THE ROLE OF CHEMOKINE RECEPTORS CCR6 AND CCR7 IN THE INITIATION AND PROGRESSION OF BREAST CANCER

A thesis submitted by

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THE UNIVERSITY *of* ADELAIDE

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ABBREVIATIONS AND DEFINITIONS

BSA	Bovine serum albumin
CCL	CC-motif chemokine ligand
CCR	CC-motif chemokine receptor
CD	Cluster of Differentiation
CD206	Mannose receptor
CD45	Leukocyte common antigen
CSC	Cancer stem cell
CXCL	CXC-motif chemokine ligand
CXCR	CXC-motif chemokine receptor
DLL1	Delta-like ligand 1
DMEM	Dulbecco's modified Eagle's medium
DNER	Delta and Notch-like epidermal growth factor-related receptor
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion marker
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FMO	Fluorescence-minus-one
GPCR	G-protein coupled receptor
H&E	Haematoxylin and eosin
HBSS	Hank's balanced salt solution
IF	Immunofluorescence/immunofluorescent
IHC	Immunohistochemistry/immunohistochemical
IL-4R	Interleukin-4 receptor
Lin^-	Lineage-negative
LN	Lymph node
M1	Classically-activated macrophage
M2	Alternatively-activated macrophage
MFE	Mammosphere-forming efficiency
MMTV	Mouse mammary tumour virus
NS	Not significant
OCT	Optimal cutting temperature (embedding medium)

PBS	Phosphate buffered saline
PyMT	Polyoma Middle T antigen
SEM	Standard error of the mean
SMA	Smooth muscle actin
TAM	Tumour-associated macrophage
Tc	Cytotoxic T cell
Th	Helper T cell
TIC	Tumour-initiating cell
Treg	Regulatory T cell
WT	Wild-type

PUBLICATIONS ARISING FROM THIS THESIS

Sarah T. Boyle and Marina Kochetkova. “Breast cancer stem cells and the immune system: Promotion, evasion and therapy” *Journal of Mammary Gland Biology and Neoplasia*, Vol. 19:2, pp 203-211, 06 July 2014 (**Appendix A**)

Sarah T. Boyle, Wendy V. Ingman, Valentina Poltavets, Jessica W. Faulkner, Robert J. Whitfield, Shaun R. McColl, Marina Kochetkova. “The chemokine receptor CCR7 promotes mammary tumourigenesis through amplification of stem-like cells” *Oncogene,* advance online publication, 16 March 2015 (**Appendix B**)

Sarah T. Boyle, Jessica W. Faulkner, Shaun R. McColl, Marina Kochetkova. “The chemokine receptor CCR6 facilitates the onset of mammary neoplasia in the MMTV-PyMT mouse model via recruitment of tumour-promoting macrophages” *Molecular Cancer,* Vol. 14:115, 06 June 2015 (**Appendix C**)

Please Note:

- Results Chapters 3 and 4, and the relevant discussion sections in Chapter 5, are taken from the articles in **Appendices B** and **C**.
- Please refer to **Appendices B** and **C** for the Materials and Methods, Figure layouts and article citations specific to each published manuscript.

CONFERENCE PROCEEDINGS ARISING FROM THIS THESIS

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ABSTRACT

Breast cancer is one of the most common causes of cancer-related death in women world-wide. Despite many advances in medical research, incidence and mortality rates still remain high. In the last decade, evidence has emerged that links components of the immune system, including various signalling mediators and cell subsets, with regulation and promotion of breast cancer. Among others, the chemokine superfamily has been heavily implicated in the pathobiology of breast cancer due to their ability to induce cellular migration, proliferation and recruitment of supporting cells to the tumour microenvironment upon binding of cognate chemokine receptors. However, the underlying mechanisms and specific details governing the function of chemokines and their receptors in regulating breast cancer development and progression are largely unknown. This study systematically examined the roles of two chemokine receptors, CCR7 and CCR6, in breast cancer initiation and progression. These receptors had previously been shown to induce cellular proliferation and migration of breast cancer cell lines upon stimulation with chemokine ligands and had been implicated in regulation of other malignancies, but *in vivo* evidence for their roles had not been shown to date.

Using the MMTV-PyMT transgenic mouse model for breast cancer, it was found that deletion of CCR7 significantly delayed mammary tumour onset and reduced both the number of primary tumours and extent of distal metastasis to the lungs. It was further found that CCR7 exerted its tumour-promoting function by maintaining populations of stem-like cancer cells. Stimulation of CCR7 in stem cell-enriched cultures induced self-renewal and the loss of this receptor resulted in a significant decrease in tumour-propagating ability of the cells. Furthermore, pharmacological blockade of CCR7 reduced proportions and activity of stem cell-like pools, indicating that this receptor can potentially be targeted therapeutically to eliminate quiescent cancer stem cells.

Deletion of CCR6 in the MMTV-PyMT model also significantly delayed tumour onset, reduced the extent of epithelial hyperplasia, and resulted in a decreased incidence of mammary tumours. However, no evidence was found of a role for CCR6 in mammary epithelium, or in maintenance

of the cell lineage hierarchy. Upon further investigation, it was discovered that CCR6 was involved in the recruitment of tumour-promoting macrophages to the mammary tumour microenvironment. CCR6 was highly expressed on tumour-associated macrophages, and the loss of CCR6 significantly reduced the numbers of macrophages present in PyMT-driven mammary tumours.

Taken together, cumulative data generated throughout the course of this project conclusively demonstrate that CCR7 and CCR6 both have important roles in the development and progression of breast cancer and may therefore have therapeutic utility in targeting both the transformed mammary epithelium and the supporting tumour microenvironment.

CHAPTER 1: Introduction

1.1. Breast cancer

Breast cancer is the most diagnosed cancer in women world-wide. Of cancer cases in women, it is also the biggest killer [1]. Overwhelmingly, the primary lesion is not responsible for death due to cancer, rather it is the progression to metastasis that is fatal. Once the cancer has spread to surrounding tissues, such as the lymph nodes, the lungs, the brain and the bone, there is no reliable or effective form of treatment [2]. Despite significant advances in medical research, the morbidity related to breast cancer is still high, and new therapeutical targets are needed to inhibit the progression of breast cancer in the early stages, before multiple mutations have occurred and the disease becomes invasive.

1.1.1. Normal mammary homeostasis and how it may contribute to the development of breast cancer

As with any neoplasm, it is necessary to understand the normal homeostatic processes in order to comprehend how cancer develops. Rudimentary mammary epithelium is formed embryonically in both mice and humans, and foetal mammary stem cells have been shown to possess a similar genetic signature to specific breast cancers, including the aggressive basal-like and Her2-enriched subtypes (discussed in more detail below) [3]. Furthermore, in a recent study, tumour cells from breast cancer patients that had been treated with various chemotherapy regimens were compared with normal and foetal mammary stem cells, and it was found that gene expression signatures of normal mammary stem cells could predict the response of tumour cells to chemotherapy [4]. As such, there is value in examining breast cancer from the perspective of developmental biology.

The mammary gland is an unusual organ – the majority of its development occurs post-natally. During puberty, hormonal changes trigger the rapid expansion of breast epithelial cells and subsequent branching morphogenesis. An epithelial “tree” then grows out from the nipple to form the adult ductal structure [5]. This process is largely driven by fibroblast growth factor

(FGF), epidermal growth factor (EGF), transforming growth factor (TGF)-β, and Jak-Stat signalling pathways, among others [6-9].

The ends of each duct are termed terminal ductal lobular units (human), or terminal end buds (mouse). These structures split as the epithelium grows, to form a dense network of ducts [10]. The ducts are lined internally by luminal cells, and externally by basal cells, the two major cell lineages within the mammary gland [11]. A complex stroma surrounds the ductal network, which is comprised mostly of adipose and connective tissue but also includes tissue-resident immune cells and fibroblasts [12]. The extracellular matrix and surrounding stroma, which is vital to proper development of the mammary gland, can also promote breast cancer due to intrinsic and extrinsic factors [13].

The first menstrual cycle in humans, known as menarche, typically occurs at around 8.5 to 13 years old, and with each cycle the mammary epithelial cell population expands and contracts. During pregnancy, the mammary gland is significantly remodelled, with the expansion of alveolar lobules capable of lactation. When lactation stops, the mammary gland undergoes involution over the course of three months, with apoptosis of alveolar cells and atrophy of lobules. This is the general homeostatic process until the event of menopause, the cessation of menstrual cycling, at the approximate age of 51 [5, 14-16]. Whilst the risk of contracting breast cancer increases significantly based on family history, consumption of alcohol, radiation exposure, use of hormone therapy or oral contraceptives, and a sedentary lifestyle, each of these normal stages in a woman's lifetime – menstruation, pregnancy, and menopause – can alone contain risk factors for breast cancer including early menarche age, late first birth age, and late menopause age [17].

As such, normal mammary development and the development of breast cancer are closely linked. Many signalling pathways involved in normal tissue homeostasis and maintenance within the breast are also involved in tumour formation [18], and signalling components that control other

normal processes – in particular those that limit growth and regulate cell death – can be damaged through mutations, which eventually can lead to onset of cancer [19, 20].

1.1.2. A brief overview of breast cancer development and progression

Malignancies in the breast often begin from uncontrolled proliferation due to mutations in key signalling and regulatory pathways, including (but not limited to) Wnt [21], hedgehog [22], Notch [23], phosphoinositide3-kinase (PI3K) [24], Jak/Stat [25] and NF-κB together with RANK and RANKL pathways [26]. If the lesion is detected early, the patient's chance of survival is relatively high. However, if left untreated, the small lesion may rapidly proliferate to form a primary tumour mass. As the primary tumour progresses, it hijacks the body's homeostatic functions. It forms its own blood vessels and corrupts the normal processes of cellular proliferation and DNA repair. Furthermore, cancer cells are capable of evading the host's immune defences, and even using them to their advantage [27].

There are five main subtypes of breast cancer, clearly defined by a distinct genetic and morphological profile: luminal A, luminal B, basal, human EGF receptor 2 (Her2)-enriched, and claudin-low. Luminal cancers (luminal A and B) are the most heterogenous, and express high levels of luminal-associated genes such as *Esr1*, *Gata3*, *Foxa1*, *Xbp1* and *Myb*. These cancers are typically oestrogen receptor (ER)-positive and Her2-negative, and present with mutations in the MAP-kinase and PI3K pathways. Luminal A tumours, due to the low proliferation rate, generally have the best prognosis for breast cancer patients [19].

The basal-like subtype is the most aggressive subtype and is predominantly negative for ER, progesterone receptor (PR), and Her2, collectively known as triple-negative breast cancer. These cancers are characterised by a high mutation rate in the TP53 and PI3K pathways, and a loss of *Rb1* and *Brca1/2* [19].

Her2 amplification in breast cancer results in a shorter overall survival and time to relapse in patients compared to those without Her2 overexpression. Upon receptor activation, the EGF-

mediated signalling cascade results in expression of genes that promote tumour growth and cell survival. Treatment of patients with trastuzumab (also known as Herceptin) specifically targets the Her2 receptor, and this therapeutic has been used relatively successfully as an adjuvant for Her2-positive cancers over the last couple of decades [28].

Claudin-low tumours have been classified as the most "stem-like" of breast cancers. They typically express high levels of markers associated with an epithelial-to-mesenchymal transition (EMT) and have low expression of proliferation genes and markers. As such, cells of these tumours are slow-cycling, a characteristic of stemness, and the low expression of adherence genes (claudins) suggests that these cancer cells are better adapted to non-adherent survival. Furthermore, the expression of stem cell-related surface markers (discussed in more detail below) and genes is higher in these tumour cells compared to cells from other tumour subtypes. A claudin-low diagnosis has a worse prognosis than luminal A, but has similar survival prognoses to luminal B, Her2-enriched and basal-like cancers. [29].

Even from an early stage, some cells that are capable of metastasis are believed to exist within the tumour [30]. Cells that have metastatic characteristics are able to extravasate into the surrounding vascular network and circulate throughout the body until they home to a suitable secondary site. Primary breast cancer cells typically metastasise to sites such as the lymph nodes, lungs, bone marrow, brain and liver [31], and the metastases formed are molecularly distinct from the primary tumours, possessing a genetic "metastatic signature" [32]. Very few drugs to target breast cancer metastatic disease exist [33], and it is a difficult area to research due to the lack of suitable *in vivo* models that accurately mimic human disease [34]. Thus, treating breast cancer at the early stages is preferable and has a greater chance of a positive clinical outcome.

1.1.3. New concepts in research

Recently, the focus of research in the field of breast cancer has somewhat shifted. As the bulk tumour mass can often be resected quite successfully, more attention has been given to

discovering other factors that are involved in tumour promotion and are necessary for cancer recurrence and spread. Based on these new concepts in research, there are now a variety of new possible therapeutic targets that have been proposed to treat breast cancer at various stages in its progression (outlined in ***Figure 1.1***), although no clinical results are available as yet [33].

The tumour is not a lone entity; whether it is the primary site or a secondary metastasis, it requires a number of other supporting untransformed cells and signalling factors if it is to survive. This includes resident cells such as fibroblasts, and certain immune cells that are recruited to the tumour microenvironment.

Cancer-associated fibroblasts (CAFs) (including myofibroblasts, mesenchymal stem cells, tissue fibroblasts and adipocytes) can be involved in many aspects of tumour progression including proliferation, angiogenesis, metastasis, and resistance to cell death. CAFs can secrete a range of factors, including growth factors, cytokines and chemokines that can drive cellular growth and induction of EMT, pro-inflammatory mediators that can activate and recruit various immune cells, and angiogenic factors. Mesenchymal stem cells are able to differentiate into myofibroblasts or adipocytes, aiding tumour growth. Most importantly, CAF numbers within the tumour correlate with poor prognosis for multiple malignancies, including breast cancer [35]. Interestingly, some findings suggest that CAFs in the tumour microenvironment may also be important for maintenance of breast cancer stem cells (discussed in detail below). Breast cancer cells co-cultured with CAFs that secrete chemokines CXCL12 and/or CCL2 had greater stem-like characteristics and higher proportions of putative cancer stem cells [36, 37]. Through remodelling of the extracellular matrix, CAFs can also enhance cancer cell survival and their ability to invade [38] through a collective invasion mechanism [39], generating circulating tumour cells.

New techniques have allowed the study of these disseminated circulating tumour cells (CTCs), which have recently been equated to cancer stem cells [40]. High numbers of CTCs in patient

blood samples correlate with increased metastasis, aggressive disease, and shorter relapse-free survival times. It has been proposed therefore that CTC levels should be used as an additional biomarker to standard biopsy procedures, due to the non-invasive nature of blood tests. However, CTCs are rare, and due to their propensity to circulate in clusters they are often trapped within small capillaries. Hence, their detection at present remains difficult, despite the clear clinical benefits of early intervention to impede metastasis [33, 41].

As with the primary site, the pre-metastatic niche is prepared for seeding of circulating tumour cells by a number of factors. Tumour-secreted factors from the primary site - including various growth factors, chemokines, tumour necrosis factor-α, hypoxia-inducible factors and granulocyte colony-stimulating factor - recruit bone marrow-derived cells and immunosuppressive cells to secondary organs, and remodel the site to promote cancer cell adhesion and invasion, hence creating microenvironments suitable for colonisation by metastatic tumour cells. Whilst it is possible that the formation of pre-metastatic niches is not essential for metastatic colonisation, it is thought to greatly enhance the chance of secondary disease occurring [42, 43]. Therefore, disruption of the metastatic niche is a potential therapeutic avenue. So-called cancer stem cells, discussed below, are the likely candidates for the preparation of the metastatic site, due to their increased expression of surface receptors involved in migration and their ability to propagate tumours in secondary sites [44].

The concept of a “cancer stem cell” (CSC) was first proposed by John Dick and colleagues, who found that in acute myeloid leukaemia there was a small subset of stem-like cells that possessed the capacity to differentiate and proliferate, and had the potential for self-renewal that was expected of a leukaemic stem cell [45]. This theory has been investigated in great detail in the last decade, in many different types of cancers. It states that a small subpopulation of cells exist within the tumour that is responsible for its maintenance and growth, termed “stem cells” for their stem-like characteristics [46]. The tumour microenvironment is believed to be responsible

for maintenance of not only the bulk tumour mass but also the CSCs [47], and so specifically targeting both the stem cells and the supporting niche simultaneously is a potentially new therapeutic approach to prevent breast cancer relapse. Despite this, the underlying biochemical pathways and cellular interactions within this biological system are currently largely unknown.

1.2. Adult stem cells and cancer stem cells in the breast

1.2.1. Properties of adult stem cells

Somatic adult stem cells reside in all tissues, and are formed from embryonic precursors during foetal development. However, unlike pluripotent embryonic stem cells that are capable of forming any cell in the body, adult tissue stem cells are generally only multipotent – they are only able to form any cell in that specific tissue. This stem cell property of differentiation is usually only employed in cases of tissue damage and repair, and stem cells are mostly quiescent in solid tissues [48]. Tissue-resident stem cells are capable of asymmetric division, similar to embryonic stem cells. Whilst terminally differentiated cells can produce two identical daughter cells, stem cells can give rise to one transit-amplifying cell (as required) and can also self-renew to form another stem cell, to maintain the pool within the tissue [49].

These stem-like properties – self-renewal, quiescence and the ability to differentiate into multiple lineages – are also the hallmarks of so-called cancer stem cells, hence the name.

1.2.2. The cancer cell-of-origin

As mentioned above, cancer stem cells are believed to be responsible for initiation, growth, maintenance and metastasis of the tumour [46, 50]. Currently however, it is unknown whether CSCs are a separate, fixed population of cells, or whether this pool of cells is in fact plastic, and any cell within a tumour can acquire stem-like characteristics depending on the circumstances [51]. Stem cells that are identified in various assays may not include those with latent regenerative capacity [52]. This is important to keep in mind when designing therapeutics that target CSCs, if new cells can easily take the place of destroyed CSCs. It is also important to note

that not all cancers may follow the stem cell model, and in breast cancer, various mutations may give rise to tumours that vary in the extent to which they follow this model [53].

Kordon and Smith first proposed a stem cell model in the mammary gland in 1998, following a series of transplantation experiments [54]. It is now understood that normal adult stem cells or bipotent progenitors that reside within epithelial ducts [55] differentiate into two cell lineages, the luminal population and the basal/myoepithelial cell population [11]. Within this hierarchy, it is still not clear from where cancer stem-like cells arise. The current consensus is that CSCs may result from mutations in normal adult stem cells and/or that more differentiated cells may be able to acquire stem-like characteristics upon mutation, and upon transplantation only these certain cells are able to form secondary tumours [46] (***Figure 1.2***).

Therefore, the "cell-of-origin" within a cancer may not be an adult stem cell, but a more committed progenitor or potentially even a terminally differentiated cell, depending on where mutations arise if the mutations are able to confer a stem-like phenotype [56]. The cell-of-origin has been putatively identified in a small number of cancers, including acute myeloid leukaemia, which originates in common myeloid progenitors [57], and prostate cancer, which originates in basal cells [58].

In breast cancer, the cell-of-origin for various subtypes of breast neoplasms (outlined above) potentially arises from different cells within the hierarchy (***Figure 1.2***), where basal-like tumours with Brca1 mutations originate from luminal progenitors [59], claudin-low are the most stem cell-like in terms of gene signatures, and luminal A and B subtypes present gene signatures overlapping with differentiated luminal cells. Her2-enriched tumours have no putative cell-of-origin defined as yet, but may arise from the luminal lineage also, as these cancers contain a high proportion of luminal progenitor cells [60]. Identification of cells-of-origin is difficult, as it is not currently possible to purify CSCs from solid tumours to near homogeneity, unlike the more defined populations in haematopoietic cancers [61].

1.2.3. Characterisation and identification of cancer stem cells

Despite being unable to completely purify CSCs, a number of methods allow for the identification and enrichment of putative CSCs within heterogeneous bulk cell populations [62]. *In vitro* and *ex vivo*, this includes flow cytometry and specific cell culture techniques. Different tumour types have different putative surface markers for CSCs, the combination of which selects for a population that has the stem-like properties of self-renewal and differentiation. Notably, positive expression of the markers CD133 and/or CD44 has been shown to enrich for the putative stem cell population in a number of solid tumours [63].

Just over a decade ago, Al Hajj *et al.* proposed a $CD44^{+}CD24^{-/lo}$ surface marker profile to detect human breast CSCs, as sorted cells with this profile had an enhanced ability to form tumours upon xenotransplantation compared to alternative phenotypes. They also noted that this population does not express a number of markers that are associated with leukocytes, endothelial cells, mesothelial cells and fibroblasts, and so the stem-like population can be further elucidated based on "lineage-negative" (Lin^{-}) gating or sorting [64].

Shortly after, Shackleton *et al.* characterised the mouse breast stem cell population and found it to be enriched in the $CD24^{+}CD29^{hi}$ subset [65]. Other groups such as Stingl and colleagues report that sorting cells based on CD49f and EpCAM expression can enrich for the stem cell population [66]. Generally these marker profiles are thought to overlap, and a certain amount of personal preference exists in terms of ability to separate out populations during flow cytometry or fluorescence-activated cell sorting (FACS). As illustrated in ***Figure 1.2***, these marker profiles select the basal subset of cells, which is believed to contain the CSCs, putative myoepithelial progenitors, and terminally differentiated basal cells [60]. Within this basal population, most cells are myoepithelial non-dividing cells, which have repopulating capacity and the ability to clonally expand. Progeny of these cells function as long-lived lineage-restricted stem cells in normal mammary glands and during pregnancy [67]. Mouse mammary epithelial cells can be

further sorted into the luminal subset based on $CD24^{+}CD29^{lo}$ [68], which can be delineated further into luminal progenitor cells ($CD61^{+}$ [69, 70]) and terminally differentiated ($Sca\text{-}1^{+}$) cells [60] (***Figure 1.2***).

In 2010, Di Fiore's group proposed a new set of markers to elucidate human mammary stem cells, in which Notch ligands delta-like ligand 1 (DLL1) and delta and Notch-like EGF receptor (DNER) were used in conjunction with CD49f ($CD49f^{+}DLL1^{+}DNER^{+}$) to enrich 530-fold for the stem-like population compared with an 85-fold enrichment when gating on $CD24^{+}CD49f^{+}$ [71]. However, since publication of this article no further evidence has been reported for the use of this marker profile in stem cell isolation, and it has not been tested in the mouse mammary gland. Putative breast CSCs may also be identified by the "side population" technique, which involves isolating a cell subset capable of Hoechst dye exclusion [72], or the ALDEFLUOR assay, based on enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) [73].

Specific cell culture techniques can also be used to enrich for stem-like cells. One of the most widely used approaches is the "sphere assay", a non-adherent culture system designed to select for cells with clonogenic and self-renewing potential, in which cells that are unable to survive without attachment undergo apoptosis [74]. This assay is based on another proposed property of stem cells – resistance to anoikis [75]. First developed to study stemness in nerve cells, the neurosphere assay [76] has been adapted for many solid malignancies, including brain, colon, prostate, lung and breast cancer [46].

Dontu *et al.* first characterised the potential of mammospheres to enrich for mammary stem cells and found colonies generated by this type of culture were clonogenic (i.e. not the product of aggregation) and capable of passage [75]. Since then, this assay has been used extensively to study normal mammary and breast cancer stem cells *in vitro* and *ex vivo.* Whilst the primary heterogeneous mammosphere culture is thought to contain a greater proportion of luminal cells and select only for cells with clonogenic potential, passage of sphere cultures selects for *bona*

fide self-renewing cells by enriching for the stem cell-containing basal population [46, 77]. Other cell cultures have also been used to study CSCs, including the colony-forming assay pioneered by Stingl's group [78], and the Matrigel assay, which also allows for identification of differentiation capacity in a semi-solid media [79].

Approaches to study mammary stem cells *in vivo* are continuously evolving. The limiting dilution assay, first developed to study breast CSCs by Clarke and colleagues, involves injection of decreasing numbers of mammary epithelial cells into the mammary fat pad, designed to determine the repopulating capacity of cells from a given population, genotype and/or treatment [64]. This assay was the foundation for two landmark papers in 2006 by Visvader's and Eave's groups, which were able to generate entirely new functional mammary glands in mice from a single cell [65, 80].

Recently, study of the cell hierarchy within the mammary gland and other tissues has significantly expanded, with the advent of cell fate-mapping by label-retention and lineage-tracing techniques [81]. However, limitations still exist in the mouse models used, and thus the conclusions drawn are not without controversy. Blanpain and colleagues, based on lineage-tracing experiments, have proposed that both basal and luminal lineages are maintained in adult life from unipotent lineage-restricted precursors. They postulated that a multipotent stem cell does not exist in normal physiological conditions and may only appear during stress conditions such as transplantation [82]. Visvader's group then counteracted these findings shortly after, as their own cell fate-mapping study suggested a bipotent precursor did indeed exist at the top of the hierarchy giving rise to both luminal and basal cell types [83]. The issue is still awaiting resolution.

Although this is the closest the field has come to identifying stem cells at the single cell level, the final picture of the mammary gland hierarchy has yet to be definitively established. This may be achieved by a combination of cell fate-mapping and prospective isolation of refined populations

of stem and progenitor cells. Despite this, the question of plasticity in the stem cell population still remains contentious as demonstrated by Van Rheenen and colleagues, using elegant intravital imaging combined with lineage-tracing experiments to show that even when a CSC disappears following asymmetric division, the loss is compensated for by clonal expansion of a neighbouring stem-like cell [84].

1.2.4. Clinical implications of cancer stem cells

As with normal adult stem cells, CSCs are believed to be mostly quiescent. This means that conventional therapeutics designed to kill rapidly-dividing bulk tumour cells may be somewhat ineffective at targeting this slow-growing and potentially plastic population, leading to relapse [52]. Furthermore, CSCs potentially up-regulate expression of ATP-binding cassette (ABC) drug transporters, and so any drugs that may happen to enter the cell are quickly eliminated [85]. Drugs designed to target cancer-specific cell surface molecules may also be useless against CSCs, which could have completely different surface marker expression. These drugs, including immunotherapies (discussed in more detail below), may also select for a more aggressive CSC, as those that do not express the target are able to survive and multiply, differentiating into bulk tumour cells that the drug is unable to recognise [86]. Furthermore, finding a drug that selectively targets cancer stem cells over normal stem cells is a significant challenge [46], although large screening experiments may make this approach viable [87, 88].

As outlined in ***Figure 1.1***, finding unique, functional CSC markers is one potential way to specifically target this small subpopulation of cells in conjunction with standard chemo- and radio-therapy. Candidate therapeutic targets may include receptors and their oncogenic forms, adhesion molecules, signalling pathway members and metabolic components [52]. Another option is to force CSCs to differentiate [53], into cells that can be readily eradicated by conventional methods. An additional avenue to explore to eliminate CSCs is to disrupt the

supporting niche, so that CSCs do not receive the survival maintenance they receive from stromal cells and the immune system [46].

1.3. The immune system and breast cancer

The immune system plays a key role in breast cancer progression. Infiltrating immune cells, which are recruited and present in many solid tumours [89], and are able to be readily identified [90], may influence many aspects of cancer, including proliferation, evasion of growth suppressors and apoptosis, avoidance of immune destruction, and induction of invasion, angiogenesis, and metastasis [38, 91]. However, the information available to date lacks consistency, and the roles of various cell subsets appear to vary depending on the tumour type and circumstance.

1.3.1. The adaptive immune system in breast cancer

Numerous reports have demonstrated that infiltration of cytotoxic T cells (Tc), helper T cells (Th), regulatory T cells (Treg) and the dendritic cells that prime them results in increased anti-tumour immunity, and infiltration of these cells into primary breast tumours has been associated with good survival prognosis in breast cancer patients [92-96]. Importantly, harnessing the actions of these immune cell subsets to create breast cancer vaccines is of particular clinical interest [97].

It is important to note however, that the role of these immune subsets in breast cancer is somewhat contradictory. For example, it has been reported that Th cells can promote early mammary tumour development via cytokine secretion [98], Tc cell infiltration has been associated with progression to metastasis [99], and dendritic cells can also have an immunosuppressive effect in breast cancer [100]. In addition, the actions of cancer-associated fibroblasts, macrophages and mast cells can block anti-tumour responses of Tc cells [38]. Treg cells in particular have been shown to play a tumour-promoting role in breast cancer, in which

the down-regulation of immune responses by Treg cells leads to evasion of the immune system by cancer cells and an increase in cancer cell invasiveness [101].

Similarly, B cells have been shown to have an anti-tumour role and can even form ectopic germinal centres within the primary tumour to activate T cell-mediated anti-cancer immunity [102, 103] but, conversely, their infiltration has also been associated with a higher tumour grade [104] and they have been found to play a pro-metastatic role through conversion of resting T cells into Treg cells [105]. To summarise, there is currently no definitive answer as to the role of active immune cell subsets in the promotion or inhibition of breast cancer, and their level of infiltration and involvement is likely to be determined by cancer subtype, stage of disease, tumour microenvironment and specific subset interplay.

1.3.2. Tumour-associated macrophages

Although thought to have a role in anti-cancer immunity under certain conditions [106], tumour-associated macrophages (TAMs) are prolific in human breast cancer [107] and in MMTV-PyMT mouse mammary tumours [90] and are consistently found to play a significant part in tumour promotion and poor patient survival [35]. Tissue-resident macrophages exist in all tissues to aid in immunity against site-specific pathogens, removal of apoptotic cells, and secretion of certain growth factors [108]. In the mammary gland they are also involved in development of the mammary epithelium and in tissue remodelling during pregnancy, lactation and involution [109-111]. These macrophages are defined as classically-activated, or of an “M1” phenotype.

However, during cancer progression, both newly recruited and resident macrophages within the cancerous tissue can be alternatively activated and switch to an “M2” phenotype [112]. This switch, induced by particular microenvironment cues, results in a decrease in pro-inflammatory chemokine and cytokine production, a decrease in antigen presentation, suppression of the T cell anti-tumour immune response, and promotion of angiogenesis, cell proliferation and tissue remodelling [113]. Furthermore, TAMs promote cancer cell survival and resistance to

chemotherapy, and together with mast cells can remodel ECM components to allow for cellular invasion [38].

M1 macrophages are induced *in vitro* by interferon-γ, sometimes in combination with microbial-derived stimuli (such as lipopolysaccharide) or cytokines. M2 macrophages can be generated by interleukin (IL)-4 and IL-13 [113]. The M1 and M2 phenotypes are identified using a number of methods. *In vitro,* different conditions can induce a change in the inducible nitric oxide synthase (iNOS) to arginase 1 (Arg1) ratio within macrophages. iNOS is involved in pro-immunogenic functions, whereas Arg1 expression has immuno-suppressive effects, which can exacerbate tumour growth. Hence, M1 macrophages can be identified based on high iNOS and low Arg1, and M2 macrophages have low expression of iNOS and elevated levels of Arg1 [113, 114]. M2 macrophages can also be identified based on positive expression of IL-4 receptor (IL-4R), mannose receptor (CD206), and IL-10 [113].

1.3.3. Immune selection and tolerance of breast cancer stem cells

The following contains excerpts from ***Appendix A***.

In addition to their role in breast cancer progression, there is some recent evidence that points to a role for various immune cells in the regulation of breast CSCs (***Appendix A*** [115]). As recently proposed [115] and outlined in ***Figure 1.3***, the "immuniche" (the components of the immune system within the tumour niche that support cancer stem cells) may be a determining factor in stem cell survival, maintenance, and subsequent tumour growth. A high infiltration of CD4- and CD8-positive immune cells has been previously correlated with increased breast cancer stem cell phenotype [116], and the presence of macrophages in mammary tumours was also found to maintain the breast CSC niche through secretion of specific cytokines, such as IL-6 and CXCL8 (also know as IL-8), enhancing the stem cell-like state and activity [117]. Cells within the immuniche may not only directly support CSCs (as in the case of TAMs and secreted factors by other stromal cells such as CAFs and mesenchymal stem cells [47]) but may potentially select

for more aggressive or evasive CSCs through the processes of immune selection (***Figure 1.3***), which could also play a part in resistance to immune-mediated therapy by cancer stem cells.

Bulk cancer cells can develop resistance to conventional cytotoxic drugs due to up-regulation of drug transporters [27], and it is thought that CSCs may have more robust mechanisms of drug efflux. The same may be true for immunotherapies, where CSCs avoid killing by cytotoxic cells. For example, it was found that treatment with trastuzumab depleted Her2-expressing breast cancer cells through a natural killer cell-mediated mechanism. However, cells that survived this natural killer cell immune selection had increased stem-like $CD44^{hi}CD24^{lo}$ surface marker expression and were able to grow more robustly as mammospheres compared with naïve cells. After six cycles of treatment, the $CD44^{hi}CD24^{lo}$ cell content was significantly increased, indicating that proportions of breast CSCs increased over time through immune selection [118].

Tumour cells are also able to avoid recognition by primed immune cells through immuno-editing, a process in which tumour cells lose or modify tumour antigens. Findings by Knutson *et al*. showed that immuno-editing may occur through the process of EMT, a route that may also result in the acquisition of a breast CSC phenotype. The authors have suggested that immuno-editing is not an incremental loss of tumour antigens but rather an active process in which cells use EMT to de-differentiate into cells of a stem-like state [119].

Immune selection and immuno-editing may result in tumour cells that would otherwise be attacked by the immune system being recognised as self, thus leading to tolerance and immune evasion. Exact mechanisms underlying these phenomena are not yet known, however glycoprotein CD200 has been previously reported to have a role in breast CSC immune evasion [120]. CD200 is commonly expressed in myeloid cell populations and has been studied as a therapeutic target due to its role in immunoregulation and immune tolerance, as deletion of CD200 in animal models can lead to development of autoimmune disorders [121]. In

tumourigenic and invasive breast cancer cells, it was found that a high percentage of $CD200^{+}$ cells were of a $CD44^{+}CD24^{-}$ phenotype [120].

It is plausible that the infiltration of immune cells into mammary tumours may stimulate more mature breast cancer cells to de-differentiate into stem-like cells through EMT, coinciding with the activation of molecules that promote tolerance of CSCs by the immune system. This in turn may enhance CSC immune evasion and the development of more aggressive tumours.

1.3.4. Chemokine receptors in cancer

The recruitment of immune cells to the tumour site is mediated by the actions of chemokines and their cognate ligands, which have also been widely linked to cancer progression and metastasis.

Chemokine receptors are G-protein coupled receptors (GPCRs), consisting of seven transmembrane domains, which bind low molecular-weight chemotactic-cytokines (chemokines). Upon binding of chemokine ligands, the receptor undergoes a conformational change leading to activation of intracellular G-proteins. This results in a signalling cascade that, depending on the context, can stimulate cellular growth, migration, pseudopodia formation, adhesion, and angiogenesis, as well as angiostasis [122]. When expressed on immune cells, the action of chemokine receptors leads to homing of an immune cell to a specific site via a chemotactic gradient. Because of the involvement of chemokine receptors in migration of cells, they have been widely implicated in metastasis of cancer cells throughout the body [123]. ***Table 1.1*** summarises the involvement of chemokine receptors in cancer pathogenesis. In breast cancer, the chemokine receptors CXCR4, CCR7, CCR6 and CXCR3 and the ligand CCL2 have been associated with metastasis [124-127].

The receptor CXCR4 is the most studied of all chemokine receptors in cancer, and its many documented roles in breast cancer encompass cell survival, proliferation, motility, invasion, angiogenesis, recruitment and activation of a number of different cell types, and metastasis [128]. CCR7 is often up-regulated together with CXCR4 in cancer [125, 129] and previously the

McColl group reported that CXCR4 together with CCR7 regulates breast cancer cell metastasis through inhibition of anoikis [130]. In addition, these two receptors form a heterodimer on metastatic breast cancer cells, which activates alternative signalling pathways and promotes a metastatic phenotype (Kochetkova; unpublished). However, CCR7 has not been as well characterised as CXCR4 in the literature.

Chemokine receptors are further involved in regulation of cancer progression through recruitment of immune cell subsets. For example, macrophages can be recruited to the tumour microenvironment through the CX_3CR1/CX_3CL1 and CCR2/CCL2 axes [131], the expression of CCR5 has both anti-tumour activity when expressed on infiltrating Tc cells and pro-tumour activity when expressed on Treg cells [132], dendritic cells can migrate to the tumour microenvironment via CXCR4, CCR6 and CCR7, and CXCL9 and CXCL10 are strong chemoattractants for CXCR3-expressing T lymphocytes [133].

Importantly, there is a paucity of information from *in vivo* experimental systems regarding the specific function of individual chemokine receptors in cancer progression, and very little data exists regarding their role in primary tumour formation.

1.3.5. Chemokine receptors and cancer stem cells

The following contains excerpts from ***Appendix A***.

Recently, a small body of evidence has also implicated chemokine receptors in maintenance of stem cells, as summarised in ***Table 1.2***. Whilst most of the available data refers to mesenchymal stem cells, some chemokines and their receptors have been suggested to play a role in maintenance of cancer stem cells [134].

Seminal work on the involvement of chemokine receptors in breast CSCs has come from Max Wicha's group, which have studied the role of CXCR1. CXCR1 is closely related to CXCR2, with which it shares multiple ligands including CXCL8. Both receptors have been heavily

implicated in the elimination of pathogens but also shown to contribute significantly to disease-associated processes, including tissue injury, fibrosis, angiogenesis and tumourigenesis [135]. It was found that CXCR1-positive cells were present in the $CD44^{+}CD24^{-}$ population in breast cancer cell lines, and were also almost exclusively contained in the ALDEFLUOR-positive population. Stimulation of CXCR1 with CXCL8 increased primary and secondary mammosphere-forming efficiency (MFE). In addition, $CXCR1^{+}ALDEFLUOR^{+}$ cells were able to form heterogenous tumours upon transplantation [136, 137], providing functional support for the role of CXCR1 in breast CSC regulation.

Treatment of breast cancer cell lines with a CXCR1 inhibitor or an anti-CXCR1 antibody resulted in a five-fold reduction in $ALDEFLUOR^{+}$ cell numbers and inhibition of the CXCR1 interaction with its ligands in sphere culture caused a massive decrease in primary and secondary MFE. Interestingly, when normal human mammary epithelial cells were cultured as mammospheres, addition of CXCL8 again increased the primary and secondary MFE, further implicating CXCR1 as a regulator of stemness in both cancer and normal breast development [137].

The CXCR2 chemokine receptor was previously shown to mediate breast cancer chemoresistance [138], an implied property of breast CSCs. Co-expression of CXCR1 and CXCR2 by cancer cells is reported to promote cancer growth [139], and as mentioned above, both are receptors for CXCL8 which is expressed by breast CSCs [140]. As well as CXCL8, CXCR2 interacts with chemokines CXCL3, CXCL5, and CXCL7. Wicha and colleagues also examined the result of neutralising CXCR2 but did not observe a difference in the breast CSC population [137].

Contrary to this, other reports showed that siRNA targeting of CXCR2 and CXCL3 decreased the viability of $CD44^{+}CD24^{-}$ basal-like breast cancer cell lines through a decrease in Stat3 activity [141], and sequestering CXCL7 blocked expression of other immune modulators from

putative breast CSCs [142]. In addition, CXCL5 stimulation of breast cancer cell lines increased the ALDEFLUOR$^+$ cell population [142]. As CXCR2 antagonists are already in the clinic for other conditions such as pulmonary disease [143], inhibiting this receptor may prove a promising breast CSC-targeting therapy, however the role of CXCR2 in breast CSCs still remains to be validated in cancer models *in vivo*.

Recently, CXCR4 was reported to contribute to the maintenance of the breast CSC population through transactivation of the aryl hydrocarbon receptor [144] and was also expressed in CD44$^+$CD24$^-$ breast cancer cell lines, which correlated with their invasiveness [145]. Furthermore, CXCR4 was detected on the surface of mammosphere cells, and CXCR4 antagonists decreased MFE and the proportion of CD44$^+$CD24$^-$ breast cancer stem-like cells [36]. Interestingly, CXCR4 expression has been putatively linked to the functionality of the CD24 surface marker (a breast CSC marker) in metastatic spread, but this finding requires further validation *in vivo* [146].

The CXCR4 antagonist plerixafor (AMD3100) has shown some promise in preclinical trials [147, 148] and could also provide an option for targeting stem cells. For example, in tamoxifen-resistant breast cancer cells, Dubrovska *et al*. showed that AMD3100 inhibited the breast CSC-enriched side population [144]. This study highlights the potential use of chemokine receptor pathway antagonists as adjuvants to conventional cytotoxic drugs, which alone cannot eradicate the quiescent cancer stem cell populations.

It has also been shown that anoikis-resistant cells (which have enhanced stem-like phenotype) up-regulate CXCR4 preferentially in breast cancer over normal mammary epithelium [149]. As the McColl group previously found that activation of CXCR4 and CCR7 inhibits anoikis [130], this suggests that both receptors have a role in promoting the breast CSC phenotype.

In a study reported in 2011, Asiedu *et al*. comprehensively characterised the chemokine and chemokine receptor profiles in breast cancer cell lines with high CD44$^+$CD24$^-$ cell content, with

the view to identifying potential breast CSC regulators among this class of immune modulators. They reported increased expression of CCR7, but unexpectedly down-regulation of CXCR4. In addition, $CD44^{+}CD24^{-}$ breast cancer cells had increased expression of CXCR7, CCL13, CCL11, CCL12, CCL2, CCL5, CCL6, CCL7 and CCL8, and down-regulated CCR3, CXCL1 and CCL20 [150]. Notably, CXCR7 also binds the ligand for CXCR4, CXCL12, and scavenges CXCL12 to promote cancer cell metastasis [151]. Increased expression of CXCR7 in the CSC population and its impact upon CXCR4 activity remains to be functionally assessed. Of the chemokines up-regulated, only CCL2 and CCL5 have been conclusively shown to have a role in breast cancer [152, 153].

Whilst CCL5 has not been examined in the context of breast cancer stem cells, CCL2 has been proposed to affect properties of stemness in breast cancer cells. In addition to secretion from mammary CSCs, treating mammosphere culture with exogenous CCL2 increased stem cell function via induction of Notch-1 signalling and resulted in more spheres with a higher number of slow-cycling stem cells, as was measured by retention of PKH67 dye [37]. Whilst there are clear indications of a potential function for CCL2, more specific *in vivo* evidence is needed to confirm its role in breast CSC regulation. Of note, CCL2 is also involved in recruitment of monocytes, memory T cells, regulatory T cells and dendritic cells to inflammatory sites [154], and to breast tumours [155] through its major receptor CCR2, which may aid breast CSCs in controlling the immune system.

The involvement of chemokine receptors appears to encompass a diverse range of roles in terms of primary cancer growth, immune infiltration, regulation of the microenvironment, metastasis, and maintenance of stem cells. However, the underlying molecular mechanisms are largely unknown, and may explain the shortage of chemokine receptor-targeting drugs in breast cancer clinical trials.

1.4. The research project

1.4.1. Project rationale

CCR7 is one of the major factors in regulation of T cell migration to secondary lymphoid organs [156], induction of tolerance through interactions with dendritic cells [157], and control of immune responses via Treg cells [158]. Cell migration mediated by CCR7 follows the gradients of its two chemokine ligands, CCL19 (EBV-induced molecule 1 ligand, ELC) and CCL21 (secondary lymphoid tissue ligand, SLC) [159]. In addition to its well-documented homeostatic role in immunity, CCR7 has also been shown to be involved in various malignancies (see ***Table 1.1***).

However, whilst the chemokine receptor CXCR4 has been widely investigated for its oncogenic properties, CCR7, which is up-regulated together with CXCR4 in breast cancer [125, 160], has not been as well-characterised. It was shown in archived human breast cancer tissue that high levels of CCR7 correlated with decreased patient survival and metastasis to the lungs and lymph nodes [125, 161]. Using *in vitro* cell line culture, CCR7 was found to promote breast cancer cell motility and invasiveness [161, 162], and intratumoural CCL21 can contribute to suppression of anti-tumour immunity [38]. Whilst these data circumstantially implicate CCR7 in breast cancer development and progression, there has been no definitive *in vivo* evidence for the role of CCR7 in the primary tumour reported so far.

CCR6 has been previously associated with higher grades of breast cancer and reported as a possible prognostic marker together with CCR7 [124, 163]. As with the CCR7 receptor, CCR6 is expressed on a variety of immune cells, and induction of CCR6-mediated pathways is stimulated upon binding to its ligand CCL20 (macrophage inflammatory protein (MIP)-3α) [164]. In particular, CCR6 is involved in dendritic cell localisation and homeostasis [165], and Treg cell migration [166]. However, a function for CCR6 in malignancies has not been previously shown,

despite being expressed in a number of cancers (see ***Table 1.1***), with no direct causal link between the expression of CCR6 and cancer progression reported to date.

It was previously found that higher CCR6 expression levels were linked with breast tumour stage and grade [163], and breast cancer metastasis to the pleura [124]. Expression of ligand CCL20 is also reported to be up-regulated in human triple-negative breast cancer cell lines [167], and stimulation of *ex vivo* peritumoural mammary cells with CCL20 increased cell proliferation, migration and invasiveness [168]. However, CCR6 has not been investigated in primary breast cancer *in vivo* and hence no functional role for CCR6 in breast cancer has been documented.

Thus, potential roles for the receptors CCR7 and CCR6 in breast cancer development and progression are implied, however the underlying mechanisms and functional relevance of their expression have not yet been established.

1.4.2. Hypothesis and Aims

1.4.2.1. Hypothesis

The hypothesis for this project was as follows:

The chemokine receptors CCR7 and CCR6 are important regulators of breast cancer development and progression.

1.4.2.2. Aims

Aim 1: To investigate the role of CCR7 in mammary tumourigenesis.

Aim 2: To investigate the role of CCR6 in mammary tumourigenesis.

1.4.3. Summary of findings

As CCR7 and CCR6 have been associated with breast cancer metastasis from analysis of archived tissue, and their stimulation induces proliferation of cancer cells *in vitro*, it was hypothesised that these receptors played a significant role in breast cancer development and progression *in vivo* via distinct molecular and cellular mechanisms.

Using a well-characterised mouse model for breast cancer, in which the polyoma middle-T transgene is activated under control of the mouse mammary tumour virus promoter (MMTV-PyMT) [169, 170], the contributions of CCR7 and CCR6 to primary breast cancer were examined *in vivo* and *ex vivo*.

When it was found that both CCR7 and CCR6 were highly expressed within the breast cancer cell population, the MMTV-PyMT transgenic mouse was crossed with both CCR7- and CCR6-null mice. Deletion of either receptor in these bigenic mouse models resulted in a decrease in mammary tumourigenesis, indicating that both receptors play a role in breast oncogenesis.

However, it was then discovered that the function of each receptor in the promotion of breast cancer pathogenesis is unique. CCR7 promoted breast cancer via amplification of cancer stem-like cells, as was found by analysing cell surface marker expression and self-renewal in sphere culture, and performing multiple transplantation approaches. Furthermore, the use of a CCR7 antagonist *in vivo* significantly depleted stem-like cell pools, making CCR7 a plausible candidate for CSC-targeting therapies (***Appendix B*** [171]).

CCR6, however, did not play any role in the epithelial compartment of the mammary gland, as its deletion did not impact upon cell proliferation, maintenance of the cellular hierarchy, or tumour propagation upon transplantation. Upon further examination, it was found that the role of CCR6 in the promotion of breast cancer is via recruitment and maintenance of pro-tumourigenic macrophages in the tumour microenvironment. The deletion of CCR6 resulted in significantly reduced pools of both total TAMs and of M2 macrophages. The negative effect of CCR6

deletion on tumourigenesis could be restored by supplementation of tumour-associated macrophages (***Appendix C*** [172]).

Results from this study imply that simultaneous assault of both the CSC pool and the tumour microenvironment by targeting specific chemokine receptors is a viable option for breast cancer therapeutic application.

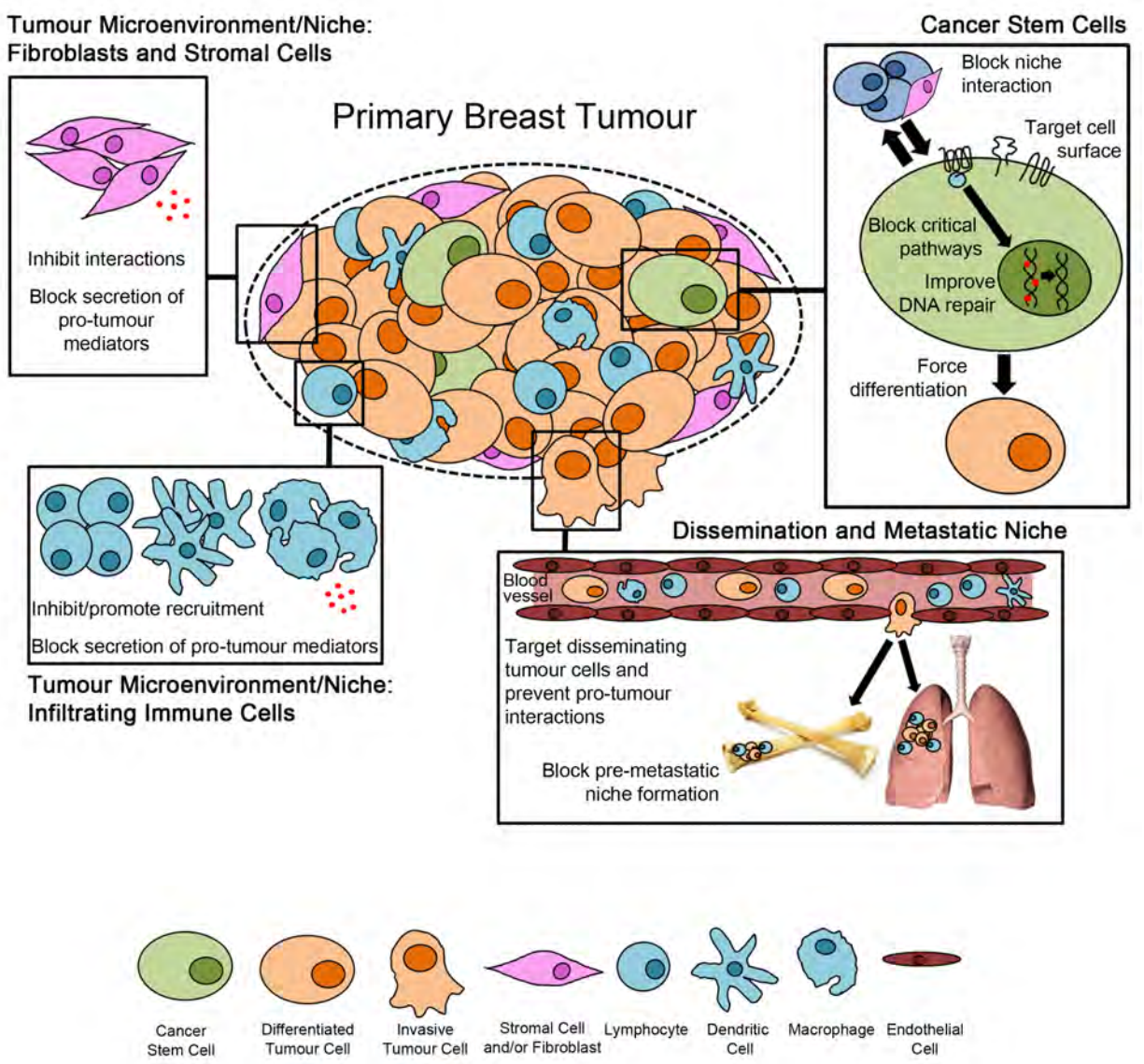

Figure 1.1: New concepts in breast cancer research and avenues of possible therapeutic targeting.

The field of breast cancer research has expanded significantly in the last decade, with investigation into a number of new concepts, including study of the tumour microenvironment, disseminated and circulating tumour cells, formation of metastatic niches, and cancer stem cells. Along with the study of these areas of breast cancer pathobiology comes new possible avenues for therapeutic application.

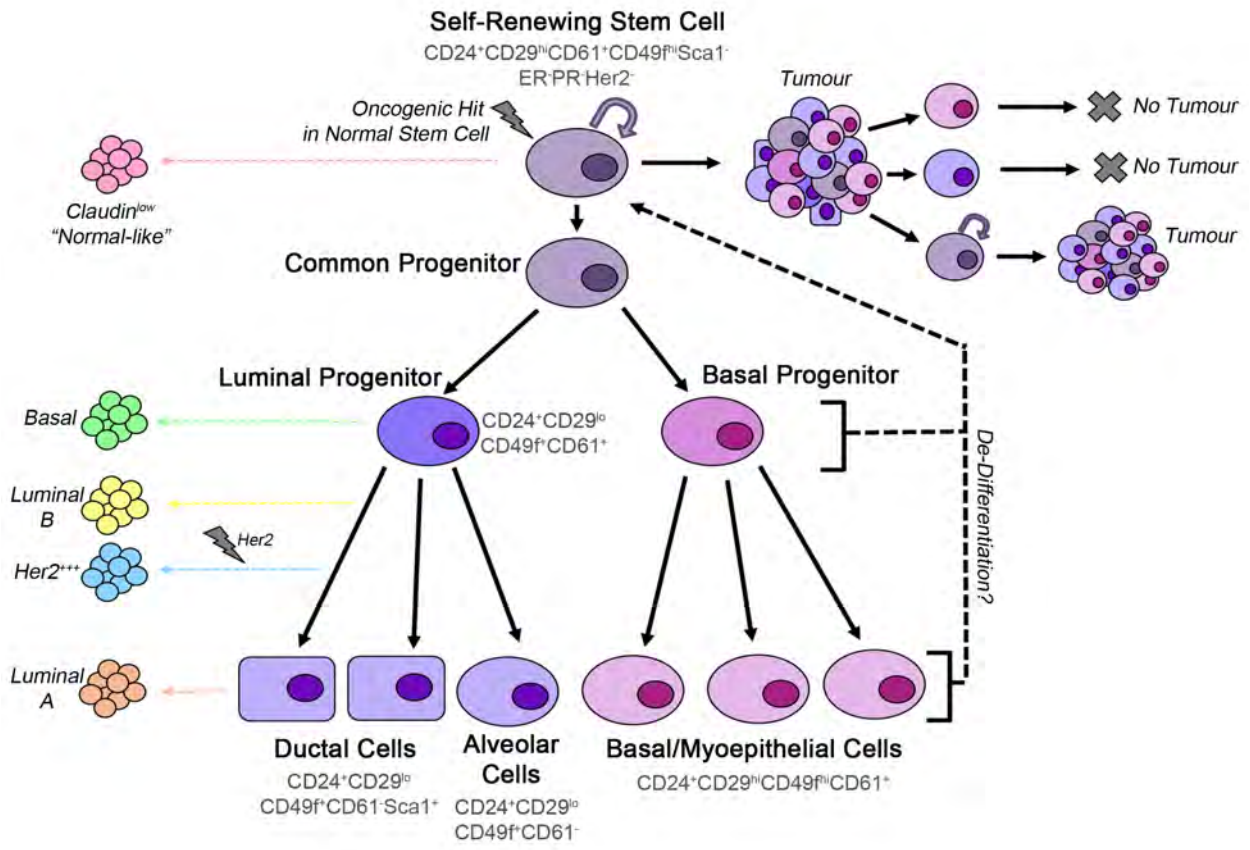

Figure 1.2: The Cancer Stem Cell Model, mammary cell hierarchy and proposed breast tumour cells-of-origin.

Mammary cells are believed to exist within a hierarchy, with a self-renewing stem cell giving rise to lineage progenitors and terminally differentiated cells of the luminal and basal lineages, which can be putatively identified based on the expression of various surface markers as indicated. Within this hierarchy, it is not clear from where cancer stem cells arise, however the cell with the most stem-like characteristics is capable of propagating secondary tumours upon serial transplantation (right). It is thought that different subtypes of breast cancer may have different cells-of-origin within this hierarchy (left), when those cells acquire stem-like characteristics.

Image adapted from [60] and [173].

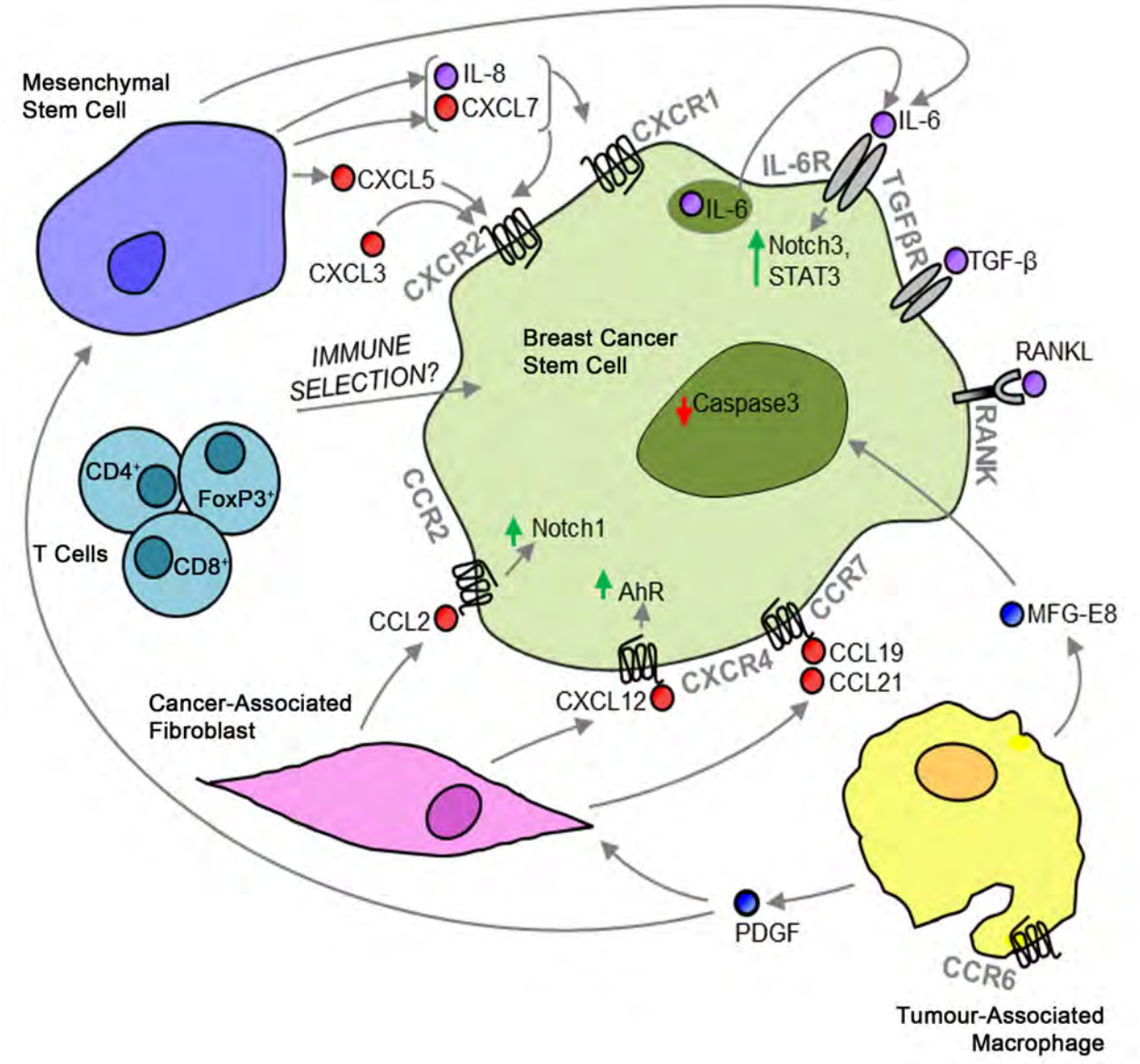

Figure 1.3: Maintenance of breast cancer stem cells by immune cells and immune modulators within the "immuniche".

Breast cancer stem cells express a variety of immune modulatory receptors on their surface. Chemokine and cytokine receptors are activated by immune modulators secreted by mesenchymal stem cells and cancer-associated fibroblasts, promoting properties of stemness. Tumour-associated macrophages promote breast cancer stem cell survival both indirectly and directly, and infiltrating T cell subsets in the immuniche provide a catalyst for immune selection.

Adapted from [115].

Table 1.1: Chemokine receptors in cancer.

Receptor	Cancer	Implication, role, mechanism	Reference
CC Chemokine Receptors			
CCR1	Colon	CCL9 and CCL15 recruit immature myeloid cells expressing CCR1 to promote metastasis to the liver	Kitamura 2010
	Liver	Promotes tumour growth together with ligand CCL3	Yang 2005, Wu 2007
CCR2	Breast	Ligand CCL2 facilitates metastasis, recruits inflammatory monocytes	Qian 2011, Yoshimura 2013
	Prostate	Expression positively correlates with tumour grade, expression of ligand CCL2 correlates with metastatic disease	Yu 2007, Yu 2009
CCR3	Kidney	Overexpression compared to normal, ligand CCL11 induces cellular proliferation	Johrer 2005
	Stomach	Expression correlates with poor survival	Sugasawa 2008
CCR4	Breast	Ligand CCL22 recruit inflammatory monocytes	Jafarzadeh 2015
	Colon	Ligand CCL17 stimulates cellular migration	Al-Haidari 2013
	Gastric	Overexpression in cancer compared to normal, promotes invasiveness	Yang 2015
	Lymphoma	Gain-of-function mutation in cancer tissues, promotes cell migration	Nakagawa 2014
CCR5	Breast	Overexpression compared to normal, activation by CCL5 promotes tumour growth via p53 signalling and metastasis	Manes 2003, Zhang 2009, Velasco-Velazquez 2012
	Colorectal	Promotes proliferation of cancer cells	Pervaiz, 2015
	Oral	Ligand CCL5 promotes cellular migration through increase of MMP-9	Chuang 2009
	Ovarian	Ligand CCL5 promotes EMT to generate stem-like cells	Long 2015
	Prostate	Promotes tumour growth and metastasis together with ligand CCL5	Vaday 2005, Sicoli 2014
	Stomach	Expression correlates with poor survival	Sugasawa 2008
CCR6	Breast	Associated with pleural metastasis	Andre 2006
	Colorectal	Receptor and ligand CCL20 both upregulated in liver metastases	Rubie 2006
	Liver	Expression correlates with metastatic disease, ligand CCL20 promotes cellular migration	Uchida 2006
	Lung	Overexpression in adrenal metastases compared to primary tumours	Raynaud 2010
	Pancreatic	Receptor and ligand CCL20 both expressed in cancer cells and promote invasion	Kimsey 2004
	Prostate	Expression correlates with metastatic disease	Ghadjar 2007
CCR7	Breast	Overexpression compared to normal, correlates with and promotes metastasis and lymphangiogenesis, prevents anoikis	Muller 2001, Cabioglu 2005, Kochetkova 2009,
		Ligand CCL21 expressed in secondary sites, CCL21 induces actin polymerisation and migration	Cunningham 2010, Huang 2015, Tutunea-Fatan 2015
	Cervical	Expression correlates with poor survival and metastasis	Kodama 2006
	Colorectal	Expression correlates with poor survival and metastasis	Gunther 2005
	Gastric	Expression correlates with poor survival and metastasis, induces EMT	Mashino 2002, Zhang 2015
	Head and Neck	Expression correlates with metastatic disease	Wang 2003
	Lung	Expression correlates with metastatic disease	Takanami 2003
	Lymphoma	Expression correlates with metastatic disease, promotes cellular migration and invasion	Yang 2011
	Melanoma	Overexpression compared to normal	Muller 2001
	Oesophageal	Expression correlates with poor survival and metastasis, CCL21 induces cell migration and pseudopodia formation	Ding 2003
	Prostate	Overexpression compared to normal, expression correlates with tumour growth and metastasis	Chen 2015
CCR8	-	*Has not been reported*	-
CCR9	Breast	Overexpression compared to normal, activation by ligand CCL25 promotes migration, invasion and cell survival	Johnson-Holiday 2011 (IJO and WJSO)
	Lung	Overexpression compared to normal, activation by ligand CCL25 promotes cell survival and tumourigenesis	Li 2015
	Melanoma	Expression correlates with metastatic disease, promotes cellular migration	Letsch 2004, Amersi 2007
	Ovarian	Overexpression compared to normal, activation by ligand CCL25 promotes migration and invasion	Johnson 2010
	Prostate	Activation by ligand CCL25 promotes migration and invasion, and inhibits apoptosis	Singh 2004, Sharma 2010
CCR10	Melanoma	Overexpression compared to normal, expression correlates with metastatic disease	Muller 2001, Simonetti 2006

Table 1.1 (Continued): Chemokine receptors in cancer.

Receptor	Cancer	Implication, role, mechanism	Reference
CXC Chemokine Receptors			
CXCR1	Breast	Maintains stem cell population	Ginestier 2010, Singh 2012
	Colon	Promotes metastasis	Varney 2011
	Lung	Expressed on cancer cells, stimulation by ligand IL-8 induces cell proliferation	Zhu 2004, Luppi 2007
	Melanoma	Expressed on cancer cells, promotes proliferation, survival and angiogenesis, stimulation by IL-8 induces cellular migration	Ramjeesingh 2003, Singh 2009
	Ovarian	Expressed on cancer cells, stimulation by ligand IL-8 induces actin polymerisation	Venkatakrishnan 1999
	Prostate	Overexpression of receptor and CXCL1 ligand compared to normal, associated with higher grade cancer, promotes cell	Murphy 2004, Maxwell 2007, Miyake 2014
CXCR2	Breast	Expression correlates with cancer risk and tumour progression, involved in chemoresistance, maintains stem cell populatio	Snoussi 2010, Acharyya 2012, Singh 2012
	Colon	Promotes metastasis	Varney 2011, Desurmont 2015
	Gastric	Ligand CXCL1 correlates with poor prognosis and tumour stage	Wei 2015
	Lung	Expressed on cancer cells, promotes tumour growth and angiogenesis, stimulation by ligand IL-8 induces cell proliferation	Keane 2004, Zhu 2004, Luppi 2007
	Melanoma	Expressed on cancer cells, promotes proliferation, survival and angiogenesis	Singh 2009
	Ovarian	Promotes tumour growth via angiogenesis and reduction in apoptosis, stimulation by IL-8 induces actin polymerisation	Venkatakrishnan 1999, Yang 2010
	Pancreatic	Promotes angiogenesis	Wente 2006
	Prostate	Overexpression compared to normal, promotes cell survival	Murphy 2004, Maxwell 2007
CXCR3	Bone	Promotes migration, proliferation, cellular survival and metastasis	Pradelli 2009, Guan 2015
	Breast	Expressed on cancer cells, associated with poor survival, promotes metastasis	Walser 2006 and Ma 2009
	Colon	Expression correlates with poor survival and metastasis, promotes metastasis	Kawada 2007
	Melanoma	Promotes metastasis, cell migration and tumourigenicity	Kawada 2004, Jenkins 2015
CXCR4	Bladder	Expression correlates with metastatic potential	Wang 2011
	Breast	Overexpression and increased activity compared to normal, correlates with and promotes metastasis, prevents anoikis, ligand CXCL12 expressed in common secondary sites, CXCL12 induces actin polymerisation and migration	Muller 2001, Liang 2004, Cabioglu 2006, Holland 2006, Kochetkova 2009
	Cervical	Expression correlates with poor survival and metastasis	Kodama 2006
	Colorectal	Expression correlates with poor survival	Speetjens 2009
	Gastric	Overexpression compared to normal, correlates with metastasis Ligand CXCL12 overexpressed in metastatic lymph nodes compared to normal, CXCL12 induces migration	Zhao 2011, Ying 2012
	Lung	Overexpression compared to normal, expression correlates with metastatic disease and promotes migration and invasion	Su 2005, Panneerselvam 2015, Zhou 2015
	Melanoma	Overexpression compared to normal, enhances metastatic potential	Muller 2001, Murakami 2002
	Oesophageal	Expression correlates with poor survival and metastasis	Kaifi 2005
	Ovarian	Expression correlates with poor survival and metastasis	Kajiama 2007
	Pancreatic	Expression correlates with poor survival, promotes cellular proliferation and migration	Marchesi 2004, Marechal 2010
	Prostate	Expression correlates with metastatic disease, induces more aggressive phenotype, enhances migration	Arya 2004, Darash-Yahana 2004
CXCR5	B-Cell Leukaemia	Overexpression compared to normal, ligand CXCL13 induces actin polymerisation and migration	Burkle 2007
	Breast	Overexpression of receptor and ligand CCL13 compared to normal	Panse 2008, Mitkin 2015
	Prostate	Overexpression compared to normal, ligand CCL13 stimulates migration and invasion	Singh 2009
CXCR6	Bladder	Overexpression compared to normal	Lee 2013
	Breast	Overexpression compared to normal, promotes invasion and metastatic ability	Xiao 2015
	Kidney	Overexpression of receptor and ligand CXCL16 compared to normal, CXCL16 possibly a marker for better survival	Gutwein 2009
	Liver	Overexpression compared to normal, promotes proliferation and invasion	Xu 2014
	Melanoma	Labels a rare aggressive cell population	Taghizadeh 2010
	Ovarian	Overexpression of receptor and ligand CXCL16 compared to normal, associated with metastasis and poor survival	Guo 2011
	Prostate	Overexpression compared to normal, promotes invasion and growth	Hu 2008, Wang 2008

Table 1.1 (Continued): Chemokine receptors in cancer.

