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A Role for CBFβ in Mammary Gland Development and Breast Carcinogenesis

Anne-Marie Michelle Mooney

A submission to the Faculty of Medicine, University of Sydney for the Degree of Doctor of Philosophy

December 2014
Declaration

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at the University of Sydney or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by others, with whom I have worked with at the University of Sydney or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistances from others in the project’s design and conception or in style, presentation and linguistic expression is acknowledged.

Anne-Marie M. Mooney
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Anne-Marie
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialo protein</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>CCAAT-enhancer-binding proteins</td>
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<tr>
<td>CBFβ</td>
<td>Core binding factor β</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELF5</td>
<td>E74-like-factor 5</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA binding protein 3</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>Hox</td>
<td>Homeobox</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus-2 kinase</td>
</tr>
<tr>
<td>LEF1</td>
<td>Lymphoid enhancing binding factor 1</td>
</tr>
<tr>
<td>MaSC</td>
<td>Mammary stem cell</td>
</tr>
<tr>
<td>MEC</td>
<td>Mammary epithelial cell</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PrL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>PrLr</td>
<td>Prolactin receptor</td>
</tr>
<tr>
<td>Pthr1</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RANK/L</td>
<td>Receptor activator of NFkB/ligand</td>
</tr>
<tr>
<td>RUNX</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and transactivator</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-type MMTV integration site</td>
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Abstract

Breast cancer carries a lifetime risk of 1 in 8, making it the most common cancer affecting Australian women. Whilst the mortality rate associated with breast cancer has decreased by approximately 30% over the last 20 years, the most recent statistics show that breast cancer accounts for 28% of all new cancers diagnosed in women. It is therefore essential to find new regulators of breast cancer cell fate in order to be able to improve therapeutics, diagnostic tools and therefore the prognosis for patients presenting with breast cancer.

The mammary gland is a complex organ that develops under the control of various hormones, regulatory genes and transcription factors. Elucidating the processes behind the mammopoiesis has shown to be crucial in increasing our understanding of how disruption of these tightly regulated systems leads to breast carcinogenesis.

Recent studies have revealed a novel role for the transcription factor Runx2 in cell fate decisions in the mammary gland development during late pregnancy and lactation. Furthermore, it has been shown that Runx2 also regulates breast cancer cell fate in vitro and is able to delay breast cancer development and prolong survival in vivo. All three Runx genes form heterodimeric complexes with core-binding factor β (CBFβ), which ensures high affinity DNA binding, protein stability and therefore increased transcriptional efficiency of downstream targets. Modifying levels of CBFβ expression may create potential to investigate the effects of perturbing RUNX2 levels whilst compensating for the redundancy that exists between members of the Runx family.

There has been no previous data linking CBFβ with either mammary gland development or breast cancer. However, three recent whole-genome sequencing studies have identified CBFβ as a gene with recurrent mutations across an array of human breast cancer patients. More specifically, CBFβ was found to be mutated in approximately 5% of luminal breast cancers, making it one of the most highly mutated genes in human breast tumours.

The overarching aim of this thesis is to determine the role of CBFβ in mammary gland cell fate specification and the mechanisms by which it controls normal mammary gland development and influence breast carcinogenesis and metastasis.

This study has now examined CBFβ expression in the mammary gland for the first time and shows that it is differentially regulated through mammary gland development.
CBFβ expression is relatively high in virgin mice and decreases as the gland develops through to late pregnancy and early lactation with expression levels recovering at the time of involution.

Mammary-specific deletion of CBFβ in an inducible mouse model clearly demonstrated an essential role for CBFβ in the early stages of mammary gland development. The CBFβ−/− glands presented with a significant decrease in ductal elongation that was attributed to a decrease in the proliferative capacity of the mammary epithelial cells.

In the context of breast cancer, the knockdown and overexpression of CBFβ in human breast cancer cell lines demonstrated that CBFβ has a critical role in controlling breast cancer cell fate. These in vitro models revealed significant changes in the ability of breast cancer cells to control phenotypes consistent with metastasis, such as proliferation and migration in response to CBFβ expression levels.

Furthermore, this thesis describes the first in vivo model capable of investigating the effects of CBFβ deletion on breast tumourigenesis and metastasis, demonstrating that deletion of CBFβ in the mouse mammary gland significantly delays tumour onset and increases overall survival in breast carcinogenesis whilst also reducing the incidence and severity of distal metastases.

This thesis reports that RUNX2 and CBFβ expression are differentially regulated throughout mammary gland development and demonstrates a novel and exciting role for CBFβ as a new regulator of both mammary gland development and breast cancer progression and metastasis.
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1. Introduction

The mammary gland is an organ that develops predominantly post partum, under the influence of various regulatory genes and hormones associated with sexual development and reproduction. The established gland will undergo repeated cycles of proliferation, differentiation and apoptosis with each pregnancy as it transitions from its nulliparous state, through to a fully functional gland capable of lactation, before undergoing remodeling to once again resemble the mature nulliparous gland. These distinct stages enable the mammary gland to be the perfect platform in which to study cell fate decisions. It is often the dysregulation of the key regulators involved in these developmental processes that lead to disease progression, including cancer. The overall aim of this thesis is to examine the role of the Runx2/CFBβ transcriptional complex in the developing mammary gland and how changes in expression and regulation affects the pathogenicity and progression of breast cancer.

Mammary Gland Development

Overview

The mammary gland is a complex organ that, unlike most other organs within the body, undergoes the majority of its development post-partum, making it quite a unique system. It was discovered in the early 1900s that the mammary gland primarily develops under the control of the ovarian hormones, estrogen and progesterone (Allen, 1924; Halban, 1900) with prolactin becoming a key regulator during pregnancy as gland differentiates into its functional form (Brisken et al., 1999; Riddle, 1933; Topper & Freeman, 1980). There are many other growth factors, regulatory genes and transcription factors that control this multifaceted developmental process and the role of those regulators more directly related to this thesis will be described in due course.

Development of the mammary gland can be compartmentalised into distinct stages that are inextricably linked to both sexual development and reproduction. Each phase of mammopoiesis is phenotypically unique, as shown in Figure 1.1. These stages have been coined embryonic, prepubertal, pubertal, pregnancy, lactation and involution (Hennighausen & Robinson, 2001).
Fig. 1.1. Stages of mammary gland development

Mammary gland development occurs in distinct and unique stages that are link to sexual and reproductive development. A) Following embryonic development, the mammary gland of the neonate consists of a rudimentary ductal tree within the fat pad. B) Pubertal growth is marked by the formation of terminal end buds (TEBs) that drive extensive proliferation and growth of the ductal network under the influence of estrogen. C) Once mature, the ductal network undergoes extensive side-branching during each estrous cycle under the influence of both estrogen and progesterone. D) During pregnancy, the gland undergoes extensive differentiation and lobuloalveolar development under the influence of both progesterone and primarily prolactin E) During lactation milk is secreted from mature alveoli in response to suckling and oxytocin. F) Post weaning, the mammary gland undergoes controlled apoptosis and re-modeling until it resembles the nulliparous gland (Adapted from Henninghausen 2001).
The mammary gland consists of two major compartments, the epithelial ductal network and the stromal fat pad through which it protrudes. The mammary stroma is comprised of endothelium, fibroblasts, extracellular matrix proteins, immune cells and adipocytes (Hennighausen & Robinson, 2001). There are complex interactions and signalling networks between the epithelial and stromal cells, which help to define the functionality of the mammary gland throughout development (Nandi, 1958; Neville & Daniel, 1987).

Much of what we know about the mechanisms behind mammary gland development has arisen from the detailed studies using mice where it is relatively simple to modify the genetic background of the animal through gene deletion and recombination approaches. Furthermore, the mouse mammary gland is very similar in architecture to the human breast and the importance of cross talk between the epithelium and stroma in both systems make the mouse a useful system in which to model human breast development and breast cancer (Plante et al., 2011; Sternlicht, 2006).

Mammary gland development in mice begins around embryonic day 10 (e10) with the formation of the milk line, which develops into 5 pairs of placodes that will house a rudimentary ductal tree at the time of birth. It is the epithelial ductal network that branches throughout the mammary fat pad and undergoes high levels of proliferation during puberty. Following puberty the mammary gland undergoes repeated cycles of proliferation and differentiation under the influence of the female steroid hormones estrogen and progesterone. The cells then differentiate into alveolar buds during pregnancy before maturing into fully functional alveoli capable of lactation post partum. Involution is the final stage of the developmental cycle, whereby the gland undergoes controlled apoptosis and remodeling, returning to a mature pre-pregnancy like state (Hennighausen & Robinson, 2005).

This brief summation of mammopoiesis will now be expanded upon to review the specific roles of the genes, hormones and growth factors that are critical for successful development and differentiation of the mammary gland.

**Structure of the Mammary Gland**

As mentioned above, two main cell types give rise to the mouse mammary gland: the epithelium that is derived from the ectoderm; and the surrounding stroma, which is derived from the mesoderm. The epithelial compartment is comprised of a branching
ductal network that does not fully begin to develop until puberty. Through the use of hormone therapy in ovariectomised, adrenalectomised and hypophysectomised mice, Nandi was able to show that estrogen is essential for ductal development during puberty, while prolactin is required later for the successful development of alveolar structures during pregnancy (Nandi, 1958). The epithelial ducts continue to branch through the stroma to form decreasingly smaller ductals that eventually form structures known as lobules. These lobules are made up of alveoli and it is this lobulo-alveolar compartment that forms the functional aspect of the gland as it develops and expands with each pregnancy. The alveoli are also comprised of 2 main cell types: the luminal epithelial cells, which undergo differentiation during pregnancy in order to produce and secrete milk; and the basal or myoepithelial cells which form a contractile mesh-like network around the ductal tree and aid in the delivery of the milk from the ducts to the nipple in response to oxytocin (Hennighausen & Robinson, 2001, 2005).

There are a host of hormones, genes, growth factors and transcription factors that controlled the developing mammary gland and it is becoming clearer that many of these are not solely confined to the mammary gland but are also important in the developmental regulation of other organs and tissues.

**Embryonic mammary gland development**

The mammary gland does not begin to form in the embryo until approximately day E10.5 with the formation of the milk line that run from the axilla to the groin along the rostro-caudal axis of the ventral body surface. By E11, the milk line is replaced by five pairs of mammary placodes, derived from the ectoderm (Balinksy, 1950; Turner & Gomez, 1933). These five placode pairs are placed where the eventual mammary glands will exist and form in an asynchronous order, beginning with the 3rd (thoracic), 4th (abdominal), 1st (cervical), 5th (inguinal) then finally the 2nd (upper thoracic) pair (Mailleux et al., 2002; Veltmaat et al., 2004).

In an interesting example of cell fate regulation, each pair of mammary placodes require different developmental cues and signals in order to control not only the time at which they develop but also the place at which they develop along the milk line. Some of the genes that are essential for the timing and placement of mammary placodes include the ERBB2 ligand NRG3, FGF10 and its receptor FGFR2B and the transcription factors TBX3, LEF1 and Gli3. When these genes are deleted separately in
the embryo, only some of the placode pairs are absent or do not develop to the stage of bud formation (Foley et al., 2001). For example, deletion of LEF1 results in a failure to develop placodes 2 and 3 (Boras-Granic et al., 2006) whilst perturbed expression of NRG3 leads to loss of placode 3 as well as duplication of the 4th placodes (Howard et al., 2005).

Many of the genes that control embryonic development of the mammary gland were identified when a genetic deletion or conditional knockout of a gene lead to an arrest of mammogenesis at a specific stage in mice (Robinson, 2004).

By E15, the placodes have developed into epithelial buds that are surrounded by a mesenchymal cell layer. This development of the placode is controlled by and dependent on epithelial to mesenchymal signalling. This was first shown by Propper when he co-cultured non-mammary ectoderm with mammary mesenchyme in vitro and showed growth of epithelial buds (Propper, 1972). This phenomena was then confirmed when Cunha and colleagues cultured dorsal epidermis and mammary mesenchyme from E13 embryos and then xenografted the tissue on to lactating mice. In this instance, not only did the epithelial buds form but they also differentiated into lobuloalveolar cells capable of secreting milk protein such as casein and α-lactalbumin (Cunha et al., 1995). There are a multitude of genes and pathways that have been shown to regulate the epithelial to mesenchymal signalling that directs mammary gland development. It is thought that targeting these interactions may lead to therapeutics that are capable of transforming malignant cancer cells from undifferentiated proliferating masses back into differentiated populations (Cunha & Hom, 1996).

One of the critical pathways in mammogenesis is the wingless-related MMTV integration site (WNT) signalling pathway and it has been found to be essential for initiation of milk line specification, successful placode development and also placode size (Chu et al., 2004; van Genderen et al., 1994; Veltmaat et al., 2004). WNT target genes Wnt10a, Wnt10b and Wnt6 can be first detected in the epithelial and mesenchymal cells of the milk line from approximately E10.25, with their signal intensifying in the placodes at E12.5 and expression is maintained as the buds develop until around E15.5 (Veltmaat et al., 2004). These target genes act through the canonical WNT/β-catenin signalling pathway to initiate placode development and drive mammary gland morphogenesis. When explants from developing embryos are cultured in WNT medium or lithium chloride (a WNT stimulator), formation of placodes is accelerated.
and the resulting size is much larger (Chu et al., 2004). Conversely, in transgenic mice expressing Dick-kopf 1 (a WNT inhibitor) in the surface ectoderm, placode formation is completely inhibited (Chu et al., 2004). This control of mammogenesis through WNT signalling seems to be mediated through expression of the lymphoid enhancer-binding factor 1 (LEF1) transcription factor, a WNT/β-catenin downstream target. Deletion of LEF1 in mice leads to a complete abolishment of WNT signalling and mice that do not develop the correct number of mammary placodes (Andl et al., 2002; Boras-Granic et al., 2006; van Genderen et al., 1994). LEF1^-/- mice are missing placode pairs 2 and 3 with the remaining placodes developing into buds, which deteriorate and therefore mice that are born with no ducts or nipples, confirming the importance of WNT signalling in mammogenesis (Boras-Granic et al., 2006).

In regards to mammary line specification, it has been shown that the fibroblast growth factor (FGF) family is also essential and acts in parallel to the WNT signalling pathway (Hens & Wysolmerski, 2005). FGF signals from the somites underlying the milk line are essential for the initiation and formation of mammary placodes (Mailleux et al., 2002). If the FGF ligand, Fgf10 or the receptor Fgfr2B are deleted in the embryo, the milk line does not form in the region where placodes two and three are supposed to form (Veltmaat et al., 2006). The 4th placode does form in these knock out mice but undergoes apoptosis by E12.5, indicating that a separate FGF family member must control the maintenance of the 4th gland though Fgfr2B, most likely Fgf7 due to its expression in the mesenchyme (Cunha & Hom, 1996; Mailleux et al., 2002). Several other FGF family members are also expressed in the formed mammary bud, including Fgf4, Fgf8, Fgf9 and Fgf17 whilst the receptors Fgfr1B and Fgfr2 are expressed in the placodes and surface ectoderm (Mailleux et al., 2002).

The Homeobox genes have also been found to act as important regulators of cell fate in the mammary gland as well as several other systems, including the skeleton and the central nervous system (Visvader & Lindeman, 2003). The Msh Homeobox (Mxs) transcription factors are part of this family and are expressed in the mammary bud alongside LEF1 (Phippard et al., 1996). Msx1 and Msx2 are initially expressed in the placode (Phippard et al., 1996) before Msx1 expression is lost and the expression of Msx2 shifts into the surrounding mesenchyme (Satokata et al., 2000). Null mutations of both Msx1 and Msx2 lead to a failure in bud formation (Satokata et al., 2000). If Msx1 alone is deleted, there is no effect on bud formation whereas specific mutation of Msx2
leads to an arrest of bud development at the sprouting stages around E16.5, proving a critical role of Msx2 in mammary branching morphogenesis (Satokata et al., 2000). Msx1 and Msx2 continue to be co-expressed in the post-natal mammary gland throughout virgin development and early pregnancy but expression is found to dramatically decrease in late pregnancy (Friedmann & Daniel, 1996; Phippard et al., 1996).

The T-box (TBX) family of proteins present another key regulatory family involved in embryonic mammary gland development that acts downstream, of both the WNT and FGF signalling pathways. More specifically, both Tbx2 and Tbx3 are expressed along the milk line from E10.5 before localising to the individual placodes as they are formed (Eblaghie et al., 2004; Jerome-Majewska et al., 2005). It has been shown that Tbx3−/− embryos do not develop mammary buds and that they do not express Wnt10b or LEF1 (Davenport et al., 2003). Tbx3 in conjunction with bone morphogenic protein 4 (BMP4) is also important for determining the dorso-ventral position of the buds through regulation of LEF1 (Cho et al., 2006). The region in the mesenchyme where Tbx3 is expressed determines the site of placode development whilst BMP4 expression localises to the area immediately ventral to the placode and acts to negatively regulate Tbx3, therefore enhancing LEF1 expression in the placode to stimulate development (Cho et al., 2006).

Similarly to the WNT pathway, Hedgehog signalling is also crucial for the regulation of mammary cell fate specification. The GLI family zinc finger (GLI) proteins, which are either activators or repressors of Hedgehog signalling depending on the context (Aza-Blanc et al., 2000) are also expressed at E11, namely Gli2 and Gli3 (Hatsell & Cowin, 2006). In the context of mammogenesis, Gli3 is acting as a repressor of Hedgehog signalling which is evident by the loss of buds two and three when GLI is forced into its activator state. It can therefore be deduced that the repression on hedgehog signalling is required in order to maintain the identity of mammary epithelial cells (Gritli-Linde et al., 2007; Hatsell & Cowin, 2006).

The final major family of proteins involved in placode development is the epidermal growth factor receptor (EGFR) family, primarily neuregulin 3 (NRG3) and its receptors v-erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2 and 4 (Erbb2 and Erbb4), which are expression in the milk line from E10.75 and confined to the epithelial placodes once they develop (Howard et al., 2005). When expression of NRG3 is not
localised to the placodes only one of the 3rd mammary buds will not develop, there is often duplication in the 4th pair and also Wnt10b and LEF1 expression is absent. This evidence points to NGR3 acting as mesenchymal signal upstream of WNT that is crucial to the positioning and number of mammary buds that are formed (Howard et al., 2005).

As well as placode and mammary bud formation, there is also a small degree of ductal growth and nipple formation that occurs during the embryonic phase of mammary gland development. Parathyroid hormone related protein (Pthrp) and parathyroid hormone related protein receptor 1 (Pthrl) are crucial to these processes as shown when mice that are null for these genes lead to ablation of LEF1 and β-catenin which are essential for mammary gland development (Foley et al., 2001; Wysolmerski et al., 1998). Furthermore, disruption of the Pthrp epithelial to mesenchymal signalling results in failure of both mammary bud and nipple formation. In this case the duct is unable to progress through the mesenchyme, the cells become more squamous and the cells lose the mammary specific keratin signals (Foley et al., 2001; Wysolmerski et al., 1998). Conversely, if Pthrp is over-expressed in the epidermis, the mammary mesenchyme undergoes forced differentiation and the nipple is formed (Foley et al., 2001). BMP4 acts downstream of Pthrp to mediated the cross talk between the epithelial bud and the surrounding mesenchyme (Hens et al., 2007). In order to stimulate ductal outgrowth, inhibit hair follicle formation and therefore allow nipple formation there is an increase in mesenchymal levels of bone morphogenic proteins (BMPs) in response to epithelial Pthrp signals (Robinson, 2007).

RASGRF1 (Ras protein-specific guanine-releasing factor1) is also involved in the induction of the mammary mesenchyme and outgrowth of the ductal epithelium as it highly expressed in the most proliferative part of the mammary gland, the terminal end buds (Chakravarty et al., 2003). It has also been shown that RASGRF1 knockout mice have significantly smaller epithelial buds by E14.5 where the epithelial cells are highly disorganised and the mesenchymal cells do no express the appropriate steroid receptors (Heckman et al., 2007).

In summary, it is evident that the control of mammary gland development during the embryonic stage is dependent on epithelial to mesenchymal signalling.

*Virgin mammary gland development*
**Ductal elongation and bifurcation**

The post-partum mammary epithelial ductal network undergoes allometric growth through the stromal fat pad from the nipple under the influence of the steroid hormone, estrogen throughout puberty. The engine that drives this growth are the terminal end buds (TEBs), which are situated at the tips of the growing ducts and enable ductal extension of the epithelium through the fat pad under the influence of both estrogen and growth hormone (Hinck & Silberstein, 2005). The presence of TEBs is the defining feature of a gland undergoing ductal extension (Williams & Daniel, 1983). TEBs are club-shaped masses made up of both proliferating and apoptosing cells that penetrate through the stromal fat pad and can undergo a process of bifurcation where two secondary ducts of equal size are formed (Silberstein, 2001).

Ductal extension is driven by the proliferating cap cells at the distal tip of the TEB combined with several layers of proliferating cells underneath. It is this secondary layer of cells that undergo selective apoptosis in order to form the lumen of the epithelial duct (Silberstein, 2001). The processes of ductal elongation and bifurcation are primarily regulated by estrogen and growth hormone but there is also interaction with the surrounding stroma.

