, there was no significant difference in the density of epithelial cells with ductal epithelium in the mammary gland between the two groups (21367.7 ± 1968.9 epithelial cells/mm^2 vs. 18191.3 ± 1763.7 epithelial cells/mm^2; unpaired t-test) (Figure 5.5 G).

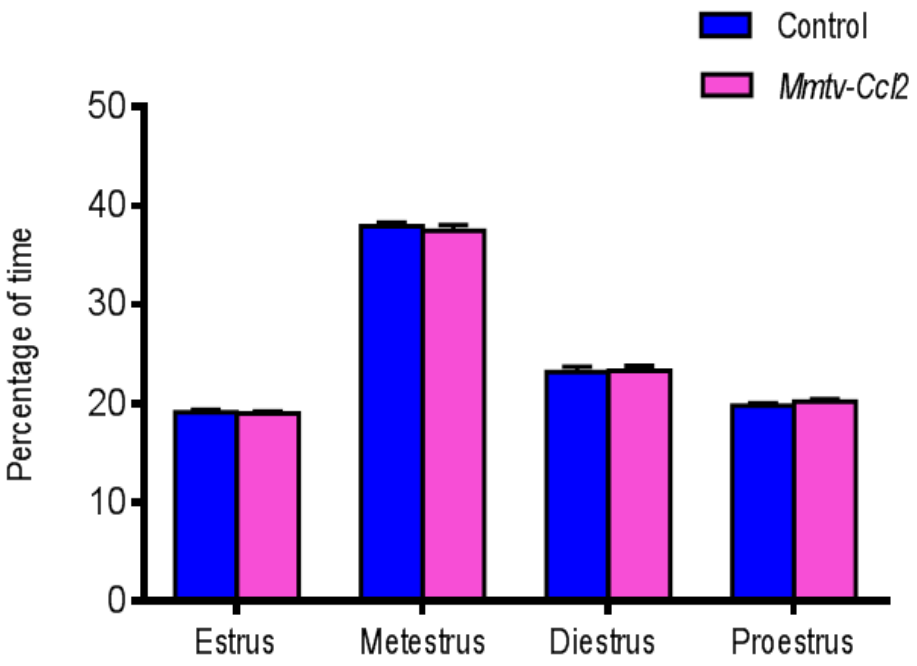

Figure 5.3 Percentage of time spent in each stage of estrous cycle in control and *Mmtv-Ccl2* adult mice.

Daily vaginal smears were analysed by phase contrast microscopy in female mice (n=29 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.

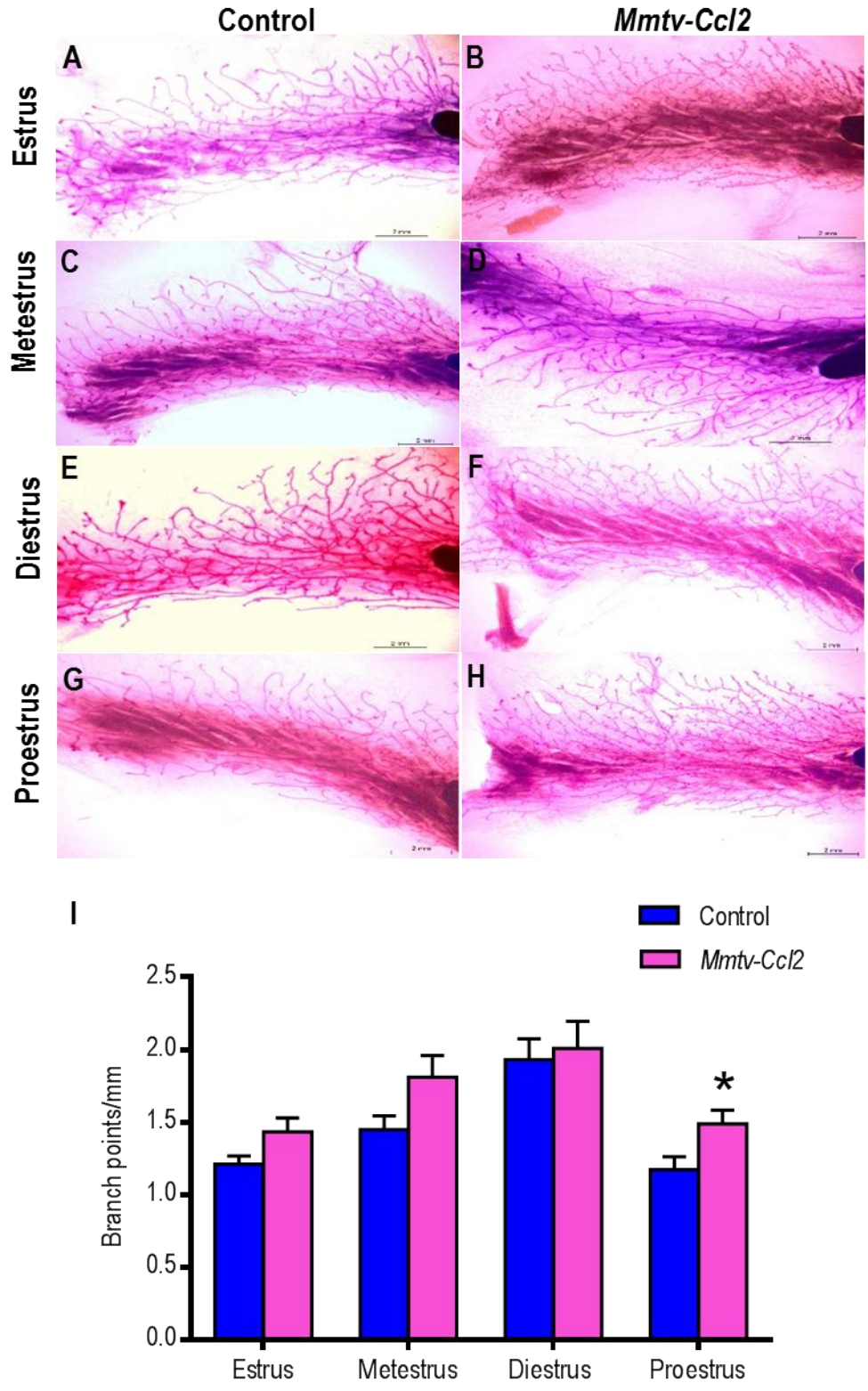

Figure 5.4 The effect of epithelial cell-derived CCL2 on mammary gland morphogenesis during ovarian cycle.

Mammary gland wholemounts of control and *Mmtv-Ccl2* were stained with carmine alum at estrus (n=6; A, B), metestrus (n=9; C, D), diestrus (n=9; E, F) and proestrus (n=6; G, H). The number of branch points/mm was calculated (G). Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

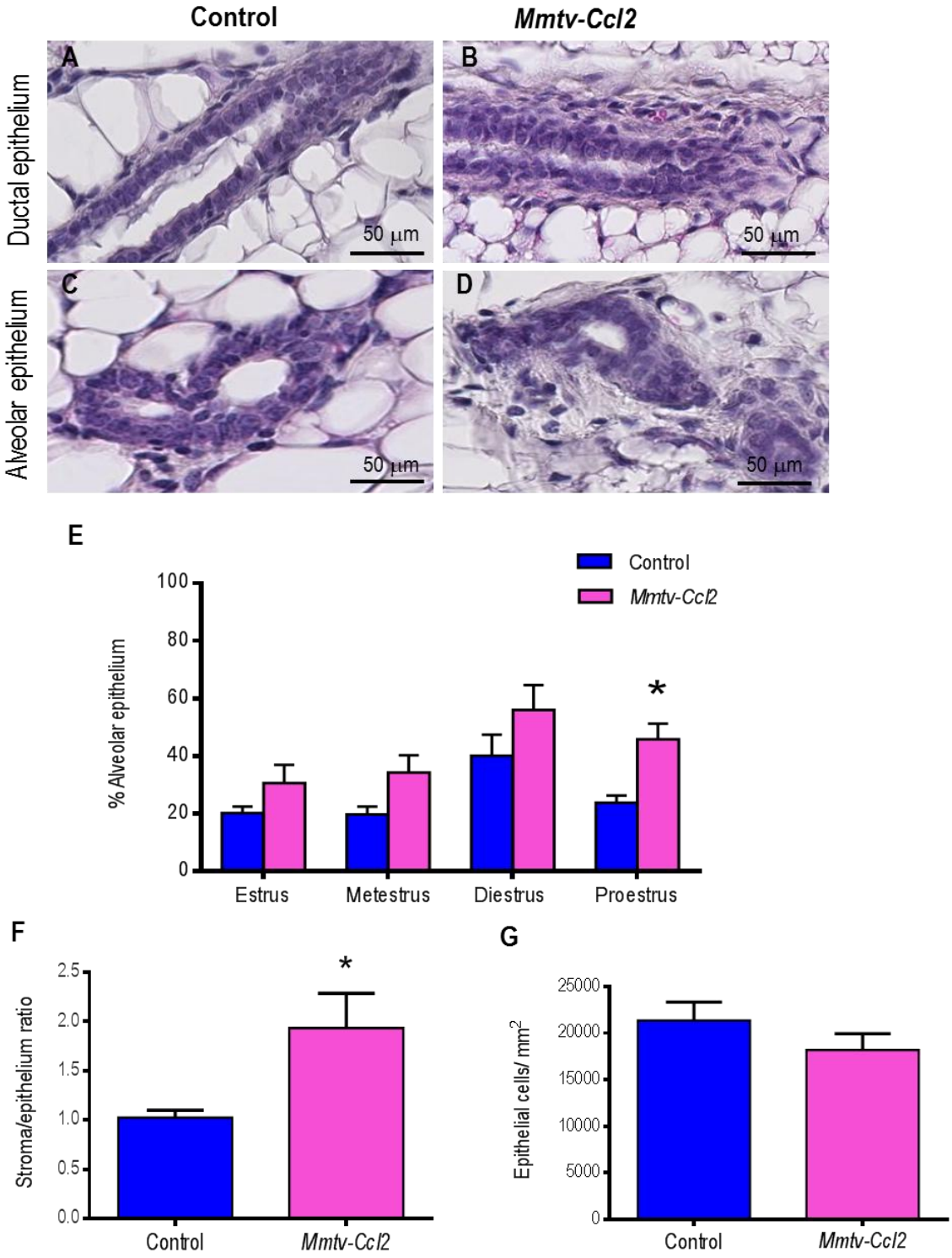

Figure 5.5 The effect of epithelial cell-derived CCL2 on mammary gland alveolar development during ovarian cycle.

Paraffin sections of mammary gland tissue of control and *Mmtv-Ccl2* mice from the 4 stages of the ovarian cycle were stained with haematoxylin and eosin (only proestrus staining shown; A and B were ductal epithelium; C and D were alveolar epithelium; n=6).The percentage of alveolar epithelium at each stages of the ovarian cycle was calculated (E). The ratio of stroma/epithelium (F) and the density of epithelial cells within ductal epithelium (G; expressed as epithelial cells/mm^2) was quantified. Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *p < 0.05.

5.5 Effect of constitutive epithelial cell-derived CCL2 on epithelial cell proliferation and apoptosis in the mammary gland at proestrus

The effect of epithelial cell-derived CCL2 on mammary epithelial cell proliferation was analysed by detection of BrdU-positive cells in control and *Mmtv-Ccl2* mammary epithelium at proestrus. BrdU-positive cells were observed in both ductal and alveolar epithelium of control (Figure. 5.6 A and C; respectively) and *Mmtv-Ccl2* (Figure. 5.6 B and D; respectively) mammary gland. There was no significant difference in the abundance of proliferating epithelial cells within control ductal epithelium (743±347 BrdU-positive cells/mm^2; p=0.91; unpaired t-test; Figure 5.6 E) compared with *Mmtv-Ccl2* ductal epithelium (694±254 BrdU-positive cells/mm^2; Figure 5.6 E). Similarly, no significant difference was observed in the abundance of proliferating epithelial cells between control alveolar epithelium (1316 ± 610 BrdU-positive cells/mm^2; p=0.68; unpaired t-test; Figure 5.6 E) and *Mmtv-Ccl2* alveolar epithelium (894±477 BrdU-positive cells/mm^2; Figure 5.6 E).

The effect of epithelial cell-derived CCL2 on mammary epithelial cell apoptosis was analysed by detection of TUNEL-positive cells in control and *Mmtv-Ccl2* mammary epithelium at proestrus. TUNEL-positive cells were observed in both the ductal and alveolar epithelium of both control (Figure. 5.7 A and C; respectively) and *Mmtv-Ccl2* (Figure. 5.7 B and D; respectively) mammary gland. There was no significant difference in the percentage of apoptotic epithelial cells within control ductal epithelium (0.94% ±0.18 TUNEL-positive cells; p=0.74; unpaired t-test; Figure 5.7 E) compared with *Mmtv-Ccl2* ductal epithelium (1.13%±0.44 TUNEL-positive cells; Figure 5.7 E). Similarly, no significant difference was observed in the percentage of apoptotic epithelial cells between control alveolar epithelium (1.37%±0.5 TUNEL-positive cells; p=0.54; unpaired t-test; Figure 5.7 E) and *Mmtv-Ccl2* alveolar epithelium (0.96% ±0.18 TUNEL-positive cells; Figure 5.7 E).

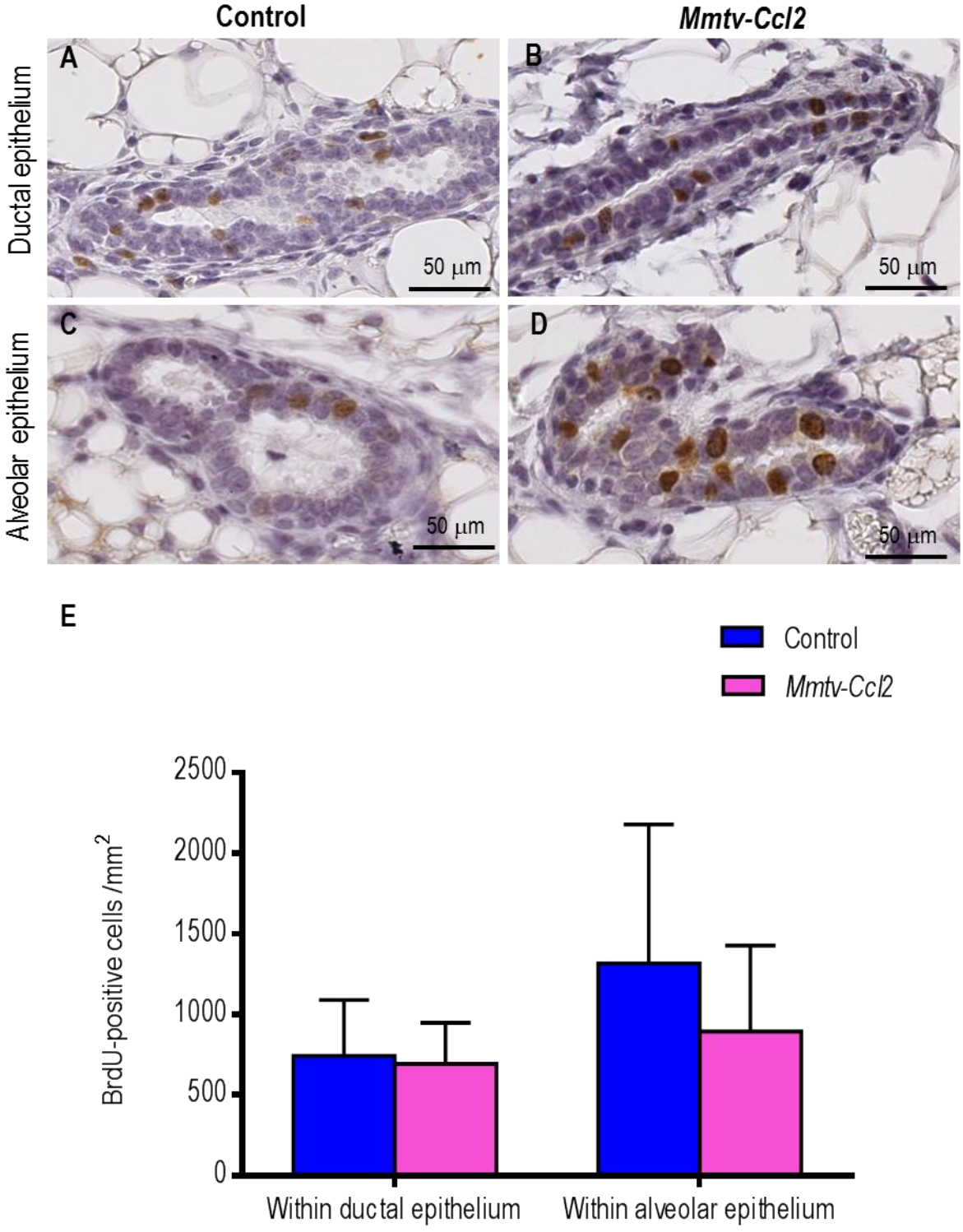

Figure 5.6 The effect of epithelial cell-derived CCL2 on mammary epithelial cell proliferation within control and *Mmtv-Ccl2* mammary epithelium at proestrus.

Paraffin sections of mammary gland tissue from control (n=6) and *Mmtv-Ccl2* (n=6) were stained with anti-BrdU antibody to detect proliferating ductal epithelial cells (A and B) and alveolar epithelial cells (C and D). The number of BrdU-positive cells (brown-stained cells) within ductal and alveolar epithelium was calculated and expressed as BrdU-positive cells/mm^2 (E). Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

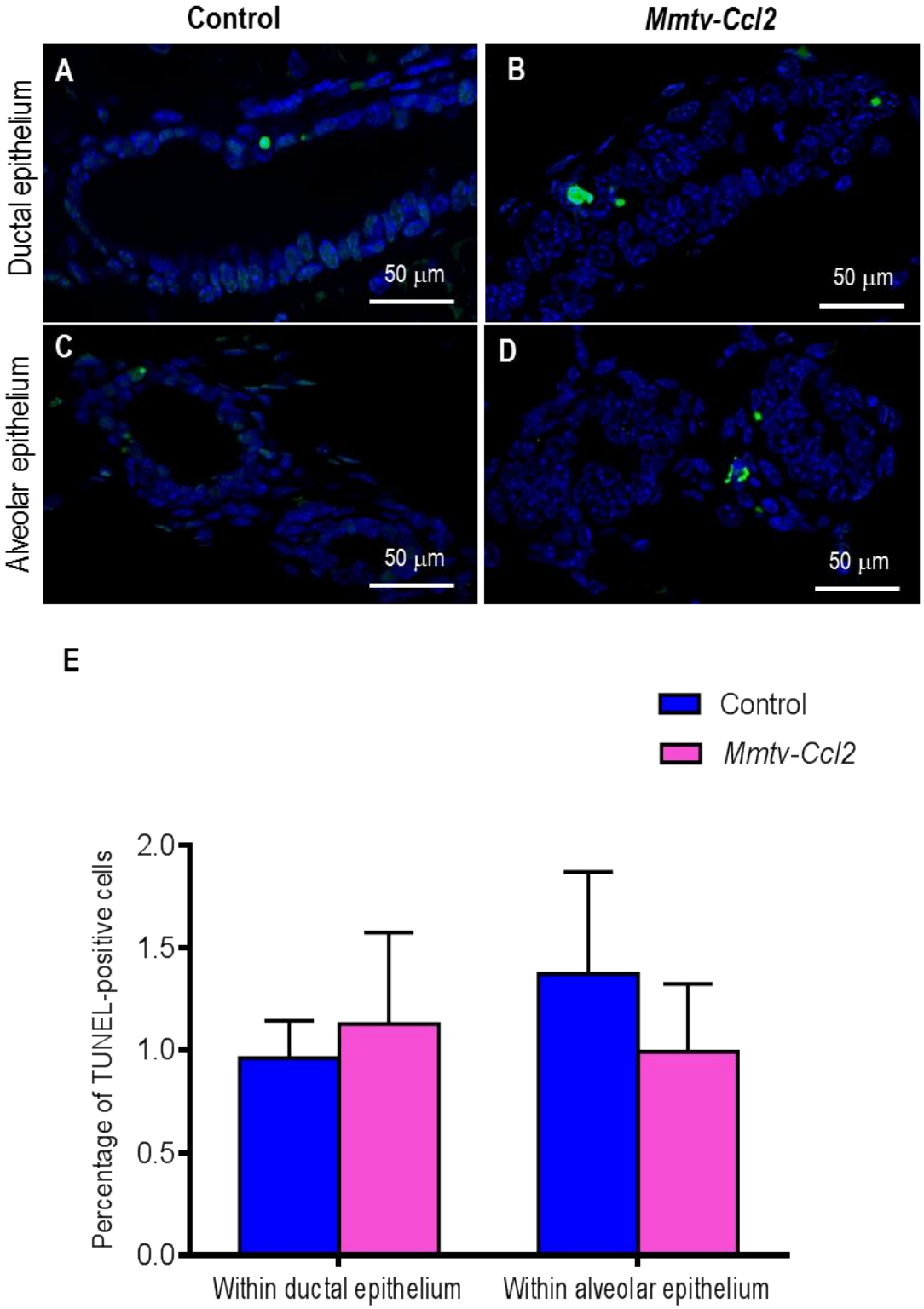

Figure 5.7 The effect of epithelial cell-derived CCL2 on mammary epithelial cell apoptosis within control and *Mmtv-Ccl2* mammary epithelium at proestrus.