Receptor	Cancer	Implication, role, mechanism	Reference
CX3C and XC Chemokine Receptors			
CX_3CR1 Fractalkine (CX_3CL1) receptor	Brain	Receptor and CX_3CL1 expression correlates with tumour grade and poor survival	Erreni 2010
	Breast	Expression associated with brain metastasis	Andre 2006
	Gastric	Overexpression compared to normal, expression associated with metastasis, proliferation and cell survival	Wei 2015
	Pancreatic	Expression correlates with earlier tumour recurrence	Marchesi 2008
	Pancreatic	Promotes bone metastasis and cellular survival	Shulby 2004, Jamieson 2008
XCR1 Lymphotactin (XCL1) receptor	Pancreatic	XCL1 has anti-tumour effects and correlates with ER expression	Emtage 2004, Keen 2004
	Pancreatic	Stimulation by XCL1 causes tumour regression and immune infiltration of neutrophils expressing XCR1	Cairns 2001
	Pancreatic	Receptor and ligand expressed on cancer cells, involved in migration, invasion, and cell proliferation	Khurram 2010
	Pancreatic	Overexpression compared to normal, stimulation leads to proliferation and migration of cancer cells	Kim 2012
Atypical Chemokine Receptors			
DARC/ACKR1	Breast	Suppresses tumour growth and metastasis, low expression correlates with increased metastatic disease and poor survival	Ou 2006, Wang 2006
	Lung	Promotes necrosis and suppresses metastasis	Addison 2004
	Prostate	Suppresses tumour growth and metastasis	Bandyopadhyay 2006, Shen 2006
D6/ACKR2	Breast	Inhibits proliferation, invasion, tumour growth and metastasis	Wu 2008
	Colon	Prevents inflammation-induced colon cancer	Vetrano 2009
	Skin	Suppresses tumour growth	Nibbs 2007
CXCR7/ACKR3	Breast	Overexpression compared to normal, scavenging of CXCL12 promotes proliferation of CXCR4+ tumour cells	Miao 2007, Luker 2012
	Glioma	Expression correlates with reduced survival, promotes proliferation, invasion and migration	Liu 2015
NB: Ability to signal is under contention	Lung	Overexpression compared to normal, upregulated after chemotherapy, expression associated with metastatic relapse	Miao 2007, Goldmann 2008, Iwakiri 2009
	Prostate	Overexpression compared to normal, may promote invasion and angiogenesis	Wang 2007
CCR11/ACKR4	Breast	Regulates metastasis via EMT	Harata-Lee 2014
PITPNM3/ACKR6	Breast	Stimulation by ligand CCL18 from tumour-associated macrophages promotes metastasis	Chen 2011
	Pancreatic	Expression of ligand CCL18 correlates with metastasis, grade, and poor survival	Meng 2015

Table 1.2: Chemokine receptors in stem cell biology.

Receptor	Implicated in…	Role, Mechanism	Reference
CC Chemokine Receptors			
CCR1	Erythroid Progenitors	Expressed on surface, involved in proliferation	Su 1997
	Mesenchymal SCs	Expressed on surface, involved in migration and protection from apoptosis	Sordi 2005, Von Luttichau 2005, Honczarenko 2009, Huang 2010
CCR2	Breast Cancer SCs	Stromal fibroblasts secrete ligand CCL2 which stimulates self-renewal of CSCs	Tsuyada 2012
	Haematopoietic SCs	Expressed on surface, involved in migration to inflammatory sites	Si 2010
	Mesenchymal SCs	Expressed on surface, involved in migration to gliomas	Ringe 2007, Xu 2010
	Neural SCs	Expressed on surface of cells and spheres, involved in migration	Widera 2004, Andres 2011
CCR3	Haematopoietic SCs	mRNA transcripts detected	Wright 2002
	Mesenchymal SCs	Expressed on surface	Ponte 2007
	Neural SCs	Activation inhibits cell proliferation and sphere formation	Krathwohl 2004
CCR4	Embryonic SCs	Expressed in small populations of embryonic and amnion-derived SCs	Miki 2006
	Mesenchymal SCs	Expressed on surface, involved in migration to skin	Von Luttichau 2005, Sasaki 2008
CCR5	Breast Cancer	Stimulation of breast cancer cells by ligand CCL5 increases CSC proportions and invasiveness, may promote cell expansion	Zhang 2009, Jiao 2010
		CCL5 from adipose-derived tissue SCs increases breast cancer cell migration in co-culture	Pinilla 2005
		CCL5 from mesenchymal SCs in breast tumours enhances cancer cell motility, invasiveness and metastasis	Karnoub 2007
	Haematopoietic SCs	Expressed on surface, involved in HIV resistance, transplantation may help treat HIV-infected individuals	Hutter 2011, Allers 2011
	Mesenchymal SCs in Skin	Expressed on surface, involved in migration and wound repair	Kroeze 2009
	Ovarian SCs	CCL5 induces EMT to generate stem-like cells	Long 2015
CCR6	Mesenchymal SCs	Expressed on surface, involved in migration	Ruster 2006
CCR7	Mesenchymal SCs	Expressed on surface, involved in migration and wound repair	Sordi 2005, Von Luttichau 2005, Honczarenko 2009, Sasaki 2008
CCR8	Mesenchymal SCs	Expressed on surface, involved in migration	Ringe 2007
CCR9	Mesenchymal SCs	Expressed on surface, involved in migration	Honczarenko 2009, Chen 2010
CCR10	Mesenchymal SCs	Expressed on surface, involved in migration to skin, salivary glands and small intestine	Von Luttichau 2005
CXC Chemokine Receptors			
CXCR1	Breast SCs	Maintains cancer SC population, stimulation by ligand IL-8 increases normal and cancer sphere formation,	Charafe-Jauffret 2009, Ginestier 2010, Singh 2012
		IL-8 increases cancer SC proportion and tumour propagating ability	
	Lung Cancer SCs	Expressed on tumour sphere cells	Levina 2008
	Mesenchymal SCs	Expressed on surface, involved in migration	Ringe 2007
CXCR2	Breast Cancer SCs	CXCR2 expression maintains cancer SC population, CXCL7/CXCL5 ligand stimulation increases SC proportions,	Liu 2007, Liu 2011, Marotta 2011, Singh 2012
		CXCR2 and ligand CXCL3 are involved in cell viability of breast cancer stem-like cell lines, CXCL7 is	
		secreted from cancer cells upon co-culture with mesenchymal SCs	
	Haematopoietic SCs	Involved in migration	Pelus 2006
	Lung Cancer SCs	Expressed on tumour sphere cells	Levina 2008
	Mesenchymal SCs	Expressed on surface, involved in migration	Ringe 2007
CXCR3	Breast Cancer	Activation of receptor on mesenchymal SCs promotes breast cancer cell migration and motility	Shin 2010
		B isoform promotes mammosphere formation	Li 2014
	Mesenchymal SCs	Receptor and ligands all expressed by cells, involved in migration	Ringe 2007, Ren 2009
CXCR4	Breast SCs	Expressed in cancer stem-like lines and correlates with invasiveness,	Sheridan 2006, Dubrovska 2012, Huang 2012
		maintains CSC population via transactivation AhR, expressed on sphere cells	
	Embryonic Stem-like Cells	Expressed and functional in a small population of adult bone marrow cells with embryonic stem cell characteristics	Kucia 2006
	Haematopoietic SCs	Expressed on cell surface, involved in migration and development, maintains SC pool	Petit 2002, Tavor 2004, Sugiyama 2006
	Liver Progenitors SCs	Expressed on surface, involved in migration	Kucia 2004
	Lung Cancer SCs	Expressed on tumour sphere cells	Levina 2008
	Mesenchymal SCs	Receptor and ligand CXCL12 expressed on/by cells and important for migration and cell survival	Ji 2004, Wynn 2004, Kortesidis 2005, Sordi 2005, Honczarenko 2009
	Muscle Progenitor Cells	Expressed on surface of satellite cells, involved in migration	Marmis 2003, Kucia 2004
	Myeloid Progenitors	Involved in cell survival and inhibition of apoptosis	Broxmeyer 2003
	Neural SCs	Expressed on surface, required for migration, proliferation and invasion	Ehtesham 2004, Imitola 2004, Kucia 2004, Xu 2009
	Renal Progenitors	Expressed on surface, required for migration and successful engraftment of renal stem/progenitor cells	Mazzinghi 2008
CXCR5	Mesenchymal SCs	Expressed on surface, involved in homing to secondary lymphoid organs	Von Luttichau 2005, Honczarenko 2009
CXCR6	Melanoma	Identifies a small population with aggressive and self-renewing phenotype	Taghizadeh, 2010
	Mesenchymal SCs	Expressed on surface, involved in migration	Sordi 2005, Brooke 2008, Honczarenko 2009

Table 1.2 (Continued): Chemokine receptors in stem cell biology.

Receptor	Implicated in…	Role, Mechanism	Reference
CX3C and XC Chemokine Receptors			
CX3CR1	Mesenchymal SCs	Expressed on surface, involved in migration, expression in MSCs has been proposed to have anti-tumour effects	Ji 2004, Sordi 2005, Lee 2006, Xin 2007
Fractalkine receptor	Neural SCs	Expressed on neurospheres in glioblastoma, stimulation with ligand CX3CL1 promotes NSC survival	Krathwohl 2004, Errede 2010
XCR1	Haematopoietic SCs	mRNA transcripts detected	Wright 2002
Lymphotactin receptor	Mesenchymal SCs	Expressed on surface	Hemeda 2010
Atypical Chemokine Receptors			
DARC/ACKR1	-	*Has not been reported*	-
D6/ACKR2	-	*Has not been reported*	-
CXCR7/ACKR3 NB: Ability to signal is under contention	Breast Cancer SCs	Expression in breast cancer cell lines with high stem-like cell content	Asiedu 2011
	Glioma Stem-like Cells	Expression induced on stem-like cells upon differentiation, stimulation results in activity of receptor and prevention of apoptosis	Hattermann 2010, Liu 2015
	Mesenchymal SCs	Required for cell viability and secretion of paracrine factors during kidney injury	Liu 2012
	Neural Progenitors	Involved in cell survival	Bakondi 2011
	Renal Progenitors	Expressed on surface, required for migration, adhesion, cell survival, and successful engraftment of renal stem/progenitor cells	Mazzinghi 2008
CCR11/ACKR4	Mesenchymal SCs	mRNA transcripts detected	Brooke 2008
PITPNM3/ACKR6	-	*Has not been reported*	-

CHAPTER 2: Materials and Methods

2.1. Models and materials

2.1.1. Mice

Mice were maintained in pathogen-free conditions in the University of Adelaide's Laboratory Animal Services facility and the University of Adelaide institutional animal ethics committee approved all experimentation.

MMTV-PyMT mice on the FVB strain background were backcrossed for 15 generations to the C57Bl/6 background, and C57Bl/6 background was confirmed by microsatellite analysis. PyMT-carrying males were then crossed with *Ccr7*$^{-/-}$ and *Ccr6*$^{-/-}$ female mice on the C57Bl/6 strain background, and the offspring were interbred to produce MMTV-PyMT *Ccr6/7*^{WT} and MMTV-PyMT *Ccr6/7*$^{-/-}$ knockout mice. See ***Figure 2.1*** for a schematic of the breeding strategy.

For the assessment of chemokine receptor expression, stimulation experiments, and antagonist function, both C57Bl/6 and FVB backgrounds were tested to eliminate any strain bias. For experiments involving knockout mice, only C57Bl/6 mice were tested.

2.1.2. Human mammary tissue

Human breast tumour samples were obtained from the Royal Adelaide Hospital (Adelaide, South Australia) and normal breast tissue samples were obtained from the Queen Elizabeth Hospital (Woodville, South Australia). All patients gave written, informed consent prior to surgery.

2.1.3. Basic solutions

- MQ H_2O: Milli-Q water was obtained from the Central Services Unit (CSU) in the School of Biological Sciences.
- PBS: 20x phosphate buffered saline (from CSU) was diluted in MQ H_2O to 1x.
- HBSS: 10x Hank's balanced salt solution (Gibco) was diluted in MQ H_2O to 1x.
- PBS/BSA: Bovine serum albumin (Sigma-Aldrich) was added at the desired concentration to 1x PBS then dissolved at 4°C before filter-sterilisation.

2.1.4. Carmine alum

Carmine alum was either obtained commercially (Stem Cell Technologies) or made by boiling 1g carmine and 2.5g aluminium potassium sulphate in 500ml distilled H_2O for 20 minutes, followed by filtration and addition of thymol. Carmine was stored at 4°C and used undiluted.

2.1.5. Formaldehyde

Formaldehyde at 37% w/v (Sigma-Aldrich) was diluted in PBS to either 3.7% or 1% for fixation of cells. Methanol-free formaldehyde at 16% w/v (Thermo Fisher Scientific) was diluted to 4% in PBS for fixation of tissues before paraffin embedding.

2.1.6. Mouse mammary tissue digestion medium

Digestion medium for mouse tissue consisted of Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with collagenase, hyaluronidase, 2% foetal calf serum (FCS), and 1% penicillin-streptomycin.

Enzymes from Stem Cell Technologies: The supplied mix of collagenase (3000U/ml) and hyaluronidase (1000U/ml) in DMEM was used at 10% of the stock concentration in digestion medium.

Enzymes from Worthington: Collagenase type III was reconstituted in in HBSS/Ca^{2+}/Mg^{2+} and used at a final concentration of 1mg/ml. Hyaluronidase was reconstituted in MQ H_2O and used at a final concentration of 100U/ml.

2.1.7. Human mammary tissue digestion medium

Digestion medium for human tissue consisted of DMEM supplemented with 20mM HEPES, collagenase and hyaluronidase (see above), 12U/ml deoxyribonuclease (DNase)I (Merck) and 1% penicillin-streptomycin.

2.1.8. Mouse red cell removal buffer (MRCRB)

MRCRB consisted of 90% 155mM NH_4Cl and 10% 170mM Tris-HCl. The pH was adjusted to 7.2 and the solution was filter-sterilised before use.

2.1.9. Complete sphere medium

Mammosphere medium consisted of 1:1 volumes of DMEM and Ham's F12 nutrient mix (Gibco) supplemented with 1xB27 (Invitrogen), 10ng/ml epidermal growth factor (EGF, R&D Systems), 20ng/ml basic fibroblast growth factor (bFGF, R&D Systems), 4ng/ml heparin (Sigma-Aldrich), 1% penicillin-streptomycin and 0.5% fungazone.

2.2. *Ex vivo* methods

2.2.1. Extraction of mammary cells

Mouse mammary glands/tumours were dissected, with removal of the lymph node if possible. Tissue was manually dissociated and then digested in mouse mammary tissue digestion medium for 3-4 hours at 37°C with gentle tilting on a HulaMixer (Invitrogen). Resultant organoids were digested for a further 15 minutes with dispase (5mg/ml from Stem Cell Technologies or 6U/ml from Gibco) and DNase I (12U/ml) at 37°C before washing in HBSS with 2% FCS and lysis of red blood cells for 2 minutes at room temperature in MRCRB. Cells were then filtered through a 70μm nylon mesh to then obtain a single cell suspension. All centrifuge washes were performed at 350x*g* for 3 minutes.

Surgical human specimens were minced and digested in human mammary tissue digestion medium. Organoids were then extensively washed with DMEM and red blood cells were lysed in MRCRB. Single cell suspensions were obtained by digesting organoids with 0.25% trypsin for 10 minutes at room temperature, with subsequent filtration through a 70μm nylon mesh.

2.2.2. Haematopoietic lineage depletion by magnetic separation

When required, freshly isolated mammary cells were pelleted and resuspended in 1ml depletion buffer (PBS/0.1% BSA and 2mM EDTA at pH 7.4) containing a cocktail of biotinylated anti-mouse antibodies against haematopoietic lineage markers CD3, TER-119, CD11b, Ly-6C/G and CD45R (panel from BioLegend). Cells were incubated for 10 minutes at 4°C with gentle tilting then resuspended in 1ml buffer and transferred to a 1.5ml centrifuge tube, together with 50µl washed biotin binder Dynabeads (Invitrogen). Samples were incubated for 30 minutes at 4°C with gentle tilting, then a further 500µl depletion buffer was added to limit trapping of unbound cells. Tubes were placed in a DynaMag-Spin magnet (Invitrogen) for 3 minutes and the lineage-negative supernatant transferred to a fresh tube for subsequent experiments.

2.2.3. Flow cytometry and fluorescence-activated cell sorting (FACS)

Experiments were performed either in FACS tubes (centrifugation at 350x*g* for 3 minutes) or 96-well round-bottomed trays (centrifugation at 400x*g* for 1 minute). Cells were pelleted and blocked for non-specific antibody binding by incubating for 15 minutes at room temperature in mouse gamma (γ) globulin in PBS/0.5% BSA. Cells were then immunostained for 30-45 minutes on ice in PBS/0.5%BSA. If intracellular staining was required, cells were permeabilised following surface antigen staining using the FoxP3 Staining Kit (eBioscience). A complete list of antibodies, manufacturer details and concentrations used is in ***Table 2***.

Samples containing biotinylated antibodies were resuspended with conjugated streptavidin in PBS/0.5% BSA for 30 minutes on ice. Fluorescence-minus-one (FMO) samples or conjugated isotypes were used as negative controls. For flow cytometry, cells were fixed in 1% formaldehyde and acquisition carried out using FACS Canto or LSR II equipment (BD Biosciences). For sorting, live cells in PBS/0.5% BSA were sorted using a FACS Aria (BD Biosciences) into DMEM, with the assistance of Mr Cameron Bastow and Ms Carly Gregor of the University of Adelaide.

2.2.4. Mammosphere assay

Cells were added at a concentration of $4x10^4$ cells/ml in complete sphere medium to polyhema-coated ultra-low attachment trays (Corning Inc.). Where indicated, CCL21, CCL19 and $CCL19_{(8-83)}$ were added at concentrations of 10ng/ml, 200ng/ml and 100ng/ml respectively. CCL20 (a gift from the late Professor Ian Clark-Lewis) was added at various concentrations as specified in Chapter 4. Spheres were cultured at 37°C and media replenished every second day. After 7-10 days, mammospheres were counted and passaged. Samples were collected, centrifuged at 200x*g* for 5 minutes and supernatant aspirated. Warm trypsin/EDTA in PBS (from CSU) was added and samples were triturated through a 19G needle to break up colonies. After 2 minutes in trypsin, tubes were topped up with PBS/2% FCS to neutralise trypsin, and centrifuged for 3 minutes at 350x*g*. Cells were filtered through 70µm filters and washed again in PBS to remove FCS, before reseeding into clean ultra-low attachment plates for secondary culture in complete sphere medium.

2.2.5. Histology

For formalin-fixed paraffin-embedded (FFPE) sections, tissue was extracted and fixed overnight in 4% formaldehyde at room temperature. Tissue was then put into cassettes and stored in 70% ethanol at 4°C, until embedding in paraffin and sectioning at 5µm (Adelaide Histology). To stain, slides were first deparaffinised by immersing in D-Limonene for 10 minutes, then rehydrated through graduated ethanol.

For frozen sections, lungs of mice were perfused and dissected, then cryoembedded in OCT and serially sectioned at 9µm. Sections were fixed in 60/40% acetone/methanol before staining.

For haematoxylin and eosin (H&E) staining, sections were rinsed through 3 changes of 1x PBS for 1 minute each before immersing in Gill's haematoxylin for 1-5 minutes. Slides were rinsed in distilled H_2O then differentiated for 3 seconds in 1% acid-alcohol (20% distilled H_2O, 10% concentrated hydrochloric acid, 70% ethanol). Slides were immersed in Scott's tapwater

substitute (2g sodium bicarbonate, 20g magnesium sulphate per 1L distilled H_2O) for 2 minutes before rinsing again in distilled H_2O. Slides were immersed in eosin for 0.5-2 minutes and rinsed in distilled H_2O briefly before completely dehydrating through graduated ethanol followed by D-Limonene. Slides were mounted with Depex (Thermo Fisher Scientific) and scanned using the NanoZoomer Digital Pathology (NDP) System (Hamamatsu Photonics). Lung sections were manually quantitated using the NDP Virtual Slide Viewer software for number of metastases and area at the largest point.

2.2.6. Immunofluorescent staining

Antigen retrieval of FFPE mouse mammary sections was performed by boiling slides in 0.1M sodium citrate buffer (pH 6.0). Slides were immuno-stained with rabbit anti-CCR7 (Epitomics) overnight at 4°C, and primary antibody was detected with Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen) for 30 minutes. All staining was performed in humid chambers. Slides were counterstained with DAPI, mounted, and analysed using the Leica TCS SP5 Confocal Microscope System.

For these experiments, Sarah Boyle extracted and fixed the mammary tissue, and analysed the results. Adelaide Histology embedded and sectioned the tissue as above. Ms Valentina Poltavets (formally) of the University of Adelaide performed the staining.

2.2.7. Immunohistochemical staining

For α-smooth muscle actin (α-SMA) staining following antigen retrieval (as for IF staining), slides were immersed in 3% hydrogen peroxide in PBS for 20 minutes with gentle agitation to inhibit endogenous peroxidase activity and blocked for 30 minutes in 10% normal rabbit serum in PBS to prevent non-specific antibody binding. For Ki67 staining, slides were blocked before antigen retrieval.

All slides were then incubated overnight at 4°C with mouse anti-α-SMA (Dako) or mouse anti-Ki67 (Vector Labs). Specific antibody binding was detected using the EnVision Dual Link System (Vector Labs) followed by incubation with diaminobenzidine (DAB) substrate (Dako). All staining was performed in humid chambers. Sections were counterstained with haematoxylin, dehydrated and mounted. Slides were scanned for analysis as specified above in Histology.

For these experiments, Sarah Boyle extracted and fixed the mammary tissue, and analysed the results. Adelaide Histology embedded and sectioned the tissue as above. Ms Natasha Pyne and Doctor Michael Samuel of the Centre for Cancer Biology, University of South Australia, performed the staining.

2.2.8. Mammary gland whole mounting, image-stitching and quantification

Intact mammary glands were extracted and spread onto glass slides before fixing in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 3-4 hours at room temperature. Slides were then transferred to 70% ethanol for at least 10 minutes before gradually changing to distilled H_2O. Whole mounts were then immersed in carmine alum overnight, then fully dehydrated through graduated ethanol followed by D-Limonene, and mounted with Permount (Thermo Fisher Scientific). Mounts were photographed using a Nikon SMZ1000 dissecting microscope connected to an Olympus DP70 camera, and the images stitched together using the Mosaic plug-in in Image J. Quantification of epithelial growth and area of hyperplasia was performed using Image J.

2.2.9. Enzyme-linked immunosorbent assay (ELISA)

Wells of a high-binding 96-well tray were coated with CCL19, CCL21, and CCL20 capture antibody (all from R&D Systems) overnight at 4°C. Non-specific binding was then blocked for 1 hour at 37°C with PBS/3% BSA. Mammary fat pad samples were weighed and then homogenised in ceramic bead tubes with 500µl ELISA buffer (10% glycerol, 1x protease inhibitor in PBS), and samples and chemokine standards in ELISA buffer were added at

100µl/well. Samples were incubated at 37°C for 90 minutes, then incubated with biotinylated detection antibody (all from R&D Systems) in PBS/1% BSA for 1 hour at 37°C. Streptavidin conjugated to horseradish peroxidase (HRP) in PBS/1% BSA was then added to samples and incubated at room temperature for 30 minutes, followed by development for up to 30 minutes in the dark with 75µl/well tetramethylbenzidine (TMB) substrate. The reaction was stopped by adding 50µl/well 1M orthophosphoric acid, and absorbance read at 490nm on a Biotrak plate reader. Between each incubation wells were washed with PBS/0.05% tween.

2.2.10. Calcium signalling analysis

The intracellular calcium mobilisation assay was performed as described [130] on cells isolated from tumours dissected from C57Bl/6 MMTV-PyMT mice at 20 weeks of age. Ligands added were CCL21 (100ng/ml) and lysophosphatidic acid (LPA) (50ng/ml).

For these experiments, Sarah Boyle extracted the mammary cells and analysed the results. Doctor Marina Kochetkova of the University of Adelaide performed the assay.

2.2.11. XTT proliferation assay

Isolated mouse mammary cells were plated in adherent culture (1:1 mixture of DMEM and Ham's F12 nutrient mix, 10% FCS, 20ng/ml EGF, 5µg/ml insulin, 0.5µg/ml hydrocortisone, 1% penicillin-streptomycin, and 0.25µg/ml fungazone) in 96-well plates and the following day were starved. The cell proliferation assay was carried out 24 hours later using the XTT Cell Proliferation Kit (ATCC) according to manufacturer's instructions, using 100ng/ml CCL19, CCL21 or CCL20. FCS (0.5%) and EGF (20ng/ml) were used as positive controls.

For these experiments, Sarah Boyle extracted the mammary cells and analysed the results. Doctor Marina Kochetkova of the University of Adelaide performed the assay.

2.3. *In vivo* methods

2.3.1. Tumourigenesis studies

Mice were monitored for onset of mammary tumours by manual palpation. For long-term tumourigenesis, mice were allowed to develop tumours for up to 24 weeks, unless the cumulative burden reached $2mm^2$ before this point. Upon sacrifice, tumours were counted, extracted, photographed, and weighed.

2.3.2. Tissue transplants

Procedures were performed on mice under isofluorane anaesthetic. Mammary gland fragments of $1mm^3$ size from donor MMTV-PyMT mice were transplanted into the fourth inguinal mammary fat pads of congenic non-PyMT recipient mice. After surgery, wounds were closed using surgical staples and either Temgesic or Rimadyl analgesic was administered subcutaneously. Mice were monitored for adverse reactions to surgery and subsequent tumour growth. Glands were either whole-mounted after a number of weeks or allowed to develop tumours. See ***Figure 2.2*** for a schematic diagram of the procedure.

2.3.3. Limiting dilution assay

Mammosphere colonies derived from 8 week-old pre-neoplastic MMTV-PyMT mice were dissociated as above and filtration was performed using endotoxin-free PBS (SA Pathology). Cells were injected in 20% Matrigel (BD Biosciences) in DMEM using a Hamilton syringe fitted to a 26G needle into the fourth inguinal mammary glands of anaesthetised recipient mice at decreasing concentrations to determine the limiting dilution as previously described [71]. Mice were sacrificed after 6 weeks and glands whole-mounted. See ***Figure 2.3*** for a diagram of the procedure.

2.3.4. Macrophage reconstitution assay

Mammary tumour cell suspensions were prepared from MMTV-PyMT *Ccr6*^{WT} at 15 weeks old and injected into the fourth inguinal mammary fat pads of anaesthetised 5 week-old *Ccr6*^{WT} and *Ccr6*$^{-/-}$ recipients in 20% Matrigel in DMEM, at 100,000 cells/gland.

Two days later, tumour-associated macrophages were sorted from MMTV-PyMT *Ccr6*^{WT} excised and dissociated mammary tumours based on CD45^{+}F4/80^{+} expression. 50,000 TAMs per gland were injected in DMEM orthotopically into the inguinal glands of *Ccr6*$^{-/-}$ tumour cell recipients. Control groups of *Ccr6*$^{-/-}$ and *Ccr6*^{WT} tumour cell recipients were sham-injected with vehicle only. Tumour development was monitored for 6 weeks, then mice were sacrificed and tumours extracted for analysis. See ***Figure 2.4*** for a diagram of the procedure.

2.3.5. Antagonist studies

CCL19$_{(8\text{-}83)}$ truncated ligand was used at a concentration of 1µg/50µl *in vivo.* The antagonist was diluted in saline and mice were injected under anaesthetic into inguinal mammary fat pads using an insulin syringe. Control mice were sham-injected with vehicle alone as previously reported [174, 175]. Glands were then allowed to develop tumours over a number of weeks. See ***Figure 2.5*** for a diagram of the procedure.

2.4. Statistics and graphing

All quantitation results were graphed using GraphPad Prism. Unless otherwise indicated, data is shown as mean ± standard error of the mean (SEM). Tumour-free survival curves for long-term tumourigenesis were graphed using the Kaplan-Meier method and distributions were compared by the log-rank statistic (Mantel-Cox test). Tumour-free survival for the reconstitution assay was compared using 2-way analysis-of-variance (ANOVA) with Tukey's multiple comparison test. Flow cytometry experiments were acquired using FACS Diva software (BD Biosciences) and compensated, gated and analysed using FlowJo (Tree Star Inc.). Data was analysed on Prism for statistically significant differences using the student's t test, ANOVA, or Chi-square (χ^2)

analysis. Results from the limiting dilution assay were analysed for tumour-initiating cell (TIC) frequency and statistically significant differences using L-Calc software (Stem Cell Technologies). P-values were used to denote statistical significance. Levels of significance were $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

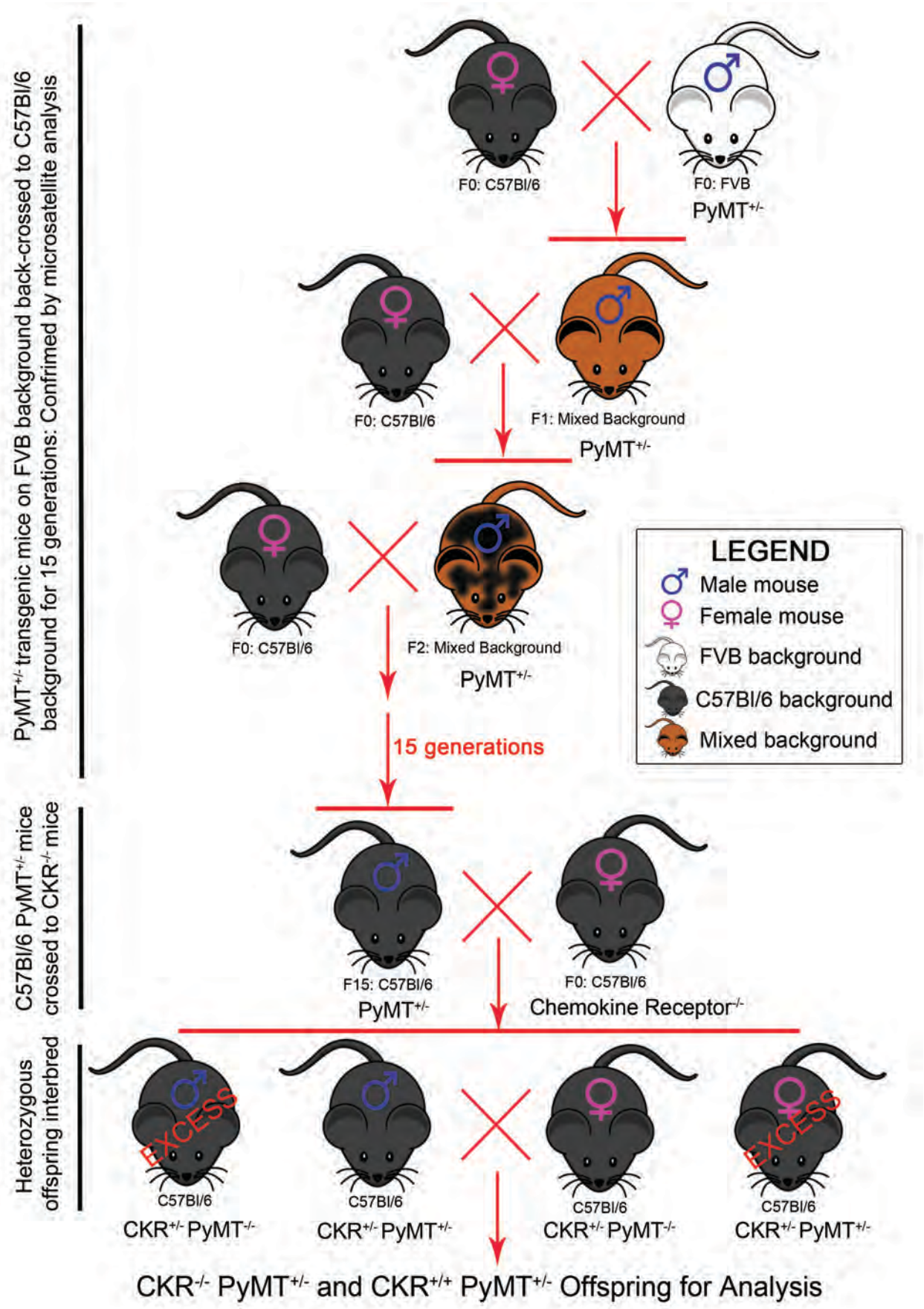

Figure 2.1: Murine breeding strategy for this study.

CKR = Chemokine receptor.

Table 2: Antibodies used in this study.

Antibody	Conjugate	Concentration	Source	Application
Chemokines and Chemokine Receptors				
Rat anti-mu CCL19 (capture)	-	2µg/ml	R&D Systems	ELISA
Goat anti-mu CCL19 (detection)	Biotin	0.3µg/ml	R&D Systems	ELISA
Goat anti-mu CCL20 (capture)	-	2µg/ml	R&D Systems	ELISA
Goat anti-mu CCL20 (detection)	Biotin	0.05µg/ml	R&D Systems	ELISA
Goat anti-mu CCL21 (capture)	-	0.5µg/ml	R&D Systems	ELISA
Goat anti-mu CCL21 (detection)	Biotin	0.75µg/ml	R&D Systems	ELISA
Rabbit anti-hu/mu CCR7	-	1:50	Abcam	Immunohistochemistry
Rat anti-mu CCR7	Alexa Fluor® 647	4µg/ml	BD Biosciences	Flow Cytometry
Arm. hamster anti-mu CCR6	Alexa Fluor® 647	2.5µg/ml	BioLegend	Flow Cytometry
Rat anti-mu CCR6	PE	1:10	R&D Systems	Flow Cytometry
Stem Cell Markers				
Rat anti-mu CD24	PE	2µg/ml	BD Biosciences	Flow Cytometry
Mouse anti-hu CD24	PE	1:20	BD Biosciences	Flow Cytometry
Arm. hamster anti-mu CD29	FITC	2.5µg/ml	BioLegend	Flow Cytometry
Mouse anti-hu CD44	FITC	1:20	BD Biosciences	Flow Cytometry
Rat anti-hu/mu CD49f	FITC or PE/Cy5	1:20	BD Biosciences	Flow Cytometry
Hamster anti-mu DLL1	PE	4µg/ml	BioLegend	Flow Cytometry
Mouse anti-hu DLL1	PE	3:50	BioLegend	Flow Cytometry
Rat anti-mu DNER	Biotin	16µg/ml	R&D Systems	Flow Cytometry
Goat anti-hu DNER	Biotin	1µg/ml	R&D Systems	Flow Cytometry
Leukocyte Markers				
Rat anti-mu B220/CD45R	BV421	0.67µg/ml	BD Biosciences	Flow Cytometry
Rat anti-mu CD11b	PE/Cy7	0.67µg/ml	BD Biosciences	Flow Cytometry
Arm. hamster anti-mu CD11c	PerCP/Cy5.5	0.83µg/ml	BioLegend	Flow Cytometry
Rat anti-mu CD124/IL4R	PE	0.83µg/ml	BD Biosciences	Flow Cytometry
Rat anti-mu CD206/mannose receptor	PerCP/Cy5.5	2µg/ml	BioLegend	Flow Cytometry
Arm. hamster anti-mu CD3e	PE/Cy7	0.83µg/ml	eBioscience	Flow Cytometry
Rat anti-mu CD4	FITC	1.67µg/ml	BD Biosciences	Flow Cytometry
Rat anti-mu CD45	APC	0.67µg/ml	BD Biosciences	Flow Cytometry, FACS
Mouse anti-mu CD45.2	FITC or Biotin	1.67µg/ml	BD Biosciences	Flow Cytometry
Rat anti-mu CD8a	BV510	0.67µg/ml	BD Biosciences	Flow Cytometry
Rat anti-mu F4/80	Biotin	0.42µg/ml	Life Technologies	Flow Cytometry
Rat anti-mu F4/80	FITC	2.08µg/ml	eBioscience	Flow Cytometry, FACS
Rat anti-mu FoxP3	PerCP/Cy5.5	1.11µg/ml	eBioscience	Flow Cytometry
Rat anti-mu Gr1/Ly6C-G	PE	0.83µg/ml	BD Biosciences	Flow Cytometry
Mouse anti-mu MHCII/I-A[6]	APC	0.83µg/ml	BD Biosciences	Flow Cytometry
Other				
Mouse anti-hu/mu α-smooth muscle actin	-	1:500	Dako	Immunohistochemistry
Mouse anti-hu/mu Ki67	-	1:20	Vector Laboratories	Immunohistochemistry
Secondary Detection Agents				
Streptavidin	PerCP/Cy5.5	0.2µg/ml	BD Biosciences	Flow Cytometry
Streptavidin	BV510	0.1µg/ml	BD Biosciences	Flow Cytometry
Streptavidin	Alexa Fluor® 488	1.8µg/ml	Jackson Immunoresearch	Flow Cytometry
Streptavidin	Alexa Fluor® 647	1.8µg/ml	Jackson Immunoresearch	Flow Cytometry
Streptavidin	HRP	0.1µg/ml	Rockland	ELISA
Goat anti-rabbit IgG	Alexa Fluor® 488	1µg/ml	Invitrogen	Immunohistochemistry

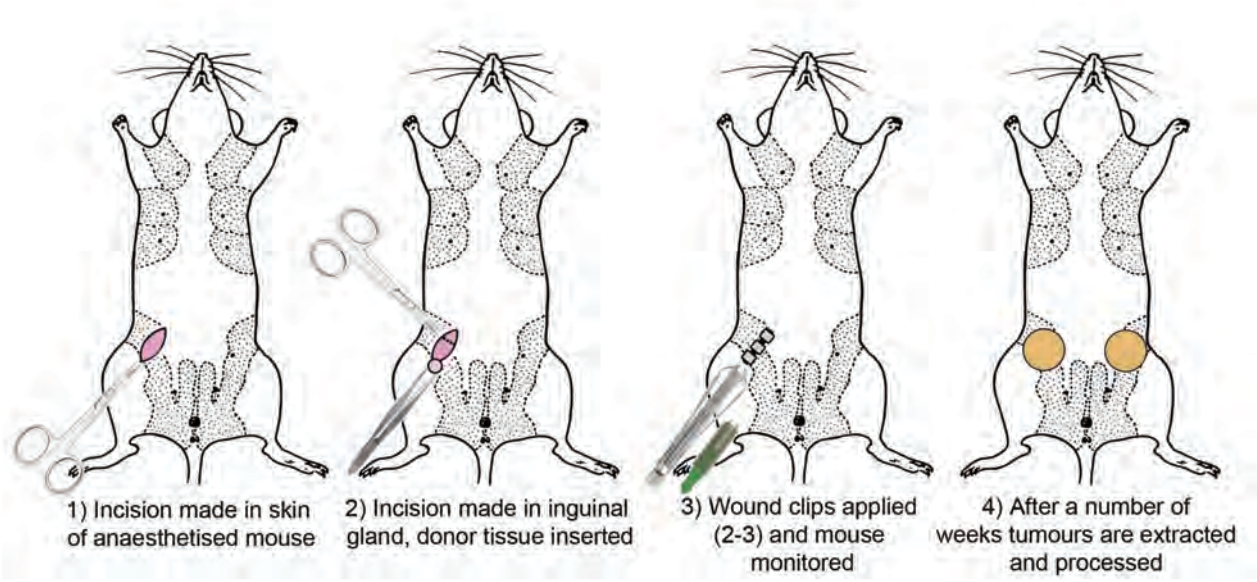

Figure 2.2: Tissue transplant procedure.

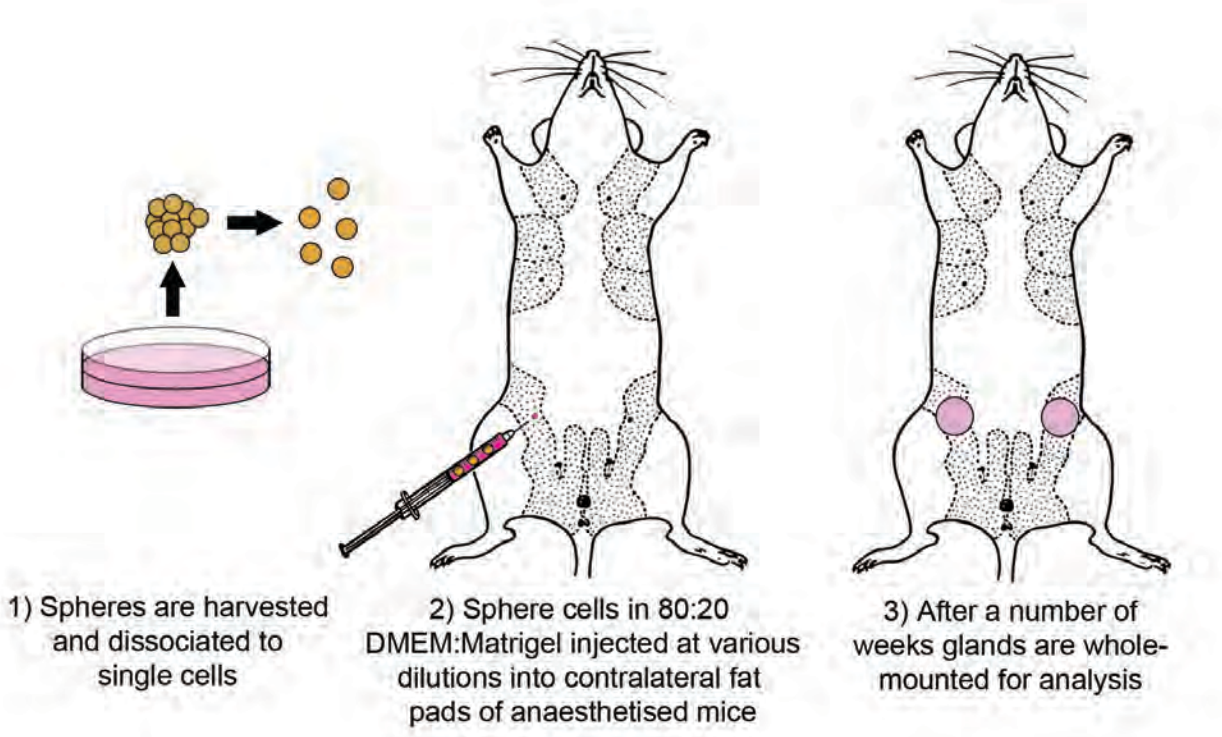

Figure 2.3: Procedure of the limiting dilution assay.

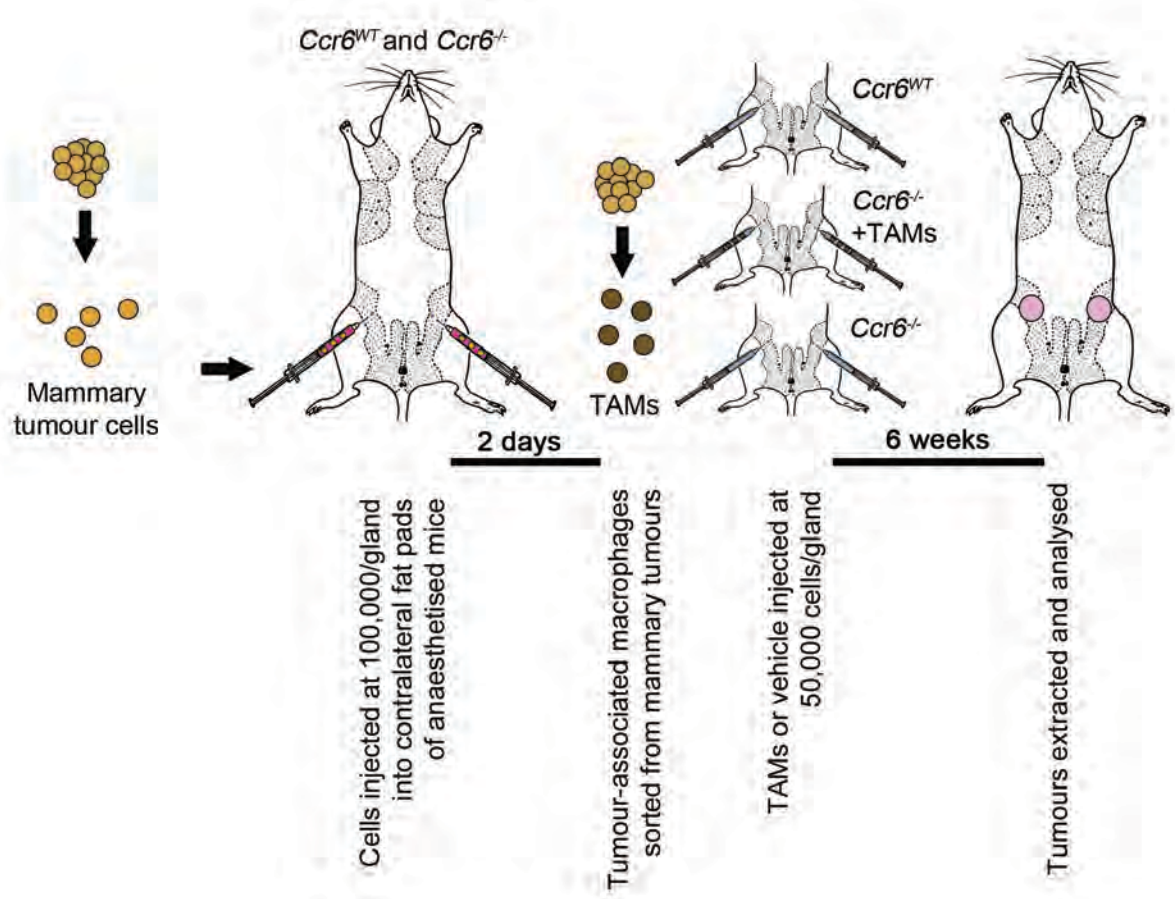

Figure 2.4: Procedure of the macrophage reconstitution assay.

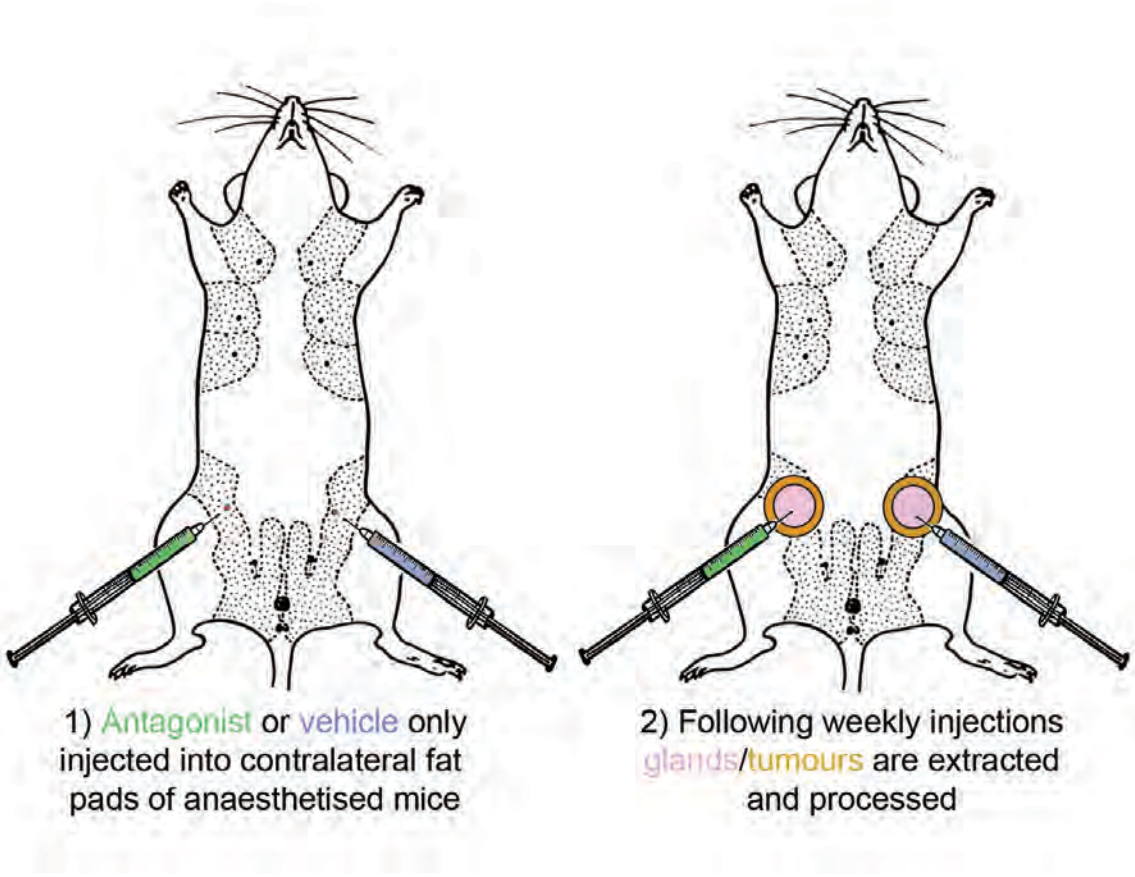

Figure 2.5: Strategy used for antagonist studies.

CHAPTER 3: The Chemokine Receptor CCR7 Promotes Mammary Tumourigenesis Through Amplification of Stem-Like Cells

3.1. Introduction

Despite concerted efforts and significant advances, breast cancer-related mortality is still a leading cause of death in women world-wide [1]. Clearly novel therapies are urgently needed. The “cancer stem cell” theory specifies that a small subset of cells in a heterogeneous tumour (termed “cancer stem cells” (CSCs)) possess stem cell-like properties of self-renewal and differentiation. CSCs are suggested to sustain and propagate tumours, and are inherently therapy-resistant [173, 176].

CSCs may originate from adult stem cells, but can also arise from more committed lineage progenitor cells if they acquire stem cell-like features due to genetic or epigenetic changes [60]. Multiple intrinsic and extrinsic factors are reported to have a role in CSC maintenance, regulation and support of stem-like characteristics. Most prominent are the Notch [177], Hedgehog [178], Wnt [179] and TGFβ [180] signalling systems. Several cytokines and chemokines have also been recently suggested as maintaining and promoting the CSC phenotype in a number of solid malignancies, including mammary tumours [181]; however, definitive *in vivo* data has been sparse.

Chemokine receptors and their cognate chemokine ligands have become widely accepted as important mediators of cancer growth and progression in many human neoplasms, being involved in tissue transformation, invasion, angiogenesis, and resistance to chemotherapy [89]. Among these, the chemokine receptor CCR7 has been implicated in metastatic spread of multiple malignancies [161]. In breast carcinogenesis, it has been attributed a number of potential functions, including promotion of cell motility, migration and adhesion, regulation of matrix metalloproteinases leading to basement membrane degradation [182], and cell survival through inhibition of anoikis [130]. Data obtained using cell lines has implicated CCR7 in breast cancer spread to the lymph nodes [162], and in human breast cancer its role was inferred from retrospective studies on archived tumour tissues [124]. High expression levels of CCR7 were

also correlated with higher grade and occurrence of secondary tumours, and poor prognosis [125, 163].

Whereas all these studies point to a role for CCR7 in malignancy, a direct function for CCR7 in cancer has not yet been established. Furthermore, its role in breast cancer in particular is unclear. In this study a novel bigenic mouse model was developed combining deletion of CCR7 with the polyoma middle-T transgene, which is under control of the mouse mammary tumour virus promoter (MMTV-PyMT), to study tumour development *in vivo*. Using this model it was shown that CCR7 deletion has a striking preventative effect on PyMT-driven mammary tumours, supporting the notion that CCR7 has a major determining role in breast oncogenesis. Moreover, the data reveal that the tumour-promoting effect of CCR7 is mediated through stem-like cells in both primary mouse and human breast tumours. These results provide new insights into the role of CCR7 in breast cancer stem-like cells and have important implications for the development of future therapeutics in breast cancer.

3.2. Results

3.2.1. CCR7 deletion arrests mammary tumourigenesis in the PyMT transgenic breast cancer mouse model

The MMTV-PyMT transgenic breast cancer mouse model has been extensively used in recent years to study various aspects of mammary neoplasia. Expression of the PyMT protein promotes the rapid epithelial transformation of mammary cells, via the corruption of various pathways including those of *Src, ras*, and PI3 kinase. This model also results in spontaneous metastasis and has been found to closely mimic the development of human breast cancer [169, 170, 183]. Representative images are shown in ***Figure 3.1***, in which α-smooth muscle actin is used to stain myoepithelial cells.

To directly assess the role of CCR7 in the multistage process of mammary tumourigenesis *in vivo*, bigenic MMTV-PyMT *Ccr7*$^{-/-}$ knockout mice were generated and the development of mammary tumours was traced relative to MMTV-PyMT *Ccr7*^{WT} mice.

Deletion of CCR7 significantly delayed PyMT-driven primary mammary tumourigenesis (representative pictures ***Figure 3.2a***). Tumour-free survival was significantly extended (***Figure 3.2b***) and total tumour burden was markedly reduced in MMTV-PyMT *Ccr7*$^{-/-}$ mice (***Figure 3.2c***) when compared with the *Ccr7*^{WT} animals. The lungs of MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ females were also examined for metastatic lesions at the time of killing. MMTV-PyMT *Ccr7*$^{-/-}$ mice developed significantly fewer and smaller metastases than MMTV-PyMT *Ccr7*^{WT} mice (***Figure 3.3***), although the number of metastases varied largely between mice of the same genotype.

As these experiments indicated a role for CCR7 in mammary gland function, normal, pre-cancerous and cancer-bearing mouse mammary glands were next examined for CCR7 expression and signalling. CCR7 was shown to be expressed on all mouse mammary epithelial cells tested, regardless of the tumour stage (***Figure 3.4,*** see also ***Figure 3.1***), and the removal of CCR7 did not affect the expression levels of its ligands CCL19 and CCL21 within the mouse mammary fat pad (***Figure 3.5***). CCR7 was also found to be functional in PyMT-driven mammary tumours, as tumour cells mobilised intracellular calcium, a hallmark of chemokine receptor activity, in response to stimulation with CCL21 (***Figure 3.6***). These data showed that CCR7 was expressed and was functional within normal and transformed mammary epithelium.

Interestingly, despite the large impact of CCR7 on overall mammary tumourigenesis, initial PyMT-driven hyperplastic growth in 8 or 11 week-old MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mice was not affected (***Figure 3.7a-b***), with similar tissue architecture in glands from both genotypes (***Figure 3.7c***). This indicated that the MMTV-PyMT *Ccr7*$^{-/-}$ mammary glands underwent the

initial oncogenic transformation leading to epithelial proliferation, but further tumourigenic transition was largely blocked by CCR7 deletion.

3.2.2. CCR7 promotes tumourigenesis by amplifying breast cancer stem-like cells

To investigate the underlying mechanisms responsible for the tumourigenic effects seen, the role of CCR7 in mammary gland development and on stem-like cells was then examined. It was found that in non-PyMT wild-type (*Ccr7*^{WT}) pubertal mice, the epithelial tree was longer with a better developed ductal structure than that in *Ccr7*$^{-/-}$ mice (***Figure 3.8***), indicating that ablation of CCR7 had a mild inhibitory effect on pubertal growth of the mammary gland epithelium. CCR7 was robustly expressed in normal mammary epithelium (***Figure 3.9***), making this receptor also potentially relevant to normal mammary development. However, development of mammary epithelium in the *Ccr7*$^{-/-}$ mice caught up with that of the *Ccr7*^{WT} mice by the age of 8 weeks, and at 12 weeks mammary glands from the two genotypes were indistinguishable (***Figure 3.10***), demonstrating that CCR7 deletion mainly delays early mammary gland development.

Because normal development and breast cancer are believed to be linked by common regulatory mechanisms, it was hypothesised that the observed promotion of PyMT-driven tumourigenesis and mammary development was due to CCR7 regulating stem/progenitor cell pools in mammary epithelium. Thus, the stem-like cell content in mice was assayed using the lineage-negative (Lin^-) $CD24^+CD29^{hi}$ cell surface marker profile [60], which was previously functionally characterised in the MMTV-PyMT model [184, 185]. CCR7 was expressed in all cell lineages in both the normal and PyMT-expressing mammary glands regardless of CD24 and CD29 status. Notably, however, higher levels of CCR7 (>90%) were observed in $Lin^-CD24^+CD29^{hi}$ normal and cancer mouse mammary stem cell-enriched populations (***Figure 3.11***). Importantly, CCR7 was also expressed on human $CD44^+CD24^-$ putative mammary stem cells [64] from both normal and breast tumour tissue (***Figure 3.12***).

Further analysis demonstrated a significantly lower content of $Lin^-CD24^+CD29^{hi}$ cells in non-PyMT *Ccr7*$^{-/-}$ mice relative to *Ccr7*^{WT} (***Figure 3.13a***). In PyMT-expressing mice at the stage of early neoplasia, when no morphological differences were found in MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ glands (***Figure 3.7***) and the stem/progenitor cell populations may, therefore, best reflect the tumour-initiating cell content, the difference in the stem cell-enriched population between MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mice was even more pronounced with the deletion of CCR7 leading to a two-fold reduction in stem-like cells (***Figure 3.13b***).

Recently Pece *et al.* have suggested a new and potentially more efficient set of markers, in which the Notch ligands delta-like ligand 1 (DLL1) and delta and notch-like epidermal growth factor-related receptor (DNER) are used in combination with CD49f ($Lin^-CD49f^+DLL1^+DNER^+$) to delineate putative stem cells in human mammary tumours [71]. It was found that the stem-like cells from both human and mouse mammary glands defined by this profile also expressed high levels of CCR7 (***Figure 3.14***). Moreover, the $Lin^-CD49f^+DLL1^+DNER^+$ cell pools were significantly smaller in both normal and PyMT-expressing *Ccr7*$^{-/-}$ murine mammary glands (***Figure 3.15***) providing further support for the findings described above.

It is generally accepted that non-adherent passaged mammosphere cultures are enriched in cells with stem-like characteristics, and secondary/tertiary mammosphere-forming efficiency (MFE) is representative of cells' potential to exhibit stem cell traits [75, 149, 186]. Stem-like activity, as measured by MFE, was then analysed in the mammary epithelium in the presence or absence of CCR7. Primary and secondary sphere formation from normal (***Figure 3.16a***) or PyMT-expressing (***Figure 3.16b***) mammary cells was substantially reduced after CCR7 ablation and, importantly, stimulation of non-PyMT *Ccr7*^{WT} and MMTV-PyMT *Ccr7*^{WT} cells with CCR7 ligands CCL19 and CCL21 significantly potentiated mammosphere growth (***Figure 3.16***).

This CCR7 stimulatory function was seen exclusively on mammosphere growth, as stimulation with CCL19 and CCL21 had no detectable effect on the proliferation of bulk mammary tumour

cells in adherent culture, a condition that supports a more differentiated phenotype (***Figure 3.17***). The addition of CCR7 ligands to sphere cultures derived from MMTV-PyMT *Ccr7*$^{-/-}$ mammary cells also had no effect on MFE (***Figure 3.18***), demonstrating a CCR7 receptor-mediated mechanism. The specificity of CCR7 was further shown by testing a panel of ligands for other tumour-associated chemokine receptors CCR6 [163], CXCR3 [126] and CXCR5 [187]. No effects were observed on MFE (***Figure 3.19***).

To extend these findings to human breast cancer, the activity of CCR7 in human primary tumour cells from resected breast cancer tissue was examined. The addition of CCL19 and CCL21 resulted in an increase in primary and secondary MFE of human breast cancer cells by two- to three-fold (***Figure 3.20***), consistent with results obtained in the mouse model.

To specifically link the deletion of CCR7 to depleted tumour-initiating cells, a limiting dilution transplantation approach was used [71] to estimate tumour-initiating cell (TIC) frequency. Secondary mammosphere-derived cells from MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mice with early neoplasia were used in this assay to address the potential of cells in mammosphere cultures to exhibit stem cell traits of self-renewal and tumour initiation *in vivo*, in the context of CCR7-dependency. Cells were injected into contralateral inguinal fat pads of non-PyMT *Ccr7*^{WT} recipients. Analysis of grafted fat pads after 6 weeks showed that MMTV-PyMT *Ccr7*^{WT} sphere cells produced much more robust growth at all dilutions (***Figure 3.21***). Most importantly, the frequency of stem-like cells capable of tumour initiation within MMTV-PyMT *Ccr7*^{WT} sphere culture (1/189) was over three-fold higher than in MMTV-PyMT *Ccr7*$^{-/-}$ (1/913) (***Table 3***), providing strong evidence for the critical role of CCR7 in the regulation and maintenance of stem-like cells and tumour-initiating cells in the mammary gland.

3.2.3. CCR7 is required for the propagation of mammary tumours

To obtain *in vivo* evidence for the role of CCR7 in tumour propagation, the PyMT mouse model was taken advantage of, as it allows tumour formation upon transplantation [183]. Expression of the PyMT oncogene results in multifocal tumours and hence can generate diverse CSC pools owing to various underlying mutations within the same gland at the late stages of tumourigenesis. Therefore, it was reasoned that if taken at the early stage of pre-neoplastic tumour development, the population of CSCs should be more homogeneous. Consequently, small $1mm^3$ fragments of pre-neoplastic mammary tissue from 8-week-old PyMT transgenic MMTV-PyMT $Ccr7^{WT}$ and $Ccr7^{-/-}$ mice were simultaneously transplanted into contralateral inguinal mammary fat pads of non-PyMT $Ccr7^{WT}$ recipients. Representative histological sections from both MMTV-PyMT $Ccr7^{WT}$ and $Ccr7^{-/-}$ 8 week-old mice, corresponding to donor tissue, are shown in ***Figure 3.22***, confirming that the glands used for transplantation were at the equivalent stage of tumourigenesis.

Analysis of tumourigenic outgrowth from transplanted tissue showed that the deletion of CCR7 almost completely blocked secondary tumour development. Only one out of six transplants from the MMTV-PyMT $Ccr7^{-/-}$ donors was able to give rise to a neoplastic lesion, whereas five out of six fragments from the MMTV-PyMT $Ccr7^{WT}$ donors produced secondary outgrowths in $Ccr7^{WT}$ recipients (***Figure 3.23***), demonstrating a key role of CCR7 in tumour propagation.

3.2.4. Physical antagonism of CCR7 *in vivo* depletes the stem-like cell population and inhibits mammary tumourigenesis

A CCR7 antagonist, $CCL19_{(8-83)}$ [188], was then used to explore the potential of targeting CCR7 for CSC-directed therapeutic intervention. Initially, the ability of $CCL19_{(8-83)}$ to block the stimulatory activity of CCR7 ligands on mammosphere-forming capacity was tested *ex vivo* and found to specifically abrogate the effect of CCL21 (***Figure 3.24***) and CCL19 on mammosphere growth, providing a rationale for *in vivo* studies.

The effect of CCR7 blockade by $CCL19_{(8-83)}$ on tumour initiation was then examined in the context of the PyMT transgenic mouse model. $CCL19_{(8-83)}$ was injected for 8 consecutive weeks into inguinal mammary glands of animals from the age of 4 weeks old. Glands were then excised and examined for the extent of tumourigenesis and stem-like cell content and function. Macroscopic analysis demonstrated that $CCL19_{(8-83)}$-injected glands had smaller lesions than their control counterparts (representative image ***Figure 3.25a***). The total weight of fat pads was not statistically different; however, the cellularity (total cell count and cells per mg of tissue) was significantly reduced by the antagonist (***Figure 3.25b-d***).

Treatment with $CCL19_{(8-83)}$ also resulted in a significant decrease in the proportion of stem-like cells ($Lin^-CD24^+CD29^{hi}$ and $Lin^-CD49f^+DLL1^+DNER^+$, ***Figure 3.26a-b***) and the function of stem and early progenitor cells (***Figure 3.26c***), without affecting the level of CCR7 receptor expression (***Figure 3.27***). PyMT transgenic mice on both FVB and C57Bl/6 backgrounds were tested, with similar results.

To determine whether treatment with $CCL19_{(8-83)}$ has an inhibitory effect on established and/or advanced later stage tumours, $1mm^3$ size fragments of MMTV-PyMT $Ccr7^{WT}$ tumours from 16 week-old mice, corresponding to the invasive ductal carcinoma stage of human breast cancer (***Figure 3.1***), were transplanted into inguinal glands of $Ccr7^{WT}$ recipients followed by 8 weekly injections of $CCL19_{(8-83)}$ or vehicle control (***Figure 3.28a***). Although no significant differences were seen between $CCL19_{(8-83)}$- or vehicle-treated tumours in size or cellularity (***Figure 3.28b***) as was observed in primary tumours, the proportions of stem-like cells determined by both conventional ($Lin^-CD24^+CD29^{hi}$) or novel ($Lin^-CD49f^+DLL1^+DNER^+$) marker sets (***Figure 3.29a-b***), as well as mammosphere growth (***Figure 3.29c***), were significantly reduced in antagonist-treated glands, demonstrating that the CCR7 axis can be blocked *in vivo* to target stem-like cells in mammary tumours.