Estrogen activity is regulated through binding interactions with its receptors ERα and ERβ, both of which are expressed in the mammary epithelium and stroma (Saji, 2000). It has been shown that ERβ is not required for ductal elongation but rather is required for the differentiation events that take place during pregnancy (Förster et al., 2002). However, when ERα is knocked out in the mammary stroma, ductal elongation fails but the same is not true if it is only knocked out in the epithelial compartment (Cunha et al., 1997). It is therefore evident that stromal ERα is crucial for outgrowth of the ductal tree. The adult animal requires both the stromal and epithelial expression of ERα in order to maintain the ductal network but this can also be achieved in the presence of stromal ERα only if doses of estrogen and progesterone are increased (Mueller, 2002).

When estrogen binds to its receptor it results in translocation of the receptor to the nucleus, which leads to interactions with co-activators and finally gene transcription (McKenna et al., 1999). ERα knock out mice and null mutants for its co-activator, steroid receptor co-activator 3 both show failed ductal elongation in response to estrogen (Xu et al., 2000).
The other crucial hormone required during pubertal development of the mammary gland is growth hormone and also members of the insulin-like growth factor (IGF) family (Kleinberg, 1997).

Growth hormone is mainly produced by the pituitary gland and its action is mediated through binding to its receptors, Ghr, located in both the mammary epithelium and stroma (Ilkbahar et al., 1999). Similar to ERα, only stromal Ghr is necessary for successful ductal elongation (Gallego et al., 2001; Walden et al., 1998) and these effects are mediated via induction of Igf1. Studies have shown that Igf1 is induced in the stroma by growth hormone (Ruan & Kleinberg, 1999; Ruan et al., 1992) and that Igf1 can substitute for growth hormone and induce ductal elongation. Furthermore, null mutants of both Igf1 and Igfr lead to impaired outgrowth of the ductal network (Bonnette & Hadsell, 2001; Ruan & Kleinberg, 1999). It can therefore be deduced that the effects of growth hormone on TEB formation and ductal elongation are mediated by stromal Ghr induction of Igf1.

Members of the epidermal growth factor (EGF) family are also important in mediating interactions between the epithelial and stromal cells in the mammary gland that are important for regulating ductal elongation. Similarly to the aforementioned hormones, it is primarily the binding of Egf to its stromal receptor, Egfr that is important of the regulation at this stage in mammopoiesis (Wiesen et al., 1999). Further to this, it has been shown that expression of a dominant negative form of Egfr in the mammary gland leads to failed outgrowth of the ducts (Xie et al., 1997). There are several ligands that are capable of binding to Egfr and therefore a multitude of binding combinations and dimerisations that will produce different signals and ultimately control different aspects of mammary gland development, from puberty through to lactation (Earp et al., 1995; Komurasaki et al., 1997; Pinkas-Kramarski et al., 1997). Another example of the role of EGF signalling importance in puberty is shown through the inactivation of one of these ligands, amphiregulain, resulting in a failure of ductal outgrowth similar to what is seen in the Egfr knockout model (Luetteke et al., 1999).

GATA3 is another important regular of cell fate that has been found to be essential in mammary gland development. GATA3 is expressed as early as E12.5 and this expression increases during puberty and pregnancy but remains confined to the luminal cells of the mammary gland (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006; Ormandy et al., 2003). Studies have shown that K14cre-mediated deletion of GATA3
results in failure of placode development (Asselin-Labat et al., 2007), which is a similar phenotype to the LEF1, Msx1, and Msx2 knockout models (Hens & Wysolmerski, 2005), indicating a potential transcriptional network that exists between GATA3 and LEF1 (Naylor & Ormandy, 2007). It has also been shown that GATA3 is highly expressed in the TEBs as well as the nascent and adult ducts and that it plays a key role in maintaining luminal cell fate in the mammary gland. A lack of GATA3 in the luminal cells of the mammary ducts leads to failure of TEB formation during puberty and a subsequent disruption of ductal elongation in the adult gland (Kouros-Mehr et al., 2006). GATA3 has now been established as a critical regulator of luminal cell differentiation within the epithelial hierarchy of the mammary gland (Asselin-Labat et al., 2007). This study has shown that a deficiency in GATA3 in the mammary gland leads an expansion of luminal progenitors and a subsequent arrest of differentiation, whilst induction of GATA3 in a stem cell enriched environment is able induce maturation of cells in the alveolar lineage (Asselin-Labat et al., 2007).

**Ductal side branching and alveolar bud formation**

During ductal elongation, the primary duct bifurcates into two new secondary ducts that are of equal size (Silberstein, 2001). Ductal side branching, which follows ductal bifurcation, is a slightly different process whereby a new ductal branch is formed at 90° from the mature duct (Wiseman & Werb, 2002). Side branching occurs during puberty and continues with each subsequent estrous cycle thereafter. This involves the mammary bud penetrating through the myoepithelial layer, the basement membrane and also the stroma and this occurs under both hormonal control and in response to epithelial to mesenchymal signalling (Wiseman & Werb, 2002). Similar to the TEBs, once the ductal side branches reach the end of the fat pad, the distal ends undergo differentiation to form alveolar buds (Hennighausen & Robinson, 1998). The process of side branching also ensures that the ducts are far enough apart to allow for the formation of lobuloalveoli during late pregnancy and lactation (Silberstein, 2001).

Estrogen is essential for mammary gland development during puberty and also into adulthood as well. ER\(\alpha\) knock out mice are born infertile and the rudimentary ductal tree is found to be lacking terminal end buds. Furthermore, these mice will fail to undergo ductal outgrowth and will have reduced levels of progesterone receptor (PR) and prolactin receptor (PRLR) (Cunha et al., 1997; Korach et al., 1996).
Progesterone is the second ovarian hormone that begins to play a role in mammary gland development primarily after puberty and into pregnancy. Through the use of PR null mice and mammary transplantation, it is evident that progesterone is essential for both ductal side branching and formation of alveolar buds (Brisken et al., 1998; Lydon et al., 1995). There are two isoforms of PR, PrA and PrB, both of which are important in the regulation of mammary gland development (Graham & Clarke, 1997). Overexpression of PrA leads to a significant increase in ductal side branching (Shyamala et al., 1998) whilst mice that are transgenic for PrB present with reduced side branching and altered alveolar development (Shyamala et al., 2000). Other studies have shown that PrA null mice show no defect in ductal side branching, which indicates that PrB is the crucial isoform for mammoipoiesis (Mulac-Jericevic et al., 2000). Tissue recombination and localisation studies have shown that epithelial rather than stromal PR is required for alveolar development (Brisken et al., 1998; Humphreys et al., 1997). There is evidence that stromal PR does play a role too, potentially through regulation of Wnt4 which acts downstream of PR and has been shown that Wnt4 is also essential for ductal side branching (Brisken et al., 2000).

There are also several negative regulators of branching morphogenesis in the mammary gland that act to ensure this process is controlled and tightly regulated. The most common examples are Tgfβ1 and Sprouty2, both of which result in accelerated ductal branching when inactivated or deleted (Daniel, 1996; Ewan et al., 2002; Lu P, 2005).

It is evident that the control and regulation of the mammary gland between birth and adulthood are both multifaceted and complex and as a result there is still much to be elucidated regarding the hormones, genes and growth factors that play key roles in mammary cell fate. It is essential to fully understand the process of ductal extension and branching morphogenesis because almost 90% of human breast cancers arise from the mammary ducts. Therefore, the regulatory factors that control these processors may be potential therapeutic targets.

**Development of the mammary gland during pregnancy**

The previous sections have summarised the complex signalling networks that exist in the mammary gland from embryonic development through to adulthood, however the mammary gland itself is regarded as relatively quiescent in the virgin state. The
mammary gland does not become a fully functioning organ until the onset of pregnancy and its complete role does not become evident until after parturition when lactation commences.

The early stages of pregnancy are similar to adolescence and involve mainly an increase in ductal side branching and alveolar bud formation. The alveolar buds then differentiate into alveoli, the precursor to the milk producing lobuloalveoli, the development of which are characteristic of mid-pregnancy. It is clear that the mammary gland has reached its functional endpoint when the lobuloalveolar cells begin to produce and eventually secrete milk proteins (Nandi, 1958; Topper & Freeman, 1980).

While estrogen and progesterone are crucial for the regulation of mammopoiesis during puberty, mammary gland development during pregnancy is largely controlled by progesterone and the peptide hormone, prolactin.

The importance of prolactin (Prl) became apparent in 1928 when Sticker & Grueuter injected pituitary peptides into artificially castrated animals and was able to induce lactation (Stricker & Grueter, 1928). It was later discovered that this peptide was in fact prolactin (Riddle, 1933) and these experiments demonstrated that mammary gland development was not purely under the control of the ovarian steroid hormones. The role of prolactin in mammary gland development was demonstrated following the generation of the Prl−/− mouse (Horseman et al., 1997). This model demonstrated that in the absence of prolactin, female mice were infertile and that mammary gland development was perturbed. Terminal end bud formation and ductal extension of the epithelial network appeared normal but the mature virgin gland did not develop alveolar buds, the precursors to lobuloalveoli (Horseman et al., 1997).

The subsequent development of mice lacking the prolactin receptor further demonstrated the importance of prolactin in the regulation of almost all aspects of female reproduction including: ovulation rates, mammary gland development, fertility, embryonic implantation, lactation and behavioural aspects such as mating frequency and maternal behaviour (Ormandy et al., 1997). Prlr homozygous mutant mice are completely infertile due to failure of the embryo to implant in the uterus so the effects of prolactin on during pregnancy and lactation was observed using mice that were heterozygous for Prlr. In these mice, a failure of lactation in the first pregnancy was observed but following subsequent estrous cycles and pregnancies, the mice regained the ability to lactate (Ormandy et al., 1997). Given the widespread affects of PRLR reduction on female reproduction it was difficult to determine exact causation of this
lactational defect so a further study by Brisken and colleagues used these PRLR homozygous mutants to perform mammary gland transplants (Brisken et al., 1999). This study showed that mammary gland development is normal up until puberty when the mammary glands of Prlr knockout mice show a reduction in the frequency and complexity of ductal side branching and the persistence of atypical TEBs through to adulthood (Brisken et al., 1999). This defect in the side branching is also observed in prolactin knockout mice, indicating that Prl and Prlr are phenocopies of each other (Horseman et al., 1997). Brisken and colleagues determined that this phenotype does not occur when PRLR−/− epithelium is transplanted into wildtype mice, indicating that PRLR regulates this aspect of mammary gland development indirectly through endocrine factors (Brisken et al., 1999) such as progesterone, which has been previously shown to be essential for side-branching (Brisken et al., 1998). During pregnancy, transplanted glands showed that deletion of PRLR leads to a complete failure of lobuloalveolar development, confirmed by a lack of β-casein production. This revealed that prolactin acts directly on the mammary epithelial cells during pregnancy to ensure lobuloalveolar development and successful lactation (Brisken et al., 1999).

**Lobuloalveolar development**

Similarly to virgin development, signalling networks between stromal ECM proteins and the mammary epithelium act to direct both mammary gland development and epithelial cell function during pregnancy.

Prolactin regulates the differentiation of alveolar cells into lobuloalveoli through binding its receptor (Prlr), which in turn activates the Janus-2 kinase JAK2/signal transducer and transactivator (STAT) signalling pathway (Hennighausen et al., 1997). Activation of Jak2 under the regulation of prolactin leads to the homodimerization and phosphorylation of both isoforms of Stat5 (Stat5a and Stat5b). There is a 96% homology between Stat5a and Stat5b but Stat5a appears to be the dominant isoform in the mammary gland as shown by the knock out mice being unable to lactate due to impaired lobuloalveolar development. The Sta5b knockout however, presents with a markedly reduced phenotype that can be rescued by forced Stat5a expression (Liu et al., 1997; Udy et al., 1997). JAK/STAT signalling can also be induced by members of the EGF family and also growth hormone (Chapman et al., 1999). Phosphorylation of Stat5
enables it to translocate to the nucleus and activate the subsequent genes that mediate proliferation and differentiation (Darnell, 1997; Ihle, 1996).

Other regulators of both Stat5 and differentiation pathways include integrins, which are an important class of ECM receptors that regulate the cross talk between the ECM and hormone signalling pathways (Giancotti & Tarone, 2003; Katz & Streuli, 2007) allowing influence over cell fate decisions such as proliferation, migration, survival and differentiation (Streuli, 2009). Integrin signalling is essential for mediating cell matrix adhesion and epithelial function in several systems, including the mammary gland (Akhtar et al., 2009). In the mammary gland, β1-integrin is necessary for activating endocrine signalling pathways and also plays an important role in the pregnant mammary gland, as shown by impaired lobuloalveolar development in the β1-integrin chimeric mouse model (Naylor et al., 2005). In this study, deletion of β1 integrin specifically in the luminal mammary epithelium, led to impaired alveologenesis and lactation in vivo as well as a detachment of the luminal epithelial cells from the ECM, indicating a crucial role for β1-integrin in maintaining cell adhesion (Naylor et al., 2005). These results were recapitulated in primary MEC cultures where ill-formed acini structures developed when grown in 3-D culture. This study highlighted the interaction between β1-integrin and hormone signalling, as demonstrated by a lack of milk secretion in β1-integrin−/− luminal MECs upon stimulation with prolactin due to a lack of Stat5 integration into the nucleus (Naylor et al., 2005). Similarly it has been shown that deletion of integrin-linked kinase (ILK) in primary culture and in vivo leads to perturbation of differentiation in the pregnant mammary gland due to inhibiting the nuclear translocation of Stat5 (Akhtar et al., 2009). The integration of integrin signalling and prolactin is clearly essential for differentiation and milk synthesis in the mammary gland and it has been shown that Rac1 is key player in linking these two systems (Akhtar & Streuli, 2006). Dominant negative Rac1 inhibits Prl-induced production of β-casein in mammary epithelial cells grown in 3-D culture whilst activation of Rac1 in β-integrin null mammary epithelial cells is conversely able to rescue β-catenin levels. Therefore it is clear that Rac1 is an essential downstream target of integrins in regards to activation of PrlR/Stat5 signaling cascade (Akhtar & Streuli, 2006). This provides further evidence of the cooperation of hormonal and integrin signalling pathways in the regulation of mammary cell fate (Naylor et al., 2005; Naylor & Streuli, 2006).
It is evident that the hormonal control of mammary gland development is fairly well understood but the ensuing transcriptional regulation of genes involved in cell fate decisions is still under investigation. There are several key players in lobuloalveolar development, whose effects have been described through the use of knockout mouse models where a failure in development of these milk-producing cells is the primary phenotype. Cyclin D1 is one such regulator that is crucial for mediating cell cycle progression and is found to be essential for lobuloalveolar development, as shown by the impairments found in the knock out animal (Fantl et al., 1995; Sicinski et al., 1995). It is also true that an upregulation of Cyclin D1 found in the transgenic Msx1 mice also leads to disrupted lobuloalveolar formation due to inhibition of differentiation (Hu et al., 2001). This model also indicates that there is a potential role for the Homeobox protein Msx1 in lobuloalveolar formation.

It has since been shown that the targeted deletion of the Hox genes, Hoxa9, Hoxb9 and Hoxd9 also results in a defect in lobuloalveolar development, which ultimately leads to a failure in lactation (Chen & Capecchi, 1999). In addition, Hoxd10 has been found to play a role in the expansion of the alveolar buds in late pregnancy and deletion of this gene also leads to impairment in lactation due to insufficient lobules (Lewis, 2000).

Another important regulator of lobuloalveolar development is the CCAAT/enhancer binding protein β (C/EBPβ), which is responsible for transcriptionally regulating several genes, including those that regulated PR-mediated proliferation in the mammary gland. In addition, C/EBPβ−/− mice present with impaired ductal outgrowth, side branching and therefore subsequently limited lobuloalveolar development (Grimm et al., 2002; Robinson et al., 1998; Seagroves et al., 2000). It is therefore feasible that C/EBPβ is able to regulate mammary cell fate, on several levels, through regulation of downstream effectors such as the progesterone receptor.

Regulators of mammary cell fate have become the focus of mammary gland biology in recent years due to their crucial role in development and the fact they are often perturbed in cancer. The ETS transcription factor ELF5 is one such example. ELF5 expression in the mammary gland increases from early pregnancy before peaking midway through gestation and remaining high through to early lactation (Zhou et al., 2005; Zhou et al., 1998). Due to embryonic lethality in ELF5−/− models, ELF5+/− mice have been used to show that this transcription factor is essential for the differentiation and
expansion of alveolar buds into lobuloalveoli (Zhou et al., 2005). ELF5 +/- mice show normal ductal elongation and side branching through virgin development and enter the proliferative phase of alveolar morphogenesis but fail to produce milk proteins and lactate, leading to starvation of pups (Zhou et al., 2005). Recapitulation of this phenotype in mammary transplants using epithelium from these mice prove that ELF5 regulation of lobuloalveolar development is epithelial cell autonomous (Naylor et al., 2003; Zhou et al., 2005). These phenotypes are also observed in the PRLR +/- mice described earlier (Ormandy et al., 1997). The PRLR deficient mice showed a reduction in ELF5 expression but this was not reciprocated in the ELF5 +/-, indicating that ELF5 is acting downstream of the prolactin signalling pathway (Zhou et al., 2005).

Furthermore, it has been shown that overexpression of ELF5 in inducible transgenic mice results in a severe disruption of ductal morphogenesis as a result of forced alveolar differentiation and production of milk proteins in virgin mice (Oakes, 2008b). This study by Oakes and colleagues also found that ELF5 knockout mammary glands have an accumulation of CD61+ luminal progenitor cells whilst the glands overexpressing ELF5 showed a marked depletion in this cell population, indicating that ELF5 controls mammary alveolar cell fate through regulation of secretory alveolar precursor cell populations (Oakes, 2008b).

Notch signalling is another crucial pathway involved in the mammary epithelial hierarchy and has been shown to regulated cell fate decisions in the. The Notch pathway has been implemented as an essential regulator of specification in many cell types (Schweisguth, 2004; Tanigaki et al., 2003) and has now been shown to be required for both mammary gland development and breast cancer (Gallahan et al., 1996; Jhappan et al., 1992; Kiaris et al., 2004; Kordon et al., 1995; Raafat et al., 2004).

Notch signalling is mediated through receptor/ligand interactions that lead to cleavage of Notch and release of the active Notch intracellular domain (NICD). NICD interacts with downstream effectors to activate transcription (reviewed in (Bouras et al., 2008)). One such effector is Recombining binding protein suppressor of hairless (RBPJ) that when down regulated in mammary stem cell (MaSC)-enriched environment, leads to increased stem cell activity and formation of aberrant end buds. Therefore it is evident that inappropriate Notch signalling leads to self-renewal and transformation of luminal progenitors (Bouras et al., 2008). Further evidence of Notch signalling luminal progenitors has been shown through the expression of downstream NICD effectors in luminal cell populations. Hey1 and Hey2 are expressed in the CD61+ luminal progenitor
populations whilst Hes6 is expressed in the CD61⁺ mature luminal cells (Bouras et al., 2008).

It is clearly evident that Notch, as well as other signalling pathways are crucial for MaSCs regulation, luminal cell commitment and overall regulation of cell fate decisions. It is therefore crucial to identify novel regulators of mammary gland development because the factors capable of influencing cell fate hold the key to understanding the dysregulation of the mammary hierarchy that leads to breast cancer.

**Lactation**

Lactation is the functional end point of mammary gland development, involving the production of milk proteins and their subsequent secretion. Milk synthesis is stimulated and maintain by prolactin in the initial stages and then a secondary release of prolactin from the pituitary gland in response to suckling signals for continued milk production (Freeman et al., 2000). The secretion of milk is mediated by oxytocin which is released in response to suckling and causes the myoepithelial cells that surround the ducts to contract and expel the milk (Hennighausen & Robinson, 1998; Jones, 1967; Van Dongen, 1967).

**Apoptosis and involution**

Apoptosis is an important aspect of mammary gland development as it occurs after each estrous cycle where there is a reduction in the complexity of side branching and the alveolar buds from the previous cycle. More importantly, apoptosis is crucial for the stage of mammary gland development known as involution, which occurs after weaning when lactation has stopped (Wagner et al., 1997; Lund et al., 1996).

Absence of suckling leads to inhibition of milk secretion and results in a loss of lobuloalveolar proliferation, followed by controlled apoptosis (Wagner et al., 1997). This is the beginning of the final phase of mammary gland development, known as involution. The second stage of involution requires the gland to undergo a process of remodeling until it resembles a nulliparous gland but with a higher degree of side branching (Lund et al., 1996).

At the time of involution a number of genes that act as markers of cell death begin to increase in expression, primarily Bax and Tgfβ3 (Li et al., 1997; Nguyen & Pollard, 2000). Overexpression of Tgfβ3 in the mammary gland is able to induce early
involution (Nguyen & Pollard, 2000) whilst knocking out Tgfβ will delay the onset of involution by preventing apoptosis (Nguyen & Pollard, 2000).

Other molecules that are required for involution include those that are found to have apoptotic roles in other systems. Deletion of the tumour suppressor gene p53 leads to a delay in involution (Jerry et al., 1998), whilst deletion of the known death inducing molecules Fas and Fas ligand leads to a failure of apoptosis of mammary epithelial cells in response to weaning (Song et al., 2000).

Members of the Stat family also play an essential role in the activation of involution, namely a loss of Stat5 and the activation of Stat3 (Chapman et al., 1999; Humphreys & Hennighausen, 1999). As reviewed earlier, Stat5 is crucial during lobuloalveolar development and milk production but acts to inhibit involution (Humphreys & Hennighausen, 1999). Stat3 however is an essential activator of involution as shown by the delay in regression of the mammary epithelial cells upon deletion of Stat3 (Chapman et al., 1999).