Paraffin sections of mammary gland tissue from control (n=6) and *Mmtv-Ccl2* (n=6) were stained with TUNEL staining kit to detect apoptotic epithelial cells within ductal epithelium (A and B) and alveolar epithelium (C and D). The percentage of apoptotic TUNEL-positive cells (green) within ductal and alveolar epithelium was quantified (E). Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

5.6 Effect of constitutive epithelial cell-derived CCL2 on macrophage recruitment and abundance in the mammary gland at proestrus

To investigate the effect of epithelial cell-derived CCL2 on the recruitment and abundance of macrophages at proestrus, F4/80 immunohistochemistry was performed to detect macrophages in the mammary glands of control and *Mmtv-Ccl2* mice. F4/80-positive macrophages were observed in close proximity to ductal and alveolar epithelium in the mammary glands from both control (Figure 5.8 A and C respectively) and *Mmtv-Ccl2* mice (Figure 5.8 B and D respectively). No positive staining was observed in the mammary glands stained with isotype matched irrelevant antibody. In both genotypes, the F4/80-positive macrophages were localised within the stroma, around the epithelium and within the epithelium. There was a 40% increase in the number of macrophages within the ductal epithelium and stroma of *Mmtv-Ccl2* mouse mammary gland (1357 ± 140 F4/80-positive cells/mm^2; p=0.046; unpaired t-test; Figure 5.8 E) compared to that of control mouse mammary gland (971 ± 95 F4/80-positive cells/mm^2; Figure 5.8 E). Additionally there was a 62% in the number of macrophages within the alveolar epithelium and stroma of *Mmtv-Ccl2* mouse mammary gland (1982 ± 191 F4/80-positive cells/mm^2; p=0.005; unpaired t-test; Figure 5.8 E) compared to that of FVB mouse mammary gland (1224 ± 90 F4/80-positive cells/mm^2; Figure 5.8 E).

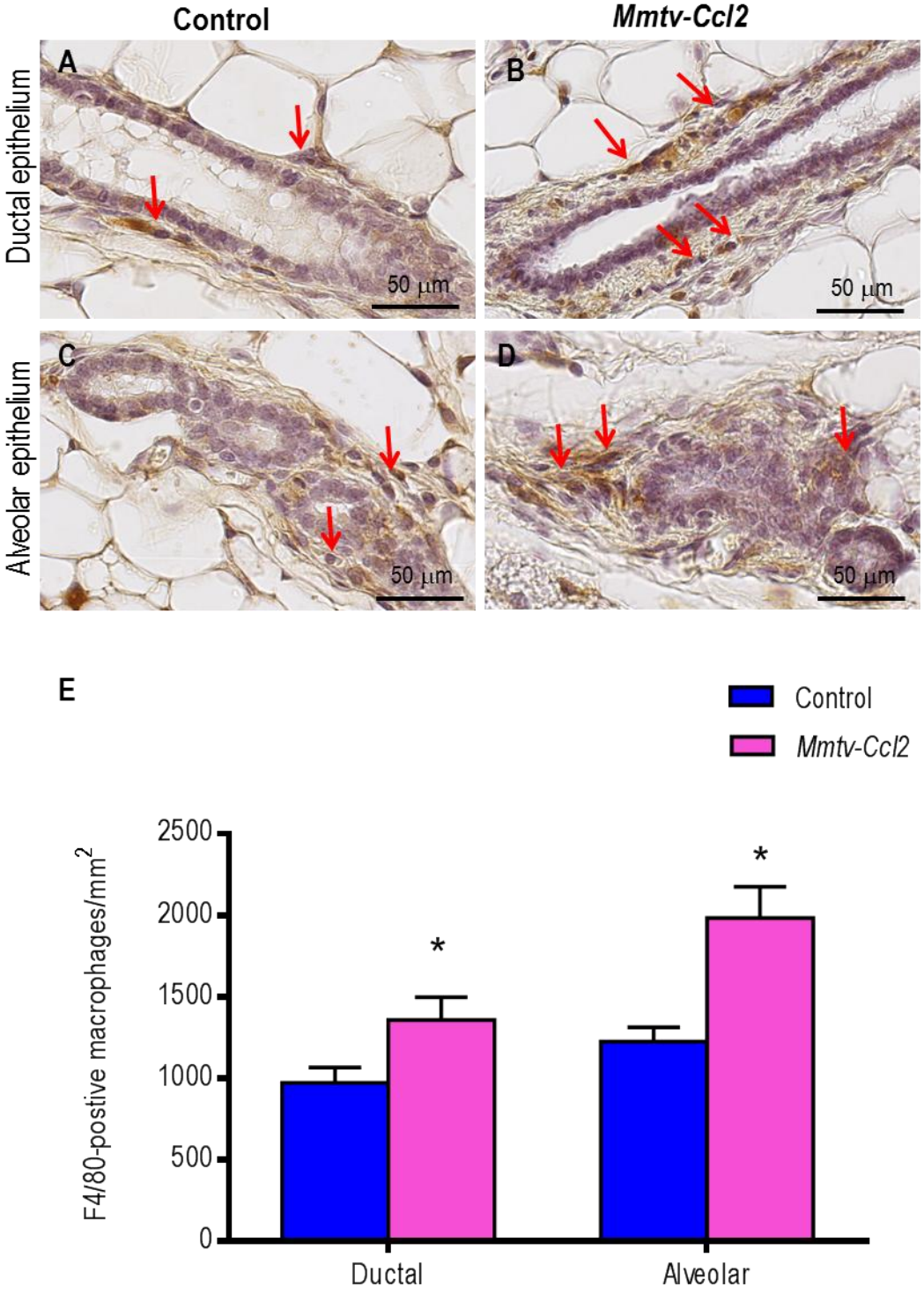

Figure 5.8 The effect of epithelial cell-derived CCL2 on macrophage abundance and location within and around mammary epithelium at proestrus.

Paraffin sections of mammary gland tissue from control (n=6) and *Mmtv-Ccl2* (n=6) were stained with anti-macrophage-specific F4/80 antibody to detect macrophages within ductal epithelium and stroma (A, B) and within alveolar epithelium and stroma (C, D) in control and *Mmtv-Ccl2* mammary glands at proestrus. Brown-stained macrophages are indicated by red arrows. The number of F4/80-positive macrophages within the epithelium and surrounding stroma was quantified and represented as F480-positive macrophages/mm^2 (E). Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

5.7 Effect of constitutive epithelial cell-derived CCL2 on collagen deposition and remodelling in the mammary gland at proestrus

To investigate the effect of epithelial cell-derived CCL2 on collagen deposition in the mammary gland at proestrus, Masson's Trichrome staining to detect the abundance of total collagen, and type 1 collagen (Collagen1) immunofluorescence to detect the density of collagen1 around the epithelium in the mammary glands of control and *Mmtv-Ccl2* mice were performed. Expression of total collagen and collagen 1 was observed around the ductal epithelium in mammary glands from both control (Figure 5.9 A, C respectively) and *Mmtv-Ccl2* (Figure 5.9 B, D respectively) mice. The thickness of total collagen (the width of collagen deposition around the ducts) as indicative of the amount of collagen deposition around each selected ductal epithelium was measured, quantified and represented as collagen thickness/μm (Figure 5.9 E). The density of collagen 1 around each selected ductal epithelium was also measured and quantified (Figure 5.9 F). There was a 53% increase in the thickness of collagen around the ductal epithelium of *Mmtv-Ccl2* mouse mammary gland (10.86 ± 1.04 μm; p=0.009; unpaired t-test) compared to that of control mouse mammary gland (7.12 ± 0.52 μm) (Figure 5.9 E). However, there was no significant difference in the density of collagen 1 around the ductal epithelium was observed between the two groups (unpaired t-test; Figure 5.9 F).

To further investigate the effect of epithelial cell-derived CCL2 on collagen synthesis and remodelling in the mammary gland at proestrus, quantitative RT-PCR was used to quantify the expression of mRNAs encoding enzymes that involves in collagen remodelling, including lysyl oxidase (*Lox*), matrix metalloproteinase 2 (*Mmp2*), matrix metalloproteinase 9 (*Mmp9*), tissue inhibitor of metalloproteinase 1 (*Timp1*), tissue inhibitor of metalloproteinase 2 (*Timp2*) and tissue inhibitor of metalloproteinase 3 (*Timp3*) (Figure 5.10). Expression of each of these genes was normalised to the house-keeping gene *Actb*. The amount of PCR product amplified by specific primers was multiplied by a constant to give an average for the control mammary gland the arbitrary value of 1. There was no significant difference in the expression level of mRNAs encoding *Mmp2*, *Mmp9*, *Timp1* and *Timp2* between the two groups. However, there was a 1.6-fold increase in the expression of mRNA encoding *Lox* in the mammary glands of *Mmtv-Ccl2* mice (1.66 ± 0.27 arbitrary units; p=0.04, unpaired t-test) compared to that of control mice (1.00 ± 0.15 arbitrary units) (Figure 5.10). Additionally there was a 1.7-fold increase in the expression of mRNA encoding *Timp3* in the mammary glands of *Mmtv-Ccl2* mice (1.72 ± 0.25 arbitrary units; p=0.02, unpaired t-test) compared to that of control mice (1.00 ± 0.17 arbitrary units) (Figure 5.10).

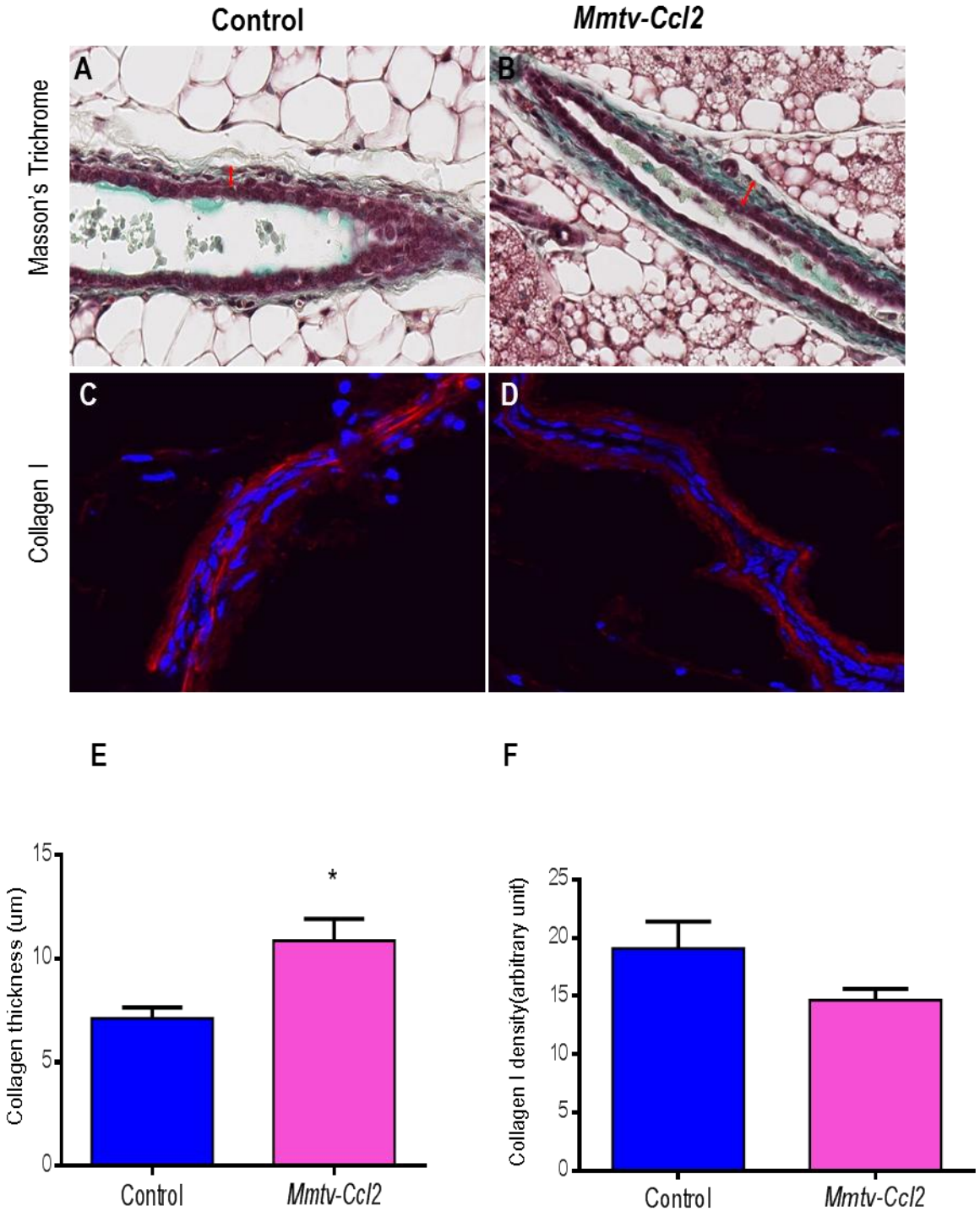

Figure 5.9 The effect of epithelial cell-derived CCL2 on collagen density around mammary epithelium at proestrus.

Masson's trichrome staining was performed to detect the density of total collagen around the ductal epithelium on paraffin sections of mammary gland tissue from control (A; n=6) and *Mmtv-Ccl2* (B; n=6) mice. Anti-collagen 1 antibody was used to detect the density of collagen 1 on frozen mammary epithelium sections from control (C; n=6) and *Mmtv-Ccl2* (D; n=6) mice .The thickness of total collagen (indicated by red arrows) around each selected ductal epithelium was measured, quantified and represented as collagen thickness/μm (E). The intensity of collagen 1 around each selected ductal epithelium was also measured, quantified and represented as collagen 1 density (arbitrary unit) (F). Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

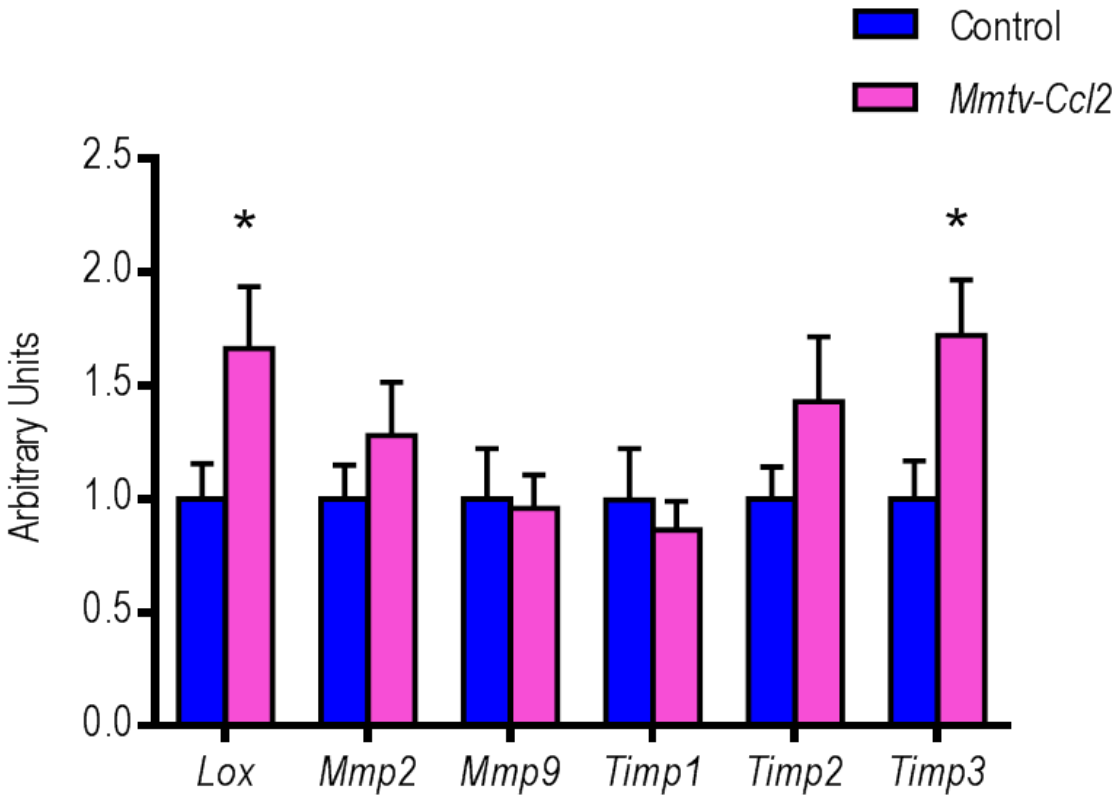

Figure 5.10 The effect of epithelial cell-derived CCL2 on collagen remodelling enzymes mRNA expression in the mammary gland at proestrus.

Mammary gland from both groups of mice (n=8) were dissected and frozen in liquid nitrogen. Messenger RNAs of collagen remodelling enzymes including *Lox*, *Mmp2*, *Mmp9*, *Timp1*, *Timp2* and *Timp3* was extracted and measured by RT-PCR. Amount of mRNA was normalised to *Actb* expression, and is given in arbitrary units where the average of the control is 1. Data are presented as mean + SEM with statistical analysis conducted using an unpaired t test. * indicates statistical significance from control. *$p < 0.05$.

5.8 Effect of constitutive expression of epithelial cell-derived CCL2 on mammary gland cancer susceptibility in mice

To investigate the effect of epithelial cell-derived CCL2 on mammary gland cancer susceptibility, control and *Mmtv-Ccl2* mice were challenged with chemical carcinogen DMBA. Mice were treated with DMBA for 6 weeks from the age of 6 weeks and monitored weekly for mammary gland tumours by palpation. A Kaplan-Meier survival plot showing the percentage of tumour-free mice following DMBA treatment for both control and *Mmtv-Ccl2* mice was generated (Figure 5.11). Tumour latency was determined as the average time to appearance of first palpable mammary tumour. There was a 28% increase in the incidence of mammary tumour formation in DMBA-treated *Mmtv-Ccl2* mice [14 out of 18 *Mmtv-Ccl2* mice developed mammary gland tumour (78%); p=0.025; Log Rank] compared to that of control mice [9 out of 18 control mice developed mammary gland tumour (50%)] (Figure 5.11).

5.9 Relationship between CCL2 and macrophage phenotype in human non-neoplastic breast tissue

After demonstrating that epithelial cell-derived CCL2 regulates macrophage recruitment and function in the mouse mammary gland, a series of experiments were conducted to investigate whether CCL2 plays a similar role in regulating macrophages in the human breast. Although many studies have shown the presence of abundant CCL2 in breast carcinomas, the expression pattern of CCL2 in the normal human mammary gland is not clear. To evaluate the expression pattern of CCL2 in human non-neoplastic breast tissue, immunohistology analysis was performed to detect CCL2 protein. The relationship between CCL2 and macrophages (immunohistology analysis with different macrophage markers was described in chapter 3) in normal human breast was also explored.

CCL2 was mainly expressed by the breast epithelium in human non-neoplastic breast tissue (Figure 5.12). However, the breast exhibited variable expression of CCL2 in the epithelium, with protein abundance ranging from low (Figure 5.12 A), medium (Figure 5.12 B) to high (Figure 5.12 C). No positive staining was observed in breast epithelium stained with isotype matched irrelevant antibody (Figure 5.12 D). There was no significant association between the expression of CCL2 and the abundance of CD68-positive macrophages (Figure 5.13 A; R^2= 0.0011, p=0.89), the abundance of CD206-positive macrophages (Figure 5.13 B; R^2= 0.1409, p=0.14), or the percentage of CCR7-positive cells (Figure 5.13 C; R^2= 0.0234, p=0.59). However, a significant negative relationship was found

between the expression of CCL2 and the abundance of stromal-associated iNOS-positive cells (Figure 5.13 D; R^2= 0.3384, p=0.018).