3.3. Summary

The chemokine receptor CCR7 is widely implicated in breast cancer pathobiology. Although recent reports correlated high CCR7 levels with more advanced tumour grade and poor prognosis, limited *in vivo* data are available regarding its specific function in mammary gland neoplasia and the underlying mechanisms involved.

To address these questions a bigenic mouse model of breast cancer combined with CCR7 deletion was generated, which revealed that CCR7 ablation results in a considerable delay in tumour onset as well as significantly reduced tumour burden. Importantly, CCR7 was found to exert its function by regulating mammary cancer stem-like cells in both murine and human tumours. *In vivo* experiments showed that loss of CCR7 activity either through deletion or pharmacological antagonism significantly decreased functional pools of stem-like cells in mouse primary mammary tumours, providing a mechanistic explanation for the tumour-promoting role of this chemokine receptor.

In conclusion, these data characterise the oncogenic properties of CCR7 in mammary epithelial neoplasia and point to a new route for therapeutic intervention to target evasive cancer stem cells.

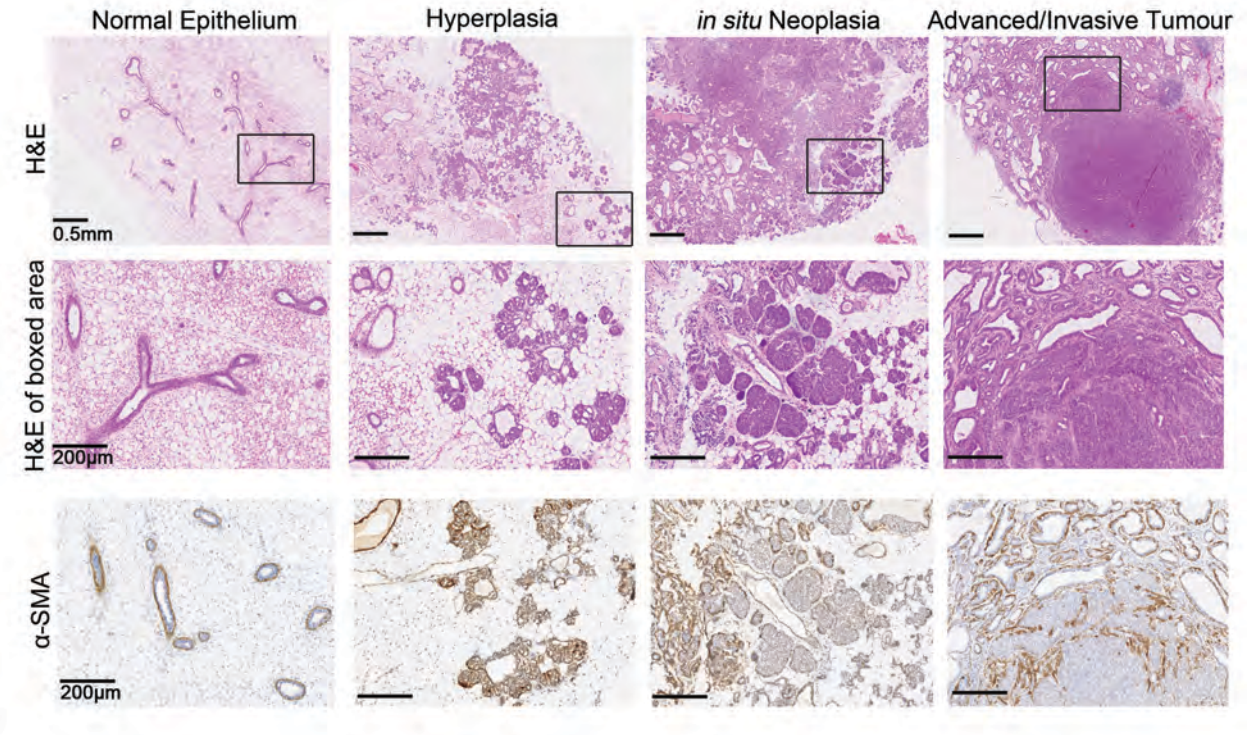

Figure 3.1: Tumour progression in the MMTV-PyMT breast cancer mouse model.

Serial sections of mouse mammary tissue from normal mice and at different tumourigenic stages stained with haematoxylin and eosin (H&E) and for α-smooth muscle actin (α-SMA) as indicated.

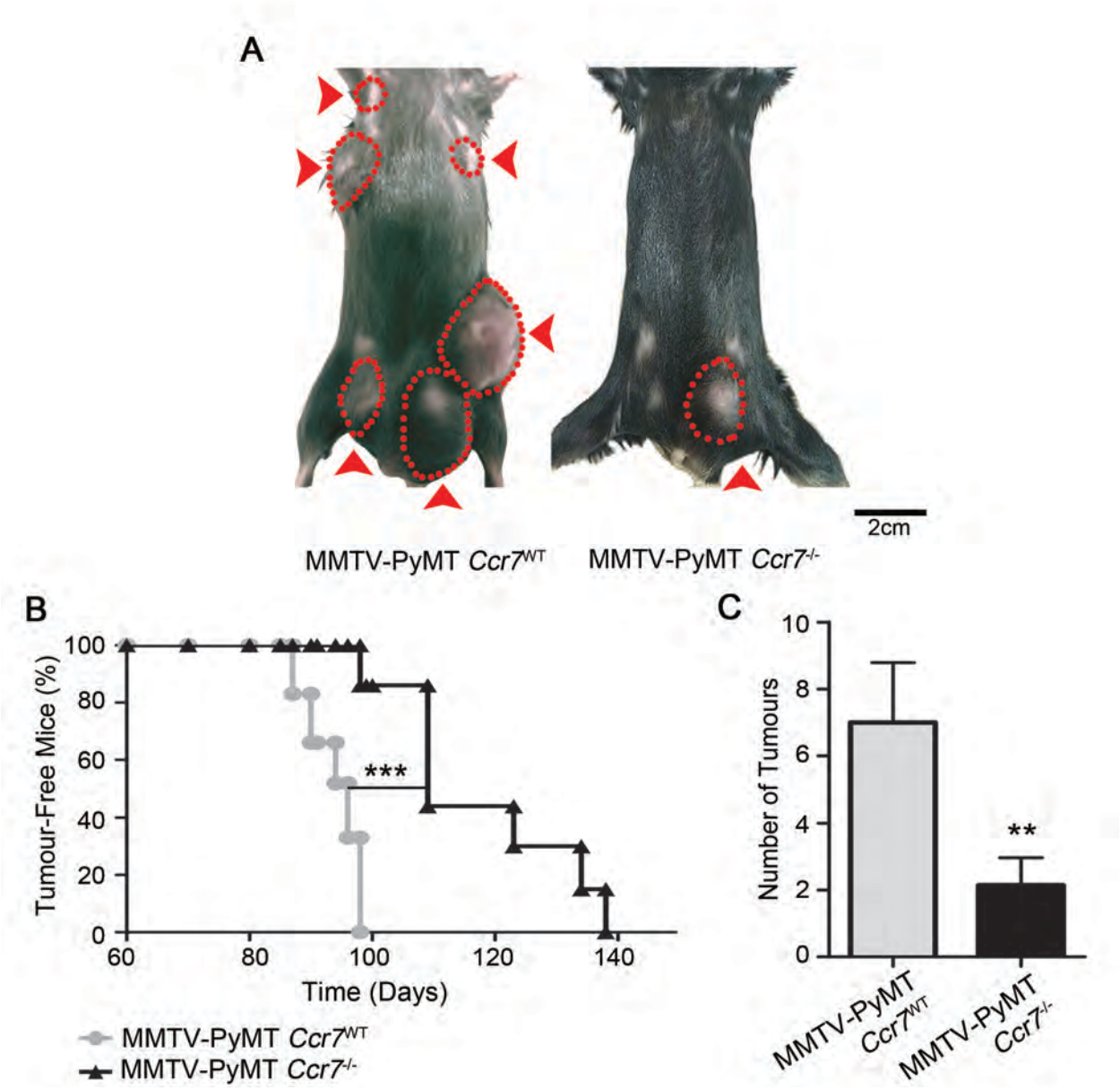

Figure 3.2: CCR7 ablation delays tumour onset and reduces tumour burden in the MMTV-PyMT-driven mouse model of breast cancer.

(A) Representative images of MMTV-PyMT *Ccr7*^{WT} and MMTV-PyMT *Ccr7*$^{-/-}$ mice at 22 weeks old, showing grossly visible tumours (demarcated by red arrows and dotted lines). **(B)** Kaplan-Meier analysis of tumour-free survival for MMTV-PyMT *Ccr7*^{WT} (n=18) and MMTV-PyMT *Ccr7*$^{-/-}$ (n=17) mice. **(C)** Number of tumours in MMTV-PyMT *Ccr7*^{WT} and MMTV-PyMT *Ccr7*$^{-/-}$ mice at the time of killing.

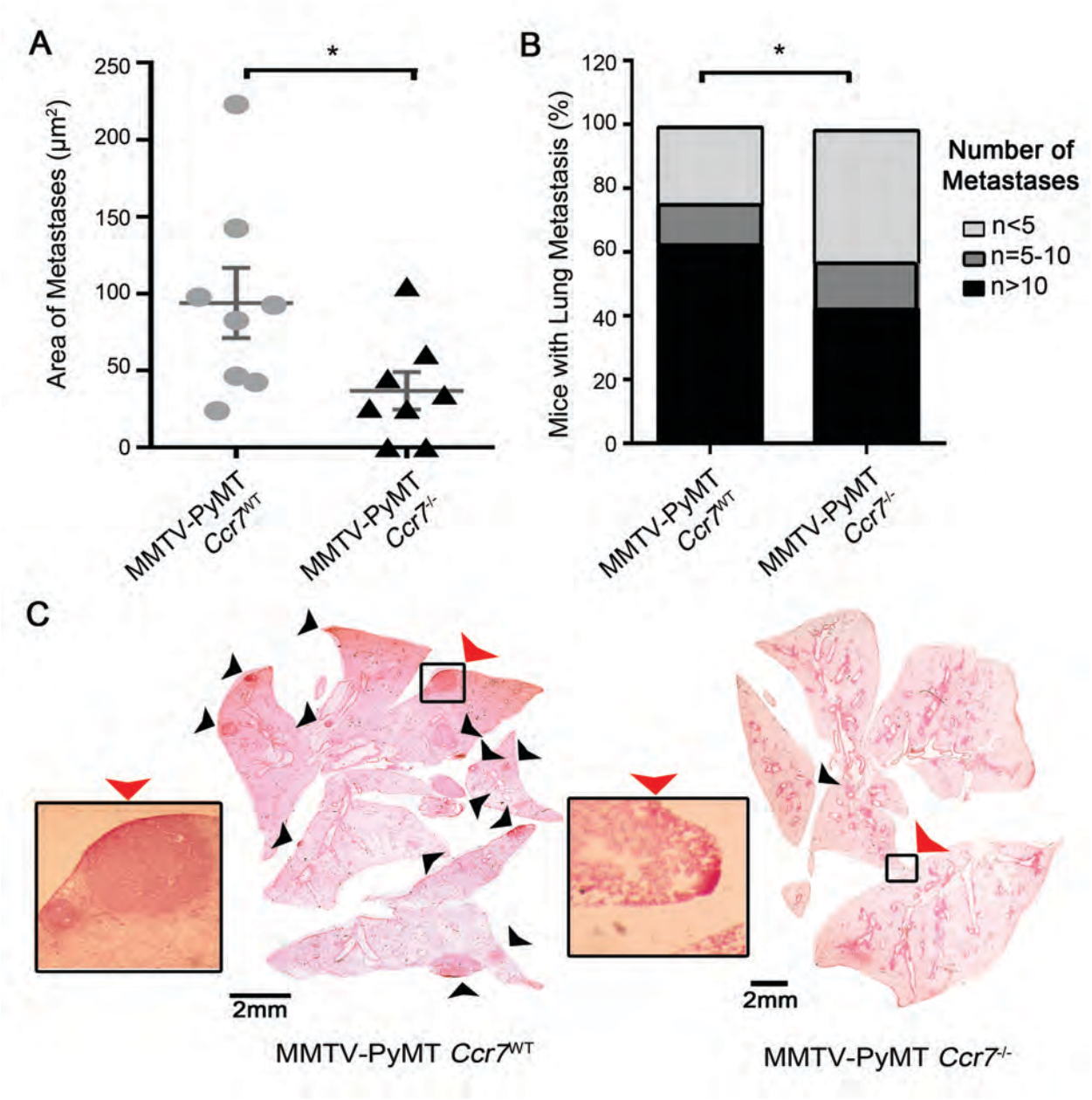

Figure 3.3: CCR7-null MMTV-PyMT mice develop less lung metastasis.

(A) Total cumulative area of lung metastatic lesions in MMTV-PyMT *Ccr7*^{WT} (n=8) and MMTV-PyMT *Ccr7*$^{-/-}$ (n=8) mice. **(B)** Distribution data of lung metastases in MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mice. **(C)** Representative images of H&E-stained lung sections from MMTV-PyMT *Ccr7*^{WT} (left) and MMTV-PyMT *Ccr7*$^{-/-}$ (right) mice with metastatic lesions (black arrowheads). Red arrowhead indicates inset magnified image.

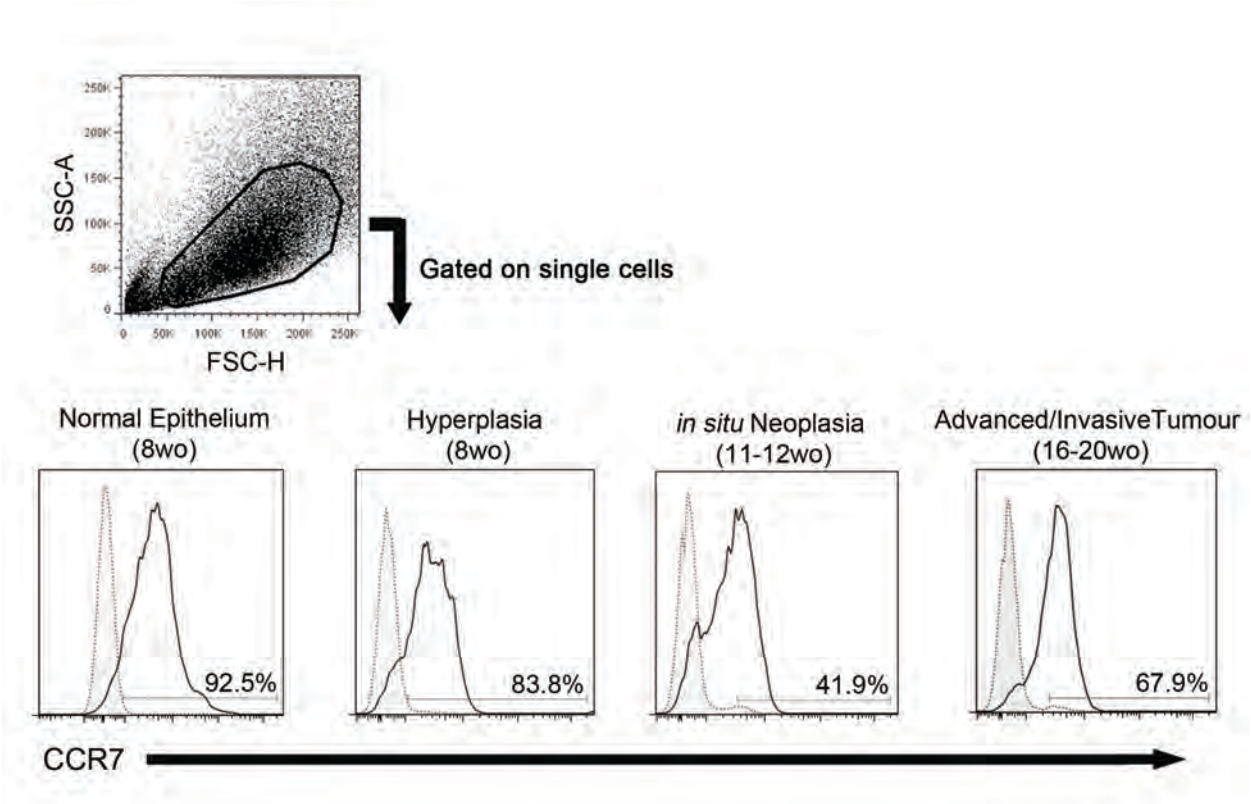

Figure 3.4: CCR7 is expressed throughout mammary gland development and cancer.

Mammary cells were gated to exclude debris, dead cells and doublets and proportions of CCR7-positive cells in mammary epithelial cell preparations were analysed by flow cytometry. Shaded histograms=FMO negative controls. wo=weeks old.

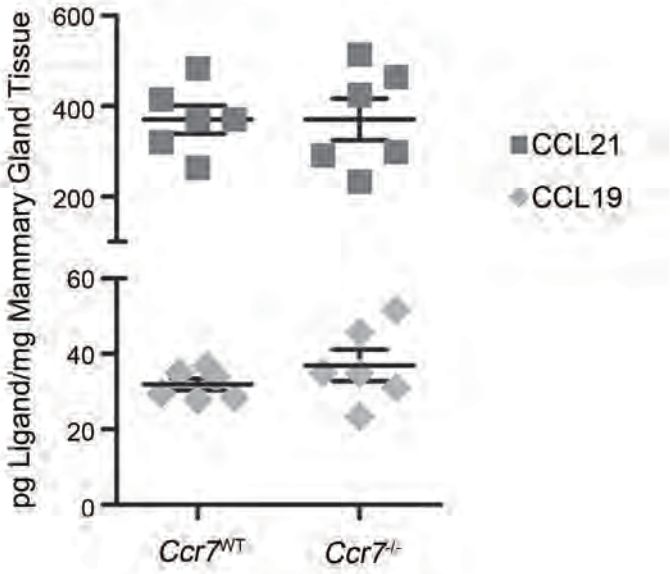

Figure 3.5: The expression of ligands CCL19 and CCL21 is not affected by CCR7 deletion.

Expression of CCR7 ligands (CCL21 and CCL19) in *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mammary fat pads with excised inguinal lymph nodes was assessed by ELISA. n=6 glands per genotype.

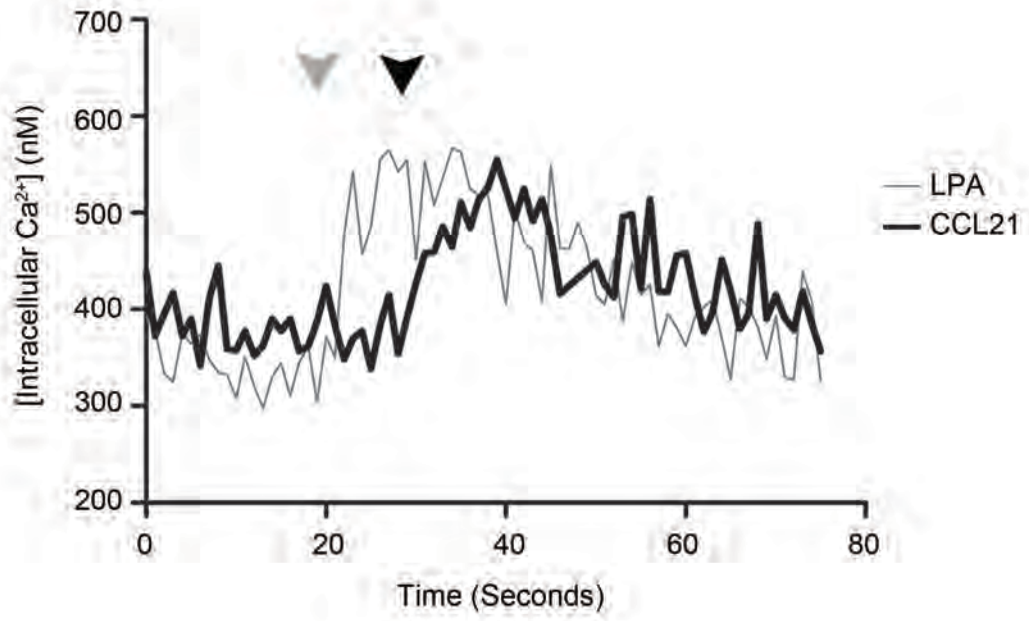

Figure 3.6: CCR7 is active in MMTV-PyMT tumour cells.

Calcium mobilisation analysis of MMTV-PyMT *Ccr7*^{WT} mouse mammary cells in response to lysophosphatidic acid (LPA) and the CCR7 ligand, CCL21. Arrowheads indicate a point of stimulus addition.

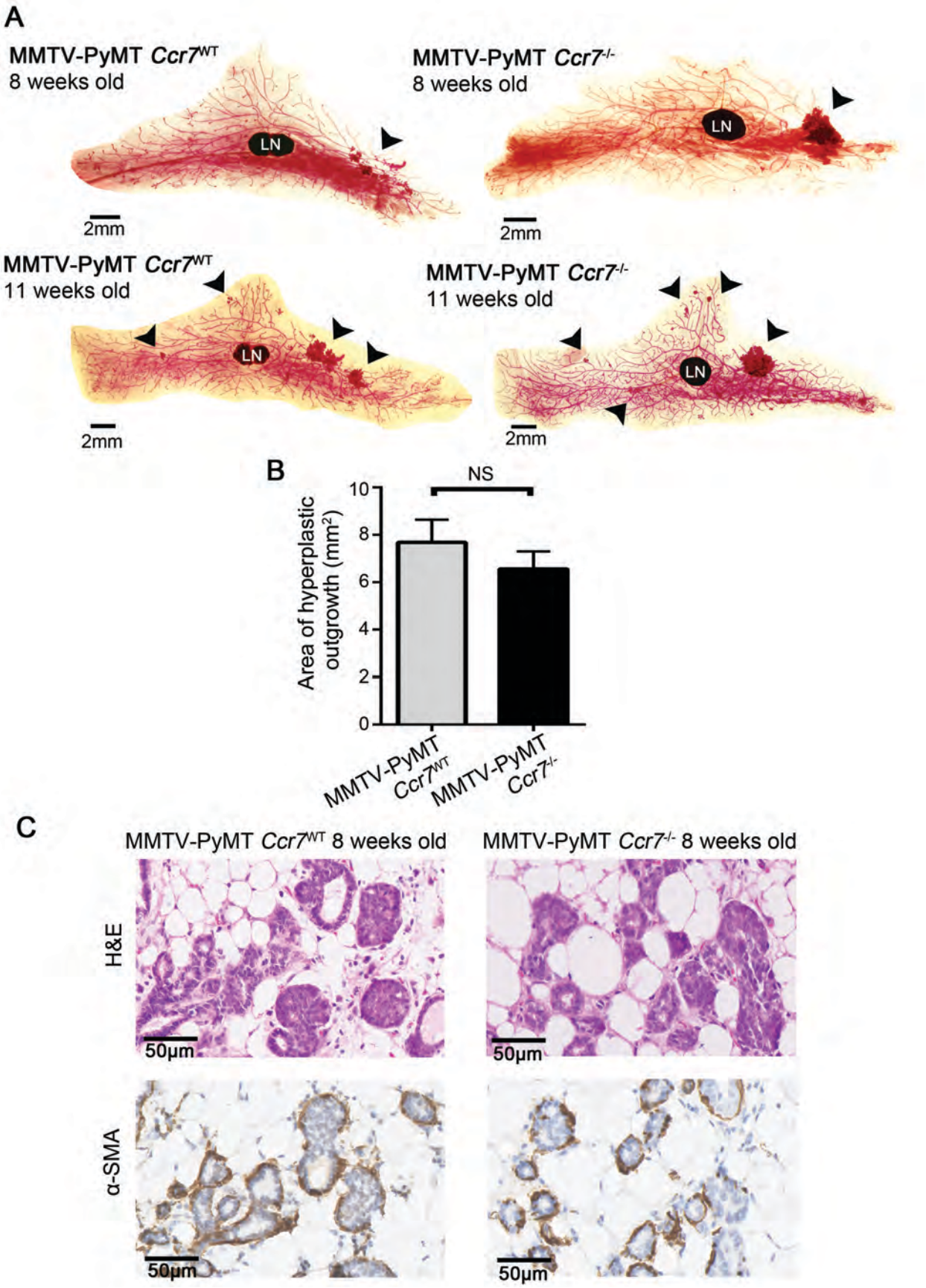
A
MMTV-PyMT Ccr7WT
8 weeks old
LN
2mm
MMTV-PyMT Ccr7-/-
8 weeks old
LN
2mm
MMTV-PyMT Ccr7WT
11 weeks old
LN
2mm
MMTV-PyMT Ccr7-/-
11 weeks old
LN
2mm
B
NS
Area of hyperplastic outgrowth (mm2)
10
8
6
4
2
0
MMTV-PyMT Ccr7WT
MMTV-PyMT Ccr7-/-
C
MMTV-PyMT Ccr7WT 8 weeks old
MMTV-PyMT Ccr7-/- 8 weeks old
H&E
50μm
50μm
α-SMA
50μm
50μm

Figure 3.7: Ablation of CCR7 has no effect on early mammary hyperplasia in MMTV-PyMT mice.

(A) Representative images of inguinal mammary glands of MMTV-PyMT *Ccr7*^{WT} (left) and MMTV-PyMT *Ccr7*$^{-/-}$ (right) mice harvested at 8 and 11 weeks of age as indicated. Arrowheads indicate areas of epithelial hyperplasia. LN=lymph node. **(B)** Quantitation of area of hyperplasia in MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mice at 8 weeks old. n=6 glands per genotype. **(C)** Serial sections of mouse mammary tissue from MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mice at 8 weeks old stained with H&E and for α -smooth muscle actin (α -SMA) as indicated.

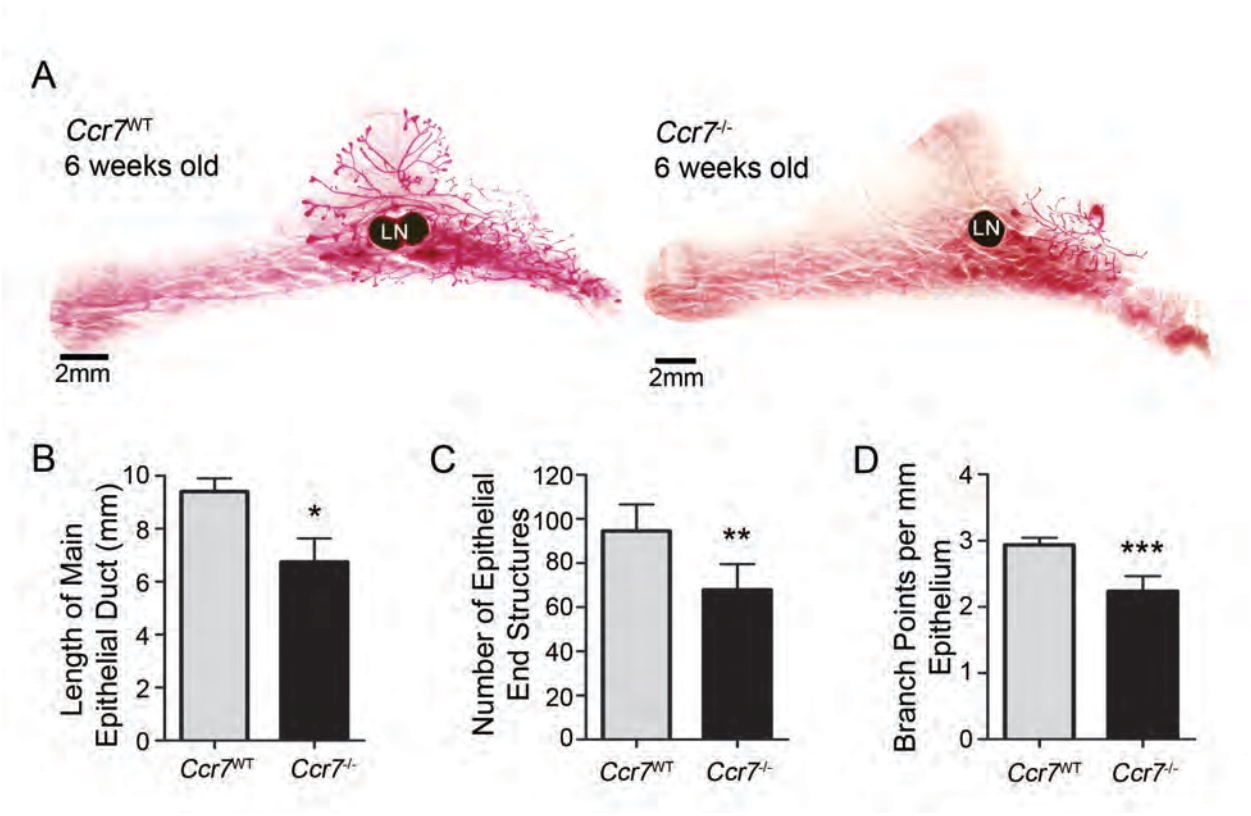

Figure 3.8: CCR7 deletion has an inhibitory effect on growth of pubertal mammary glands.

Development of the mammary ductal tree was evaluated in *Ccr7*^{WT} and *Ccr7*$^{-/-}$ C57Bl/6 mice at 6 weeks of age. **(A)** Representative whole mount images of mammary glands, with apparent reduction in the size of ductal trees in *Ccr7*$^{-/-}$ mice (n=6) compared with *Ccr7*^{WT} mice (n=7). LN=lymph node. **(B-D)** Quantitation of the length of main epithelial duct **(B)**, total number of mammary epithelial terminal structures **(C)**, and branching within mammary epithelium as determined by quantifying branch points per mm along three individual ducts **(D)**.

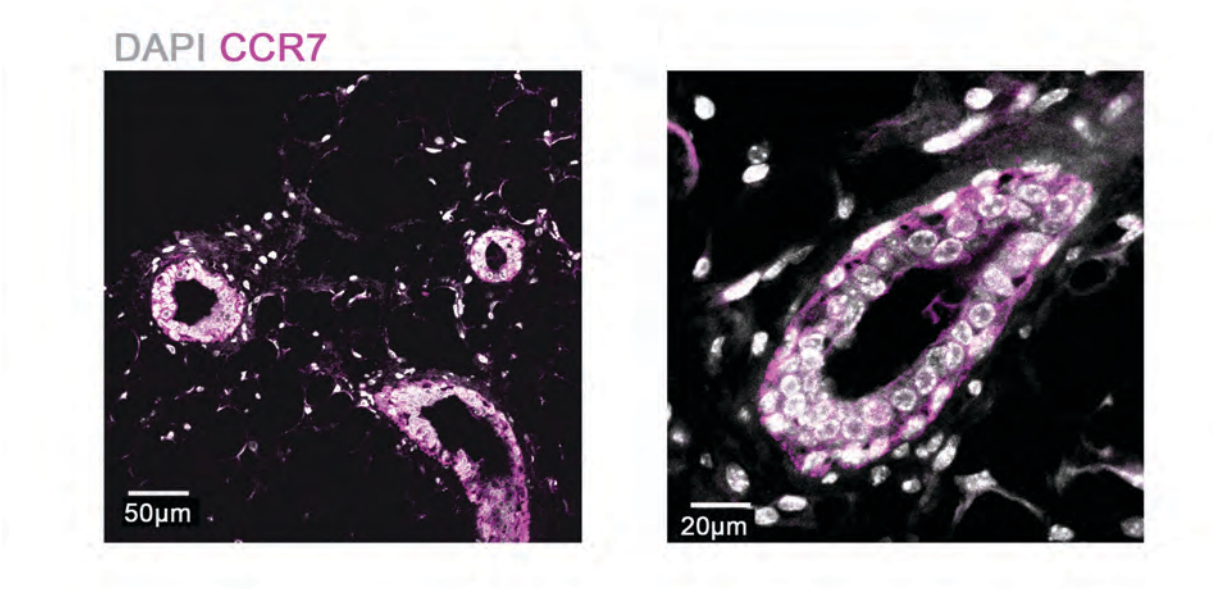

Figure 3.9: CCR7 is robustly expressed in normal mammary epithelium.

Formalin-fixed paraffin-embedded sections of normal pubertal mouse mammary glands were stained for CCR7 expression (magenta). Shown are two individual terminal end buds. Nuclei are counterstained with DAPI (grey).

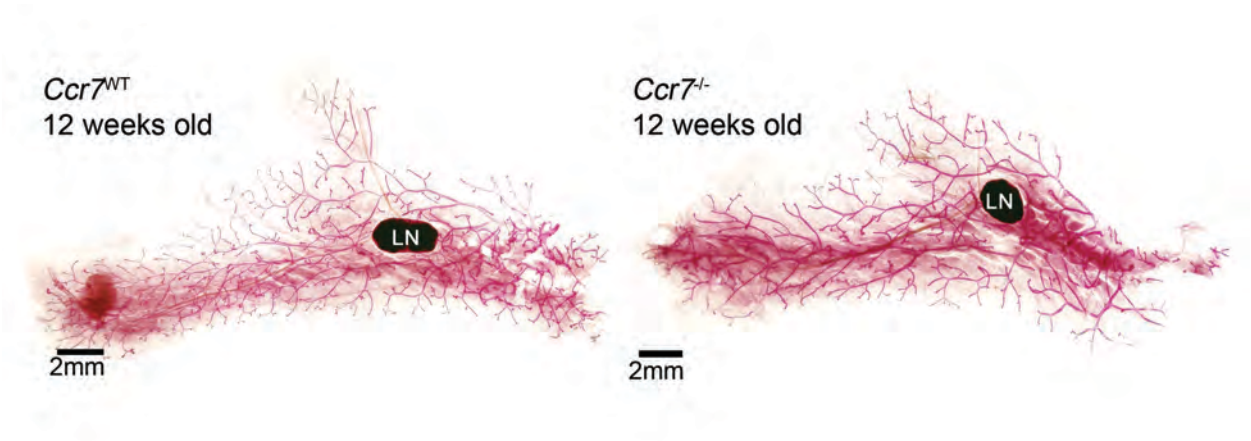

Figure 3.10: The effect of CCR7 on normal mammary development is not present in the adult mammary gland.

Representative whole mount images of mammary glands taken from adult *Ccr7*^{WT} (n=6) and *Ccr7*$^{-/-}$ (n=6) mice at 12 weeks of age with no apparent differences in the size and architecture of the mammary tree. LN=lymph node.

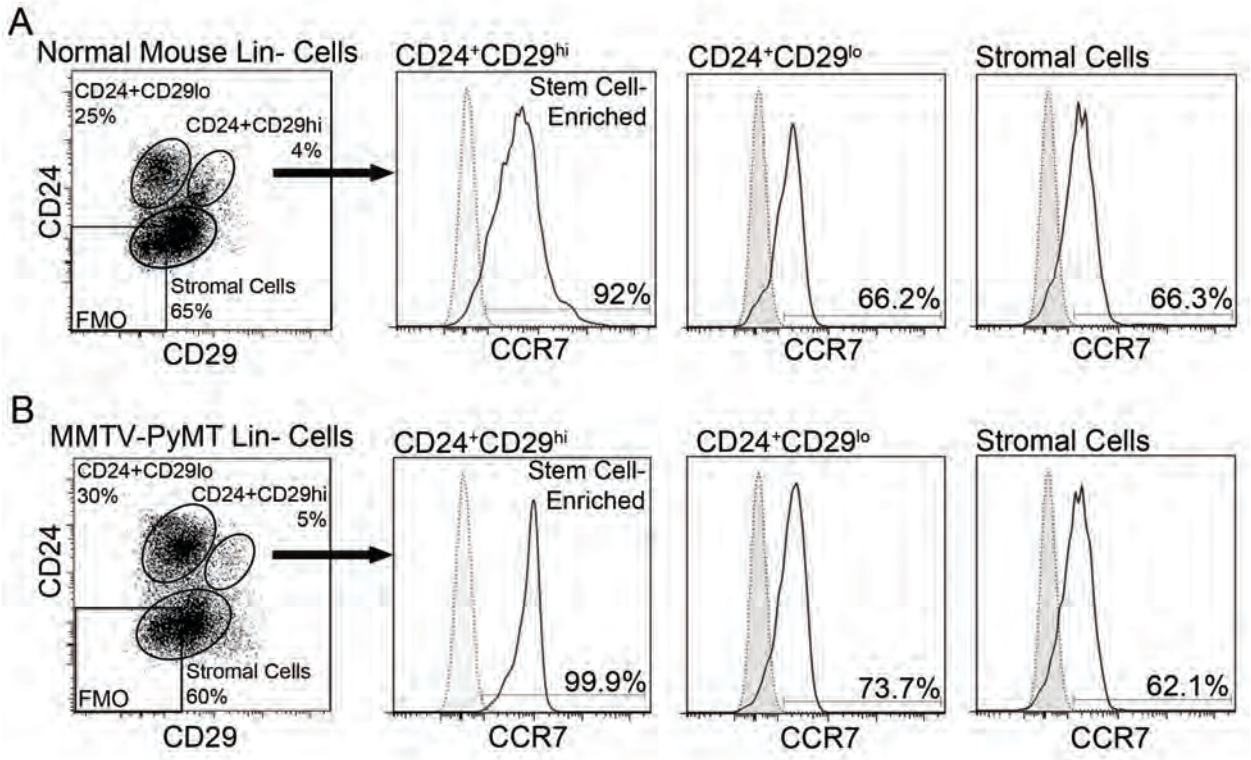

Figure 3.11: CCR7 expression in mammary cell lineages as defined by CD24 and CD29.

All cells were pre-gated to exclude debris, dead cells and doublets. Shown is the proportion of cells positive for CCR7 in lineage-negative (Lin^-) cell populations in normal mouse mammary epithelium **(A)** and PyMT-expressing glands **(B)**, as denoted by CD24 and CD29 surface marker expression. Shaded histograms=FMO negative controls. Results are representative of at least three independent experiments, n=6-10 mice per experiment.

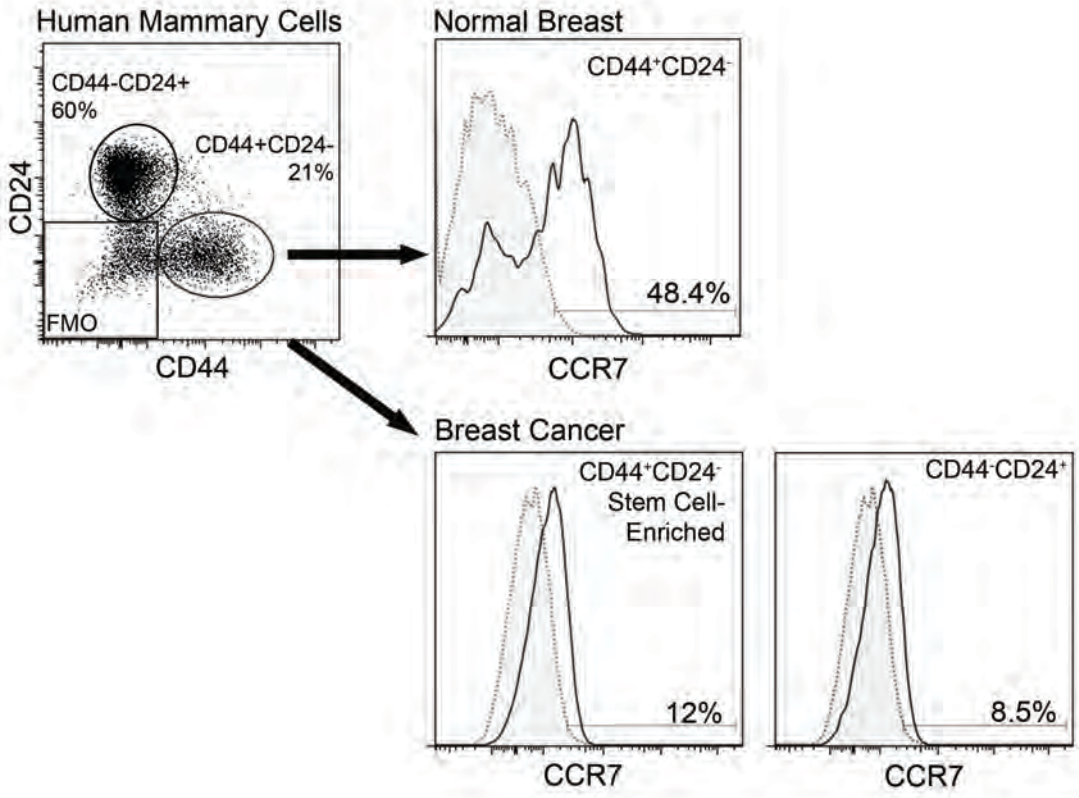

Figure 3.12: CCR7 expression in normal and cancerous human breast cells as defined by CD44 and CD24.

All cells were pre-gated to exclude debris, dead cells and doublets. Shown is the proportion of cells positive for CCR7 in different cell populations in normal human mammary epithelium and breast cancer as indicated, denoted by surface marker expression of CD44 and CD24. Shaded histograms=FMO negative controls. Results are representative of two normal and four independent tumour samples.

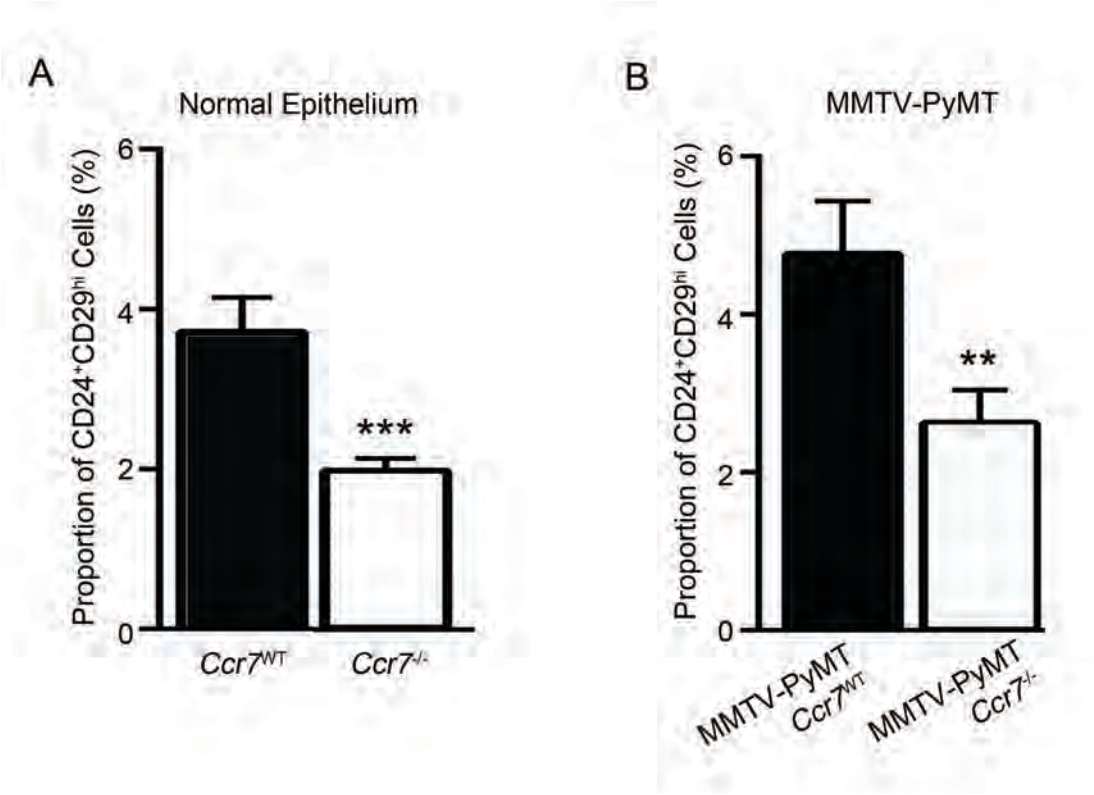

Figure 3.13: CCR7 deletion decreases the mammary stem cell-enriched pool as defined by CD24^{+}CD29^{hi} expression.

Cells from normal mammary glands **(A)** and PyMT-driven mammary tumours **(B)** were analysed by flow cytometry for stem-like cell content based on the Lin^{-}CD24^{+}CD29^{hi} marker profile. Results are representative of at least three independent experiments, n=6-10 mice per group per experiment.

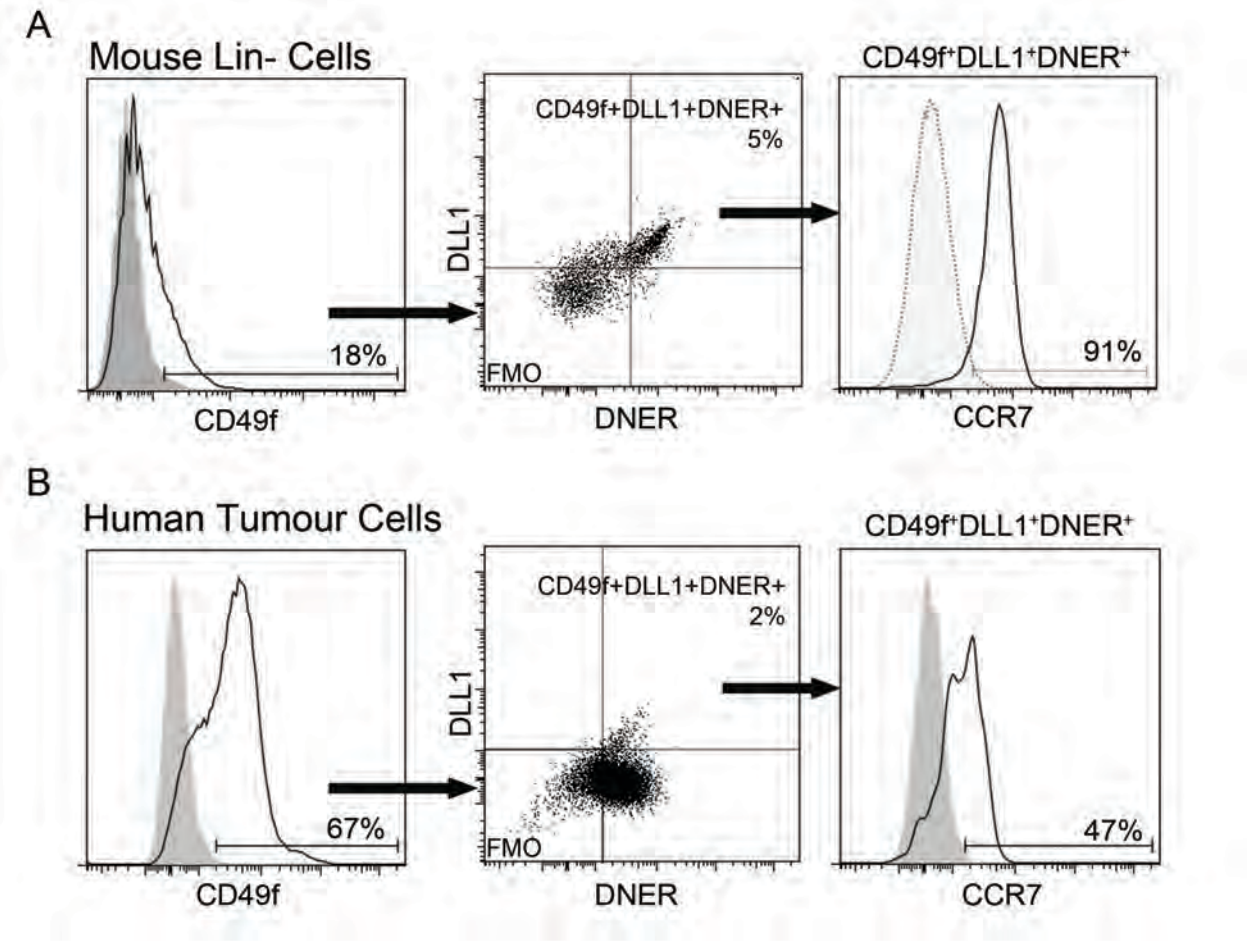

Figure 3.14: CCR7 expression in the CD49f$^+$DLL1$^+$DNER$^+$ cancer stem cell-enriched population.

Flow cytometry gating strategy for delineating the mouse **(A)** and human **(B)** stem cell-enriched populations based on alternative CD49f$^+$DLL1$^+$DNER$^+$ surface marker expression, together with representative plots demonstrating proportions of CCR7 positive cells within these populations. Shaded histograms=FMO negative controls. Results in **(A)** are representative of at least three independent experiments, n=6 MMTV-PyMT mice per experiment. Results in **(B)** are representative of four independent human tumour samples.

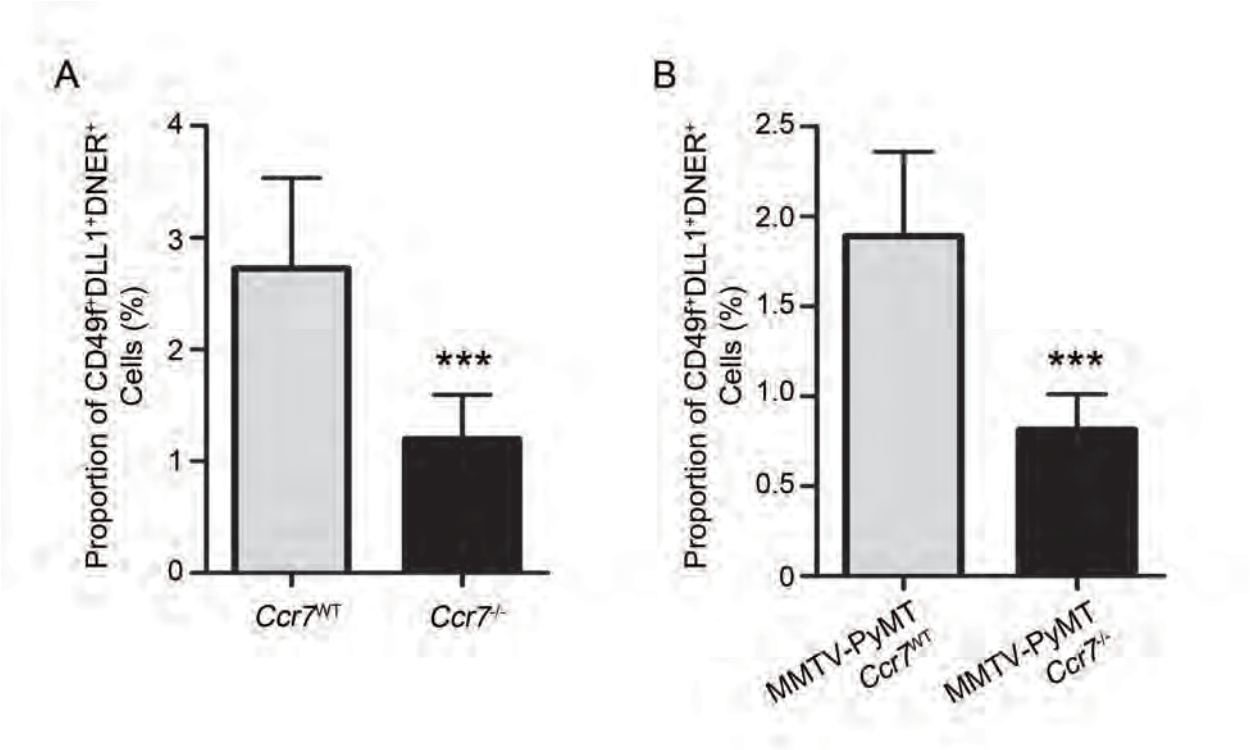

Figure 3.15: CCR7 deletion decreases the mammary stem cell-enriched pool as defined by CD49f^{+}DLL1^{+}DNER^{+}.

Cells from normal mammary glands **(A)** and PyMT-driven mammary tumours **(B)** were analysed by flow cytometry for stem-like cell content based on the putative marker profile of Lin^{-} CD49f^{+}DLL1^{+}DNER^{+}. Data are representative of at least three independent experiments, n=6 mice per genotype per experiment.

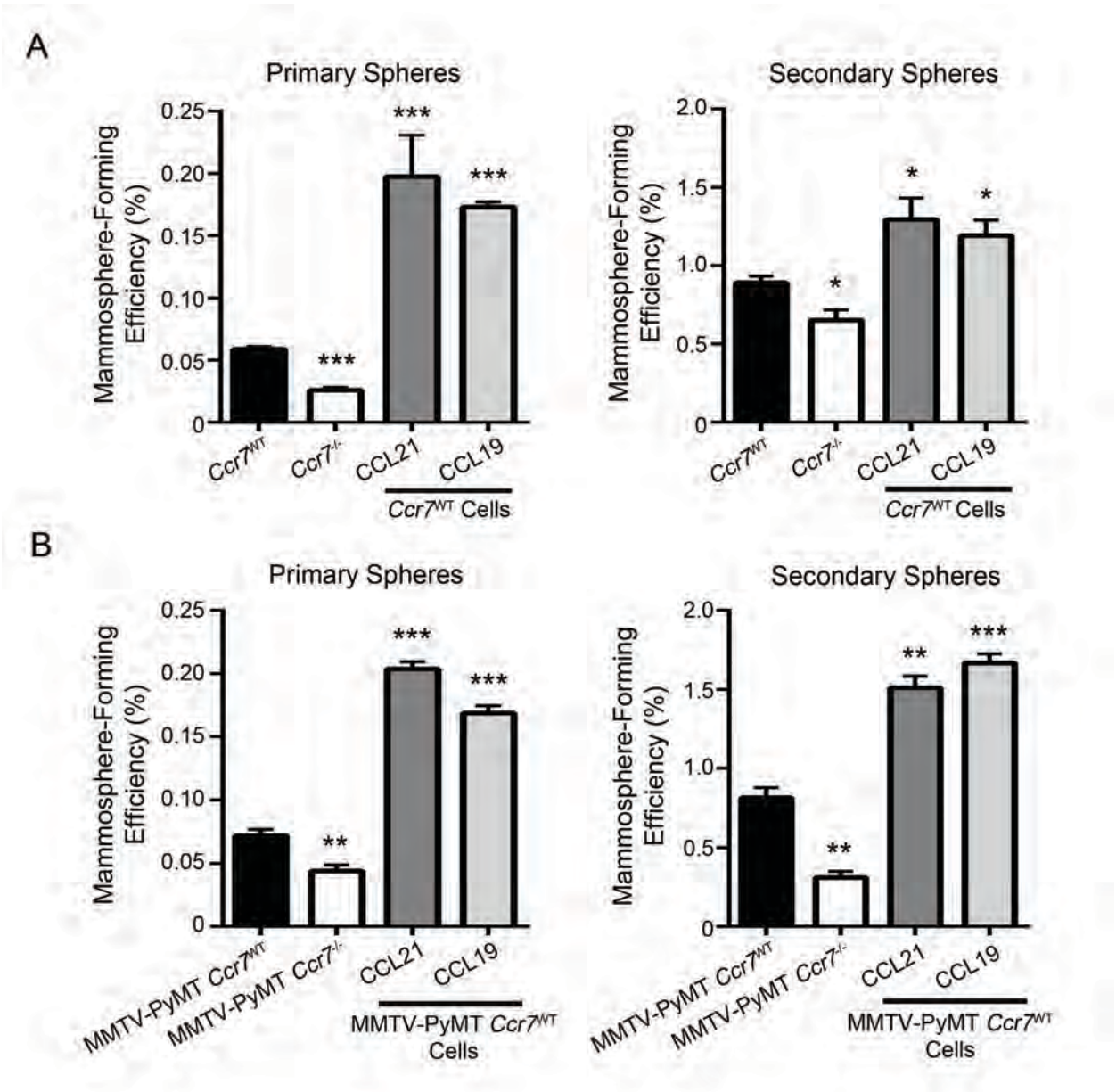

Figure 3.16: Self-renewal in normal and PyMT-expressing mouse mammary cells is regulated by CCR7.

The effect of CCR7 deletion/activation in primary and passaged secondary mammosphere culture was assessed. Shown are mammosphere-forming efficiencies of cells derived from normal mouse mammary glands **(A)** and PyMT-expressing mouse mammary glands **(B)**. Cells were stimulated with CCL21 and CCL19 as indicated. Results are representative of at least three independent experiments, n=6 mice per genotype per experiment.

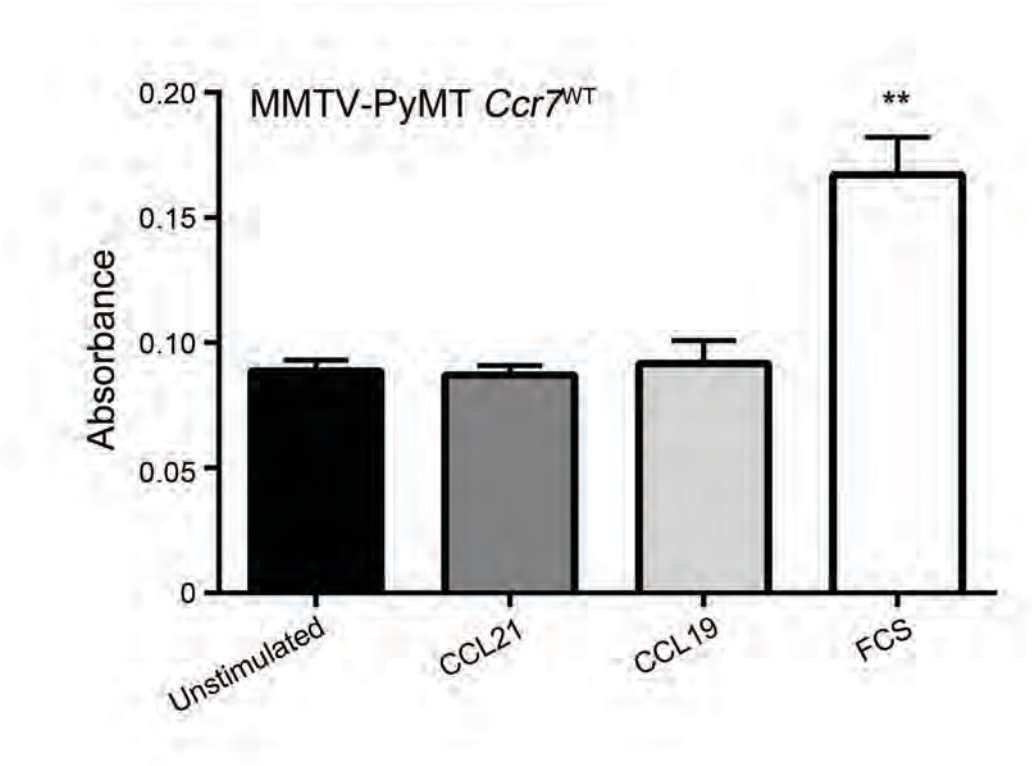

Figure 3.17: The effect of CCR7 in culture is specific to non-adherent conditions.

Results of XTT proliferation assay on MMTV-PyMT *Ccr7*^{WT} (n=3) mouse mammary cells in adherent culture with and without addition of CCR7 ligands, showing no effect on proliferation. Foetal calf serum (FCS) was used as a positive control.

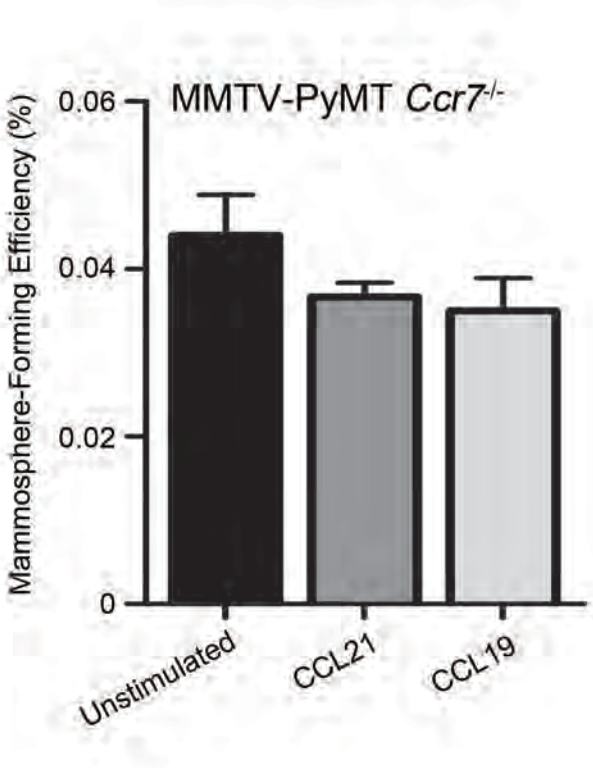

Figure 3.18: The potentiation of mammosphere formation is mediated by CCR7.

Primary mammosphere cultures derived from MMTV-PyMT *Ccr7*$^{-/-}$ (n=4) mouse mammary cells were stimulated with CCR7 cognate chemokines CCL19 and CCL21. No differences were seen in mammosphere-forming efficiency.

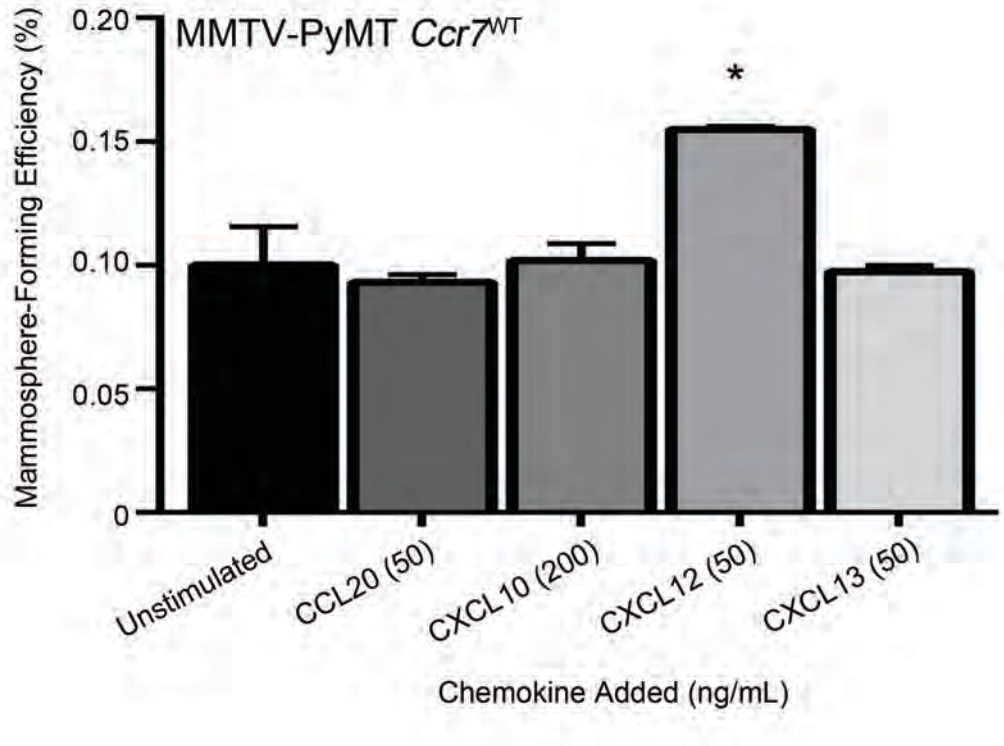

Figure 3.19: The effect on mammosphere formation by stimulation with various chemokines.

Primary mammosphere cultures derived from MMTV-PyMT *Ccr7*^{WT} (n=6) mouse mammary cells were stimulated with chemokine ligands for receptors CCR6, CXCR3, CXCR4 and CXCR5 respectively.

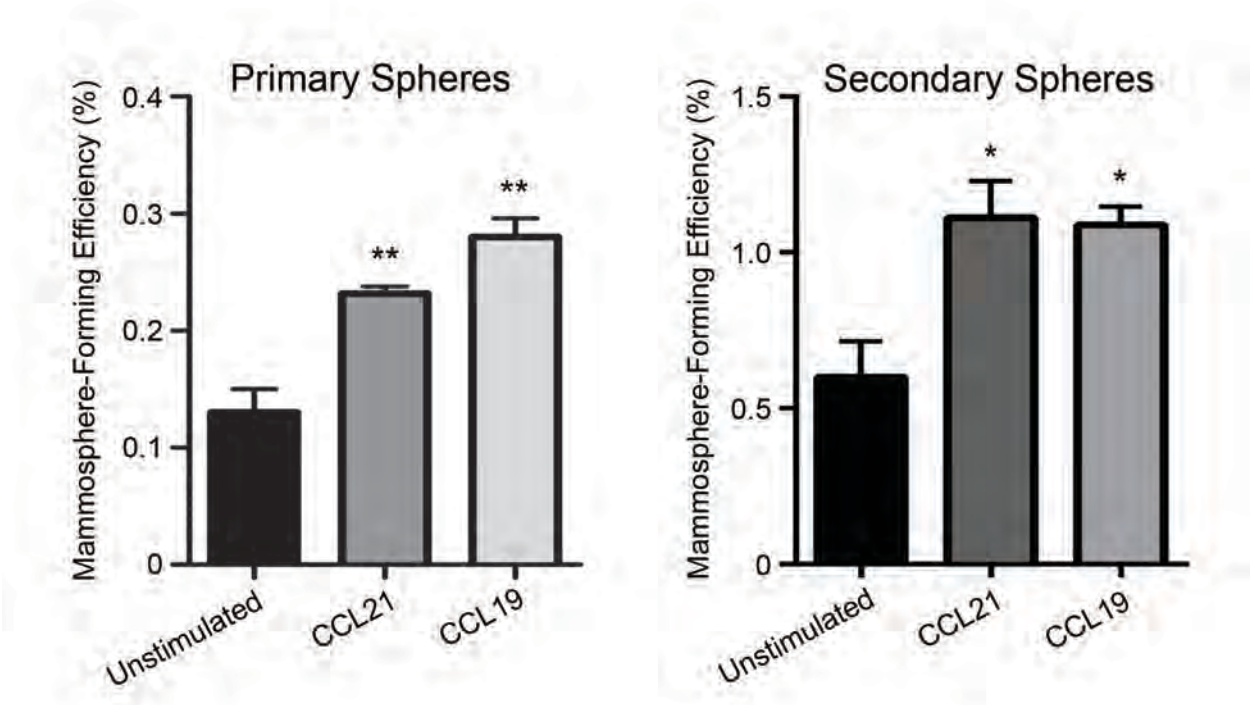

Figure 3.20: Self-renewal in human breast cancer cells is regulated by CCR7.

The effect of CCR7 activation in primary and secondary mammosphere culture was assessed. Shown are mammosphere-forming efficiencies of primary cells from human patient-derived breast tumours, where cells were stimulated with CCL21 or CCL19. Results are representative of four tumour samples.