As seen throughout embryonic and virgin mammary gland development, interactions between the mammary epithelium and stroma are necessary throughout involution. Extracellular matrix (ECM) proteins such as the matrix metalloproteinases (MMPs) are recruited to assist in involution and work to remodel the mammary microenvironment by cleaving extracellular matrix and other scaffolding proteins (Wiseman & Werb, 2002). Lysyl oxidase-like 2 (LOXL2), a member of the LOX family of ECM modifying enzymes, is also upregulated in the mammary gland during lactation and involution and has been shown to co-localise with MMP9 and tissue inhibitor metalloproteinase 1 (TIMP1) (Barker et al., 2011). LOXL2 acts to remodel the mammary gland through regulation of TIMP1 and MMP9 activity and also through its action as actin cross-linking ECM protein (Barker et al., 2011).

**Stems cell and the mammary gland**

The mammary gland is an organ that is capable of undergoing dramatic cyclical changes with each estrous cycle and pregnancy in response to an array of regulator genes, hormones and transcription factors. It is also evident that regulators of cell fate are crucial in mammapoiesis and furthermore, they provide clear links between development and disease. The ability of the gland to essentially be able to self-renew during and after each estrous cycle and pregnancy has been attributed to the mammary
stem cells (MaSCs) that exist in, not only the developing gland, but the adult gland as well (Daniel et al., 1968; Hoshino & Gardner, 1967; Smith & Medina, 1988). MaSCs are essential for organogenesis and homeostasis and fall into two lineages, luminal and myoepithelial (basal). The luminal lineage gives rise to the ductal cells that line the epithelial ducts and establish the secretory alveolar components capable of milk production, and the luminal cells that line the epithelial ducts (Visvader, 2009) (Fig. 1.2).

The initial in vivo experiments showing the these stem cell phenotypes of the breast were established by De Ome and colleagues in 1959 where donor epithelium is transplanted into the cleared fat pad of a recipient mouse and the ensuing result is a completely reconstituted mammary gland capable of differentiation under the influences of pregnancy (De Ome et al, 1959). It has since been shown that these transplants can be performed using both explants and also cell suspension of dissociated mammary glands (Daniel et al., 1968; Hoshino & Gardner, 1967; Smith, 1996). Furthermore, explants from various regions of the ductal network have all shown full reconstitution of a mammary gland through up to seven generations, which is evidence that persistent and long-lived stem cells exist throughout the entire adult mammary ductal network (Daniel & Young, 1971; Smith & Medina, 1988).
Fig. 1.2. Mammary stem cell hierarchy

The regenerative capabilities of the mammary gland can be attributed to the existence of the mammary stem cell hierarchy. Luminal and basal are the two main lineages that arise from a common multi-potent progenitor. The luminal lineage gives rise to the ductal and secretory alveolar cells whilst the basal progenitors give rise to the contractile myoepithelial cells. In red are the major transcription factors involved in the regulation of stem cell populations and in blue are the breast cancer subtypes that arise from each stem cell population (Adapted from Visvader 2009).
The regulation of MaSCs is controlled by a number of major signalling pathways and transcription factors including Hedgehog, WNT, GATA3, Notch (Liu et al., 2005) and more recently Runx2 (Owens et al., 2014), which have been described in previous sections. The principal concept behind the heterogeneity of breast cancer is known as the “cell of origin” theory and states that breast cancers arise from different mammary epithelial subtypes. Therefore, a better understanding of the properties of these different subtypes will further our knowledge about the heterogeneity of breast cancer (Visvader, 2009) (Fig. 1.2).

More recently, techniques have been developed that allow the formation of single cell suspensions of mammary epithelial cells (MECs), which can be analysed using fluorescent-activated cell sorting (FACS). The populations of stem cells are sorted using specific cell surface markers (Shackleton et al., 2006; Sleeman et al., 2005; Stingl et al., 2006). For example, MECs enriched in the CD49f<sup>hi</sup>, CD29<sup>hi</sup>, CD24<sup>+</sup>, Sca<sup>-</sup> subset and when transplanted, generate an extensive ductal network and a whole gland that is capable of forming alveolar components that produce milk (Shackleton et al., 2006; Sleeman et al., 2005; Stingl et al., 2006). FACS-sorting of MECs allows lineage tracing of stem cells involved in both mammopoiesis and breast cancer and indicate the potential to identify the “cell of origin” of a particular breast tumour, which would allow a more in-depth perspective in regards to treatment options.

There has been ongoing debate in the field as to whether bipotent or unipotent MaSCs are responsible for the coordination of the extensive ductal branching during both estrous and pregnancy and the subsequent maintenance of the mature gland (van Amerongen et al., 2012; Van Keymeulen et al., 2011). A recent study has shown through extensive lineage tracing experiments, that bipotent MaSCs exist and that these are responsible for producing long-lived progenitor cells (Rios et al., 2014). This study was also able to determine that bipotent MaSCs exist in extensive clonal domains of the mammary epithelium and actively work to control physiological processes in the gland rather than lay dormant until injury or transplantation requires them to function, as was previously thought (Rios et al., 2014).

Research into MaSCs has been critical to increasing our knowledge of how the gland is able to undergo repeated and drastic changes and it provides us with the potential to understand how aberrations in the regulation of such processes lead to disease progression and cancer. Cell fate mapping will ultimately be needed in order to elucidate the complex cell hierarchy that exists within the mammary gland.
Summary

It is abundantly clear that the process of mammary gland development involves a complex signalling network of hormones, growth factor ligands and their receptors and transcription factors. Whilst many of these pathways and functions have been elucidated, we are still yet to unravel the complete story behind mammary gland development. It is essential to fully understand the developmental regulation of the mammary gland in order to be able to comprehend how perturbation of these signalling networks leads to disease progression.

Breast Carcinogenesis

Overview

The primary structures within the breast are the lobules that produce milk and the ducts that transport the milk to the nipple. A network of connective, lymphatic and fatty tissue surrounds both of these structures. Breast cancer is able to form in both the lobules and the ducts and is found to be the point of origin for 10-15% and 50-75% of breast cancers respectively (Youk et al., 2009). If the cancer remains within these structures it is classified as in situ carcinoma however if it spreads into the surrounding breast tissue, it is known as invasive carcinoma.

Breast cancer is a heterogeneous disease that has a good prognosis in the initial phase but this changes dramatically if the cancer has spread to distal organs. The molecular subtyping of breast cancer has played a significant role in both the successful treatment of patients and the development of new therapeutic targets. The five major subtypes of breast cancer are Luminal A, Luminal B, Basal/Triple Negative (TNBC), “Normal-like” and HER2 type (Perou et al., 2000) and are characterised in regards to their hormone receptor status, specifically the expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).

The Luminal A and B subtypes are defined as being positive for both ER and PR, while Luminal B is also positive for HER2. These are the most prevalent types of breast cancer diagnosed, accounting for a combined total of 60% of all cases. These tumours form in the luminal cells lining the ducts in the mammary gland and have the highest survival rates and the best prognoses when compared to other subtypes due to the
effectiveness of hormonal therapy. Basal breast cancers, including triple negative cancers, are associated with a poor patient prognosis and more aggressive tumours that arise from the basal cells of the mammary ducts. Basal breast cancers often carry mutations in both the P53 and the BRCA genes whilst the TNBCs are also negative for all three main hormone receptors, which, combined with a lack of targeted therapeutic options, leads to significantly lower survival rates and increased rates of recurrence when compared to ER/PR+ luminal breast cancers (Dent et al., 2007). HER2 type breast cancers lack both ER and PR expression and also have a mutation in the P53 gene, but can be treated with trastuzumab because the majority of these tumours are HER2+. These tumours are still associated with poor prognosis and do have high recurrence rates, compared to luminal breast cancers. “Normal-like” breast cancers have, as the name suggests, similar hormone receptor status to normal breast cells but have a similar prognosis to basal breast cancers.

There is a high degree of tumour heterogeneity that exists in breast cancers, meaning that that there will be tumour cells that do not fit the classification of the diagnosed subtype and as such may evade therapeutics and result in relapses or metastasis. In order to improve the clinical outcomes of breast cancer we need to fully understand the reasons behind tumour heterogeneity and the cells type from which breast cancers originate. There is also an crucial need to identify novel regulators of mammary cell fate that act to direct breast cancer phenotypes such as increased proliferative, migratory and invasive capacities.

**Prevalence of breast cancer**

More than 1,300,000 people across the world suffer from breast cancer each year making it one of the most common forms of cancer and despite advances in detection methods and therapeutics approximately 450,000 will die due to this disease each year (CGAN 2012).

The most recent statistics for breast cancer in Australia show that whilst the risk of developing the disease before 85 is 1 in 8, the survival rate has significantly increased over the last 20 years. In New South Wales alone, there were 4,606 cases of breast cancer diagnosed in 2009, accounting for 27.4% of all new cancers diagnosed in women and 28.8% cancers diagnosed in women overall. In the same year 13,668 patients were
diagnosed across the country, making it the most the common cancer detected in Australian women and the third most common cancer overall.

In terms of breast cancer mortality, in Australia it was found to be the second leading cause of cancer-related death in women with 2,840 patients succumbing to the disease in 2010, which equated to 15.3% of all cancer related death in women.

Whilst the incidence of breast cancer in Australia is high, the prognosis for patients presenting with localised disease is extremely good and they have a 97% five-year survival rate. Unfortunately, for patients presenting with distal metastasis, this five-year survival rate is greatly reduced to only 40%, and more dramatically so in regards to long-term survival in which the survival rate is only 5-10%. These poor statistics highlight importance of elucidating the mechanism behind breast cancer progression and investigate new key regulators of breast cancer cell fate.

Breast Cancer Metastasis

Overview

As stated above, the survival rates for breast cancer, both short term and long term, are severely reduced in patients who present with distal metastasis. Over 90% of all breast cancer-related deaths can be attributed to metastasis, most commonly detected in the liver, lung and bone.

Even though improvement in treatment options and early detection methods have significantly increased the survival rates for primary breast cancer over the last two decades, the poor statistics associated with the metastatic phase of the disease have remained largely unchanged for 25 years. For this reason, it is integral that research into the mechanism behind which breast cancer cells are able to travel to and colonise these secondary sites continues.

Metastasis is a multistage process whereby cancer cells acquire the ability to escape the primary tumour environment and colonise a secondary organ (Steeg, 2006). There are six main phenotypes that cancer cells exhibit that enable them to become metastatic and these “hallmarks” of cancer have been outlined by Hanahan and Weinberg (Hanahan & Weinberg, 2000, 2011) and are summarized in Figure 1.3.
Fig. 1.3. **Hallmark of cancer**

The six major functional capabilities cancer cells acquire in order to grow, spread and colonise secondary organs are found in the center, surrounded by the more recent additions in red (adapted from Hanahan & Weinberg 2011).
In order to metastasise, the primary cancer cell must be able to invade the local environment before undergoing intravasation and extravasation, a process by which they enter the blood stream in order to circulate around the body before exiting the blood vessel and finally establishing a new colony in a distal organ (Steeg, 2006). In breast cancer where tumours are comprised of epithelial cells, these cells need to undergo a transformation in order to become more aggressive by losing such traits as cell adhesion and apical-planar cell polarity and gaining traits such as apoptotic resistance and motility, this process is collectively known as the epithelial-mesenchymal transition (EMT) (Kalluri & Weinberg, 2009).

There are numerous genes involved in the progression of breast cancer from a primary tumour to metastatic disease. One of the initial steps is to evade detection by the immune system and to do this breast cancer cells secrete compounds such as Fas death receptor ligand, which tricks the immune cells into destroying each other instead of the cancer cells (Mann et al., 1999). Once “invisible” the cancer cells have to be able to detach in order to metastasise and do this by altering expression of genes that regulate cell adhesion such as selectins (Krause & Turner, 1999), integrins (Kumar, 1998), lectins (Raz & Lotan, 1987) and cadherins (Perl et al., 1998) (Akimoto et al., 1999). In order to cross basement membranes and separate tissue, breast cancer cells upregulate proteolytic enzymes such as metalloproteinases (MMPs), serine proteinases and cathepsins (Andreasen et al., 1997; Brew et al., 2000; Chambers & Matrisian, 1997; Koblinski et al., 2000; Nelson et al., 2000) as well as genes that increase cell motility such as autotaxin and hepatocyte growth factor (Lamszus et al., 1997; Stracke et al., 1997). The organotropism of breast cancer metastasis is potentially aided by the upregulation of specific chemoattractants and receptors (Müller et al., 2001). As previously mentioned, breast cancer preferentially metastasises to the bone and this process has been reviewed below.

*Breast cancer bone metastasis and the “vicious cycle”*

Bone metastasis is one of the most painful and unfortunately most common outcomes of advanced breast cancer. Almost 70% of patients who are diagnosed with metastatic breast cancer present with bone lesions. This preference of breast cancer cells to metastasise to the bone contributes to the high morbidity and mortality associated with breast cancer (Coleman & Rubens, 1987; McNeil, 1984).
Bone metastases that arise from breast cancer are osteolytic and this resorption of bone leads to severe complications such as hypercalcemia, long bone fractures, spinal cord compression and osteoporosis (Coleman & Rubens, 1987). Current treatments for bone metastasis are considered to be palliative rather than curative, aiming at reducing pain and improving quality of life. These treatments include radiation therapy and occasionally surgery to reduce tumour size and slow tumour growth and also the administration of bisphosphonates, which treat the ensuing osteoporosis and slow tumour growth by preventing bone resorption (Lipton et al., 2000; Paterson et al., 1993).

Paget’s “seed and soil” theory postulates that the bone microenvironment acts a fertile “soil” factor that attracts the breast cancer cells and then provides a suitable environment where the cancer cells are able to thrive and proliferate (Paget, 1889). It is therefore pertinent to investigate the mechanisms of genes and pathways that regulate both bone development and homeostasis and also mammary gland development. The most prominent example is receptor activator of NF-κB (RANK) and its ligand RANKL. RANKL is an essential regulator of osteoclastogenesis in the bone (Lacey et al., 1998) whilst it is also crucial in the mammary gland for lobuloalveolar development as it controls epithelial cell proliferation and apoptosis along with RANK (Fata et al., 2000). Due to having essential functions in both bone and mammary development, it is evident that RANK/RANKL may be acting as a “soil” factor in what is known as the “vicious cycle” of breast cancer bone metastasis.

The “vicious cycle” bone destruction hypothesis states that tumour cells activate and perturb bone cell function by secreting growth factors resulting in bone destruction which in turn leads to the secretion and release of subsequent growth factors that support the growth of the tumour cells (Roodman & Dougall, 2008; Steeg, 2006). When breast cancer cells colonise the bone it results in secretion of tumour growth factors such as OPN, IL-6, IL-8, IL-11, PTHrP, TGF-β and MMPs, which in turn activate osteoblasts that will secrete RANKL and OPG. This causes a shift in the balance of osteoblastogenesis and osteoclastogenesis and eventually leads to excess osteoclasts and therefore bone destruction (Roodman & Dougall, 2008; Steeg, 2006). To complete the cycle, the damaged bone release osteoblast-activating growth factors such as BMPs, TGF-β, IGF and FGFs which aid tumour growth and exacerbate the excessive osteolysis (Roodman & Dougall, 2008; Steeg, 2006).
Another potential key player in this cycle is the osteogenic transcription factor, Runx2. Runx2 is known as a master regulator of bone development and homeostasis due to its regulation of cell fate and lineage determination in the bone (Ali et al., 2008) (Young et al., 2007a; Young et al., 2007b). Runx2 both directly regulates and is regulated by many of the genes and growth factors involved in bone metastasis including OPN, TGF-β, collagenase-3, BSP, MMP13 and RANKL (Steeg, 2006) (Roodman & Dougall, 2008). Runx2 is also considered to be a master regulator of osteoclastogenesis because it regulates both RANKL expression and osteoblast differentiation (Enomoto et al., 2003). It is clear that Runx2 controls cell fate in the bone through a number of regulatory functions and therefore dysregulation in Runx2 expression would lead disruption of the balance of bone resorption and formation, thus leading to bone lesions.

Furthermore, a recent study by our lab has indicated that Runx2 may be a novel regulator of both mammary gland development and breast cancer cell fate (Owens et al., 2014). This new evidence may provide the tangible link between these two seemingly disparate systems and may lead development of novel therapies against breast cancer bone metastasis.

**Summary**

Breast cancer, in its primary form, is a disease with a good prognosis and an ever-increasing array of treatment options. However, the complex mechanisms by which breast cancers are able to metastasise, and location to which they preferentially colonise, often lead to poor prognosis, reduced quality of life and eventual death for the patient. It is important that we continue to investigate the missing pieces of the puzzle in order to find ways to treat and circumvent the unrelenting process that is breast cancer metastasis by studying both the cancer itself and also the regulatory factors involved in the formation and function of the health mammary gland. This will allow the tools to design improved diagnostic, prognostic and treatment options for people suffering from breast cancer.

Our lab has recently identified Runx2 as a potential link between breast cancer and bone metastasis, however the fetal lethal phenotypes of Runx2−/− mice and the potential redundancy that exists in the Runx family have presented difficulties regarding full elucidation of the role of Runx2 in the breast. This thesis suggests that investigation into
the binding partner, CBFβ, may not only negate these issues but provide the field with an exciting and novel regulator of mammary gland development and breast carcinogenesis.

**CBF Complex**

The Core Binding Factor (CBF) complex is essential for the successful regulation of developmental processes (Kundu et al., 2002; Yoshida, 2002) and also stem cell homeostasis (Kurosaka et al., 2011; Okuda et al., 1996; Wang et al., 1996). In addition, this complex plays key roles in the aetiology of several diseases, namely leukaemias (Blyth et al., 2005; Speck & Gilliland, 2002).

The CBF complex is a heterodimeric transcription factor that is made up of a DNA binding subunit, CBFα, and the non-DNA binding subunit, CBFβ (E Ogawa, 1993b; Ogawa et al., 1993a; Wang et al., 1993). The CBFα subunit is encoded by the genes in both mice and humans Runx1, Runx2 and Runx3 (Bae et al., 1995; E Ogawa, 1993b; Levanon et al., 1994; Miyoshi et al., 1991). These Runx genes are differentially expressed across tissue types (Levanon & Groner, 2004) whilst the non-DNA binding subunit on the other hand is only encoded by one gene in humans, CBFβ, and it is ubiquitously expressed across most tissue types (Adya et al., 2000; Hajra & Collins, 1995; Ogawa et al., 1993a; Wang et al., 1993).

The CBF complex can both activate or repress transcription of key regulators of growth, survival and differentiation pathways (Blyth et al., 2005) and its most widely known for its role in both osteogenesis and hematopoiesis (Huang et al., 1999; Nagata et al., 1999) (Fig. 4). Aberrations in the CBF complex during osteogenesis leads to disorders such as cleidocranial dysplasia and similarly, translocation events during haematopoiesis implicate CBF in a large percentage of acute human leukaemias (Huang et al., 1999; Nagata et al., 1999).
All members of the Runx family form heterodimeric complexes with the same co-transcription factor, CBFβ. This complex can both activate and repress transcription of key regulators of growth, survival and differentiation pathways depending on context (adapted from Blyth 2005).

Fig. 1.4. **The CBF complex**
There is new evidence that the CBF complex, primarily via Runx2, plays an important role in the developing mammary gland and potentially breast cancer (McDonald et al., 2014; Owens et al., 2014). Our lab has recently shown a novel role for Runx2 in lobuloalveolar development through regulation of progenitor cell populations. We have also shown a potential role for Runx2 in the regulation of breast cancer cell fate (Owens et al., 2014). This novel Runx2 data will be reviewed throughout this thesis and furthermore, how it lead to the discovery of CBFβ as a key player in both mammary development and breast carcinogenesis.

Runx

Runx gene family

The Runx family is made up of three closely related yet phenotypically diverse transcription factors which are primarily known for their critical roles in tissue development and haematopoietic lineages (Coffman, 2003).

Runx genes 1, 2 and 3 are essential for the regulation of cell fate processes in both a lineage and stage specific context. All members of this family are integral to decisive processes whereby a cell will either continue to proliferate or exit the cell cycle or alternatively, whether it continues along the self-renewal pathway or undergoes functional differentiation. The Runx genes have also been implicated in several diseases involving skeletal and neurological defects as well as several types of cancer, such as leukaemia, bone and gastric cancer (Reviewed in (Blyth et al., 2005)).

Runx 1, 2 and 3 have highly conserved and essential roles in important developmental processes such as haematopoiesis, osteogenesis and neurogenesis respectively (Reviewed in (Blyth et al., 2005)). In terms of disease progression, Runx1 has a well-defined role in acute myeloid leukaemia (Okuda et al., 1996) whilst Runx2 has severe implications in bone development and related conditions such as cleidocranial dysplasia (Otto et al., 1997). Runx3 on the other hand has been implicated in gastric and other epithelial cancers (Li et al., 2002).

It is a well-understood paradigm that aberrant expression or dysregulation of genes involved in critical developmental processes often leads to disease progression and cancers. Due to this reasoning, there has been a significant increase of interest of the Runx genes in recent years, not only because of their role in development but also due to their somewhat paradoxical roles in cancer, whereby they are capable on acting as...
both tumour repressors or oncogenes depending on context (Reviewed in (Blyth et al., 2005)).