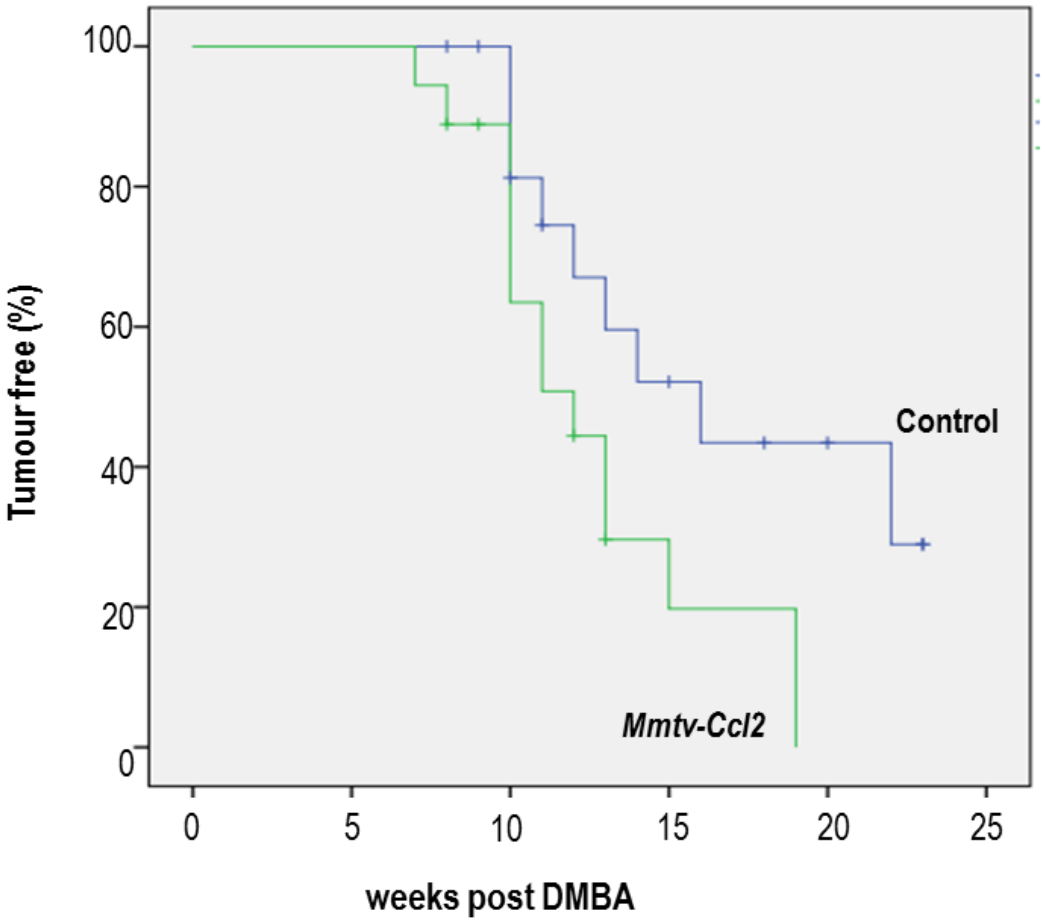

Figure 5.11 The effect of epithelial cell-derived CCL2 on mammary gland cancer susceptibility in mice.

A Kaplan-Meier survival plot showing the percentage of tumour-free mice as a function of post-DMBA treatment in weeks for both control and *Mmtv-Ccl2* mice was generated. Tumour latency was determined as the time to first appearance of palpable mammary tumour. The censored-mice (mice were killed because of other tumours or sickness) were represented by a vertical line on the plot. n=18 per group, statistical analysis was conducted using SPSS Statistics Version 17.0, *p=0.025, Log Rank.

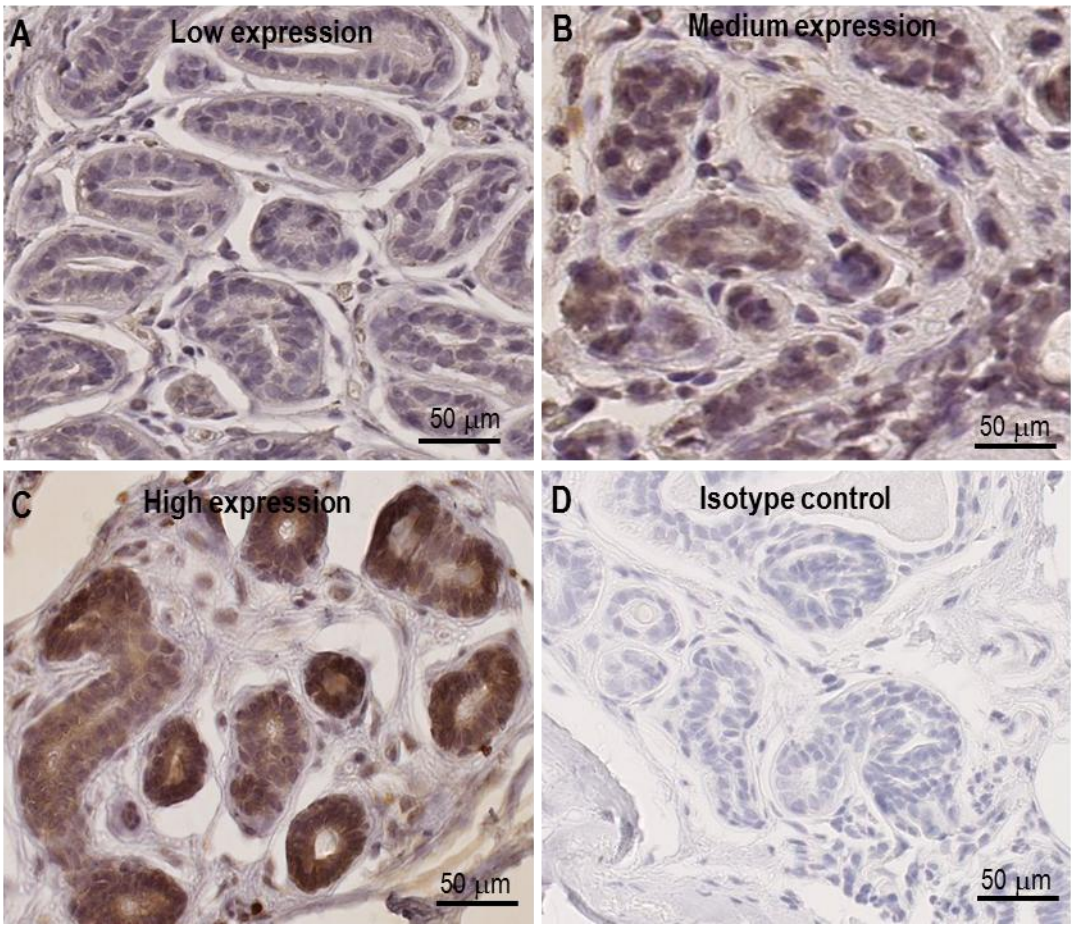

Figure 5.12 Detection of the abundance of CCL2 in non-neoplastic human breast tissue.

Anti-human CCL2 antibody was used to detect the expression of CCL2 within human breast epithelium. The breast exhibits variable expression of CCL2 in the epithelium, with protein abundance ranging from low (A), moderate (B) to high (C). Isotype matched irrelevant antibody negative control (D) was included.

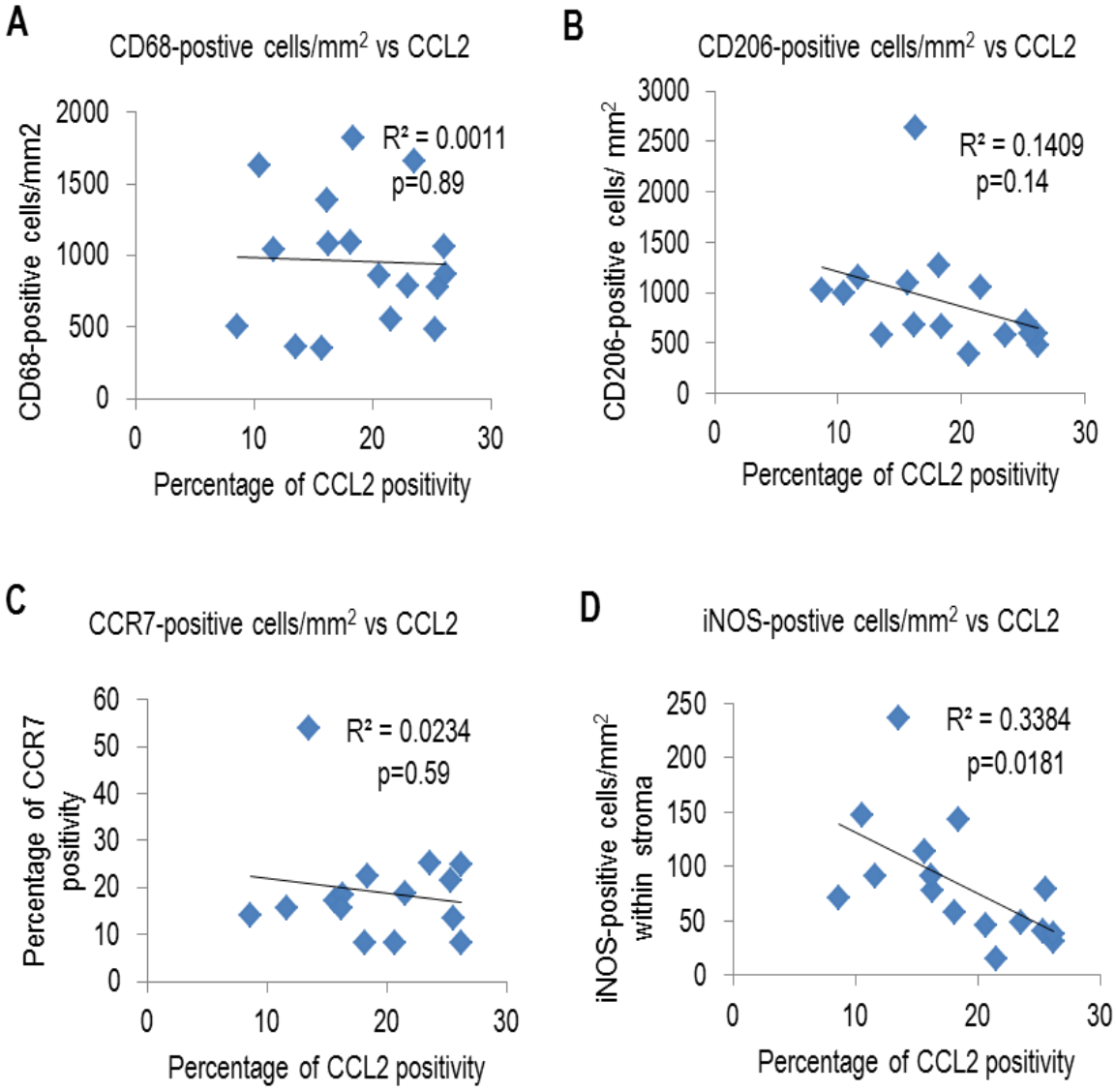

Figure 5.13 Relationship between the expression of CCL2 and the abundance of macrophages in non-neoplastic human breast tissue.

No significant association was found between the expression of CCL2 and CD68-positive macrophage density (A). No significant association was found between the expression of CCL2 and the abundance of CD206-positive cells (B). No significant association was found between the expression of CCL2 and percentage of CCR7-positive cells (C). However, the expression of CCL2 was significantly negatively associated with the abundance of iNOS-positive cells within the stroma (D). Linear regression analysis was conducted using SPSS Statistics Version 17.0, Significant differences were inferred when p values were ≤0.05, n=19.

5.10 Discussion

A number of *in vitro* and *in vivo* studies have demonstrated the chemotactic ability of CCL2 in recruiting macrophages to tissue during inflammation (Matsushima et al., 1989, Yoshimura et al., 1989, Carr et al., 1994, Allavena et al., 1994, Fuentes et al., 1995, Rutledge et al., 1995). Although over-expression of CCL2 and increased infiltration of macrophages have been found in breast carcinomas, the role of CCL2 in regulation of macrophages in mammary gland development and cancer susceptibility is still unclear. By using *Mmtv-Ccl2* transgenic mouse model whereby CCL2 is constitutively expressed by the mammary epithelium, the experiments presented in this chapter have clearly demonstrated that epithelial cell-derived CCL2 in the mammary gland have multiple functions: (1) regulation of mammary epithelium development, (2) regulation of macrophage recruitment and (3) regulation of collagen deposition and remodelling. Furthermore, our results also suggest that constitutive expression of CCL2 in the mammary gland can increase mammary tumour susceptibility in mice, and the presence of CCL2 in the epithelium of non-neoplastic human breast tissue suggests CCL2 may have a similar role in women.

5.10.1 Epithelial cell-derived CCL2 inhibits mammary gland epithelium regression

In cycling non-pregnant mice, the mammary epithelium undergoes ductal development and regression over the course of each ovarian cycle under the influence of ovarian hormones and various cytokines (Fata et al., 2001, Hodson et al., 2013, Chua et al., 2010). For the mammary gland to undergo these dynamic developmental processes, whilst maintaining regulation of tissue homeostasis, cell proliferation, differentiation and apoptosis is essential. Active mammary epithelium branching and alveolar budding occurs during the ovarian cycle in mice, with the greatest alveolar bud development at diestrus (Fata et al., 2001, Chua et al., 2010). Subsequently, if pregnancy does not proceed, the alveolar buds undergo regression, there is apoptosis of newly formed epithelium and tissue remodelling at proestrus, which returns the mammary epithelium to its basic architecture at estrus, ready for the onset of the next ovarian cycle of development (Chua et al., 2010).

Increased ductal branch points and alveolar bud development at proestrus suggests that despite normal estrous cyclicity, mammary epithelium regression was inhibited in mice. However, at estrus, the mammary epithelium exhibited a basic ductal structure, implying that regression was delayed rather than completely inhibited. A recent study has shown although CCL2 is not normally constitutively expressed by the mammary epithelium, an increased expression of CCL2 was observed during

involution when rapid tissue remodelling was occurred. It was demonstrated that CCL2 expression was at highest at the early stage of involution, and its expression was rapidly decreased at the later stage of involution during normal mammary gland involution (O'Brien et al., 2010a). This study, together with our finding, suggests CCL2 is likely to play a role in regulating tissue remodelling in the mammary gland, however, the expression of CCL2 needs to be tightly regulated to maintain appropriate rate of tissue remodelling and regression in the mammary gland.

Mammary gland regression involves a series of processes, including remodelling of mammary extracellular matrix, apoptosis of the mammary epithelial cells and clearance of dying epithelial cells by phagocytosis (O'Brien et al., 2010b, Khokha and Werb, 2011, Chua et al., 2010). Epithelial cell proliferation and apoptosis was unaffected by constitutive expression of CCL2 by mammary epithelium, which suggests that CCL2 might not affect epithelial cell turnover in the adult cycling mammary gland. However, the stroma area around ductal mammary epithelium was significantly larger in the presence of epithelial cell-derived CCL2, and therefore, CCL2 might promote stroma deposition around mammary epithelium. Mammary gland stroma is composed of fibrous connective tissue extracellular matrix proteins (e.g. collagens), and a wide variety of cells, including adipocytes, fibroblasts and leukocytes such as macrophages and eosinophils (Schwertfeger et al., 2006, Gouon-Evans et al., 2000, Lin et al., 2002, Muschler and Streuli, 2010). The observation of increased stroma deposition with constitutive expression of CCL2 implies a possible role for CCL2 in mediating remodelling of extracellular matrix proteins which might impact on mammary gland regression.

An increased abundance of macrophages was observed in the mammary gland with overabundant CCL2 at proestrus. Macrophages are required for tissue remodelling during mammary gland regression (Chua et al., 2010), the observation of increased abundance of macrophages and increased stroma deposition in the presence of constitutive expression of epithelial cell-derived CCL2 at proestrus suggests CCL2 might regulate mammary gland regression at proestrus through directing the extracellular matrix remodelling function of stromal macrophages.

5.10.2 Epithelial cell–derived CCL2 promotes macrophage recruitment in the mammary gland

The chemoattractant activity of CCL2 in regulating macrophage recruitment was demonstrated in studies using genetically modified mouse models, in which deficiency in CCL2 or CCR2 cause perturbed recruitment of monocytes and macrophages, whereas overexpression of CCL2 results in

increased macrophage infiltration (Fuentes et al., 1995, Lu et al., 1998, Rutledge et al., 1995). Consistent with the known role of CCL2 in macrophage recruitment, macrophages within the epithelium and stroma was increased in mammary gland with constitutive expression of epithelial cell-derived CCL2.

In the naturally cycling mammary gland, particularly at proestrus, macrophages are recruited to the mammary epithelium where they assist in the breakdown of alveolar buds through phagocytosis of apoptotic cells and tissue remodelling (Chua et al., 2010). As CCL2 promotes macrophage recruitment to mammary epithelium at proestrus, it is likely that CCL2 promotes the regulation of mammary epithelium regression through modulating macrophage recruitment and phagocytosis. However, perturbed mammary epithelium regression was observed in the presence of over-expression of CCL2, which is implying that even though CCL2 leads to increase recruitment of macrophages to mammary epithelium, the phenotypes of these macrophages could be altered and become less involved in tissue remodelling due to the presence of abundant CCL2. Further investigations are required to dissect the phenotypes of these macrophages and provide further information on the role of CCL2 in regulating macrophage functions in the mammary gland.

5.10.3 Epithelial cell-derived CCL2 promotes collagen deposition and remodelling in the mammary gland

Mammary extracellular matrix is a major component of mammary gland stroma (Fata et al., 2004). Collagen is a prominent component of extracellular matrix in mammary gland stroma which contributes significantly in supporting tissue integrity and elasticity; furthermore; the deposition and remodelling of collagen has found to be highly associated with normal mammary gland development and tumourigenesis (Maller et al., 2010, Maller et al., 2013, Levental et al., 2009). Increased collagen deposition was observed around ductal mammary epithelium during puberty and during involution in mice (Keely et al., 1995, O'Brien et al., 2010a), suggesting that increased collagen deposition might be associated with increased tissue remodelling during these two stages of mammary gland development.

Evidence for involvement of CCL2 in collagen deposition was demonstrated in previous studies in which increased CCL2 expression was observed in human pulmonary fibrosis (Antoniades et al., 1992), bleomycin-induced pulmonary fibrosis in rats and mice (Brieland et al., 1993, Baran et al., 2007), and

interstitial renal fibrosis in mice (Lloyd et al., 1997). Furthermore, increased CCL2 expression was also observed in involuting mammary gland with increased collagen deposition coincident with increased macrophage infiltration (O'Brien et al., 2010a). This is consistent with our finding as increased total collagen deposition and macrophage density was found around ductal epithelium with constitutive expression of CCL2. Many studies have also shown that macrophages can promote collagen synthesis *in vitro* (Weitkamp et al., 1999, Schnoor et al., 2008, Schmid-Kotsas et al., 1999), moreover, it was demonstrated *in vivo* that macrophages promote collagen fibrillogensis around mammary epithelium during development (Ingman et al., 2006). These studies strongly suggest that constitutive expression of CCL2 in mammary gland epithelium could promote collagen deposition via increasing macrophage recruitment to mammary epithelium and this dysregulates extracellular matrix synthesis and perturbs mammary gland regression.

It is well established that the extracellular matrix is a highly dynamic structure and the balance between its deposition and remodelling is crucial for development and the maintenance of tissue homeostasis (Bonnans et al., 2014). To maintain normal mammary gland branching morphogenesis and development, the remodelling of extracellular matrix is tightly controlled by remodelling enzymes, including matrix metalloproteinases (MMPs) such as MMP2, MMP9 and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Fata et al., 2004, Simian et al., 2001). TIMPs inhibit extracellular matrix proteolysis, to avoid dysregulaion of excessive tissue degradation and remodelling (Bonnans et al., 2014). MMPs and TIMPs are present at many stages of mammary gland development, particularly at puberty, pregnancy and involution (Green and Lund, 2005). However, the underlying mechanism of how these remodelling enzymes are activated and regulated in the mammary gland is yet to be elucidated.

Analysis of these remodelling enzymes in mammary glands with constitutive expression of epithelial cell-derived CCL2 suggest that CCL2 might not regulate collagen deposition through direct regulation of MMPs as the expression of *Mmp2* and *Mmp9* mRNA was unaffected by over-expression of CCL2. However, increased *Timp3* mRNA expression was observed in the presence of epithelial cell-derived CCL2, indicating CCL2 could be enhancing the inhibitory effect of TIMPs on MMP-mediated collagen degradation, resulting in overly increased collagen deposition in these mice. Moreover, high expression of epithelial cell-derived CCL2 also causes increased expression of lysyl oxidase (*Lox*), a collagen crosslinking enzyme involved in cross-linking elastin and collagen in extracellular matrix and hence enhancing tissue integrity (Levental et al., 2009). This could also contribute to the increased collagen deposition around mammary epithelium in the presence of abundant epithelial cell-derived CCL2, as

increased LOX expression could lead to increase mammary gland stromal stiffness and collagen cross-linking, which could further inhibit MMP-mediated collagen degradation.