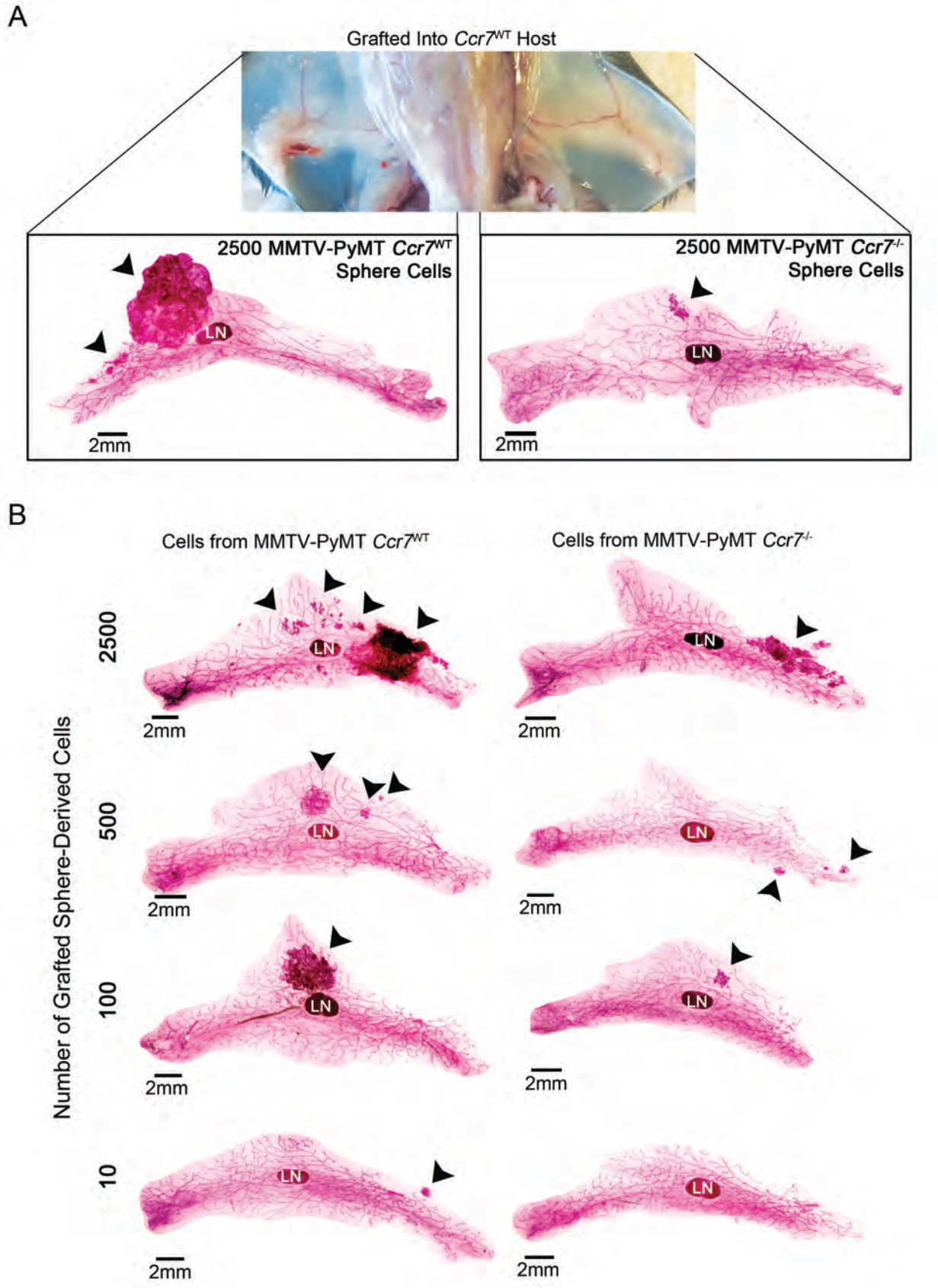
A
Grafted Into Ccr7WT Host
2500 MMTV-PyMT Ccr7WT Sphere Cells
2500 MMTV-PyMT Ccr7-/- Sphere Cells
LN
2mm
B
Cells from MMTV-PyMT Ccr7WT
Cells from MMTV-PyMT Ccr7-/-
Number of Grafted Sphere-Derived Cells
2500
500
100
10

Figure 3.21: The loss of CCR7 reduces the size of secondary lesions upon transplantation, regardless of cell concentration.

(A) Representative image of intact and respective whole-mounted contralateral mammary glands engrafted with 2500 MMTV-PyMT *Ccr7*^{WT} or *Ccr7*$^{-/-}$ mammosphere-derived cells. Black arrowheads indicate areas of outgrowth from engrafted cells. LN=lymph node. **(B)** Representative images of *Ccr7*^{WT} recipient glands engrafted with MMTV-PyMT *Ccr7*^{WT} or *Ccr7*$^{-/-}$ sphere-derived cells as indicated. Shown are contralateral glands in which both MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ cells produced outgrowths (black arrowheads), to demonstrate differences in size between lesions. At the lowest dilution, only MMTV-PyMT *Ccr7*^{WT} cells produced any secondary outgrowth. LN=lymph node.

Table 3: The presence of CCR7 in MMTV-PyMT mammosphere cells maintains the frequency of tumour-initiating cells.

Results of limiting dilution assay indicating frequency of tumour-initiating cells (TIC) in MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ mammosphere cultures. Fractions indicate the number of fat pads with lesion(s) per total number of recipient fat pads. SE=standard error.

	Number of Grafted Sphere-Derived Cells					
Donor	2500	500	100	10	TIC Frequency	Range ± SE
MMTV-PyMT *Ccr7*^{WT}	4/4	3/4	3/5	1/4	1/189*	117-306
MMTV-PyMT *Ccr7*$^{-/-}$	3/4	3/4	1/5	0/4	1/913	563-1480

*p=0.0104

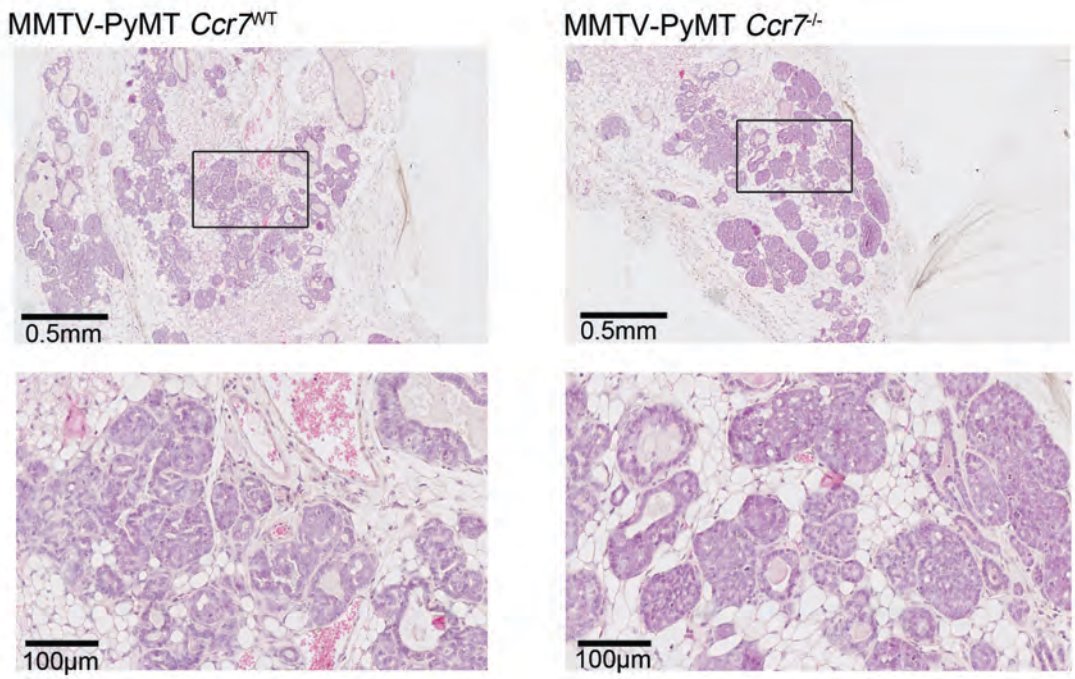

Figure 3.22: Representative H&E-stained sections from mice of transplant donor age.

Representative H&E-stained sections of pre-neoplastic mouse mammary glands at the MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ donor age of 8 weeks old. Bottom: magnified images of boxed area.

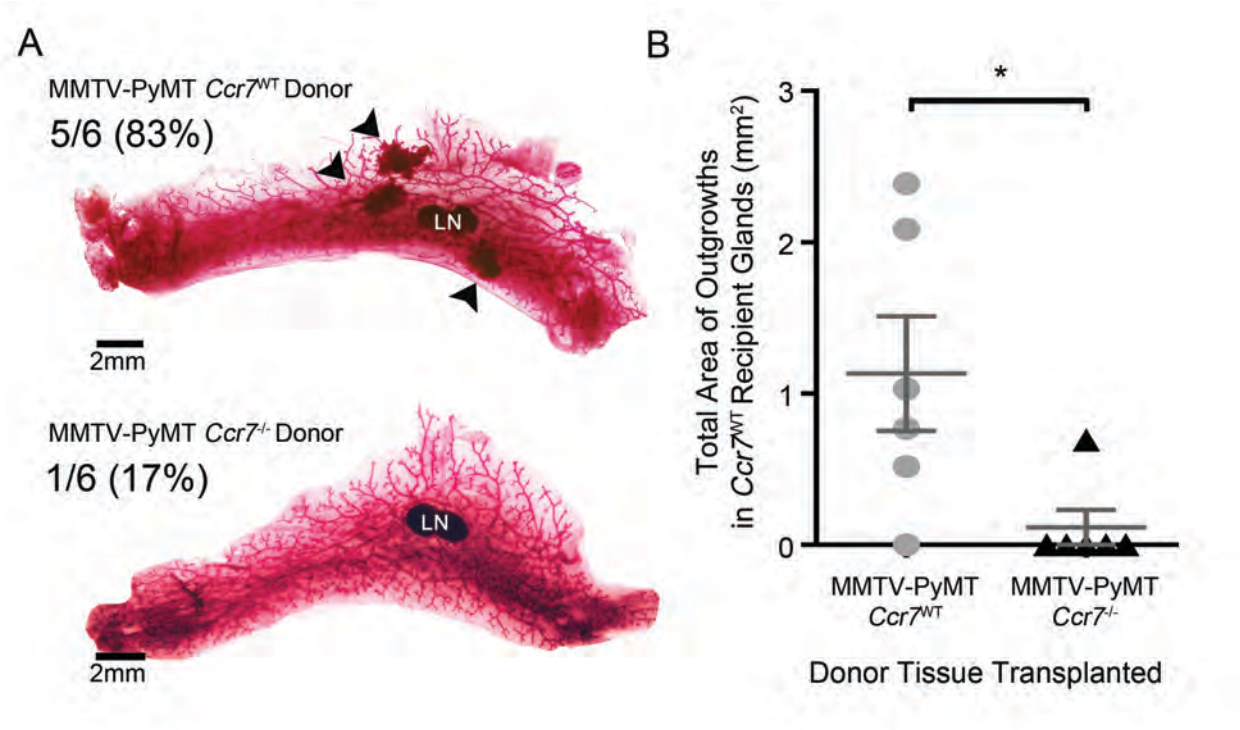

Figure 3.23: CCR7 is required for the propagation of mammary tumours.

(A) Representative whole-mount images of *Ccr7*^{WT} recipient glands after transplantation of pre-neoplastic mammary tissue from MMTV-PyMT *Ccr7*^{WT} (top) and *Ccr7*$^{-/-}$ (bottom) donor mice at 8 weeks of age. Black arrowheads indicate areas of outgrowth from donor tissue. Fractions indicate the number of fat pads with lesion(s) per total number of recipient fat pads. LN=lymph node. **(B)** Cumulative area of transplant outgrowth in recipient mammary glands. n=6 glands per group.

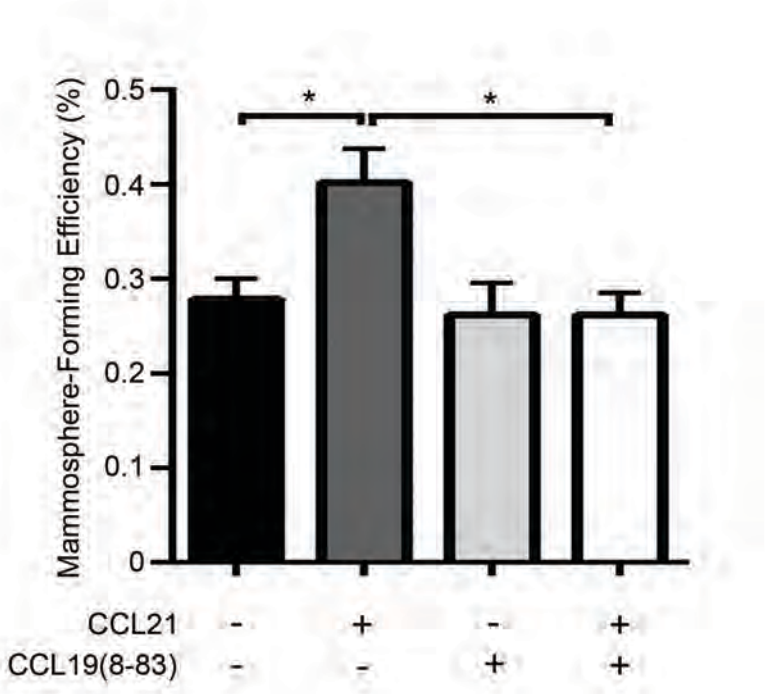

Figure 3.24: Addition of a CCR7 antagonist inhibits CCR7-mediated mammosphere formation.

Mammosphere-forming efficiency of Lin^- mammary cells from MMTV-PyMT *Ccr7*^{WT} mice (n=9), untreated or treated with CCL21 and/or the CCR7 antagonist $CCL19_{(8-83)}$.

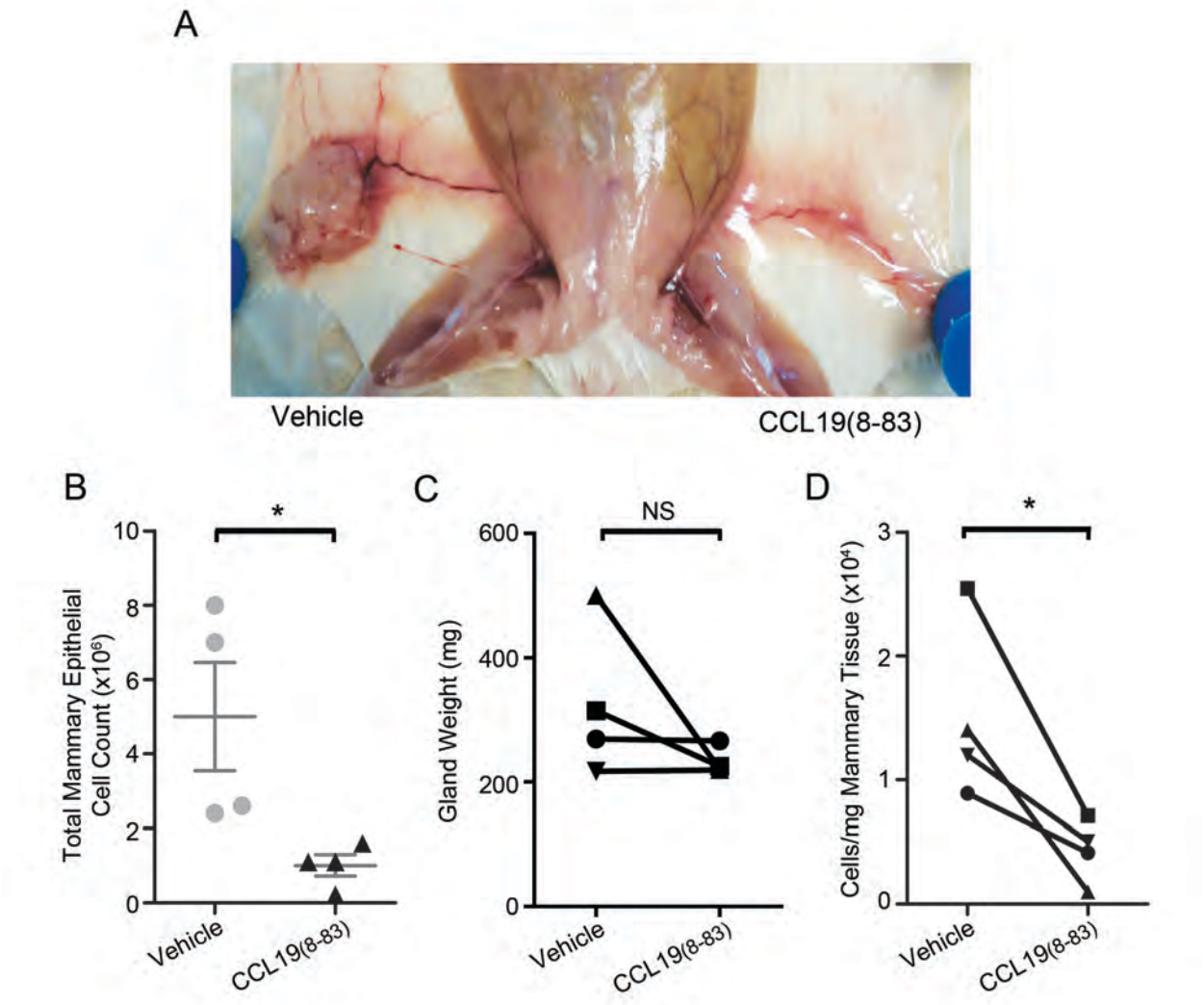

Figure 3.25: Administration of a CCR7 antagonist reduces tumourigenesis in the MMTV-PyMT mouse model.

(A) Representative image of intact MMTV-PyMT mammary glands treated with vehicle or $CCL19_{(8-83)}$ as indicated, at the time of sacrifice. **(B-C)** Total mammary epithelial cell count **(B)** and weight of mammary glands **(C)** in MMTV-PyMT mice following injection with vehicle or $CCL19_{(8-83)}$ antagonist for 8 weeks in contralateral inguinal mammary fat pads. **(D)** Cellularity of contralateral vehicle- or $CCL19_{(8-83)}$-treated glands. Data are representative of two independent experiments, n=4-6 mice per experiment.

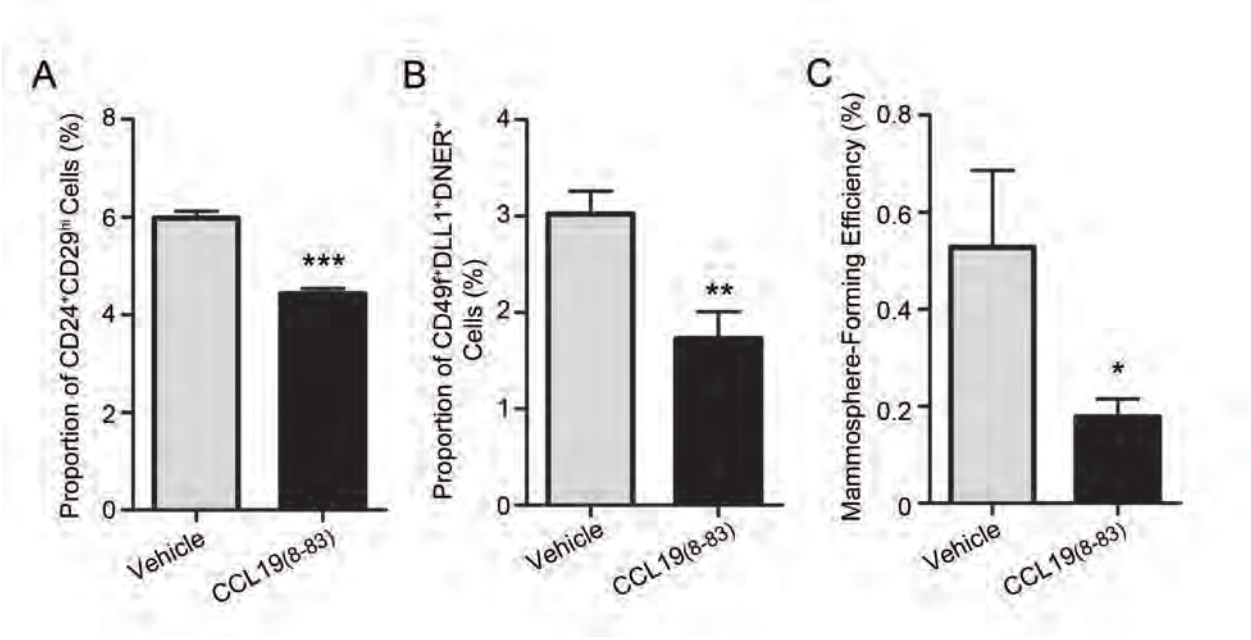

Figure 3.26: Administration of a CCR7 antagonist to PyMT-expressing mammary glands decreases the proportions and activity of stem-like cells.

(A-B) Proportions of putative stem cells as identified by $Lin^{-}CD24^{+}CD29^{hi}$ **(A)** or $Lin^{-}CD49f^{+}DLL1^{+}DNER^{+}$ **(B)** in mammary cells from vehicle- or $CCL19_{(8-83)}$-treated MMTV-PyMT glands. **(C)** Mammosphere-forming efficiency of cells from vehicle- or $CCL19_{(8-83)}$-treated mammary glands.

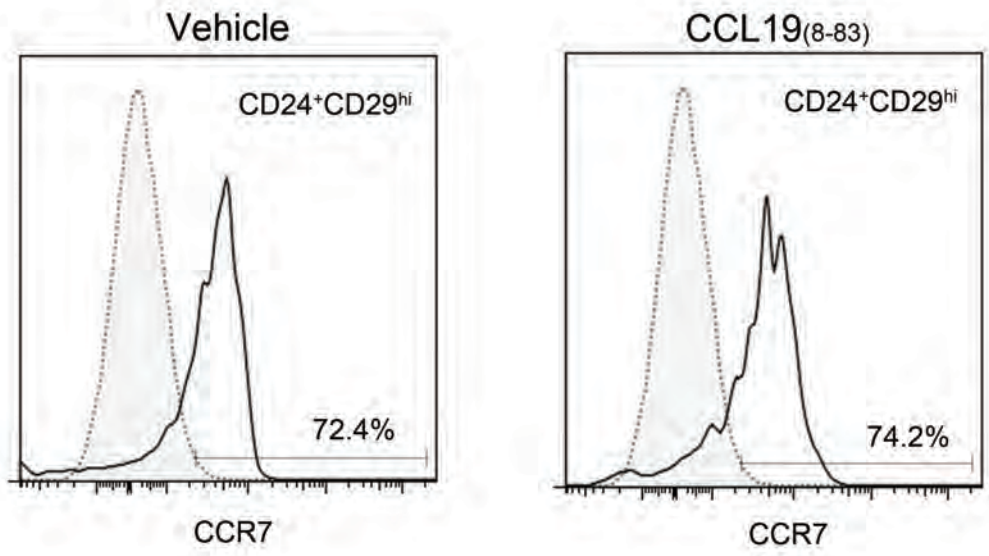

Figure 3.27: Blocking CCR7 function in PyMT-expressing mammary glands with a peptide antagonist does not affect expression of the receptor.

CCR7 expression levels in the $Lin^{-}CD24^{+}CD29^{hi}$ population as assessed by flow cytometry, following treatment of MMTV-PyMT mammary glands with vehicle control (left) or $CCL19_{(8-83)}$ (right). Shaded histograms=FMO negative controls.

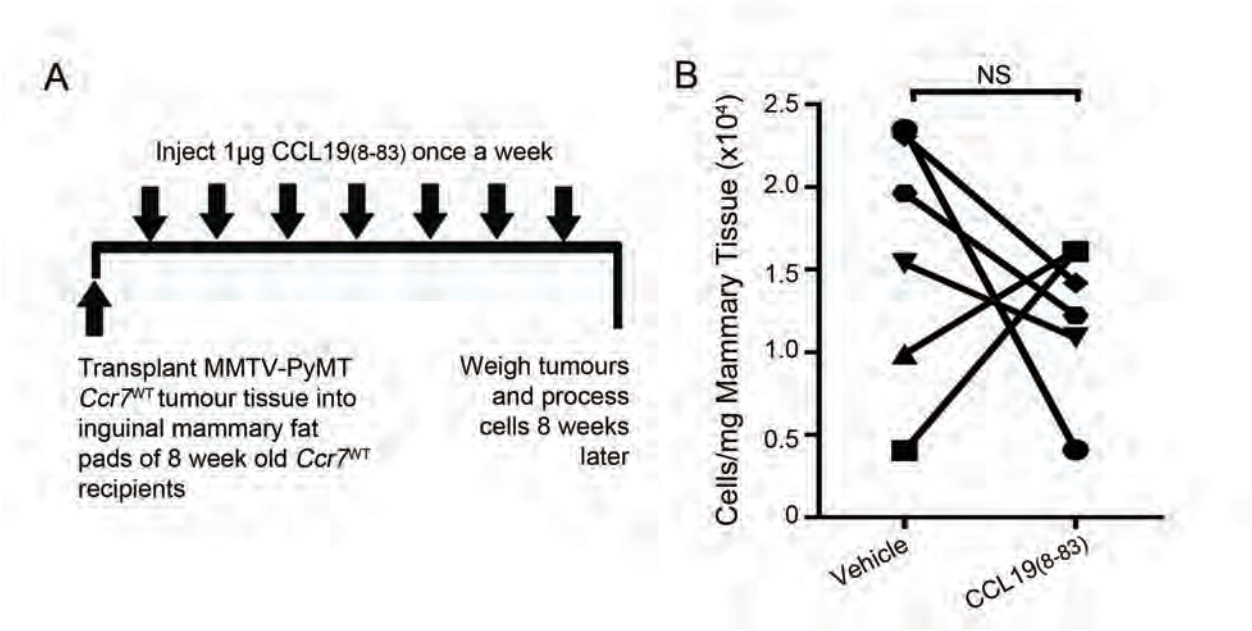

Figure 3.28: The results of CCR7 antagonism in a mammary tumour transplant model.

A transplant model was used to examine the effects of treating advanced mammary tumours with the CCR7 antagonist $CCL19_{(8-83)}$. **(A)** Experimental strategy. **(B)** Cellularity of transplant recipient glands, showing no effect following treatment with $CCL19_{(8-83)}$. n=6 recipient glands per group.

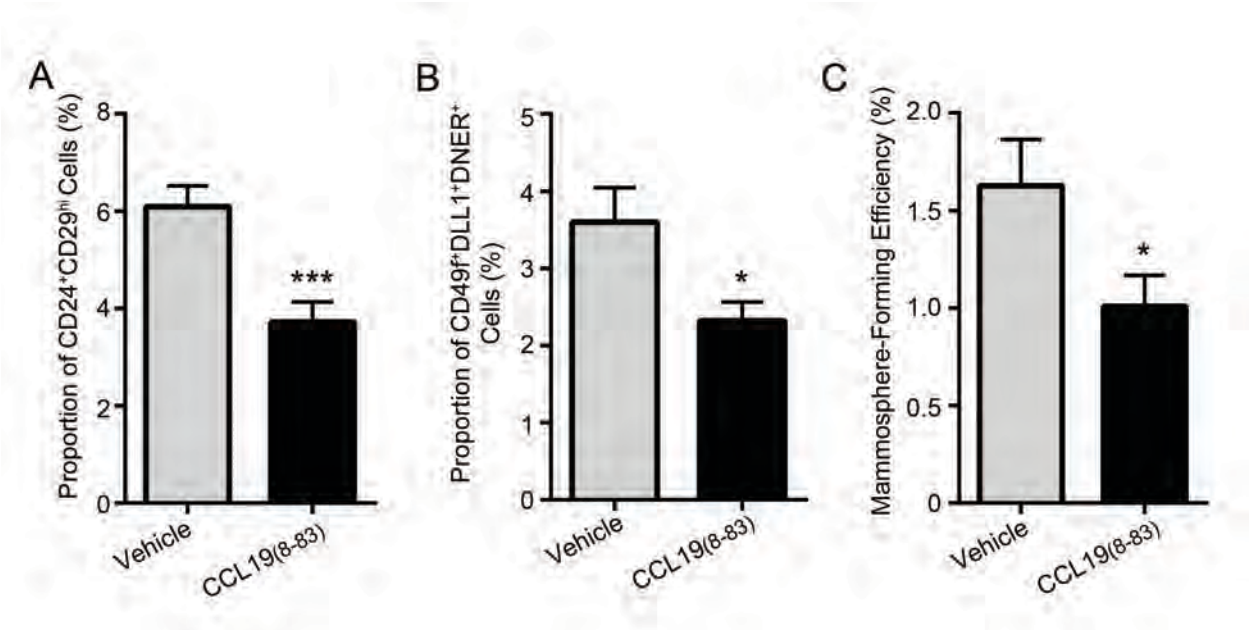

Figure 3.29: Administration of a CCR7 antagonist decreases the mammary stem cell-enriched pool and self-renewal in a transplant model.

(A-B) Proportions of putative stem cells as identified by $Lin^{-}CD24^{+}CD29^{hi}$ **(A)** or $Lin^{-}CD49f^{+}DLL1^{+}DNER^{+}$ **(B)** in mammary cells from vehicle- or $CCL19_{(8-83)}$-treated transplant recipient glands. **(C)** Mammosphere-forming efficiency of cells from vehicle- or $CCL19_{(8-83)}$-treated transplanted recipient mammary glands.

CHAPTER 4: The Chemokine Receptor CCR6 Facilitates the Onset of Mammary Neoplasia in the MMTV-PyMT Mouse Model via Recruitment of Tumour-Promoting Macrophages

4.1. Introduction

Breast cancer is one of the leading causes of cancer-related death in women world-wide. Evasion of the immune system is a hallmark of cancer, and aids tumour cells to survive, intravasate, and potentially form distal metastases [27]. As such, the tumour microenvironment has a profound effect on the development and progression of malignancies, and it has been suggested that levels of infiltrating immune cells correlate with stage and aggressiveness of human breast cancer [189]. In particular, tumour-associated macrophages (TAMs) have been found to play an important part in facilitating breast tumour development [190] through polarisation from a classically-activated "M1" anti-tumour resident cell within adult mammary tissue to an alternatively-activated "M2" pro-tumour phenotype [112]. This "switch" results in shifts in cell metabolism, a decrease in pro-inflammatory chemokine/cytokine production, poor antigen-presentation ability, and suppression of T cell responses. In addition, M2 TAMs promote angiogenesis, cell proliferation and tissue remodelling [113].

Chemokines and their cognate receptors are involved in the development, migration and activation of many different types of immune cells, both adaptive and innate. Low molecular-weight proteins, chemokines bind to their cognate seven-transmembrane domain G-protein coupled receptors (GPCRs), activating a multitude of signalling pathways, which mediate many different homeostatic and inflammatory functions. Importantly, a large body of literature in the last decade has linked the action of chemokines and chemokine receptors to cancer progression and metastasis [89].

The CC-chemokine receptor CCR6 is expressed on dendritic cells [191, 192], regulatory T cells and various T helper lymphocyte subsets [166, 193], and mediates their migration and function via stimulation with its ligand CCL20 (also known as macrophage inflammatory protein (MIP)-3α [164]). CCR6 is also expressed on natural killer cells, B lymphocytes, neutrophils [194] and macrophages [193, 195]. Despite the significant role of TAMs in breast cancer, the expression

and function of CCR6 within the macrophage population has not been shown within the mammary gland.

Interestingly, together with CCL20, CCR6 expression has been correlated with stage and prognosis in a variety of cancers including hepatocellular carcinoma [196, 197], colorectal carcinoma [198-200], glioma [201], and non-small cell lung cancer [202], and a function for CCR6 in regulation of cancer progression has been putatively demonstrated using cell lines and xenograft models [198, 199, 203, 204]. In breast cancer, higher CCR6 expression levels were linked with tumour stage and grade [163], and incidence of metastasis to the pleura [124]. Stimulation of *ex vivo* mammary peritumoural cells with CCL20 was found to increase their proliferation rate, invasiveness and migration [168]. CCL20 is also up-regulated in human triple negative breast cancer cell lines [167]. Moreover, it was recently proposed that the presence of CCR6 may act as a prognostic factor for breast cancer patient survival [163]. However, no causative or functional link between the CCR6-CCL20 axis and progression of breast cancer has been documented to date.

In this study, a well-characterised transgenic model for breast cancer was utilised, in which the polyoma middle-T oncogene is activated under control of the mouse mammary tumour virus promoter (MMTV-PyMT) [169]. This transgenic model has been shown to closely mimic the stages of human breast disease from initial hyperplasia, through to ductal carcinoma *in situ* and invasive ductal carcinoma [170]. Crossing this transgenic mouse with a CCR6-null mouse to generate a bigenic MMTV-PyMT *Ccr6*$^{-/-}$ animal model has allowed direct assessment of the role of CCR6 in mammary tumourigenesis *in vivo*. The results demonstrated that CCR6 promotes breast cancer initiation and progression through maintenance of pro-tumourigenic TAMs within tumour-bearing mammary glands, warranting further investigation of CCR6 as a possible therapeutic target.

4.2. Results

4.2.1. CCR6 expression increases throughout cancer development and results in a higher number of mammary tumours

To first determine whether CCR6 may play a role in the regulation of mammary neoplasia, expression of the receptor was investigated in CD45-negative normal mouse mammary cells, and cells from various tumour stages (representative H&E pictures in ***Figure 4.1***). CCR6 was expressed on a low proportion of normal mammary cells, but this proportion was greatly amplified in accordance with increasingly higher grades of MMTV-PyMT cancer including initial hyperplasia, early carcinoma and late carcinoma as indicated (***Figure 4.1***). This is consistent with human breast cancer [163] and other mouse models of cancer [200]. Additionally, in both non-PyMT *Ccr6*^{WT} and MMTV-PyMT *Ccr6*^{WT} mammary tissues, the ligand for CCR6, CCL20, was highly expressed at concentrations over 50ng/mg tissue (***Figure 4.2***). These data raised the possibility of a role for CCR6 in breast cancer development.

To next establish the role of CCR6 deletion on mammary tumourigenesis, the rate and total extent of PyMT-driven neoplasia between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice was compared. Tumour onset was significantly delayed in MMTV-PyMT *Ccr6*$^{-/-}$ mice (***Figure 4.3a***), with some mice not developing palpable tumours until 150 days old (21 weeks) compared to a maximum onset age of 130 days old (18 weeks) for MMTV-PyMT *Ccr6*^{WT} counterparts (***Figure 4.3b***).

In order to assess the impact of CCR6 on the later stages of cancerogenesis, MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice were sacrificed at 22-24 weeks of age and the total number of mammary tumours per mouse was determined. It was found that MMTV-PyMT *Ccr6*$^{-/-}$ mice had significantly decreased tumour incidence compared to MMTV-PyMT *Ccr6*^{WT} animals (***Figure***

4.3c). Together, these results implicated CCR6 as being an important player in breast oncogenesis.

4.2.2. CCR6 deletion significantly delays tumour initiation *in vivo*

It was then examined as to whether CCR6 influenced early hyperplasia of mammary glands during tumour initiation as well as late stage tumourigenesis. Glands from 8 week-old MMTV-PyMT *Ccr6*$^{-/-}$ and *Ccr6*^{WT} mice were extracted and whole-mounted for quantification of hyperplastic/early-neoplastic lesion area (representative images from both genotypes shown in ***Figure 4.4a***). It was found that the deletion of CCR6 significantly reduced the initial hyperplastic outgrowth within the gland (***Figure 4.4b***), a common indicator of future breast cancer development. As the total area of PyMT-driven hyperplastic outgrowth per gland was reduced by three-fold in CCR6-null animals, it was concluded that the effect of CCR6 on mammary tumourigenesis is manifested very early on in cancer development.

The difference seen in early tumour initiation between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice can potentially result from a difference in normal mammary development, which may then have translated into decreased hyperplasia. Therefore, pubertal mammary glands were extracted from non-PyMT 6 week-old *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice. Representative glands are shown in ***Figure 4.5a***. When ductal epithelial growth was quantitated, no statistically significant difference was observed in ductal length, number of terminal end structures or branching between *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice (***Figure 4.5b-d***), and hence the overall effect of CCR6 deletion on normal mammary gland biology appeared to be minimal and was unlikely to account for differences in PyMT-driven tumour development between the two genotypes. Furthermore, the levels of CCL20 were not statistically different between non-PyMT *Ccr6*^{WT} and MMTV-PyMT *Ccr6*^{WT} mammary tissues (***Figure 4.2***), demonstrating that the expression of CCL20 was not affected by the process of tumourigenesis. Taken together, these data showed that early stage tumourigenesis is mediated by a CCR6-dependent mechanism, without affecting normal mammary morphogenesis.

4.2.3. CCR6 promotes mammary gland neoplasia independently of cancer epithelial cells or stem-like cells

To investigate the mechanism underlying CCR6-driven mammary tumourigenesis, the epithelial cell population was studied to determine if CCR6 was having a direct effect on cell proliferation. Cells at the stage of early neoplasia from MMTV-PyMT *Ccr6*^{WT} mammary glands were assayed for proliferation upon stimulation with CCL20. No differences in cell proliferation were observed (***Figure 4.6***). Furthermore, Ki67 staining of sectioned hyperplastic mammary glands from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice showed that epithelial cells in knockout mice were still able to adequately proliferate and staining of Ki67 was equal to that in the *Ccr6*^{WT} (***Figure 4.7***). This suggested that the role of CCR6 in breast cancer is independent of epithelial cells.

It was then determined whether CCR6 may exert its effect by skewing distinct cell populations within the bulk epithelium, as reported previously for the chemokine receptor CCR7 [171]. The current prevailing paradigm has mammary epithelial and breast cancer cells hierarchically organised with a self-renewing, quiescent, multipotent progenitor (or stem-like cell) population giving rise to basal and luminal progenitors which in turn differentiate into specific lineages making up the mammary gland and heterogenous breast tumours [205]. Recently, a number of immune mediators including chemokine receptors have been implicated in maintenance of the cancer stem-like cells within mammary tumours [115]. Therefore, the potential link between the tumour-promoting function of CCR6 and breast cancer stem-like cell pools was tested.

Freshly isolated MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mammary cells from pre-neoplastic mice at 8-9 weeks old were assayed by flow cytometry for expression of cell surface markers CD24 and CD29 [60] (representative plots shown in ***Figure 4.8a***), which were previously used to define stem cells in the MMTV-PyMT [184, 185] and other breast cancer mouse models [65, 206]. It was found that the deletion of CCR6 did not alter the proportions of the stem cell-enriched basal

population ($CD24^{+}CD29^{hi}$, ***Figure 4.8b***) nor the luminal population ($CD24^{+}CD29^{lo}$, ***Figure 4.8c***) in hyperplastic mammary glands.

The effect of CCR6 ablation on functional stem-like mammary cancer cell pools was also investigated using the mammosphere assay, which is used to select for colonies of early stem-like progenitors [75]. Pre-neoplastic MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mammary cells were seeded into non-adherent mammosphere culture and allowed to grow for 7 days. The deletion of CCR6 did not alter mammosphere-forming efficiency (***Figure 4.9a***), and when sphere cultures were stimulated with varying concentrations of CCL20, no change in their ability to form mammospheres (***Figure 4.9b***) was observed, supporting results obtained using flow cytometric analysis.

Having found no difference in the proportion or function of stem cell-like pools within early tumourigenic lesions, a *bona fide* property of cancer stem cells was then tested – their ability to propagate tumours upon transplantation. MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ donor mammary tissue was transferred into recipient fat pads of syngeneic non-PyMT *Ccr6*^{WT} mice. Using this approach, no significant difference was found between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ tissue in the ability to form outgrowths when transplanted into *Ccr6*^{WT} recipients (***Figure 4.10***), indicating that the deletion of CCR6 did not reduce the tumour-propagating capability of mammary epithelium.

Altogether, these results demonstrated that the role of CCR6 in breast cancer is independent of breast epithelial and progenitor cells, raising the possibility that its mechanism of action involves the tumour microenvironment.

4.2.4. CCR6 mediates the recruitment of pro-tumourigenic macrophages to the mammary tumour microenvironment

To test whether the reduced mammary tumourigenesis caused by the deletion of CCR6 was due to an effect of the CCR6-CCL20 axis on the tumour microenvironment, flow cytometry was used to investigate the levels and identity of tumour-infiltrating leukocytes in mammary tumours at the stage of early carcinoma (see ***Figure 4.1***). Tumour-associated macrophages have been extensively implicated in tumour promotion both in the mammary gland and elsewhere, due to their role in angiogenesis, cell proliferation and tissue remodelling [113]. To initially examine the polarisation of TAMs in the MMTV-PyMT mice, macrophages were assessed for expression of prototypic markers interleukin-4 receptor (IL-4R) and mannose receptor (CD206) (gating strategy shown in ***Figure 4.11***), which have been used previously in flow cytometric analysis to distinguish alternatively-activated M2 macrophages from classically-activated M1 [107, 207-210]. It was estimated using these markers that a high proportion of TAMs were of an M2-like phenotype (***Figure 4.11***), as has been suggested previously for MMTV-PyMT mammary tumours [211]. Interestingly, CCR6 was found to be highly expressed within the TAM population as it was detected on greater than 60% of total macrophages (***Figure 4.12a***). Most importantly, CCR6 was expressed at higher levels and on a significantly higher proportion of putative M2 macrophages (up to 90%) than M1 (***Figure 4.12a-b***), using both IL-4R and CD206 to delineate the populations. This strong correlation potentially implicates CCR6 in the regulation of pro-tumourigenic macrophages within the mammary gland microenvironment.

The levels of macrophages were then assessed in mammary tumours from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice, and it was found that the proportion and overall numbers of TAMs were significantly reduced in MMTV-PyMT *Ccr6*$^{-/-}$ mammary tumours relative to *Ccr6*^{WT} (***Figure 4.13***). Furthermore, the deletion of CCR6 resulted in a shift towards an M1 macrophage phenotype, defined by both IL-4R and CD206 prototypic markers (***Figure 4.14***).

The deletion of CCR6 also resulted in reduced trafficking of dendritic cells to the tumour site, consistent with previous studies, which demonstrated a reduced overall migratory ability of dendritic cells in CCR6-null mice [165, 212]. Although, overall numbers of tumour-infiltrating dendritic cells were much lower than the corresponding macrophage population. Notably, the majority of tumour-infiltrating dendritic cells were CCR6-positive, consistent with previous findings [194] (***Figure 4.15***). CCR6 expression was also assessed on B cells and specific T cell subsets (helper T cells (Th), cytotoxic T cells (Tc), and regulatory T cells (Treg)) within mammary tumours. In agreement with earlier reports for various biological settings [194, 213], all tested infiltrating leukocyte subsets expressed CCR6 at varying levels. When CCR6 was ablated, only the Tc cell subset showed a slight increase in the MMTV-PyMT *Ccr6*$^{-/-}$ as a proportion of CD45^{+} tumour-infiltrating cells, however no significant differences were found in total cell numbers between the two genotypes (***Figures 4.16-4.20***).

These findings thus suggest that CCR6 promotes mammary tumourigenesis through an epithelium-independent mechanism involving tumour-infiltrating macrophages.

4.2.5. CCR6-mediated pro-tumourigenic macrophages promote breast cancer *in vivo*

To provide definitive evidence for the macrophage-mediating function of CCR6 in mammary tumour promotion, an *in vivo* macrophage reconstitution assay was used. Reconstitution assays, sometimes referred to as “add-back” assays, are frequently used to underscore a role for various cellular subsets in multiple pathological settings, and macrophage reconstitution has been used previously in mammary gland studies [214]. A schematic of the experimental setup is shown in ***Figure 4.21a***. MMTV-PyMT mammary tumour cells from *Ccr6*^{WT} donor mice were purified and transplanted into the inguinal mammary fat pads of non-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ recipients at 5 weeks of age. Two days post-transplantation, TAMs (CD45^{+}F4/80^{+}) were sorted from excised and dissociated MMTV-PyMT *Ccr6*^{WT} tumours (***Figure 4.21b***) and orthotopically injected into a

group of *Ccr6*$^{-/-}$ recipients as specified in ***Figure 4.21a***. All other recipients received sham injections with vehicle only, followed by assessment of mammary tumour growth 6 weeks later.

In agreement with the results from spontaneous tumourigenesis studies, it was found that tumours grew significantly slower in the *Ccr6*$^{-/-}$ hosts compared to *Ccr6*^{WT}, indicating that CCR6 was required in the mammary stroma for robust tumour development. However, when the reduced macrophage phenotype was restored in *Ccr6*$^{-/-}$ mice through orthotopical injections, the tumour latency was significantly shortened, approaching that of the *Ccr6*^{WT} mice (***Figure 4.22***).

It was further found that the supplementation of *Ccr6*$^{-/-}$ mice with TAMs restored the efficiency of tumour growth (measured by weight of tumour-bearing mammary glands) within these mice to that seen in the *Ccr6*^{WT}, whilst *Ccr6*$^{-/-}$ mice that received sham injections displayed reduced tumourigenesis (***Figure 4.23***) as seen in the spontaneous model (see ***Figure 4.3***).

Enumeration of macrophages within grafted tumours in *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice (***Figure 4.24***) paralleled the results seen in spontaneous mammary tumours (see ***Figure 4.13***). Whilst there was an upward trend towards increased TAMs in the *Ccr6*$^{-/-}$ mice that received TAM injections, the difference was not statistically significant (***Figure 4.24***). This, combined with the decreased tumour growth in the CCR6-null mice, indicated that the support of macrophages is essential at the early stages of tumour growth.

Thus, these data established an essential role for CCR6 in the tumour microenvironment, providing a causative link between this receptor, infiltrating macrophages and mammary tumour development. Hence, therapeutic opportunities may be explored to control breast cancer progression, via manipulation of the CCR6-CCL20 axis to control tumour-promoting macrophages.

4.3. Summary

The expression of the chemokine receptor CCR6 has been previously correlated with higher grades and stages of breast cancer and decreased relapse-free survival. Also, its cognate chemokine ligand CCL20 has been reported to induce proliferation of cultured breast epithelial cells. This study investigated whether CCR6 plays a functional role in mammary tumourigenesis, using a bigenic MMTV-PyMT CCR6-null mouse to assess mammary tumour development. Levels of tumour-infiltrating immune cells within tumour-bearing mammary glands from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice were also analysed.

Deletion of CCR6 delayed tumour onset, significantly reduced the extent of initial hyperplastic outgrowth, and decreased tumour incidence in PyMT transgenic mice. CCR6 was then shown to promote the recruitment of pro-tumourigenic macrophages to the tumour site, facilitating the onset of neoplasia. In conclusion, this study delineated for the first time a role for CCR6 in the development of breast cancer, and demonstrated a critical function for this receptor in maintaining the pro-tumourigenic cancer microenvironment.

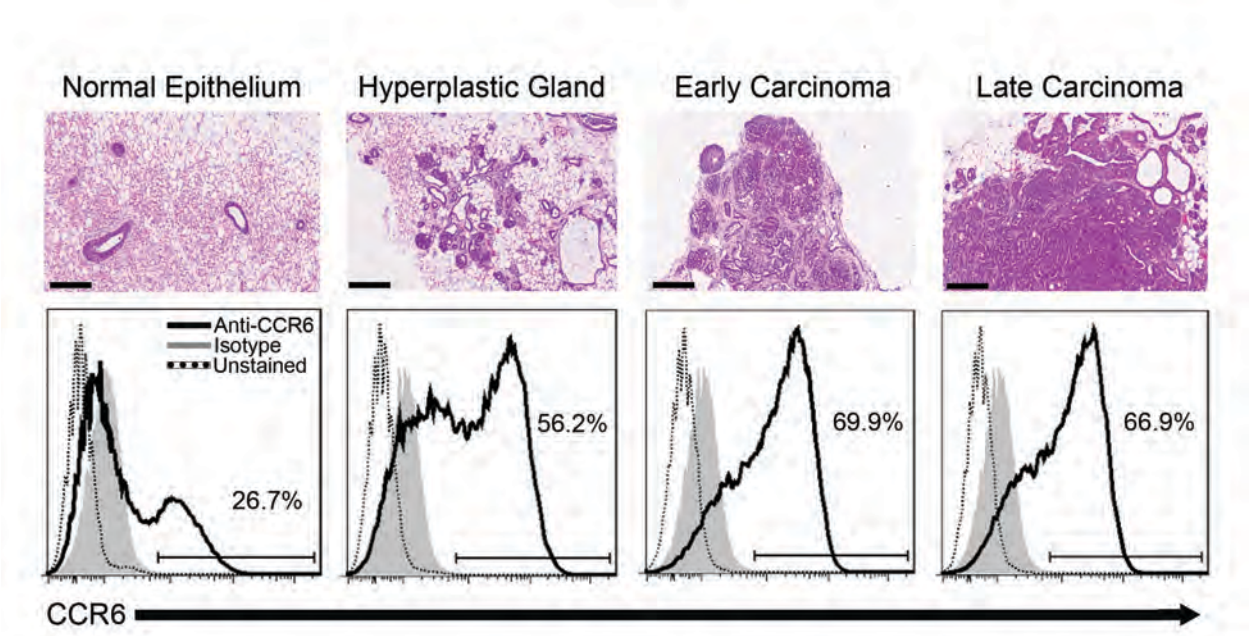

Figure 4.1: CCR6 expression increases throughout progression of mammary cancer.

Top: Representative H&E images of mammary tissue from normal gland and various stages of PyMT-driven tumourigenesis as indicated. Scale bar is 200µm. Bottom: Proportion of CCR6-positive epithelial cells (CD45-negative) purified from mammary glands at respective stages of tumourigenesis. 18 normal samples (2 glands/sample), 7 hyperplastic samples (2 glands/sample), 6 early carcinomas and 3 late carcinomas were analysed.

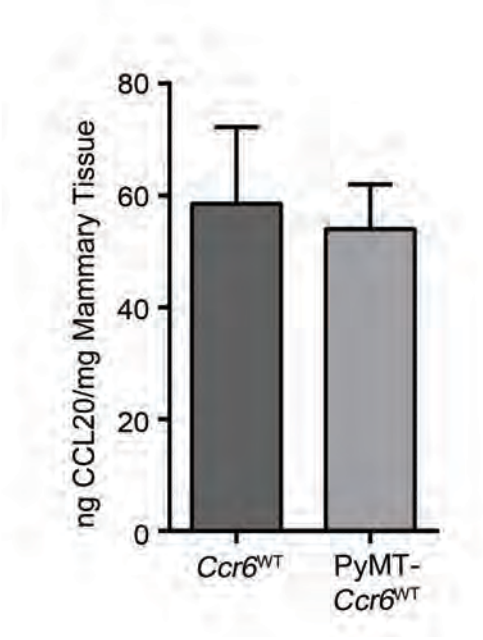

Figure 4.2: The ligand for CCR6, CCL20, is expressed in both normal and cancerous mammary glands.

Expression of CCL20, the ligand for CCR6, in normal and early neoplastic MMTV-PyMT *Ccr6*^{WT} mammary glands, as determined by ELISA. n=4 samples per genotype.

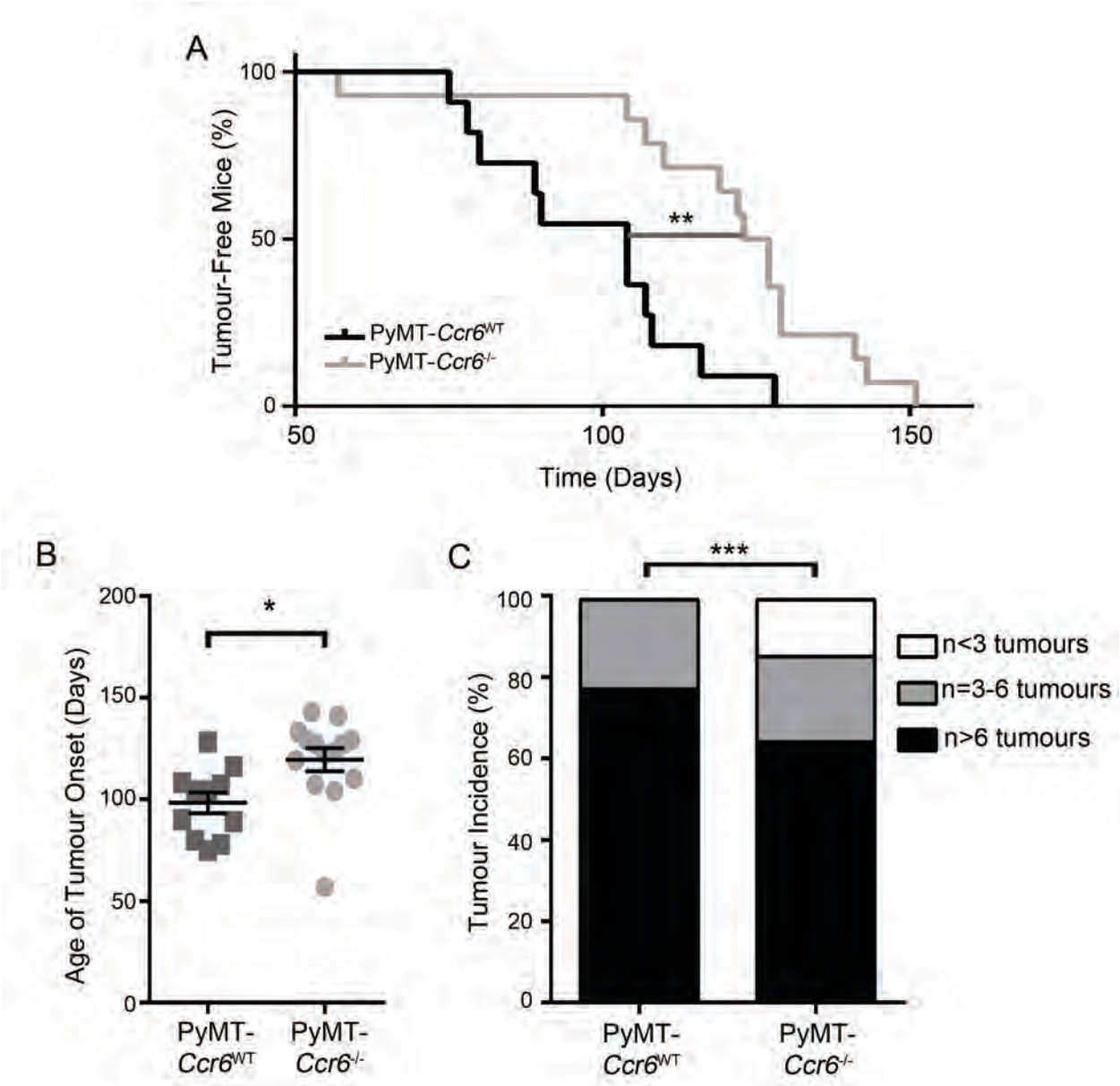

Figure 4.3: CCR6 deletion reduces mammary tumourigenesis.

(A) Kaplan-Meier analysis of the palpable tumour onset in MMTV-PyMT *Ccr6*$^{-/-}$ mice (n=14) compared to MMTV-PyMT *Ccr6*^{WT} mice (n=11). **(B)** Age of tumour onset in MMTV-PyMT *Ccr6*$^{-/-}$ mice and MMTV-PyMT *Ccr6*^{WT} mice. **(C)** Mammary tumour incidence at the time of sacrifice in MMTV-PyMT *Ccr6*^{WT} (n=9) and *Ccr6*$^{-/-}$ (n=14) mice.

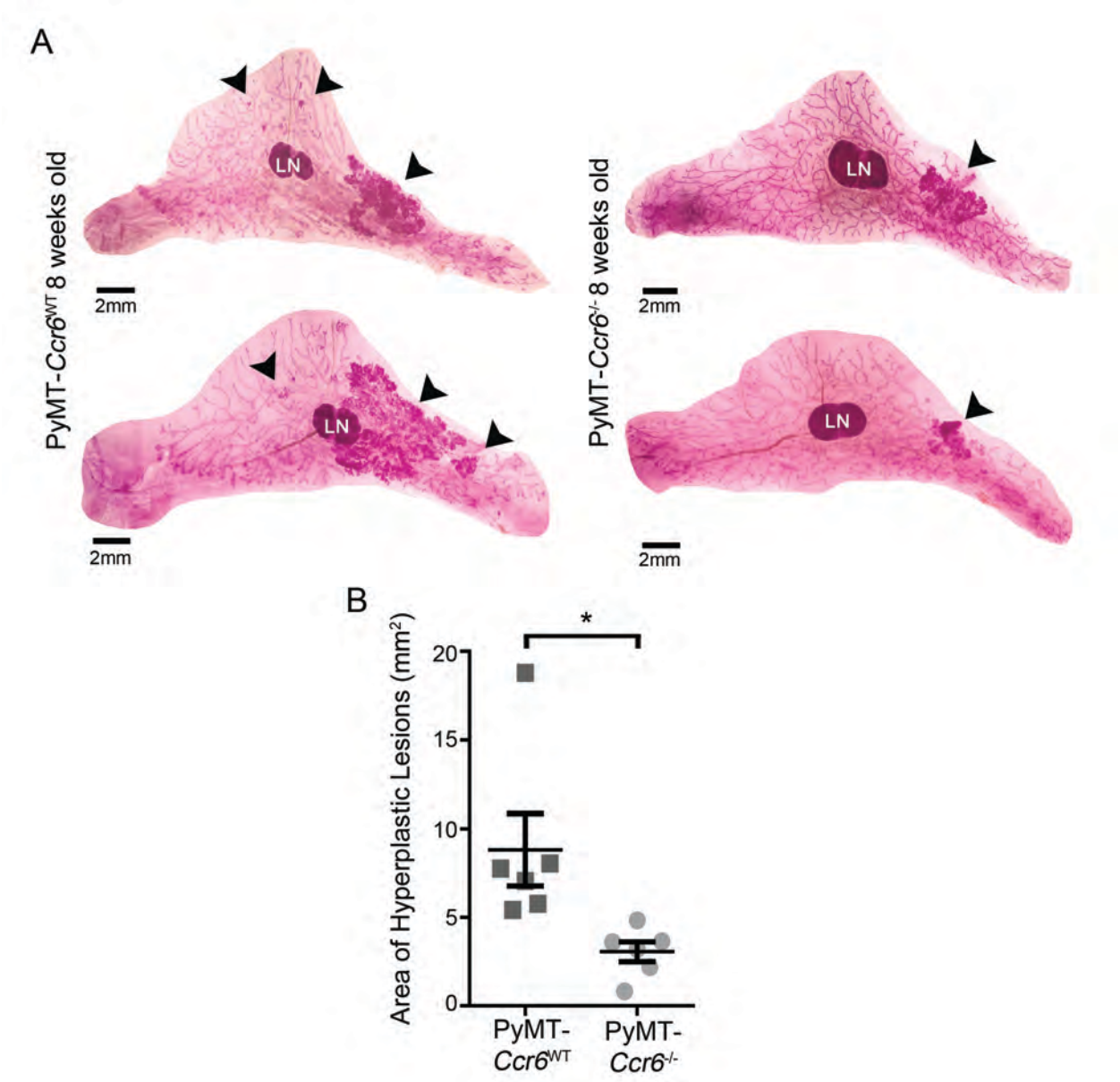

***Figure 4.4: CCR6 deletion significantly delays tumour initiation* in vivo.**

(A) Representative whole-mount images of MMTV-PyMT *Ccr6*^{WT} (n=6) and *Ccr6*$^{-/-}$ (n=6) mammary glands from mice at 8 weeks of age. LN=lymph node. Black arrowheads indicate hyperplastic lesions within the gland. **(B)** Quantitation of area of pre-neoplastic lesions in 8 week-old MMTV-PyMT mouse mammary glands.

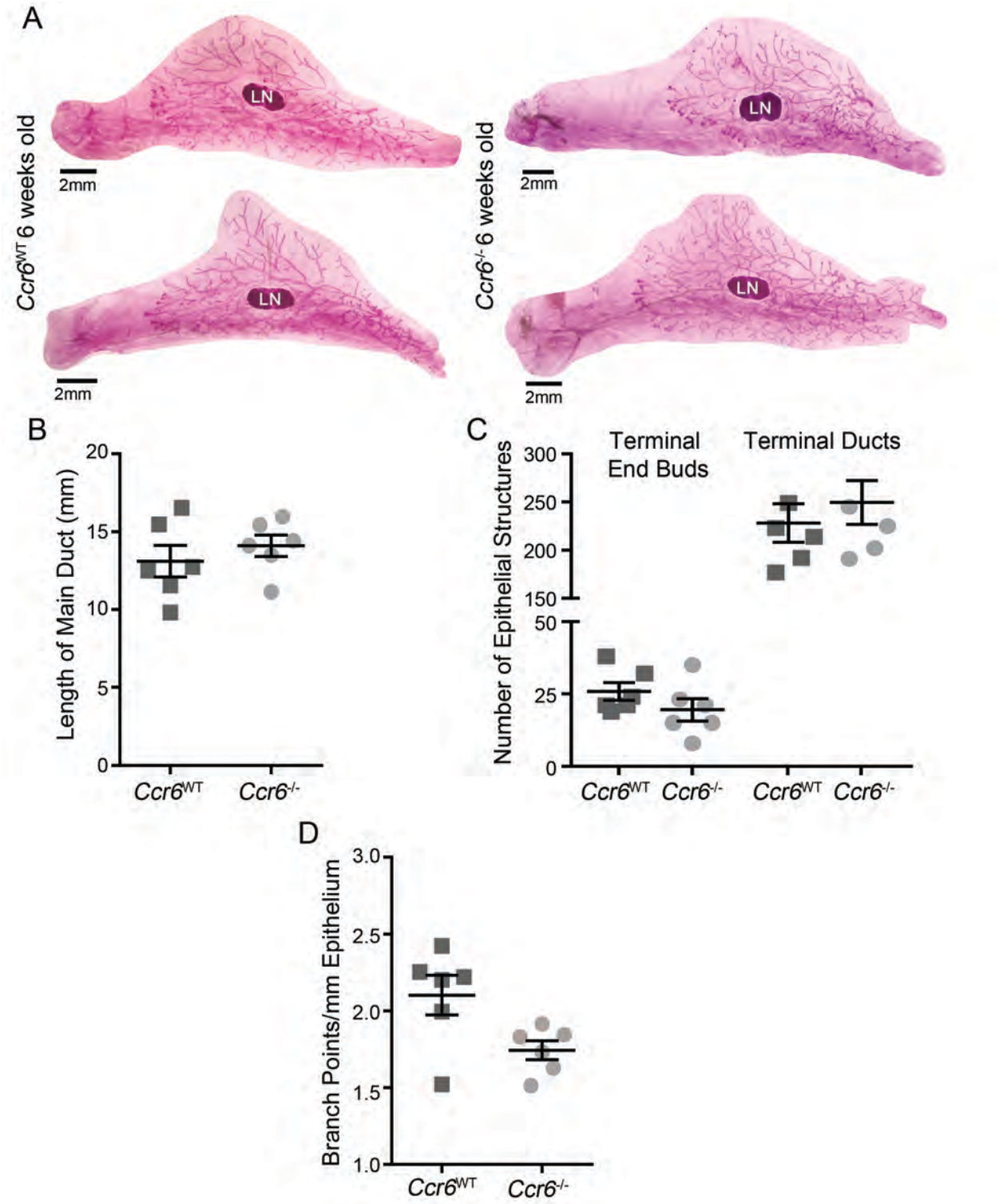

Figure 4.5: The role of CCR6 in normal mammary development.

(A) Representative whole-mount images of *Ccr6*^{WT} (n=6) and *Ccr6*$^{-/-}$ (n=6) mammary glands from mice at 6 weeks of age showing development of mammary epithelium. LN=lymph node. **(B-D)** Quantitation of epithelial growth, by length of main duct **(B)**, number of end structures **(C)** and branching **(D)**.

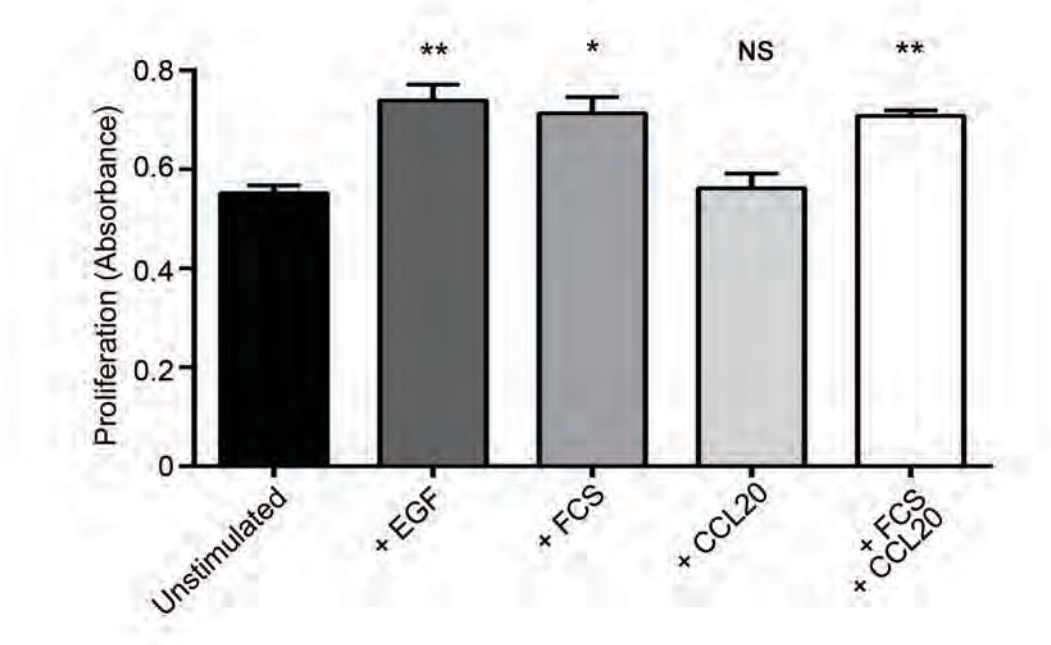

Figure 4.6: Stimulation of CCR6 does not affect proliferation of epithelial tumour cells.

Results of XTT proliferation assay on mammary epithelial cells purified from MMTV-PyMT *Ccr6*^{WT} mice at the stage of early neoplasia with and without stimulation by ligand CCL20 (100ng/ml). Foetal calf serum (FCS, 0.5%) and epidermal growth factor (EGF, 20ng/ml) were used as positive controls. All statistical analyses are relative to Unstimulated. Data are representative of 3 independent experiments, n=3 mice per experiment.