Genes that are implicated in cancer generally fall either into the category of oncogenes or tumour suppressors, the Runx genes however refuse to conform to this model (Reviewed in (Blyth et al., 2005)). Runx1 and Runx3 in particular highlight the tumour suppressor function of this family, promoting gene deletion, dominant negative function, hypermethylation events and inactivating mutations in some cancers including both haematological and gastric cancers (Li et al., 2002; Osato et al., 1999; Song et al., 1999; Tanaka et al., 1995). There is also evidence of the dominant oncogenic function of all three Runx genes, principally in lymphomas where Runx genes are often targeted for viral insertion (Kim et al., 2003; Li et al., 1999; Stewart et al., 1997).

Runx1 is found to be the most targeted gene in leukaemias with over 30 translocations that fall into 3 main classes of fusion proteins; RUNX1-ETO, RUNX1-EVI1 and RUNX1-TEL. These fusion proteins act as a dominant negative across several subtypes of leukaemias including AML, B-ALL and ALL (Gamou et al., 1998; Golub et al., 1995; Roulston et al., 1998; Shurtleff et al., 1995; Slovak et al., 2002) (Romana et al., 1995) (Fears et al., 1996). RUNX1 also forms another fusion protein with CBFβ and MYH11, which also presents with dominant negative functions and significantly increases the binding affinity of Runx2 to its cofactors (Adya et al., 1998; Lutterbach et al., 1999).

Runx3 on the other hand is found to be highly methylated in epithelial cancers such as gastric cancer which provides strong evidence for Runx3, like Runx1, to be acting in a tumour suppressor-like capacity (Goel et al., 2004; Kim et al., 2004; Ku et al., 2004) (Li et al., 2004). This theory is supported by the fact that mice that are null for Runx3 developed epithelial hyperplasia, particularly in the gastric tract (Li et al., 2002).

Runx2 differs from its family members as it acts predominantly as an oncogene when expression is perturbed. This is evidenced by its amplification in several cancers, primarily osteosarcoma but also leukaemia and more recently, both primary and secondary prostate and breast cancers (Forus et al., 1995; Selvamurugan et al., 2004) (Barnes et al., 2003; Lau et al., 2004; McDonald et al., 2014; Owens et al., 2014; Yang et al., 2001; Yeung et al., 2002).
Runx2 is the master regulator of bone development and homeostasis

Runx2 is one of the most critical transcription factors involved in skeletal morphogenesis and osteoblast differentiation (Komori, 2011). Its primary known function is as a master regulator of osteoblast differentiation and bone development and homeostasis (Komori et al., 1997; Otto et al., 1997).

Deletion of Runx2 in the mouse leads to a severe inhibition of bone development through an ablation of the osteoblast population and impaired bone mineralisation (Komori et al., 1997; Otto et al., 1997) whilst a haploinsufficiency in humans results in the autosomal dominant skeletal condition known as cleidocranial dysplasia (Mundlos et al., 1997). In regards to the role of Runx2 in cancer, numerous studies show that Runx2 is amplified in in several cancers, especially osteosarcomas, indicating a primarily oncogenic role (Forus et al., 1995; Lau et al., 2004). This oncogenic phenotype is reiterated by cell culture studies which have shown that the overexpression of Runx2 in both endothelial and osteoblastic cells drives the cells towards a more metastatic phenotype where cell migration, invasion, survival and angiogenesis are enhanced (Fujita et al., 2004; Sun et al., 2001; Sun et al., 2004; Zelzer et al., 2001).

It is evident from these studies that Runx2 is integral to the normal development of the skeletal system and that its dysregulation has severe implications on the aetiology of disease. This paradigm has shaped the course of this thesis in the context of the mammopoiesis and breast carcinogenesis.

Runx2 and mammary gland development

As stated previously, Runx2 is best known for its role as a master regulator of bone development and homeostasis, but in recent years the role Runx2 plays in non-osseous tissue has become an increasing point of intrigue (Reviewed in (Ferrari et al., 2013)).

Recent studies by our lab and others have shown a novel role for this osteogenic transcription factor in both mammary gland development and carcinogenesis (Blyth et al., 2010; Owens et al., 2014; Pratap et al., 2011; Shore, 2005).

The first evidence of a relationship between Runx2 and the mammary gland was established when Otto and colleagues (Otto et al., 1997) discovered it was expressed in the nascent mammary gland through β-galactosidase staining of the CBFα1−/− embryos that housed the LacZ reporter gene (Otto et al., 1997). It was later shown that Runx2 is particularly highly expressed in the terminal end buds, which are the driving force
behind extending the ductal network through the mammary fat pad (Kendrick et al., 2008; Kouros-Mehr et al., 2006) confirmed the role of Runx2 in the mammary gland when they found that it was expressed specifically in the basal/myoepithelial sub-population (Kendrick et al., 2008). This was an interesting discovery as it was previously shown that mammary stems cells exist in the basal/myoepithelial sub-population (Shackleton et al., 2006; Stingl et al., 2006).

Not only is there now evidence that Runx2 is expressed in the mammary gland, there have also been several studies reported in recent years that have focused on the role this transcription factor is playing in mammopoiesis.

It has now been shown that the expression of Runx2 in the mammary gland fluctuates over the course of development, indicating critical involvement (Blyth et al., 2010; Owens et al., 2014) and it is required for regulation of genes that are essential for mammary function (Inman & Shore, 2003).

It has been shown that Runx2 regulates genes that are essential for mammary gland function in both primary mammary epithelial cells and in HC11 cells, which are derived from the lactating mouse mammary gland (Inman et al., 2005; Inman & Shore, 2003). Inman & Shore found that Runx2 is not only expressed in HC11 cells but that it binds directly to the osteopontin (OPN) promoter (Inman & Shore, 2003). OPN is an extracellular matrix protein that acts to stimulate cell migration, invasion adhesion and protection against apoptosis and is a known target gene of Runx2 in osteoblasts (reviewed in (Shore, 2005)). OPN is also expressed in the mammary gland and is important for lactation. Transgenic mice that expressed OPN anti-sense RNA that are deficient in OPN are found to show a deficiency in lactation due to an absence of mammary alveolar structures and reduced synthesis of both β-catenin and whey acidic milk protein (WAP).

The binding interaction between Runx2 and the OPN promoter is required for activation of OPN in mammary epithelial cells (Inman & Shore, 2003). Furthermore it has been shown, through transient transfections, that dominant negative Runx proteins can inhibit the actions of both OPN and its promoter in mouse mammary epithelial cells (Inman & Shore, 2003). These findings were pivotal in this field because it was the first indication that Runx2 is able to regulate gene transcription in normal mammary cells where most of the previous data had indicated it role more inline with cancer due to
Runx2 transcripts being discovered in the MDA-MB-231 and MCF7 breast cancer cell lines (Selvamurugan & Partridge, 2000; Shore, 2005).

Our lab has recently confirmed that Runx2 is differentially expressed across development of the mouse mammary gland. Runx2 expression is relatively high in virgin glands and throughout early pregnancy but then levels are significantly reduced during late pregnancy and into early lactation before returning to virgin-like levels during involution of the gland (McDonald et al., 2014; Owens et al., 2014). Our lab went on to produce two different Runx2 knockout mouse models in order to examine the role of Runx2 throughout mammopoiesis for the first time in vivo. Two approaches were used, a whole body knockout combined with mammary transplantation and a mammary gland specific Runx2 knockout. The Runx2-/- transplantation model showed no difference in the ability of the gland to undergo ductal extension whilst the mammary-specific knockout displayed only transient delay of ductal morphogenesis during puberty or in early to mid pregnancy. This indicates that Runx2 may not be essential for development of the ductal epithelium. This study did not investigate the effects of Runx2 deletion during the early stages of puberty; therefore there is a need to determine if there is a role for Runx2 during ductal extension in order to fully comprehend the role of Runx2 in mammopoiesis. There was however a severe impairment of lobuloalveolar development noticed during late-mid pregnancy, which continued into early lactation in both knockout models (Owens et al., 2014). These alveoli had no discernable lumen but were found to be correctly polarized and capable of lactation. We then showed, through lineage analysis that a loss of Runx2 during mid to late pregnancy results in a shift in the balance of luminal progenitors to alveolar progenitors in the pregnant mammary glands of both mouse models. This increase of alveolar progenitors explains the impaired development in the lumens of Runx2 knockout mice and suggests Runx2 plays a role in mammary progenitor cell specification (Owens et al., 2014). Another study showed a similar inhibition of lobuloalveolar development in a Runx2 overexpressing transgenic mouse rather than a knockout model (McDonald et al., 2014). However, the transgenic mouse used in this study was reported to have supraphysiological overexpression of Runx2 (McDonald et al., 2014) complicating the interpretation of this data.

It is evident from the conflicting data that there is a need for further investigation into the role of Runx2 and the entire CBF complex in mammary gland development.
Runx2 in breast cancer

Breast cancer has a good prognosis and survival rate in the localised phase of the disease but the survival rate drops dramatically if the cancer is able to metastasise.

There is certainly a dual purpose for many genes that are involved in both mammary gland development and breast cancer including RANK, RANKL, vitamin D, bone sialoprotein and osteopontin (Martin & Gillespie, 2001) and current evidence has confirmed that Runx2 is not only essential for regulating gene transcription in the developing mammary gland, but it also a key regulator of breast cancer cell fate (Ferrari et al., 2013; Owens et al., 2014).

It is widely known that breast cancers will preferentially metastasise to bone (Juan Juan et al., 2005; Yoneda & Hiraga, 2005) but the mechanism behind this has remained a key question in breast cancer research. Many genes involved in mammary gland development are also involved in bone homeostasis, such as those listed below and it is believed that Runx2 could be the missing link that drives breast cancer to bone and allows the cancer cells to maintain this colonisation as it is known to regulate these “bone” genes that are also found in metastatic breast cancer (Martin & Gillespie, 2001) (Blyth et al., 2005; Shore, 2005).

It has been shown that, when compared to normal mammary epithelial cells, Runx2 is upregulated in several breast cancer cell lines (Lau et al., 2006). The involvement of Runx2 in breast cancer was further evidenced when Nagaraja et al identified, through a comparative screen, it as being on of the most regulated genes in invasive breast cancers versus non-invasive breast cancers (Nagaraja et al., 2006).

Several studies have used the highly invasive MDA-MB-231 cell lines to assess the function of Runx2 in terms of its ability to increase the invasiveness of human breast cancer cells (Ferrari et al., 2013; Owens et al., 2014; Pratap et al., 2011; Shore, 2005). More specifically, down regulating Runx2 in MDA-MB-231 cells leads to a reduction in the invasive capacity of the cells in vitro. Furthermore, when these cells are injected into mice, there is a reduction in subsequent osteolytic disease and tumour growth in vivo (Barnes, 2004; Javed, 2005; Mendoza-Villanueva et al., 2010; Pratap, 2009).

Converse studies have also shown that forcing Runx2 expression in the less aggressive breast cancer cell line, MCF7, leads to a distinct epithelial to mesenchymal transition (Chimge et al., 2011) and the cells also become more invasive (Pratap, 2006). Similarly, ectopic expression of Runx2 in the “normal” breast cell line, MCF10A
induces cancer-like phenotypes when grown in 3D (Pratap, 2009). Our lab has also shown that forced expression of Runx2 in MCF-10A cells leads to an upregulation of Cyclin D1 and N-Cadherin as well as a decrease in E-Cadherin, further evidencing that the cells undergo EMT when exposed to ectopic levels of Runx2 (Owens et al., 2014). We also went on to show that Runx2 over-expression in the normal mouse mammary epithelial HC11 cells lead to a perturbation in differentiation which resulted in a transition of these epithelial cells to a more “cancer-like” phenotype with increased E-Cadherin and phosphor-FAK levels and an increased ability to migrate compared to control cells (Owens et al., 2014).

Current evidence suggests that Runx2 is able to enhance the metastatic potential of breast cancer cells through the regulation of target genes that known key players in the metastatic cascade (Reviewed in (Pratap, 2006)). These target genes include vascular endothelial growth factor (VEGF) which requires Runx2 for activation and is essential for tumour vascularisation (Zelzer et al., 2001) and bone matrix protein such as bone sialo protein (BSP) and osteopontin (OPN) which are required for recruiting cancer cells to the bone and increasing resorptive activity respectively (Natasha et al., 2006) (Ohyama et al., 2004). As stated previously, Runx2 is known to regulate OPN in the developing mammary gland, through binding of the OPN receptor, (Inman & Shore, 2003) and also in cancers, including some melanomas and colorectal cancers (Riminucci et al., 2003; Wai et al., 2006). Barnes et al confirmed the role of Runx2 in metastatic cancer by showing that inhibition Runx2 expression in MDA-MB-231 cells leads to decreased expression in both OPN and BSP which suggests that Runx2 may be driving breast cancer cells to preferentially metastasise to bone by enhancing both bone targeting and adhesion genes (Barnes, 2004).

Runx2 also directly regulates genes that are associated with the ability of breast cancer cells to escape the primary tumour and colonise a secondary site, such as the matrix metalloproteinases (MMPs), in particular MMP9 and MMP13 (Pratap et al., 2005) (Selvamurugan et al., 2004). Studies have shown that suppressing Runx2 in MDA-MB-231 cells causes a decrease in OPN, MMP9 and MMP13 expression which in turn leads to decreased invasive capability of the cells (Mendoza-Villanueva et al., 2010; Mendoza-Villanueva et al., 2011; Pratap et al., 2005).

Runx2 also has a known oncogenic role in the T-cell lineage and has been shown by our lab and others to be upregulated in human breast cancer cell lines, correlating with invasiveness so it is an enticing candidate for a potential prognostic marker in human
breast cancer (McDonald et al., 2014). One study has attempted to examine the expression of Runx2 in human breast cancer TMAs but due to a limited sample number they were unable to draw concrete conclusions linking Runx2 expression to overall survival, tumour size or grade or lymph node status. This study does however show that Runx2 expression is associated with ER/PR/HER2 negative breast cancers and could possibly distinguish patient prognosis within this specific subtype (McDonald et al., 2014).

To date, all of the data describing the role of Runx2 in breast cancer has been collected using either cell lines or tissue sections. In order to further examine the role Runx2 plays in breast tumourigenesis, our lab generated the first in vivo model capable of discerning the function of this essential transcription factor. We crossed the Runx2+/− mouse on to the MMTV-PyMT luminal breast cancer mouse model and transplanted mammary epithelium from these mice into immunocompromised recipients. This was the first in vivo model to look into the role Runx2 plays in breast tumourigenesis and it showed that a lack of Runx2 in the mammary gland lead to an increase in both tumour latency and an increase in overall survival of the mice (Owens et al., 2014). We also report a down regulation of Cyclin D1 accompanied reduced Runx2 expression, indicating a reduction in the proliferative capacity of the Runx2−/− tumour cells. Unfortunately this model did not examine metastases due to the fact that the recipient mice are immunocompromised, and a functioning immune system is required for metastasis (Olkhanud et al., 2009). While a transplantation approach was required due to the embryonic lethality that occurs in Runx2 knockout mice, it is clear that there is a need for a model in which the metastatic potential of tumour cells can be observed in vivo. This is one of the issues that has been addressed in this thesis.

Summary

It is clear that the Runx genes are extremely important in both the normal development and disease progression of several systems including haematopoiesis, neurogenesis, osteogenesis and now the mammary gland.

Runx2 has long been a prime suspect in breast cancer, particularly in regards to metastasis, and now recent studies have outlined a novel role for Runx2 in mammopoiesis as well. It is also understood that the Runx genes act as one part of the CBF complex, however there is no data describing the role its binding partner, CBFβ,
may be playing in either mammary gland development or breast carcinogenesis. Given the critical role that Runx2 in these systems coupled with the limitations of working in a system where there is potential for redundancy and fetal lethal phenotypes, there is a clear need for further investigation and establishment of models that address these issues. The results of this thesis show that the investigation of the role of CBFβ is not only able to circumvent these issues but has also uncovered an exciting and novel role for this gene in mammary gland development and breast cancer.

**CBFβ**

The role of the Runx genes as critical regulators of cell fate in both developmental processes and cancer is well established but they are not able to execute this role in isolation.

Whilst the Runx genes determine tissue specificity, CBFβ is the transcriptional co-activator that increases the stability and the DNA binding affinity of the complex by triggering structural changes (Ito, 2008; Tahirov et al., 2001; Zhang et al., 2003). Not only does the binding of CBFβ alter the confirmation of the complex to make to increase DNA binding affinity but studies also suggest that this structural shift may also provide protection to the Runx proteins from proteolytic degradation (Huang et al., 2001; Huang et al., 1999).

**CBFβ in development**

The heterodimeric binding of Runx proteins and CBFβ is able to bind co-factors that able to either activate or repress transcription of key regulators of growth, survival and differentiation pathways (Tanaka et al., 1995). More specifically, CBFβ has found to be integral in the control of haematopoiesis and skeletal development through the regulation of stem cell proliferation and differentiation (Kurosaka et al., 2011).

It was originally thought that the function of CBFβ was purely to increase the DNA binding affinity of the Runx genes (Wang et al., 1996) but it has since been discovered that CBFβ collaborates with the Runx genes in order to successfully regulate transcription (Davis et al., 2010). It has now been shown that CBFβ null mice show the same embryonic lethality at day 11.5 as Runx1 null mice due severe haemorrhaging as a result of defective haematopoiesis in the fetal liver. Interestingly, over expression of
Runx genes in the CBFβ null mice leads to only a partial rescue of the phenotypes reported (Okuda et al., 1996; Wang et al., 1996). This indicates that there are functions of CBFβ and Runx2 that are independent of each other. Furthermore, CBFβ null mice present with a delayed bone formation phenotype, where the fontanelles and sutures of the skull remain open and the clavicles are hypoplastic. These phenotypes are similar to Runx2 null mice and have shown that it is possible to rescue the CBFβ null mice in a haematopoietic-specific context (Kundu et al., 2002; Yoshida, 2002). This data also indicates that CBFβ is able to enhance the transcriptional effectiveness of the Runx genes in order to control developmental processes (Davis et al., 2010). In the context of skeletal development it has been shown that CBFβ is necessary for the efficient DNA binding of Runx2 and therefore the subsequent Runx2-dependent transcriptional activation (Yoshida, 2002). A more recent study from the same lab has now shown that CBFβ is also able to stabilise all Runx proteins through induction of structural changes, which in turn leads to enhanced transcriptional activation of downstream target genes (Qin et al., 2014).

**CBFβ in the mammary gland**

There is currently no data regarding the role of CBFβ in the developing mammary gland, or in the mammary gland at all. We do know however that it is a crucial transcription factor in development of other systems such as haematopoiesis and osteogenesis (Kundu et al., 2002; Yoshida, 2002) in conjunction with Runx proteins through the regulation of stem cell homeostasis (Okuda et al., 1996; Wang et al., 1996) (Kurosaka et al., 2011).

It has now been shown that Runx2 plays an integral role in the development of the mammary gland (Owens et al., 2014) and when this is coupled with the fact that CBFβ is critical for enhancing the DNA binding affinity and overall stabilisation of the Runx genes (Ito, 2008; Tahirrov et al., 2001; Zhang et al., 2003), it is evident that CBFβ may also be playing a key role in mammopoiesis.
**CBFβ in cancer**

It is well known that the CBF complex is capable of binding co-factors that able to either activate or repress transcription of key regulators of growth, survival and differentiation pathways (Tanaka et al., 1995) and many of these targets are now known to be compromised in cancer, including those which involved in reprogramming cell fate (Blyth et al., 2010).

The first cases of CBF perturbation were described in acute myeloid leukaemias (AML) where it was shown that both Runx1 and CBFβ are the targets of the most common chromosomal translocations found in AML (Look, 1997). CBFβ has also been found to form fusion proteins through inversion events in mye-lomonocytic (M4Eo) leukaemias, for example CBFβ-MYH11, which affects the binding of the CBF complex in a dominant negative fashion (Adya et al., 1998; Lutterbach et al., 1999). There is further evidence of the oncogenic properties of CBFβ as it has also been implemented in both human B-cell acute lymphoblastic leukaemia and also granulocytic sarcoma where expression is upregulated along with Runx1 (Mallo et al., 2007; Niini et al., 2000).

Whilst CBFβ is primarily known for its role in leukaemias, there has been a growing interest in its involvement in solid tumours, a role that is yet to be fully elucidated (Davis et al., 2010). Recent publications have provided evidence that CBFβ may play a role in prostate and ovarian cancers (Davis et al., 2010), may be involved in breast cancer invasion in vitro (Mendoza-Villanueva et al., 2011) and is found to be down regulated in gastric cancer along side Runx1 and Runx3 (Sakakura et al., 2005).

**CBFβ in breast cancer**

As previously mentioned, many genes that are involved in developmental systems are often perturbed in disease progression. This thesis essentially aims to examine this concept in regards to the mammary gland and breast cancer. Evidence has been provided by several studies and has indicated a potential role of Runx2 in both mammopoiesis and breast carcinogenesis.

With the exception of a single in vitro study focusing on a single breast cancer cell line, there has been no data suggesting a role for CBFβ, the non-DNA binding partner of Runx2 in breast cancer. During the course of this thesis, three separate whole
genome/exon sequencing studies sought to identify genes that are significantly mutated in human breast cancers. These studies examined 77, 103 and 510 human breast tumours and identified 18, 6 and 23 significantly mutated genes respectively (Banerji et al., 2012; CGAN, 2012; Ellis et al., 2012). From three independent studies, only five genes were found to be commonly mutated, GATA3, PIK3CA, MAP2K1, TP53 and CBFβ. The first four genes in this list have known key players with well-documented roles in breast cancer, CBFβ on the other hand is the outlier of the group as it has no previous ties to breast cancer (Banerji et al., 2012; CGAN, 2012; Ellis et al., 2012). Even more remarkably, CBFβ was found to be mutated in approximately 5% of all luminal tumours, making it one of the most frequently mutated genes in human breast cancer (CGAN, 2012).