Taken together, these findings suggest that epithelial cell-derived CCL2 increases collagen deposition and inhibits collagen remodelling around the mammary epithelium. Through promotion of macrophage recruitment and the activities of TIMP3 and LOX, high CCL2 expression would cause imbalance in the turnover of collagen, which in turn could impair mammary gland regression during proestrus phase of the ovarian cycle.

5.10.4 Constitutive expression of epithelial cell-derived CCL2 increases mammary cancer susceptibility in mice

Population studies have demonstrated that the risk of developing metastasis is significantly increased in breast cancer patients carrying the CCL2-2518 A/G promoter polymorphism compared to A/A homozygotes (Ghilardi et al., 2005), as the A/G genotype causes increased CCL2 production in monocytes after stimulation compared to the A/A genotype (Rovin et al., 1999). Although the statistical power of these two studies was limited by the cohort size of patients, these findings suggest a role of CCL2 in promoting cancer metastasis. Increased incidences of mammary gland tumour formation and decreased tumour latency was observed in transgenic mice that constitutively express epithelial cell-derived CCL2 challenged with DMBA carcinogen, implying CCL2 expression could increase mammary cancer susceptibility. The experiments described in this chapter were the first to model the increase risk of breast cancer observed in population studies.

The expression of CCL2 in breast carcinomas is highly associated with macrophage infiltration (Fujimoto et al., 2009, Arendt et al., 2013, Ueno et al., 2000, Qian et al., 2011). This is likely to be a causal relationship, as administration of a neutralising antibody to CCL2 to mice xenografted with human breast tumour cells resulted in a significant reduction in the number of infiltrated macrophages and reduced tumour growth (Fujimoto et al., 2009). This causal relationship is further supported by a recent study showing that discontinuation of CCL2 inhibition in tumour-bearing mice resulted in increased monocytes infiltration (Bonapace et al., 2014). Hence, it is reasonable to suggest that increased CCL2-regulated macrophage recruitment in the mammary gland with constitutive expression

of epithelial cell-derived CCL2 might be responsible for the accelerated tumour progression in these mice.

However, increased macrophage infiltration is not the only factor that associates with increased mammary cancer susceptibility. Collagen deposition has long been recognised as a critical factor that linked with increased risk of breast cancer (McCormack and dos Santos Silva, 2006, Boyd et al., 2001). Recent animal studies have further illustrated the causal relationship between collagen deposition and mammary tumour progression. Transgenic mice with abundant stromal collagen deposition in the mammary gland exhibited increased mammary tumour formation and tumour metastasis when cross-bred with MMTV-PyMT mice, indicating increased collagen deposition in the mammary gland directly promotes mammary gland tumourigenesis (Provenzano et al., 2008). Further, increased collagen deposition was found in postpartum involuting mammary gland xenografted with human breast cancer cells, in which a more invasive tumour phenotype was observed with increased collagen density (Lyons et al., 2011). These findings suggest that increased collagen deposition could be another factor responsible for the increased mammary tumour incidences we observed in transgenic mice with constitutive expression of epithelial cell-derived CCL2.

The remodelling and stiffness of extracellular matrix is also involved in cancer progression (Bonnans et al., 2014). MMPs and TIMPs are found to be overly-expressed in breast carcinomas (Bergamaschi et al., 2008, Mylona et al., 2006, Jodele et al., 2006), indicating the balance between collagen deposition and break down affects tumour progression. A recent study has demonstrated that mammary tumourigenesis was greatly suppressed in *Timp3*$^{-/-}$ mice cross-bred with MMTV-PyMT or MMTV-Neu transgenic mice, suggesting that loss of *Timp3* could result in mammary tumour suppression (Jackson et al., 2015). Increased expression of *Timp3* but not *Mmps* was observed in mammary glands with constitutive expression of epithelial cell-derived CCL2, suggesting abundant CCL2 might inhibit collagen turnover through promoting the expression of *Timp3*, and therefore increases mammary cancer susceptibility in these mice.

Increased extracellular matrix stiffness has also been associated with cancer progression (Paszek et al., 2005, Chaudhuri et al., 2014). Stiffness is a measure of the ability of a material to resist deformation, and extracellular matrix stiffness is an important mechanical force that regulates cell functions during different physiological processes (Wells, 2008). LOX is a key enzyme that mediates collagen cross-

linking, and its expression is elevated in many cancers (Erler and Weaver, 2009). LOX-mediated collagen cross-linking increases tissue stiffening and mammary tumour invasion, conversely, inhibition of LOX activity using a LOX polyclonal antibody induced reduced fibrosis and mammary tumour incidence in mice (Levental et al., 2009). Herein we have demonstrated there was increased *Lox* expression in the presence of epithelial cell-derived CCL2; implying CCL2 might induce collagen stiffening via up-regulating the expression of *Lox* and hence drive tumour development.

It is well established that chronic inflammation is highly associated with cancer formation. A recent study has shown that increased LOX expression and collagen deposition with increased macrophage infiltration was observed in a mouse model of chronic bacterial-induced prostatic inflammation (Wong et al., 2014), and these observations is in mirrored with our observations in mice with constitutive expression of epithelial cell-derived CCL2, suggesting high abundance of CCL2 is likely to induce chronic inflammation. Given that chronic inflammation is directly associated with cancer formation (Shacter and Weitzman, 2002) and high expression of pro-inflammatory enzyme COX2 is linked with accelerated tumour invasion (Lyons et al., 2011), therefore, these evidence strongly suggest that the presence of epithelial cell-derived CCL2 is likely to induce chronic inflammation in the mammary gland, thus resulting in increased macrophage infiltration and perturbed collagen remodelling, which in turn promotes increased risk of mammary gland tumourigenesis.

Taken together, these findings suggest that constitutive expression of epithelial cell-derived CCL2 by mammary epithelium increased mammary cancer susceptibility in mice through inducing a chronic inflammation in the mammary gland, which is characterised by increased macrophage infiltration and fibrosis associated with perturbed collagen deposition and remodelling.

5.10.5 Epithelial cell-derived CCL2 affects macrophage functions in the human breast

We observed highly variable abundance of CCL2 in human non-neoplastic breast tissue, ranging from low, medium to high. Previous studies have shown that the human breast epithelium expresses low but detectable quantities of CCL2 protein (Soria and Ben-Baruch, 2008, Ueno et al., 2000), whilst this is the first study to observe CCL2 protein in human breast epithelium. In the breast, macrophages are primarily located around the epithelium, within the epithelium and the stroma, which suggests that macrophages are in close proximity to CCL2, and therefore CCL2 could be playing a similar role in

regulating macrophages in the human breast as they do in the mouse mammary gland. However, we did not observe increased macrophage recruitment in the presence of increased abundant CCL2 expression, and this observation is not consistent with what we found in mouse mammary gland. As we mentioned earlier, increased CCL2 expression is induced during inflammation and under disease conditions accompanied with increased macrophage recruitment (Yadav et al., 2010). Little is known about the role of CCL2 in the normal breast, and it is not clear whether the CCL2 expression we observe is transient or chronic. Chronic CCL2 expression may result in adaptive changes in the breast which dampen the chemotactic effects of CCL2 such that the abundance of macrophages is overtly normal.

The abundance of CD206-positive macrophages and CCR7-positive macrophages was not significantly associated with CCL2 expression by the epithelium in human breast, however, interestingly; the abundance of iNOS-positive macrophages was decreased with the presence of increased abundant epithelial cell-derived CCL2 in the human breast. iNOS is considered to be an "M1" macrophage marker and it is highly expressed by macrophages involved in inflammation (Mantovani et al., 2002). Hence, it is surprising that increased CCL2 expression is associated with decreased abundance of iNOS-positive macrophages, as both CCL2 and iNOS are up-regulated during inflammation. As discussed above, transient and chronic CCL2 expression may result in different tissue responses, transient CCL2 may recruit inflammatory macrophages to respond to a specific immune challenge, whereas chronic CCL2 may result in a more fibrotic phenotype which is likely to be associated with different macrophage phenotype. Our cohort of breast tissue samples were taken at one time point only from each participant and therefore would not be sufficiently representative to fully evaluate the relationship between CCL2 and "M1" macrophage. Further investigations are required to explore the associations between CCL2 and different macrophage markers, perhaps including breast tissue at multiple time points from each participant with a larger sample size of participants.

Mammographic density (MD) is an important risk factor for breast cancer (McCormack and dos Santos Silva, 2006), and high MD is associated with increased breast cancer risk (Boyd et al., 1995). High MD tissue is composed of greater stroma deposition and increased collagen deposition (Ghosh et al., 2012, Barcus et al., 2013, Provenzano et al., 2008), and these characterisations are strikingly similar to the mammary glands of transgenic mice with constitutive expression of epithelial cell-derived CCL2, suggesting a possible role of CCL2 in promoting stroma deposition and therefore increasing MD in women. Furthermore, increased mammary tumour susceptibility in these transgenic mice is also

reminisced with increased breast cancer risk in high MD women, implying the abundance of CCL2 in breast epithelium could be associated with increased cancer risk in women with high MD. Recently, by analysing the location and abundance of macrophages in paired high MD and low MD tissue, our laboratory has demonstrated that high MD tissue is associated with lesser abundance of CD206+ "M2" macrophages, however, the total abundance of macrophages is not affected by the degree of MD (Huo., et al, unpublished). The identity of these increased macrophages is unknown, and it is reasonable to postulate that these macrophages could be associated with CCL2, and which in turn driving the deposition of stroma and collagen in the breast and thus increase in risk of cancer.

Together, these findings suggest a possible role of epithelial cell-derived CCL2 in regulating macrophages in the human breast; however, it is difficult to conclude if CCL2 play a similar role in humans as they do in mice, as our study is limited by cohort size, and a limited understanding of the regulation of CCL2 expression in breast epithelium. However, our transgenic mouse model has shed light on a likely mechanical link between CCL2 and MD in the breast, and further studies are required to investigate the underlying mechanisms of how CCL2 regulates stroma deposition in the breast and thus affects the status of MD in women.

5.11 Conclusions

In summary, the findings reported in this chapter have revealed the significance of CCL2 in regulating macrophage recruitment and collagen remodelling in the mammary gland and in cancer susceptibility. We propose that constitutive expression of epithelial cell-derived CCL2 induces chronic inflammation in the mammary gland and increases mammary cancer susceptibility in mice via (1) the promotion of macrophage recruitment, and (2) the perturbation of collagen deposition and remodelling. Future studies will examine how CCL2 is regulated in mouse and human epithelium, and how macrophage phenotypes and functions are regulated by epithelial cell-derived CCL2. Understanding the underlying mechanisms of how CCL2 regulates macrophages and collagen remodelling will shed light on the cellular mechanisms that underpin susceptibility of the breast to cancer.

Chapter 6 General discussion and conclusions

6.1 Introduction

It is widely acknowledged that dynamic and complex interactions between mammary gland epithelium and the surrounding stromal cells are pivotal for proper development and functioning of the mammary gland. Macrophages are a major component of the mammary gland stroma and are capable of diverse roles in mammary gland development and maintenance of homeostasis. The extraordinary plasticity of these cells enables them to function and respond appropriately to the diverse signals they receive within the local cytokine microenvironment. TGFB1 and CCL2 are two key cytokines that are expressed by the mammary epithelium, which thus may affect macrophage phenotypes and functions in the mammary gland. Irregularities in these interactions are found to be associated with different stages of tumourigenesis and may increase the risk of tumour formation. Numerous *in vitro* and *in vivo* studies have demonstrated the significance of TGFB1 and CCL2 in regulating the functions of macrophage in many other tissues; however, the cross-talk between these two epithelial cell-derived cytokines with macrophages in mammary gland development and tumourigenesis has not so far been elucidated.

Immunohistochemical analysis revealed that both TGFB1 and CCL2 were expressed by the mouse and human mammary epithelium, where these cytokines are in close proximity to macrophages within the mammary gland stroma. The close spatial vicinity between these epithelial cell-derived cytokines and macrophages suggests cross-talk between them is likely to occur in the mammary gland. The studies described in this thesis are the first to investigate the significance of this cross-talk in normal mammary gland development and mammary cancer susceptibility. The experiments reported herein provide compelling evidence of the critical roles of epithelial cell-derived TGFB1 and CCL2 in regulating macrophage phenotypes and functions in maintaining normal mammary gland morphogenesis, and suggest that cytokine-mediated epithelial cell-macrophage interactions could significantly influence mammary cancer susceptibility in mice. In addition, these experiments also reveal the importance of cytokine-macrophage regulatory networks in the human breast, as it appears that both TGFB1 and CCL2 could be playing similar roles in the human breast as they do in the mouse mammary gland. In this chapter, the roles of epithelial cell-derived TGFB1 and CCL2 in modulating macrophages in both mouse and human mammary gland are discussed. Opportunities for future research to improve our understanding of the significance of cytokine-macrophage regulatory networks in mammary gland morphogenesis are highlighted, as well as prospects for improved therapeutic approaches to breast cancer prevention and treatment.

6.2 Epithelial cell-derived cytokines regulate macrophage functions in mouse mammary gland development

As has been demonstrated for epithelial cell-derived CSF1 previously (Gouon-Evans et al., 2002, Ingman et al., 2006), these studies show that both TGFB and CCL2 produced by mammary gland epithelium have profound effects on adjacent macrophage recruitment and phenotype. TGFB and CCL2 have profoundly different effects on regulating immune system responses. Overall, however, TGFB1 is widely considered a primarily anti-inflammatory cytokine, whereas CCL2 is considered as a pro-inflammatory cytokine. By investigating the two side-by-side, we can address the question of how the balance between pro- and anti-inflammatory stimuli influences mammary gland morphogenesis and cancer risk.

Direct regulation of macrophages by epithelial cell-derived TGFB1 was demonstrated via mammary gland transplantation of *Tgfb1* null and wild-type epithelium into the cleared mammary gland of wild-type recipients. Deficiency in epithelial cell-derived TGFB1 caused increased invasion of macrophages to mammary epithelium and increased abundance of iNOS-positive and CCR7-positive "M1" macrophages. The significance of TGFB1 in regulating macrophages in the mammary gland was further supported in studies utilising the *TGF-Mac* transgenic mouse model, where impaired TGFB signalling in macrophages also resulted in increased invasion of macrophages into the mammary epithelium and increased abundance of "M1" macrophages. Although the phenotypes of macrophages recruited under control of constitutive expression of CCL2 are yet to be elucidated, these studies also demonstrate that epithelial cell-derived CCL2 increases the abundance of macrophages. The increased macrophage recruitment seen in our model is consistent with several previous studies which reported transgenic mice with deficiency in CCL2 or CCR2 resulted in perturbed recruitment of monocytes and macrophages, and overexpression of CCL2 caused increased macrophage infiltration (Fuentes et al., 1995, Lu et al., 1998, Rutledge et al., 1995), implying a role of CCL2 in promoting macrophage recruitment in the mammary gland. These findings suggest that the abundance of TGFB and CCL2 in mammary epithelium could alter the balance between different macrophage populations. Abundant CCL2 promotes the recruitment of macrophages to the mammary gland in mice, while the presence of abundant TGFB restrains the immune regulatory activities of macrophages and inhibits invasion of macrophages into the epithelium.

Epithelial cell-derived TGFB and CCL2 both exerted regulatory influences on mammary gland development during the estrous cycle, albeit at different stages of the cycle. Impaired TGFB signalling in macrophages resulted in increased ductal branching and alveolar development in naturally cycling mice at diestrus, suggesting an inhibitory role of TGFB-regulated macrophages in mammary gland morphogenesis. This is in line with studies which have employed transgenic mice with over-expression of TGFB1 in the mammary epithelium, where retardation of ductal development and alveolar development was observed during puberty and pregnancy (Pierce et al., 1993, Jhappan et al., 1993). On the other hand, constitutive expression of epithelial cell-derived CCL2 resulted in increased ductal branch points and alveolar bud development observed at proestrus, suggesting that CCL2 could exert an inhibitory effect on mammary epithelium regression. Overtly normal estrous cyclicity was observed in both transgenic mouse models, suggesting that the effect on mammary gland morphogenesis was the direct result of the cytokine acting on the mammary gland microenvironment. However, circulating estrogen and progesterone were not evaluated so indirect hormonal effects cannot be discounted.

Macrophages exert profound effects on both proliferation and development of alveolar buds at diestrus and mammary gland regression at proestrus, and it is likely that TGFB and CCL2 direct macrophage function during development and regression respectively. Although the effect of TGFB signalling in modulating mammary epithelial cell proliferation was not evaluated in these studies, we have recently demonstrated that epithelial cell-derived TGFB1 regulates mammary epithelial cell turnover at diestrus in an autocrine manner (Sun et al., 2013). Impaired TGFB signalling to macrophages caused increased alveolar bud development at diestrus, suggesting TGFB restrains mammary gland alveolar bud development. Therefore, both autocrine and paracrine effects of TGFB are clearly significant in regulating appropriate mammary gland morphogenesis. Regression and remodelling of mammary epithelium requires proper remodelling of mammary extracellular matrix, apoptotic death of the mammary epithelial cells and clearance of dying epithelial cells by phagocytosis (O'Brien et al., 2010b, Khokha and Werb, 2011, Chua et al., 2010). Immunohistochemical analysis revealed that mammary epithelial cell turnover was unaffected by CCL2, however, increased stroma deposition was observed in the presence of constitutive expression of CCL2. These findings suggest that expression of CCL2 by mammary epithelial cells affects mammary epithelium regression through mediating components of mammary gland stroma, such as extracellular matrix proteins (collagen) and other stromal cells including macrophages, rather than through its modulation of mammary epithelial cell turnover. Nonetheless, it was noted that an overall normal mammary gland ductal development was observed in mice with altered TGFB signalling and with constitutive expression of CCL2, which suggests that these

transient effects on morphogenesis could perhaps be rescued or compensated by other growth factors, cytokines or signalling pathways.

CSF1-regulated macrophages are reported to be involved in promoting collagen fibrillogensis around mammary epithelium during development (Ingman et al., 2006), and our findings provide supporting evidence of a role for CCL2 in enhancing collagen deposition through increasing macrophage recruitment and macrophage-assisted collagen fibrillogensis in the mammary gland. The involvement of CCL2 in promoting collagen deposition observed in our model is in line with previous studies demonstrated that increased CCL2 expression is found in human pulmonary fibrosis (Antoniades et al., 1992), bleomycin-induced pulmonary fibrosis in rats and mice (Brieland et al., 1993, Baran et al., 2007), and interstitial renal fibrosis in mice (Lloyd et al., 1997). To more definitively decipher whether CCL2 directly promotes collagen deposition via macrophages, macrophages would need to be cultured *in vitro* in the presence of CCL2, and the production of collagen-associated mRNA or proteins examined. TGFB is reported to induce collagen synthesis and fibrosis *in vivo* (Roberts et al., 1986), however, we did not observe the overt alteration in stroma in *TGF-Mac* transgenic mice, and did not investigate collagen synthesis and extracellular matrix remodelling. Epithelial cell-derived TGFB is found to suppress branching morphogenesis in the mammary gland through its regulation of extracellular matrix (Silberstein et al., 1992), hence, it would be of interest to further dissect the direct role of TGFB and TGFB-regulated macrophages in mediating extracellular matrix in mammary gland development.

These studies demonstrate an absolute requirement of a dynamic and balanced cytokine-macrophage regulatory network for normal mammary gland development across the ovarian cycle. Dysregulaion of cytokine expression in the mammary gland could perturb mammary gland development and also affect mammary tumourigenesis. Therefore, the expression and actions of these cytokines need to be tightly regulated to direct the equilibrium between macrophage populations and phenotypes at different stages of development. However, the upstream mechanisms of how these cytokines are regulated is still unclear. Further studies dissecting the upstream signalling pathways that regulate cytokine expression in mammary epithelial cells such as NOTCH (Yumimoto et al., 2015) and ovarian hormones would be informative in unravelling the molecular mechanisms behind epithelial cell-macrophage cross-talk.