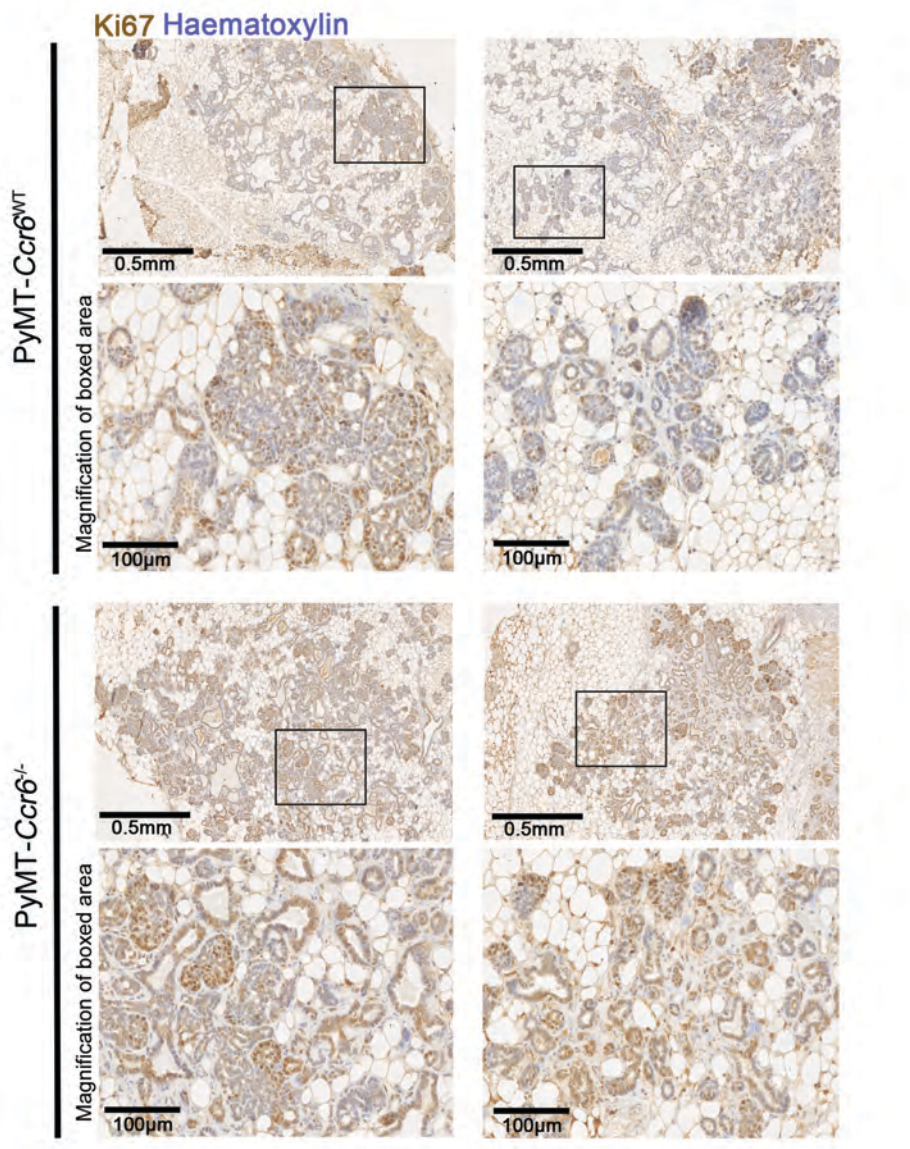

Figure 4.7: CCR6 deletion does not affect proliferation in the mammary gland as assessed by Ki67 staining.

Analysis of Ki67-positive proliferating cells within MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice at the stage of early neoplasia. Shown are representative fields from 2 separate tumours per genotype, displaying equal distribution of cells positive for Ki67.

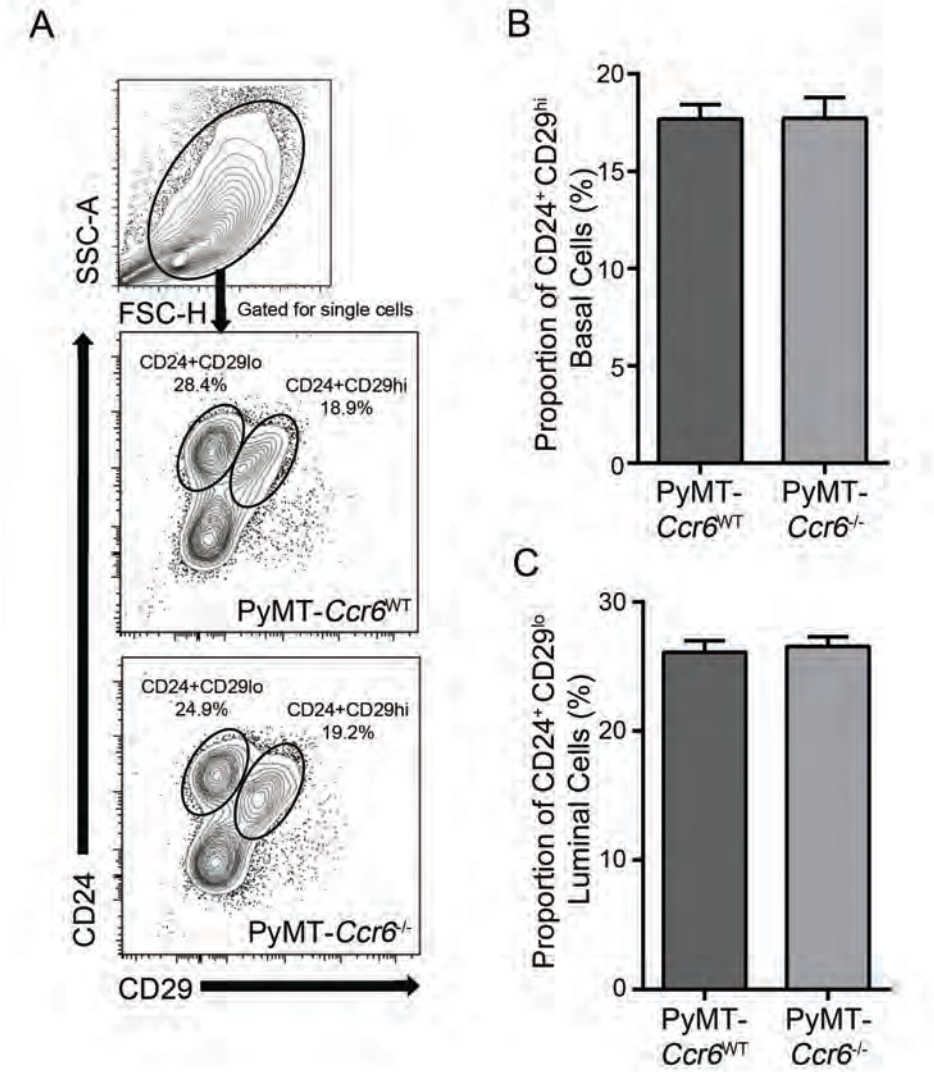

Figure 4.8: CCR6 deletion does not impact upon the mammary cell hierarchy.

Single cell suspensions (CD45-negative) from mammary glands with early neoplasia from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice were analysed by flow cytometry for the expression of CD24 and CD29. **(A)** Representative flow cytometry contour plots of gating strategy for CD24 and CD29 for both genotypes. **(B-C)** Analysis of proportions of the basal cell **(B)** and luminal cell **(C)** lineages. Data are representative of 3 independent experiments, n=6 mice per genotype per experiment.

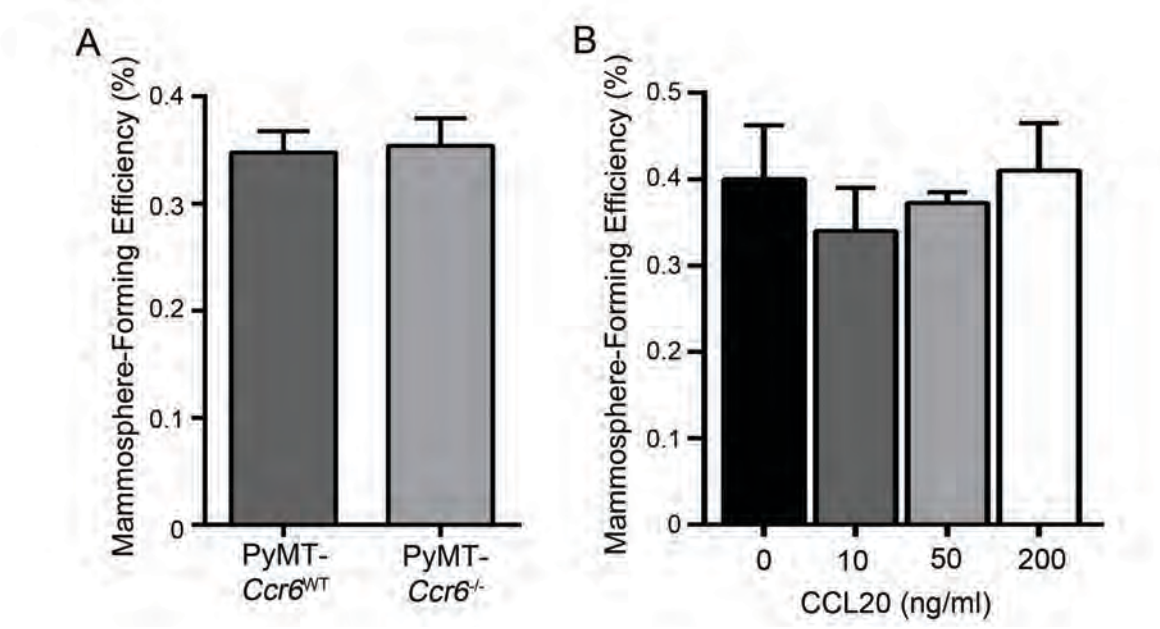

Figure 4.9: The CCR6 receptor does not influence self-renewal of cancer stem-like cells.

(A) Mammosphere-forming efficiency of cells isolated from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mammary glands. Data are representative of 3 independent experiments, n=6 mice per genotype per experiment. **(B)** Mammosphere-forming efficiency of MMTV-PyMT *Ccr6*^{WT} cells stimulated with varying concentrations of CCL20. Data are representative of 3 independent experiments, n=3 individual mice per experiment.

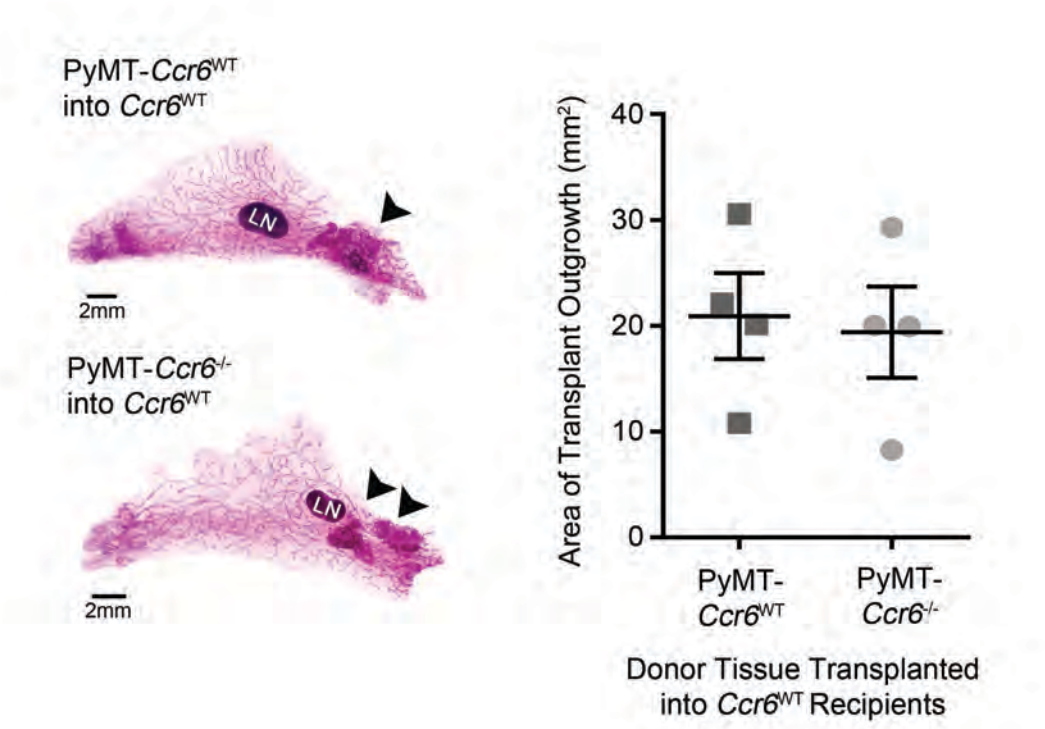

Figure 4.10: CCR6 in donor epithelium does not promote mammary tumour propagation.

Representative whole-mount images and quantification of the neoplastic outgrowth area in *Ccr6*^{WT} recipient glands of MMTV-PyMT *Ccr6*^{WT} and MMTV-PyMT *Ccr6*$^{-/-}$ mammary tumour tissue transplants, n=4 recipient mice per group. LN=lymph node. Black arrowheads indicate donor transplant outgrowth within the glands.

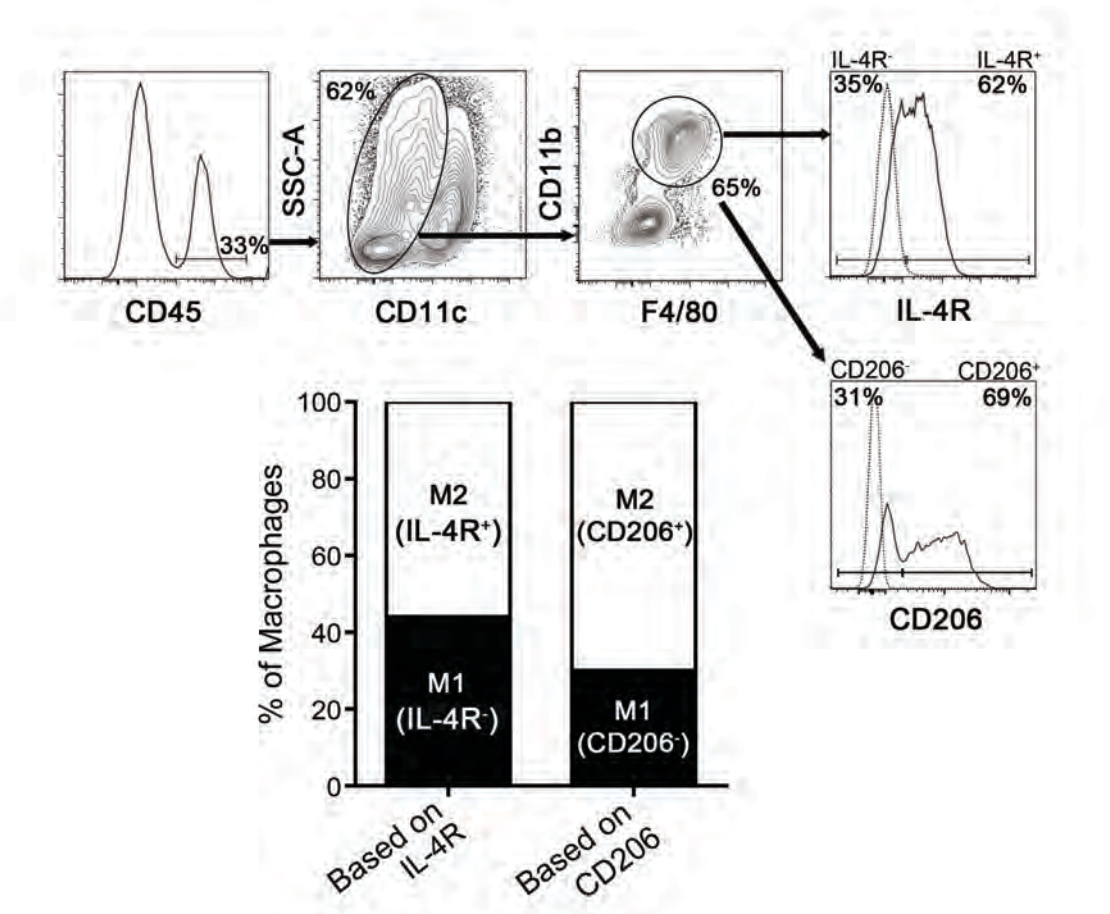

Figure 4.11: Analysis of tumour-associated macrophage phenotype in MMTV-PyMT mammary tumours.

MMTV-PyMT *Ccr6*^{WT} mammary tumour cells were analysed by flow cytometry for tumour-associated macrophage (TAM) phenotype. Analysis of M1 and M2 macrophages within the total macrophage population ($CD45^{+}CD11c^{-}CD11b^{+}F4/80^{+}$) was based on IL-4R expression and CD206 expression as indicated, where M1 macrophages were classed as IL-4R or CD206-negative and M2 were IL-4R or CD206-positive. Dotted lines=FMO negative controls. M1 and M2 cell proportions (bottom) are presented as percentage of the total macrophage population. Representative results from 3 independent experiments.

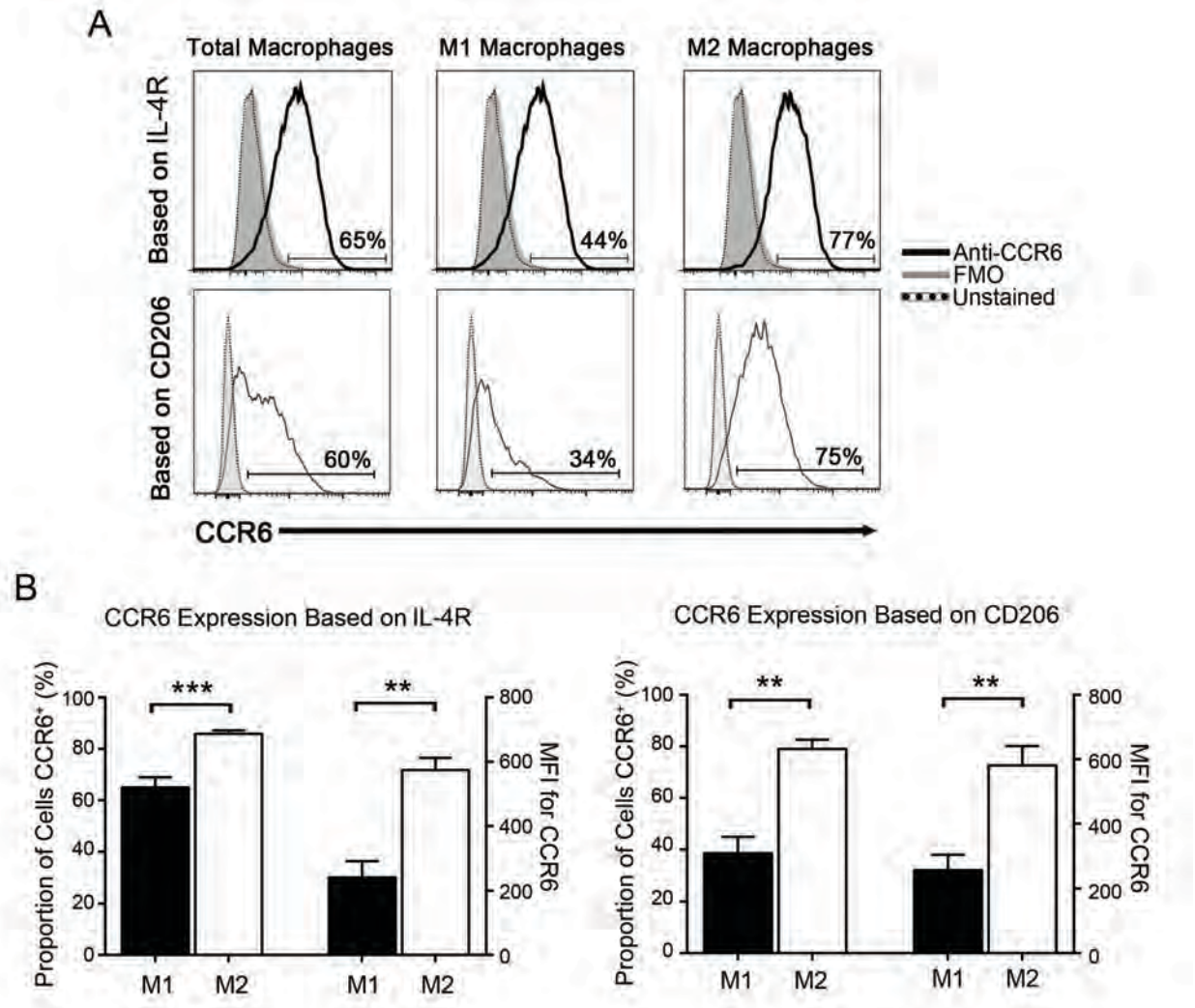

Figure 4.12: CCR6 expression in macrophage populations in MMTV-PyMT tumours.

(A) Representative flow cytometry plots of CCR6 expression within the total, M1 and M2 macrophage populations as based on IL-4R and CD206. All cells were gated for $CD45^{+}CD11c^{-}CD11b^{+}F4/80^{+}$ as in Figure 4.11. **(B)** Comparison of proportions of CCR6-positive cells and levels of CCR6 within putative M1 and M2 macrophage populations, based on IL-4R (left) and CD206 (right) expression. MFI=mean fluorescence intensity. Data are representative of 3 independent experiments.

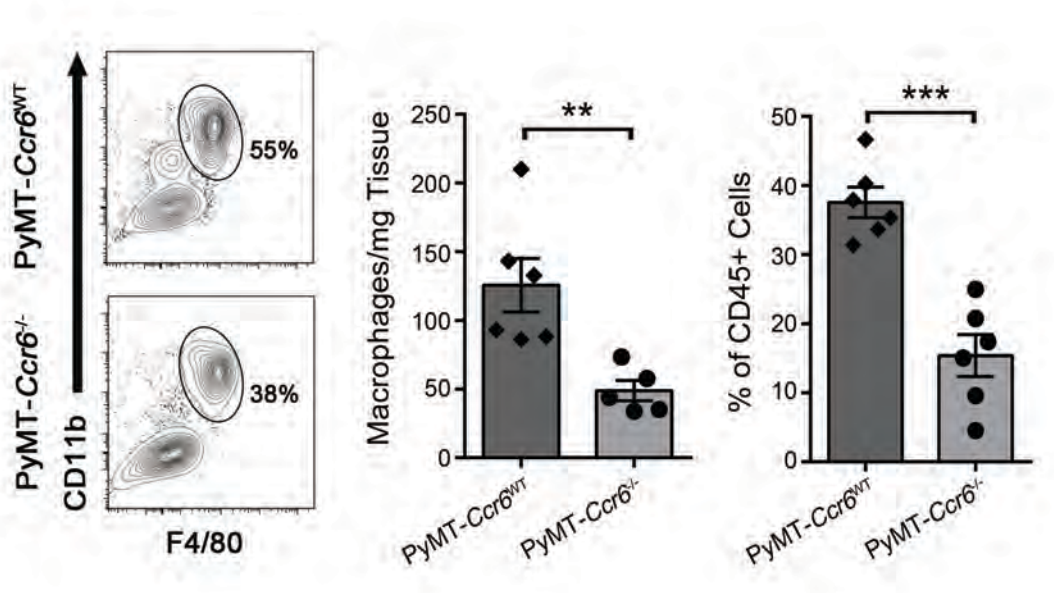

Figure 4.13: CCR6 deletion reduces the numbers and proportions of tumour-associated macrophages in mouse mammary cancer.

Numbers and proportions of tumour-associated macrophages in mammary tumours from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice as determined by flow cytometry. All cells were gated for CD45^{+}CD11c^{-}CD11b^{+}F4/80^{+} as in Figure 4.11. Data are representative of 3 independent experiments, n=5-7 mice per genotype per experiment.

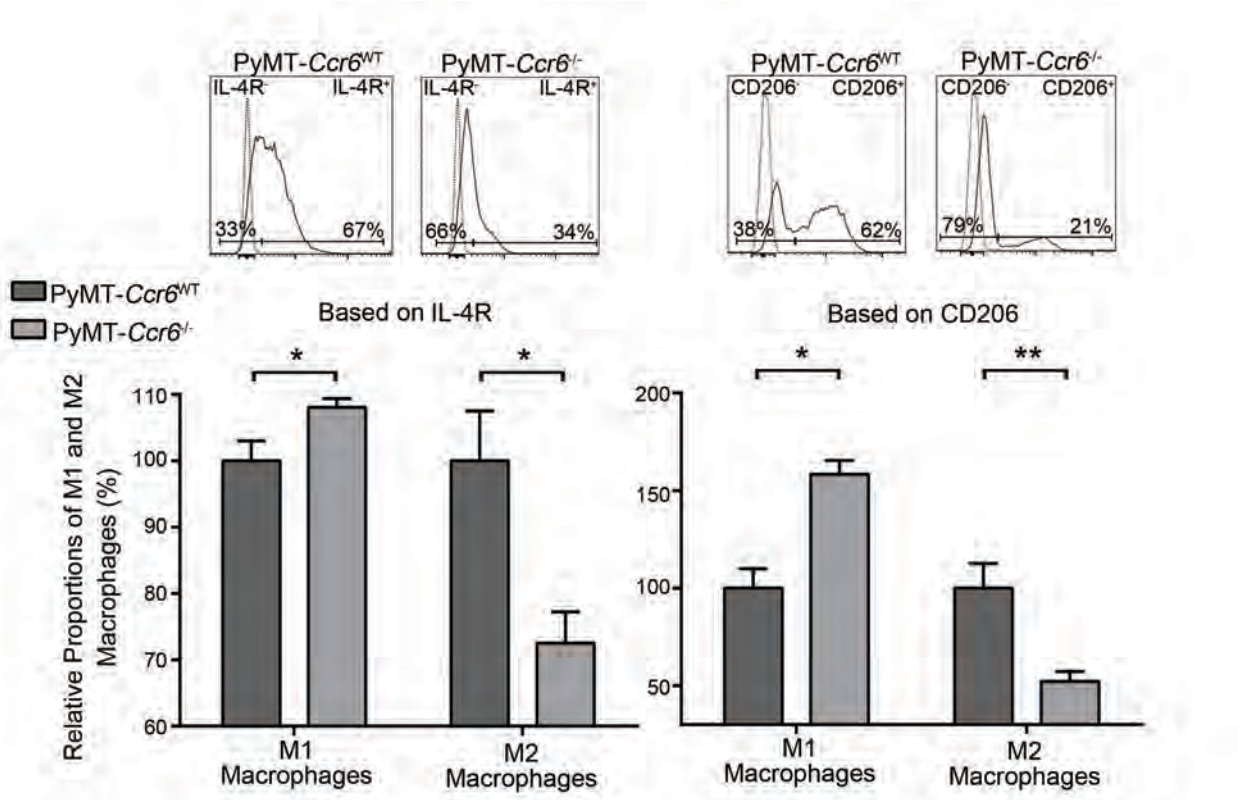

Figure 4.14: CCR6 mediates the recruitment of pro-tumourigenic macrophages to the mammary tumour microenvironment.

Proportions of putative M1 and M2 macrophage subtypes within the TAM population in mammary tumours from MMTV-PyMT *Ccr6*$^{-/-}$ mice relative to *Ccr6*^{WT} as determined by flow cytometry. All cells were gated for CD45^{+}CD11c^{-}CD11b^{+}F4/80^{+} (as in Figure 4.11), and M1/M2 status determined based on IL-4R (left) and CD206 (right). Dotted lines=FMO negative controls. Data are representative of 4 independent experiments, n=3 mice per genotype per experiment.

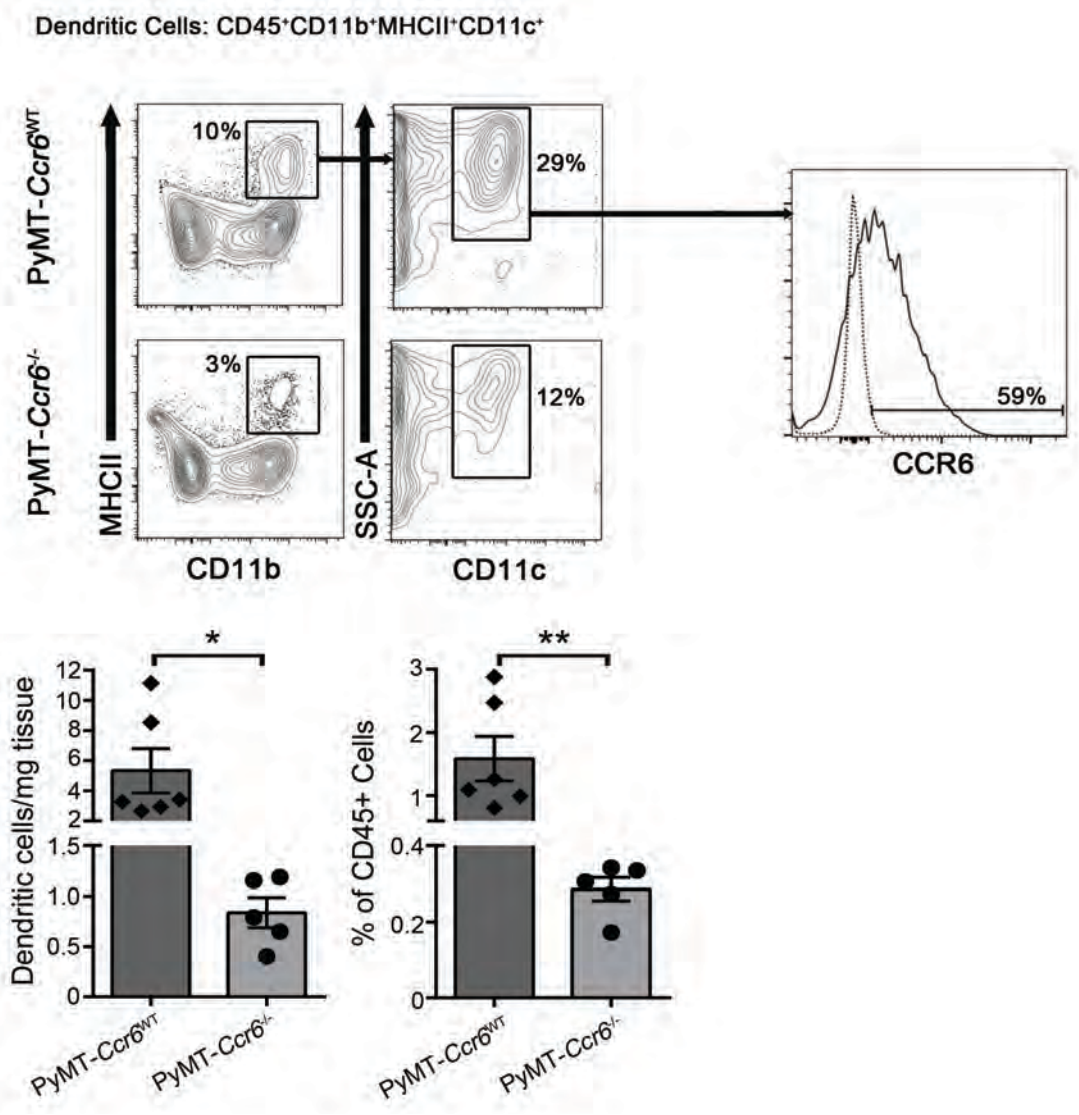

Figure 4.15: CCR6 expression and effect of CCR6 deletion on tumour-infiltrating dendritic cells.

MMTV-PyMT *Ccr6*^{WT} dendritic cells (CD45^{+}CD11b^{+}MHCII^{+}CD11c^{+}) were assessed for CCR6 expression (right), and numbers and proportions of dendritic cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (bottom). Dotted line=FMO negative control. n=5-7 mice per genotype.

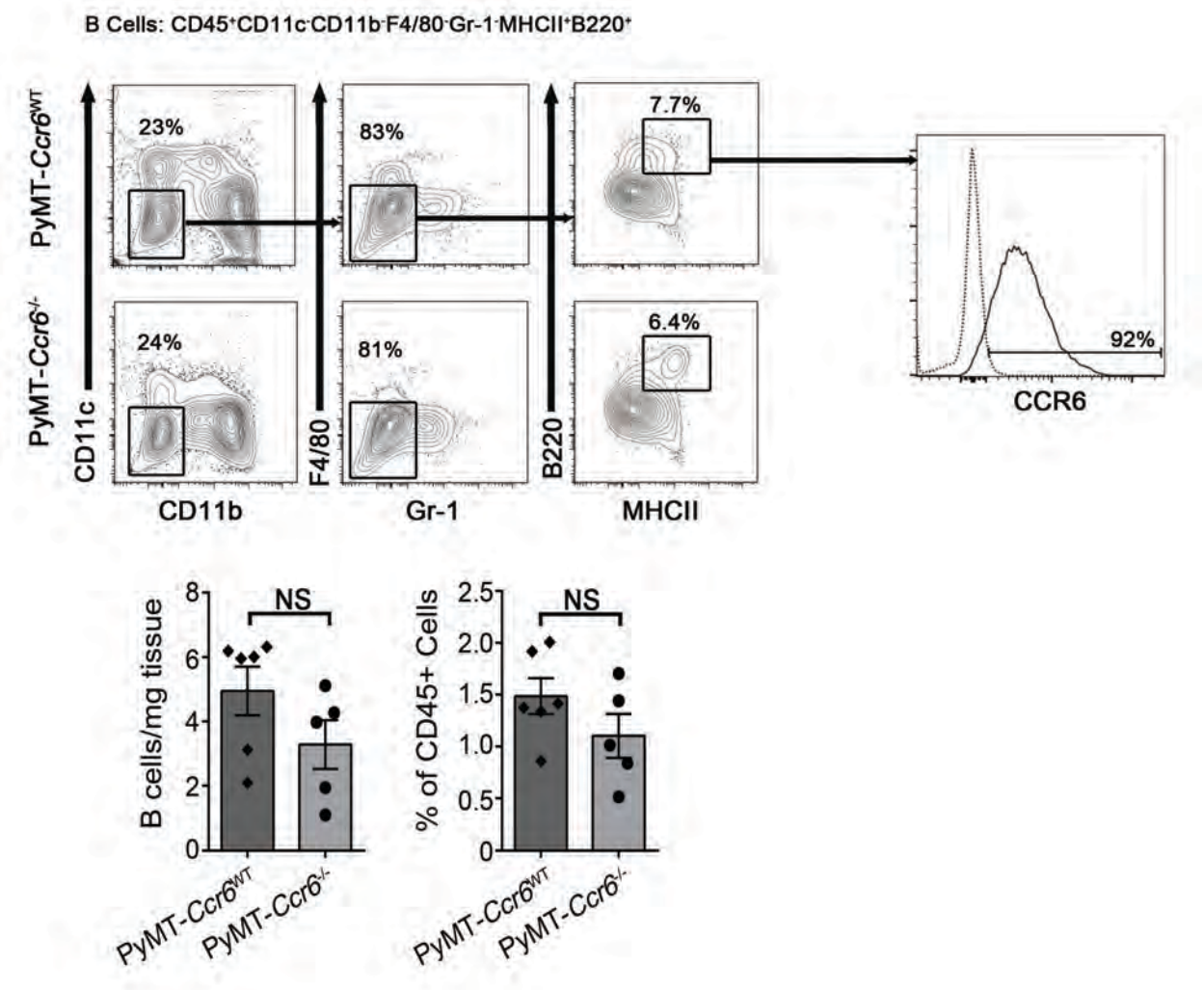

Figure 4.16: CCR6 expression and effect of CCR6 deletion on tumour-infiltrating B cells.

MMTV-PyMT *Ccr6*^{WT} B cells (CD45^{+}CD11c^{-}CD11b^{-}F4/80^{-}Gr-1^{-}MHCII^{+}B220^{+}) were assessed for CCR6 expression (right), and numbers and proportions of B cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (bottom). Dotted line=FMO negative control. n=5-7 mice per genotype.

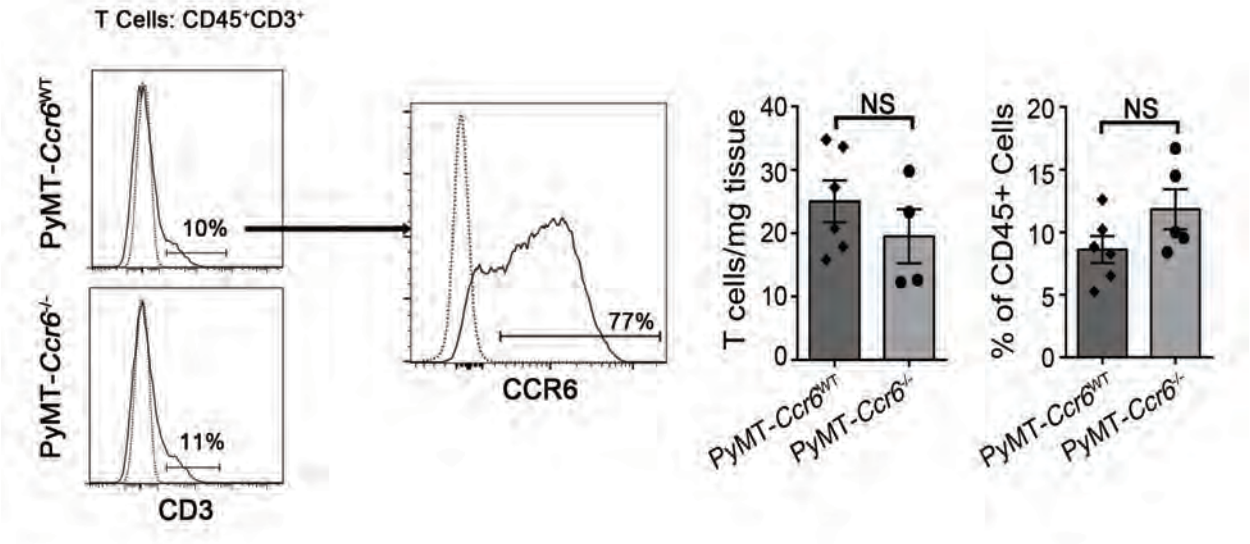

Figure 4.17: CCR6 expression and effect of CCR6 deletion on tumour-infiltrating T cells.

MMTV-PyMT *Ccr6*^{WT} T cells (CD45^{+}CD3^{+}) were assessed for CCR6 expression (middle), and numbers and proportions of T cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (right). Dotted lines=FMO negative controls. n=5-7 mice per genotype.

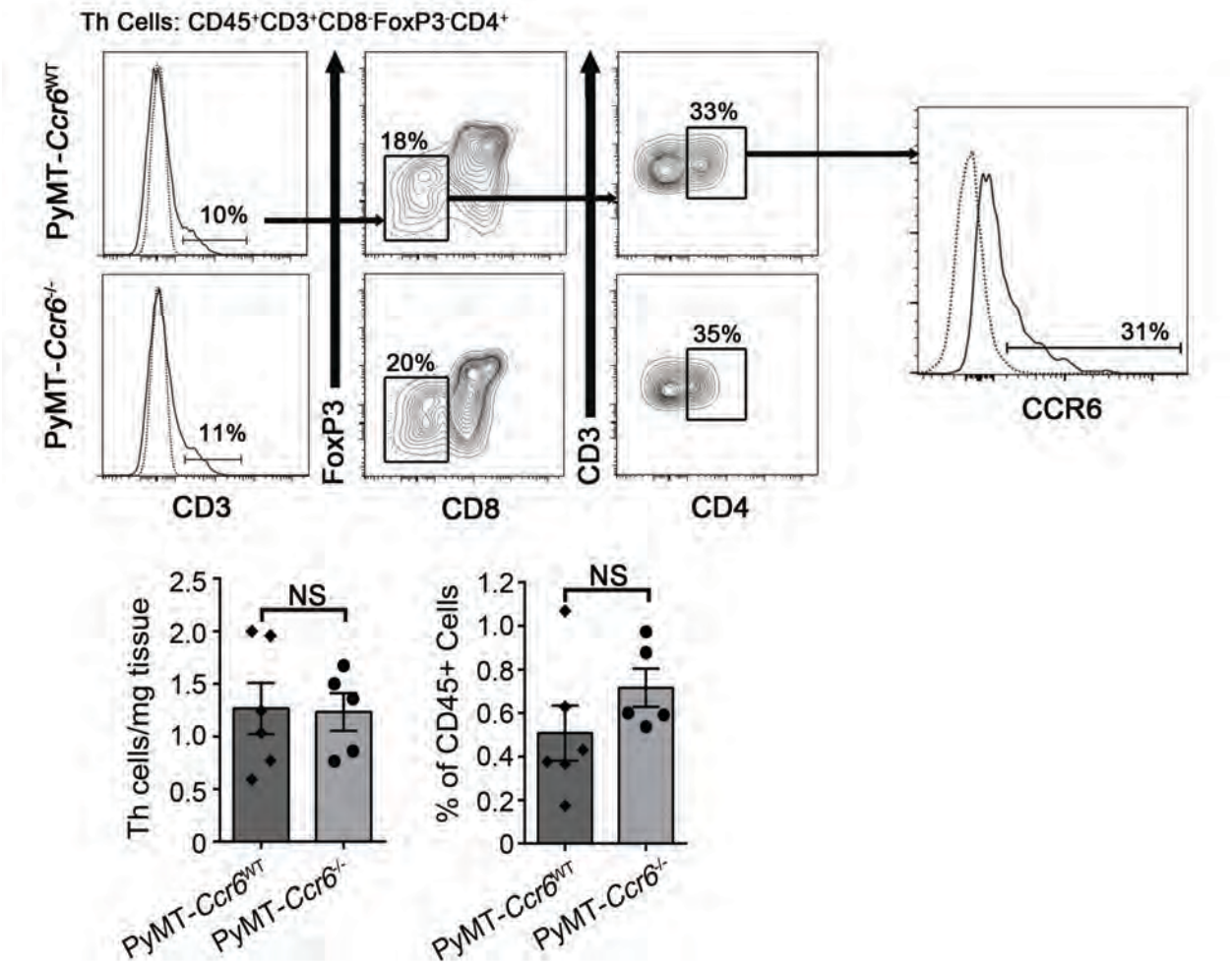

Figure 4.18: CCR6 expression and effect of CCR6 deletion on tumour-infiltrating helper T cells.

MMTV-PyMT *Ccr6*^{WT} Th cells (CD45^{+}CD3^{+}CD8^{-}FoxP3^{-}CD4^{+}) were assessed for CCR6 expression (right), and numbers and proportions of Th cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (bottom). Dotted lines=FMO negative controls. n=5-7 mice per genotype.

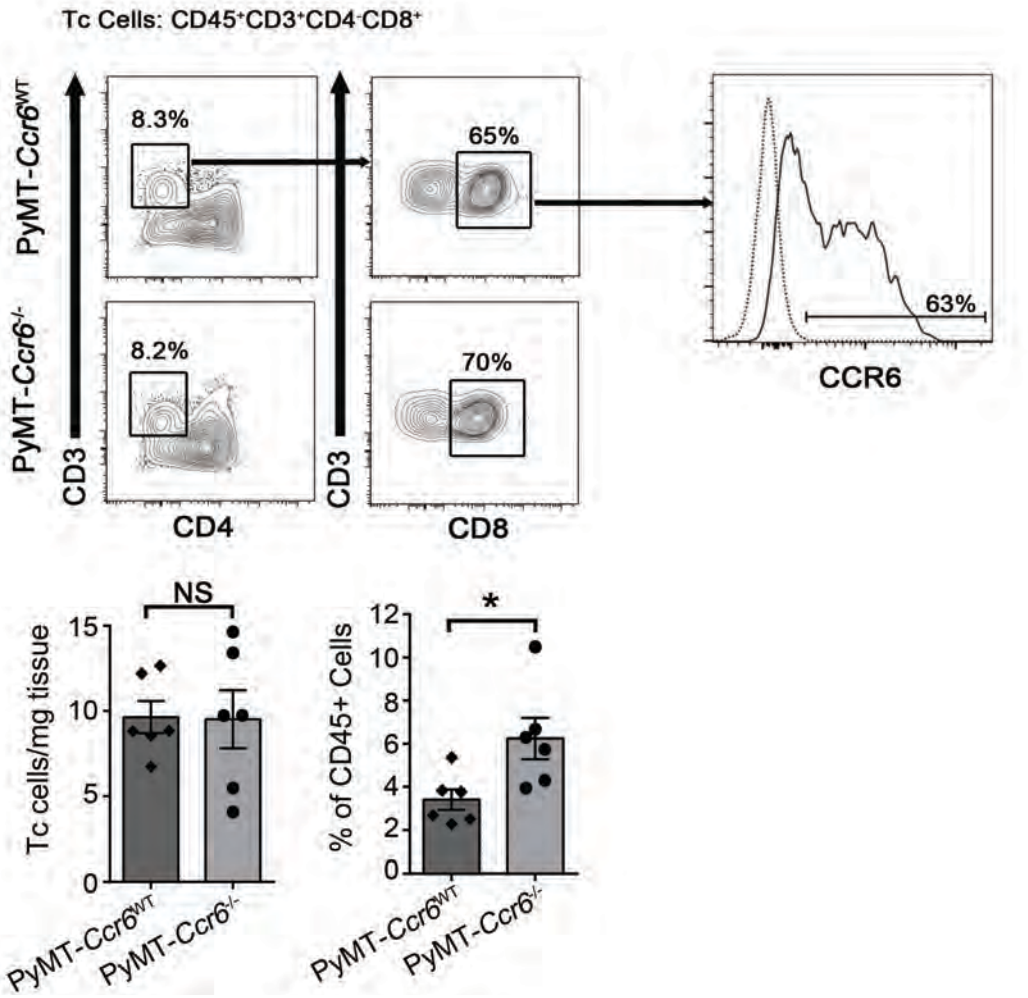

Figure 4.19: CCR6 expression and effect of CCR6 deletion on tumour-infiltrating cytotoxic T cells.

MMTV-PyMT *Ccr6*^{WT} Tc cells ($CD45^+CD3^+CD4^-CD8^+$) were assessed for CCR6 expression (right), and numbers and proportions of Tc cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (bottom). Dotted line=FMO negative control. n=5-7 mice per genotype.

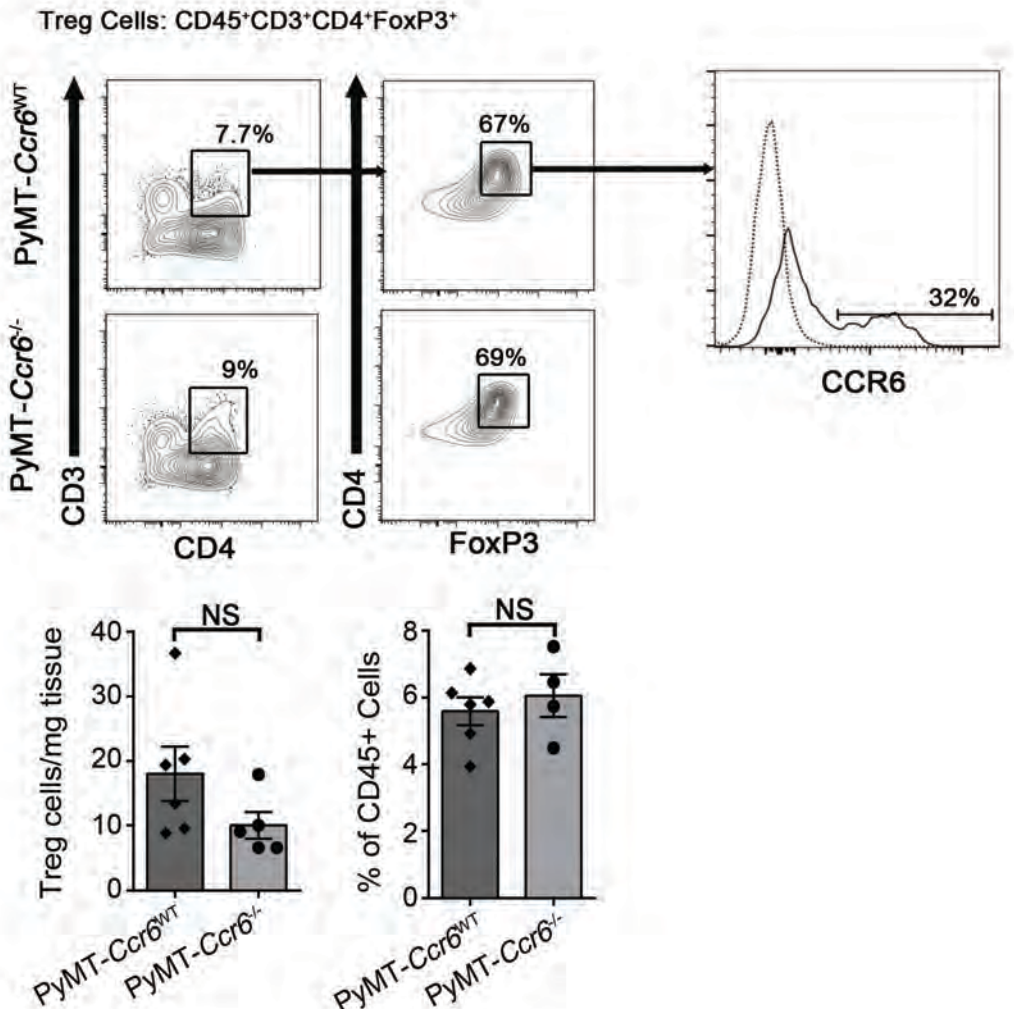

Figure 4.20: CCR6 expression and effect of CCR6 deletion on tumour-infiltrating regulatory T cells.

MMTV-PyMT *Ccr6*^{WT} Treg cells (CD45$^+$CD3$^+$CD4$^+$FoxP3$^+$) were assessed for CCR6 expression (right), and numbers and proportions of Treg cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (bottom). Dotted line=FMO negative control. n=5-7 mice per genotype.

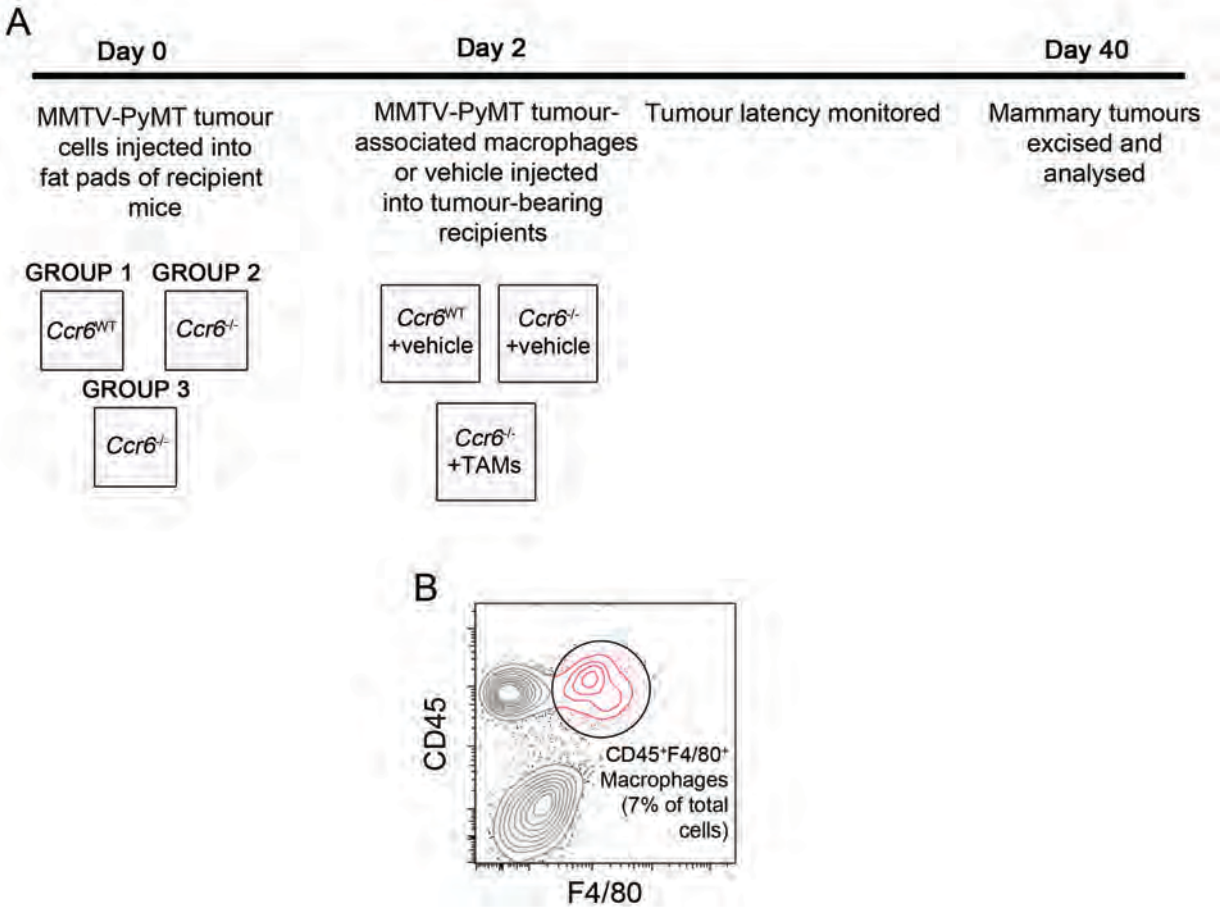

Figure 4.21: Experimental setup of the macrophage reconstitution assay.

(A) Schematic of macrophage reconstitution assay to determine contribution of macrophages to mammary tumourigenesis in the context of CCR6 deletion. TAM=tumour-associated macrophage. **(B)** FACS plot showing the sorted macrophage population used for reconstitution.

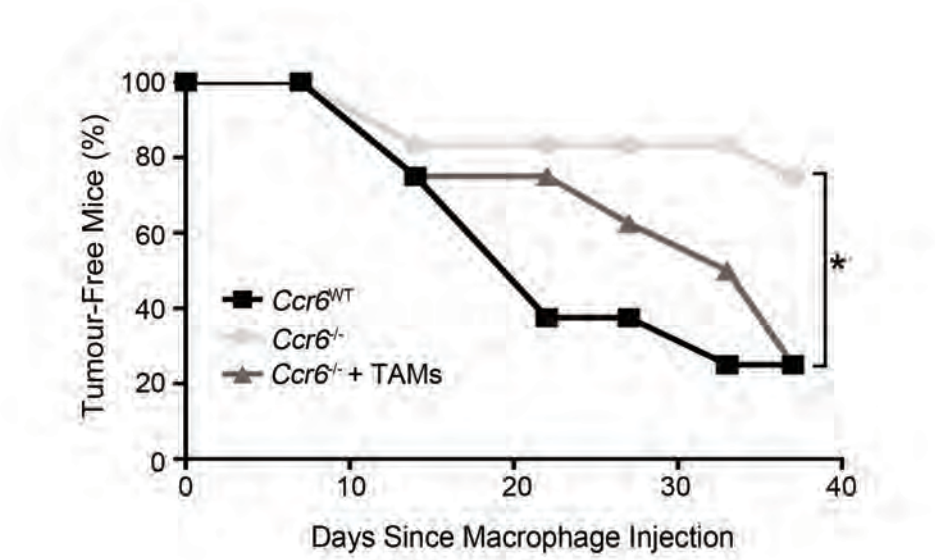

Figure 4.22: CCR6-mediated pro-tumourigenic macrophages promote mammary cancer progression.

Tumour-free survival curves over the course of the macrophage reconstitution assay.

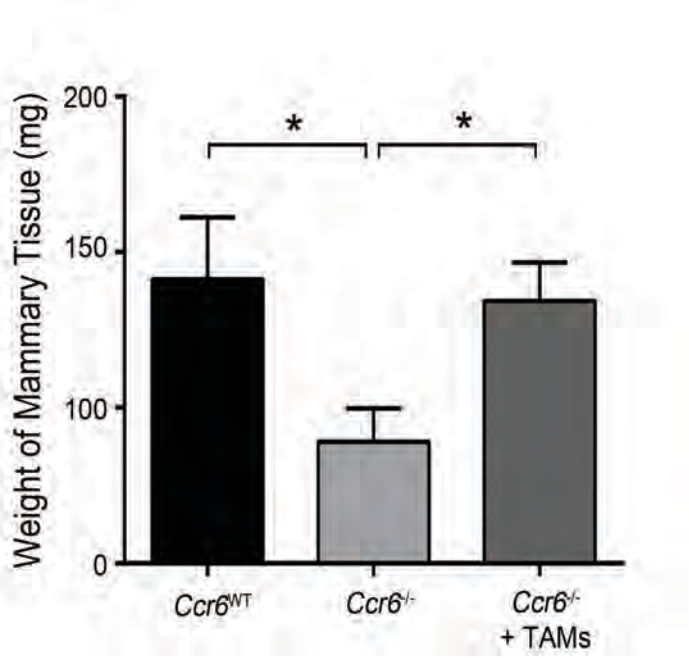

Figure 4.23: CCR6-mediated pro-tumourigenic macrophages promote mammary cancer growth.

Tumour weight of control tumours generated in *Ccr6*^{WT} recipients and in *Ccr6*$^{-/-}$ recipients ± tumour-associated macrophages at the time of sacrifice. n=6-8 tumours per group.

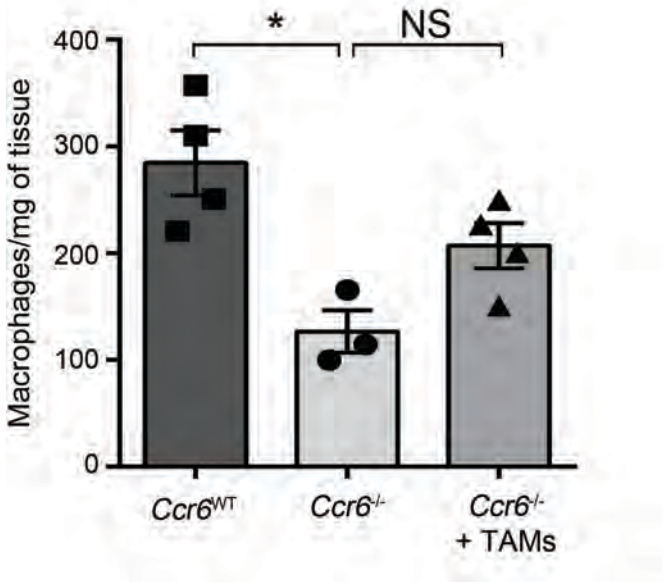

Figure 4.24: Assessment of tumour-associated macrophages in macrophage reconstitution recipients.

Macrophage numbers in experimental groups at end-point, as assessed by flow cytometry. All cells were gated for $CD45^{+}CD11c^{-}CD11b^{+}F4/80^{+}$ as in Figure 4.11 n=3-4 samples per group.

CHAPTER 5: Discussion

5.1. Introduction

This project investigated the roles played by the chemokine receptors CCR6 and CCR7 in the progression of mammary tumourigenesis, using novel bigenic mouse models that combine expression of the PyMT oncogene under the control of the MMTV promoter, with chemokine receptor deletion. It was evident that deletion of either receptor resulted in a decrease in mammary neoplastic development, as shown by an extended tumour latency and a decrease in tumour incidence. However, further studies demonstrated that CCR7 and CCR6 have distinct roles in mammary gland neoplasia.

Loss of CCR7 does not decrease initial hyper-proliferative activity within the mammary gland, however tumour growth is significantly inhibited from this point onwards. CCR6 deletion, adversely, results in a very early stage decrease in mammary hyperplasia, and so the effects of CCR6 are present before tumourigenesis has progressed to advanced stages. One possible explanation for this is that CCR7 may play a more prominent role in mammary homeostasis at early stages of cancer development rather than as an oncogenic driver. Indeed, when normal mammary glands were examined, the deletion of CCR7 significantly slowed pubertal ductal growth within the epithelial tree and reduced the normal adult mammary stem cell pool. CCR6 has no such effect on the normal mammary gland. Potentially, as normal stem cells transition into “cancer” stem cells, driving tumourigenesis, the oncogenic properties of CCR7 become more significant, promoting tumour development from the stage of early neoplasia onwards.

The CCR6 receptor in the transformed mammary gland has a different function to that of CCR7. Despite being expressed on cancer cells, it has no significant role in cell proliferation, or maintenance of the mammary cellular hierarchy. Instead, its primary function appears to be to recruit tumour-promoting macrophages to the microenvironment, as the loss of CCR6 specifically depletes the levels of alternatively-activated M2 macrophages.

As both CCR7 and CCR6 have been found to have important and distinct roles in mammary tumour progression, they are worth exploring both individually and in combination to potentially develop targeted therapeutics to treat breast cancer in its early stages.

5.2. The role of CCR7 in breast cancer progression

The contribution of CSCs to tumour initiation is a major issue in tumour biology, yet one of the least understood processes [176]. In this study, it was shown that ablating CCR7 in mammary cancer using a bigenic MMTV-PyMT *Ccr7*$^{-/-}$ mouse model significantly depleted the breast CSC-enriched pool. Using the surface marker profiles CD24^{+}CD29^{hi} [60] and CD49f^{+}DLL1^{+}DNER^{+} [71] it was shown that the underlying mechanism involves a decrease in the ability of stem-like cells and early progenitor cells to self-renew and initiate neoplasia. Significantly, exogenously targeting CCR7 with a peptide antagonist led to a decrease in tumourigenesis.

CCR7 has been extensively studied for its role in adaptive immunity and secondary lymphoid organogenesis, and CCR7-null mice display disrupted architecture of the thymus and lymph node, as well as a reduced ability to mount a primary immune response [215]. The role of CCR7 in mediating anti-tumour immunity is also slowly emerging [216]. In this context, the fact that abrogation of CCR7 severely affected mammary tumourigenesis provides definitive evidence of CCR7 as a pro-tumourigenic driver. Furthermore, numerous transplantation approaches used in this study underscore an immune system-independent role of this chemokine receptor in maintaining stem-like cell pools in breast cancer.

Interestingly, hyperplastic outgrowth, widely believed to be a precursor of mammary tumours [183], was found in 100% of MMTV-PyMT *Ccr7*^{WT} and *Ccr7*$^{-/-}$ glands examined. However, the majority of MMTV-PyMT *Ccr7*$^{-/-}$ glands were unable to sustain this initial proliferative burst of tumour cells and progress to the next stage in tumour development. Therefore, the delay in mammary tumourigenesis appears to be due to CCR7 maintaining specialised hierarchical sub-

populations of cancer stem and progenitor-like cells that are thought to be crucial for tumour initiation and advanced tumourigenesis [173].

The fact that both CCL19 and CCL21 stimulated mammosphere growth from both human and mouse tumour cells strongly suggests that CCR7 plays a global role in sustaining properties of stemness in mammary epithelium. The specificity of CCR7 in this process was validated by testing a panel of chemokine receptor ligands, where only CCL19 and CCL21 showed an ability to significantly increase MFE.

Stimulation of CXCR4, the chemokine receptor that is consistently found to be up-regulated together with CCR7 in a number of cancers [125], did potentiate sphere formation but to a lesser extent (***Figure 3.19***). Recently, Clarke and colleagues demonstrated that stimulation of CXCR4 also increased MFE preferentially in malignant breast cancer cell lines compared to normal breast cell lines [149]. It is interesting to speculate that as CCR7 is less important for homeostasis than CXCR4, as has been inferred from animal models [217], CCR7 may represent a more attractive target for future CSC-targeting therapies.

As stimulation of CCR7 had no effect on proliferation of the bulk population of cells when seeded into adherent culture, compared to a highly significant effect in non-adherent culture, it is likely that CCR7 predominantly mediates specific cellular properties of stemness. Moreover, it was previously reported by the McColl group that CCR7 activation on breast cancer cells inhibits anoikis [130], a characteristic of breast and other CSCs [75, 218]. Therefore, it is plausible that CCR7 supports CSC survival without attachment to the extracellular matrix, a hypothesis that may form the basis for future studies.

CCR7 appeared to play a quantitative rather than a qualitative role in normal mammary stem cells compared with CSCs. When CCR7 was deleted, a mild effect was observed on the normal mammary gland. In contrast, a major effect was seen in mammary tumourigenesis. Interestingly, whereas the morphological effect on normal mammary gland development was not extensive,

CCR7 deletion discreetly affected normal mammary gland stem-like cells. Therefore, it is possible that CCR7 has a role in regulating the properties of stemness within the mammary epithelial cell population, an effect that appears more prominent during cancer progression. As highlighted in a recent study by Cheresh and colleagues [219], dysregulation of normal stem cells may contribute to breast cancer progression and stemness, and CCR7 may emerge as a novel mediator of this transition.

Translation of these findings from the mouse model to human disease is of particular significance, considering that there is currently no clear consensus on the markers that define functional mammary stem cells in both mice and humans. Thus these results show that CCR7 not only has a role in mouse mammary tissue but is also expressed, is functional and is highly responsive in the stem-like populations within human breast cancer tissue. Intriguingly, circulating tumour cells, an indicator of metastatic spread and poor outcome in breast and other cancers, have been recently equated to CSCs [40]. In the last decade numerous studies also suggested a role for CCR7 in malignant dissemination of mammary tumours to distant sites [124, 125, 130]. Taken together, these results suggest a novel causative link between CCR7 activity on stem-like populations and metastatic breast cancer.

To seek proof-of-principle on the utility of pharmacologically targeting CCR7, the receptor antagonist $CCL19_{(8\text{-}83)}$ [188] was tested. It was found that pharmacological inhibition of CCR7 through direct mammary fat pad injection of $CCL19_{(8\text{-}83)}$ afforded a significant reduction in early stage primary mammary tumourigenesis. As the relative contribution of the malignant lesions to the weight of the whole mammary fat pad was very small at this early stage the reduction in total weight between antagonist and vehicle-treated glands was not statistically significant. However, the cellularity, a characteristic that directly reflects the extent of epithelial malignant outgrowth and is used in clinical pathology to evaluate the response to chemotherapy in breast cancer, was strongly impacted by treatment with the CCR7 antagonist.

More importantly, directly targeting CCR7 using the antagonist significantly depleted the stem-like cell pools in both early and late-stage mammary neoplasia as was shown using the transplantation approach. These findings strongly suggest that the CCR7 receptor axis is a potential point of intervention in stem cell-targeting therapies. Furthermore, the results of this study provide a rationale for the use of antagonists of the CCR7 pathway as adjuvants to conventional cytotoxic drugs unable to eliminate quiescent CSCs [173].

Thus, the characterisation of CCR7 in primary breast tumourigenesis *in vitro* and *in vivo*, and in mouse and human tissue, strongly suggests a role for this molecule in breast cancer development and progression. These insights raise the possibility of pharmacologically targeting CCR7 for the development of new therapies in breast cancer.

5.3. The role of CCR6 in breast cancer progression

The results of this study show that the deletion of the chemokine receptor CCR6 caused a delay in tumour onset and decreased mammary tumour incidence *in vivo* in the MMTV-PyMT transgenic mouse model. It was determined that the underlying basis of the CCR6 oncogenic function is the increase in numbers of infiltrating pro-tumourigenic macrophages.

Multiple functional roles have been suggested for members of the chemokine family and their receptors in breast cancer pathophysiology [89], however little data using animal models is available to support these observations. The expression of CCR6 has been reported to correlate with higher stage and grade of human breast cancer, and has been proposed as a prognostic tool for determining relapse-free survival in breast cancer patients [163]. However, a causative link *in vivo* has yet to be demonstrated. The well-characterised MMTV-PyMT transgenic mouse model of breast cancer was employed in this study, and it was found that CCR6 facilitates an earlier tumour onset and an increased incidence of mammary tumours. Of note, CCR6 affects mammary tumourigenesis from as early as the hyperplastic, or hyper-proliferative, stage. This initial phase of tumour development remains largely uncharacterised, despite being the most treatment-

effective stage of cancer progression. Therefore, a better understanding of tumour initiation is crucial in order to develop therapies that target the tumourigenic process at the early stages of breast cancer.