The outcome of this study highlights that whilst we have identified many of the regulatory factors that are crucial for the development of breast cancer, there are clearly still essential elements that are yet to be examined. The fact that CBFβ is involved in regulation of cell fate processes in both development and cancer makes it a tantalising new key player in breast carcinogenesis. The aim of this thesis is to investigate this potential novel role for CBFβ.

A previously stated, there has been almost a complete lack of published data linking CBFβ to breast cancer other than the whole genome/exon sequencing results. As part of their research into the role of Runx2 in breast cancer metastasis, Mendoza-Villanueva et al found that CBFβ was expressed in the aggressive MDA-MB-231 cell line (Mendoza-Villanueva et al., 2010), which is consistent with previous data showing higher levels of CBFβ mRNA in cancer cell lines compared to normal breast cells (Lau et al., 2006). CBFβ expression was also found in cell lines for osteosarcoma (UMR-106), HeLa cells and also MCF7 cells, which shows CBFβ is present in both metastatic and non-metastatic cancer cell lines. Interestingly, when the nuclear fraction of the cell lysate was examined, it was found that CBFβ was only present in the nucleus of cells that also expressed Runx2. Therefore CBFβ was present in both the MDA-MB-231s and the UMR-106 cells but not in the Runx2 negative HeLa and MCF7 cells (Mendoza-Villanueva et al., 2010).

Mendoza-Villanueva et al examines the potential involvement of both CBFβ and Runx2 for the invasive potential of MDA-MB-231 cells by using siRNA and shRNA against both genes. The results showed that deletion of either gene led to a decrease in
the invasive potential of the breast cancer cell (Mendoza-Villanueva et al., 2010). Mendoza and colleagues then investigated the effects of CBFβ knockdown on a subset of Runx2 target genes that are involved in cell invasion, it was observed that CBFβ expression was required for expression of some but not all of these targets. The downstream targets that were tested were the matrix metalloproteases MMP9 and MMP13, osteopontin (OPN), Galectin-3 and osteocalcin (OC) and it was found that a down regulation of CBFβ led to a decrease in the expression of all these genes except for Galectin-3. This indicates that the functions of Runx2 and CBFβ are not mutually exclusive and together with the sequencing data, highlight a potentially novel and exciting role of CBFβ in breast cancer (Mendoza-Villanueva et al., 2010; Mendoza-Villanueva et al., 2011). Furthermore, chromatin precipitation (ChIP) was performed to see if the DNA binding interactions of CBFβ with the promoters of both OPN and Galectin-3 and it was discovered that CBFβ is recruited to both of the promoters but is not essential for Galectin-3 activation (Mendoza-Villanueva et al., 2010).

A reduced level of CBFβ in MDA-MB-231 cells leads to a reduction in not only genes required for invasion but the invasive capacity of the cells as well. This invasive defect and also the expression levels of both MMP13 and OPN can be rescued by the restoration of CBFβ expression, which proves that CBFβ is a positive regulator of metastasis in breast cancer cells (Mendoza-Villanueva et al., 2010).

An additional study from this group used a microarray to identify the genes which were common as well as differentially regulated between MDA-MB-231 cells with either Runx2 or CBFβ knocked down using siRNA. The results showed that out of 38,500 genes in the array they identifies 357 and 340 genes in the Runx2 and CBFβ respectively that were differentially expressed between MDA-MB-231 transfected with Runx2 or CBFβ siRNA and those transfected with non-specific siRNA (Mendoza-Villanueva et al., 2011). They found that there was no significant change in Runx2 expression in the CBFβ knockdown cells or in Runx2 expression in CBFβ knockdown cells, indicating that the changes in the 161 genes that were common to both sets were not due to downstream changes in just one of these transcription factors. Further analysis found that Runx2/CBFβ regulate some of the genes that play a role in mediating the activation of osteoclasts and the inhibition of osteoblasts, namely IL-11, CSF-2 and SOST (Mendoza-Villanueva et al., 2011).
This work has helped to provide some insight into the potential links between breast cancer and bone metastasis. However, this data has been accumulated using *in vitro* techniques and focuses on one breast cancer cell line. It is essential to see how CBFβ mediates metastatic phenotypes in, not only additional cell lines, but also in an *in vivo* context.

**Summary**

Transcription factors, regulatory genes and hormones act to influence cell fate and are essential for mammary gland development. It is pertinent to fully elucidate the mechanism of cell fate regulation in the mammary gland to be able to understand the implication of their dysregulation in breast cancer.

The role of the CBF complex in its entirety is undoubtedly crucial for development of numerous systems, including neurogenesis, haematopoiesis and osteogenesis. All members of this complex have been shown to influence cell fate decisions and hence dysregulation or aberrant expression of either CBFβ or the Runx genes has severe implications in terms of disease, including several cancers. Recent studies have now identified CBFβ as one of the most frequently mutated genes in human breast tumours which fits with other published data stating that the functions of the Runx proteins and CBFβ in breast cancer are not always mutually exclusive. With this in mind, we can start to explore the role of CBFβ as a novel regulator of cell fate in the in the developing mammary gland and examine the implication of this role in the context of breast carcinogenesis.
Aims of this Thesis

The overall aim of this project is to examine a potential role for CBFβ as a regulator of mammary cell fate specification and determine if it plays a role in the regulation of normal mammary gland development and can influence breast carcinogenesis and metastasis.

The mammary gland is a dynamic organ that undergoes dramatic cyclical changes under the influence of female hormones and given the complexity of this system it is clear that there may be more transcription factors involved then is currently known. Runx2, one of the binding partners of CBFβ, has recently has been implicated in the mammopoiesis and given that we know CBFβ is integral to the functions of all Runx proteins, this thesis aims to provide insight into the role CBFβ itself may have in mammary gland development. Chapter 3 therefore specifically examines the effects of CBFβ deletion during mammopoiesis through the use of two independent inducible and conditional mouse models as well as through analysis of protein and mRNA levels through the developmental time course of the gland.

Furthermore, we aim to elucidate the role CBFβ is playing in human breast cancer. Breast cancer is a heterogeneous disease with good prognosis in the primary phase of the disease but a high morbidity and mortality rate in the metastatic phase, due in the most part to the preference of breast cancer cells to colonise the bone. This is a mechanism that still evades us but it is thought that Runx2 plays a role due to its involvement in both osteogenesis and now mammopoiesis. Although closely linked, it has already been suggested that the functions of Runx2 and CBFβ may not be mutually exclusive so there is potential for a novel role CFBβ in its own right. Recent sequencing studies have now identified CBFβ, for the first time, as one of the most frequently and significantly mutated genes in human breast tumours. Chapter 4 therefore aims to examine the expression patterns of CBFβ in human breast cancer cell lines then determine how the augmentation of CBFβ expression affects the growth and phenotype of these cells. In addition, we aim to provide insight into the potential mechanism behind CBFβ regulation of breast cancer through microarray analysis of breast cancer cells with altered levels of CBFβ expression.

As mentioned previously, it is the metastatic phase of breast cancer that has poor prognosis and survival rates and unfortunately, this is the area of research that poses the
most questions. Chapter 5 is therefore dedicated to studying the outcomes of deleting CBFβ in an in vivo model of breast carcinogenesis and metastasis. We will investigate if deletion of CFBβ affects tumour latency, overall survival of the animals and also on the incidence and severity of metastasis.

Overall, it is evident from the literature that there is definite potential for CBFβ to be deemed an important player and key regulator in both the developing mammary gland and also in breast carcinogenesis and it is the definitive aim of this thesis to reveal this novel function of CBFβ.
2. Materials and Methods

In Vivo Techniques

Mice

The development of CBFβ floxed mice (Naoe et al., 2007), CreERT2 transgenic mice (Ruzankina et al., 2007), BLG-Cre transgenic mice (Selbert et al 1998), Polyoma Middle T (PyMT) transgenic mice (Lin et al., 2003) and Rag1\(^{-/-}\) mice (Mombaerts et al., 1992) have been previously described. FVBN mice were used for time mating experiments and were purchased from the Animal Research Centre (ARC) Perth, Australia. Rag1\(^{-/-}\) mice of the C57BL/6J strain were purchased from ARC, Perth, Australia. The CBFβ floxed, CreERT2 and BLG-Cre mice used in these studies were of the mixed background 129/C57. The PyMT mice in these studies were of the FVBN background. Mice were housed in the Bosch Rodent Facility at the University of Sydney in accordance with ethics approvals K00/6-11/3/5545. All animals were housed with food and water \textit{ad libitum} at 22°C and 80% relative humidity with a 12-hour day/night cycle.

Time mating

Time mating is a technique used to assess changes in the mouse mammary gland across all stages of development. Breeding pairs were set up and then mammary glands were harvested at representative time points from virgin development, pregnancy, lactation and involution. The stage of the estrous cycle was determined through staining of a vaginal smear using the Shandon Kwik Diff\textsuperscript{TM} Stain (Thermo Scientific, Australia). Early stages of pregnancy were confirmed after post-mortem examination of the uterus. For the purpose of this thesis, the 4\textsuperscript{th} mammary glands were harvested from mice at the following developmental stages: virgin (estrous); 4 days post coitus (d.p.c); 7 d.p.c; 12 d.p.c; 18 d.p.c; the 2\textsuperscript{nd} day of lactation; and the 5\textsuperscript{th} day of involution. Both of the inguinal 4\textsuperscript{th} mammary glands were harvested, one was used for protein and RNA analysis and the second was used for whole mount analysis.
Mammary epithelium transplants

Mammary epithelium transplants were performed as previously described (De Ome, 1959). Succinctly, the procedure involved excising a 1mm³ (approximately) piece of mammary gland epithelium from between the nipple and lymph node of the 4th mammary gland from 12-week-old donors. The 3rd mammary gland was also taken from the donor and stained with carmine to be used as a histological reference. The donor mammary gland tissue was transplanted into the 4th mammary fat pad of 3-week-old Rag1⁻/⁻ animal once it was cleared of all endogenous epithelium. The epithelium was cleared from the recipient fat pad by cauterisation of the lymph node, nipple and the bridge between the 4th and 5th mammary gland. This section of the mammary gland is then removed which left the remaining fat pad that the ductal tree had yet to penetrate. The transplanted epithelium forms a new association with the host stroma as it grows out in to the recipient fat pad. Transplanted gland were examined by whole mount analysis which confirmed the formation of a new functional mammary gland that had been derived from the transplanted epithelium and not the endogenous gland. Ductal structures arising from endogenous epithelium originate from the side of the gland whilst ductal outgrowth from transplanted epithelium originates from the centre (Lewis et al., 1999).

Tamoxifen injections to activate Cre expression

In order to drive Cre expression and activate deletion of CBFβ in the CBFβ⁺/creERT2 line, the mice were injected with a Tamoxifen solution. The Tamoxifen powder (Sigma) was added to corn oil (Sigma) and incubated in the dark for three hours at 37°C until dissolved and filter sterilised to make a 20mg/mL stock solution. Mice were administered with a 50µL intra-peritoneal injection of 1mg of Tamoxifen over three consecutive days.

Tumour measurement and harvesting

In the PyMT tumourigenesis study, mice were examined three times a week for tumour development, weight and overall health. Once mammary tumours were palpable, digital calipers were used to measure the minor and major axis of each mammary tumour to determine the volume, which has been found to have a good correlation to the
weight of the tumour in grams (Attia & Weiss, 1966). Mice were euthanised when the tumour burden reached the ethical endpoint, which was when the combined tumour burden reached 10% of the animal’s weight. After the mice were euthanised, tumours and organs were collected for histological analysis and the remaining mammary glands were collected for whole mount analysis.

*Organ and tumour harvesting*

In the PyMT tumourigenesis study, mice were examined post-mortem for signs of metastasis and any organs found to be affected were surgically removed and placed in 10% neutral buffered formalin (NBF) for 4 hours before being transferred to 70% ethanol and then processed for histological analysis.

*In Vitro Techniques*

*Cell culture*

All cell lines were obtained from the American Type Culture Collection (Rockville, MD) through the Garvan Institute of Medical Research (Sydney, Australia). All cell lines were cultured in a humidified incubator at 37°C with 5% carbon dioxide. MDA-MB-231 and Hs578T cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Gibco) with 10% fetal bovine serum (Bovogen) and PlatE cells were culture in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) with 10% fetal bovine serum.

*siRNA transfection*

Two different and specific human CBFβ siRNAs (On-TARGETplus CBFβ siRNAs) and a negative control (On-TARGETplus RISC-free siRNA) were purchased from Dharmaco (USA). MDA-MB-231 cells were seeded at 1x10^5 cells/mL in 6-well plates to ensure they were 30-40% confluent 24 hours later. The cells were transfected with a final concentration of 5nM CBFβ or RISC-free siRNA diluted in Opti-MEM (Gibco). The transfection reagent, Lipofectamine 2000 (Invitrogen) was added to the diluted siRNA and incubated for 20 minutes at room temperature before being added to the cells. Each transfection was conducted in triplicate and cells were incubated for 48 hours before the cells were harvested.
Retroviral synthesis

Retroviral constructs to knockdown and overexpress CBFβ were generated as part of this thesis. In order to synthesise retrovirus, PlatE cells were seeded at 8x10^6 cells/15cm dish and left to grow for 24 hours at 37°C. The following day, 180µL of Fugene6 (Promega) to 5mL of serum-free media and incubated at room temperature for 5 minutes before the addition of 60µg of vector DNA and a further 20 minutes of incubation. The majority of growth media was removed from the cells and replaced with the 5mL Fugene/media/DNA solution and incubated overnight at 37°C. The media was changed the next day and then cells left to incubate overnight before the viral supernatant harvested and filter sterilised (0.45µm) the following day. The filtered virus was then aliquoted, frozen in liquid nitrogen and stored at -80°C until use.

Retroviral infection of human breast cancer cells

Human breast cancer cells were plated at 2x10^5 cells/10cm dish and left to incubate overnight at 37°C before the majority of media was removed and they were infected with a mixture of 1mL retrovirus, 3mL growth media and 4µL polybrene (8mg/mL stock) and incubated at 37°C. After 6 hours incubation, a further 6mL of growth media was added and the cells left to incubate overnight at 37°C. The process for infection was repeated for a second time before the cells populations were sorted by FACS sorting.

Proliferation assays

In order to assess the rate of proliferation, cells were seeded at a low density in 6-well plates. Cells were counted over a 96 hour period at 3-8 time points a day as the cells moved through the exponential growth phase. MDA-MB-231 cells expressing CBFβ or non-specific shRNA were initially seeded at 2x10^4 cells/mL and Hs78T cells containing the CBFβ overexpression construct or empty vector were seeded at 5x10^3 cells/mL.

Migration assays

Migration assays were performed using the xCelligence DP system (Roche) by measuring increasing electrical impedance as cells migrated across electrodes towards a chemoattractant, in this case fetal bovine serum. MDA-MB-231 cells expressing CBFβ
or non-specific shRNA were initially seeded at $4 \times 10^4$ cells/well and Hs78T cells containing the CBFβ overexpression construct or empty vector were seeded at $1 \times 10^4$ cells/well.

**Cloning of CBFβ shRNA retroviral constructs**

*Primer design*

Sense and anti-sense shRNA primers were designed based on the siRNA sequences and were made to include both the hairpin and an additional MLU1 restriction enzyme that was used to confirm the identity of the insert. The sense primers used can be found in the table below:

<table>
<thead>
<tr>
<th>hCBFβ5shRNA</th>
<th>GATCCGCCGAGAGTATGTCGACTTATTCAAGAGAAGTCGACATATTCTCCTCGGTCTTTTACGCGTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCBFβ6shRNA</td>
<td>GATCCGCAATTAGGTTGGTGATTTCAGAGAAATCCACCACCTAAAATGTTTTTACGCGTG</td>
</tr>
</tbody>
</table>

_Annealing and ligation_

The CBFβ shRNA sense and anti-sense primers were annealed in a 1:1 ratio in the following reaction: 30 seconds at 95°C, 2 minutes at 72°C, 2 minutes at 37°C and then 2 minutes at 25°C before storage at -20°C. The annealed oligos were then ligated into the linearised pSiren-retroQ-ZsGreen vector. The amount of vector and insert DNA was calculated using the following formula:

$$\frac{(\text{ng vector } \times \text{ insert size})}{\text{vector size}} \times \text{molar ratio of insert to vector}$$

Ligation reactions were performed using 1:1 and 1:3 vector:insert ratios containing: 50ng of vector, the appropriate amount of insert, 1 unit of T4 DNA ligase (Promega), 2x DNA ligase buffer in a 10µL reaction. Ligation reactions were incubated at room temperature for three hours.

_Transformation_

The plasmid DNA was transformed into the competent DH5α *E. coli* strain, which were thawed on ice. Approximately 15ng of the ligated DNA was mixed gently with 100µL of bacteria then incubated on ice for 30 minutes. The mixture was then heated to
42°C for 1 minute to heat shock the cells, before being placed immediately on ice for a further 2 minutes. 500µL of Luria Bertani (LB) medium was added and incubated on an orbital shaker for 1 hour at 37°C and 220 rpm. The entire mixture was then plated onto selective LB plates containing 50µg/mL of ampicillin and incubated overnight at 37°C.

DNA isolation and sequencing

Several colonies were picked from the selective LB plates and the plasmid DNA was then purified using the Wizard Plus SV Miniprep DNA Purification System (Promega) according to the manufacturer’s instructions. The purified DNA was sent for sequencing at the ACRF facility at the Garvan Institute according to their recommendations and requirements. Once sequences were confirmed, Endo-free Maxipreps (Qiagen) were performed and plasmids were eventually used to generate MDA-MB-231 cells that stably expressed CBFβ shRNA.

Cloning of CBFβ overexpression retroviral vector

A CBFβ human cDNA ORF clone was ordered from Origen (NM_022845) and the insert was excised from the pCMV6-Entry vector is arrived in through restriction enzyme digests using AsiS1 and Fse1. The CBFβ insert was then ligated (as per previous description) into the linearised pRetroX-IRES-DsRedExpress retroviral vector. A diagnostic digest was performed using the restriction enzyme PshA1 before the construct was sent for sequencing. Once the sequence was confirmed, this retroviral vector was used to generate Hs578T cells that stably overexpressed CBFβ.

Histological Analysis

Mammary gland whole mounts

Mammary gland whole mounts were generated by spreading the gland over a charged glass slide and were fixed immediately in 10% NBF solution (Sigma) for a minimum of 4 hours or overnight. The glands were then defatted through three changes of acetone before carmine alum staining overnight (0.2% carmine, 0.5% aluminum sulfate). The whole mount was then dehydrated using a graded ethanol series and then treated with Histolene (Thermofisher) for 60 minutes and then stored in methyl
salicylate. Metamorph software was used to determine the extent of ductal elongation in whole mounted mammary glands.

**Haemotoxylin and eosin staining**

Five micron sections of mammary glands and lungs were incubated for 5 minutes in two changes of Histolene (Thermofisher). Sections were then rehydrated before a 2 minute incubation in Harris Haemotoxylin (Thermofisher), washed with water, dehydrated and stained for 30 seconds in Eosin (Thermofisher). Sections were then incubated in 95% ethanol and two changes of 100% ethanol before two final changes of Histolene before coverslips were mounted.

**Immunohistochemistry**

IHC for CBFβ and Ki-67 was performed on 5µm sections of mammary gland that were de-waxed as per standard protocol before antigen retrieval using S1699 buffer (DAKO) in a 95°C water bath for 20 minutes. A 5% hydrogen peroxide (DAKO) in methanol solution was used as an enzyme block for both Ki-67 and CBFβ and an additional protein block (DAKO) was used in the CBFβ IHC only. Sections were incubated in primary antibodies (anti-Ki-67 (Rb) from Thermofisher (LBVRM-9106-S) and anti-CBFβ (Rb) from Abcam (ab133600)) at room temperature for 60 minutes and the Envision rabbit/mouse secondary (DAKO) for 30 minutes. Counterstaining and was performed according to standard protocol before coverslips were mounted.

**RNA Analysis**

**RNA extraction**

TriZol reagent (Life Techonologies) was used, according to the manufacturer’s instructions, to extract RNA from the 4th mouse mammary glands that were frozen in liquid nitrogen and stored at -80°C prior to use.

**cDNA synthesis**

RNA samples were DNase treated using the RQ1 DNase kit (Promega) before cDNA synthesis was performed using Multiscribe reverse transcriptase (Life Technologies) according to the manufacturer’s instructions.
Quantitative RT-PCR

RT-qPCR was performed on the Rotogene 3000 (Roche) using TaqMan® probes (Applied Biosciences) and the TaqMan® Universal Master Mix (Life Technologies). Thermal cycling conditions include an initial UNG activation step for 2 minutes at 50°C, followed by 10 minutes at 95°C, then 40 cycles at 95°C for 15 seconds with an annealing temperature of 60°C. The following probes were used: CBFβ (Hs00903431_g1), Runx2 (Hs00231692_m1), SatB1 (Hs00161515_m1), MMP9 (Hs00957562_m1), MMP13 (Hs00233992_m1), BgLap1 (Hs01587814_g1), Pthlp (Hs00174969_m1), Rankl (Hs00243522_m1) and β-actin (Hs99999903_m1).