6.3 Epithelial cell-derived cytokines affect macrophage abundance and functions in the human breast

High abundance of TGFB1 produced by the breast epithelium was associated with fewer macrophages invaded into the breast epithelium and reduced abundance of macrophages within the stroma, and this was consistent with our results from mice studies. Although epithelial cell-derived TGFB1 did not appear to constrain the abundance and function of iNOS-positive and CCR7-positive "M1" macrophages in the human breast, the abundance of CD206-positive "M2" macrophages was increased with increasing abundance of epithelial cell-derived TGFB1; suggesting TGFB1 might promote the activities of "M2" macrophages in human breast. This result concurs with a previous study showing that TGFB signalling promotes expression of genes associated with "M2" macrophages and expression of "M2" phenotypic marker Arginase I (Arg1) on macrophages (Gong et al., 2012). Therefore our findings suggest that epithelial cell-derived TGFB1 inhibits macrophage invasion into the epithelium and stroma in the human breast. Overabundance of TGFB1 could up-regulate pro-tumourigenic "M2" macrophage activity in the breast, which in turn may skew the immune environment in favour of tolerance and tumourigenesis. However, the size of our cohort was relatively small (n=19) and the samples of human breast tissue exhibited great degree of variation in age, menopausal status and hormonal environment, all of which could confound our interpretation of the results. Thus the relationship between TGFB1 and macrophages with different phenotypic markers needs to be further validated in a larger cohort with more controlled sample collection criteria to eliminate confounding factors.

Interestingly, the abundance of macrophages was not associated with the abundance of CCL2 in breast epithelium, which was not consistent with what we observed in the mouse mammary gland. Little is known about the role of CCL2 in the normal breast, and it is not clear whether the expression of CCL2 we observe is acute or constitutive. Acute CCL2 expression is likely to promote the recruitment of macrophages temporarily due to acute inflammation (Yadav et al., 2010), however, chronic CCL2 expression may lead to adaptive changes in the breast which reduce the chemotactic effects of CCL2 such that the abundance of macrophages is overtly normal. In addition, other factors such as hormones, CSF1 and TGFB might have more significant roles in recruiting macrophages in human compared to CCL2. Interestingly, a negative association was observed between CCL2 expression and the abundance of iNOS-positive macrophages, and no association was found between CCL2 expression and the abundance of "M2" macrophages in the human breast. Given that both CCL2 and iNOS are considered to be up-regulated during inflammation, it was surprising that increased CCL2 expression was associated with decreased abundance of iNOS-positive macrophages. Preliminary analysis

investigating the inflammatory condition of each participant revealed that circulating serum C-reactive protein (CRP), which is increased during acute and chronic inflammation was positively correlated with CCL2 in the majority of women in the cohort (Ingman et al., unpublished). However, small (n=5) subset of women exhibited very low CRP and very high CCL2, suggesting that CCL2 expression may become uncoupled from inflammatory stimuli that ordinarily restrain its abundance, and in this small proportion of women CCL2 may be constitutively expressed regardless of other inflammatory cues. Chronic inflammation that caused by constitutive expression of CCL2 could alter various factors in the microenvironment, including cytokines and hormones, which could subsequently affect the phenotype and polarization of macrophages. A larger cohort of participants with detailed disease and inflammatory status recorded through multiple time points is required to further validate the relationship between CCL2 and different populations of macrophages.

The associations between cytokines secreted by the breast epithelium and macrophage populations suggest TGFB1 and CCL2 could be playing similar roles in regulating macrophages in the human breast as they do in the mouse mammary gland. The relationship between abundance of epithelial cell-derived TGFB and the surrounding macrophage population in the human breast bares striking resemblance to the impact of altered TGFB-macrophage signalling in mice. Although differences have been noted in the relationship between epithelial cell-derived CCL2 and the surrounding macrophage population in mouse versus human studies, we do not yet sufficiently understand the effect of acute and constitutive cytokine expression or why CCL2 is increased in some women. Overall however, these studies suggest that cytokine-mediated epithelial cell-macrophage interactions are likely to be involved in normal human breast development and homeostasis.

6.4 Cytokine-macrophage regulatory networks affect mammary cancer susceptibility

The significant roles of TGFB and CCL2 in normal mammary gland development raised our interest to investigate how these cytokines affect mammary cancer susceptibility. The DMBA carcinogen mammary cancer model was utilised to examine the effect of TGFB and CCL2 on mammary gland tumourigenesis as this model is able to evaluate susceptibility, unlike other models such as transgenic MMTV-PyMT in which tumours arise with 100% penetrance. These studies generated enlightening results, suggesting that an appropriate balance between inflammation and immune suppression is required to reduce

cancer susceptibility, as anti-inflammatory TGFB and pro-inflammatory CCL2 both appear to increase mammary cancer susceptibility in mice.

Impaired TGFB signalling in macrophages resulted in reduce susceptibility to carcinogen-induced mammary tumour development, suggesting that the TGFB-macrophage signalling axis increases breast cancer risk. This is supported by findings made by The International Breast Cancer Consortium, which reported that increased abundance of TGFB1 is linked with increased breast cancer risk (Cox et al., 2007); implying abundant expression of TGFB1 could promote breast carcinogenesis. Given that our studies demonstrate that TGFB prevents macrophage invasion and down-regulates the abundance of pro-tumourigenic "M1" macrophages, it appears that TGFB signalling to macrophages dampens the immunosurveillance in the breast and skews the immune environment in favour of tolerance and tumourigenesis. However, the role of TGFB in breast cancer is complex and conflicting, as it can act as both tumour suppressor and promoter. Many *in vivo* studies have shown that perturbation of TGFB signalling in mammary epithelium resulted in increased mammary tumour formation and enhanced metastasis (Forrester et al., 2005, Gorska et al., 2003, Bottinger et al., 1997), suggesting an inhibitory role of TGFB signalling in mammary gland tumourigenesis. Nonetheless, it should be highlighted that our study is the first to identify the importance of TGFB signalling to macrophages in mammary gland tumourigenesis and perturbation of TGFB signalling to macrophages could impair immune system protection from tumour formation. Inflammatory COX2 expression and macrophage infiltration was increased when TGFB signalling is attenuated in mammary epithelial cells (Bierie et al., 2008), suggesting that TGFB can be highly anti-inflammatory and subsequently cause immune suppression. The studies herein suggests that the pro-tumourigenic effects of TGFB signalling may be a result of a number of autocrine and paracrine interactions within the tumour microenvironment, particularly in modulating macrophage activities and dampening anti-tumour immunity.

Despite our findings on the role of anti-inflammatory TGFB in promoting increased mammary cancer susceptibility, pro-inflammatory cytokines and increased immune cell activity in the breast can also increase cancer risk. High expression of pro-inflammatory CCL2 is implicated in early relapse and poor prognosis of breast cancer (Ueno et al., 2000, Fujimoto et al., 2009), and numerous studies have demonstrated that increased infiltration of macrophages are linked with increased risk of breast cancer (McCormack and dos Santos Silva, 2006, Boyd et al., 2001, Qian and Pollard, 2010, Martinez et al., 2009, Mahmoud et al., 2012, Provenzano et al., 2008, Bonapace et al., 2014). Therefore, our findings provide supportive evidence of a critical role of pro-inflammatory CCL2 in increasing mammary gland

cancer susceptibility, potentially through up-regulation of macrophage recruitment and promotion of collagen deposition in the mammary gland. It should be noted that our observations of increased infiltration of macrophages and collagen deposition in the presence of constitutive expression of CCL2 is mirrored with a previous study using a mouse model of chronic bacterial-induced prostatic inflammation (Wong et al., 2014), suggesting high abundance of CCL2 is likely to induce chronic inflammation. Given that chronic inflammation is directly associated with cancer formation (Shacter and Weitzman, 2002), our findings highlight the potential role of CCL2 in driving tumourigenesis by inducing chronic inflammation in the mammary gland, which is characterised by up-regulation of macrophage infiltration and perturbation of collagen deposition and remodelling. The striking similarities between mammary glands with overabundant CCL2 and high mammographic density (MD) tissue also suggest that CCL2 could contribute to the increased breast cancer risk in women with high MD. However, little is understood on the biology that underpins MD, or whether high MD tissue possesses a more inflammatory microenvironment. Interestingly, perturb TGFB signalling appears to be a feature of mammographically dense breast tissue (Yang et al., 2010b), suggesting the balance between anti-inflammatory and pro-inflammatory immune response may be skewed. Further research into the relationship between macrophages, epithelial cell-derived CCL2 and TGFB, and extracellular matrix deposition in mice and human breast tissue will shed light on the increased breast cancer risk in women with high MD.

In summary, these findings have demonstrated that cytokine-macrophage regulatory networks are vital in modulating the balance between inflammation and tolerance in the breast, which in turn affects breast cancer risk in women. Both epithelial cell-derived TGFB and CCL2 have profound effects on macrophages, and can skew the abundance and phenotypes of macrophages during normal mammary gland development and mammary gland tumourigenesis. These studies demonstrate the importance of a tightly regulated immune system to reduce the risk of cancer formation and highlight the significance of the mammary epithelium in directing this balance in breast tissue.

6.5 Future research directions

The experiments reported herein clearly demonstrated the significance of epithelial cell-derived TGFB and CCL2 in regulation of macrophages in mammary gland development and tumourigenesis (Figure 6.1); and these novel findings could potentially provide new insights into potential therapeutic treatments and prevention of breast cancer. However, there are several aspects of the cytokine-macrophage

regulatory networks in the mammary gland that remain to be explored, including (1) the specific mechanisms whereby these epithelial cell-derived cytokines modulate mammary gland tumour initiation; (2) whether the identification of different macrophage populations directed by TGFB and CCL2 might be a useful biomarker of breast cancer diagnosis and treatment; and (3) the potential of interfering of one or both signalling pathways in immunotherapy.

Both TGFB and CCL2 are reported to promote mammary tumour metastasis (Pardali and Moustakas, 2007, Qian et al., 2011), however, their roles in regulating immune cell populations in tumour initiation is still unclear. To explore whether epithelial cell-derived TGFB1 and CCL2 affect mammary tumour initiation, both *TGF-Mac* and *Mmtv-Ccl2* mice and their controls could be challenged by DMBA carcinogen weekly for a period of 6 weeks. However, instead of monitoring tumour progression in these mice weekly, the mammary glands, lymph nodes, serums and spleens from these mice could be collected for flow cytometry after the final dose of DMBA treatment. DMBA is presumed to cause DNA damage in cells thereby to initiate tumour formation. It would be of interest to determine the activation state of T cells and macrophage populations upon DMBA challenge, as both T cells and macrophages are highly involved in tumourigenesis, and in particular whether TGFB and CCL2 would alter the population of immune cells during tumour initiation. Different immune cell populations could be identified using different markers through flow cytometry, and of interest in susceptibility to tumourigenesis are T cell markers CD4, CD8, FOXP3 and CD25, as well as macrophage surface receptors NKG2D, CD204 and MHCII. The measurement of interferon gamma (IFNG) production would provide further information on immunosurveillance as IFNG plays crucial roles in promoting innate and adaptive immune responses (Ikeda et al., 2002). This could be assessed by flow cytometry following intracellular cytokine staining of cells with anti- IFNG antibody.

Macrophages express many receptors in response to change of the local tissue microenvironment, and different subsets of macrophages have been proposed as potential biomarkers for many diseases (Bargagli et al., 2011, Yang et al., 2014, Adams et al., 2014). Breast tumours frequently contain a high abundance of infiltrating macrophages (Qian and Pollard, 2010, Wu and Zheng, 2012), and the presence of tumour-associated macrophages (TAM) has been found to correlate with poor prognosis in breast cancer (Mahmoud et al., 2012). A number of studies have proposed to use TAM as potential diagnostic and prognostic biomarkers in breast cancer (Tang, 2013, Mukhtar et al., 2011). TAM are primarily considered to be “M2” macrophages, and they are also associated with other markers in cancer development, such as “M2” marker CD163 (Medrek et al., 2012), vascular endothelial growth

factor (VEGF) (Leek et al., 2000) and proliferating cellular nuclear antigen (PCNA) (Campbell et al., 2011). However, whether TGFB and CCL2 could direct the phenotypes and populations of cancer-associated macrophages is yet to be elucidated. Our observations in mice and humans raise the question of whether the presence of abundant TGFB or CCL2 could be an indication of increased breast cancer risk in some women, and more importantly, whether TGFB-regulated and CCL2-regulated macrophages could be used as biomarkers for detection of breast cancer. To address this, immunohistochemistry and flow cytometry can be used to detect different subsets of macrophages using different macrophages phenotypic markers in *TGF-Mac* and *Mmtv-Ccl2* mice. These approaches could be informative as it would provide a profile of TGFB-regulated and CCL2-regulated macrophages in the absence or overabundance of certain cytokine. These markers could also be explored in human breast tissue to further investigate the relationship between cytokine and macrophage phenotypes in the breast, and thus to further explore the potential of using TGFB and CCL2 expression in addition to macrophages as a prognostic tool, and to understand or alleviate cancer risk.

As a result of a wide variety of effects of TGFB and CCL2 on promoting tumour progression and metastasis in mammary gland cancer based on numerous *in vivo* studies, both cytokines may constitute a therapeutic target for cancer therapy. Currently, there are three main approaches to block TGFB signalling that are under development at both the pre-clinical and clinical stages, including neutralising the ligand, inhibition of TGFB expression using antisense molecules or blocking the receptor mediated signaling cascade with inhibitors (Yingling et al., 2004, Connolly et al., 2012). Despite attenuation of TGFB signalling being shown to cause a significant reduction in metastasis in mouse models, clinical results have been inconclusive so far and this approach requires further evaluation (Connolly et al., 2012). Similarly, clinical therapeutics targeting CCL2 or CCR2 are also currently being conducted, however, the therapeutic value is still too early to be conclusive (Mitchem and DeNardo, 2012). A recent study however has provided new insights of the role of CCL2 signalling in cancer metastasis (Yumimoto et al., 2015); this study identified F-box protein FBXW7, which has tumour-suppressive effect through down-regulation of the expression of CCL2 through NOTCH signalling. Moreover, inhibition of CCL2/CCR2 signaling by administration of a CCL2 receptor antagonist resulted in a significant reduction of metastasis in the FBXW7-deficient mice, suggesting the potential of FBXW7/CCL2 pathway as a therapeutic target for breast cancer therapy. As increased mammary tumour formation was observed in our *Mmtv-Ccl2* transgenic mouse model, it would be of interest to determine whether administration of F-box protein FBXW7 could reduce mammary gland tumourigenesis in these mice. Although animal models have provided significant insights into TGFB and CCL2 signalling in cancer progression, altered TGFB or CCL2 signalling in human breast cancer could be far less definitive and targeting only one

signalling pathway in cancer therapy might lead to a less robust result. Hence, combinatorial therapy of targeting both TGFB and CCL2 signalling pathways might increase the efficacy of cancer treatment. This could be tested in our transgenic mouse models as administration of CCL2 antagonist into *TGF-Mac* transgenic mice could impaired both TGFB and CCL2 signalling in these mice. The effect of impaired TGFB and CCL2 signalling in mammary gland cancer susceptibility could be determined by challenging the mice with DMBA, or alternatively by xenografting human breast cancer cells into these mice. Investigating the potential benefits of targeting both the TGFB and CCL2 signalling pathways in breast cancer therapy could shed light on future development of cancer treatments.

Besides conventional targeted therapy in cancer treatment, the development of cancer immunotherapy has provided new opportunities in treating cancer. Immunotherapy is designed to manipulate and boost a host immune response to direct an effective and long-lasting immune response against cancer antigens (Vanneman and Dranoff, 2012, Milani et al., 2014). As the abundance of TGFB and CCL2 in mammary epithelium appears to contribute substantially to cancer susceptibility, it would be of interest to investigate whether vaccines could be developed to stimulate anti-tumour T cell immunity that incorporate manipulation of one or both of these cytokines, and thus to restore the balance of cytokine microenvironment which could in turn be beneficial for long-term cancer treatment. Our studies in mice and humans have revealed that the orchestration between macrophage population and function is crucial in regulating immunosurveillance, tolerance and inflammation in cancer progression. TGFB and CCL2 are both key players in regulating macrophages in the mammary gland; hence, personalised immunotherapy treatments that manipulate TGFB and CCL2-regulated macrophage function via vaccines or adoptive cell transfer that designates to achieve a balanced immune system would be of great benefit to future cancer treatment.

6.6 Conclusions

These studies have revealed that epithelial cell-derived TGFB and CCL2 play significant roles in regulating the functions and phenotypes of macrophages in mammary gland development and tumourigenesis (Figure 6.1). By using a mammary gland transplant model and a *TGF-Mac* double transgenic mouse model, we have demonstrated that epithelial cell-derived TGFB1 restrains the immune regulatory activities of macrophages and inhibits invasion of macrophages into the epithelium. Through suppression of iNOS and CCR7, high TGFB1 expression appears to dampen inflammatory “M1” macrophage activity, which in turn could impair immune surveillance and increase mammary cancer

susceptibility. On the other hand, our studies also revealed the significance of CCL2 in regulating macrophage recruitment and collagen remodelling in the mammary gland. We have established that constitutive expression of epithelial cell-derived CCL2 induces chronic inflammation in the mammary gland and increases mammary cancer susceptibility in mice via promoting macrophage recruitment and perturbing collagen deposition and remodelling. Moreover, we also revealed that both TGFB1 and CCL2 could be playing similar roles in human breast as they do in mouse mammary gland. Collectively, the current work and previous studies suggest that a finely tuned cytokine-macrophage regulatory network is crucial for normal mammary gland development, and that epithelial cell-macrophage cross-talk through expression of specific cytokines can increase the risk of cancer formation. Further studies are required to dissect the underlying mechanisms of how specific cell types and determine how cytokine signalling pathways regulate mammary gland morphogenesis and cancer progression, and to explore how this knowledge might be incorporated into the development of novel therapeutic approaches to breast cancer prevention and treatment.

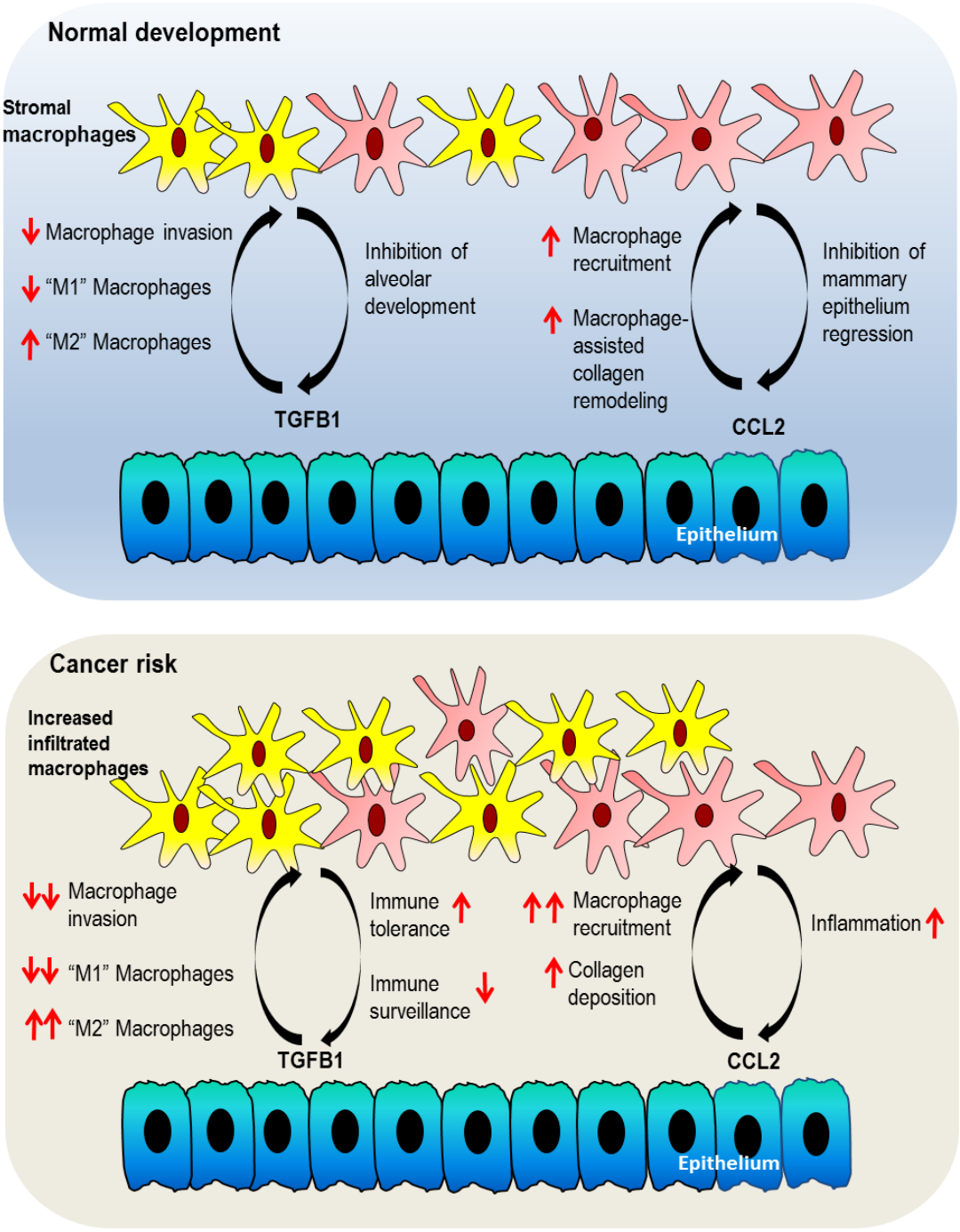

Figure 6.1 Schematic illustration of mechanisms involving epithelial cell-derived TGFB1 and CCL2 in regulation of macrophages in normal breast and breast cancer.