When CCR6 was deleted in the MMTV-PyMT mouse, tumour latency was significantly extended, and these mice developed fewer mammary tumours than their *Ccr6*^{WT} counterparts. However, CCR6 deletion did not affect tumourigenic properties of the epithelium as was found with the chemokine receptor CCR7 [171]. Stimulation with CCL20 did not result in an increased proliferation rate of purified mammary epithelial cells from hyperplastic glands or tumourous lesions in contrast to previous studies with primary human breast peritumoural cells [168]. Furthermore, the deletion of CCR6 did not lead to decreased numbers of Ki67-positive proliferating cells within intact tumour-bearing mammary glands, pointing to an epithelial-independent function of this receptor in breast cancer.

It was also observed that the loss of CCR6 did not alter the numbers and functional properties of mammary cancer stem-like cells. Transplantation experiments in particular demonstrated that the presence of CCR6 in donor epithelium was not required for tumour propagation in recipient mammary glands.

Further investigation demonstrated that CCR6 functions via organisation of the immune system during the early stage of mammary carcinogenesis. It was shown that the levels of tumour-associated macrophages (TAMs) are reduced by almost three-fold when CCR6 is deleted. TAMs, which have been previously identified in MMTV-PyMT tumours [220], are widely reported to support the development of cancer [190, 221] and in the tumour microenvironment they are generally thought to polarise towards an alternatively-activated M2 pro-tumour phenotype relative to the classic M1 anti-tumour phenotype [112]. Whilst the TAMs in MMTV-PyMT tumours are polarised towards an M2-like subtype, these results show that the presence of CCR6 maintains M2 TAMs as the predominant phenotype. Therefore, it is plausible to suggest

that CCR6 in breast cancer functions to recruit pro-tumourigenic macrophages to the tumour immuniche [115], to support growth of transformed epithelial cells and cancer stem cells, as TAMs in the MMTV-PyMT model have also been shown to also maintain stem-like cells [117].

CCR6 is not expressed on peripheral blood monocytes, and is thought to only be acquired upon their differentiation into macrophages, induced by the tumour microenvironment [194]. In accordance with this, it was found that a high proportion of macrophages within PyMT-driven mammary tumours express CCR6, which has not been previously demonstrated in breast cancer. Also of potential importance is the fact that up to 90% of pro-tumourigenic M2-like TAMs expressed CCR6. These findings parallel results from a recent study which showed that CCR6-null mice bearing the adenomatosis polyposis coli $(APC)^{min}$ transgene (a well-characterised model for gastro-intestinal tumourigenesis) developed fewer intestinal adenomas and polyps, and that the effect of CCR6 was also linked to a significant reduction in $F4/80^{+}$ macrophages [200]. Interestingly, Liu *et al* also recently demonstrated that the ligand CCL20 is secreted from both macrophages and tumour cells in another mouse model of colorectal cancer, potentially suggesting common regulatory mechanisms and a universal role for CCR6 in tumours of various etiology [222].

MMTV-PyMT cancer cell transplant experiments showed that tumour growth in a CCR6-null microenvironment was significantly inhibited compared to wild-type microenvironment conditions, directly demonstrating that the mammary stroma is dependent upon CCR6 for adequate tumour initiation and growth support. The reconstitution of this CCR6-negative microenvironment with MMTV-PyMT $Ccr6^{WT}$ TAMs restored the tumour-promoting properties of mammary stroma, indicating that breast cancer can be therapeutically targeted through manipulation of the CCR6-CCL20 axis to control tumour-infiltrating macrophages.

CCR6 deletion has also impeded recruitment of dendritic cells into PyMT-driven mammary tumours. Recruitment of dendritic cells into various solid tumours has been well-documented

[223], and their role in tumour progression is mainly centred around tumour antigen presentation to lymphocyte subsets leading to anti-tumour immune responses [224, 225]. Furthermore, there is some evidence supporting direct tumouricidal activity of dendritic cells [224]. As previous studies have reported an intrinsic requirement for CCR6 in migration and fundamental functions of dendritic cells [165, 212], this finding of the reduced infiltration of dendritic cells in mammary tumours may not be a facet of cancer development in MMTV-PyMT *Ccr6*$^{-/-}$ mice, but is an inherent property of dendritic cell migration at a slower rate after CCR6 deletion.

In conclusion, results of this study show that CCR6 plays a significant role in tumour initiation and at the early stage of breast cancer development *in vivo* by mediating recruitment of pro-tumourigenic macrophages to the tumour site, and thus facilitating further progression to advanced stages of mammary neoplasia. Results presented here therefore suggest CCR6 as a potential target for therapeutic intervention in early breast cancer.

5.4. Implications of this study

This is the first *in vivo* evidence for the important roles of CCR6 and CCR7 in promotion of breast cancer. This work builds on previous observations based on *in vitro* models and xenotransplantation studies, and creates a strong baseline for further research in this area. The area of cancer immunology is largely uncharacterised and investigation into mechanisms underlying the functional role of these and other receptors in breast cancer should be the next step forward.

This work contributes significantly to the knowledge about the roles of the chemokine receptor family in breast cancer. Importantly, the work with CCR7 reveals that chemokine receptors may have an important function in regulation of breast cancer stem cells, an area that is still largely in its infancy.

5.5. Clinical predictions and future work

The studies with the $CCL19_{(8-83)}$ antagonist in the PyMT mouse model suggest that CCR7 may be an interesting candidate for further pre-clinical evaluations into targeting of evasive cancer stem cells. In addition to $CCL19_{(8-83)}$, studies have been undertaken using the truncated ligand $CCL21_{(8-110)}$. As with $CCL19_{(8-83)}$, injection of this peptide antagonist in PyMT-expressing mammary glands also resulted in a significant decrease in tumour weight and size compared to vehicle controls (data not shown). Another approach may be to investigate the potential of cancer stem cell vaccines in the context of CCR7. Wicha's group has undertaken preliminary studies into the use of CSC vaccines, by pulsing dendritic cells with CSC lysates (based on expression of aldehyde dehydrogenase) to generate B cell-mediated immunity against CSCs in mouse models of melanoma and squamous cell carcinoma. Application of the dendritic cell vaccine reduced development of primary tumours and metastasis, and interestingly, this was associated with a significant decrease in expression of CCR7 and its ligand CCL21 [226]. Therefore, inhibition of CCR7 may potentially be used in combination with conventional chemotherapeutics in a multi-pronged approach to eliminate both rapidly-dividing bulk tumour cells and quiescent stem-like cells.

The role of the tumour microenvironment in controlling cancer progression is highly dynamic, and the chemokine receptor family is emerging as a key regulator of this process. In addition to these studies with CCR6, Pollard and colleagues have recently demonstrated that CCR1 and CCR2 signalling in mammary tumour-infiltrating macrophages promotes metastatic seeding of breast cancer cells in the lungs [227]. Most likely, various chemokine receptors are involved in every aspect of breast pathogenesis, and function not only on the cancer cells, but also on stromal and circulating cells. Identifying these receptors and their specific roles is a major challenge for medical research, but due to the physiological nature of GPCRs – being expressed on the cell surface – developing drugs that target them is somewhat more straightforward. This

brings up the possibility of generating carefully tailored therapeutics for individual patients based on their chemokine receptor profile.

As TAMs have recently been shown to maintain the CSC niche within MMTV-PyMT tumours [117], it would be worth investigating the result of blocking CCR7 and CCR6 together pharmacologically to treat mammary cancer, as hypothetically, loss of two components that are separately driving the epithelial hierarchy and the recruitment of major tumour-promoting cells within the cancer niche could result in an even greater reduction than that that is seen by deletion of the single receptors. A follow-up study in this area would be of specific clinical relevance.

However, investigation into biochemical mechanisms behind the actions of CCR7 and CCR6 is necessary before any clinical work could be carried out, as the genes that are turned on or switched off by these chemokine receptor pathways in these scenarios have not yet been elucidated. In models used in this study, no redundancy seems to exist between different chemokine receptors, however crosstalk between chemokine receptor pathways and others (such as Wnt and Notch) would need to be thoroughly examined.

In terms of breast cancer stem cell regulation, chemokine receptors are only one aspect of an extremely complicated regulatory system. Other aspects of the immune system, most importantly presence or absence of various immune cell subsets, may also contribute to the maintenance of this important population. With various genetically-modified mouse models now at the disposal of researchers, and using a similar strategy to that applied in this study, it will be possible to examine in more detail the aspects of tumour immunology which until now have remained somewhat elusive.

5.6. Conclusion

The chemokine receptors CCR6 and CCR7 play distinct but important roles in mammary cancer development. As the loss of these receptors largely blocks tumourigenesis, antagonists towards CCR6 and CCR7 should be explored further as potential therapeutics, to target both the tumour microenvironment and the cancer stem cell population respectively.

APPENDICES

Appendix A:

ST Boyle, M Kochetkova. “Breast cancer stem cells and the immune system: Promotion, evasion and therapy” *Journal of Mammary Gland Biology and Neoplasia*, Vol. 19:2, pp 203-211, 06 July 2014

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Appendix B:

ST Boyle, WV Ingman, V Poltavets, JW Faulkner, RJ Whitfield, SR McColl, M Kochetkova. “The chemokine receptor CCR7 promotes mammary tumorigenesis through amplification of stem-like cells” *Oncogene* advance online publication, 16 March 2015

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Appendix C:

ST Boyle, JW Faulkner, SR McColl, M Kochetkova. “The chemokine receptor CCR6 facilitates the onset of mammary neoplasia in the MMTV-PyMT mouse model via recruitment of tumor-promoting macrophages” *Molecular Cancer*, Vol. 14:115, 06 June 2015

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APPENDIX A

Statement of Authorship

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Author Contributions

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Contribution to the Paper	Wrote the manuscript		
Signature	[redacted]	Date	20 / 03 /2015

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Appendices

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Breast Cancer Stem Cells and the Immune System: Promotion, Evasion and Therapy

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Abstract Cancer stem cells are believed to be a subset of heterogeneous tumour cells responsible for tumour initiation, growth, local invasion, and metastasis. In breast cancer, numerous factors have been implicated in regulation of cancer stem cells, but there is still a paucity of information regarding precise molecular and cellular mechanisms guiding their pathobiology. Components of both the adaptive and the innate immune system have been shown to play a crucial role in supporting breast cancer growth and spread, and recently some immune mediators, both molecules and cells, have been reported to influence breast cancer stem cell biology. This review summarises a small, pioneering body of evidence for the potentially important function of the "immuniche" in maintaining and supporting breast cancer stem cells.

Keywords Breast cancer · Cancer stem cells · Immune system · Chemokine · Cytokine · Immunotherapy

Abbreviations

ALDH1	Aldehyde dehydrogenase 1
BCSC	Breast cancer stem cell
CAF	Cancer-associated fibroblast
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CSC	Cancer stem cell
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
EMT	Epithelial-to-mesenchymal transition
IL-6	Interleukin-6
IL-8	Interleukin-8
MFE	Mammosphere-forming efficiency
MMTV	Mouse mammary tumour virus
MSC	Mesenchymal stem cell
PyMT	Polyoma middle T antigen
RANK	Receptor activator of NFκB
RANKL	Receptor activator of NFκB ligand
TAM	Tumour-associated macrophage
TGF-β	Transforming growth factor β

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Introduction

The evolution of the cancer stem cell (CSC) theory [1] has provided a new paradigm for how we view cancer initiation, progression and metastasis. A large body of evidence has been accumulated to support this theory following the first identification of stem-like cells in acute myeloid leukaemia by John Dick and colleagues [2]. This theory states that within a hierarchically organised yet heterogenous tumour mass, there resides a small subset of cells with specific characteristics generally attributed to adult and embryonic stem cells such as quiescence and pluripotency among others, which are responsible for initiation and maintenance of the tumour and subsequent re-seeding to a secondary location [3, 4].

Targeting these quiescent cancer stem cells for therapeutic intervention has come to the forefront of medical research [3], and many of their regulatory mechanisms have been discovered. The major molecular pathways implicated in CSC pathobiology include Notch, Hedgehog, Wnt and TGF-β signalling systems [5–8]. For a recent detailed review see [9]. Importantly, the hypothesis that CSCs exist in a specific stem cell "niche" has recently been supported by a small body of evidence. The CSC niche includes nerves, mesenchyme, and extracellular matrix, as well as stromal and adipose cells [10].

Breast cancer stem cells (BCSCs) have been putatively identified based on the expression of specific surface markers $CD44^{+}CD24^{-/lo}$ (human) and $CD24^{+}CD29^{hi}CD49f^{hi}$ (mouse) [11–13] or methods such as the side population technique (a

Hoechst dye-effluxing cell subset [14]) or ALDEFLUOR assay (based on enzymatic activity of aldehyde dehydrogenase 1, ALDH1 [15]). The ability to sort stem cells based on these markers and to assay their function through use of non-adherent mammosphere assays, in which sphere-forming efficiency can be a measure of self-renewal capability [16], has provided the basis for recent important discoveries in this area.

The immune system plays an important role in cancer progression. Through a series of mutagenesis events, cancer cells may acquire the ability to evade the immune system and overcome anti-tumour host defences. This allows tumour cells to escape T cell-mediated cytotoxicity in primary lesions and potentially extravasate into circulation where they are capable of metastasising [17]. The immune system can be a double-edged sword during cancer progression, as immune cells have been proposed to promote cancer development through secretion of inflammatory cytokines, chemokines, autoantibodies, proteases and induction of angiogenesis [18, 19]. Tumours are known to also dampen the anti-tumour response by activation of suppressive immune cell subsets, such as regulatory T cells and myeloid-derived suppressor cells which are readily recruited and infiltrate the tumour mass [20–22]. Immune modulatory cytokines, chemokines and other molecules are also secreted and/or expressed by immune and cancer cells alike, stimulating cancer cell motility, survival and proliferation [23]. In breast and other cancers, the overall role that the immune system and immune modulators have in facilitating or preventing malignant progression has been summarised elsewhere [24, 25] and is outside the scope of this article.

Recently, literature has begun to emerge that specifically implicates molecules and cells of the immune system in the biology of cancer stem cells. Below, we discuss how certain immune-associated cells and molecules may regulate breast cancer stem cells and maintain the BCSC immunological niche, which we have termed the "immuniche", outlined in Fig. 1. We will also examine how BCSCs may be phenotypically selected for by immune cells and discuss recently proposed cell-based immunotherapies to target BCSCs.

Promotion of Breast Cancer Stem Cells by Immune Modulators

Chemokines and Chemokine Receptors

Chemokines and chemokine receptors have been widely shown to regulate immune cell homeostasis, inflammation and/or responses to infection [26]. In the last two decades, chemokines and their receptors have been implicated in the pathogenesis of various malignancies including colon, lung, pancreatic, skin and many other cancers (for an excellent overview of the multifaceted functions of the chemokine family in cancer see [23]). In breast cancer development, this receptor/ligand family is reported to affect multiple stages of progression, including initial cellular transformation, local invasion, angiogenesis and metastasis [27–30].

Seminal work on the involvement of chemokine receptors in BCSCs has come from Max Wicha's group which have studied the role of CXCR1. CXCR1 is closely related to CXCR2, with which it shares multiple ligands including chemokine interleukin-8 (IL-8, also known as CXCL8). Both receptors have been heavily implicated in the elimination of pathogens but also shown to contribute significantly to disease-associated processes, including tissue injury, fibrosis, angiogenesis and tumourigenesis [31]. It was found that CXCR1-positive cells were present in the $CD44^{+}CD24^{-}$ population in breast cancer cell lines, and were also almost exclusively contained in the ALDEFLUOR-positive population. Stimulation of CXCR1 with IL-8 increased primary and secondary mammosphere-forming efficiency (MFE). In addition, $CXCR1^{+}ALDEFLUOR^{+}$ cells were able to form heterogenous tumours upon transplantation [32, 33], providing functional support for the role of CXCR1 in BCSC regulation. Treatment of breast cancer cell lines with a CXCR1 inhibitor or an anti-CXCR1 antibody resulted in a 5-fold reduction in $ALDEFLUOR^{+}$ cells and inhibition of the CXCR1 interaction with its ligands in sphere culture caused a massive decrease in primary and secondary MFE. Interestingly, when normal human mammary epithelial cells were cultured as mammospheres, IL-8 again increased the primary and secondary MFE, further implicating CXCR1 as a regulator of stemness in both cancer and normal breast development [33].

The CXCR2 chemokine receptor was previously shown to mediate breast cancer chemoresistance [34], an implied property of BCSCs. Co-expression of CXCR1 and CXCR2 by cancer cells is reported to promote cancer growth [35], and as mentioned above, both are receptors for IL-8 which is expressed by BCSCs [36]. As well as IL-8, CXCR2 interacts with chemokines CXCL3, CXCL5, and CXCL7. Wicha and colleagues also examined the result of neutralising CXCR2 but did not observe a difference in the BCSC population [33]. Contrary to this, other reports showed that siRNA targeting of CXCR2 and CXCL3 decreased the viability of $CD44^{+}CD24^{-}$ basal-like breast cancer cell lines through a decrease in STAT3 activity [37], and sequestering CXCL7 blocked expression of other immune modulators from putative BCSCs [38]. In addition, CXCL5 stimulation of breast cancer cell lines increased the $ALDEFLUOR^{+}$ population [38]. As CXCR2 antagonists are already in the clinic for other conditions such as pulmonary disease [39], inhibiting this receptor may prove a promising BCSC-targeting therapy, however the role of CXCR2 in BCSCs still remains to be validated in cancer models in vivo.

Whilst the chemokine receptor CCR7 is upregulated during breast cancer metastasis and has been associated with poor prognosis for breast cancer patients [30], its function in mammary tumourigenesis is still largely uncharacterised. We have

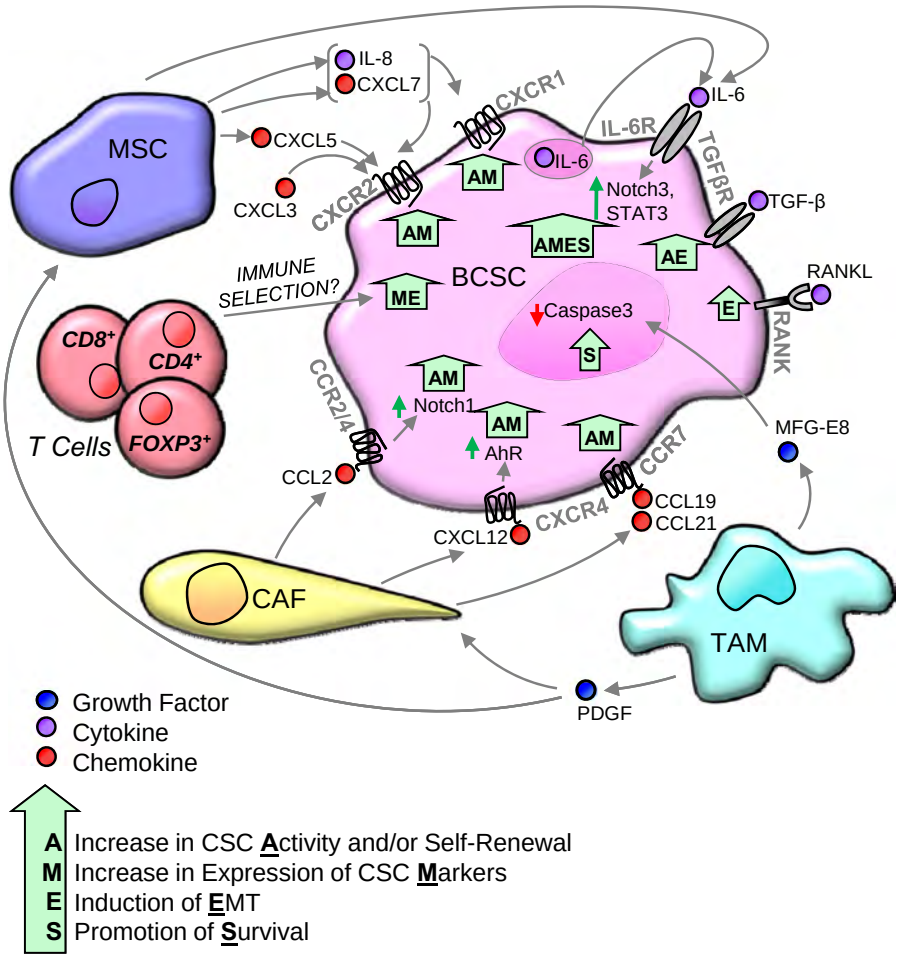

Fig. 1 Maintenance of breast cancer stem cells by immune cells and immune modulators within the immuniche. Breast cancer stem cells (BCSCs) express a variety of immune modulatory receptors on their surface, which correlates with biomarker expression, survival, EMT, and mammosphere-forming ability. Chemokine receptors on the BCSC surface are activated by chemokines secreted by mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs), promoting properties of stemness. Autocrine and MSC-secreted IL-6 stimulates multiple functions in the BCSC, and stimulation by other cytokines such as RANKL and TGF-β result in EMT, an implied characteristic of BCSCs. Tumour-associated macrophages (TAMs) promote BCSC survival both indirectly and directly, and infiltrating T cell subsets in the immuniche provide a catalyst for immune selection of the BCSC

recently shown that CCR7 is expressed on BCSCs from both mice and humans (manuscript submitted for publication). Deletion of CCR7 reduced the BCSC content and self-renewal activity in an MMTV-PyMT mouse model of breast cancer, providing a mechanistic basis for the observed delay in tumour onset and severely reduced tumour burden. We have also demonstrated that pharmacological inhibition of CCR7 in vivo leads to impairment of primary tumour growth via BCSC-related mechanisms, supporting the hypothesis that the CCR7 receptor pathway is a point of intervention in stem cell-targeting therapies.

The CCR7 and CXCR4 receptors are often concurrently upregulated in cancer [30, 40] and we have shown previously that CXCR4 together with CCR7 promotes cancer cell metastasis through inhibition of anoikis, the process of detachment-mediated cell death [41]. Interestingly, whilst CXCR4 has been heavily implicated in various malignancies, its involvement in BCSC pathobiology has not been well defined.

Recently however, CXCR4 was reported to contribute to the maintenance of the BCSC population through transactivation of the aryl hydrocarbon receptor [42] and was also expressed in $CD44^+CD24^-$ breast cancer cell lines, which correlated with their invasiveness [43]. Furthermore, CXCR4 was detected on the surface of mammosphere cells, and CXCR4 antagonists decreased MFE and the proportion of putative $CD44^+CD24^-$ BCSCs [44]. The CXCR4 antagonist plerixafor (AMD3100) has shown some promise in preclinical trials [45, 46] and could also provide an option for targeting stem cells. For example, in tamoxifen-resistant breast cancer cells, Dubrovska et al. showed that AMD3100 inhibited the BCSC-enriched side population [42]. This study, together with our own observations, highlights the potential use of chemokine receptor pathway antagonists as adjuvants to conventional cytotoxic drugs, which alone cannot eradicate the quiescent cancer stem cell populations.

In a recent study, Asiedu et al. comprehensively characterised the chemokine and chemokine receptor profiles in breast cancer cell lines with high $CD44^+CD24^-$ cell content, with the view to identify potential BCSC regulators among this class of immune modulators. They reported increased expression of CCR7, but unexpectedly down-regulation of CXCR4. In addition, $CD44^+CD24^-$ breast cancer cells had increased expression of CXCR7, CCL13, CCL11, CCL12, CCL2, CCL5, CCL6, CCL7 and CCL8, and down-regulated CCR3, CXCL1 and CCL20 [47]. Notably, CXCR7 also binds the ligand for CXCR4, CXCL12, and scavenges CXCL12 to promote cancer cell metastasis [48]. Increased expression of CXCR7 in the CSC population and its impact upon CXCR4 activity remains to be functionally assessed. Of the chemokines upregulated, only CCL2 and CCL5 have been conclusively shown to have a role in breast cancer [49, 50].

CCL2 has been proposed to affect properties of stemness in breast cancer cells. In addition to secretion from BCSCs, treating mammosphere culture with exogenous CCL2 increased stem cell function via Notch-1 signalling and resulted in more spheres with a higher number of slow-cycling stem cells, as was measured by retention of PKH67 dye [51]. Whilst there are clear indications of a potential function for CCL2 from both BCSCs and the immuniche, more specific in vivo evidence is needed to confirm its role in BCSC regulation. Of note, CCL2 is also involved in recruitment of monocytes, memory T cells, regulatory T cells and dendritic cells to inflammatory sites [52], and to breast tumours [22] through its receptors CCR2 and/or CCR4, which may aid BCSCs in controlling the immune system.

Interleukin-6

Interleukin-6 (IL-6) regulates a number of immune functions and stimulates many inflammatory and auto-immune processes. As yet, it is one of the most studied cytokines in BCSC biology with significant evidence accumulated to support its multi-faceted role in this specialised subset of tumour cells. Thus, NFκB-mediated upregulation of IL-6 was reported for mammosphere cells when compared to the bulk cell population [53, 54], and trastuzumab-resistant CSCs also expressed high levels of IL-6 [36]. Stem-like cells were found to express higher levels of IL-6 than differentiated cells and the addition of IL-6 to differentiated cell culture could force cells to rapidly de-differentiate. When an anti-IL-6 antibody was added, this de-differentiation was blocked [55]. As de-differentiation was recently proposed as an important mechanism in the generation of BCSCs [56], this novel finding may provide new options in specifically targeting the BCSC axis.

Similarly, blocking the interaction of IL-6 with its receptor (IL-6R) in mammosphere cultures also inhibited cell propagation through down-regulation of Notch-3 [53, 54], and blocking IL-6 activity via siRNA or neutralising antibody decreased viability of $CD44^+CD24^-$ putative BCSCs [36, 37]. Furthermore, it was found that IL-6 is able to increase stem cell activity, via Notch-3 mediated up-regulation of Jagged-1, and STAT-3. It was proposed that IL-6 induces an autocrine loop and stem cell-linked aggressiveness in breast cancer cells [54, 57]. The fact that in the presence of high levels of IL-6, drugs such as trastuzumab fail to eliminate cancer stem cells in culture and xenograft models [36] makes the IL-6 axis a firm candidate for stem cell directed therapeutic intervention.

Circulating tumour cells are believed to possess properties of stemness such as anoikis resistance and a more mesenchymal phenotype. High levels of IL-6 have been reported to promote the epithelial-to-mesenchymal transition (EMT) in breast cancer cells, leading to a conversion to the BCSC phenotype [58]. Thus, addition of IL-6 to adherent human breast cancer cells was found to increase the proportion of $CD44^+$ putative BCSCs, which had greater ability to form mammospheres as well as increased in vivo tumourigenic potential over $CD44^-$ cells. The acquisition of CD44 also coincided with a change in cell morphology from cobblestone to spindle-like, increased invasiveness, and the induction of a gene expression profile consistent with EMT, including decreased E-cadherin, and increased vimentin and twist [36, 59, 60].

RANK and RANKL

Receptor activator of NFκB ligand (RANKL) is a member of the Tumour Necrosis Factor cytokine family. It is expressed by T cells and stromal cells, and is thought to be involved in dendritic cell maturation [61, 62]. Activation of its receptor RANK in breast cancer cells through administration of RANKL inhibited cell differentiation, induced EMT, and promoted expression of stem cell surface markers and stemness genes (such as SOX2, NANOG and OCT4). Furthermore, the RANK-RANKL interaction was found to increase self-

renewing capacity and augment tumour development both in transplant models and spontaneously [63–65].

More direct evidence for the role of RANK and RANKL in BCSCs was obtained using the medroxyprogesterone acetate (MPA) mouse model of mammary cancer. Schramek et al. found that MPA-induced tumour formation triggered an induction of RANKL, with a corresponding expansion of $Lin^{-}CD24^{+}CD49f^{hi}$ BCSCs in $Rank^{+/+}$ mice, but not in MMTV-$Rank^{-/-}$ mice. While both $Rank^{+/+}$ and MMTV-$Rank^{-/-}$ breast cancer cells were able to form primary spheres, secondary spheres could only be propagated when RANK was present [66], demonstrating the role of RANK and RANKL in BCSC self-renewal.

Cancer and normal mammary stem cells have been proposed to follow similar regulatory routes [67, 68]. Interestingly, RANK and RANKL also have important roles in the regulation of normal mammary stem cells, in adulthood and in alveologenesis during pregnancy. Thus, in response to progesterone signalling, RANKL is expressed in the mature luminal cell subset and RANK is up-regulated in the mammary stem and basal progenitor cell compartment. The interaction of RANKL on luminal cells and RANK on stem/basal cells potentially creates a paracrine loop, in which RANKL acts as a key hormone mediator for mammary stem cells in mice [69, 70].

These findings warrant further investigation in potentially important functions of both RANK and RANKL in regulation of human BCSCs. A small number of other factors have been shown to play a role in promotion of the BCSC phenotype, however the evidence for their role may be circumstantial. The only other well-documented player in regulation of BCSCs is TGF-β, the role of which has been comprehensively reviewed elsewhere [71].

The "Immuniche": The Role of the Immune System in the BCSC Niche

Breast cancer stem cells are thought to reside within a stem cell niche composed of nerves, mesenchyme and extracellular matrix [10]. To some extent, each of these components supports the turnover and survival of this sub-population. More recently, certain immune cells and stromal cells secreting cytokines, chemokines, and other immune modulators, have been identified as playing a specific role in the BCSC niche, to form an "immuniche" that provides stem cells with necessary growth signals and factors to maintain their stemness properties, avoid immuno-surveillance and propagate tumours.

Stroma-Derived Cells and the Immuniche

Mesenchymal stem cells (MSCs) are heavily involved in support of both active and innate immune cells [72, 73]. They have been shown previously to have a role in breast cancer progression and metastasis through secretion of, or migration in response to chemokines CCL5 and CCL2 [74, 75]. It was recently found that the co-culture of human breast cancer cells with bone marrow-derived MSCs also led to an increase in $ALDEFLUOR^{+}$ and $CD44^{+}CD24^{-}$ putative BCSCs. This co-culture induced the expression of CXCL1, CXCL5, CXCL6 and CXCL7, as well as IL-6 and IL-8, from both the MSCs and BCSCs [38]. Therefore MSCs in the immuniche are potentially an important source of secreted specific immune modulators that are required to maintain stem cell activity by BCSCs.

Cancer-associated fibroblasts (CAFs), another notable component of the immuniche, have been well documented for their function in breast cancer promotion via secretion of a milieu of cytokines and chemokines [76–78]. Whilst direct evidence for the role of CAFs in BCSC regulation is yet to be established, some recent findings suggest that CAFs may be important for maintaining optimal conditions in the BCSC immuniche, as it was shown that breast cancer cells co-cultured under mammosphere conditions with CAFs that secrete CXCL12 and/or CCL2 have higher MFE and proportions of $CD44^{+}CD24^{-}$ putative BCSCs. Furthermore, neutralising CCL2 secreted from CAFs with antibody completely inhibits MFE of the breast cancer cells, therefore disrupting the BCSC immuniche [44, 51].

Immune Cells and the Immuniche

Macrophages are innate immune cells that are often recruited to the tumour microenvironment. As tumour cells may be recognised as self, these tumour-associated macrophages (TAMs), rather than aid the anti-tumour response, promote breast cancer cell growth and metastasis by the secretion of immune modulators [79, 80]. Their role in the CSC immuniche is largely uncharacterised, but limited evidence has come to light that implicates TAMs in regulation of BCSCs.

In the study of breast cancer metastasis to the bone, TAMs were generated by stimulating human monocytes with cytokines and conditioned media and co-injected into tibial bones of mice with magnetically isolated $CD44^{+}CD24^{-}$ cells from the 231BoM breast cancer cell line which is highly metastatic to the bone. This co-injection significantly augmented tumour growth compared to injection of $CD44^{+}CD24^{-}$ cells alone, strongly supporting the idea that TAMs may play an important role in BCSC maintenance. Using an antibody array and immunofluorescent microscopy, it was further found that platelet-derived growth factor (PDGF) was specifically released from TAMs upon co-culture and interaction with $CD44^{+}CD24^{-}$ cells. The authors showed that TAMs had a dual role in the immuniche, additionally stimulating stromal cells such as fibroblasts and MSCs to secrete FGF and other growth factors, that act directly on BCSCs [81].

TAMs were found to produce large amounts of the growth factor milk fat globule (MFG)-E8, and colon and lung CSCs

were found to specifically induce MFG-E8 expression from macrophages to support their survival when inoculated into syngeneic mice. MFG-E8 secreted from TAMs suppressed caspase-3 activation, inhibiting apoptosis in the lung and colon cancer stem cell populations. This was then tested in putative BCSCs, with similar results [82]. Considering the fact that the presence of TAMs in the BCSC immuniche has a number of direct and indirect functions, these immune cells may be targeted by anti-BCSC therapies.

T cells can participate in breast cancer promotion when they are recruited to the tumour microenvironment [18, 20, 22]. Breast cancers of higher histological grade and with highly aggressive steroid receptor-negative status have high levels of $CD8^+$ cytotoxic T cell infiltration and pro-inflammatory cytokines associated with a $CD4^+$ helper T cell response [83–85]. In addition, $FOXP3^+$ regulatory T cells, which have been implicated in breast cancer aggressiveness, are recruited to the tumour microenvironment by CCL22 [22, 86]. There is little evidence available to date on the direct role of different T cell subsets on BCSCs, however emerging data suggest that T cells may also be active players in the BCSC immuniche.

Thus, it has been reported that high grade estrogen receptor-negative and highly aggressive triple-negative breast cancers had increased levels of cytotoxic, helper and regulatory T cell tumour infiltrations. This was also correlated with increased $CD44^+CD24^-ALDH1^+$ tumour cell content and expression of EMT-associated markers vimentin, osteonectin and smooth muscle actin [85]. Additionally, it has been suggested that EMT induction to generate BCSCs may be dependent on cytotoxic T cells [87]. However, this increase in BCSC content upon tumour infiltration by T cells may also be due to stem cell specific immune selection, discussed in more detail below.

Although the role of active immune cells in regulation of BCSCs is currently not well defined, some recent advances in delineating a function for specific T cell subsets within the BCSC immuniche should encourage more research in this area. Moreover, given the large part that T cells can play in breast cancer development, it is thought that this field will soon expand significantly.

Immune Selection and Tolerance of Breast Cancer Stem Cells

One of the hallmarks of cancer is the evasion of immune clearance [17] and the ability of rapidly-dividing bulk cancer cells to do this has been well characterised [88]. Immune evasion by quiescent cancer stem cells, including breast CSCs, is an interesting paradigm that remains to be elucidated. Currently, the exact origin of BCSCs is yet to be uncovered. It is thought that a *bona fide* cancer stem cell, with acquired stem-like properties, evolves from the tumour cell-of-origin through survival of the fittest [56]. The presence of immune cells within the immuniche may aid cancer cells in acquiring a more stem-like and immune-resistant phenotype through a process termed "immune selection" (Fig. 1).

Immune selection may play a part in resistance to immune-mediated therapy of cancer stem cells. Bulk cancer cells can develop resistance to conventional cytotoxic drugs due to up-regulation of drug transporters [17], and it is thought that CSCs may have more robust mechanisms of drug efflux. The same may be true for immunotherapies, where CSCs avoid killing by cytotoxic cells. For example, it was found that treatment with trastuzumab depleted HER2-expressing breast cancer cells through a natural killer (NK) cell-mediated mechanism. However, cells that survived this NK immune selection had increased $CD44^{hi}CD24^{lo}$ surface marker expression and were able to grow more robustly as mammospheres compared with naïve cells. After six cycles of treatment, the $CD44^{hi}CD24^{lo}$ cell content was significantly increased, indicating that proportions of BCSCs increased over time through immune selection [89].

Tumour cells are also able to avoid recognition by primed immune cells through immuno-editing, a process in which tumour cells lose or modify tumour antigens. Findings by Knutson et al. showed that immuno-editing may occur through the process of EMT, a route that may also result in the acquisition of a BCSC phenotype. The authors have suggested that immuno-editing is not an incremental loss of tumour antigens but rather an active process in which cells use EMT to de-differentiate into cells of a stem-like state [90].

Immune selection and immuno-editing may result in tumour cells that are recognised as self by immune cells, thus leading to tolerance and evasion of the immune system. Exact mechanisms underlying these phenomena are not yet known, however glycoprotein CD200 (OX2) has been reported to be an important player in BCSC immune evasion. OX2 is commonly expressed in myeloid cells and has been studied as a therapeutic target for its role in tolerance. In tumourigenic and invasive breast cancer cells, it was found that a high percent of $OX2^+$ cells were $CD44^+CD24^-$ [91].

In summary, it is plausible that the infiltration of immune cells into mammary tumours may stimulate more mature breast cancer cells to de-differentiate into stem-like cells through EMT, coinciding with the activation of molecules that promote tolerance of CSCs by the immune system. This in turn may lead to CSC immune evasion and the development of more aggressive tumours. This hypothesis warrants specifically designed future studies, which should examine the interplay between immune infiltrates and BCSC populations in animal models and primary human tumour samples.

Cell-Based Immunotherapy and Breast Cancer Stem Cells

Numerous cancer-targeted immunotherapies are currently being explored and considerable progress has been made in the

development of cell-based novel therapeutics, also known as "activation" immunotherapy, for the treatment of breast cancer (for a recent review, see [92]). Whilst the use of cell-based immunotherapy to treat multiple malignancies has progressed to the advanced stages of preclinical development, the concept of CSC-directed immunotherapy is still in its infancy. Bearing in mind that CSCs are largely resistant to conventional therapeutics, the generation of new cell-based approaches aimed at specifically eliminating this evasive cancer cell subset may provide a potential cure for as yet incurable cancers.

Recently, Wang et al. developed an irradiated whole cell vaccine from $CD24^{+}CD29^{+}$ mammosphere cells, originally from HER2/neu transgenic mice with breast cancer. Once mice were immunised with the vaccine, both cytotoxic and helper T cells were isolated from draining lymph nodes, separated, and activated by cytokines in vitro. When the primed T cells were adoptively transferred into mice with established lung metastasis, there was near complete eradication of lung tumours [93], demonstrating the applicability of this cell-based approach.

Another group found that in drug-resistant breast cancer cell lines which have a high proportion of cells expressing BCSC markers, the balance of stem cell-associated molecules Notch and its antagonist Numb shifted towards Numb. In culture, Numb-1 peptides were able to activate $Numb1^{+}CD8^{+}$ immune cells, and when these $Numb1^{+}CD8^{+}$ cells were incubated with breast cancer cells, the proportion of $CD44^{hi}CD24^{-/lo}$ cells was reduced. This implies that cells with BCSC markers could be eliminated by Numb-1-peptide complex specific cytotoxic T cells to induce cell-mediated cytotoxic immunity [94]. Adoptive transfer experiments may show whether this approach is a viable in vivo option.

Pulsed dendritic cell vaccines may also hold potential as BCSC vaccines. At the time of writing this review, no literature was available on this topic. However, it is feasible that a similar approach as described in melanoma could be applied to breast cancer. Recently, Ning et al. showed that pulsing dendritic cells with $ALDH1^{+/hi}$ melanoma cells to obtain a CSC vaccine, and subsequent immunisation of mice followed by challenge with bulk tumour cells, induced both humoral and cell-mediated protective immunity. Treatment of mice with this vaccine led to reduced tumour growth and metastasis in murine xenograft models of melanoma [95].

The latest study by Knutson and colleagues proposed a multi-peptide vaccination in combination with antibody therapy to simultaneously target various cell subsets within the tumour and the immuniche including TAMs and BCSCs. However, these results do not directly assess the impact of this therapeutic approach on the BCSC populations [96].

These approaches are only just evolving and there remains the need to verify and validate their feasibility in more diverse disease models, particularly in mouse models of breast cancer and human preclinical studies. Whilst vaccines specifically targeting BCSCs are a promising avenue for more effective future treatments, a great deal of work is still required as there are no currently available data for their use in primary settings. Also, the possibility of immune selection of cells able to acquire stem-like characteristics remains a cause for concern.

Conclusions

There is considerable evidence that the maintenance and activity of breast cancer stem cells is dependent to a large extent on immune modulators expressed not only by BCSCs themselves but also by stromal and immune cells within the immuniche. The presence of cancer-associated fibroblasts, mesenchymal stem cells, macrophages and T cells may be necessary for BCSC self-renewal and maintenance of the stem cell phenotype through secreted factors. Furthermore, the impact of T cells and other immune cells must be carefully balanced to prevent tolerance and immune selection of BCSCs which are able to survive the onslaught of conventional therapies. In the near future, the development of novel agents and approaches targeting the components of the immuniche may provide more effective cancer stem cell-directed therapies for breast cancer patients.

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ORIGINAL ARTICLE

The chemokine receptor CCR7 promotes mammary tumorigenesis through amplification of stem-like cells

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The chemokine receptor CCR7 is widely implicated in breast cancer pathobiology. Although recent reports correlated high CCR7 levels with more advanced tumor grade and poor prognosis, limited *in vivo* data are available regarding its specific function in mammary gland neoplasia and the underlying mechanisms involved. To address these questions we generated a bigenic mouse model of breast cancer combined with CCR7 deletion, which revealed that CCR7 ablation results in a considerable delay in tumor onset as well as significantly reduced tumor burden. Importantly, CCR7 was found to exert its function by regulating mammary cancer stem-like cells in both murine and human tumors. *In vivo* experiments showed that loss of CCR7 activity either through deletion or pharmacological antagonism significantly decreased functional pools of stem-like cells in mouse primary mammary tumors, providing a mechanistic explanation for the tumor-promoting role of this chemokine receptor. These data characterize the oncogenic properties of CCR7 in mammary epithelial neoplasia and point to a new route for therapeutic intervention to target evasive cancer stem cells.

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INTRODUCTION

Despite concerted efforts and significant advances, breast cancer-related mortality is still a leading cause of death in women worldwide.[1] Clearly novel therapies are urgently needed. The 'cancer stem cell' theory specifies that a small subset of cells in a heterogeneous tumor (termed 'cancer stem cells' (CSCs)) possess stem cell-like properties of self-renewal and differentiation. CSCs are suggested to sustain and propagate tumors, and are inherently therapy-resistant (for the latest reviews see[2] and[3]).

CSCs may originate from adult stem cells, but can also arise from more committed lineage progenitor cells if they acquire stem cell-like features owing to genetic or epigenetic changes.[4] Multiple intrinsic and extrinsic factors are reported to have a role in CSC maintenance, regulation and support of stem-like characteristics. Most prominent are the Notch,[5] Hedgehog,[6] Wnt[7] and TGFβ[8] signaling systems. Several cytokines and chemokines have also been recently suggested as maintaining and promoting the CSC phenotype in a number of solid malignancies, including mammary tumors (reviewed in[9]); however, definitive *in vivo* data has been sparse.

Chemokine receptors and their cognate chemokine ligands have become widely accepted as important mediators of cancer growth and progression in many human neoplasms, being involved in tissue transformation, invasion, angiogenesis and resistance to chemotherapy.[10] Among these, the chemokine receptor CCR7 has been implicated in metastatic spread of multiple malignancies.[11] In breast carcinogenesis, it has been attributed a number of potential functions, including promotion of cell motility, migration and adhesion, regulation of matrix metalloproteinases leading to basement membrane degradation,[12] and cell survival through inhibition of anoikis.[13] Data obtained using cell lines has implicated CCR7 in breast cancer spread to the lymph nodes,[14] and in human breast cancer its role was inferred from retrospective studies on archived tumor tissues.[15] High expression levels of CCR7 were also correlated with higher grade and occurrence of secondary tumors, and poor prognosis.[16,17]

Whereas all these studies point to a role for CCR7 in malignancy, a direct function for CCR7 in cancer has not yet been established. Furthermore, its role in breast cancer in particular is unclear. We show here the development of a novel bigenic mouse model combining deletion of CCR7 with the polyoma middle-T transgene, which is under control of the mouse mammary tumor virus promoter (MMTV-PyMT), to study tumor development *in vivo*. Using this model we show that CCR7 deletion has a striking preventative effect on PyMT-driven mammary tumors, supporting the notion that CCR7 has a major determining role in breast oncogenesis. Moreover, our data reveal that the tumor-promoting effect of CCR7 is mediated through stem-like cells in both primary mouse and human breast tumors. These results provide new insights into the role of CCR7 in breast cancer stem-like cells and have important implications for the development of future therapeutics in breast cancer.

RESULTS

CCR7 deletion arrests mammary tumorigenesis in the PyMT transgenic breast cancer mouse model

The MMTV-PyMT transgenic breast cancer mouse model has been extensively used in recent years to study various aspects of

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mammary neoplasia. Expression of the PyMT protein promotes the rapid epithelial transformation of mammary cells via the corruption of various pathways including those of *Src, ras* and PI3 kinase. This model also results in spontaneous metastasis and has been found to closely mimic the development of human breast cancer.[18–20] Representative images are shown in Supplementary Figure 1a, in which α-smooth muscle actin is used to stain myoepithelial cells.

To directly assess the role of CCR7 in the multistage process of mammary tumorigenesis *in vivo*, we generated bigenic MMTV-PyMT *Ccr7*$^{-/-}$ knockout mice (further referred to as CCR7 KO+) and traced the development of mammary tumors relative to MMTV-PyMT *Ccr7*^{WT} mice (further referred to as WT+).

Deletion of CCR7 significantly delayed PyMT-driven primary mammary tumorigenesis (representative pictures Figure 1a). Tumor-free survival was significantly extended (Figure 1b) and total tumor burden was markedly reduced in CCR7 KO+ mice (Figure 1c) when compared with the WT+ animals. The lungs of WT+ and CCR7 KO+ females were also examined for metastatic lesions at the time of killing. CCR7 KO+ mice developed significantly fewer and smaller metastases than WT+ mice (Figures 1d–f), although the number of metastases varied largely between mice of the same genotype.

As these experiments indicated a role for CCR7 in mammary gland function, we next examined normal, pre-cancerous and cancer-bearing mouse mammary glands for CCR7 expression and signaling. CCR7 was shown to be expressed on all mouse mammary epithelial cells tested, regardless of the tumor stage (Supplementary Figure 1b), and the removal of CCR7 did not affect the expression levels of its ligands CCL19 and CCL21 within the mouse mammary fat pad (Supplementary Figure 2a). CCR7 was also found to be functional in PyMT-driven mammary tumors, as tumor cells mobilized intracellular calcium, a hallmark of chemokine receptor activity, in response to stimulation with CCL21 (Supplementary Figure 2b). These data showed that CCR7 was expressed and was functional within normal and transformed mammary epithelium.

Interestingly, despite the large impact of CCR7 on overall mammary tumorigenesis, initial PyMT-driven hyperplastic growth in 8- or 11-week-old WT+ and CCR7 KO+ mice was not affected (Supplementary Figure 3a), with similar tissue architecture in glands from both genotypes (Supplementary Figure 3b). This indicated that the CCR7 KO+ mammary glands underwent the initial oncogenic transformation leading to epithelial proliferation, but further tumorigenic transition was largely blocked by CCR7 deletion.

CCR7 promotes tumorigenesis by amplifying breast cancer stem-like cells

To investigate the underlying mechanisms responsible for the tumorigenic effects seen, we examined the role of CCR7 in mammary gland development and on stem-like cells. We found that in non-PyMT wild-type (WT) pubertal mice, the epithelial tree was longer with a better developed ductal structure than that in *Ccr7*$^{-/-}$ (CCR7 KO) mice (Figures 2a and b), indicating that ablation of CCR7 had a mild inhibitory effect on pubertal growth of the mammary gland epithelium. CCR7 was robustly expressed in normal mammary epithelium (Figure 2c), making this receptor also potentially relevant to normal mammary development. However, the development of mammary epithelium in the CCR7 KO mice caught up with that of the WT mice by the age of 8 weeks, and at 12 weeks mammary glands from the two genotypes were indistinguishable (Figure 2d), demonstrating that CCR7 deletion mainly delays early mammary gland development.

Because normal development and breast cancer are believed to be linked by common regulatory mechanisms, we hypothesized that the observed promotion of PyMT-driven tumorigenesis and mammary development was due to CCR7 regulating stem/progenitor cell pools in mammary epithelium. Thus, we next assayed the stem-like cell content in mice using the lineage-negative (Lin$^-$) CD24$^+$CD29^{hi} cell surface marker profile,[4] which was previously functionally characterized in the MMTV-PyMT model.[21,22] CCR7 was expressed in all cell lineages in both the normal and PyMT-expressing mammary glands regardless of CD24 and CD29 status. Notably, however, higher levels of CCR7 (>90%) were observed in Lin$^-$CD24$^+$CD29^{hi} normal and cancer mouse mammary stem cell-enriched populations (Figure 3a, Supplementary Figure 4a and b). Importantly, CCR7 was also expressed on human CD44$^+$CD24$^-$ putative mammary stem cells[23] from both normal and breast tumor tissue (Figure 3b, Supplementary Figure 4c).

Further analysis demonstrated a significantly lower content of Lin$^-$CD24$^+$CD29^{hi} cells in non-PyMT CCR7 KO mice relative to WT (Figure 3c left panel). In PyMT-expressing mice at the stage of early neoplasia, when no morphological differences were found in WT+ and CCR7 KO+ glands (Supplementary Figure 3) and the stem/progenitor cell populations may, therefore, best reflect the tumor-initiating cell content, the difference in the stem cell-enriched population between WT+ and CCR7 KO+ mice was even more pronounced with the deletion of CCR7 leading to a twofold reduction in stem-like cells (Figure 3c right panel).

Recently Pece *et al.* have suggested a new and potentially more efficient set of markers, in which the notch ligands delta-like ligand 1 (DLL1) and delta and notch-like epidermal growth factor-related receptor (DNER) are used in combination with CD49f (Lin$^-$CD49f$^+$DLL1$^+$DNER$^+$) to delineate putative stem cells in human mammary tumors.[24] We found that the stem-like cells from both human and mouse mammary glands defined by this profile also expressed high levels of CCR7 (Supplementary Figure 4d and e). Moreover, the Lin$^-$CD49f$^+$DLL1$^+$DNER$^+$ cell pools were significantly smaller in both normal and PyMT-expressing CCR7 KO murine mammary glands (Supplementary Figures 5a and b), providing further support for the findings described above.

It is generally accepted that non-adherent passaged mammosphere cultures are enriched in cells with stem-like characteristics, and secondary/tertiary mammosphere-forming efficiency (MFE) is representative of cells' potential to exhibit stem cell traits.[25–27] Stem-like activity, as measured by MFE, was then analyzed in the mammary epithelium in the presence or absence of CCR7. Primary and secondary sphere formation from normal (Figure 3d) or PyMT-expressing (Figure 3e) mammary cells was substantially reduced after CCR7 ablation and, importantly, stimulation of WT and WT+ cells with CCR7 ligands CCL19 and CCL21 significantly potentiated mammosphere growth (Figures 3d and e).

This CCR7 stimulatory function was seen exclusively on mammosphere growth, as stimulation with CCL19 and CCL21 had no detectable effect on the proliferation of bulk mammary tumor cells in adherent culture, a condition that supports a more differentiated phenotype (Supplementary Figure 5c). The addition of CCR7 ligands to sphere cultures derived from CCR7 KO+ mammary cells also had no effect on MFE (Supplementary Figure 5d), demonstrating a CCR7 receptor-mediated mechanism. The specificity of CCR7 was further shown by testing a panel of ligands for other tumor-associated chemokine receptors CCR6,[16] CXCR3[28] and CXCR5.[29] No effects were observed on MFE (Supplementary Figure 5e).

To extend these findings to human breast cancer we next examined the activity of CCR7 in human primary tumor cells from resected breast cancer tissue. The addition of CCL19 and CCL21 resulted in an increase in primary and secondary MFE of human breast cancer cells by two- to threefold (Figure 3f), consistent with results obtained in the mouse model.

To specifically link the deletion of CCR7 to depleted tumor-initiating cells, a limiting dilution transplantation approach was used[24] to estimate tumor-initiating cell (TIC) frequency. Secondary

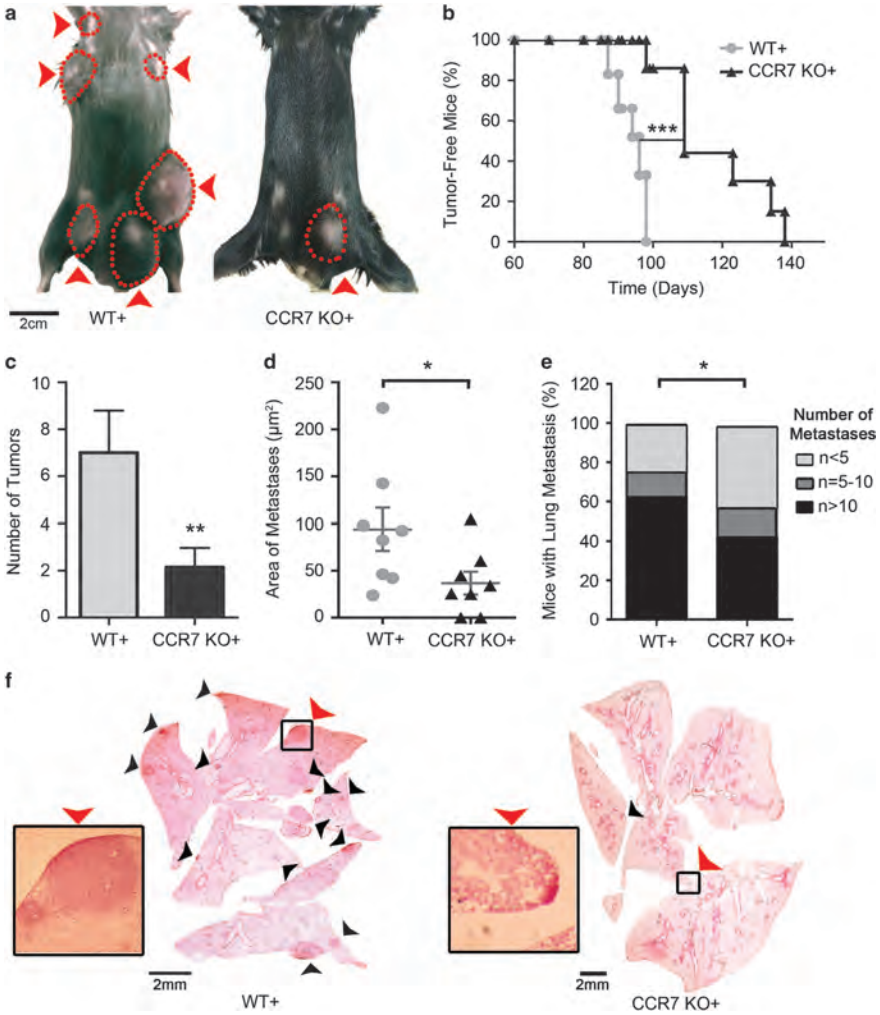


Figure 1. CCR7 deletion arrests mammary tumorigenesis in the PyMT transgenic breast cancer mouse model. (**a–c**) CCR7 ablation delays tumor onset and reduces tumor burden in the MMTV-PyMT-driven mouse model of breast cancer. (**a**) Representative images of MMTV-PyMT $Ccr7^{WT}$ (WT+) and MMTV-PyMT $Ccr7^{-/-}$ (CCR7 KO+) mice at 22-weeks-old, showing grossly visible tumors (demarcated by red arrows and dotted lines). (**b**) Kaplan–Meier analysis of tumor-free survival for WT+ ($n = 18$) and CCR7 KO+ ($n = 17$) mice. (**c**) Number of tumors in WT+ and CCR7 KO+ mice at the time of killing. (**d–f**) CCR7 KO+ mice developed less lung metastases than their WT+ counterparts. (**d**) Total cumulative area of lung metastatic lesions in WT+ ($n = 8$) and CCR7 KO+ ($n = 8$) mice. (**e**) Distribution data of lung metastases in WT+ and CCR7 KO+ mice. (**f**) Representative images of H&E-stained lung sections from WT+ (left) and CCR7 KO+ (right) mice with metastatic lesions (black arrowheads). Red arrowhead indicates inset magnified image. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mammosphere-derived cells from WT+ and CCR7 KO+ mice with early neoplasia were used in this assay to address the potential of cells in mammosphere cultures to exhibit stem cell traits of self-renewal and tumor initiation *in vivo*, in the context of CCR7-dependency. Cells were injected into contralateral inguinal fat pads of non-PyMT WT recipients. Analysis of grafted fat pads after 6 weeks showed that WT+ sphere cells produced much more robust growth at all dilutions (Figure 4a and Supplementary Figure 6). Most importantly, the frequency of stem-like cells capable of tumor initiation within WT+ sphere culture (1/189) was over threefold higher than in CCR7 KO+ (1/913) (Figure 4b), providing strong evidence for the critical role of CCR7 in the regulation and maintenance of stem-like cells and tumor-initiating cells in the mammary gland.

CCR7 is required for the propagation of mammary tumors

To obtain *in vivo* evidence for the role of CCR7 in tumor propagation, we took advantage of the PyMT mouse model, which allows tumor formation upon transplantation.[20] Expression of the PyMT oncogene results in multifocal tumors and hence can

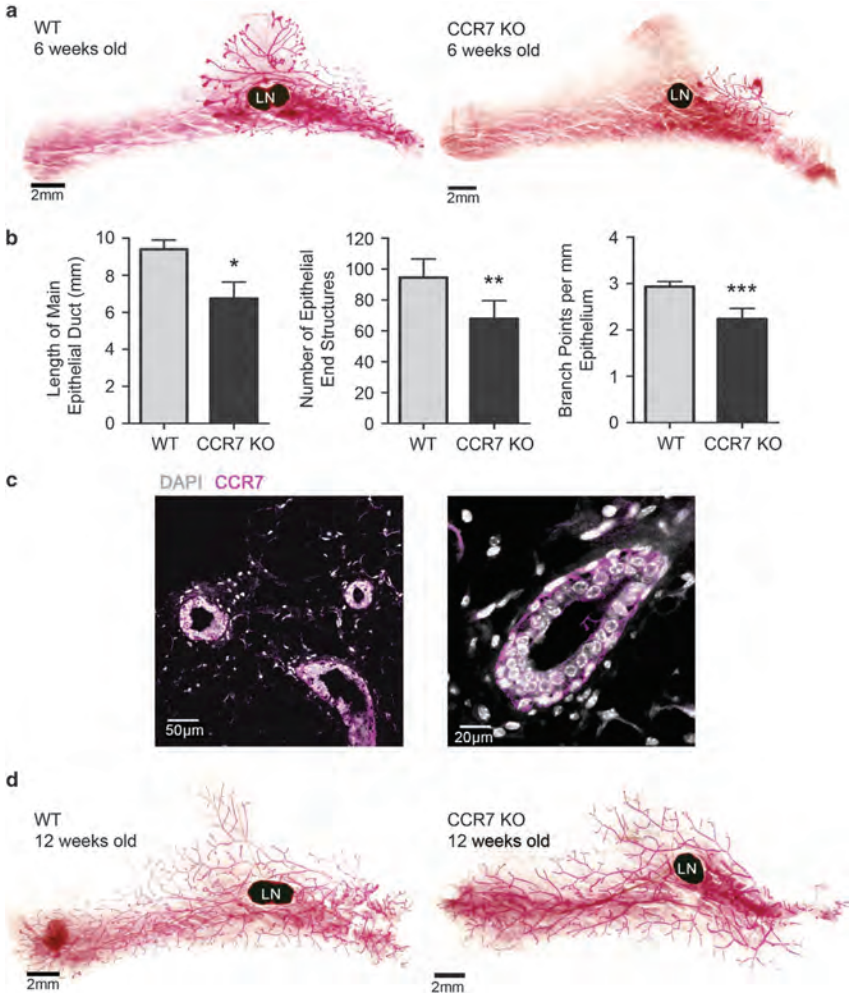
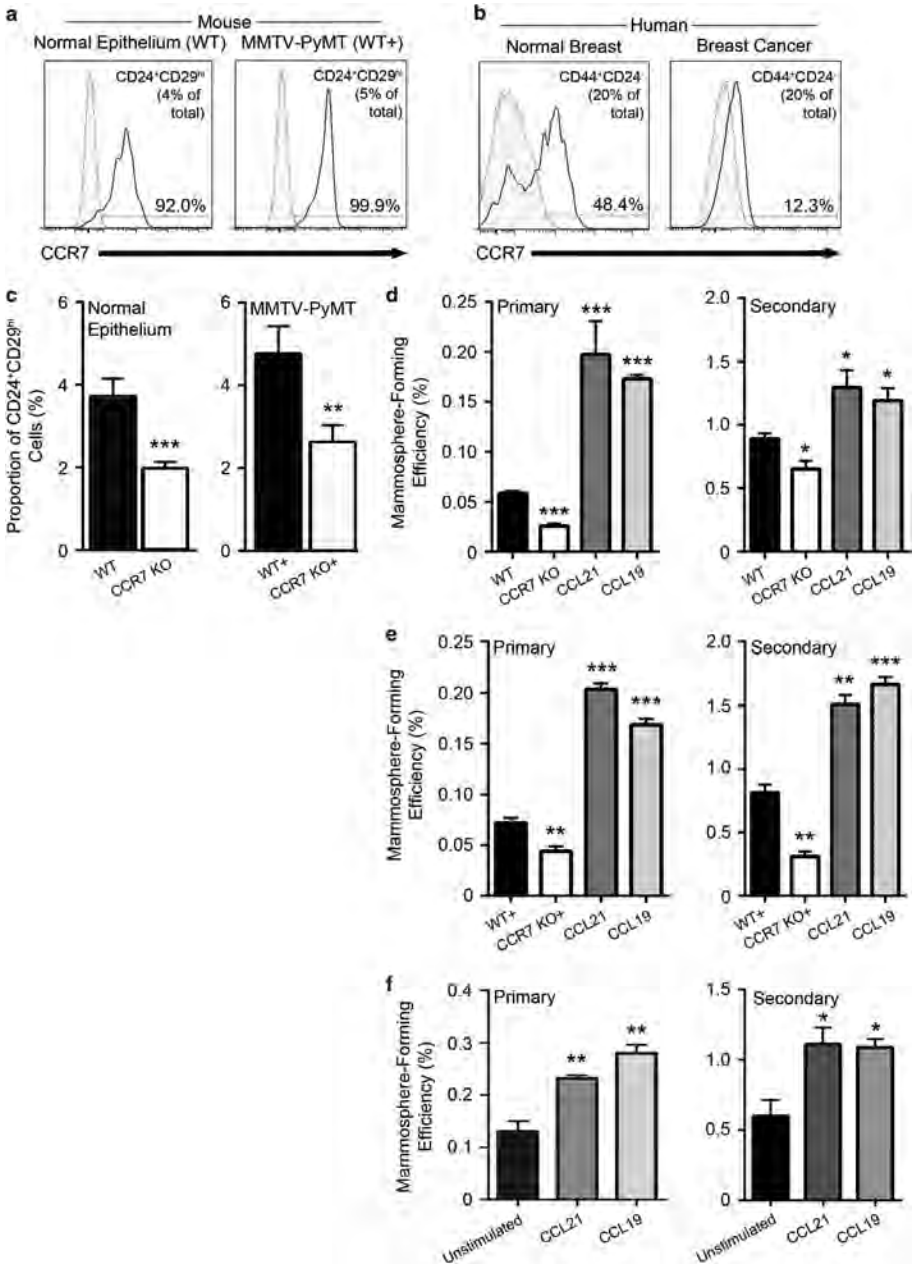
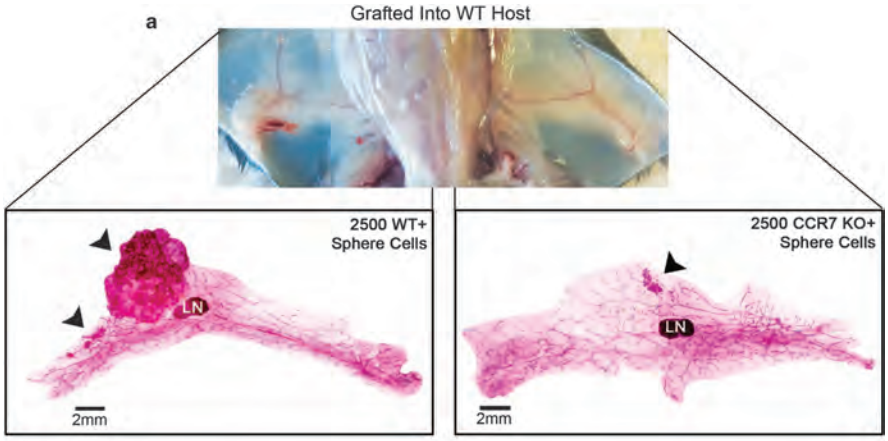


Figure 2. The effect of CCR7 on normal mammary development. (**a** and **b**) Development of the mammary ductal tree was evaluated in *Ccr7*^{WT} (WT) and *Ccr7*$^{-/-}$ (CCR7 KO) C57Bl/6 mice at 6 weeks of age. (**a**) Representative whole mount images of mammary glands, with apparent reduction in the size of ductal trees in CCR7 KO (right, $n=6$) compared with WT (left, $n=7$). LN = lymph node. (**b**) Quantitation of the length of the main epithelial duct (left), total number of mammary epithelial terminal structures (center), and branching within mammary epithelium determined by quantifying branch points per mm along three individual ducts (right). *$P<0.05$, **$P<0.01$, ***$P<0.001$. (**c**) CCR7 expression (magenta) in normal mouse mammary terminal end buds of pubertal mice. Nuclei are counterstained with DAPI (gray). (**d**) Representative whole mount images of mammary glands taken from adult WT (left, $n=6$) and CCR7 KO (right, $n=6$) mice at 12 weeks of age with no apparent differences in the size and architecture of the mammary tree.

Figure 3. CCR7 activity amplifies stem-like cells in both mouse and human mammary glands. (**a–c**) Normal mammary glands and mammary tumors were analyzed by flow cytometry for stem-like cell content. (**a**) Proportion of cells positive for CCR7 in Lin$^-$ stem cell-enriched populations in normal mouse mammary epithelium (left) and PyMT-expressing glands (right), as denoted by surface marker expression CD24$^+$ CD29^{hi}. Shaded histograms = fluorescence-minus-one negative gates. (**b**) Proportion of cells positive for CCR7 in putative stem-like cell populations in normal human mammary epithelium (left) and breast cancer (right), as denoted by surface marker expression CD44$^+$CD24$^-$. Shaded histograms, fluorescence-minus-one negative gates. (**c**) CCR7 deletion decreases the proportion of mouse Lin$^-$ stem cell-enriched populations in normal (left) and PyMT-expressing (right) mammary glands as indicated. (**d–f**) The effect of CCR7 deletion/activation on primary and secondary mammosphere formation was assessed. Shown are mammosphere-forming efficiencies of cells derived from normal mouse mammary glands (**d**), PyMT-expressing mouse mammary glands (**e**), and human patient-derived breast tumors (**f**). WT mouse cells and primary human cells were stimulated with CCL21 and CCL19 as indicated. (**a–f**) Mouse data are representative of at least three independent experiments, $n=6$–10 mice per group. Human results are representative of two normal and four independent tumor samples. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

generate diverse CSC pools owing to various underlying mutations within the same gland at the late stages of tumorigenesis. Therefore, we reasoned that if taken at the early stage of preneoplastic tumor development, the population of CSCs should be more homogeneous. Consequently, small 1mm^3 fragments of preneoplastic mammary tissue from 8-week-old PyMT transgenic WT+ and CCR7 KO+ mice were simultaneously transplanted into contralateral inguinal mammary fat pads of non-PyMT WT





b

Number of Grafted Sphere-Derived Cells

Donor	2500	500	100	10	TIC Frequency	Range ± SE
WT+	4/4	3/4	3/5	1/4	1/189*	117-306
CCR7 KO+	3/4	3/4	1/5	0/4	1/913	563-1480

*p=0.0104

Figure 4. CCR7 increases *in vivo* tumor-initiating capacity of sphere cells. (**a**) Representative images of intact and respective whole-mounted contralateral mammary glands engrafted with 2500 WT+ or CCR7 KO+ sphere-derived cells. Black arrowheads indicate areas of outgrowth from engrafted cells. LN = lymph node. (**b**) Results of limiting dilution assay indicating frequency of tumor-initiating cells (TIC) in WT+ and CCR7 KO+ sphere cultures. Fractions indicate the number of fat pads with lesion(s) per total number of recipient fat pads.

recipients. Representative histological sections from both WT+ and CCR7 KO+ 8-week-old mice, corresponding to donor tissue, are shown in Figure 5a, confirming that the glands used for transplantation were at the equivalent stage of tumorigenesis.