Microarray Analysis

RNA extraction for microarray analysis was performed using the RNeasy Mini Kit from Qiagen according to the manufacturer’s instructions. For each sample, 1 µg of RNA at a concentration of 50ng/µl was submitted for microarray analysis. The quality of the RNA was assessed on the Nanodrop and Aglient Bioanalyzer 2100. Probe labeling and hybridisation on to the Affymetrix GeneChip PrimeView Human Gene Expression Array was performed at the Ramaciotti Centre for Gene Function Analysis (UNSW, Australia). Microarrays were normalised using the RMA method of normalisation through the use of Garvan GenePattern NormalizeAffymetrix3prime (version 2.1). Hierarchical clustering was performed to validate the results using GENE-E software (version 3.0.230, Broad Institute, Inc). Differentially expressed genes were detected using Garvan GenePattern LimmaGP (v20.0) using the positive false discovery rate (FDR) multiple hypothesis test correction. Gene set enrichment analysis (GSEA) was run in pre-ranked mode using Garvan GenePattern GSEApreranked module (version 3.2) and GSEA (version 2.0.13). The GSEA data was visualised using Cytoscape software (version 3.2.0).

Protein Analysis

Protein extraction
Protein was extracted from mouse mammary tissue using the TriZol reagent according to the manufacturer’s instructions, with the exception that the protein pellet was dissolved in 4M urea and 1% SDS. Protein was extraction from cell lines using NET lysis buffer supplemented with protease inhibitor (Roche), 1mM sodium orthovanadate and 10mM sodium fluoride. The cell lysate was incubated at 4°C for 30 minutes, centrifuged and then the supernatant was used for protein analysis. Quantification of protein was performed using the Bradford reagent (Bio-Rad) according to the manufacturer’s instructions.

Western blotting

Protein samples were denatured in laemmli sample buffer (Bio-Rad) supplemented with β-mercaptoethanol by heating to 95°C and then separated on 12% SDS-PAGE gels depending on the molecular weight of the protein being examined. Proteins were transferred on to PVDF membrane (Bio-Rad) and blocked for 2-3 hours at room temperature or overnight at 4°C in a solution containing 5% skim milk, 10mM Tris pH 7.6, 150mM NaCl and 0.2% v/v Tween-20. Membranes were incubated in one of the following primary antibodies for 1-2 hours at room temperature or 4°C overnight: α-CBFβ (Abcam), α-CBFβ (kindly provided by Ichiro Taniuchi, RIKEN, Japan), α-Runx2 (Cell Signaling Technology), α-cytokeratin 18 (Sapphire Biosciences), α-Cre (Covance) or α-β-actin (Sigma). Membranes were washed a minimum of three times for 10-15 minutes between antibody incubations in TBS/Tween (10mM Tris pH 7.6, 150mM NaCl, 0.2% v/v Tween-20). Specific binding was detected using Horseradish Peroxidase conjugated secondary antibodies (VWR) and Western Clarity chemiluminescence reagent (Bio-Rad) using the Bio-Rad ChemiDoc instrument.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5 software.
3. A novel role for CBFβ in mammary gland development

Introduction

Regulatory genes and hormones such as estrogen, progesterone, ERBB2, WNT and Notch have been implicated in the developmental processes of the mammary gland. It is these key players that then act on a smaller sub-set of transcription factors, which in turn regulate cell fate decisions such as whether or not a cell undergoes proliferation, differentiation or apoptosis (Hennighausen & Robinson, 2005). Investigation into the role of these factors that regulate mammary gland cell fate has been fundamental in increasing our current understanding of the processes that control the development of this complex organ and by extension, the implications of their dysregulation in regards to breast cancer.

The mammary gland is an incredibly dynamic organ that undergoes dramatic cyclical changes under the influence of female hormones (Hennighausen & Robinson, 2005). Several transcription factors, including Gata3, Elf5, Stat5A, ER and PR have been identified as essential regulators of mammary gland development and their roles have been studied in detail (Kouros-Mehr et al., 2006; Kouros-Mehr & Werb, 2006; Naylor & Ormandy, 2007; Oakes, 2008b; Siegel & Muller, 2010). Given the complexity of this developmental system, much of which is yet to be fully understood, it is clear that there may be more transcription factors involved.

The Runx family of transcription factors have become an increasingly intriguing point of interest due to their critical involvement in both developmental processes and disease progression. The Runx genes are well known as essential regulators of cell fate, with lineage and stage specific roles. The importance of Runx2 in the regulation of cell-fate and lineage-maintenance of non-osseous tissue has been highlighted by recent studies, particularly its potential role in mammary gland development (Blyth et al., 2005; Ferrari et al., 2013; McDonald et al., 2014; Owens et al., 2014; Shore, 2005).

Our lab recently demonstrated that Runx2 is expressed in the mammary ductal epithelium and that expression is differentially regulated throughout mammopoiesis (Owens et al., 2014). Furthermore, we have shown that Runx2 controls lobuloalveolar development in vivo through regulation of luminal progenitors during alveolar development (Owens et al., 2014). Another recent study reports that overexpression of Runx2 also results in perturbed lobuloalveolar development (McDonald et al., 2014).
This is an intriguing conundrum and indicates that further work is required to determine the mechanism of Runx2 function in the mammary gland.

Whilst the Runx genes are critical for the regulation of cell fate and essential for normal development, they require a binding partner in order to carry out transcriptional regulation. All members of the Runx family form heterodimeric complexes with the same non-DNA binding transcriptional co-activator, core-binding factor β (CBFβ) (Ito, 2008). The binding of CBFβ to the Runx genes causes a structural change that increases the DNA-binding affinity of the entire complex (Tahirov et al., 2001; Zhang et al., 2003). It is this combined CBF complex that can bind co-factors capable of both activating and repressing transcription of key regulators of growth, survival and differentiation pathways (Tanaka et al., 1995).

It has been shown in other developmental systems such as osteogenesis and haematopoiesis that CBFβ is essential for the regulation of stem cell differentiation and proliferation (Kurosaka et al., 2011). Furthermore, there is now evidence that CBFβ is able to boost the transcriptional effectiveness of the Runx genes and their downstream targets by both enhancing their DNA-binding efficiency and structural stability, allowing for successful regulation of cell fate in multiple systems (Davis et al., 2010; Qin et al., 2014; Yoshida, 2002).

Given the novel role recently discovered for Runx2 in the mammary gland and the knowledge that CBFβ is able to enhance Runx2 function and DNA-binding affinity, possibly through stem cell control, there is a strong indication that CBFβ may be essential to the developing mammary gland and potentially in its own right.

The aim of this chapter was to examine, for the first time, the potential role of CBFβ in mammary gland development.
Results

**CBFβ is developmentally regulated in the mammary gland**

Several studies have demonstrated that whilst there is a close relationship between the Runx genes and CBFβ, their functions are not always mutually exclusive, (Mendoza-Villanueva et al., 2011; Okuda et al., 1996; Wang et al., 1996). We have previously shown that Runx2 is differentially regulated throughout mammary gland development at both the protein and mRNA levels, and plays an essential role in the regulation of mammopoiesis (Owens et al., 2014). This study aimed to explore whether CBFβ expression was also differentially regulated throughout mammary gland development and whether CBFβ was required for normal mammary gland development and epithelial cell function.

To investigate the potential role of CBFβ during mammopoiesis, expression levels of CBFβ were measured throughout all stages of development. Analysis of mRNA from the mouse mammary gland showed that CBFβ expression levels decreased in late pregnancy through to early lactation, before increasing again during involution as the glands returns to a virgin-like state (Fig.3.1.A). This expression pattern mirrors that of Runx2 and is a strong indication that CBFβ is involved in mammary gland development.

**CBFβ expression is restricted to the mammary epithelial cells**

It has been shown that Runx2 is expressed in the basal or myoepithelial cell sub-populations in the mammary gland where the mammary stem cells are thought to reside (Kendrick et al., 2008). This is a further indication that Runx2 plays a critical role in the mammary gland, perhaps at the stem cell level. Since we have now shown that CBFβ is also developmentally regulated in the mammary gland in conjunction with Runx2, we performed IHC for CBFβ and have shown that CBFβ expression in the mammary gland is also restricted to the luminal and myoepithelial cells of the mammary epithelium (Fig. 3.1.B).

This data therefore provides further evidence that CBFβ is involved in mammary gland development and that it may potentially be involved in regulation of stem cell populations, similar to its function in haematopoiesis and skeletal development (Kurosaka et al., 2011).
Fig. 3.1. **CBFβ is expressed in mammary epithelial cells and is developmentally regulated during mammopoiesis.**

CBFβ expression in the mammary gland is exclusively found in the luminal and myoepithelial cells layers of the epithelial ductal network. Furthermore CBFβ is developmentally regulated throughout mammary gland development, whereby expression levels are decreased in late pregnancy through to early lactation. A) mRNA expression of CBFβ across a mammary gland development series relative to the virgin gland and normalised to β-actin as shown by RT-qPCR (P = pregnancy, L = lactation, I = involution). Error bars represent SEM (N=3). B) Immunohistochemistry staining showing exclusive localisation of CBFβ to the epithelium of the virgin mammary gland.
CBFβ expression is required in the early stages of mammary gland development for successful outgrowth of the ductal epithelium

Given that CBFβ is differentially regulated throughout mammopoiesis and expression is confined to the mammary epithelium, we next sought to investigate the potential role of CBFβ in mammary gland development. In order to achieve this, we generated a CBFβ knockout mouse by crossing CBFβ floxed (CBFβf/f) mice (Naoe et al., 2007) with CreERT2tg/+ mice (Ruzankina et al., 2007) resulting in CBFβf/+;CreERT2tg/+. These mice were then backcrossed to CBFβf/f mice to produce either CBFβf/+CreER+/+ or CBFβf/+CreERtg/+ mice. Crossing the CBFβf/+ mice with the CreERT2tg/+ mice resulted in an inducible model where Cre expression is activated in the presence of Tamoxifen, resulting in the subsequent deletion of CBFβ ubiquitously throughout the mouse.

In order to investigate the temporal and spatial effects of CBFβ in the developing mammary gland, mammary epithelium from CBFβf/+CreER+/+ and CBFβf/+CreERtg/+ mice was transplanted into the opposite inguinal fat-pads, cleared of endogenous epithelium, of 3-week-old immunocompromised Rag1−/− mice. Mammary transplantation enables reconstitution of a mammary gland with epithelium derived from the donor gland which interacts with the host stroma as the ductal network extends throughout the recipient fat pad (De Ome, 1959). These mice were subsequently injected with Tamoxifen four days post transplant for three consecutive days to activate Cre-mediated deletion of CBFβ from the epithelium of the transplanted mammary glands.

Mice were sacrificed six weeks post transplantation surgery and the transplanted glands were harvested and whole mounted for carmine staining and histological analysis. In order to confirm that mammopoiesis progressed normally in the Rag1−/− recipient mice, an endogenous 3rd mammary was also collected as a control.

At six weeks post transplant, CBFβf/+CreERtg/+ glands showed a significant reduction in the development of the ductal tree when compared to the CBFβf/+CreER+/+ gland from the same mouse (Fig. 3.2. A). The majority of CBFβf/+CreERtg/+ glands showed a 50% reduction in epithelial ductal outgrowth compared to the CBFβf/+CreER+/+ glands, as shown by image analysis using Metamorph software (Fig. 3.2. B).
This result clearly demonstrates that deletion of CBFβ at the onset of post-natal mammary gland development leads to a marked reduction in the successful outgrowth of the ductal network. This *in vivo* data has demonstrated for the time that CBFβ plays a critical role in mammary gland development.
A) CBFβ\textsuperscript{+/−}CreER\textsuperscript{+/−} virgin glands  CBFβ\textsuperscript{−/−}CreER\textsuperscript{+/−} virgin glands

B) Mask of CBFβ\textsuperscript{+/−}CreER\textsuperscript{+/−} virgin glands  Mask of CBFβ\textsuperscript{−/−}CreER\textsuperscript{+/−} virgin glands
Fig. 3.2. 

Spatial and temporal deletion of CBFβ in the mammary gland leads to reduced ductal elongation in virgin mice.

Deletion of CBFβ expression in the mammary gland leads to a reduction in outgrowth of the ductal tree through the fat pad. A) Representative whole mount images of virgin mammary glands 6 weeks post transplant showing delayed ductal elongation in the CBFβ<sup>+/+</sup>CreER<sup>tg/+</sup> glands when compared to the CBFβ<sup>+/+</sup>CreER<sup>-/-</sup> glands (N=6). These mice were injected with Tamoxifen 4 days post transplant in order to activate Cre-induced deletion of CBFβ in the mammary gland. B) Representative images of the binary mask generated in order to analysis ductal extension in CBFβ<sup>+/+</sup>CreER<sup>tg/+</sup> and CBFβ<sup>+/+</sup>CreER<sup>-/-</sup> glands. C) Histograms quantifying the percentage of the fat pad that is filled with ductal epithelium in the CBFβ<sup>+/+</sup>CreER<sup>tg/+</sup> glands compared to the CBFβ<sup>+/+</sup>CreER<sup>-/-</sup> glands in five individual animals.
Characterisation of CBFβ deletion in the CBFβcreER mouse model

Our novel mouse model of mammary-specific CBFβ deletion has shown an undisputable role for CBFβ in this mammary gland development. In order to confirm that the phenotype observed was due to a lack of CBFβ in the mammary gland, western blotting for CBFβ was performed on protein from the transplanted CBFβ^{f/f}CreER^{tg/+} and CBFβ^{f/f}CreER^{+/+} glands. Western blot analysis for CBFβ showed a clear deletion of CBFβ in the CBFβ^{f/f}CreER^{tg/+} glands compared to the CBFβ^{f/f}CreER^{+/+} glands (Fig. 3.3.A).

CBFβ is required for the proliferation of mammary epithelial cells during ductal elongation

CBFβ has been shown to regulate both skeletal and haematopoietic development through the regulation of both stem cell proliferation and differentiation (Kurosaka et al., 2011). The virgin gland undergoes high levels of proliferation during the process of ductal extension (Hennighausen & Robinson, 2005), which corresponds to the stage of mammary gland development where we observe a defect in the CBFβ^{f/f}CreER^{tg/+} glands. In order to examine whether this phenotype was due to a defect in proliferation, we stained 6-week-old CBFβ^{f/f}CreER^{tg/+} and CBFβ^{f/f}CreER^{+/+} transplanted mammary glands for Ki-67, a marker of cell proliferation and quantified the percentage of Ki-67 positive cells between the two genotypes.

There are significantly less Ki-67 positive cells in the CBFβ^{f/f}CreER^{tg/+} glands compared to the CBFβ^{f/f}CreER^{+/+} glands as seen in Figure 3.3.B. Quantification of cell counts confirmed a marked decrease Ki-67 staining in the CBFβ^{f/f}CreER^{tg/+} glands (p = 0.003) with a total of 53.5% (± 4.8) Ki67 positive cells in the CBFβ^{f/f}CreER^{+/+} glands and only 18.9% (± 5.1) Ki67 positive cells in the CBFβ^{f/f}CreER^{tg/+} glands (Fig. 3.3.C).

This data demonstrates a novel role for CBFβ in the regulation of mammary epithelial cell proliferation during ductal elongation.
Fig. 3.3. **CBFβ** regulates mammary epithelial cell proliferation during ductal elongation

CBFβ deletion leads to a decrease proliferation of mammary epithelial cells during ductal elongation. A) Western blot for CBFβ confirming CBFβ knockout in the CBFβ<sup>fl/fl</sup>CreER<sup>tg/+</sup> glands compared to the CBFβ<sup>fl/fl</sup>CreER<sup>g/+</sup> glands, loaded to the epithelial marker cytokeratin 18. B) IHC staining showing a reduction in the proliferative marker Ki-67 in the CBFβ<sup>fl/fl</sup>CreER<sup>tg/+</sup> glands compared to the CBFβ<sup>fl/fl</sup>CreER<sup>g/+</sup> 6-week-old transplanted glands (N=6). C) Histogram quantitating a significant increase in Ki-67 positive cells in CBFβ<sup>fl/fl</sup>CreER<sup>g/+</sup> compared to the CBFβ<sup>fl/fl</sup>CreER<sup>g/+</sup> 6-week-old transplanted glands (p = 0.003, students unpaired t-test). Error bars represent SEM.
Ductal elongation is not affected in 14-week-old CBFβ^{ff}CreERTg^{tg/+} mammary glands

In order to determine if the stunted ductal outgrowth observed in the 6-week-old CBFβ^{ff}CreERTg^{tg/+} transplanted glands was due to a delay in epithelial outgrowth or if proliferation was being completely blocked, transplanted glands were left to develop for a further 8 weeks.

Mammary glands collected 14 weeks post transplant and showed no difference between the CBFβ^{ff}CreERTg^{tg/+} and CBFβ^{ff}CreERTg^{tg/+} glands in regards to ductal elongation and outgrowth, as seen in the whole mount images in Figure 3.4. This result indicates that deletion of CBFβ results in a delay in ductal extension of the mammary epithelium but does not lead to a permanent arrest.
Fig. 3.4. 14-week-old CBFβ+/CreERtg/+ mammary glands show no delay in ductal elongation.

Representative whole mount images of virgin CBFβ+/CreERtg/+ mammary glands 14 weeks post transplant showing a recovery in ductal outgrowth (N=10). These mice were injected with Tamoxifen 4 days post transplant in order to activate Cre-mediated deletion of CBFβ in the mammary gland.
CBFβ deletion does not perturb lobuloalveolar development in CBFβ^f/fCreER^tg/+ mice

We have now shown that CBFβ plays a critical role in the early development of the mammary gland, a phenotype that is recovered as the gland reaches maturity. We have determined that this is due to a reduction in the number of proliferating mammary epithelial cells in the CBFβ^f/fCreER^tg/+ glands, leading to a significant delay in the elongation of the ductal network.

The second important aspect of cell fate that CBFβ is known to regulate in other developmental systems is differentiation (Kurosaka et al., 2011). The highest levels of differentiation occur in the mammary gland during pregnancy, when lobuloalveolar development occurs and the functional alveoli are formed (Hennighausen & Robinson, 2005).

In order to investigate whether CBFβ plays a role in the regulation of differentiation in the mammary gland, we examined CBFβ^f/fCreER^tg/+ transplanted glands at both mid and late pregnancy time points. Mammary glands were harvested at both 12 and 18 days post coitus (d.p.c). Whole mount analysis showed that neither alveolar bud formation or lobuloalveolar development was affected in the CBFβ^f/fCreER^tg/+ glands compared with CBFβ^f/fCreER^+/+ glands (Fig. 3.5). This data indicates that CBFβ is an important regulator of proliferation but not differentiation in the developing mammary gland.
CBFβ does not regulate alveolar bud formation or lobuloalveolar development in CBFβ<sup>CreER<sub>tg</sub></sup><sup>WT</sup> mice.

Whole mount images of CBFβ<sup>CreER<sub>tg</sub></sup><sup>WT</sup> and CBFβ<sup>CreER<sub>tg</sub></sup><sup>WT</sup><sup>+/−</sup> mammary glands at mid and late pregnancy showing a recovery of the phenotype seen in the 6-week-virgin glands on no defects in alveolar bud formation or lobuloalveolar development.
*CBFβ does not regulate virgin development in CBFβ^{fl/fl}BLGCre^{tg/+} mice*

In order to further investigate the role of CBFβ in mammopoiesis we generated a second CBFβ knockout mouse model by crossing the CBFβ^{fl/fl} mouse (Naoe et al., 2007) on to a BLG-Cre^{tg/+} mouse (Selbert & Bentley, 1998). The resulting CBFβ^{fl/+};BLG-Cre^{tg/+} mice were then backcrossed on to the CBFβ^{fl/fl} mice to generate both CBFβ^{fl/fl}BLGCre^{tg/+} and CBFβ^{fl/+}BLGCre^{tg/+} mice. Crossing the CBFβ^{fl/fl} mice with the BLGCre^{tg/+} mice resulted in a mammary epithelium-specific, constitutive CBFβ knockout model where Cre-mediated deletion of CBFβ is driven off the beta lactoglobulin (BLG) promoter, which is expressed mostly highly during pregnancy and lactation.

To further examine the effects of CBFβ deletion on virgin development, CBFβ^{fl/fl}BLGCre^{tg/+} and CBFβ^{fl/+}BLGCre^{tg/+} mammary glands were collected at 5, 7 and 9 weeks of age, representing pre-pubescent, pubescent and mature virgin glands respectively.

Whole mount analysis of the glands at each time point showed no differences in the ductal outgrowth, terminal end bud formation or side branching of the ductal network in either the CBFp^{fl/fl}BLGCre^{tg/+} or CBFβ^{fl/+}BLGCre^{tg/+} mice (Fig 3.6).

This data indicates that CBFβ is not required to regulate virgin mammary development and morphogenesis in CBFβ^{fl/fl}BLGCre^{tg/+} mice.
**Fig. 3.6.** Late onset CBFβ deletion does not affect development of the virgin mammary gland.