The illustration depicts the known and proposed roles of TGFB and CCL2-regulated macrophages in in normal mammary gland development and tumourigenesis. Studies described in this thesis have demonstrated that TGFB regulates macrophage invasion and phenotype in mammary gland development, in particular through promoting the function of development-associated "M2" macrophages; whereas CCL2 regulates macrophage recruitment and maintain proper remodelling of collagen during normal development. Moreover, our studies also highlight that excessive epithelial cell-derived TGFB1 could inhibit macrophage invasion into the mammary epithelium, dampen inflammatory "M1" macrophage activity and promote pro-tumourigenic activities of "M2" macrophages, and hence impair tumour immune surveillance and promote tumourigenesis in the mammary gland. On the other hand, overabundance of CCL2 could induce chronic inflammation in the mammary gland mainly through promotion of macrophage recruitment and collagen deposition, which in turn could also increase mammary cancer susceptibility.

Chapter 7 Bibliography

7.1 References

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Chapter 8 Appendix

Publications Contributing to This Thesis

1. **Sun X**, Ingman WV. *Cytokine Networks That Mediate Epithelial Cell-Macrophage Crosstalk in the Mammary Gland: Implications for Development and Cancer*. J Mammary Gland Biol Neoplasia. 2014; 19:191–201.

2. **Sun X**, Robertson SA, Ingman WV. *Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland*. Cytokine. 2013; 61(2):377–88.

Sun, X. & Ingman, W.V. (2014). Cytokine Networks That Mediate Epithelial Cell-Macrophage Crosstalk in the Mammary Gland: Implications for Development and Cancer.
Journal of Mammary Gland Biology and Neoplasia, 19(2), 191-201.

Cytokine 61 (2013) 377–388

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland

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ARTICLE INFO

Article history:
Received 11 January 2012
Received in revised form 7 November 2012
Accepted 4 December 2012
Available online 3 January 2013

Keywords:
Transforming growth factor beta1
Mammary gland
Macrophage
Epithelium

ABSTRACT

Transforming growth factor beta1 (TGFB1) is a multi-functional cytokine that regulates cell proliferation, apoptosis and immune system responses. In the breast, the mammary epithelium is the primary source of TGFB1 and increased expression is associated with increased breast cancer risk. This study was conducted to investigate the roles of epithelial cell-derived TGFB1 in regulation of epithelial cell activity and macrophage phenotype in the mammary gland. *Tgfb1* null mutant and wildtype mammary epithelium was transplanted into contra-lateral sides of the cleared mammary gland of TGFB1 replete scid mice. Transplanted tissue was analysed for markers of proliferation and apoptosis to determine the effect of *Tgfb1* null mutation on epithelial cell turnover, and was analysed by immunohistochemistry to investigate the location, abundance and phenotype of macrophages. The number of proliferating and dying ductal epithelial cells, determined by BrdU and TUNEL, was increased by 35% and 3.3-fold respectively in mammary gland transplanted with *Tgfb1* null epithelium compared to wildtype epithelium ($p < 0.05$). Abundance of F4/80+ macrophages in between *Tgfb1* null epithelial cells compared to wildtype epithelial cells was increased by 50%. The number of iNOS+ and CCR7+ cells in the stroma surrounding *Tgfb1* null alveolar epithelium was increased by 78% and 2-fold respectively, and dendriform MHC class II+ cells within ductal epithelium were decreased by 30%. We conclude that epithelial cell-derived TGFB1 in the mammary gland has two functions: (1) regulation of cellular turnover of epithelial cells, and (2) regulation of local macrophage phenotype. These findings shed new light on the diversity of roles of TGFB1 in the mammary gland which are likely to impact on breast cancer risk.

1. Introduction

Transforming growth factor beta1 (TGFB1) is a multifunctional cytokine that controls many aspects of cellular function, including proliferation, differentiation, migration, apoptosis, and the immune response [1–3]. A strong association between breast cancer risk and the TGFB1 L10P gene polymorphism has been reported by the Breast Cancer Association Consortium [4,5]. The TGFB1 L10P gene is linked to both increased cellular expression of TGFB1 and elevated circulating TGFB1 [5,6] suggesting that TGFB1 expression is an important determinant of breast cancer risk.

TGFB1 has both stimulatory and inhibitory roles in regulating tissue homeostasis, development, remodelling, and cancer progression [3,7]. TGFB1 acts as a tumour suppressor in the early phase of cancer development, and promotes invasion and metastasis during the later stages of cancer progression [7]. TGFB1 can suppress tumour development through inhibition of cell proliferation, induction of apoptosis, and suppression of growth factor, cytokine and chemokine production [8,9]. However, as tumours progress, resistance to TGFB1 is acquired through mutations or inhibition of TGFB1 signalling pathways. At this time, tumour cells begin to secrete large quantities of TGFB1 which appears to further promote tumour progression [7,10]. This surge in TGFB1 production by tumour cells mediates epithelial-mesenchymal transition, increased angiogenesis and impairs immune surveillance, thereby promoting tumour invasion and metastasis [7–10].

TGFB1 is produced by many cell types. It is secreted as an inactive latent complex (LTGFB1) consisting of the active mature TGFB1 dimer non-covalently bound to a latency associated peptide (LAP) [11–13]. This large complex is associated with latent TGFB binding protein (LTBP) [12]. Active TGFB1 has a half life of 2 min, whereas TGFB1 associated with its latent complex is substantially more stable, with a half life of 90 min [14]. Activation of latent TGFB1 through heat, acid or alkaline treatment, proteolysis or irradiation [11,12,15,16] is necessary before TGFB1 can exert

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http://dx.doi.org/10.1016/j.cyto.2012.12.002

biological effects [11]. Therefore, activation of TGFB1 from the latent form is a key regulatory event that controls its biological availability [13,14,17].

Latent TGFB1 is expressed by mammary epithelium during puberty, in cycling virgin mice, and during pregnancy [17–19]. The highest expression of both latent and active TGFB1 is observed at the diestrus phase of the ovarian cycle [17]. TGFB1 has a largely inhibitory role in regulating mammary gland epithelial cell proliferation and ductal development. Exogenous TGFB1 released from pellets implanted into the developing mammary gland causes cessation of DNA synthesis in the highly proliferative terminal end buds, which results in complete inhibition of ductal growth. Transgenic mice expressing constitutive active TGFB1 exhibit significant retardation in ductal development [20,21]. On the other hand, transgenic mice carrying a dominant negative receptor in the mammary epithelium exhibit mammary epithelial hyperplasia together with inappropriate alveolar development [22] and delayed epithelial cell death during involution [23]. Accelerated ductal development during puberty is observed in heterozygous *Tgfb1* null mutant mice [17], and *Tgfb1* homozygous null mutant epithelium transplanted into wildtype hosts also results in accelerated ductal development [24]. Thus TGFB1 has an important autocrine function in constraining inappropriate epithelial cell proliferation, development and survival, which is likely to affect breast cancer risk. However, as a multi-functional cytokine, it is highly likely that TGFB1 in the mammary gland acts on other cell types within the stroma, which in turn may also influence breast cancer risk, and these effects are yet to be investigated.

Key target cells for TGFB1 action are macrophages, which are bone-marrow derived cells present in the mammary gland stroma that regulate epithelial cell function in normal development. In estrous cycling adult mice, macrophages promote epithelial cell proliferation, alveolar development, phagocytose dying epithelium and promote tissue remodelling [25]. In addition to their function in regulation of epithelial cell turnover during the ovarian cycle, colony-stimulating factor1 (CSF1)-regulated macrophages contribute to development during puberty [26,27], pregnancy [28] and the switch to a lactational state [28]. Macrophages have also been shown to support stem cell activity in the mammary gland [29]. In breast cancer, macrophages play multiple roles in metastasis, and promote growth and survival of tumour cells, angiogenesis and cell invasion [8,30].

Tgfb1 and *Csf1* null mutant mice have strikingly similar reproductive phenotypes, including perturbation in mammary gland development and function [31], suggesting the possibility of a mechanistic link between TGFB1 and macrophages in the mammary gland. Furthermore, in established mammary gland tumours, TGFB signalling in the epithelium regulates recruitment of macrophages and expression of inflammatory markers [32]. This study seeks to investigate cross-talk between epithelial cell-derived TGFB1 and macrophages, to better understand the role of TGFB1 in regulation of normal mammary gland function. Through analysis of mammary gland tissue from *Tgfb1* null mutant and wildtype mice transplanted into TGFB1 replete recipients, we have shown that epithelial cell-derived TGFB1 regulates both the rate of epithelial cell turnover, and macrophage phenotype in the mammary gland. These findings provide new information on the diversity of roles of TGFB1 in the mammary gland which potentially contribute to breast cancer risk.

2. Methods and materials

2.1. Animals

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th ed., 2004). All mice were maintained in specific pathogen-free conditions with controlled light (12 h light, 12 h dark cycle) and temperature at the Laboratory Animal Services Medical School facility.

2.1.1. FVB and BalbC Prkdc^{scid} mice

Female FVB mice and 24 day old, wild-type BalbC *Prkdc*^{scid} female mice were obtained from the Waite campus of the University of Adelaide, South Australia. The diestrus stage of the estrous cycle in FVB mice was determined by daily histological analysis of vaginal smears [33].

2.1.2. Tgfb1 null mutant and wild-type mice

Heterozygous (*Tgfb1*+/−) breeding pairs on a mixed CF1/129/C3H background produced progeny that were homozygous for a targeted null mutation in the *Tgfb1*gene (*Tgfb1*−/−), heterozygous (*Tgfb1*+/−), or homozygous wild type (*Tgfb1*+/+). All mice in the colony are homozygous for the *Prkdc*^{scid} mutation. The *Tgfb1* genotype of each mouse was determined by diagnostic PCR of tail DNA, using the forward primer 5′-GAGAAGAACTGCTGTGCG together with the reverse primers (1) 5′-GTGTCCAGGCTCCAAATATAGG to detect the intact *Tgfb1* gene, or (2) 5′-CTCGTCCTGCAGTTCATTCA, to detect the mutant *Tgfb1* null gene [34].

2.2. Mammary gland transplants

Donor mammary gland tissue was obtained from 8-week-old *Tgfb1*+/+ and *Tgfb1*−/− female mice. The mice were killed and the inguinal pair of mammary glands was dissected. Single tissue fragments (1-mm^3) isolated from the donor mammary glands were rinsed and maintained in ice-cold PBS. Under 2% Avertin anaesthesia, donor mammary gland tissue was transplanted into the cleared fat pad of recipient 24 day old BalbC *Prkdc*^{scid} female mice. To clear the fat pad, the portion of mammary gland from the nipple to the mammary gland lymph node was dissected and removed. In each recipient, donor tissue fragments were inserted into the cleared fat pad, one side of the fat pad was transplanted with *Tgfb1*+/+ mammary tissue; the contra-lateral side of the fat pad was transplanted with *Tgfb1*−/− mammary tissue [24]. The transplants were performed using *Tgfb1*−/− and *Tgfb1*+/+ donors (n = 3 per genotype) to generate a total of 20 recipient mice.

Expression of TGFB1 by mammary gland epithelium is highest at diestrus [17], and therefore this stage was selected to analyse the effect of epithelial cell-derived TGFB1 on macrophage function. However, between mouse variation in progesterone secretion at diestrus could confound these experiments, as progesterone also regulates macrophage phenotype and function [25]. In order to standardise the hormonal environment and mimic diestrus, a model employing hormone replacement in ovariectomised mice was utilised. This model has been shown to support mammary gland ductal and alveolar development and mimics the diestrus phase of the ovarian cycle [35], (Hodson and Ingman, in preparation). The mice were ovariectomised (OVX) at the age of 12 weeks (i.e. 9 weeks after transplantation) and allowed to recover for 1 week. The mice were injected daily for 3 days with 17β-estradiol (10 μg/ml in sesame oil) (Sigma, MO, USA) and progesterone (10 mg/ml in sesame oil) (Sigma) administered subcutaneously (OVX + PE) to mimic the diestrus stage of the natural cycle. The OVX + PE mice were killed 1 day after the final hormone injection (diestrus-like stage) and the transplanted mammary glands were dissected. Some mice were injected with 100 μl i.p. of a 10 mg/ml solution of bromodeoxyuridine (BrdU) (Sigma) before they were killed. The mammary glands were either embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at −80 °C, or fixed in 4% paraformaldehyde and embedded in paraffin.

2.3. Immunohistochemistry

Haemotoxylin and eosin staining was performed on paraffin-embedded sections. The sections were dewaxed in Safsolv (Ajax, Finechem, and Australia) and passed sequentially through 100%, 95%, 80% and 70% ethanol for rehydration. The sections were stained with haemotoxylin and counterstained with eosin and then mounted on coverslips.

Proliferating cells were identified by BrdU immunostaining of paraffin-embedded mammary gland tissue. Sections were stained for BrdU incorporation into DNA using a BrdU In Situ Detection Kit (BD Biosciences Pharmingen) according to the manufacturer's instructions.

Apoptotic cells were identified by TUNEL staining of paraffin-embedded mammary gland tissue. Sections were stained with the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Only BrdU and TUNEL positive cells clearly located within the ductal or alveolar epithelium were included in the analysis.

Macrophage abundance and phenotype was determined by F4/80, CCR7, iNOS and MHCII antibody staining. Five micrometer paraffin-embedded sections mounted on glass slides were incubated with rat anti-F4/80 monoclonal antibody (1:100 dilution; overnight at 4 °C) (Caltag Laboratories, Burlingame, CA) followed by biotinylated rabbit anti-rat IgG (1:200 dilution; 40 min at room temperature) (Vector Laboratories) and ABC Elite kit (Vector Laboratories) with DAB peroxidase (DAKO, Denmark). For CCR7 immunohistochemistry, the paraffin-embedded sections were immunostained with rat anti-CCR7 monoclonal antibody (1:200 dilution; overnight at 4 °C) (Clone 4B12; kindly donated by Dr. Marina Kochetkova, the University of Adelaide) followed by rabbit anti-rat HRP (DAKO) (1:200 dilution, 60 min at room temperature) with DAB peroxidase (DAKO). For MHC class II immunohistochemistry, 10 μm frozen sections mounted on glass slides were incubated with anti-MHC class II supernatant (neat) (ATCC number TIB120) (overnight at 4 °C) followed by rabbit anti-rat HRP (DAKO) (1:100 dilution, 120 min at room temperature) and DAB peroxidase (DAKO). For iNOS immunohistochemistry, 10 μm frozen sections mounted on glass slides were incubated with polyclonal rabbit anti-iNOS/NOS type2 (BD Biosciences Pharmingen) (1:400 dilution, 60 min at room temperature) followed by goat anti-rabbit HRP (DAKO) (1:400 dilution, 30 min at room temperature) and DAB peroxidase (DAKO). APC rat IgG2b isotype control (BD Biosciences Pharmingen) or rabbit IgG (Chemicon international) was used in parallel as negative controls. Images of stained sections were captured using a Hamamatsu Protonics Nanozoomer.

2.4. Immunofluorescence

The co-localisation of macrophages and TGFB1 in the mammary gland was investigated in FVB mice killed at diestrus. Mammary glands were dissected and frozen in OCT compound. Ten micrometer frozen sections mounted on glass slides were incubated simultaneously overnight with polyclonal affinity-purified chicken anti-TGFB1 (1:40 dilution) (R&D Systems) and rat anti-F4/80 monoclonal macrophage-specific antibody (1:100 dilution) (Caltag Laboratories) at 4 °C, followed by corresponding secondary antibodies, anti-chicken IgY FITC conjugate (1:200 dilution, 60 min at room temperature) (Sigma) and Alexa Fluor 594 conjugated rabbit anti-rat IgG (1:200 dilution, 60 min at room temperature) (Invitrogen). The mammary gland tissue from a *Tgfb1*−/− mouse, and a TGFB1 replete mouse transplanted with *Tgfb1*−/− mammary epithelium were used as negative controls for active TGFB1 antibody.

The dual-label immunofluorescence of epithelial cells and macrophages in the mammary gland was investigated in adult FVB mice. Mammary glands were dissected and frozen in OCT compound. Ten micrometer frozen sections mounted on glass slides were incubated with monoclonal anti TROMA-1 antibody (1:50) (Developmental Studies Hybridoma Bank, University of Iowa; kindly donated by Dr. Carmela Ricciardelli, the University of Adelaide) at 4 °C overnight, followed by secondary antibody Alexa Fluor 594 conjugated rabbit anti-rat IgG (1:200 dilution, 60 min at room temperature) (Invitrogen). The sections were then subsequently incubated with biotin anti-mouse F4/80 antibody (1:50 dilution) (eBioscience, San Diego, USA) or biotin anti-mouse MHCII antibody (1:50 dilution) (eBioscience) at 4 °C overnight, followed by secondary antibody strepavidin FITC (1:200 dilution, 60 min at room temperature) (eBioscience). The mammary gland tissue stained with secondary antibodies only or with isotype matched antibody was included as negative controls. All sections were mounted in fluorescent mounting medium (DAKO) with 4′, 6-diamidino-2-phenylindol (DAPI) (Vector Laboratories). Sections were visualised using a Leica SP5 spectral scanning confocal microscope (Adelaide Microscopy) and images were captured at 63× magnification using the AMIRA and analySIS softwares.

2.5. Analysis of stained sections

All quantification analysis was performed by an assessor blinded to donor mouse genotype. To quantify extent of alveolar development, epithelium was categorized as ductal (single epithelium layer) or alveolar (clusters of epithelial structures containing alveolar lumens) as described previously [25,36]. The numbers of ductal and alveolar epithelial structures were counted manually. The total number of alveolar buds was expressed as percent of total epithelial structures (ductal and alveolar combined) as described previously [25]. To quantify immunostaining of F4/80, CCR7, iNOS and MHC class II positive cells in diestrus-like tissue, five ducts and five clusters of alveolar buds in each section were randomly chosen. Only positive cells with visible haematoxylin stained nuclei were included. For F4/80 staining, F4/80 positive macrophages were distinguished from F4/80 stained eosinophils on the basis of nuclear morphology [37]. All results were expressed as positive cells/mm^2. The mean density of positive cells within the five ductal, five alveolar stroma and within the five ductal, five alveolar epithelium was calculated, and grouped by donor genotype (*Tgfb1*+/+ vs. *Tgfb1*−/−).

2.6. Statistical analysis

Data were analysed using independent samples *t*-test with SPSS 17 for Windows software. Significance was inferred at $p < 0.05$.