Analysis of tumorigenic outgrowth from transplanted tissue showed that the deletion of CCR7 almost completely blocked secondary tumor development. Only one out of six transplants from the CCR7 KO+ donors was able to give rise to a neoplastic lesion, whereas five out of six fragments from the WT+ donors produced secondary outgrowths in WT recipients (Figures 5b and c), demonstrating a key role of CCR7 in tumor propagation.

Pharmacological antagonism of CCR7 *in vivo* depletes the stem-like cell population and inhibits mammary tumorigenesis

A CCR7 antagonist, $CCL19_{(8-83)}$,[30] was used to explore the potential of targeting CCR7 for CSC-directed therapeutic intervention. Initially, the ability of $CCL19_{(8-83)}$ to block the stimulatory activity of CCR7 ligands on mammosphere-forming capacity was tested *ex vivo* and found to specifically abrogate the effect of CCL21 (Figure 6a) and CCL19 (data not shown) on mammosphere growth, providing a rationale for *in vivo* studies.

The effect of CCR7 blockade by $CCL19_{(8-83)}$ on tumor initiation was then examined in the context of the PyMT transgenic mouse model. $CCL19_{(8-83)}$ was injected for eight consecutive weeks into inguinal mammary glands of animals from the age of 4-weeks-old. Glands were then excised and examined for the extent of tumorigenesis and stem-like cell content and function. Macroscopic analysis demonstrated that $CCL19_{(8-83)}$-injected glands had smaller lesions than their control counterparts (representative image Figure 6b). The total weight of fat pads was not statistically different; however, the cellularity (total cell count and cells per mg of tissue) was significantly reduced by the antagonist (Figure 6c, Supplementary Figures 7a and b).

Treatment with $CCL19_{(8-83)}$ also resulted in a significant decrease in the proportion of stem-like cells ($Lin^-CD24^+CD29^{hi}$ Figure 6d left panel, and $Lin^-CD49f^+DLL1^+DNER^+$ Supplementary Figure 7c) and the function of stem and early progenitor cells (Figure 6d right panel), without affecting the level of CCR7 receptor expression (Supplementary Figure 7d). PyMT transgenic mice on both FVB and C57Bl/6 (not shown) backgrounds were tested, with similar results.

To determine whether treatment with $CCL19_{(8-83)}$ has an inhibitory effect on established and/or advanced later stage tumors, $1mm^3$ size fragments of MMTV-PyMT WT+ tumors from 16-week-old mice, corresponding to the invasive ductal carcinoma stage of human breast cancer (Supplementary Figure 1a), were transplanted into inguinal glands of WT recipients followed by eight weekly injections of $CCL19_{(8-83)}$ or vehicle control (Figure 6e left panel). Although no significant differences were seen between $CCL19_{(8-83)}$- or vehicle-treated tumors in size or cellularity (Supplementary Figure 7e) as was observed in primary tumors, the proportions of stem-like cells determined by both

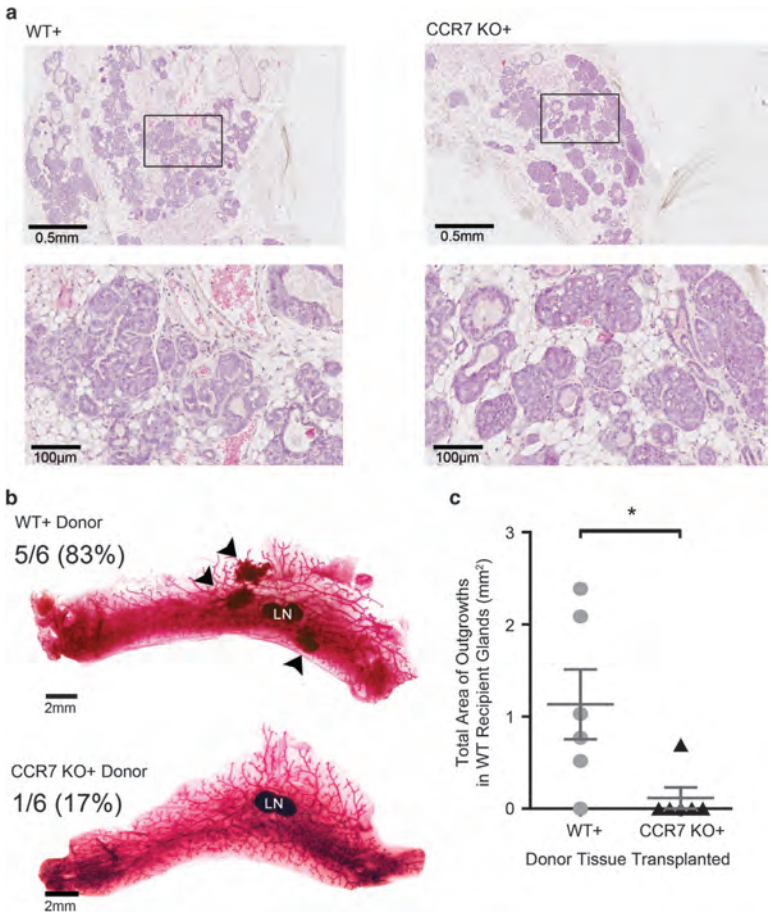


Figure 5. CCR7 is required for the propagation of mammary tumors. (**a**) Representative H&E-stained sections of pre-neoplastic mice at the WT+ and CCR7 KO+ donor age of 8 weeks. Bottom: magnified images of boxed area. (**b**) Representative whole-mount images of WT-recipient glands after transplantation of pre-neoplastic mammary tissue from WT+ (top) and CCR7 KO+ (bottom) donor mice at 8 weeks of age. Black arrowheads indicate areas of outgrowth from donor tissue. Fractions indicate the number of fat pads with lesion(s) per total number of recipient fat pads. LN = lymph node. (**c**) Cumulative area of transplant outgrowth in recipient mammary glands. $n = 6$ mice per group. $^*P < 0.05$.

conventional ($Lin^-CD24^+CD29^{hi}$ Figure 6e center panel) or novel ($Lin^-CD49f^+DLL1^+DNER^+$ Supplementary Figure 7f) marker sets, as well as mammosphere growth (Figure 6e right panel), were significantly reduced in antagonist-treated glands, demonstrating that the CCR7 axis can be blocked *in vivo* to target stem-like cells in mammary tumors.

DISCUSSION

The contribution of CSCs to tumor initiation is a major issue in tumor biology, yet one of the least understood processes.[3] We show here that ablating CCR7 using a bigenic MMTV-PyMT $Ccr7^{-/-}$ model significantly depleted the breast CSC-enriched pool. Using the surface marker profiles $CD24^+CD29^{hi}$ [4] and $CD49f^+DLL1^+DNER^+$ [24] we showed that the underlying mechanism involves a decrease in the ability of stem-like cells and early progenitor cells to self-renew and initiate neoplasia. Significantly, exogenously targeting CCR7 with a peptide antagonist led to a decrease in tumorigenesis.

CCR7 has been extensively studied for its role in adaptive immunity and secondary lymphoid organogenesis, and CCR7-null mice display disrupted architecture of the thymus and lymph node, as well as a reduced ability to mount a primary immune response.[31] The role of CCR7 in mediating anti-tumor immunity is also slowly emerging.[32] In this context, the fact that abrogation of CCR7 severely affected mammary tumorigenesis provides definitive evidence of CCR7 as a pro-tumorigenic driver. Furthermore, numerous transplantation approaches used in this study underscore an immune system-independent role of this chemokine receptor in maintaining stem-like cell pools in breast cancer.

Interestingly, hyperplastic outgrowth, widely believed to be a precursor of mammary tumors,[20] was found in 100% of WT and

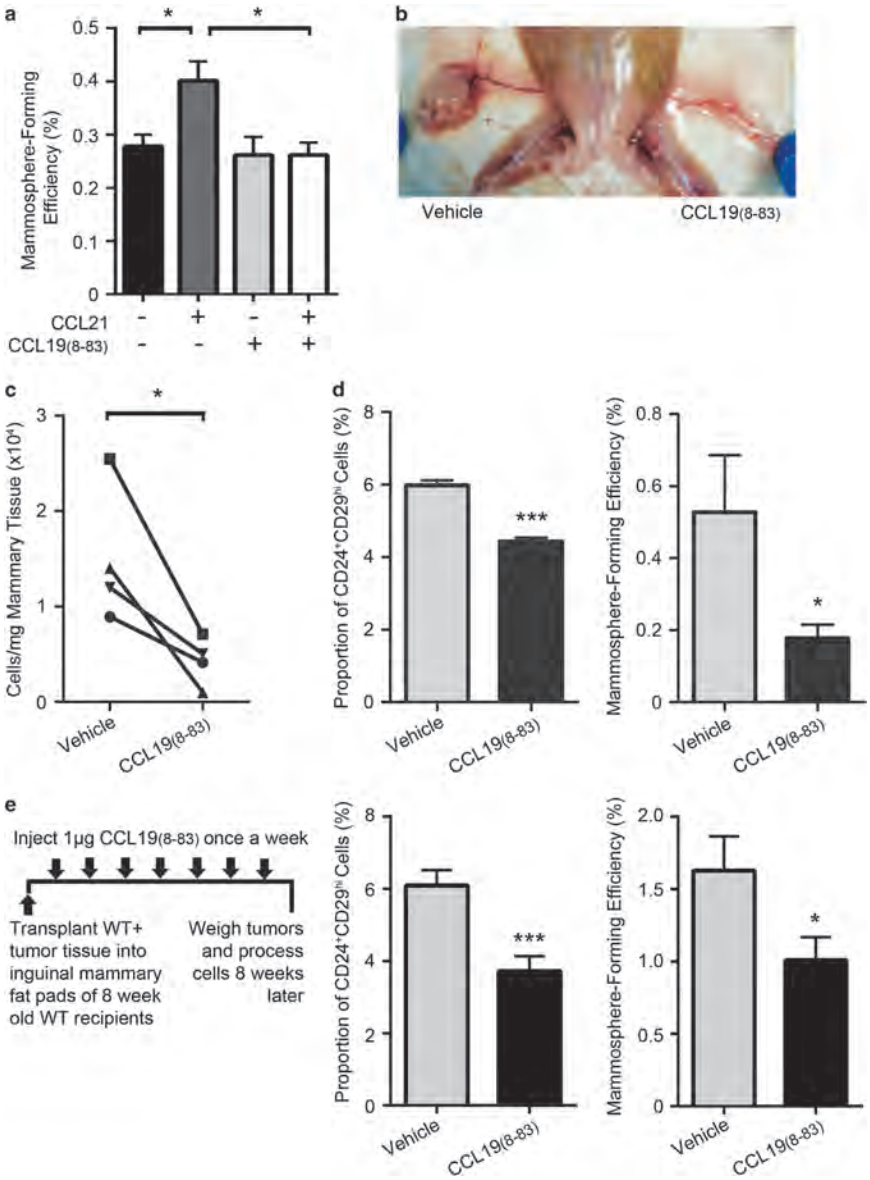


Figure 6. Pharmacological antagonism of CCR7 *in vivo* depletes the stem-like cell population and inhibits mammary tumorigenesis. (**a**) MFE of Lin^- mammary cells from WT+ mice ($n = 9$), untreated or treated with CCL21 and/or the CCR7 antagonist $CCL19_{(8–83)}$. $^*P < 0.05$. (**b–d**) Treatment of mice with $CCL19_{(8–83)}$ reduces tumorigenesis in MMTV-PyMT WT+ mice. (**b**) Representative image of intact mammary glands treated with vehicle or $CCL19_{(8–83)}$ as indicated. (**c**) Cellularity of contralateral vehicle- or $CCL19_{(8–83)}$-treated glands. (**d**) Proportions of Lin^- $CD24^+CD29^{hi}$ cells (left) and MFE (right) of cells from vehicle- or $CCL19_{(8–83)}$-treated glands. (**e**) Treatment of mice with $CCL19_{(8–83)}$ reduces the stem cell-enriched population in a transplant model. Experimental strategy (left), proportion of $Lin^-CD24^+CD29^{hi}$ cells (center) and MFE (right) of cells from transplanted tumors with or without $CCL19_{(8–83)}$ treatment. (**b–e**) Data are representative of two independent experiments, $n = 4–6$ mice per experiment. $^*P < 0.05$, $^{***}P < 0.001$.

CCR7-null PyMT-carrying glands examined. However, the majority of CCR7 KO+ glands were unable to sustain this initial proliferative burst of tumor cells and progress to the next stage in tumor development. Therefore, the delay in mammary tumorigenesis appears to be due to CCR7 maintaining specialized hierarchical sub-populations of cancer stem and progenitor-like cells that are thought to be crucial for tumor initiation and advanced tumorigenesis.[2]

The fact that both CCL19 and CCL21 stimulated mammosphere growth from both human and mouse tumor cells strongly suggests that CCR7 has a global role in sustaining properties of stemness in mammary epithelium. The specificity of CCR7 in this process was validated by testing a panel of chemokine receptor ligands, where only CCL19 and CCL21 showed an ability to significantly increase MFE.

Stimulation of CXCR4, the chemokine receptor that is consistently found to be upregulated together with CCR7 in a number of cancers,[17] did potentiate sphere formation but to a lesser extent (Supplementary Figure 5e). Recently, Clarke and colleagues demonstrated that stimulation of CXCR4 also increased MFE preferentially in malignant breast cancer cell lines compared with normal breast cell lines.[26] It is interesting to speculate that as CCR7 is less important for homeostasis than CXCR4, as has been inferred from animal models,[33] CCR7 may represent a more attractive target for future CSC-targeting therapies.

As stimulation of CCR7 had no effect on proliferation of the bulk population of cells when seeded into adherent culture, compared with a highly significant effect in non-adherent culture, it is likely that CCR7 predominantly mediates specific cellular properties of stemness. Moreover, we have previously reported that CCR7 activation on breast cancer cells inhibits anoikis,[13] a characteristic of breast and other CSCs.[25,34] Therefore, it is plausible that CCR7 supports CSC survival without attachment to the extracellular matrix, a hypothesis that may form the basis for future studies.

CCR7 appeared to have a quantitative rather than a qualitative role in normal mammary stem cells compared with CSCs. When CCR7 was deleted we saw a mild effect on the normal mammary gland. In contrast, a major effect was seen in mammary tumorigenesis. Interestingly, whereas the morphological effect on normal mammary gland development was not extensive, CCR7 deletion discreetly affected normal mammary gland stem-like cells. Therefore, it is possible that CCR7 has a role in regulating the properties of stemness within the mammary epithelial cell population, an effect that appears more prominent during cancer progression. As highlighted in a recent study by Cheresh and colleagues,[35] dysregulation of normal stem cells may contribute to breast cancer progression and stemness, and CCR7 may emerge as a novel mediator of this transition.

Translation of our findings from the mouse model to human disease is of particular significance, considering that there is currently no clear consensus on the markers that define functional mammary stem cells in both mice and humans. Thus, we show here that CCR7 not only has a role in mouse mammary tissue but is also expressed, is functional and is highly responsive in the stem-like populations within human breast cancer tissue. Intriguingly, circulating tumor cells, an indicator of metastatic spread and poor outcome in breast and other cancers, have been recently equated to CSCs.[36] In the last decade numerous studies also suggested a role for CCR7 in malignant dissemination of mammary tumors to distant sites.[13,15,17] Taken together, these results suggest a novel causative link between CCR7 activity on stem-like populations and metastatic breast cancer.

To seek proof-of-principle on the utility of pharmacologically targeting CCR7 we tested the receptor antagonist $CCL19_{(8-83)}$.[30] We found that pharmacological inhibition of CCR7 through direct mammary fat pad injection of $CCL19_{(8-83)}$ afforded a significant reduction in early-stage primary mammary tumorigenesis. As the relative contribution of the malignant lesions to the weight of the whole mammary fat pad was very small at this early stage the reduction in total weight between antagonist and vehicle-treated glands was not statistically significant. However, the cellularity, a characteristic that directly reflects the extent of epithelial malignant outgrowth and is used in clinical pathology to evaluate the response to chemotherapy in breast cancer, was strongly impacted by treatment with the CCR7 antagonist.

More importantly, directly targeting CCR7 using the antagonist significantly depleted the stem-like cell pools in both early and late-stage mammary neoplasia as was shown using the transplantation approach. These findings strongly suggest that the CCR7 receptor axis is a potential point of intervention in stem cell-targeting therapies. Furthermore, the results of this study provide a rationale for the use of antagonists of the CCR7 pathway as adjuvants to conventional cytotoxic drugs unable to eliminate quiescent CSCs.[2]

In conclusion, the characterization of CCR7 in primary breast tumorigenesis *in vitro* and *in vivo*, and in mouse and human tissue, strongly suggests a role for this molecule in breast cancer development and progression. These insights raise the possibility of pharmacologically targeting CCR7 for the development of new therapies in breast cancer.

MATERIALS AND METHODS

Mice

Mice were maintained in pathogen-free conditions in the University of Adelaide's Laboratory Animal Services facility. $Ccr7^{-/-}$ mice were purchased from Jackson Laboratory. FVB MMTV-PyMT (+) mice were backcrossed for 14 generations to C57Bl/6 background, and C57Bl/6 background was confirmed by microsatellite analysis. PyMT-carrying males were then crossed with $Ccr7^{-/-}$ females, and the offspring were interbred to produce MMTV-PyMT $Ccr7^{WT}$ and MMTV-PyMT $Ccr7^{-/-}$ mice. The University of Adelaide institutional animal ethics committee approved all experimentation. For the assessment of CCR7 expression and $CCL19_{(8-83)}$ function both C57Bl/6 and FVB backgrounds were tested to eliminate any strain bias. For experiments involving knockout mice, only C57Bl/6 mice were tested. Nomenclature used for genotypes is as follows: $Ccr7^{WT}$ = WT, $Ccr7^{-/-}$ = CCR7 KO, MMTV-PyMT $Ccr7^{WT}$ = WT+, MMTV-PyMT $Ccr7^{-/-}$ = CCR7 KO+.

Human mammary tissue

Ethical approval was granted by the Royal Adelaide Hospital Ethics Committee and all patients gave written, informed consent prior to surgery. Pathology reports for tumors used are available on request. Normal breast tissue samples were obtained from the Queen Elizabeth Hospital, Adelaide.

Whole mount staining

Mammary glands were fixed in Carnoy's and were stained overnight in Carmine Alum before dehydration and mounting. Image 'stitching' and analysis was performed using Image J.

Histology

Lungs of MMTV-PyMT WT+ or CCR7 KO+ mice were perfused and dissected, then cryoembedded in optimal cutting temperature (OCT) reagent and serially sectioned at 9μm. Formalin-fixed paraffin-embedded mammary glands/tumors were sectioned at 5μm. All slides were stained using haemotoxylin and eosin, dehydrated and mounted. Slides were scanned using the NanoZoomer Digital Pathology System (Hamamatsu Photonics, Shizuoka, Japan) and lung sections were manually quantitated using the NanoZoomer Digital Pathology Virtual Slide Viewer software for number of metastases and area at the largest point.

Immunofluorescent microscopy

Antigen retrieval of formalin-fixed paraffin-embedded mouse mammary sections was performed by boiling slides in 0.1 M sodium citrate buffer (pH 6.0). Slides were stained with rabbit anti-CCR7 (Epitomics, Burlingame, CA, USA) overnight at 4 °C, and primary antibody was detected with Alexa

Fluor 488-conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA, USA) for 30 min. Slides were counterstained with DAPI (4',6-diamidino-2-phenylindole), mounted and analyzed using the Leica TCS SP5 Confocal Microscope System.

Processing mouse mammary tissue to single cell suspension

Mouse mammary glands/tumors were dissected, with removal of the lymph node if possible. Tissue was manually dissociated and then digested in 10% collagenase/hyaluronidase (Stem Cell Technologies, Vancouver, BC, Canada) in Dulbecco's Modified Eagle's medium (DMEM) for 3-4 h with gentle tilting. Single cells were extracted as previously described[37] and filtered through a 70 μm nylon mesh. To remove contaminating infiltrating cells of hematopoietic origin, Biotin Binder Dynabeads (Invitrogen) in combination with a biotinylated anti-mouse lineage antibody panel (BioLegend, San Diego, CA, USA) were used as suggested by the manufacturer.

Processing human mammary tissue to single cell suspension

Surgical specimens were minced and digested in 10% collagenase/hyaluronidase (Stem Cell Technologies) in DMEM supplemented with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), penicillin–streptomycin and 0.25 μg/ml fungazone. Organoids were then extensively washed with DMEM, and red blood cells were lysed by isotonic lysis buffer (150 mM NH_4Cl in 17 mM Tris-HCl, pH 7.2). Single cell suspensions were obtained by digesting organoids with trypsin for 10 min at room temperature, with subsequent filtration through a 70 μm nylon mesh.

Flow cytometry

Cells were fixed in 4% formaldehyde and immunostained for 45 min on ice in phosphate-buffered saline/0.5% bovine serum albumin (PBS/0.5% BSA). Antibodies used were: Alexa Fluor 647-conjugated anti-mouse CCR7, phycoerythrin (PE)-conjugated anti-mouse/anti-human CD24, fluorescein isothiocyanate (FITC)/PECy5-conjugated anti-human CD49f, FITC-conjugated anti-human CD44 (all from BD, North Ryde, NSW, Australia), FITC-conjugated anti-mouse CD29, PE-conjugated anti-mouse/anti-human DLL1 (all from BioLegend), and biotinylated anti-mouse/anti-human DNER (R&D Systems, Minneapolis, MN, USA). Samples containing biotinylated antibodies were resuspended in PerCP/Cy5.5 or Alexa Fluor 488-conjugated streptavidin in PBS/0.5% BSA for 30 min on ice. Fluorescence-minus-one samples or conjugated isotypes were used as negative controls. Flow cytometry was carried out using FACSCanto equipment (BD). Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Mammosphere assay

Cells were seeded in mammosphere medium (1:1 mixture of DMEM and Ham's F12 medium (Gibco, Life Technologies, Mulgrave, VIC, Australia) supplemented with 1 × B27 (Invitrogen), 20ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor, 4 μg/ml heparin (Sigma-Aldrich, St Louis, MO, USA), penicillin–streptomycin, and 0.25 μg/ml fungazone) at 4×10^4 cells/ml to an ultra-low attachment tray (Corning Inc., Corning, NY, USA). Where indicated, CCL21, CCL19 and $CCL19_{(8–83)}$ were added at concentrations of 10 ng/ml, 200 ng/ml and 100 ng/ml, respectively. Media was replenished every second day. After 7–10 days, mammospheres were counted and passaged.

Limiting dilution assay

Mammosphere colonies derived from 8-week-old pre-neoplastic MMTV-PyMT WT+ and CCR7 KO+ mice were dissociated using trypsin and triturated through a 19G needle. Following filtration, cells were injected in 20% Matrigel (BD):80% DMEM into the fourth inguinal mammary glands of anaesthetized WT-recipient mice (8-weeks-old) at limiting dilutions as previously described.[24] Mice were killed after 6 weeks and glands whole-mounted. Tumor-initiating cell-frequency and statistical calculations were performed using L-Calc software (Stem Cell Technologies).

Tissue transplants

Mammary gland fragments of $1mm^3$ size from donor MMTV-PyMT mice were transplanted into contralateral sides of anaesthetized congenic non-PyMT WT-recipient mice (8-weeks-old) within the fourth inguinal mammary glands. Mice were monitored for adverse reactions to surgery and subsequent tumor growth.

In vivo treatment with CCR7 antagonist

Mice were injected under anesthetic into an inguinal mammary fat pad, with 1 μg $CCL19_{(8–83)}$ truncated ligand in 50 μl saline vehicle, as indicated. As a control, mice were injected in the contralateral inguinal mammary fat pad with vehicle alone as previously reported.[38,39]

Statistical analysis

Unless otherwise indicated, analyses were carried out using GraphPad Prism and data is shown as mean ± s.e.m. Significant statistical difference was estimated using student's *t*-tests, or χ^2-tests for distribution analysis. Tumor-free survival curves were graphed using the Kaplan–Meier method and distributions were compared by the log-rank statistic (Mantel–Cox test). All measurements were done in triplicate. *P*-values were used to denote statistical significance. Levels of significance were $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Appendices

SUPPORTING INFORMATION

Supplementary Experimental Procedures

Immunohistochemistry (IHC)

Immunohistochemical analysis was performed using standard procedures. Briefly, antigen retrieval of FFPE mouse mammary sections was carried out by boiling slides in 0.1M sodium citrate buffer (pH 6.0) under pressure. Slides were immersed in 3% hydrogen peroxide in PBS for 20 minutes with gentle agitation to inhibit endogenous peroxidase activity and blocked for 30 minutes in 10% normal serum in PBS to prevent non-specific antibody binding. Slides were then incubated overnight at 4°C with mouse anti-α-smooth muscle actin (α-SMA, Dako). Specific antibody binding was detected using the EnVision Dual Link System (Vector Labs) followed by incubation with diaminobenzidine (DAB) substrate (Dako). Sections were counterstained with haemotoxylin, dehydrated and mounted.

Calcium (Ca+) Signaling Analysis

Intracellular calcium mobilization assay was performed on cells isolated from tumors dissected from C57Bl/6 MMTV-PyMT mice at 20 weeks of age as described (1). Ligands added were CCL21 (100ng/ml) and lysophosphatidic acid (LPA) (50ng/ml).

Enzyme-Linked Immunosorbent Assay (ELISA)

Wells were coated in CCL21 and CCL19 capture antibody (R&D) and receptors blocked using PBS/3%BSA for 1 hour at 37°C. After determining WT and CCR7 KO mammary gland weight, fat pad samples were homogenized in PBS buffer (10% glycerol, 1x protease inhibitor) and aliquotted to coated wells for 90 minutes at 37°C. Detection antibody (R&D) was added for 1 hour then streptavidin-HRP added for 30 minutes.

Proliferation Assay

Isolated lineage-negative mouse mammary cells were plated in adherent culture (DMEM:F12/10% FCS, supplemented with 20ng/ml EGF, 5µg/ml insulin, 0.5µg/ml hydrocortisone, penicillin-streptomycin, and 0.25µg/ml fungazone) in 96-well plates and the following day were serum-starved for 4 hours. The cell proliferation assay was carried out 24 hours later using the XTT Cell Proliferation Kit (ATCC) according to manufacturer's instructions, using 100ng/ml CCL19 and CCL21. FCS was used at a concentration of 5%.

Supplementary References

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Appendices

SUPPLEMENTARY FIGURES

Supplementary Figure 1 (Related to Figure 1)

(**a**) Serial sections of mouse mammary tissue from normal mice and at different tumourigenic stages stained with haemotoxylin and eosin (H&E) and for α-smooth muscle actin (α-SMA) as indicated. (**b**) Mammary cells were gated to exclude debris, dead cells and doublets (top) and proportions of CCR7-positive cells in mammary epithelial cell preparations from corresponding stages in (a) were analyzed by flow cytometry(bottom). Shaded histograms= fluorescence-minus-one negative gate. wo= weeks old.

Supplementary Figure 2 (Related to Figure 1)

(**a**) Expression of CCR7 ligands (CCL21 and CCL19) in WT and CCR7 KO mammary fat pads with excised inguinal lymph nodes was assessed by ELISA. n=6 glands per genotype. (**b**) Calcium mobilization analysis of PyMT-expressing WT+ mouse mammary cells in response to lysophosphatidic acid (LPA) (positive control) and the CCR7 ligand, CCL21. Arrowheads indicate a point of stimulus addition.

Supplementary Figure 3

Ablation of CCR7 has no effect on early mammary tumourigenesis in MMTV-PyMT mice. (**a**) Top: Representative images of inguinal mammary glands of WT+ (left) and CCR7 KO+ (right) mice harvested at 8 and 11 weeks of age as indicated. Arrowheads indicate areas of epithelial hyperplasia. LN= lymph node. Bottom: Quantitation of area of hyperplasia in WT+ and CCR7 KO+ at 8 weeks old. n=6 glands per genotype. (**b**) Serial sections of mouse mammary tissue from WT+ and CCR7 KO+ mice at 8 weeks old stained with haemotoxylin and eosin (H&E) and for α-smooth muscle actin (α-SMA) as indicated.

Supplementary Figure 4 (Related to Figure 3)

All cells were pre-gated to exclude debris, dead cells and doublets. (**a-b**) Flow cytometry gating strategy for delineating the stem cell-enriched population ($CD24^{+}CD29^{hi}$) and non-stem cell populations ($CD24^{+}CD29^{lo}$ cells and stromal cells) and proportion of CCR7 positive cells within these populations, in normal mice (**a**) and from MMTV-PyMT mice (**b**). (**c**) Flow cytometry gating strategy for delineating the stem cell-enriched population ($CD44^{+}CD24^{-}$) and non-stem cell population ($CD44^{-}CD24^{+}$) in human mammary epithelium, and proportion of CCR7 positive cells within these populations as indicated. (**d-e**) Flow cytometry gating strategy for delineating mouse (**d**) and human (**e**) stem cell-enriched populations based on alternative $CD49f^{+}DLL1^{+}DNER^{+}$ surface marker expression, together with representative plots demonstrating proportion of CCR7 positive cells within these populations. “FMO” and shaded histograms= fluorescence minus one negative gates.

Supplementary Figure 5 (Related to Figure 3)

(**a-b**) CCR7 deletion decreases proportion of the stem cell-enriched population in normal (**a**) and PyMT-expressing mice (**b**) designated by a putative marker profile of $Lin^{-}CD49f^{+}DLL1^{+}DNER^{+}$. Data are representative of at least three independent experiments, n=6 mice per genotype. (**c**) CCR7 stimulation does not have any effect on proliferation of WT+ mammary cells in adherent culture. Shown are results of XTT proliferation assay with and without addition of CCR7 ligands. FCS was used as a positive control. (**d**) The stimulatory effect of CCL19 and CCL21 on MFE of MMTV-PyMT cells is dependent on CCR7. The primary MFE of CCR7 KO+ (n=4 mice) mouse mammary cells was not affected by the addition of these chemokines. (**e**) Effect of stimulation of primary WT+ (n=6 mice) mammosphere culture with chemokine ligands for receptors CCR6, CXCR3, CXCR4 and CXCR5 respectively.

Appendices

Supplementary Figure 6 (Related to Figure 4)

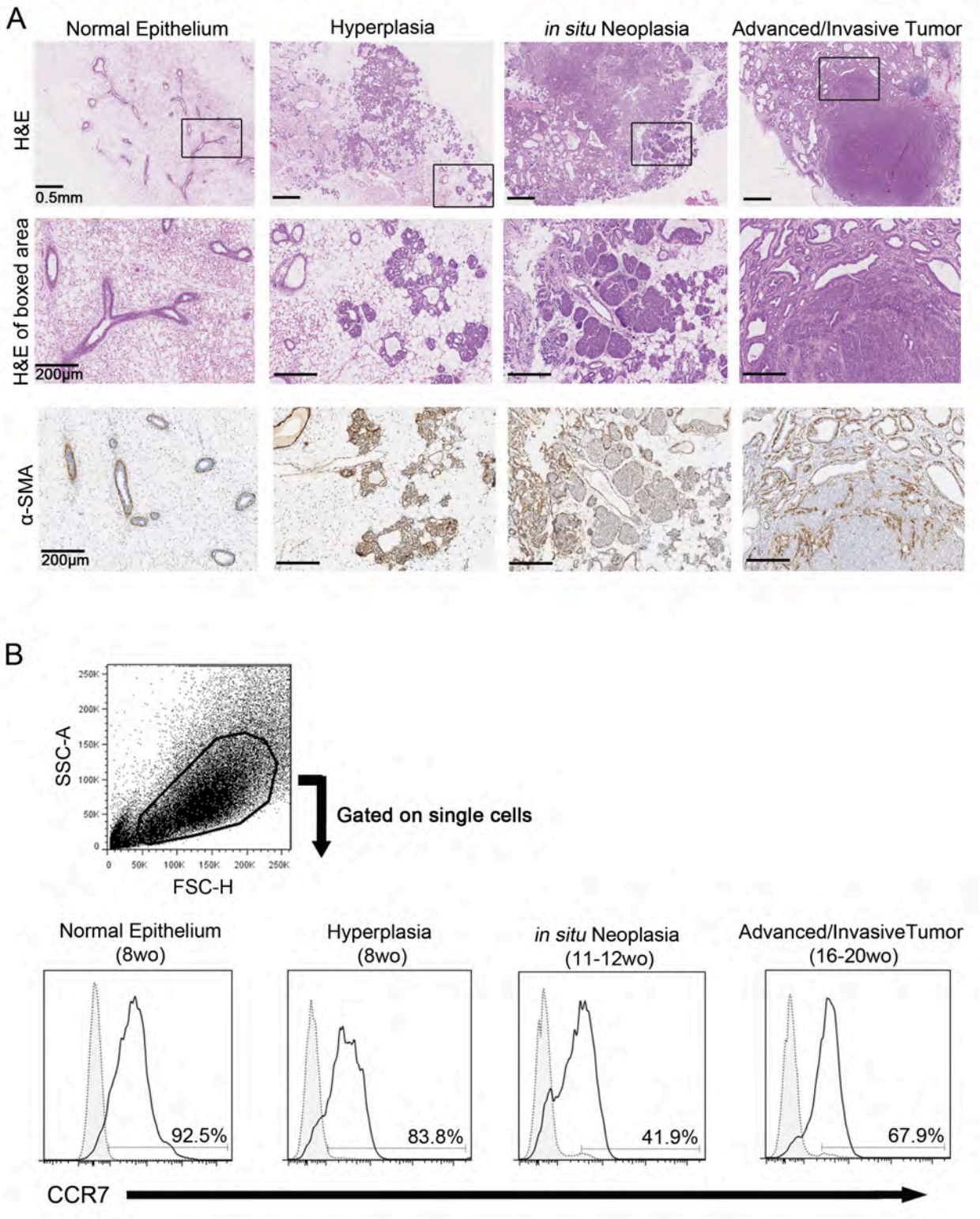
Representative images of WT recipient glands engrafted with WT+ or CCR7 KO+ mammosphere-derived cells as indicated. Shown are contralateral glands in which both WT+ and CCR7 KO+ cells produced outgrowths (black arrowheads), to demonstrate differences in size between lesions. At the lowest dilution, only WT+ cells produced any outgrowth. LN= lymph node**.**

Supplementary Figure 7 (Related to Figure 6)

(**a**) Total mammary epithelial cell count in MMTV-PyMT mice following injection with vehicle or $CCL19_{(8\text{-}83)}$ antagonist for 8 weeks in contralateral inguinal mammary fat pads. (**b**) Weight of mammary glands. (**c**) Comparison of stem cell-enriched population in MMTV-PyMT glands delineated by alternative marker set $Lin^-CD49f^+DLL1^+DNER^+$. (**d**) Treatment of mammary glands with vehicle control (left) or $CCL19_{(8\text{-}83)}$ (right) does not alter CCR7 expression levels in the $Lin^-CD24^+CD29^{hi}$ population. Shaded histograms = fluorescence minus one negative gates. (**e**) Treatment with $CCL19_{(8\text{-}83)}$ does not change cellularity of transplanted tumor tissue despite affecting the stem cell-like pool. (**f**) Comparison of the stem cell-enriched population in transplanted glands treated with $CCL19_{(8\text{-}83)}$ or vehicle, as delineated by alternative marker set $Lin^-CD49f^+DLL1^+DNER^+$.

Appendices

Supplementary Figure 1



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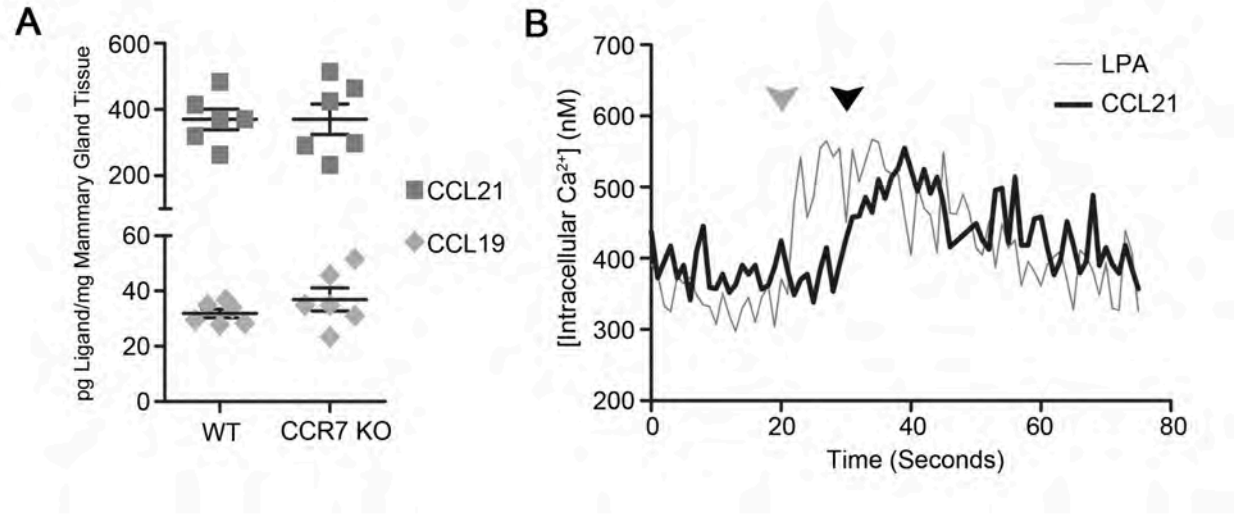
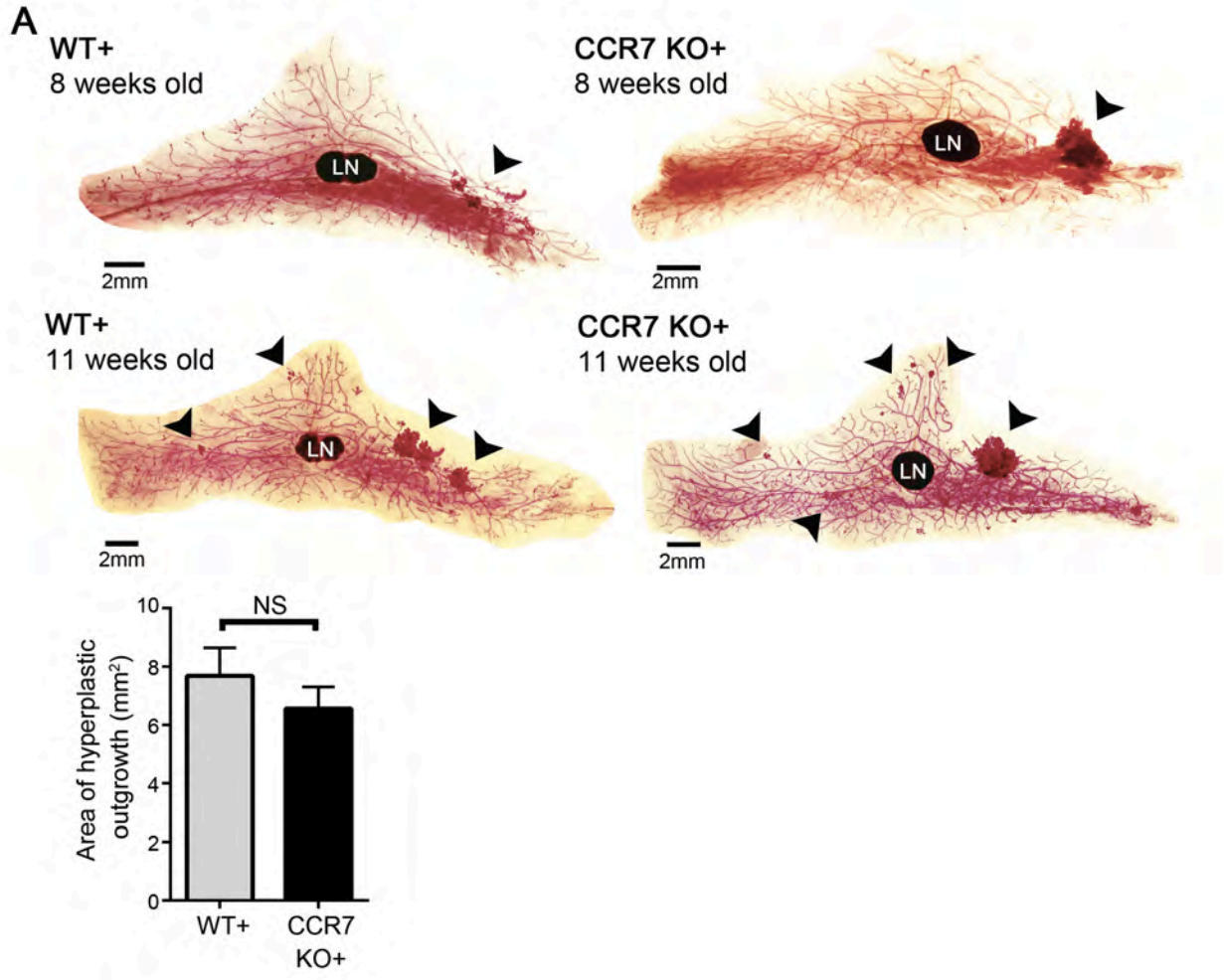
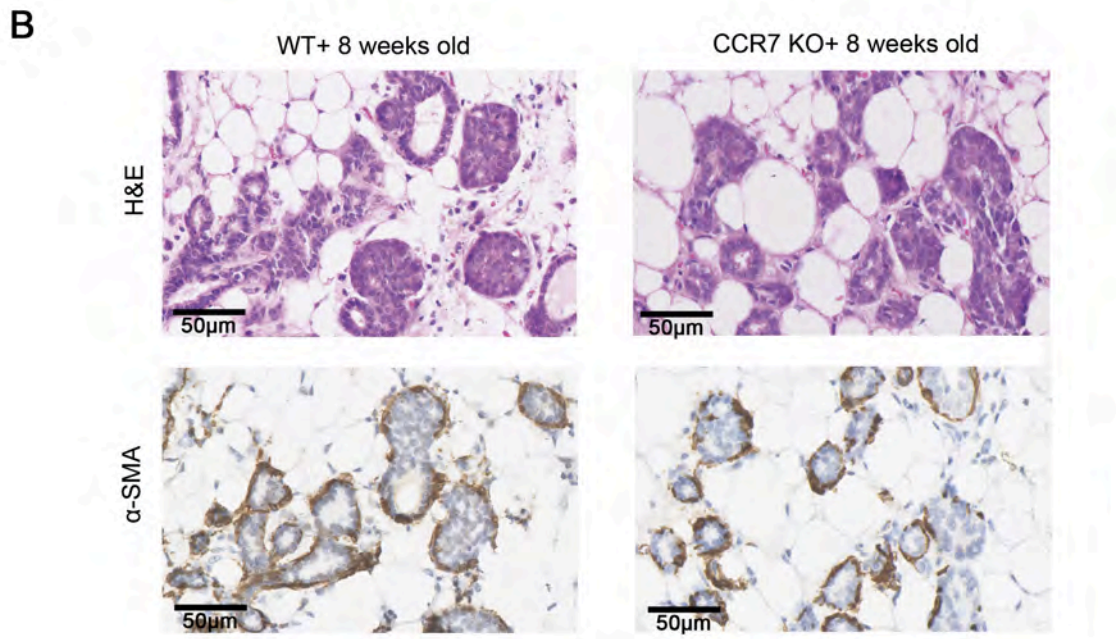
Supplementary Figure 2



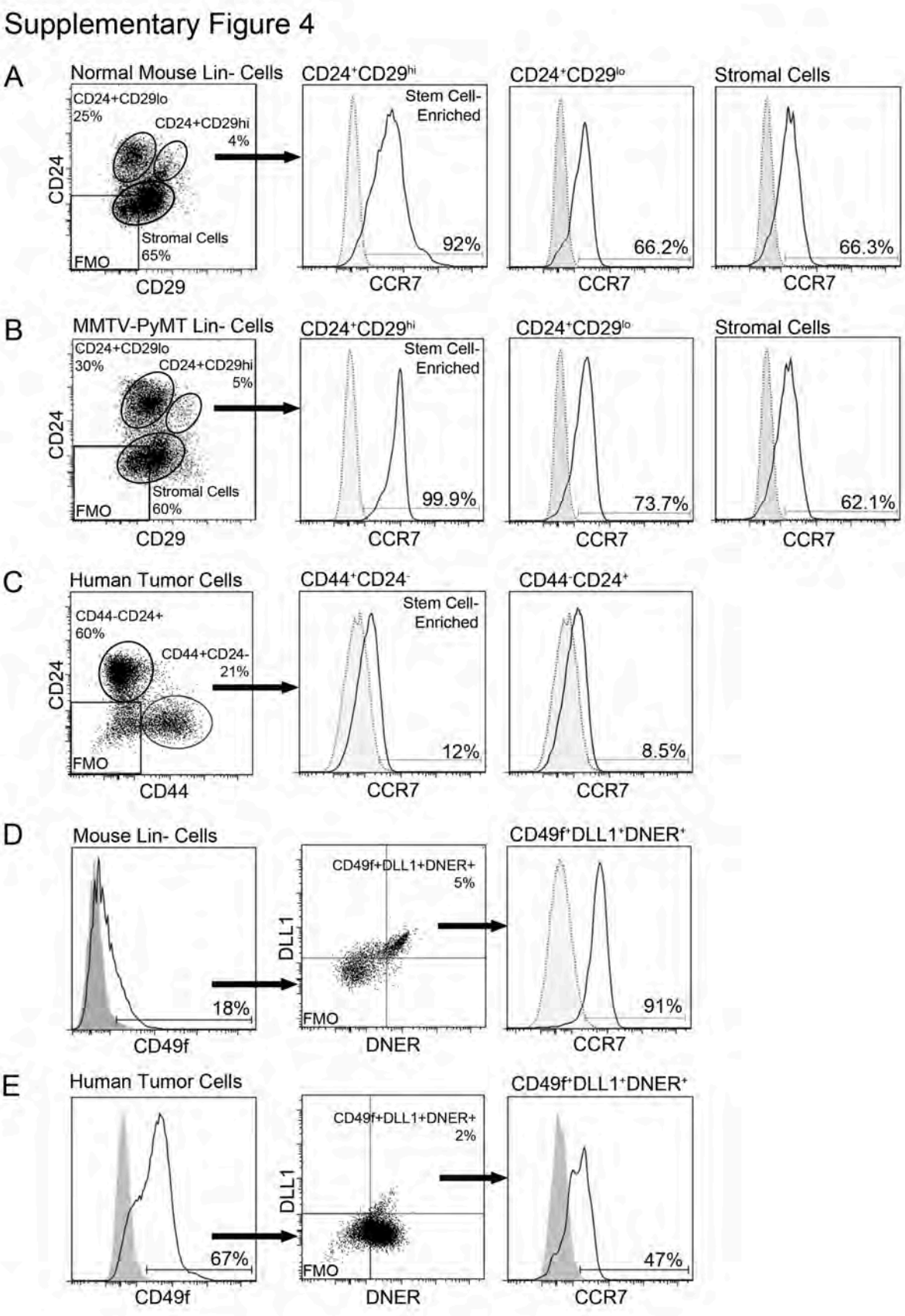
Appendices

Supplementary Figure 3



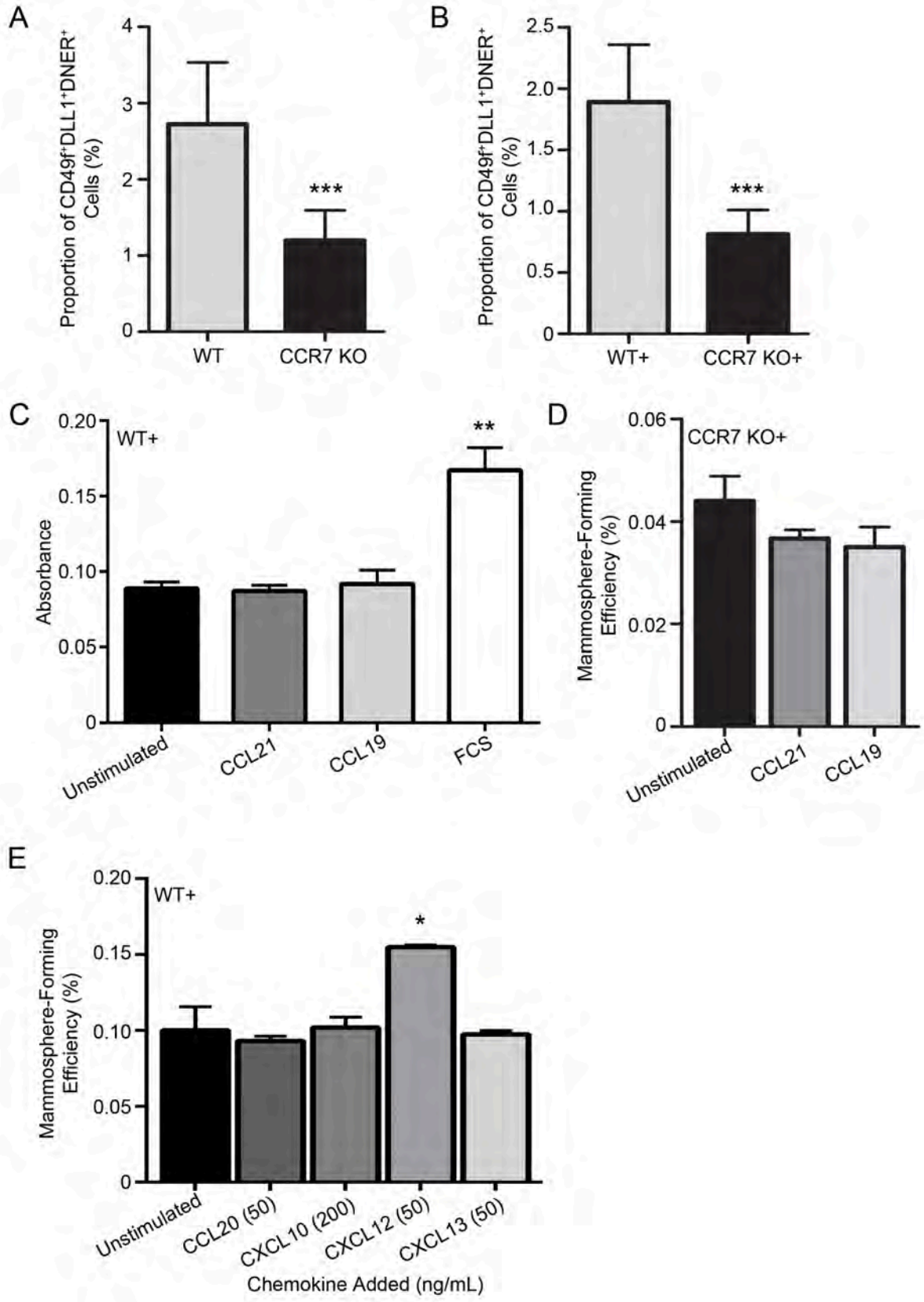


Appendices


Supplementary Figure 4
A
Normal Mouse Lin- Cells
CD24+CD29lo
25%
CD24+CD29hi
4%
Stromal Cells
65%
FMO
CD24
CD29
CD24+CD29hi
Stem Cell-
Enriched
92%
CCR7
CD24+CD29lo
66.2%
CCR7
Stromal Cells
66.3%
CCR7
B
MMTV-PyMT Lin- Cells
CD24+CD29lo
30%
CD24+CD29hi
5%
Stromal Cells
60%
FMO
CD24
CD29
CD24+CD29hi
Stem Cell-
Enriched
99.9%
CCR7
CD24+CD29lo
73.7%
CCR7
Stromal Cells
62.1%
CCR7
C
Human Tumor Cells
CD44-CD24+
60%
CD44+CD24-
21%
FMO
CD24
CD44
CD44+CD24-
Stem Cell-
Enriched
12%
CCR7
CD44-CD24+
8.5%
CCR7
D
Mouse Lin- Cells
18%
CD49f
CD49f+DLL1+DNER+
5%
FMO
DLL1
DNER
CD49f+DLL1+DNER+
91%
CCR7
E
Human Tumor Cells
67%
CD49f
CD49f+DLL1+DNER+
2%
FMO
DLL1
DNER
CD49f+DLL1+DNER+
47%
CCR7

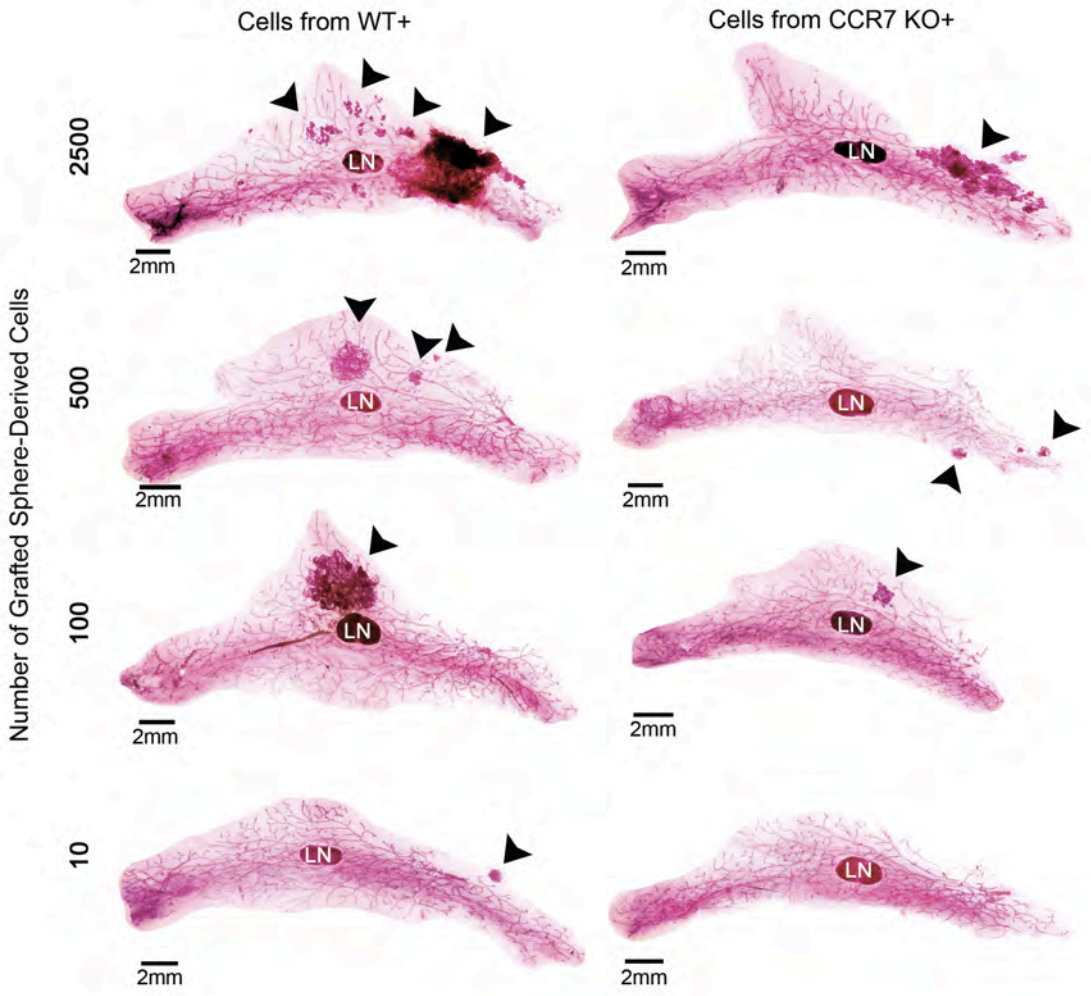
Appendices

Supplementary Figure 5

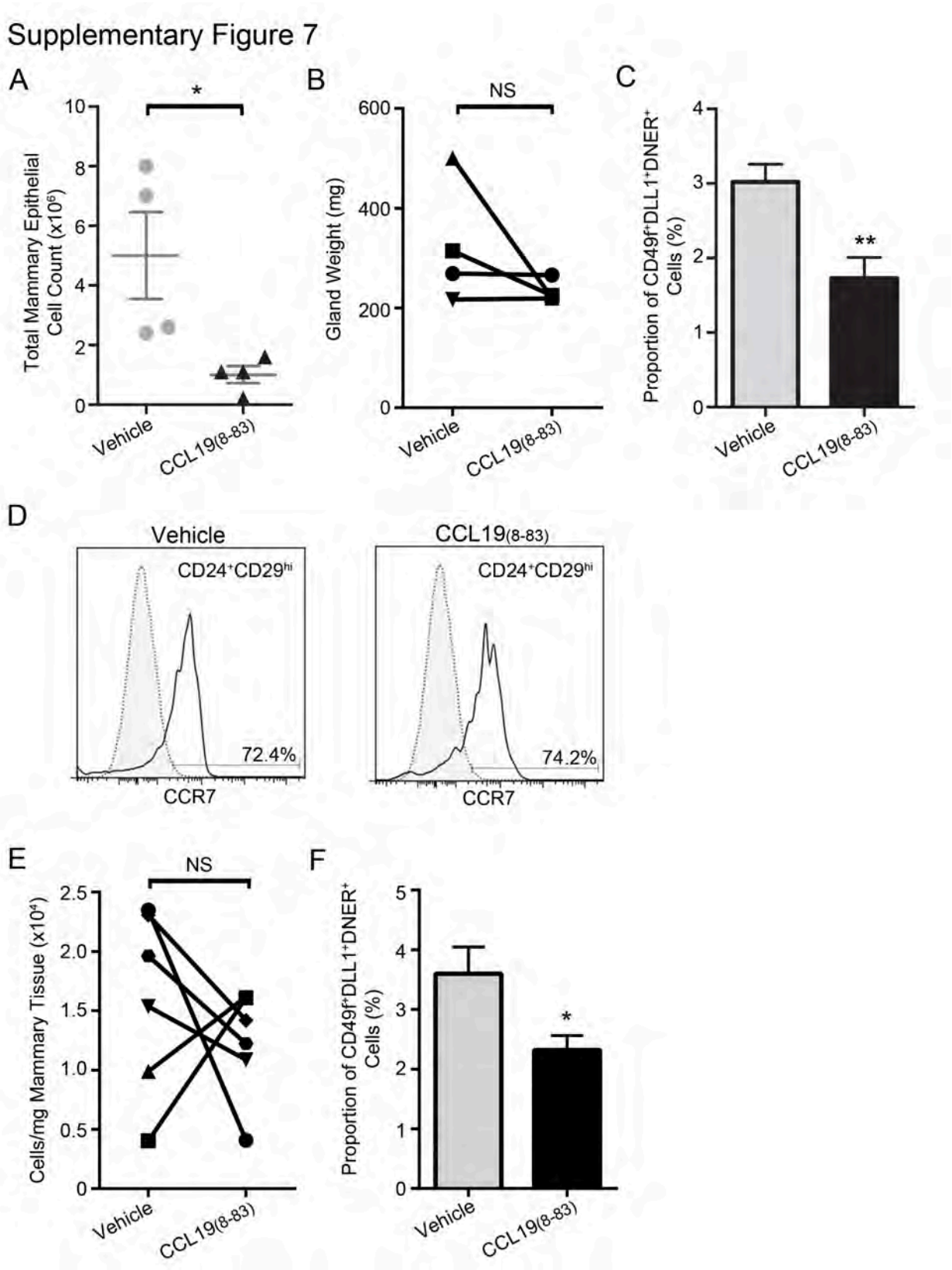


Appendices

Supplementary Figure 6



Appendices


Supplementary Figure 7
A
*
Total Mammary Epithelial Cell Count (x10⁶)
10
8
6
4
2
0
Vehicle
CCL19(8-83)
B
NS
Gland Weight (mg)
600
400
200
0
Vehicle
CCL19(8-83)
C
Proportion of CD49f⁺DLL1⁺DNER⁺ Cells (%)
4
3
2
1
0
**
Vehicle
CCL19(8-83)
D
Vehicle
CD24⁺CD29ʰⁱ
72.4%
CCR7
CCL19(8-83)
CD24⁺CD29ʰⁱ
74.2%
CCR7
E
NS
Cells/mg Mammary Tissue (x10⁴)
2.5
2.0
1.5
1.0
0.5
0
Vehicle
CCL19(8-83)
F
Proportion of CD49f⁺DLL1⁺DNER⁺ Cells (%)
5
4
3
2
1
0
*
Vehicle
CCL19(8-83)

Statement of Authorship

Title of Paper	The chemokine receptor CCR6 facilitates the onset of mammary neoplasia in the MMTV-PyMT mouse model via recruitment of tumour-promoting macrophages
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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)	Miss Sarah T. Boyle		
Contribution to the Paper	Designed and conceived of the study Carried out all experiments except those specified below Maintained animal models Wrote the manuscript		
Signature		Date	17 / 06 /2015

Name of Co-Author	Miss Jessica W. Faulkner		
Contribution to the Paper	Technical assistance in performing experiments Maintained animal models		
Signature		Date	18 / 06 /2015

Name of Co-Author	Professor Shaun R. McColl		
Contribution to the Paper	Supervised the study Provided reagents and animal models Edited the manuscript		
Signature		Date	18 / 06 /2015

Name of Co-Author	Doctor Marina Kochetkova		
Contribution to the Paper	Assistance in design of and supervised the study Provided technical assistance Performed proliferation assay experiments Edited the manuscript		
Signature		Date	21 / 07 /2015

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MOLECULAR CANCER

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The chemokine receptor CCR6 facilitates the onset of mammary neoplasia in the MMTV-PyMT mouse model via recruitment of tumor-promoting macrophages

Sarah T. Boyle[1], Jessica W. Faulkner[1], Shaun R. McColl[1,2] and Marina Kochetkova[1*]

Abstract

Background: The expression of the chemokine receptor CCR6 has been previously correlated with higher grades and stages of breast cancer and decreased relapse-free survival. Also, its cognate chemokine ligand CCL20 has been reported to induce proliferation of cultured human breast epithelial cells.

Methods: To establish if CCR6 plays a functional role in mammary tumorigenesis, a bigenic MMTV-PyMT CCR6-null mouse was generated and mammary tumor development was assessed. Levels of tumor-infiltrating immune cells within tumor-bearing mammary glands from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice were also analyzed.

Results: Deletion of CCR6 delayed tumor onset, significantly reduced the extent of initial hyperplastic outgrowth, and decreased tumor incidence in PyMT transgenic mice. CCR6 was then shown to promote the recruitment of pro-tumorigenic macrophages to the tumor site, facilitating the onset of neoplasia.

Conclusions: This study delineated for the first time a role for CCR6 in the development of breast cancer, and demonstrated a critical function for this receptor in maintaining the pro-tumorigenic cancer microenvironment.

Keywords: Breast Cancer, Mammary Gland, Chemokine Receptor, CCR6, Transgenic Mouse Model, Immune System, Macrophages

Introduction

Breast cancer is one of the leading causes of cancer-related death in women world-wide. Evasion of the immune system is a hallmark of cancer, and aids tumor cells to survive, intravasate, and potentially form distal metastases [1]. As such, the tumor microenvironment has a profound effect on the development and progression of malignancies, and it has been suggested that levels of infiltrating immune cells correlate with stage and aggressiveness of human breast cancer [2]. In particular, tumor-associated macrophages (TAMs) have been found to play an important part in facilitating breast tumor development [3] through polarization from a classically-activated "M1" anti-tumor resident cell within adult mammary tissue to an alternatively-activated "M2" pro-tumor phenotype [4]. This "switch" results in shifts in cell metabolism, a decrease in pro-inflammatory chemokine/cytokine production, poor antigen-presentation ability, and suppression of T cell responses. In addition, M2 TAMs promote angiogenesis, cell proliferation and tissue remodeling (reviewed in [5]).

Chemokines and their cognate receptors are involved in the development, migration and activation of many different types of immune cells, both adaptive and innate. Small molecular-weight proteins, chemokines bind to their cognate seven-transmembrane domain G-protein coupled receptors (GPCRs), activating a multitude of signaling pathways, which mediate many different homeostatic and inflammatory functions. Importantly, a large body of literature in the last decade has linked the action of chemokines and chemokine receptors to cancer progression and metastasis [6].

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The CC-chemokine receptor CCR6 is expressed on dendritic cells [7, 8], regulatory T cells and various T helper lymphocyte subsets [9, 10], and mediates their migration and function via stimulation with its ligand CCL20 (also known as macrophage inflammatory protein (MIP)-3α [11]). CCR6 is also expressed on natural killer cells, B lymphocytes, neutrophils [12] and macrophages [10, 13]. Despite the significant role of TAMs in breast cancer, the expression and function of CCR6 within the macrophage population has not been shown within the mammary gland.

Interestingly, together with CCL20, CCR6 expression has been correlated with stage and prognosis in a variety of cancers including hepatocellular carcinoma [14, 15], colorectal carcinoma [16–18], glioma [19], and non-small cell lung cancer [20], and a function for CCR6 in regulation of cancer progression has been putatively demonstrated using cell lines and xenograft models [16, 18, 21, 22]. In breast cancer, higher CCR6 expression levels were linked with tumor stage and grade [23], and incidence of metastasis to the pleura [24]. Stimulation of *ex vivo* mammary peritumoral cells with CCL20 was found to increase their proliferation rate, invasiveness and migration [25]. CCL20 is also upregulated in human triple negative breast cancer cell lines [26]. Moreover, it was recently proposed that the presence of CCR6 may act as a prognostic factor for breast cancer patient survival [23]. However, no causative or functional link between the CCR6-CCL20 axis and progression of breast cancer has been documented to date.

In this study we have utilized a well-characterized transgenic model for breast cancer, in which the polyoma middle-T oncogene is activated under control of the mouse mammary tumor virus promoter (MMTV-PyMT) [27]. This transgenic model has been shown to closely mimic the stages of human breast disease from initial hyperplasia, through to ductal carcinoma *in situ* and invasive ductal carcinoma [28]. Crossing this transgenic mouse with a CCR6-null mouse to generate a bigenic MMTV-PyMT $Ccr6^{-/-}$ animal model has allowed us to directly assess the role of CCR6 in mammary tumorigenesis *in vivo*. The results demonstrated that CCR6 promotes breast cancer initiation and progression through maintenance of pro-tumorigenic TAMs within tumor-bearing mammary glands, warranting further investigation of CCR6 as a possible therapeutic target.