Images of whole mounted images virgin mammary glands from CBFβBLG mice showing no change in morphology at 5, 7 and 9 weeks of age (N=3 for CBFβBLGCre°/° and CBFβBLGCre°/+ at each time point).
**CBFβ is not required for lobuloalveolar development or alveologenesis**

To examine whether CBFβ deletion in CBFβ^ffBLGCre^g/+ mice affected development during pregnancy, we harvested glands from CBFβ^ffBLGCre^g/+ and CBFβ^ffBLGCretg/+ mice at the key developmental stages of mid pregnancy and early lactation, where CBFβ is deleted in the majority of mammary epithelial cells in this model (Selbert & Bentley, 1998).

Whole mount analysis of CBFβ^ffBLGCre^g/+ and CBFβ^ffBLGCretg/+ mammary glands at day 12 d.p.c and at the first day of lactation show no difference in morphology (Fig. 3.7.A). As per the virgin CBFβ^ffBLGCre^g/+ mice, ductal elongation and side branching also remains unchanged under BLGCre-mediated deletion of CBFβ. In addition, there is also no change in lobuloalveolar development during pregnancy or alveoli formation in the lactating glands.

This data indicates that CBFβ is not required for regulating differentiation during pregnancy and lactation.

**Characterisation of CBFβ deletion in the CBFβBLG mouse model**

To confirm that CBFβ was deleted in the CBFβ^ffBLGCre^g/+ mice, mammary glands were collected for protein expression analysis by western blotting. There was clearly a significant reduction of CBFβ protein expression in the CBFβ^ffBLGCre^g/+ glands when compared to the glands from CBFβ^ffBLGCre^+/+ mice, as shown in Figure 3.7.B.
Fig. 3.7. **Constitutive CBFβ deletion does not effect development of the pregnant mammary gland.**

A) Whole mount analysis of CBFβ^{f/f}BLGCre^{tg/+} mammary glands at key stages of pregnancy shows no change in morphology between the CBFβ^{f/f}BLGCre^{tg/+} and CBFβ^{f/f}BLGCre^{+/+} glands (N=3 for each genotype). B) Analysis of CBFβ protein levels, normalised to cytokeratin 18, confirming CBFβ knockout in the CBFβ^{f/f}BLGCre^{tg/+} mouse line (p = 0.01, student’s unpaired t-test).
Discussion

The mammary gland is a complex and dynamic organ that undergoes cyclical rounds of proliferation and differentiation under the influence of female hormones (Hennighausen & Robinson, 2001), making it an excellent system in which to study regulators of cell fate. There are a number of elements of mammary gland development that are well understood, such as placode development and placement in the embryonic mammary gland or the signals required to induce lactation. There are however, many aspects of virgin development and development during pregnancy that are yet to be fully elucidated.

It is well understood that, as part of the CBF complex, CBFβ is crucial for the regulation of cell fate in haematopoiesis and skeletal development (Coffman, 2003; Huang et al., 1999; Nagata et al., 1999). This however, is the first study to show a role for the CBFβ in the mammary gland. Given the importance of the binding process of CBFβ to the Runx genes, we hypothesised that this role for CBFβ would be in either a dependent or supportive context.

We observed that CBFβ expression levels follow the same differentially regulated pattern as Runx2 throughout mammary developmental which is intriguing as it has been shown in other systems that expression of the Runx genes is tissue-specific whilst CBFβ is more ubiquitously expressed (Adya et al., 2000; Hajra & Collins, 1995; Ogawa et al., 1993a; Wang et al., 1993). Therefore, it was exciting to see that CBFβ levels are differentially expressed in the mammary gland in conjunction with Runx2 even though unpublished data from our lab shows that Runx and Runx3 levels stay consistent. This is a clear indication that CBFβ is plays a key role in this developmental process.

The decrease in CBFβ expression in the mammary gland during pregnancy was not as dramatic as the observed decrease in Runx2 expression (unpublished data), which may provide an explanation for the apparent lack of lobuloalveolar defect in both the CBFβ<sup>fl/fl</sup>Cre<sup>ER<sup>tg</sup>+</sup> and the CBFβ<sup>fl/fl</sup>BLGCre<sup>tg</sup>/+ mammary glands during pregnancy and lactation. Rather we observe a proliferative defect in the pubertal CBFβ<sup>fl/fl</sup>Cre<sup>ER<sup>tg</sup>+</sup> glands at a time point where expression levels of CBFβ are constitutively higher in wildtype mammary glands.

Whilst our lab has not examined the effects of Runx2 deletion at the onset of mammary gland development, there appears to be no discernable effect on Runx2 deletion in virgin mammary glands, rather major defects during lobuloalveolar
development (Owens et al., 2014). Runx2 deletion may not affect the onset of mammary gland development because CBFβ expression would remain unchanged and therefore it would still act to stabilise Runx1 and Runx3 proteins in the same way it has been shown to stabilise Runx2 during skeletal development (Qin et al., 2014; Yoshida, 2002). This would lead to a protective effect and would allow Runx1 and 3 to compensate for Runx2 function, resulting in normal virgin development. Alternatively, Runx1 or Runx3 may be regulating early mammary gland development, which would also explain the phenotypic differences observed between the Runx2 and CBFβ knockout mouse models. This hypothesis could be tested via generation of Runx1 and Runx3 mammary-specific mouse models.

The evidence our lab has produced demonstrating a novel role for Runx2 in the mammary gland has formed the basis for this thesis. This study has shown a crucial role for Runx2 in lobuloalveolar development during pregnancy through regulation of the luminal stem cell populations (Owens et al., 2014).

Interestingly, a separate study has shown a similar lobuloalveolar defect through the use of a Runx2 transgenic mouse rather than a knockout model (McDonald et al., 2014). As described by the authors, the levels of Runx2 are expressed at supraphysiological levels in the transgenic mouse (McDonald et al., 2014) and as such, the validity of these results may be compromised. This Runx2 overexpression study also shows a delay in ductal elongation in the developing virgin gland (McDonald et al., 2014) and whilst our lab did not examine earlier time points in the Runx2 knockout mouse, the delayed ductal extension observed in the CBFβ^{f/f}CreER^Tg/+ glands is clear. Traditionally, the roles of genes and transcription factors involved in mammary gland development have been described through targeted deletion in vivo (reviewed in (Robinson, 2004; Visvader & Lindeman, 2003)). This is the approach used by our group in both the Runx2 and CBFβ studies as it allows greater control over temporal and spatial expression levels than the transgenic model used by McDonald and colleagues (McDonald et al., 2014). However, the discrepancies observed, particularly in regards to Runx2 function, indicate that further investigation is required to discern the mechanism by which the CBF complex regulates mammary cell fate.

Both Runx2 studies do demonstrate that the decreased levels of Runx2 observed throughout normal mammary gland development indicates that Runx2 down regulation is required in order to induce differentiation (McDonald et al., 2014; Owens et al.,
2014). It has been shown that forcing Runx2 expression at this time (both in vivo and in vitro) leads to poorly differentiated lobuloalveolar structures (McDonald et al., 2014) and results in HC11 cells that are no longer able to differentiate and produce milk proteins (Owens et al., 2014). These studies show that the overexpression of Runx2 leads to changes in the mammary epithelium that are consistent with tumourigenesis, an area that will be discussed in the following chapters. It is therefore clear that Runx2 is essential for the control cell fate decisions in the mammary gland.

Whilst there is virtually no previous data describing a role for CBFβ in the developing mammary gland, there have been suggestions by studies investigating breast cancer, that CBFβ is not just playing a supportive role to Runx2 but may have independent functions as well (Mendoza-Villanueva et al., 2010). The stark difference in the phenotypes observed in CBFβ−/− and Runx2−/− mammary glands supports this hypothesis.

Previous studies in other developmental models such as haematopoiesis and skeletal development indicate that CBFβ is an important regulatory element in a range of developmental processes (Qin et al., 2014; Tanaka et al., 1995; Yoshida, 2002). The common hypothesis formed from these studies is that CBFβ is controlling the function of Runx proteins through regulating the proliferation and differentiation of stem cell populations (Kurosaka et al., 2011). This thesis, for the first time, has detected CBFβ expression in the mammary gland and shows that it is expressed in both the luminal and also in myoepithelial cells of the mammary gland, which is where Runx2 is expressed and also where mammary stem cells are derived from (Kendrick et al., 2008; Molyneux et al., 2010; Owens et al., 2014; Rios et al., 2014). This supports the hypothesis that CBFβ may be involved in the control of mammary stem cell populations.

The in vivo data from this chapter demonstrates a significant delay in ductal elongation of the developing mammary gland due to decreased numbers of proliferating mammary epithelial cells in CBFβ+/CreERtg/+ mammary glands. This defect however, is only observed in virgin mice when CBFβ is deleted from the onset of post-natal mammary gland development. In this model, CreERT2-mediated deletion of CBFβ targets all cells, including stem and progenitor cells. In contrast in BLG mice CBFβ deletion is restricted to more differentiated luminal populations.

Given the phenotypes observed in these in vivo models of CBFβ deletion, our data indicates a potential and novel role for CBFβ as a potential regulator of mammary
progenitor/stem cell populations. This is a hypothesis supported by previous published data describing roles for both CBFβ and the Runx genes in stem cell regulation in other developmental systems (Kurosaka et al., 2011).

In order to confirm the effects of CBFβ on the proliferative capacity of mammary gland stem cells, it would be interesting to perform additional mammary transplants using the CBFβ^{f/f}\text{CreER}^{tg/+} mice and then use flow cytometry analysis on total cell populations to see if there is a shift in progenitor populations in the CBFβ^{f/f}\text{CreER}^{tg/+} glands compared to the CBFβ^{f/f}\text{CreER}^{+/+} glands.

In regards to the effects of CBFβ deletion on mammary gland during pregnancy, no effect was seen in either CBFβ^{-/-} mouse model. There was no difference in the ability of the ductal tree to undergo successful lobuloalveolar development during pregnancy or alveoli formation during lactation. This indicates that CBFβ is not required for regulating differentiation in the mammary gland during pregnancy or lactation.

This is the first study to show that CBFβ is both present and essential for the development of the mammary gland and highlights a novel and exciting role for this transcription factor.
4. CBFβ plays an important regulatory role in breast cancer cells and can drive metastatic phenotypes such as proliferation and migration

Introduction

Our lab has now shown that Runx2 plays a definitive role in lobuloalveolar development via regulation of luminal progenitor cells populations (Owens et al., 2014). Data from this thesis has also determined that CBFβ also plays a role in mammary gland development, through regulating the proliferation of mammary epithelial cells during ductal elongation. The importance of this novel role for CBFβ in mammary gland development is the potential implication for CBFβ in breast cancer. It is a well-understood paradigm that regulatory genes and transcription factors that are involved in development and cell fate regulation are often perturbed in cancer.

Most genes that are implicated in cancer can be easily defined as either oncogenes or tumour suppressors however, this is not the case for the Runx genes and CBFβ, which have been shown to function as either in a context-dependent manner (Blyth et al., 2005). Runx2 is found to be amplified in several cancers, especially osteosarcoma, and therefore is primarily considered to be an oncogene (Forus et al., 1995; Lau et al., 2004). When Runx2 is overexpressed in endothelial cells and osteoblasts it leads to enhanced cell migration, survival, invasion and angiogenesis, all of which are indicative of oncogenic transformation (Fujita et al., 2004; Sun et al., 2001; Sun et al., 2004; Zelzer et al., 2001).

Our lab has now shown that overexpressing Runx2 in the MCF10a normal breast cells leads to induction of epithelial to mesenchymal transition (EMT), characterised by increased expression of N-Cadherin and Cyclin D1, loss of E-Cadherin expression and a distinct shift in the morphology of the cell from a cobblestone epithelial shape to a more fibroblastic shape consistent with breast cancer cells. Similarly, forced expression of Runx2 in the HC11 mouse mammary epithelial cells leads to an increase in both the proliferative and migrational capacity of the cells, classic hallmarks of cancer (Owens et al., 2014). We and others have also shown that reducing Runx2 expression in the aggressive MDA-MB-231 cells breast cancer cell line leads to decreased migration and invasion (Mendoza-Villanueva et al., 2010; Mendoza-Villanueva et al., 2011; Owens et al., 2014). This collective data demonstrates a novel for Runx2 as a regulator of breast
cancer cell fate and also highlights that there are many aspects of breast cancer that require further investigation.

The focus of this thesis is to determine whether CBFβ also plays a role in breast cancer, although to date there has been very little data suggesting a causal relationship between the two. As part of their research into the role of Runx2 in breast cancer metastasis, Mendoza-Villanueva et al found that CBFβ was expressed in the aggressive MDA-MB-231 and found evidence to suggest a potential role for CBFβ in the invasive potential of these cells (Mendoza-Villanueva et al., 2010). This study indicates that CBFβ may be involved in breast cancer but further investigation is clearly needed.

Recent whole genome/exon signaling sequencing, aiming to find genetic alterations, has recently found that only 5 genes were found to be commonly mutated; GATA3, PIK3CA, MAP3KI, TP53 and CBFβ. The first four genes are known key players in breast cancer, however as previously stated, there has been very little data associating CBFβ with breast cancer these studies present an enticing new link (Banerji et al., 2012; CGAN, 2012; Ellis et al., 2012). In addition, CBFβ was identified as one of the most frequently mutated genes, being mutated in approximately 5% of all luminal breast cancers (CGAN, 2012). This data suggests that CBFβ may play a critical role in breast cancer and further investigation is needed in order to elucidate this potential new function.

The aim of this chapter therefore, is to explore the role of CBFβ in human breast cancer in the context of an in vitro model.
Results

*CBFβ is expressed across all sub-types of human breast cancer*

Until recently, very little has been known about the role of CBFβ in the context of breast cancer, as such we examined the expression levels of CBFβ across a panel of human breast cancer cell lines in order to determine whether CBFβ expression correlated with a particular subtype. This panel included multiple cell lines from each of the following breast cancer subtypes; Luminal, Basal A, Basal B and also cell lines that represented a normal breast cell population.

The mRNA expression analysis shows that CBFβ expression does not distinctly associate with a particular breast cancer (Fig. 4.1.A). Similarly, CBFβ expression also does not increase with invasiveness or correlate with estrogen receptor status as shown in Figures 4.1.B and C respectively.

Our lab has previously shown that Runx2, unlike its binding partner CBFβ, has distinct expression patterns in relation to subtype and estrogen receptor status (Fig. 4.1.D). Runx2 levels are higher in the more aggressive basal A and B subtypes lower in the luminal cancers that are associated with better prognosis. Runx2 levels also increase with invasiveness and are higher in cell lines that are estrogen receptor negative. This indicates that CBFβ is it always present but potentially only directly affected when Runx2 is activated.

In addition to performing CBFβ expression analysis in breast cancer cell lines, we have also shown through immunohistochemistry that CBFβ is expressed in a range of human breast tumours (Fig. 4.1.E). The data is the first evidence of CBFβ expression in the human breast and is a clear indication that CBFβ may be playing an important role in breast cancer.
Relative CBFβ Expression as Invasiveness Increases

Relative CBFβ Expression and ER Status

ER +ve  ER -ve
D) 

![Bar graph showing relative expression for Luminal, BaA, and BaB subtypes.](image)

![Relative expression for ER+ve and ER-ve subtypes.](image)
Fig. 4.1. Expression of CBFβ in human breast cancer cell lines

A) mRNA expression of CBFβ, relative to MCF10a cells and normalised to GAPDH, across a panel of human breast cancer cell lines as shown by RT-qPCR. Error bars represent SEM (N=3). B) mRNA analysis shows that CBFβ expression does not correlate with invasiveness. C) mRNA analysis shows that CBFβ expression does not correlate with estrogen receptor status. D) mRNA analysis shows that Runx2 is significantly upregulated in the aggressive basal breast cancer subtype (p = 0.02, Student’s t-test) and that Runx2 expression also correlates with ER negative breast cancers using a panel of human breast cancer cell lines. E) Immunohistochemistry was used to show that CBFβ is expressed in a range human breast tumours from the Royal Brisbane & Women’s Hospital breast cancer cohort (Supplied by Peter Simpson).
CBFβ regulates the expression of crucial downstream targets involved in cancer progression

A previous study in human breast cancer cells showed that knocking down CBFβ expression in MDA-MB-231 cells lead to a subsequent reduction of several downstream targets of the Runx2-CBFβ complex (Mendoza-Villanueva et al., 2010). We have now shown that CBFβ is expressed across all breast cancer subtypes and that expression varies between individual cell lines.

Based on this comparative analysis we selected two cell lines in which to study the function of CBFβ; one that has high levels of CBFβ expression and one that has low expression. We chose to focus on the highly metastatic MDA-MB-231 cells, which have high levels of both Runx2 and CBFβ and also another basal breast cancer cell line, Hs578T cells which have high Runx2 expression but low expression of CBFβ.

CBFβ expression was transiently knocked down in the MDA-MB-231 cells using two individual siRNAs against CBFβ, achieving over 90% decrease in expression (p < 0.0001 for both siRNAs) as shown by western blotting and RTqPCR (Fig. 4.2.A). Similarly, two siRNAs were used to knockdown the protein and mRNA levels of Runx2 by 50% (p = 0.001, p <0.0001) (Fig. 4.2.B). Non-specific siRNAs were used as negative controls for both CBFβ and Runx2.

Expression analysis of key downstream targets of the Runx2/CBFβ complex when the levels of either CBFβ or Runx2 were significantly down regulated was undertaken using RT-qPCR. The genes examined were MMP9, MMP13, RankL, SatB1, BgLap1 and PTHrP. This cohort includes gene that are involved in the maintenance and development of the mammary gland (PTHrP); genes that control bone metabolism and homeostasis (SatB1 and BgLap1); and also genes that contribute to breast cancer growth and disease progression (MMP9, MMP13 and SatB1) (Duffy et al., 2000; Pratap et al., 2005; Pratap, 2006 ; Shore, 2005).

Knocking down the expression of CBFβ or Runx2 in MDA-MB-231s reduces the expression of these keys target genes, however Runx2 levels are unaffected when CBFβ is knocked down and the same is true for CBFβ expression levels when Runx2 is knocked down (Figure 4.2.C). Mendoza-Villanueva and colleagues have also observed this pattern of expression (Mendoza-Villanueva et al., 2010) and it indicates that any downstream effects are not due to a reduction in expression of the entire Runx2/CBFβ complex.
complex but that these co-transcription factors also have critical individual roles in breast cancer.
Fig. 4.2. Deletion of CBFβ in MDA-MD-231 breast cancer cells by siRNA results in downregulation of key downstream targets

A) Western blot and RT-qPCR showing a 90% knockdown of CBFβ protein and mRNA levels in MDA-MB-231 cells transfected with CBFβ siRNA (CBFβ5 & CBFβ6 p <0.0001, Student’s unpaired t-test). Error bars represent SEM. B) RT-qPCR showing a 50% knockdown of Runx2 protein and mRNA levels in MDA-MB-231 cells transfected with Runx2 siRNA (Runx2A p <0.0001; Runx2B p <0.0001, Student’s unpaired t-test). Error bars represent SEM. C) RT-qPCR data showing downregulation of key downstream targets of Runx2/CBFβ in MDA-MB-231 cells as a result of CBFβ siRNA knockdown. Expression of BgLap1, MMP9 and MMP13 is significantly decreased (p <0.05, Student’s t-test) Error bars represent SEM.
**Down regulation of CBFβ reduces the metastatic potential of human breast cancer cells**

It has been shown that Runx2 regulates several genes that are implicated in the metastatic potential of breast cancer cells (Pratap et al., 2005; Pratap, 2006; Shore, 2005) and whilst there is some evidence to suggest that CBFβ is potentially required for invasion of breast cancer cells (Mendoza-Villanueva et al., 2010), our aim is to further characterise the role for CBFβ in breast cancer cell fate.

After validation, the CBFβ siRNA sequences were used to generate CBFβ shRNA constructs. MDA-MB-231 breast cancer cells were retrovirally infected with these constructs in order to generate three stable cohorts; two expressing different CBFβ shRNAs and one expressing a luciferase shRNA as a control. Validation of shRNA-mediated knockdown of CBFβ in the MDA-MB-231 cells was shown through western blotting and RT-qPCR as seen in Figure 4.3.A and B respectively.

In order to determine whether CBFβ is able to regulate the growth rate of breast cancer cells, the proliferation rate of these MDA-MB-231 cohorts was assessed using proliferation assays. The results of these assays showed that the two cell lines expressing the CBFβ shRNA underwent proliferation at a significantly slower rate than the cells expressing the non-specific luciferase as shown by the graph in Figure 4.3.C (p < 0.001).

Migration assays were performed using the xCelligence DP system to ascertain whether reducing CBFβ expression in MDA-MB-231 cells also affected the ability of the cells to migrate towards a chemoattractant. The data obtained showed, once again, that in the two cell lines containing CBFβ shRNA migration was significantly impaired compared to the luciferase shRNA control (p < 0.01) (Fig. 4.4).

The collective data from the proliferation and migration assays indicates a critical role for CBFβ as a regulator of breast cancer cell fate, not only complemented but also furthering previously described results from Mendoza-Villanueva and colleagues (Mendoza-Villanueva et al., 2010).
Fig. 4.3. Down regulation of CBFβ in MDA-MB-231 breast cancer cells leads to a reduction in cell proliferative capacity.