3. Results

3.1. Effect of epithelial cell-derived TGFB1 on alveolar bud development at diestrus

The effect of epithelial cell-derived TGFB1 on development of alveolar buds was determined by analysis of haematoxylin and eosin stained paraffin sections from mammary gland transplanted with *Tgfb1*+/+ and *Tgfb1*−/− epithelium. The diestrus stage of the cycle was mimicked by ovariectomy followed by administration of progesterone and oestogen (OVX + PE). Two distinct types of ducts were observed in the OVX + PE transplanted mammary gland: ductal epithelium with a single epithelial cell layer (Fig. 1A and C) and alveolar epithelium (Fig. 1B and D) identified as clusters of epithelial cells containing an alveolar lumen [36]. We have previously reported that in whole mounts of *Tgfb1*−/− epithelium transplanted into TGFB1 replete hosts, the extent of ductal branching is normal compared with wild-type controls

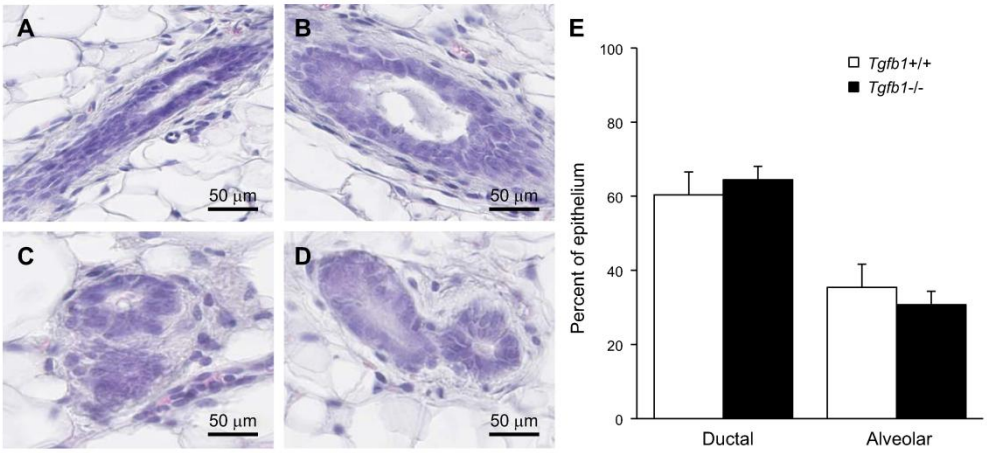

Fig. 1. The effect of epithelial cell-derived TGFB1 on the development of transplanted mammary epithelium. The ductal epithelium was identified on the basis of a single epithelial layer of epithelial cells (A and B) and alveolar epithelium was observed in clusters (C and D) in H and E stained thin sections. The percent of epithelium classified as ductal and alveolar was quantified in H and E stained sections in the transplanted *Tgfb1*+/+ ($n = 6$) and *Tgfb1*−/− ($n = 7$) epithelium (E). The effect of epithelial cell-derived TGFB1 on mammary epithelium development was analysed by independent samples *t*-test. Data are presented as mean + SEM.

[24]. Both ductal and alveolar epithelium was examined in *Tgfb1*+/+ (Fig. 1A and B) and *Tgfb1*−/− tissue (Fig. 1C and D). There was no significant difference in the relative proportion of ductal or alveolar epithelium between the two groups (Fig. 1E).

3.2. Effect of epithelial cell-derived TGFB1 on epithelial cell proliferation and apoptosis at diestrus

The effect of epithelial cell-derived TGFB1 on mammary epithelial cell proliferation in tissue from OVX + PE mice was analysed by detection of BrdU-positive cells in the transplanted mammary tissue. BrdU-positive cells were observed in both the ductal and alveolar epithelium of mammary gland transplanted with both *Tgfb1*+/+ (Fig. 2A and C; respectively) and *Tgfb1*−/− epithelium (Fig. 2B and D; respectively). There was no significant difference in the number of proliferating epithelial cells within the *Tgfb1*−/− alveolar epithelium (mean ± SEM = 3315 ± 177 BrdU-positive cells/mm^2; $p = 0.072$) compared to *Tgfb1*+/+ alveolar epithelium (2771 ± 211 BrdU-positive cells/mm^2) (Fig. 2E). However, there was a 35% increase in the number of proliferating epithelial cells within *Tgfb1*−/− ductal epithelium (3141 ± 129 BrdU-positive cells/mm^2; $p = 0.007$) compared to *Tgfb1*+/+ ductal epithelium (2326 ± 220 BrdU-positive cells/mm^2) (Fig. 2E).

The effect of epithelial cell-derived TGFB1 on mammary epithelial cell apoptosis in tissue from OVX + PE mice was analysed by detection of TUNEL-positive cells in the transplanted mammary tissue. TUNEL-positive cells were observed in both ductal and alveolar epithelium of mammary glands transplanted with *Tgfb1*+/+ (Fig. 2F and H; respectively) and *Tgfb1*−/− epithelium (Fig. 2G and I; respectively). There was a 3.3-fold increase in the number of apoptotic cells within *Tgfb1*−/− ductal epithelium (157 ± 47 apoptotic cells/mm^2; $p = 0.01$) compared to *Tgfb1*+/+ ductal epithelium (47 ± 14 apoptotic cells/mm^2) (Fig. 2J). There was a 2.7-fold increase in the number of apoptotic cells within *Tgfb1*−/− alveolar epithelium (191 ± 33 apoptotic cells/mm^2; $p = 0.0365$) compared to *Tgfb1*+/+ alveolar epithelium (71 ± 17 apoptotic cells/mm^2) (Fig. 2J).

We observed a greater proportion of proliferating BrdU-positive epithelial cells compared to apoptosing TUNEL-positive epithelial cells. This is consistent with previous studies in naturally cycling mice [36] and our previous finding of rapid macrophage-mediated phagocytosis of apoptotic cells in the mammary gland [25].

3.3. Co-localisation of active TGFB1 and macrophages in the mammary gland

In order for TGFB1 to regulate macrophage function, active TGFB1 must be localised in the immediate vicinity of macrophages within the stroma surrounding mammary epithelial cells. To examine the spatial relationship between active TGFB1 and macrophages in the mammary gland, dual-label immunofluorescence was performed on frozen sections from wild-type FVB mice at diestrus. Active TGFB1 was observed throughout the mammary gland epithelium and stroma (Fig. 3A, C, E and F), as previously described [17]. The sections were also counterstained with Dapi (Fig. 3D). Macrophages were localised immediately subjacent to the mammary epithelium within the stroma and were in close proximity to active TGFB1 (Fig. 3B, C, E and F). Positive staining with active TGFB1 antibody was not observed in *Tgfb1* null mutant mouse mammary tissue (Fig. 3G), or in sections stained with isotype matched negative control antibodies (Fig. 3H). No positive staining of active TGFB1 was observed in TGFB1 replete mammary gland transplanted with *Tgfb1*−/− epithelium (Fig. 3I), confirming previous reports that the epithelium is the primary source of TGFB1 production in the mammary gland [17–19].

3.4. Dual-label immunofluorescence of mammary epithelial cells and macrophages in the mammary gland

To investigate whether mammary epithelial cells express macrophage markers, which would confound interpretation of our results, dual-label immunofluorescence was performed on frozen sections from adult FVB mice using the luminal epithelial cell marker TROMA-1 (cytokeratin 8) and macrophage markers F4/80 and MHC II. F4/80-positive macrophages and MHCII-positive macrophages were mainly located within the mammary gland stroma (Fig. 4A and B respectively), whereas mammary epithelial cells expressing cytokeratin 8 were clearly a distinct population

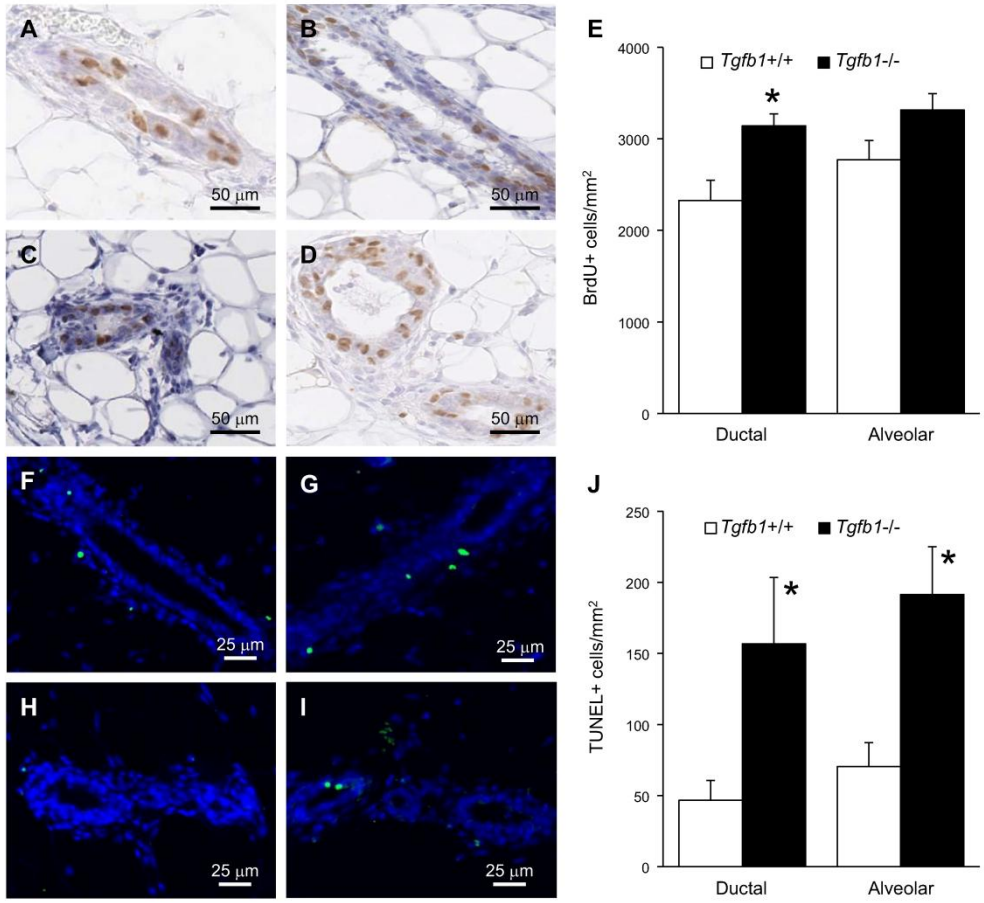

Fig. 2. The effect of epithelial cell-derived TGFB1 on epithelial cell proliferation and apoptosis within transplanted mammary epithelium. Paraffin sections of mammary gland tissue transplanted from *Tgfb1*+/+ mice ($n = 6$) and *Tgfb1*−/− mice ($n = 7$) were stained with anti-BrdU antibody to detect proliferating ductal epithelial cells (A and B) and alveolar epithelial cells (C and D). Sections were also stained with TUNEL staining kit to detect apoptotic epithelial cells within ductal epithelium (F and G) and alveolar epithelium (H and I). The number of BrdU-positive cells and apoptotic cells within ductal and alveolar epithelium were calculated and expressed as BrdU-positive cells/mm^2 (E) and apoptotic cells/mm^2 (J) respectively.

(Fig. 4D and E). Neither macrophage marker F4/80 nor MHCII was expressed byTROMA-1-positive epithelial cells; however F4/80-positive macrophages and MHCII-positive macrophages were in close physical contact with the mammary epithelium (Fig. 4G, H, J and K). Positive staining was not observed in mammary gland tissue stained with isotype-matched negative control antibodies (Fig. 4C, F, I and L).

3.5. Effect of epithelial cell-derived TGFB1 on macrophage abundance and phenotype in the mammary gland at diestrus

To investigate the effect of epithelial cell-derived TGFB1 on the abundance and phenotype of macrophages, immunohistochemistry was performed to detect F4/80, CCR7, iNOS and MHC class II positive cells from mammary gland transplanted with *Tgfb1*+/+ or *Tgfb1*−/− epithelium.

F4/80-positive macrophages were observed in close proximity to ductal and alveolar epithelium in mammary glands transplanted with *Tgfb1*+/+ (Fig. 5A and E respectively) and *Tgfb1*−/− epithelium (Fig. 5B and F respectively). No positive staining was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Fig. 5C and G). In both genotypes and epithelial cell subpopulations, the F4/80-positive macrophages were localised both within the stroma surrounding the epithelium, as well as in between epithelial cells. There was no significant difference in macrophage density within the stroma between *Tgfb1*+/+ and *Tgfb1*−/− epithelium (Fig. 5D and H). However, there was a 48% increase in the number of macrophages within *Tgfb1*−/− ductal epithelium (1304 ± 142 macrophage density/mm^2; $p = 0.026$) compared to *Tgfb1*+/+ ductal epithelium (884 ± 125 macrophage density/mm^2) (Fig. 5D and H). Additionally there was a 50% increase in the number of macrophages within *Tgfb1*−/− alveolar

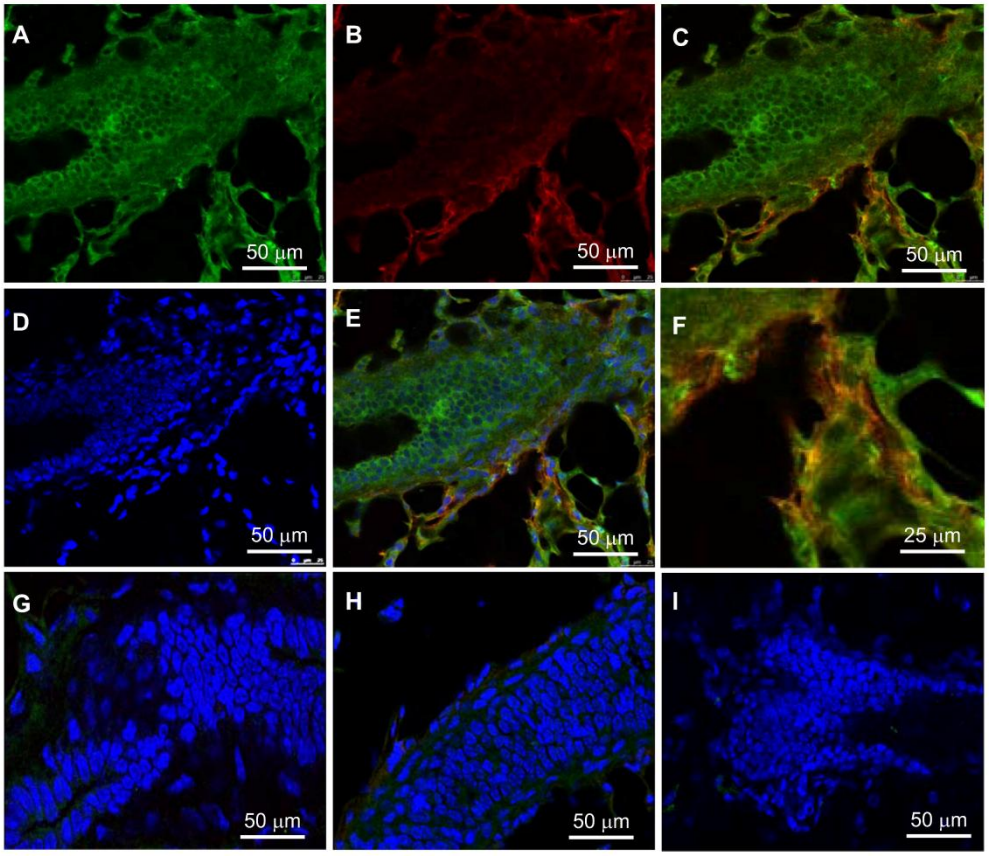

Fig. 3. The co-localisation of active TGFB1 and macrophages in the mammary gland at diestrus. Frozen mammary gland tissue sections from adult diestrus FVB mice were stained simultaneously with antibody reactive with active TGFB1 and F4/80 antibody to detect macrophages. Active TGFB1 (green) (A) and F4/80 (red) (B) were visualised simultaneously under a confocal microscope (C: merged A and B). The sections were also counter stained with DAPI (D, E: merged A, B and D). F is a higher magnification of A and B to visualise active TGFB1 and F4/80 antibody co-localisation. Active TGFB1antibody on mammary gland tissue from an adult *Tgfb1* null mutant mouse (G), isotype-matched negative control on mammary gland tissue from a diestrus FVB mouse (H) and active TGFB1antibody on transplanted *Tgfb1*−/− tissue into TGFB1 replete recipient (I) were used as negative controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epithelium (1327 ± 154 macrophage density/mm^2; $p = 0.028$) compared to *Tgfb1*+/+ alveolar epithelium (882 ± 54 macrophage density/mm^2) (Fig. 5D and H).

iNOS expression was observed in both the mammary gland epithelium and stromal compartment. Previous studies report that iNOS is expressed by most of the epithelial cells in normal breast tissue as well as by macrophages [38,39]. Therefore, it is not possible to distinguish between iNOS-positive macrophages and iNOS-positive epithelial cells within the epithelium, and hence only iNOS-positive macrophages within the stroma surrounding ductal and alveolar epithelium in mammary gland transplanted with *Tgfb1*+/+ (Fig. 6A and D respectively) and *Tgfb1*−/− (Fig. 6B and E respectively) could be quantified. No positive staining was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Fig. 6C and G).There was a 54% increase in the number of iNOS-positive macrophages within the ductal stroma of *Tgfb1*−/− epithelium (174 ± 16 iNOS-positive cells/mm^2; $p = 0.014$) compared to that of *Tgfb1*+/+ epithelium (113 ± 7 iNOS-positive cells/mm^2) (Fig. 6G). There was a 78% increase in the number of iNOS-positive macrophages within the alveolar stroma associated with *Tgfb1*−/− epithelium (208 ± 14 iNOS-positive cells/mm^2; $p = 0.005$) compared to alveolar stroma of *Tgfb1*+/+ epithelium (117 ± 8 iNOS-positive cells/mm^2) (Fig. 6G).

CCR7-positive cells were located in the stroma surrounding ductal and alveolar epithelium of mammary glands transplanted with *Tgfb1*+/+ (Fig. 7A and D respectively) and *Tgfb1*−/− epithelium (Fig. 7B and E respectively). No positive staining was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Fig. 7C and F). There was no significant difference in the number of CCR7-positive cells within the ductal stroma associated with *Tgfb1*+/+ epithelium (236 ± 72 CCR7-positive cells/mm^2) compared with *Tgfb1*−/− epithelium (378 ± 66 CCR7-positive cells/mm^2; $p = 0.17$) (Fig. 7G). However, there was a 2-fold increase in the number of CCR7-positive

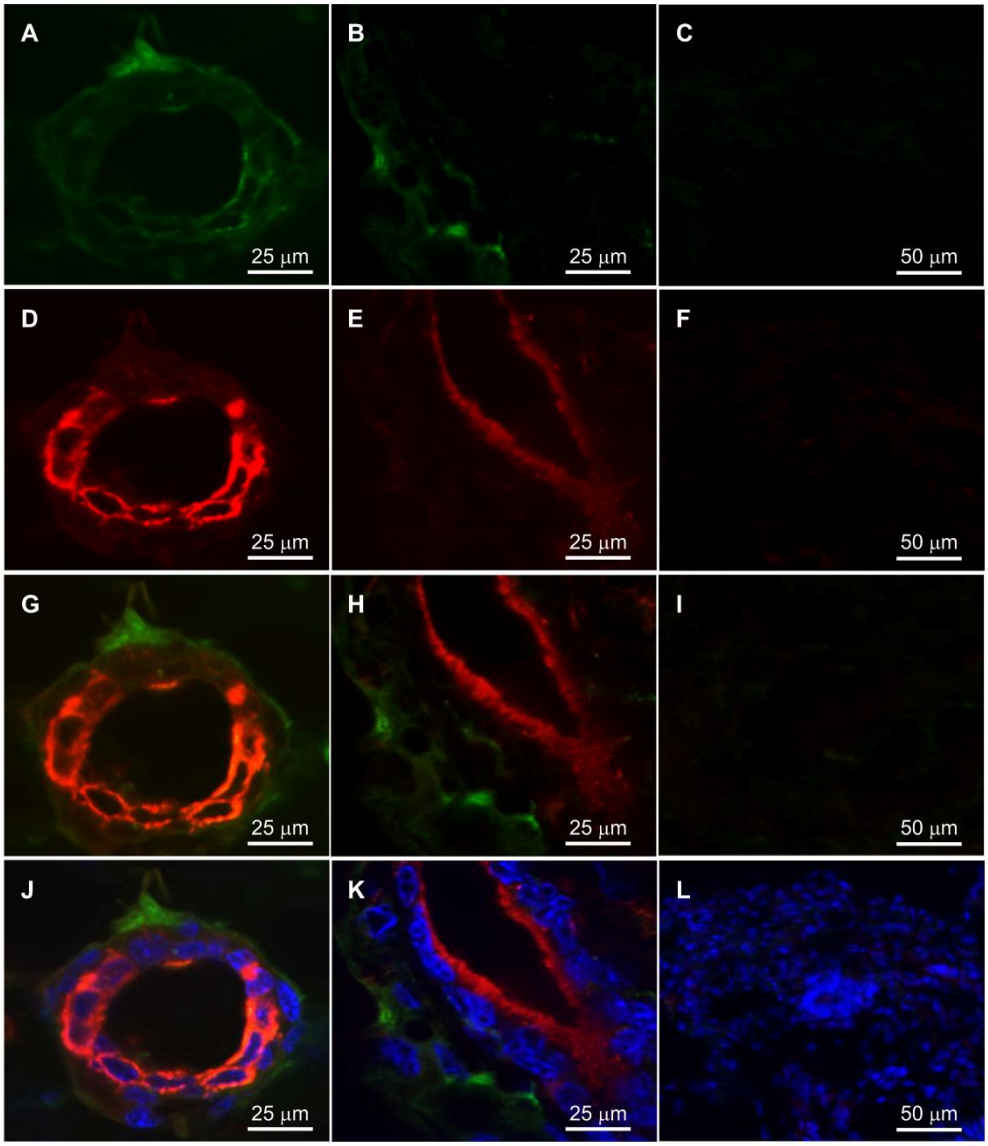

Fig. 4. The dual-label immunofluorescence of mammary epithelial cells and F4/80$^+$/ MHCII$^+$ macrophages in the mammary gland. Frozen mammary gland tissue sections from adult FVB mice were stained sequentially with TROMA-1 antibody reactive with mammary luminal epithelial cells and F4/80 (A, D, G, and J) or MHCII (B, E, H, and K) antibody. F4/80$^+$ macrophages (green) (A) or MHC II$^+$ macrophages (green) (B) and mammary luminal epithelial cells (red) (D and E) were visualised simultaneously under a confocal microscope (G: merged A and D, H: merged B and E). Isotype-matched negative control on mammary gland tissue from FVB mouse (C: control for F4/80 and MHCII antibody; F: control for TROMA-1 antibody; I: merge picture of C and F). All the sections were counter stained with DAPI (J: merge of G and DAPI; K: merge of H and DAPI; L; merge picture of I and DAPI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

macrophages within the alveolar stroma of *Tgfb1*−/−epithelium (563 ± 80 CCR7-positive cells/mm^2) compared with *Tgfb1*+/+ epithelium (256 ± 89 CCR7-positive cells/mm^2; $p = 0.026$) (Fig. 7G).