Results

CCR6 expression increases throughout cancer development and results in a higher number of mammary tumors

To first determine whether CCR6 may play a role in the regulation of mammary neoplasia, we investigated expression of the receptor in CD45-negative normal mouse mammary cells, and cells from various tumor stages (representative H&E pictures in Fig. 1a). CCR6 was expressed on a low proportion of normal mammary cells, but this proportion was greatly amplified in accordance with increasingly higher grades of MMTV-PyMT cancer including initial hyperplasia, early carcinoma and late carcinoma as indicated (Fig. 1a). This is consistent with human breast cancer [23] and other mouse models of cancer [17]. Additionally, in both non-PyMT $Ccr6^{WT}$ and MMTV-PyMT $Ccr6^{WT}$ mammary tissues the ligand for CCR6, CCL20, was highly expressed at concentrations over 50 ng/mg tissue (Fig. 1b). These data raise the possibility of a role for CCR6 in breast cancer development.

To next establish the role of CCR6 deletion on mammary tumorigenesis, we compared the rate and total extent of PyMT-driven neoplasia between MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice. Tumor onset was significantly delayed in MMTV-PyMT $Ccr6^{-/-}$ mice (Fig. 1c), with some mice not developing palpable tumors until 150 days old (21 weeks) compared to a maximum onset age of 130 days old (18 weeks) for MMTV-PyMT $Ccr6^{WT}$ counterparts (Fig. 1d).

In order to assess the impact of CCR6 on the later stages of cancerogenesis, MMTV-PyMT $Ccr6^{WT}$and $Ccr6^{-/-}$ mice were sacrificed at 22–24 weeks of age and the total number of mammary tumors per mouse was determined. We found that MMTV-PyMT $Ccr6^{-/-}$ mice had significantly decreased tumor incidence compared to MMTV-PyMT $Ccr6^{WT}$ animals (Fig. 1e). Together, these results implicate CCR6 as being an important player in breast oncogenesis.

CCR6 deletion significantly delays tumor initiation *in vivo*

We then sought to examine whether CCR6 influenced early hyperplasia of mammary glands during tumor initiation as well as late stage tumorigenesis. Glands from 8-week-old MMTV-PyMT $Ccr6^{-/-}$ and $Ccr6^{WT}$ mice were extracted and whole mounted for quantitation of hyperplastic/early-neoplastic lesions (representative images from both genotypes shown in Fig. 2a). We found that the deletion of CCR6 significantly reduced the initial hyperplastic outgrowth within the gland (Fig. 2b), a common indicator of future breast cancer development. As the total area of PyMT-driven hyperplastic outgrowth per gland was reduced by threefold in CCR6-null animals, we concluded that the effect of CCR6 on mammary tumorigenesis is manifested very early on in cancer development.

The difference seen in early tumor initiation between MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice can potentially result from a difference in normal mammary development, which may then have translated into decreased hyperplasia. We therefore extracted pubertal mammary

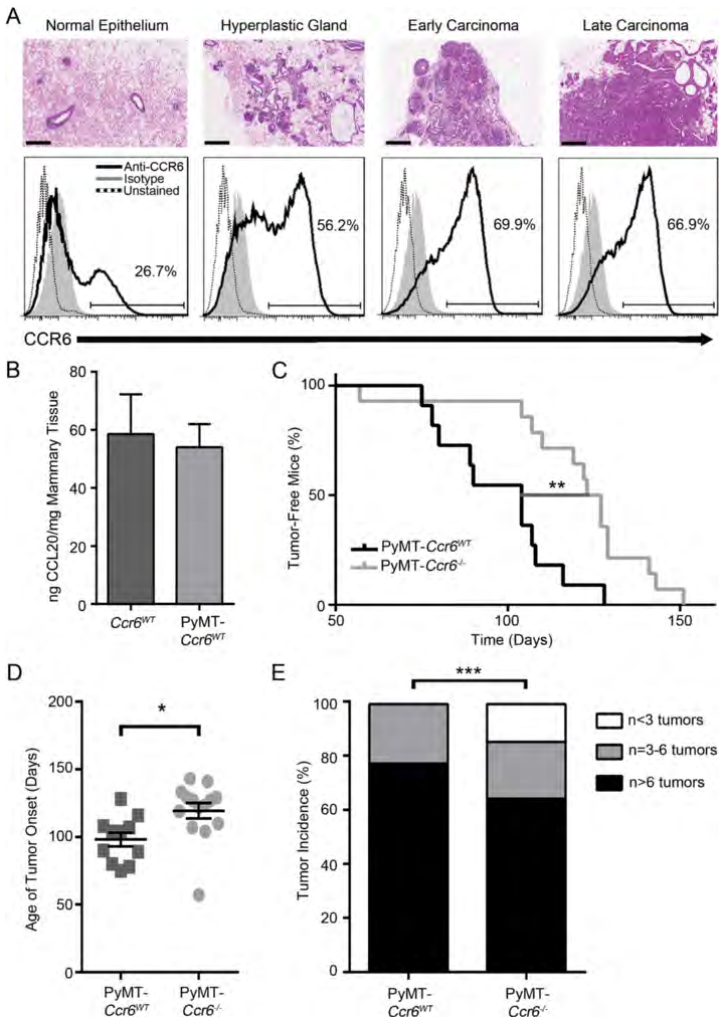

Fig. 1 CCR6 expression increases throughout cancer development and results in a higher number of mammary tumors. **a** Top: Representative H&E images of mammary tissue from normal gland and various stages of PyMT-driven tumorigenesis as indicated. Scale bar is 200 μm. Bottom: Proportion of CCR6-positive epithelial cells (CD45-negative) purified from mammary glands at respective stages of tumorigenesis. 18 normal samples (2 glands/sample), 7 hyperplastic samples (2 glands/sample), 6 early carcinomas and 3 late carcinomas were analyzed. **b** ELISA for CCL20, the ligand for CCR6, in normal (left) and early neoplastic MMTV-PyMT $Ccr6^{WT}$ (right) mammary glands. n = 4 samples per genotype. **c** Kaplan-Meier analysis of the palpable tumor onset in MMTV-PyMT $Ccr6^{-/-}$ mice (n = 14) vs MMTV-PyMT $Ccr6^{WT}$ mice (n = 11). **d** Age of tumor onset in MMTV-PyMT $Ccr6^{-/-}$ mice and MMTV-PyMT $Ccr6^{WT}$ mice. **e** Mammary tumor incidence in MMTV-PyMT $Ccr6^{WT}$ (n = 9) and $Ccr6^{-/-}$ (n = 14) mice

glands from non-PyMT 6-week-old $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice. Representative glands are shown in Additional file 1: Figure S1a. When ductal epithelial growth was quantitated, we observed no statistically significant difference in ductal length, number of terminal end structures or branching between $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice (Additional file 1: Figure S1b-d), and hence the overall effect of CCR6 deletion on normal mammary

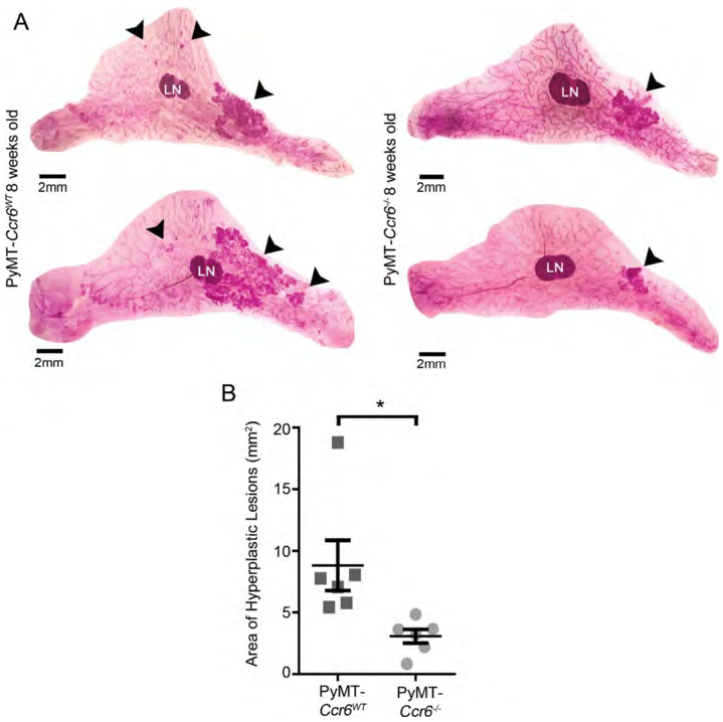

Fig. 2 CCR6 deletion significantly delays tumor initiation *in vivo*. **a** Representative whole mount images of MMTV-PyMT $Ccr6^{WT}$ (n = 6) and $Ccr6^{-/-}$ (n = 6) mice at 8 weeks of age. LN = lymph node. Black arrowheads indicate hyperplastic lesions within the glands. **b** Quantitation of area of pre-neoplastic lesions in 8 week-old MMTV-PyMT mice

gland biology appears to be minimal and is unlikely to account for differences in PyMT-driven tumor development between the two genotypes. Furthermore, the levels of CCL20 were not statistically different between non-PyMT $Ccr6^{WT}$ and MMTV-PyMT $Ccr6^{WT}$ mammary tissues (Fig. 1b), demonstrating that the expression of CCL20 is not affected by the process of tumorigenesis. Taken together, these data show that early stage tumorigenesis is mediated by a CCR6-dependent mechanism, without affecting normal mammary morphogenesis.

CCR6 promotes mammary gland neoplasia independently of cancer epithelial cells or stem-like cells

To investigate the mechanism underlying CCR6-driven mammary tumorigenesis, we studied the epithelial cell population to determine if CCR6 was having a direct effect on cell proliferation. Cells at the stage of early neoplasia from MMTV-PyMT $Ccr6^{WT}$ mammary glands were assayed for proliferation upon stimulation with CCL20. No differences in cell proliferation were observed (Fig. 3a). Furthermore, Ki67 staining of sectioned hyperplastic mammary glands from MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice showed that epithelial cells in knock-out mice are still able to adequately proliferate and staining of Ki67 is equal to that in the $Ccr6^{WT}$ (Fig. 3b). This suggests that the role of CCR6 in breast cancer is independent of epithelial cells.

We next determined whether CCR6 may exert its effect by skewing distinct cell populations within the bulk epithelium, as we have reported previously for the chemokine receptor CCR7 [29]. The current prevailing paradigm has mammary epithelial and breast cancer cells hierarchically organized with a self-renewing, quiescent, multipotent progenitor (or stem-like cell) population giving rise to basal and luminal progenitors which in turn differentiate into specific lineages making up the mammary gland and heterogenous breast tumors [30]. Recently, a number of immune mediators including chemokine receptors have been implicated in maintenance of the cancer stem-like cells within mammary tumors (reviewed in [31]). We therefore tested the

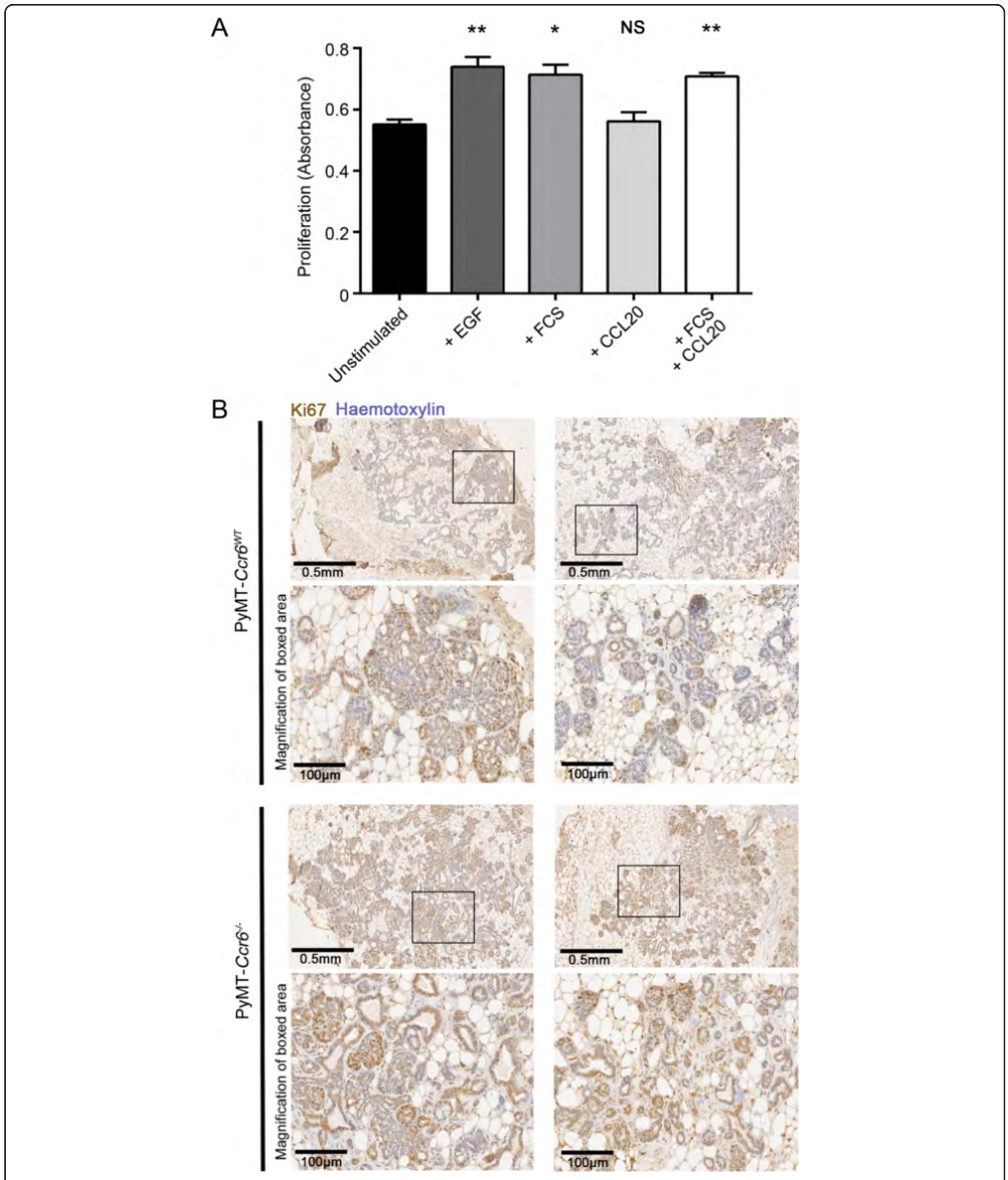

Fig. 3 CCR6 promotes mammary gland neoplasia independently of cancer epithelial cells. **a** Proliferation assay of mammary epithelial cells purified from MMTV-PyMT $Ccr6^{WT}$ mice at the stage of early neoplasia with and without stimulation by recombinant CCL20 (100 ng/ml). Fetal calf serum (0.5 %) and EGF (20 ng/ml) were used as positive controls. Data are representative of 3 independent experiments, n = 3 mice per experiment. **b** Analysis of Ki67-positive proliferating cells within MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice at the stage of early neoplasia. Shown are representative fields from 2 separate tumors per genotype, displaying equal distribution of cells positive for Ki67

potential link between the tumor-promoting function of CCR6 and breast cancer stem-like cell pools.

Freshly isolated MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mammary cells from pre-neoplastic mice at 8–9 weeks-old were assayed by flow cytometry for expression of cell surface markers CD24 and CD29 [32] (representative plots shown in Fig. 4a), which were previously used to define stem cells in the MMTV-PyMT [33, 34] and other breast cancer mouse models [35, 36]. We found that the deletion of CCR6 did not alter the proportions

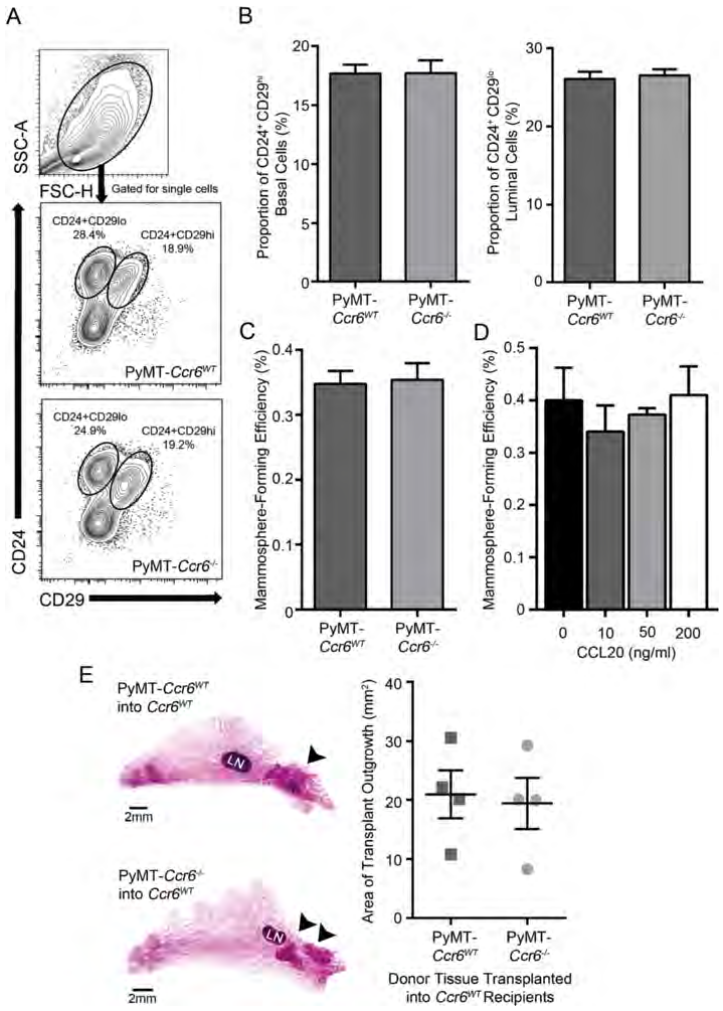

Fig. 4 CCR6 promotes mammary gland neoplasia independently of cancer stem-like cells **a-b** Single cell suspensions (CD45-negative) from mammary glands with early neoplasia from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice were analyzed by flow cytometry for the expression of CD24 and CD29 to quantitate the proportions of basal and luminal cell lineages. **c** Mammosphere-forming efficiency of cells isolated from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mammary glands. **a-c** Data are representative of 3 independent experiments, n = 6 mice per genotype per experiment. **d** Mammosphere-forming efficiency of MMTV-PyMT *Ccr6*^{WT} cells stimulated with varying concentrations of CCL20. Data are representative of 3 independent experiments, n = 3 individual mice per experiment. **e** Representative whole mount images and quantitation of the neoplastic outgrowth area in *Ccr6*^{WT} recipients of MMTV-PyMT *Ccr6*^{WT} and MMTV-PyMT *Ccr6*$^{-/-}$ mammary tumor tissue transplants; n = 4 recipient mice per group, LN = lymph node. Black arrowheads indicate donor transplant outgrowth within the glands

of the stem cell-enriched basal population ($CD24^{+}CD29^{hi}$) nor the luminal population ($CD24^{+}CD29^{lo}$) (Fig. 4b) in hyperplastic mammary glands.

We also investigated the effect of CCR6 ablation on functional stem-like mammary cancer cell pools using the mammosphere assay, which is used to select for colonies of early stem-like progenitors [37]. Pre-neoplastic MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ mammary cells were seeded into non-adherent mammosphere culture and allowed to grow for 7 days. The deletion of CCR6 did not alter mammosphere-forming efficiency (Fig. 4c), and when sphere cultures were stimulated with varying concentrations of CCL20, no change in their ability to form mammospheres (Fig. 4d) was observed, supporting results obtained using flow cytometric analysis.

Having found no difference in the proportion or function of stem cell-like pools within early tumorigenic lesions, we next tested a *bona fide* property of cancer stem cells – their ability to propagate tumors upon transplantation. MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ donor mammary tissue was transferred into recipient fat pads of syngeneic non-PyMT $Ccr6^{WT}$ mice. Using this approach, we found no significant difference between MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ tissue in the ability to form outgrowths when transplanted into $Ccr6^{WT}$ recipients (Fig. 4e), indicating that the deletion of CCR6 did not reduce the tumor-propagating capability of mammary epithelium.

Altogether, these results demonstrate that the role of CCR6 in breast cancer is independent of breast epithelial and progenitor cells, raising the possibility that its mechanism of action involves the tumor microenvironment.

CCR6 mediates the recruitment of pro-tumorigenic macrophages to the mammary tumor microenvironment

To test whether the reduced mammary tumorigenesis caused by the deletion of CCR6 was due to an effect of the CCR6-CCL20 axis on the tumor microenvironment we next investigated by flow cytometry the levels and identity of tumor-infiltrating leukocytes in mammary tumors at the stage of early carcinoma (see Fig. 1a). Tumor-associated macrophages have been extensively implicated in tumor promotion both in the mammary gland and elsewhere, due to their role in angiogenesis, cell proliferation and tissue remodeling [5]. To initially examine the polarization of TAMs in the MMTV-PyMT mice, macrophages were assessed for expression of prototypic markers interleukin-4-receptor (IL4-R) and mannose receptor (CD206), which have been used previously in flow cytometric analysis to distinguish alternatively-activated M2 macrophages from classically-activated M1 [38–42]. We estimated using these markers that a high proportion of TAMs were of an M2-like phenotype (Additional file 1: Figure S2a), as has been suggested previously for MMTV-PyMT mammary tumors [43]. Interestingly, CCR6 was found to be highly expressed within the TAM population as it was detected on greater than 60 % of total macrophages (Additional file 1: Figure S2b). Most importantly, CCR6 was expressed at higher levels and on a significantly higher proportion of putative M2 macrophages (up to 90 %) than M1 (Fig. 5a and Additional file 1: Figure S2b), using both IL4-R and CD206 to delineate the populations. This strong correlation potentially implicates CCR6 in the regulation of pro-tumorigenic macrophages within the mammary gland microenvironment.

We then assessed the levels of macrophages in mammary tumors from MMTV-PyMT $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice, and found that the proportion and overall numbers of TAMs were significantly reduced in MMTV-PyMT $Ccr6^{-/-}$ mammary tumors relative to $Ccr6^{WT}$ (Fig. 5b). Furthermore, the deletion of CCR6 resulted in a shift towards an M1 macrophage phenotype, defined by both IL4-R and CD206 prototypic markers (Fig. 5c).

The deletion of CCR6 also resulted in reduced trafficking of dendritic cells to the tumor site, consistent with previous studies, which demonstrated a reduced overall migratory ability of dendritic cells in CCR6-null mice [44, 45]. Although, overall numbers of tumor-infiltrating dendritic cells were much lower than the corresponding macrophage population. Notably, the majority of tumor-infiltrating dendritic cells were CCR6-positive, consistent with previous findings [12] (Additional file 1: Figure S3). We have also assessed CCR6 expression on B cells and specific T cell subsets (helper T cells (Th), cytotoxic T cells (Tc), and regulatory T cells (Treg)) within mammary tumors. In agreement with earlier reports for various biological settings [12, 46], all tested infiltrating leukocyte subsets expressed CCR6 at varying levels. When CCR6 was ablated, only the Tc cell subset showed a slight increase in the MMTV-PyMT $Ccr6^{-/-}$ as a proportion of $CD45^{+}$ tumor-infiltrating cells, however no significant differences were found in total cell numbers between the two genotypes (Additional file 1: Figures S4 and S5).

Our findings thus suggest that CCR6 promotes mammary tumorigenesis through an epithelium-independent mechanism involving tumor-infiltrating macrophages.

CCR6-mediated pro-tumorigenic macrophages promote breast cancer *in vivo*

We then sought to provide definitive evidence for the macrophage-mediating function of CCR6 in mammary tumor promotion using an *in vivo* macrophage reconstitution assay. Reconstitution assays, sometimes referred to as “add-back” assays, are frequently used to underscore a role for various cellular subsets in multiple pathological settings, and macrophage reconstitution has been used

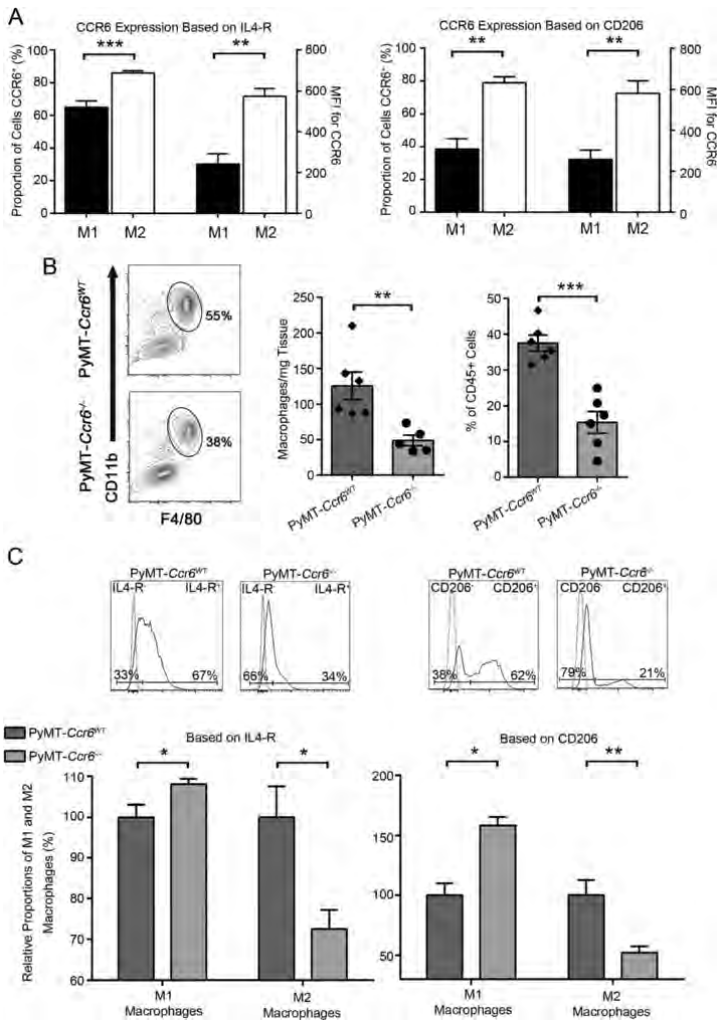

Fig. 5 CCR6 mediates the recruitment of pro-tumorigenic macrophages to the mammary tumor microenvironment. **a** Comparison of proportions of CCR6-positive cells and levels of CCR6 within putative M1 and M2 macrophage populations, based on IL4-R (left) and CD206 (right) expression. MFI = mean fluorescence intensity. Representative results from 3 independent experiments. **b** Proportion of TAMs in mammary tumors from MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mice as determined by flow cytometry. Data are representative of 3 independent experiments, n = 5-7 mice per genotype per experiment. **c** Proportions of putative M1 and M2 macrophage subtypes within the TAM population in mammary tumors from MMTV-PyMT *Ccr6*$^{-/-}$ mice relative to *Ccr6*^{WT}, as determined by flow cytometry. Dotted line = FMO control. Data are representative of 4 independent experiments, n = 3 mice per genotype per experiment

previously in mammary gland studies [47]. A schematic of the experimental setup is shown in Fig. 6a. MMTV-PyMT mammary tumor cells from *Ccr6*^{WT} donor mice were purified and transplanted into the inguinal mammary fat pads of non-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ recipients at 5 weeks of age. Two days post-transplantation, TAMs ($CD45^+F4/80^+$) were sorted from excised and dissociated MMTV-PyMT *Ccr6*^{WT} tumors (Fig. 6b) and orthotopically injected into a group of *Ccr6*$^{-/-}$ recipients as specified in Fig. 6a. All other recipients received sham

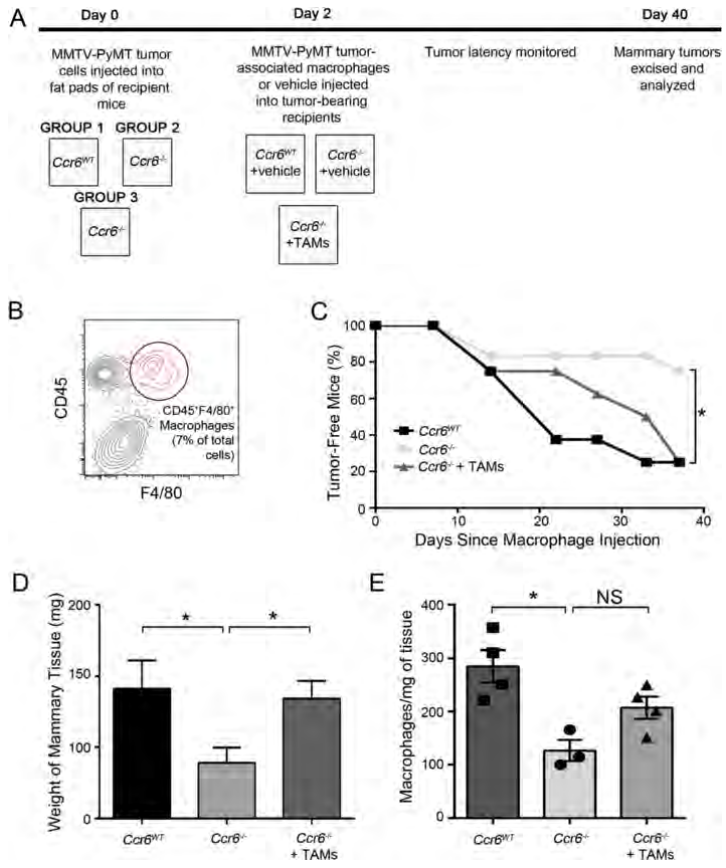

Fig. 6 CCR6-mediated pro-tumorigenic macrophages promote breast cancer *in vivo*. **a** Schematic of macrophage reconstitution assay to determine contribution of macrophages to mammary tumorigenesis in the context of CCR6 deletion. TAM = tumor-associated macrophage. **b** FACS plot showing the sorted macrophage population used for reconstitution. **c** Tumor-free survival curves over the course of the experiment. **d** Tumor weight of control tumors generated in $Ccr6^{WT}$ recipients and in $Ccr6^{-/-}$ recipients ± TAMs. n = 6-8 tumors per group. **e** Macrophage numbers in experimental groups at end-point, as assessed by flow cytometry. n = 3-4 samples per group

injections with vehicle only, followed by assessment of mammary tumor growth 6 weeks later.

In agreement with the results from spontaneous tumorigenesis studies, we have found that tumors grew significantly slower in the $Ccr6^{-/-}$ hosts compared to $Ccr6^{WT}$, indicating that CCR6 is required in the mammary stroma for robust tumor development. However, when the reduced macrophage phenotype was restored in $Ccr6^{-/-}$ mice through orthotopical injections, the tumor latency was significantly shortened, approaching that of the $Ccr6^{WT}$ mice (Fig. 6c).

It was further found that the supplementation of $Ccr6^{-/-}$ mice with TAMs restored the efficiency of tumor growth (measured by weight of tumor-bearing mammary glands) within these mice to that seen in the $Ccr6^{WT}$, whilst $Ccr6^{-/-}$ mice that received sham injections displayed reduced tumorigenesis (Fig. 6d) as seen in the spontaneous model (see Fig. 1).

Enumeration of macrophages within grafted tumors in $Ccr6^{WT}$ and $Ccr6^{-/-}$ mice (Fig. 6e) paralleled the results seen in spontaneous mammary tumors (see Fig. 5b). Whilst there was an upward trend towards increased TAMs in the $Ccr6^{-/-}$ mice that received TAM injections, the difference was not statistically significant (Fig. 6e). This, combined with the decreased tumor growth in the CCR6-null mice, indicated that the support

of macrophages is essential at the early stages of tumor growth.

We have thus established an essential role for CCR6 in the tumor microenvironment, providing a causative link between this receptor, infiltrating macrophages and mammary tumor development. Hence, therapeutic opportunities may be explored to control breast cancer progression, via manipulation of the CCR6-CCL20 axis to control tumor-promoting macrophages.

Discussion

We show here that the deletion of the chemokine receptor CCR6 caused a delay in tumor onset and decreased mammary tumor incidence *in vivo* in the MMTV-PyMT transgenic mouse model. We have determined that the underlying basis of the CCR6 oncogenic function is the increase in numbers of infiltrating pro-tumorigenic macrophages.

Multiple functional roles have been suggested for members of the chemokine family and their receptors in breast cancer pathophysiology [6], however little data using animal models is available to support these observations. The expression of CCR6 has been reported to correlate with higher stage and grade of human breast cancer, and has been proposed as a prognostic tool for determining relapse-free survival in breast cancer patients [23]. However, a causative link *in vivo* has yet to be demonstrated. We have employed the well-characterized MMTV-PyMT transgenic mouse model of breast cancer, and have found that CCR6 facilitates an earlier tumor onset and an increased incidence of mammary tumors. Of note, CCR6 affects mammary tumorigenesis from as early as the hyperplastic, or hyper-proliferative, stage. This initial phase of tumor development remains largely uncharacterized, despite being the most treatment-effective stage of cancer progression. Therefore, a better understanding of tumor initiation is crucial in order to develop therapies that target the tumorigenic process at the early stages of breast cancer.

When CCR6 was deleted in the MMTV-PyMT mouse, tumor latency was significantly extended, and these mice developed fewer mammary tumors than their $Ccr6^{WT}$ counterparts. However, CCR6 deletion did not affect tumorigenic properties of the epithelium as we have found with the chemokine receptor CCR7 [29]. Stimulation with CCL20 did not result in an increased proliferation rate of purified mammary epithelial cells from hyperplastic glands or tumorous lesions in contrast to previous studies with primary human breast peritumoral cells [25]. Furthermore, the deletion of CCR6 did not lead to decreased numbers of Ki67-positive proliferating cells within intact tumor-bearing mammary glands, pointing to an epithelial-independent function of this receptor in breast cancer.

We have also observed that the loss of CCR6 did not alter the numbers and functional properties of mammary cancer stem-like cells. Transplantation experiments in particular demonstrated that the presence of CCR6 in donor epithelium was not required for tumor propagation in recipient mammary glands.

Further investigation demonstrated that CCR6 functions via organization of the immune system during the early stage of mammary carcinogenesis. We have shown that the levels of TAMs are reduced by almost threefold when CCR6 is deleted. TAMs, which have been previously identified in MMTV-PyMT tumors [48], are widely reported to support the development of cancer [3, 49] and in the tumor microenvironment they are generally thought to polarize towards an alternatively-activated M2 pro-tumor phenotype relative to the classic M1 anti-tumor phenotype [4]. Whilst the TAMs in MMTV-PyMT tumors are polarized towards an M2-like subtype, we have shown that the presence of CCR6 maintains M2 TAMs as the predominant phenotype. Therefore, it is plausible to suggest that CCR6 in breast cancer functions to recruit pro-tumorigenic macrophages to the tumor immuniche [31], to support growth of transformed epithelial cells and cancer stem cells, as TAMs in the MMTV-PyMT model have also been shown to also maintain stem-like cells [50].

CCR6 is not expressed on peripheral blood monocytes, and is thought to only be acquired upon their differentiation into macrophages, induced by the tumor microenvironment [12]. In accordance with this, we found that a high proportion of macrophages within PyMT-driven mammary tumors express CCR6, which has not been previously demonstrated in breast cancer. Also of potential importance is the fact that up to 90 % of pro-tumorigenic M2-like TAMs expressed CCR6. Our findings parallel results from a recent study which showed that CCR6-null mice bearing the adenomatosis polyposis coli $(APC)^{min}$ transgene (a well-characterized model for gastrointestinal tumorigenesis) developed fewer intestinal adenomas and polyps, and that the effect of CCR6 was also linked to a significant reduction in $F4/80^{+}$ macrophages [17]. Interestingly, Liu *et al.* also recently demonstrated that the ligand CCL20 is secreted from both macrophages and tumor cells in another mouse model of colorectal cancer, potentially suggesting common regulatory mechanisms and a universal role for CCR6 in tumors of various etiology [51].

MMTV-PyMT cancer cell transplant experiments showed that tumor growth in a CCR6-null microenvironment was significantly inhibited compared to wild-type microenvironment conditions, directly demonstrating that the mammary stroma is dependent upon CCR6 for adequate tumor initiation and growth support. The reconstitution of this CCR6-negative

microenvironment with MMTV-PyMT $Ccr6^{WT}$ TAMs restored the tumor-promoting properties of mammary stroma, indicating that breast cancer can be therapeutically targeted through manipulation of the CCR6-CCL20 axis to control tumor-infiltrating macrophages.

CCR6 deletion has also impeded recruitment of dendritic cells into PyMT-driven mammary tumors. Recruitment of dendritic cells into various solid tumors has been well-documented (reviewed in [52]), and their role in tumor progression is mainly centered around tumor antigen presentation to lymphocyte subsets leading to anti-tumor immune responses [53, 54]. Furthermore, there is some evidence supporting direct tumoricidal activity of dendritic cells [53]. As previous studies have reported an intrinsic requirement for CCR6 in migration and fundamental functions of dendritic cells [44, 45], our finding of the reduced infiltration of dendritic cells in mammary tumors may not be a facet of cancer development in MMTV-PyMT $Ccr6^{-/-}$ mice, but is an inherent property of dendritic cell migration at a slower rate after CCR6 deletion.

Conclusions

In conclusion, we show here that CCR6 plays a significant role in the initiation and at the early stage of breast cancer development *in vivo* by mediating recruitment of pro-tumorigenic macrophages to the tumor site, and thus facilitating further progression to advanced stages of mammary neoplasia. Results presented in this study therefore suggest CCR6 as a potential target for therapeutic intervention in early breast cancer.

Methods

Mice

Mice were maintained in pathogen-free conditions in the University of Adelaide's Laboratory Animal Services facility. $Ccr6^{-/-}$ mice have been described previously [44]. $Ccr6^{-/-}$ females were crossed with C57BL/6 MMTV-PyMT males and the heterozygous offspring were interbred to produce MMTV-PyMT $Ccr6^{WT}$ (wild-type for CCR6) and bigenic MMTV-PyMT $Ccr6^{-/-}$ mice on the C57Bl/6 background. The University of Adelaide institutional animal ethics committee approved all animal experimental protocols.

Histology

Mouse mammary tissues were extracted, fixed in formalin and embedded in paraffin before sectioning at 5 μm. Haemotoxylin and eosin staining was carried out according to standard protocols. For immunohistochemical analysis of Ki67, slides were immersed in 0.5 % hydrogen peroxide in methanol for 10 minutes to inhibit endogenous peroxidase activity, followed by antigen retrieval by boiling slides in 0.1 M sodium citrate buffer under pressure. Slides were blocked for 20 minutes in 5 % normal rabbit serum in TBS/0.1 % Tween to prevent non-specific antibody binding, and then incubated overnight at 4 °C with mouse anti-Ki67 antibody (Vector Labs) according to the manufacturer's instructions. Specific antibody binding was detected using the EnVision Dual Link System (Vector Labs), followed by incubation with diaminobenzidine (DAB) substrate (Dako). Sections were counterstained with haemotoxylin, dehydrated and mounted.

Enzyme-linked immunosorbent assay

Wells were coated with anti-CCL20 capture antibody (R&D Systems) at 2 μg/ml overnight followed by a blocking step in PBS/3%BSA. Homogenized mammary tissue lysates (in PBS containing 10 % glycerol and 1x protease inhibitor) were added for 1.5 hours at 37 °C. Biotinylated anti-CCL20 detection antibody (R&D Systems) was added at 50 ng/ml for 1 hour at 37 °C followed by incubation with streptavidin-HRP (Rockland) for 30 minutes at room temperature. Wells were washed with PBS/0.05 % Tween after each incubation.

Whole mount staining

Mammary glands were mounted on slides, fixed in Carnoy's (30 % glacial acetic acid, 30 % absolute ethanol, 10 % chloroform), stained overnight in Carmine Alum (Stem Cell Technologies), then dehydrated and mounted using Permount (ThermoFisher Scientific). Image "stitching" and analysis were performed using Image J software.

Processing mouse mammary tissue to single cell suspension

Mouse mammary gland/tumor tissue was minced and then digested in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 1 mg/mL collagenase III, 100U/mL hyaluronidase (both from Worthington), 2 % fetal calf serum (FCS) and penicillin-streptomycin for 3–4 hours with gentle tilting. Organoids were further digested for 15 minutes with 6U/mL dispase (Gibco) in PBS and 20U/mL DNase I (Merck), and red blood cells were lysed by isotonic lysis buffer (150 mM NH_4Cl in 17 mM Tris–HCl, pH 7.2). Single cells were obtained by filtration through a 70 μm nylon mesh.

Proliferation assay

Isolated mouse mammary cells were plated in adherent culture (1:1 mixture of DMEM and Ham's F12 medium (Gibco) with 10 % FCS, supplemented with 20 ng/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, penicillin-streptomycin, and 0.25 μg/ml fungazone) in a 96-well plate. The following day the medium was replaced by DMEM with no additives, and after 2 hours of starvation cells were stimulated with FCS (0.5 %) ±

CCL20 (a gift from the late Professor Ian Clark-Lewis) at a concentration of 100 ng/ml. Stimulation with EGF at 20 ng/ml was used as a positive control. The cell proliferation assay was carried out 24 hours later using the XTT Cell Proliferation Kit (ATCC) according to manufacturer's instructions.

Flow cytometry

Single cell suspensions from processed mammary glands were incubated for 30 minutes on ice in PBS/0.5%BSA with anti-mouse primary antibodies to cell surface markers as indicated. Antibodies used were as follows: PE-conjugated anti-CCR6 (R&D), AlexaFluor647-conjugated anti-CCR6, PerCP/Cy5.5-conjugated anti-CD11c, PerCP/Cy5.5-conjugated anti-CD206, FITC-conjugated anti-CD29 (all from BioLegend), BV421-conjugated anti-B220, PE/Cy7-conjugated anti-CD11b, PE-conjugated anti-CD24, FITC-conjugated anti-CD4, APC-conjugated anti-CD45, biotinylated anti-CD45.2, FITC-conjugated anti-CD45.2, BV510-conjugated anti-CD8a, PE-conjugated anti-IL4-R (all from BD Biosciences), PE/Cy7-conjugated anti-CD3e, FITC-conjugated anti-F4/80 (both from eBioscience), and biotinylated anti-F4/80 (Life Technologies). When required, cells were also permeabilized using the FoxP3 Staining Kit, and incubated with PerCP/Cy5.5-conjugated anti-FoxP3 (both from eBioscience).

Samples containing biotinylated antibodies were further stained with BV510-conjugated streptavidin (BD Biosciences) in PBS/0.5 % BSA for 30 minutes. Fluorescence-minus-one (FMO) samples or cells stained with conjugated isotype control antibodies only were used as negative controls. After staining cells were fixed in 1 % paraformaldehyde and flow cytometry carried out using FACSCanto or LSRII equipment (BD). Data analysis was performed using FlowJo software (Tree Star Inc.). All flow cytometry data presented has been gated to exclude dead cells and debris using FSC-A/SSC-A, and to exclude doublets using FSC-A/FSC-H plots.

Mammosphere assay

Freshly isolated mammary cells were seeded into ultra-low attachment plates (Corning Inc.) at a concentration of $4x10^4$/ml, in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 1xB27 (Invitrogen), 20 ng/ml FGF, 20 ng/ml EGF, 4 μg/ml heparin (Sigma Aldrich), penicillin-streptomycin and 0.25 μg/ml fungazone. Mammosphere cultures were incubated at 37 °C for 7 days ± CCL20 at varying concentrations before manual enumeration under a light microscope.

Mammary Fat Pad transplants

Mammary gland fragments of 1 mm^3 size from donor MMTV-PyMT mice (18 weeks-old) were transplanted into contralateral sides of anaesthetized congenic non-PyMT recipient mice as indicated (8 weeks-old) within the inguinal mammary glands, and were monitored for adverse reactions to surgery. After 7 weeks, recipient glands were extracted and whole mounted for quantification.

Macrophage reconstitution assay

Mammary tumor cell suspensions were prepared from MMTV-PyMT $Ccr6^{WT}$ mice at 15 weeks-old as described above and injected into the fourth inguinal mammary fat pads of anaesthetized 5 week-old $Ccr6^{WT}$ and $Ccr6^{-/-}$ recipients in 80:20 % DMEM:Matrigel (BD), at 100,000 cells/gland.

Two days later, tumor-associated macrophages were sorted from MMTV-PyMT $Ccr6^{WT}$ excised and dissociated mammary tumors based on $CD45^+F4/80^+$ expression. 50,000 TAMs per gland were injected in DMEM orthotopically into the inguinal glands of $Ccr6^{-/-}$ tumor cell recipients. Control groups of $Ccr6^{-/-}$ and $Ccr6^{WT}$ tumor cell recipients were sham-injected with vehicle only.

Tumor development was monitored for 6 weeks, then mice were sacrificed and tumors extracted for analysis.

Statistical analysis

Analyses were carried out using GraphPad Prism and data is presented as mean ± SEM unless otherwise indicated. Significant statistical difference was estimated using student's t-tests, ANOVA for multiple comparisons, or chi-square tests for distribution analysis. Tumor-free survival curves for spontaneous tumors were graphed using the Kaplan-Meier method and compared by the log-rank statistic (Mantel-Cox test). Tumor-free survival curves for the reconstitution assay were compared using 2-way ANOVA with Tukey's multiple comparison test. P-values were used to denote statistical significance. Levels of significance were $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

Additional file

Additional file 1: Figures S1-S5. Accompanies the manuscript.

Abbreviations

FMO: Fluorescence-minus-one; GPCR: G-Protein coupled receptor; IL4-R: Interleukin-4-receptor; MFI: Mean fluorescence intensity; MIP-3α: Macrophage inflammatory protein-3α; MMTV: Mouse mammary tumor virus; PyMT: Polyoma middle-T; TAM: Tumor-associated macrophage; Tc: Cytotoxic T cell; Th: Helper T cell; Treg: Regulatory T cell; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

STB designed the study, performed and analyzed all experiments, and wrote the manuscript. JWF provided experimental and technical assistance in acquisition of data. SRM supervised the study, provided reagents, and edited the manuscript. MK designed the study, provided experimental assistance, supervised the study, and wrote the manuscript.

Appendices

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Appendices

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Boyle, ST *et al*. The chemokine receptor CCR6 facilitates the onset of mammary neoplasia in the MMTV-PyMT mouse model via recruitment of tumor-promoting macrophages.

Additional File 1:
Supplementary Figures S1-S5

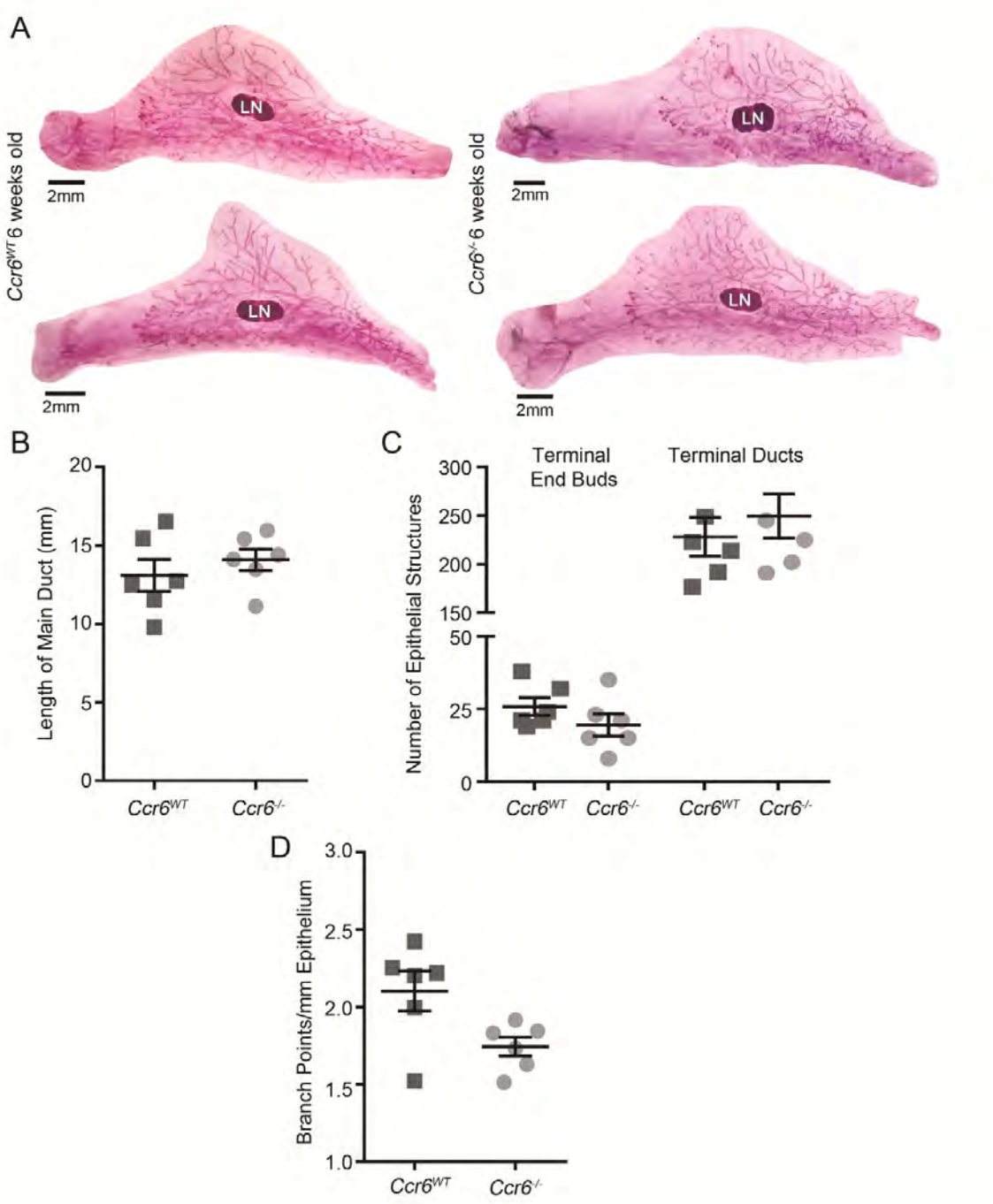

Figure S1: The role of CCR6 in normal mammary development
(a) Representative whole mount images of *Ccr6*^{WT} (n=6) and *Ccr6*$^{-/-}$ (n=6) mice at 6 weeks of age showing development of mammary epithelium. LN=lymph node. **(b-d)** Quantitation of epithelial growth, by length of main duct **(b)**, number of end structures **(c)** and branching **(d)**.

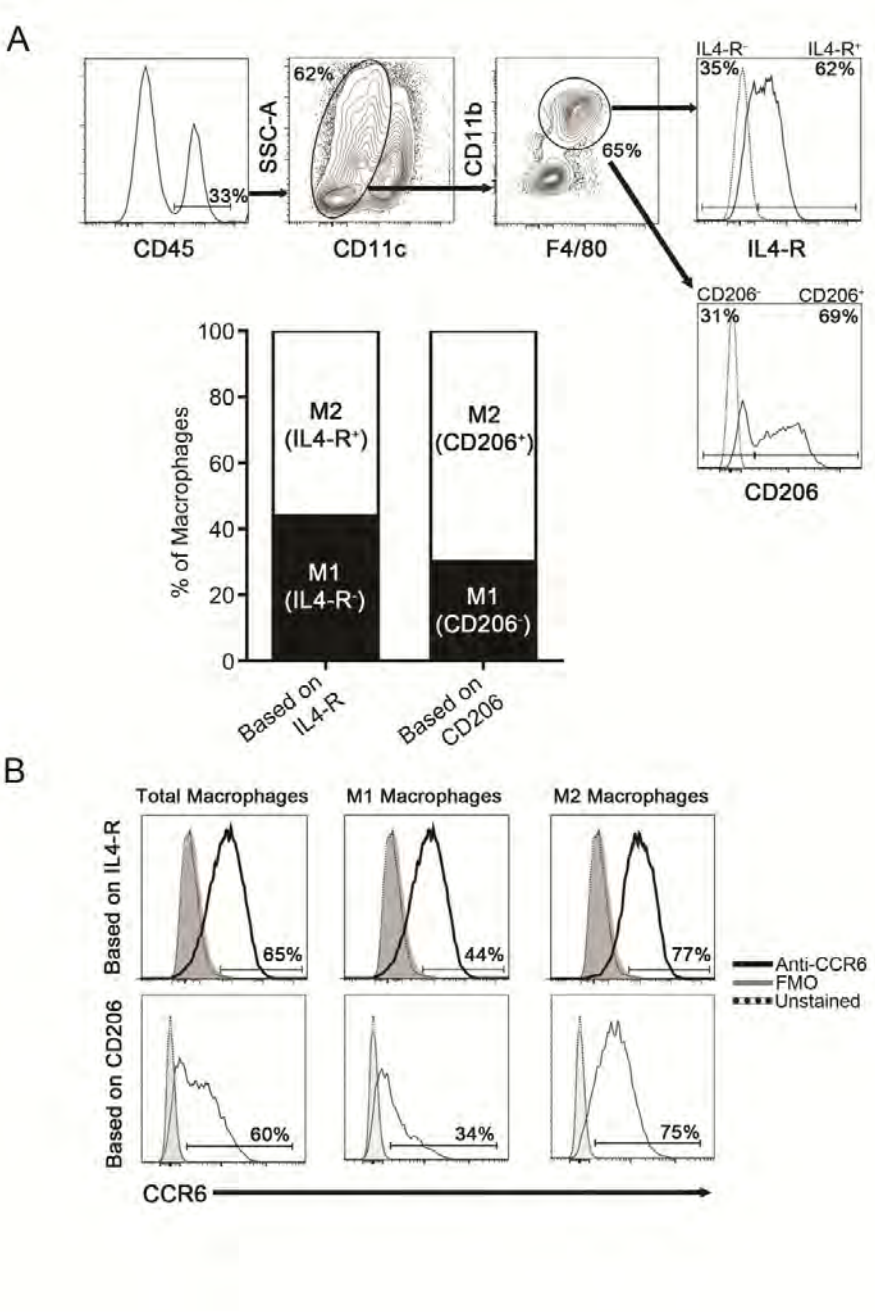

Figure S2: Analysis of infiltrating macrophages in MMTV-PyMT mammary tumors
(a) Analysis of M1 anti-tumor and M2 pro-tumor macrophages within the total macrophage population ($CD45^+CD11c^-CD11b^+F4/80^+$), based on IL4-R expression and CD206 expression as indicated, where M1 macrophages are classed as IL4-R or CD206-negative and M2 are IL4-R or CD206-positive. Dotted line=FMO control. M1 and M2 cell proportions (bottom) are presented as percentage of the total macrophage population. **(b)** Representative flow cytometry plots of CCR6 expression within the total, M1 and M2 macrophage populations as based on IL4-R and CD206 (gated as in (a)). **(a-b)** Representative results from 3 independent experiments.

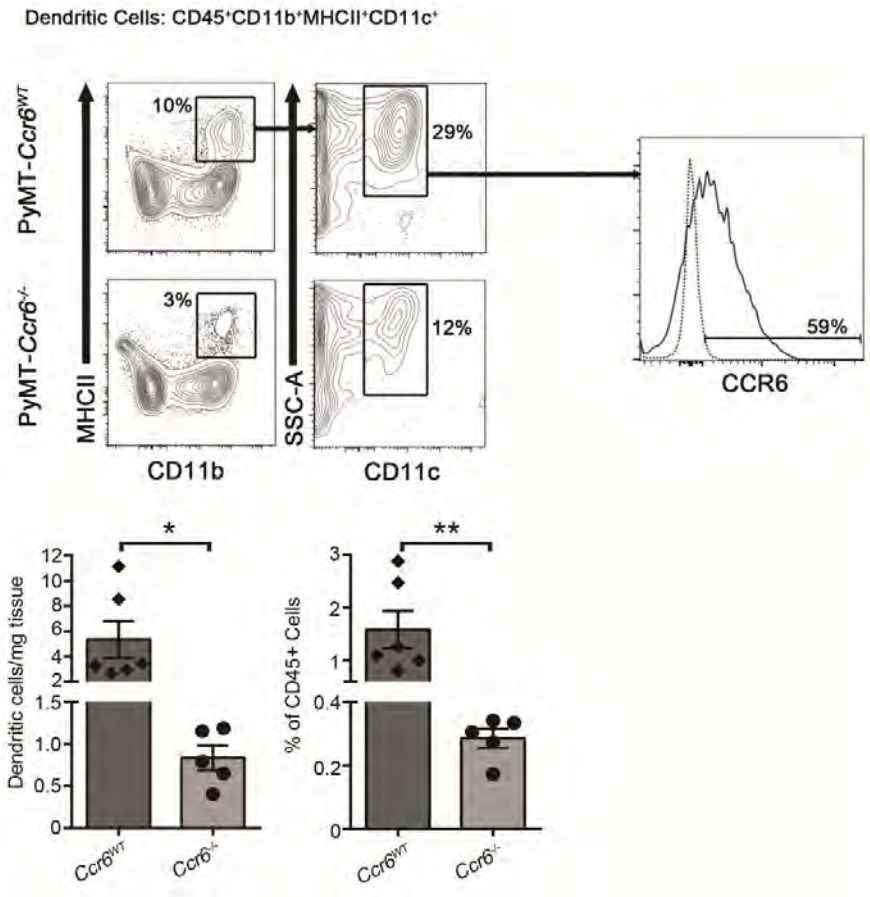

Figure S3: Infiltrating dendritic cells in MMTV-PyMT *Ccr6^{WT}* and *Ccr6$^{-/-}$* mammary tumors as determined by flow cytometry

All cells were gated for CD45^{+}. MMTV-PyMT *Ccr6^{WT}* dendritic cells were assessed for CCR6 expression (right), and proportions and numbers of dendritic cells compared between MMTV-PyMT *Ccr6^{WT}* and *Ccr6$^{-/-}$* (bottom). Dotted line=FMO control. n= 5-7 mice per genotype.

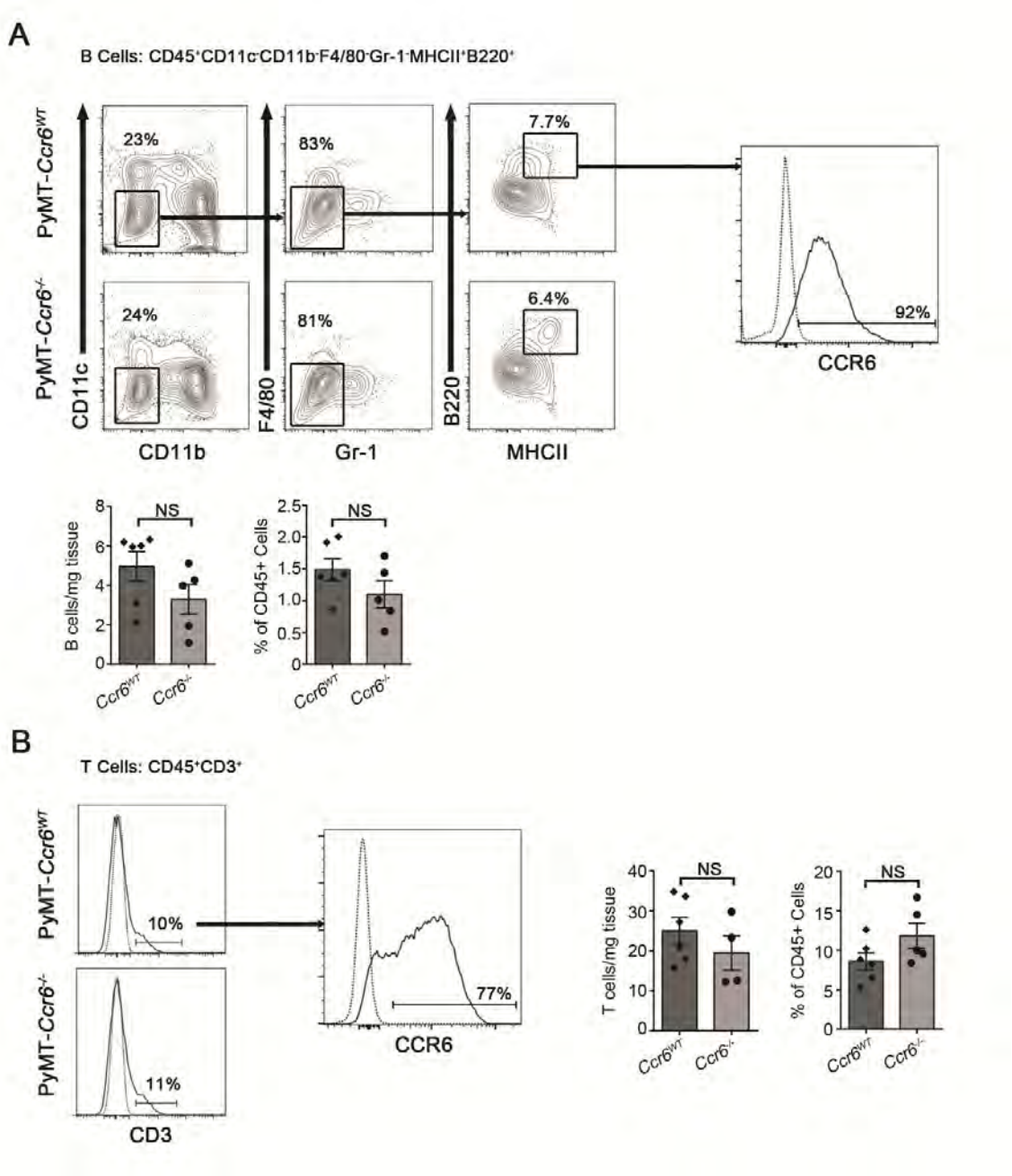

Figure S4: Infiltrating B and T immune cells in MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ mammary tumors as determined by flow cytometry

All cells were gated for CD45^{+}. **(a)** MMTV-PyMT *Ccr6*^{WT} B cells were assessed for CCR6 expression (right), and proportions and numbers of B cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (bottom). **(b)** MMTV-PyMT *Ccr6*^{WT} T cells were assessed for CCR6 expression (middle), and proportions and numbers of T cells compared between MMTV-PyMT *Ccr6*^{WT} and *Ccr6*$^{-/-}$ (right). Dotted lines=FMO controls. n= 5-7 mice per genotype.

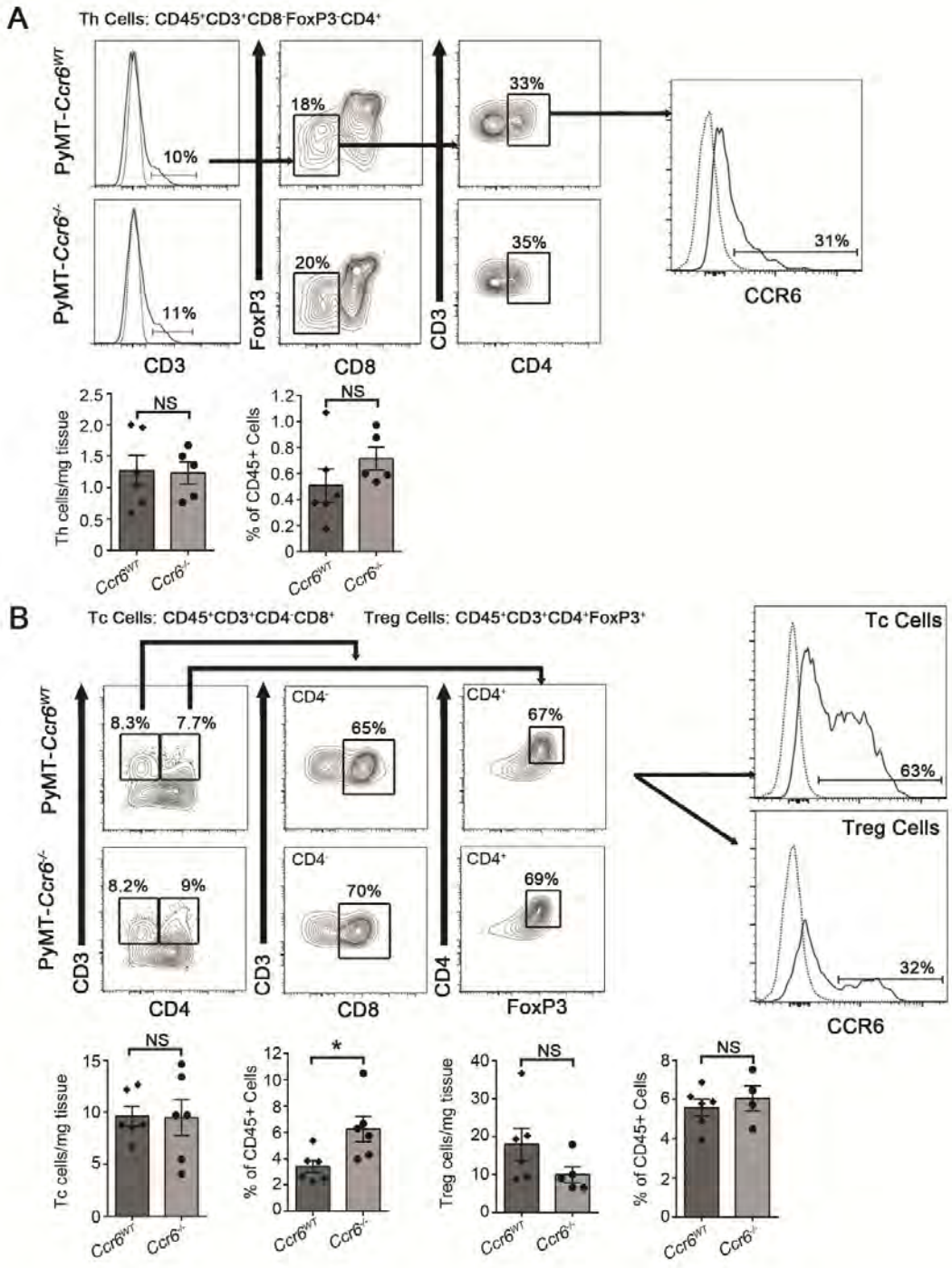

Figure S5: Infiltrating T cell subsets in MMTV-PyMT *Ccr6^{WT}* and *Ccr6$^{-/-}$* mammary tumors as determined by flow cytometry

All cells were gated for CD45^{+}. **(a)** MMTV-PyMT *Ccr6^{WT}* helper T cells (Th) were assessed for CCR6 expression (right), and proportions and numbers of Th cells compared between MMTV-PyMT *Ccr6^{WT}* and *Ccr6$^{-/-}$* (bottom). **(b)** MMTV-PyMT *Ccr6^{WT}* cytotoxic T cells (Tc) and regulatory T cells (Treg) were assessed for CCR6 expression (right), and proportions and numbers of Tc/Treg cells compared between MMTV-PyMT *Ccr6^{WT}* and *Ccr6$^{-/-}$* (bottom). Dotted lines=FMO controls. n= 5-7 mice per genotype.

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