MHC class II-positive cells were observed in both the stroma and epithelium, however, due to the poorly resolved tissue histology of the frozen sections stained with MHC class II antibody, it was not possible to accurately quantify the number of MHC class II-positive cells within the stroma. Therefore only MHC class II-positive cells within the ductal and alveolar epithelium of mammary glands transplanted with *Tgfb1*+/+ (Fig. 8A and E respectively) and

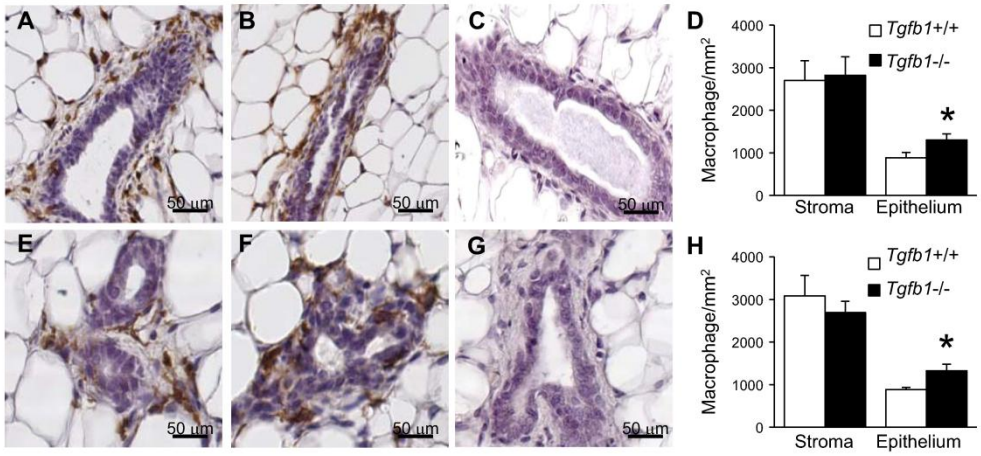

Fig. 5. The effect of epithelial cell-derived TGFB1 on macrophage abundance and location within and around mammary epithelium. Paraffin sections of mammary gland tissue transplanted from *Tgfb1+/+* mice ($n = 6$) and *Tgfb1−/−* mice ($n = 7$) were stained with macrophage-specific F4/80 antibody to detect macrophages within ductal epithelium and stroma (A and B) and within alveolar epithelium and stroma (E and F). Isotype matched irrelevant antibody negative controls (C and F are ductal and alveolar epithelium respectively). The number of F4/80-positive cells within ductal epithelium, and within alveolar epithelium and stroma were calculated and expressed as macrophage density/mm^2 (D and H).

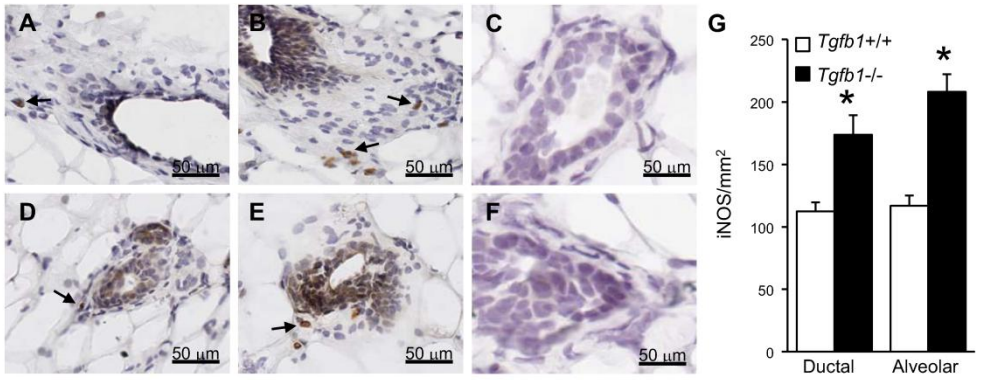

Fig. 6. The effect of epithelial cell-derived TGFB1 on abundance and location of iNOS-positive cells within mammary epithelium stroma. Frozen sections of mammary gland tissue transplanted from *Tgfb1+/+* mice ($n = 5$) and *Tgfb1−/−* mice ($n = 8$) were stained with anti-iNOS antibody to detect iNOS-positive macrophages (arrows) within mammary ductal epithelium stroma (A and B) and alveolar epithelium stroma (D and E). Isotype matched irrelevant antibody negative controls (C and F are ductal and alveolar epithelium respectively). The number of iNOS-positive cells within mammary epithelium stroma were calculated and expressed as iNOS-positive cells/mm^2 (G).

Tgfb1−/− epithelium (Fig. 8B and F respectively) were quantified. No positive staining was observed in the transplanted mammary epithelium stained with isotype matched irrelevant antibody (Fig. 8C and G). Within the MHC class II-positive cell population, two distinct cell morphologies were observed. One type were characteristically round in shape (white arrows), the other type displayed clearly visible dendrites projecting from the surface of the cell (black arrows). When separated into the two distinct cell types, there was no significant difference in the number of round MHC class II-positive cells between mammary glands transplanted with *Tgfb1+/+* and *Tgfb1−/−* epithelium within ductal epithelium (Fig. 8D). However, there was a 30% decrease in dendriform MHC class II-positive cells within *Tgfb1−/−* ductal epithelium (600 ± 48 MHC class II-positive cells/mm^2; $p = 0.031$) compared to *Tgfb1+/+* ductal epithelium (853 ± 106 MHC class II-positive cells/mm^2) (Fig. 8D). No significant differences were observed in the number of round or dendriform MHC class II-positive cells within the alveolar epithelium between mammary glands transplanted with *Tgfb1+/+* and *Tgfb1−/−* epithelium (Fig. 8H).

4. Discussion

Studies utilising a variety of genetically modified mouse models have shown both macrophages and TGFB1 play essential roles in mediating the complex developmental processes characteristic of the mammary gland [21,22,24,26,28,40,41]. Although macrophages are implicated as a key target cell for TGFB1 action based

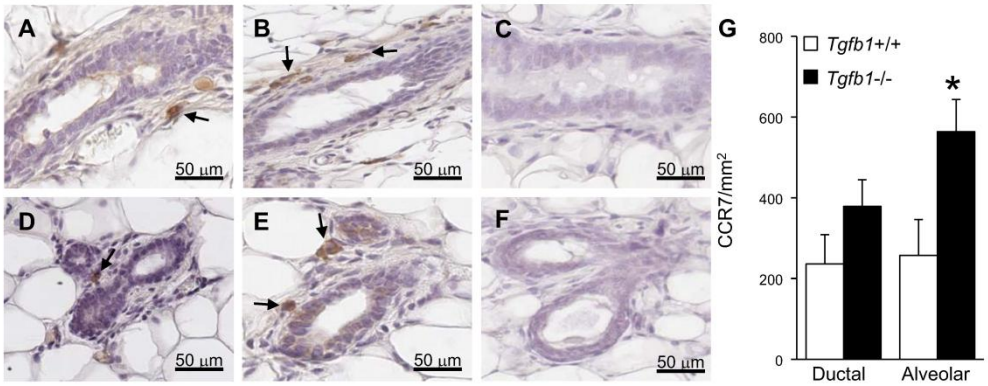

Fig. 7. The effect of epithelial cell-derived TGFB1 on abundance and location of CCR7-positive cells within mammary epithelium stroma. Paraffin sections of mammary gland tissue transplanted from *Tgfb1*+/+ mice ($n = 6$) and *Tgfb1*−/− mice ($n = 7$) were stained with anti-CCR7 antibody to detect CCR7-positive macrophages (arrows) within mammary ductal epithelium stroma (A and B) and alveolar epithelium stroma (D and E). Isotype matched irrelevant antibody negative controls (C and F are ductal and alveolar epithelium respectively). The number of CCR7-positive cells within mammary epithelium stroma were calculated and expressed as iNOS-positive cells/mm^2 (G).

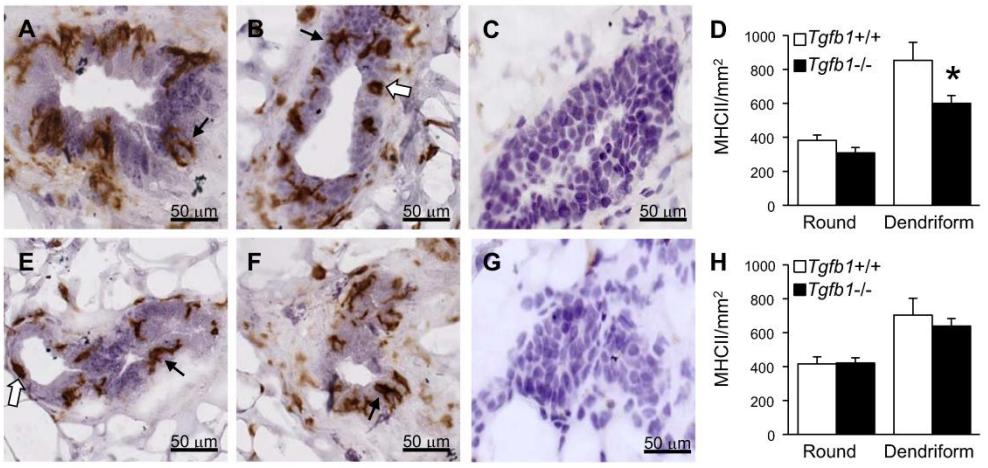

Fig. 8. The effect of epithelial cell-derived TGFB1 on abundance and location of MHC class II-positive cells within mammary epithelium. Frozen sections of mammary gland tissue transplanted from *Tgfb1*+/+ mice ($n = 5$) and *Tgfb1*−/− mice ($n = 8$) were stained with anti-MHC class II antibody to detect MHC class II-positive cells within ductal and alveolar epithelium (A, B, E, and F respectively). Isotype matched irrelevant antibody negative controls (C and G are ductal and alveolar epithelium respectively). MHC class II-positive cells were classified as round (white arrows) or dendriform (black arrows). The number of the round and dendriform MHC class II-positive cells and within ductal and alveolar epithelium were calculated and expressed as MHC class II-positive cells/mm^2 (D and H respectively).

on studies in other tissues, the cross-talk between epithelial cell-derived TGFB1 and macrophages in the mammary gland has not previously been investigated. Here, we report that epithelial cell-derived TGFB1 in the cycling non-pregnant gland has two functions: (1) regulation of cellular turnover of epithelial cells, and (2) regulation of local macrophage abundance and phenotype.

4.1. Epithelial cell-derived TGFB1 regulates epithelial cell turnover

A number of studies have demonstrated an inhibitory role for TGFB1 in mammary gland development during puberty [18,19,21], whereby diminished synthesis or signalling capacity of TGFB1 results in increased ductal elongation. Transplantation of TGFB1 deficient and replete mammary gland epithelium did not reveal an overt effect of deficiency in epithelial cell-derived TGFB1 on the amount of alveolar epithelium present in ovariectomised mice treated with progesterone and oestogen to induce artificial diestrus. It should be noted that the classification system utilised here and previously [25,36] may occasionally misclassify ductal epithelium as alveolar, however quantification of both TGFB1 replete and deficient epithelium will be equally affected as the analysis was done blinded. Further analysis suggested that both epithelial cell proliferation and apoptosis were increased within ductal epithelium in the absence of epithelial cell-derived

TGFB1. Although these dying cells were identified as epithelial in origin on the basis of their location within ductal epithelium, it remains a possibility that they are cells of stromal origin. Apoptosis was also increased within the alveolar epithelial cell population, however proliferation in this compartment was not significantly affected.

Alveolar progenitor cells within the ductal epithelial cell population give rise to alveolar buds [42], and it is possible that it is this cell type that undergoes increased proliferation in the absence of TGFB1. Increased proliferation, together with increased incidence of apoptosis in *Tgfb1* null mutant epithelial cells transplanted into TGFB1 replete hosts, suggests that epithelial cell-derived TGFB1 inhibits epithelial cell turnover in the mammary gland. The increased activation of TGFB1 observed at diestrus [17] may occur in response to the progesterone-mediated increase in rate of proliferation and apoptosis that occurs at diestrus compared to estrus [36].

4.2. The co-localisation of active TGFB1 and macrophages in the mammary gland during diestrus

TGFB1 is produced in a latent form and must be activated to exert a biological effect [11,43]. Active TGFB1 has a short half life and its site of action is therefore likely to be limited to the immediate vicinity [43]. In the mammary gland, *Tgfb1* mRNA is mainly expressed by mammary epithelial cells [19]. Previous studies have shown macrophages have close spatial association with the mammary epithelium and they are highly abundant in the cycling non-pregnant gland during diestrus [25,41]. Dual-staining immunofluorescence confirms the co-localisation of epithelial cell-derived TGFB1 and macrophages at diestrus, implying that TGFB1 would be available to target macrophages and other stromal cells in the non-pregnant mammary gland.

4.3. Epithelial cell-derived TGFB1 regulates macrophage invasion and inhibits "M1" macrophage activity

Macrophages are considered as key players in immune system responses through their actions as professional antigen-presenting cells involved in phagocytosis of tumour cells and processing and presentation of tumour antigens [44]. Macrophages are also implicated in promotion of tumourigenesis in many tissues, particularly tumours of epithelial cell origin in the breast, ovary and endometrium [45,46]. Anti- and pro-tumourigenic properties of macrophages have been generally assigned as "M1" and "M2" macrophage functions respectively [47]. In broad terms, M1 macrophages are mainly engaged in antigen presentation and immune surveillance, while M2 macrophages participate in tissue development, wound healing and repair [47–50]. Although this paradigm is likely to be a simplification of the physiological situation [51], it is clear that the diversity of macrophage phenotypes permits their direction of distinct biological processes, with the balance of tumour-promoting or tumour-inhibiting effects being dependent on signals emanating from the local tissue microenvironment.

Increased penetration of macrophages into both the ductal and alveolar epithelium was observed in mammary glands deficient in epithelial cell-derived TGFB1. In the naturally cycling mammary gland, macrophages invade into the epithelium particularly at proestrus, where they assist in the breakdown of alveolar buds through phagocytosis of apoptotic cells and tissue remodelling [25]. Increased invasion of macrophages may be the consequence of signals emanating from the increased numbers of apoptotic epithelial cells when TGFB1 is absent [25]. Alternatively, increased macrophage invasion might be due to variations in the chemoattractant signals produced by TGFB1 deficient epithelium or altered composition of the extracellular matrix between the epithelium and stroma.

The abundance of stromal cells expressing C–C chemokine receptor 7 (CCR7), and inducible nitric oxide synthase (iNOS) was increased by deficiency in epithelial cell-derived TGFB1. iNOS is highly expressed by activated cytotoxic macrophages involved in antigen presentation and immune surveillance [49] and is considered a marker for M1 type macrophages [47]. TGFB1 acts to down-regulate the expression of iNOS in activated macrophages and thus resolves inflammation by deactivating macrophages [52,53]. This increased abundance of cytotoxic macrophages when TGFB1 is deficient suggests epithelial cell-derived TGFB1 might play a role in inhibiting macrophage activation and immune surveillance.

CCR7 is a chemokine receptor involved in lymphocyte homing [54] that is highly expressed by breast cancer cells and promotes metastasis [55,56]. CCR7 is also a phenotypic marker of M1 macrophages and is uniquely expressed on adipose tissue macrophages, which promote inflammation in obese mice [47,57]. The present study is the first to identify CCR7-positive macrophages in the mammary gland. Deficiency in epithelial cell-derived TGFB1 caused an increased abundance of CCR7-positive macrophages within the alveolar stroma, implying that TGFB1 suppresses immune-associated activities of CCR7-postive macrophages in the mammary gland during diestrus. Further studies are needed to investigate the role of CCR7-positive macrophages in the mammary gland.

Together, these findings suggest that epithelial cell-derived TGFB1 restrains the immune regulatory activities of macrophages and inhibits invasion of macrophages into the epithelium. Through suppression of iNOS and CCR7, high TGFB1 expression would dampen inflammatory M1 macrophage activity, which in turn would be expected to impair tumour immune surveillance in the mammary gland.

4.4. Antigen-presenting cells in the mammary gland are regulated by TGFB1

Professional antigen-presenting cells, including both macrophages and dendritic cells, take up foreign or tumour-associated antigens, then traffic to the draining lymph node and present these to naive T cells via interaction between MHC class II and the T cell receptor. MHC class II-positive dendritic cells in lactating mammary gland were first reported in 1985 and were designated epithelial-associated resident dendritic cells, known as Langerhans cells [58]. In the skin, epidermal Langerhans cells promote tolerance [59] and facilitate carcinogen-mediated tumorigenesis [60], and their infiltration into breast carcinoma tissue implies a similar role in the mammary gland [61].

In the absence of epithelial cell-derived TGFB1, we observed increased penetration of F4/80-positive macrophages into the ductal epithelium together with decreased abundance of dendriform MHC class II-positive cells. We hypothesise that these dendriform MHC class II-positive cells are dendritic cells, although we were not able to identify them directly in our study. Epithelial cell-derived TGFB1 might promote differentiation of macrophages into Langerhans cell-like dendritic cells within the ductal epithelium, consistent with previous findings that TGFB1 promotes Langerhans cell differentiation from macrophages in vitro [62–64]. The functional significance of Langerhans cells in the mammary gland and in mammary tumorigenesis requires further investigation.

4.5. Significance of TGFB1 and macrophages in breast cancer risk

Although previous studies have shown that TGFB1 has both stimulatory and inhibitory roles in cancer progression [7,31], the increased risk of breast cancer conferred by the presence of the

TGFB1 L10P gene polymorphism suggests that increased production of TGFB1 is associated with increased susceptibility to breast cancer [5]. Macrophages play multiple roles in tumour development and progression, promoting growth and survival of tumour cells, angiogenesis and cell invasion, and their function is regulated by a variety of cytokines including TGFB1 [8,30,50]. Our findings suggest high expression of TGFB1 might impair immune surveillance in the breast via suppression of the immune regulatory activities of macrophages, leading to increased risk of breast cancer.

5. Conclusions

These studies show that the function of epithelial cell-derived TGFB1 in the non-pregnant cycling mammary gland is two-fold: regulation of epithelial cell turnover, and regulation of macrophage phenotype. These findings demonstrate that resident tissue macrophages are key targets of epithelial cell-derived TGFB1 expression and indicate that TGFB1-regulated macrophage function may be a pivotal pathway in explaining how the TGFB1 L10P polymorphism leads to increased breast cancer risk in women. The significance of macrophages as intermediary cells through which TGFB1 affects tumorigenesis in the breast warrants further investigation.

Acknowledgements

Thank you to Dr. Marina Kotchetkova for provision of CCR7 antibody, Dr. Carmela Ricciardelli for provision of TROMA-1 antibody and Leigh Hodson for technical assistance. This study was supported by NHMRC Project Grant 565313 awarded to WVI, University of Adelaide Graduate Research Scholarship (XS), Florey Medical Research Foundation Postgraudate Cancer Research Top Up Scholarship (XS), and NHMRC Fellowship (SAR). WVI is an NBCF/THRF Fellow.

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