A) Western blot showing successful knockdown of CBFβ via stable shRNA integration into MDA-MB-231 cells. B) RT-qPCR results showing knock down of CBFβ mRNA levels in MDA-MB-231 cell (N=3, p = 0.032, Student’s unpaired t-test). Error bars represent SEM. C) Results from proliferation assays using MDA-MB-231 cells stably expressing CBFβ shRNA showing significantly decreased proliferation with a non-specific shRNA used as a control. A two-way ANOVA of N=3 assays resulted in the following: Time: F (8,162.5) = 83.48, p < 0.0001; Treatment: F (2,32.51) = 7.57, p = 0.0006; Interaction: F (16, 51.05) = 5.24, p < 0.0001. Error bars represent SEM.
Fig. 4.4. Down regulation of CBFβ in MDA-MB-231 breast cancer cells leads to a reduction in cell migratory capacity

Results from migration assays using MDA-MB-231 cells stably expressing CBFβ shRNA showing significantly decreased migration with a non-specific shRNA used as a control. A two-way ANOVA of N=3 assays resulted the following: Time: F (95, 127.6) = 61.08, p < 0.0001; Treatment: F (92, 10.53) = 21.88, p < 0.01; Interaction: F (190, 8.293) = 7.94, p < 0.0001. Error bars represent SEM.
**Overexpression of CBFβ increases the metastatic potential of human breast cancer cells**

We have now shown that knocking down CBFβ expression in MDA-MB-231 cells leads to a significant decrease in the ability of these cells to proliferate and migrate. There is currently no published data documenting the effects of CBFβ overexpression on breast cancer cell fate. In order to investigate this, we developed a CBFβ overexpression construct and retrovirally infected Hs578T breast cancer cells, which has constitutively low levels of CBFβ. Hs578T cells expressing the vector-only construct were used as a control. The overexpression of CBFβ compared to the vector only in the Hs578T cells was confirmed by both western blot and RT-qPCR analysis (Figures 4.5.A and B respectively).

In order to examine the effects of CBFβ overexpression on the growth rate of Hs578T cells, proliferation assays were performed using cells expressing either the CBFβ or vector-only construct. Overexpression of CBFβ led to an increase in the rate of proliferation when compared to the control cell line (p <0.05) (Fig. 4.5.C).

Migration assays were performed using the xCelligence DP system to determine if overexpressing CBFβ in Hs78T cells had any affect on the ability of the cells to migrate towards a chemoattractant. The results of these experiments showed that there was a substantial increase in the ability of the Hs578T cells overexpressing CBFβ to migrate when compared to the cells containing only the empty vector.

These results show a clear and novel role for CBFβ in the regulation of breast cancer cell fate, specifically in terms of proliferation and migration. As these two phenotypes are classic characteristics of breast cancer pathogenesis, it can be deduced that CBFβ also has a potential and important role in breast cancer metastasis.
Fig. 4.5. Overexpression of CBFβ in Hs578T breast cancer cells leads to an increase in cell proliferative capacity.

A) Western blot showing successful overexpression of CBFβ via stable integration into Hs578T cells. B) RT-qPCR results showing increased CBFβ mRNA levels in Hs578T cells (N=3, p = 0.001, Student’s unpaired t-test). Error bars represent SEM. C) Results from proliferation assays using Hs578T cells stably overexpressing CBFβ showing significantly increased proliferation compared to a vector only control. A two-way ANOVA from N=3 assays resulted in the following: Time: F (8, 61.46) = 73.47, p < 0.0001; Treatment: F (1, 20.70) = 13.43, p = 0.0104; Interaction: F (8, 4.785) = 5.72, p = 0.0006. Error bars represent SEM.
Fig. 4.6. Overexpression of CBFβ in Hs-578T breast cancer cells leads to an increase in cell migratory capacity.

Results from migration assays using Hs578T cells stably overexpressing CBFβ showing increased migration compared to a vector only control (N=4). Error bars represent SEM.
CBFβ affects the regulation of genes involved in mammary gland development and breast cancer

The results described in this chapter demonstrate that perturbation of CBFβ expression leads to altered metastatic phenotypes in breast cancer cells, clearly indicating that CBFβ is able to regulate breast cancer cell fate.

In order to elucidate a potential mechanism by which CBFβ may be able to control proliferation and migration of breast cancer cells, we performed microarray analysis on mRNA lysates from MDA-MB-231 cells containing CBFβ shRNA, Hs578T cells overexpressing CBFβ and their respective controls, using the GeneChip PrimeView Human Gene Expression Array (N=3 for each sample). Hierarchical clustering and gene expression profiling was performed to confirm that the levels of CBFβ were reduced in the MDA-MB-231 cells and increased in the Hs587T cells compared to their respective control groups (Figure 4.7.A and B). Gene expression profiling for the Hs578T cells did not show a clear increase in CBFβ expression levels even though cluster analysis showed clear separation of the two groups. This was mostly likely do to the probes not being specific enough, therefore independent RT-qPCR validation was performed and clearly confirmed significant overexpression of CBFβ in the Hs587T cells compared to the control group. CBFβ expression was clearly decreased in MDA-MB-231 cells expressing CBFβ shRNA but initial cluster analysis identified one of the luciferase shRNA samples as an outlier and it was therefore been removed from subsequent analyses.

Further analysis was performed using Garvan GenePattern LimmaGP (v20.0) in order to determine the number of differentially expressed genes in each experimental group. When CBFβ was overexpressed in Hs587T cells, a total of 725 of the 20548 genes in the array were significantly regulated by CBFβ (with an q-value < 0.05). Of these 725 genes, we found that 451 were significantly up regulated and 275 were significantly down regulated (Figure 4.7.C). In the opposing experimental conditions where CBFβ expression was knocked down in the MDA-MB-231 cells, we found that of the 20548 genes in the array 58 were significantly regulated by CBFβ (q-value < 0.05), with 29 genes significantly up regulated and 29 genes significantly down regulated (Figure 4.7.C). This data is evidence that CBFβ is responsible for differentially regulating genes in human breast cancer cells.
Fig. 4.7. Validation of experimental designs for microarray analysis and evidence that CBFβ differentially regulates gene expression in human breast cancer cells.

A) Expression profile for Hs578T cells containing the CBFβ or vector only construct indicates that the probes used in the array were not specific enough to detect an increase in CBFβ expression. Therefore RT-qPCR was used as further validation (N=3 for experimental group and control group).

B) Expression profile for MDA-MB-231 cells expressing CBFβ or luciferase shRNA shows reduced CBFβ expression in the experimental group, as confirmed by RT-qPCR analysis (N=3 for experimental group and N=2 for control group).

C) Table summarising the number of genes found to be differentially regulated by CBFβ.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs587T_CBFβ vs Hs587T_empty vector</td>
<td>725</td>
<td>451</td>
<td>275</td>
</tr>
<tr>
<td>MM231_shCBFβ vs MM231_control</td>
<td>58</td>
<td>29</td>
<td>29</td>
</tr>
</tbody>
</table>
Once the array was validated and it was clear that CBFβ was regulating genes involved breast cancer, the top fifty genes were collated for both the Hs578T CBFβ overexpression and MDA-MB-231 CBFβ knock down data sets. These results reveals that CBFβ regulates several genes are important for both mammary gland development as well as genes that have been implicated in breast cancer, including: components of both the WNT and Hedgehog signalling pathways; insulin, epidermal and fibroblastic growth factors; and ECM components (Figure 4.8.A & B). It is therefore clear that CBFβ regulates multiple aspects of breast development and carcinogenesis.

Interestingly, there was minimal correlation between the differentially regulated genes identified in the CBFβ overexpression samples when compared with the samples where CBFβ expression was knocked down. This dissimilarity is highlighted in Figure 4.9 where the data has been summarised to show that, of the 8 genes common to both studies, 7 were found to be oppositely regulated genes, 1 commonly upregulated and there were no genes that were commonly down regulated.

Comparisons were also made between data from this array and from a previous microarray undertaken by our lab, where MCF10a cells overexpressing Runx2 were compared to MCF10a containing the empty pMIG vector. Two separate comparisons were made; between the CBFβ and Runx2 overexpression data and also between the CBFβ knock down study and the Runx2 overexpression data. These analyses also showed little correlation in differential gene expression with 10 commonly regulated genes between the two overexpression studies and only 1 gene in the Runx2 overexpression versus CBFβ knockdown comparison (Figure 4.9). This lack of correlation indicates that CBFβ function is driven by the genetic landscape of individual breast cancers and highlights the heterogeneity of breast cancer, which ultimately contributes to the poor prognosis associated with certain tumour types.

These comparisons show that, whilst CBFβ is clearly regulating breast cancer at the gene level, the minimal correlation between experimental groups indicate that it is necessary to investigate the changes occurring at the gene-set level in order to fully elucidate how CBFβ affects breast carcinogenesis and metastasis.
Fig. 4.8. List of genes most significantly regulated by CBFβ

Microarrays performed on human breast cancer cells with altered levels of CBFβ demonstrated that CBFβ regulates genes involved in normal breast development and breast cancer. A) The top 50 most significantly differentially regulated genes in response to CBFβ expression in Hs578T cells. B) The top 50 most significantly differentially regulated genes in response to CBFβ down regulation in MDA-MB-231 cells.
Fig. 4.9. Comparison between the number of differentially expressed genes in relation to CBFβ and Runx2 expression.

A summary of the number of genes differentially regulated by Runx2 and CBFβ, showing that there is little correlation between the different experimental conditions at the gene level.

<table>
<thead>
<tr>
<th>Experimental Comparisons (A vs B)</th>
<th>Common</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
<th>Opposite</th>
<th>Unique (A)</th>
<th>Unique (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBFβ overexpression vs CBFβ knockdown</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>50</td>
<td>715</td>
</tr>
<tr>
<td>CBFβ overexpression vs Runx2 overexpression</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>87</td>
<td>715</td>
</tr>
<tr>
<td>CBFβ knockdown vs Runx2 overexpression</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>57</td>
<td>96</td>
</tr>
</tbody>
</table>
CBFβ regulated expression of genes set involved in breast cancer formation, progression and metastasis

At the gene level, there does not appear to be significant correlation of differentially regulated genes in response to CBFβ knock down, overexpression or Runx2 overexpression in regards to breast cancer. In order to further elucidate the role of CBFβ in the regulation of breast cancer cell fate we analysed the effects of altering CBFβ expression in human breast cancer cells at the gene set level.

Gene Set Enrichment Analysis (GSEA) was used to compare the molecular profiles of the sample data with published gene set databases in order to determine which gene sets were enriched in response to either CBFβ over expression or down regulation. For the purpose of this thesis, the sample data was compared to the “c2: curated gene sets”, which encompass published gene sets from pathway databases.

A comparison of the differentially regulated genes sets found as a result of either CBFβ down regulation or overexpression can be seen in the Cytoscape maps in Figure 4.10. In these maps, the outer circles represent CBFβ knockdown in MDA-MB-231 samples and the inner circles represent CBFβ overexpression in Hs578T cells. Each circle represents a different gene set, those in red are significantly upregulated and those in blue are significantly down regulated.

An overview of gene set expression can be seen in Figure 4.10.A, showing that between the two experimental conditions, CBFβ overexpression or down regulation, the majority of gene sets are oppositely regulated. Upon closer inspection of the Cytoscape maps, it is clear that, at the gene set level, CBFβ is involved in the regulation of several cancers, including numerous breast cancer-specific gene sets. In addition, it is clear that CBFβ is also involved in the regulation of many other gene sets that are essential to carcinogenesis and cancer progression, including; EMT pathways, ECM components, metastasis, integrin signalling, embryonic stem cells growth and cell cycle pathways (Fig. 4.10.B). This finding is highlighted in the GSEA plots in Figures 4.10.C & D, which also clearly show that reducing CBFβ expression in MDA-MB-231 cells let to an upregulation of these breast cancer gene sets whilst they were down regulated when CBFβ was overexpressed in Hs578T cells.
The opposite regulation of breast cancer, metastasis and cell cycle gene sets complements the functional assay data described and indicates a definite role for this transcription factor in breast cancer cell fate.
D) Fig. 4.10. CBFβ regulates gene sets that involved in breast development, carcinogenesis and metastasis.

Several genes sets involved in the development and progression of breast cancer are regulated by CBFβ. A) A Cytoscape map showing an overview of the major gene sets that are significantly up (red) or down (blue) regulated in response to CBFβ expression. The size of the circle increases with the number of genes being differentially expressed in a particular gene set. B) Differentially expressed gene sets that are directly related to breast cancer, cell cycle, embryonic development, EMT and EMC pathways have to been highlighted to emphasis the wide-spread effects of CBFβ perturbation. C) GSEA plots showing upregulation of gene sets related to breast cancer when CBFβ expression is knocked down in MDA-MB-231 cells. D) GSEA plots showing down regulation of gene sets related to breast cancer when CBFβ is overexpressed in Hs578T cells.
**CBFβ and Runx2 regulate genes involved in breast cancer and metastasis**

It is well established that the relationship between Runx2 and CBFβ is essential for the normal development of several systems and that the CBF transcriptional complex has been implicated in several diseases. To this effect, we compared the data from both the CBFβ microarrays and microarray data where Runx2 was overexpressed in MCF10a normal breast cells. Whilst we did not observe a significant amount of correlation at the gene level (Fig. 4.9), we see a marked increase in correlation between experimental groups when we interrogated that data at the gene set level.

Separate comparisons were made between the MCF10a cells where Runx2 was overexpressed (inner circles) and either the MDA-MD-231 cells where CBFβ was knocked down (outer circles) (Fig.4.11.A) or the Hs578T cells where CBFβ was overexpressed (out circles) (Fig.4.11.B).

The Cytoscape maps in Figure 4.11.A shows that the down regulation of CBFβ and the overexpression of Runx2 leads to opposite regulation of several gene sets involved in breast cancer. Interestingly, there are several instances where gene sets are only significantly differentially regulated in response to either CBFβ or Runx2 perturbation but not both conditions (white circles indicate no significant up or down regulation in response to the experimental condition). This supports the hypothesis that functions of CBFβ and Runx2 are not mutually exclusive.

The effects of overexpressing CBFβ and Runx2 was also assessed and showed a greater degree of similarity in the expression pattern of the gene set. It can be seen in Figure 4.11.B that the expression of majority of gene sets, including those involved in breast cancer, are significantly down regulated. A major exception is the upregulation of two gene sets that are specific to invasive breast cancer, which is in alignment with the data from this thesis and published studies suggesting that both CBFβ and Runx2 play a role in driving aggressive and metastatic phenotypes in breast cancer cells (McDonald et al., 2014; Mendoza-Villanueva et al., 2010; Owens et al., 2014).

Overall these comparisons highlight that Runx2 and CBFβ, both independently and in conjunction with each other, are involved in the regulation of genes essential for breast carcinogenesis, metastasis, cell cycle, EMT and multiple signalling pathways in the ECM.
CBFβ and Runx2 regulate genes involved in breast cancer progression, cell fate and metastasis.

A) Gene set comparisons between CBFβ knockdown and Runx2 overexpression show that a large subset of the gene sets involved in breast cancer are oppositely regulated as a result of these two experimental conditions. There are also a number of gene sets that are only significantly regulated by either Runx2 or CBFβ, indicating that their functions are not always mutually exclusive. The size of the circle increases with the number of genes being differentially expressed in a particular gene set. Red indicates up regulation and blue indicates down regulation.
Discussion

Transcriptional regulation of breast cancer cell fate has become an important aspect of breast cancer research and several important regulators of normal mammary gland development have been found to be implicated in cancer, including Elf5, ER, PR, WNT and Notch. Recent studies by our lab and others have now identified the osteogenic transcription factor Runx2 as being essential for mammary gland development during pregnancy (Owens et al., 2014) and its expression both correlates and contributes to invasive breast cancer cell phenotypes and tumourigenesis in vivo (McDonald et al., 2014; Owens et al., 2014). There has been little investigation into the role of CBFβ in breast cancer and as such, this study provides one of the first in depth interrogations of the role of this non-DNA binding component of the CBF complex.

Previous studies have identified increased CBFβ levels in the aggressive MDA-MB-231 breast cancer cell line and have indicated that CBFβ may contribute to the invasive capacity of these cells (Mendoza-Villanueva et al., 2010). We have now shown that CBFβ is expressed in a wide range of human breast cancer cell lines from each of the major subtypes. Unlike Runx2, CBFβ expression does not correlate with a particular subtype, which was not unexpected given that CBFβ is generally more ubiquitously expressed than the Runx genes (Adya et al., 2000; Hajra & Collins, 1995; Ogawa et al., 1993a; Wang et al., 1993).

This study however, definitively shows that CBFβ is able to influence the ability of breast cancer cells to proliferate and migrate, two of the most common hallmarks of cancer (Hanahan & Weinberg, 2000). Previous studies have shown that CBFβ inhibition leads to reduced invasiveness in MDA-MB-231 cells (Mendoza-Villanueva et al., 2010), but the effects of forced CBFβ expression have not been tested. This chapter addressed this issue and has now shown, using multiple cell lines, that knocking down CBFβ expression reduces the aggressive phenotypes of breast cancer cell lines and that the overexpression of CBFβ leads to an increase in both proliferation and migration. This data therefore suggests that CBFβ plays a role in controlling breast cancer cell fate.

In order to begin to understand the mechanisms by which CBFβ is able to regulate the metastatic potential of breast cancer cells, microarrays were performed using the cell lines in which CBFβ levels were either increased or decreased. At the gene level, the data from these arrays show that CBFβ is influencing expression of several genes that
are involved in critical pathways such as WNT and Hedgehog signalling pathways. Both the WNT and Hedgehog signalling pathways play an important role in the normal breast development, from embryogenesis to adulthood, and have been implicated in several cancers, including breast cancer (Howe & Brown, 2004; Kasper et al., 2009). CBFβ mediated regulation of these critical signalling pathways may indicate a potential mechanisms by which CBFβ is able to drive mammary cell fate decisions.

Furthermore, CBFβ was shown to regulate ECM proteins and growth factors that are required for normal mammary gland development and functionality have been implicated in breast cancer formation and progression including FGF12, IGFBP5, MEFG6 and CADM1. IGF and FGF signalling pathways in particular, are associated with the ability of breast cancer cells to colonise the bone function as a part of the “vicious cycle” of bone metastasis which also heavily relies on Runx2 expression (Roodman & Dougall, 2008; Steeg, 2006). The fact that CBFβ regulates members of these crucial signalling pathways may indicate it is acting independently or though Runx2 to promote breast cancer metastasis through regulation of IGF and FGF signalling.

This data provides further evidence that CBFβ is an important regulator of breast carcinogenesis and progression and that dysregulation of this transcription factor has severe implications on the genetic interactions of the breast.

Whilst we were able to identify downstream target genes of CBFβ that are involved in breast cancer, there was minimal correlation at the gene level between CBFβ overexpression, CBFβ knockdown or following Runx2 overexpression. Breast cancers are grouped into subtypes that correlate to hormone receptor status and mutations in genes such as P53 and BRCA, which ultimately leads to differences in genetic landscapes of individual breast tumours. Therefore, the minimal correlation observed between breast cancer cells with altered CBFβ levels suggests that CBFβ function is influenced by the heterogeneity that exists in breast cancer.

Therefore to further understand the influence of CBFβ control in the context of breast cancer, we interrogated the microarray data using Gene Set Enrichment analysis and Cytoscape software and found that a significant number of gene sets were oppositely regulated in response to CBFβ and Runx2 levels, including; Kegg Pathways in Cancer, Smid Breast Cancer Luminal A Up, Smid Breast Cancer Normal Like Up, Zhang Breast Cancer Progenitors, Biocarta ECM Pathway, Biocarta Met Pathway, Zhou
Cell Cycle and Pujana BrCa Centered Network. These gene sets identified as differentially regulated represent pathways and genes associated with multiple cancers, growth factors, stem cells, ECM components and cell cycle pathways. More importantly, a large number of these gene sets are specific to breast cancer formation, progression and metastasis. This analysis conclusively demonstrates that CBFβ is implicated in all aspects of breast cancer and an in depth analysis is required to determine the direct mechanism driving this regulation.

A previous study has indicated a role for CBFβ and Runx2 in the ability of breast cancer cells to mediate osteoblast differentiation through regulation of the WNT-antagonist sclerostin (Mendoza-Villanueva et al., 2011) and we have now confirmed through microarray analysis, that perturbation of CBFβ differentially regulates components of the WNT signalling pathway, such as β-catenin interacting protein 1. Together this data indicates that CBFβ may be regulating breast cancer cell fate through interactions with the WNT signalling cascade. Furthermore, a recent study in osteoblasts has shown that there is cross talk between WNT and TGFβ pathways that converges on Runx2 (McCarthy & Centrella, 2010), which is an interesting development as microarray analysis has also shown that CBFβ regulates several Smad proteins in breast cancer cells. Given our knowledge of Runx2 and CBFβ interactions, it is therefore possible that CBFβ is acting to stabilise and increase the DNA-binding affinity of Runx2 leading to transcriptional regulation of these crucial signalling pathways and subsequent dysregulation in the context of breast cancer.

CBFβ may also be directing breast cancer cell fate through control of integrins and other ECM proteins as we observed differential expression of gene sets involved in integrin signalling and the FAK pathway. It has been previously shown that FAK interacts with MMP9 to promote cell migration and invasion (Das et al., 2009) and SMAD3 interacts with Runx2 in order to regulate TGFβ-mediated expression of MMP13 in human breast cancer cells (Selvamurugan et al., 2004). Therefore, as CBFβ is able to regulate FAK signalling and TFGβ signalling through control of Smad protein expression it is evident that CBFβ plays an important role in directing breast cancer cell fate. Further analysis is required to determine the precise mechanism through which downstream targets of CBFβ are directly and indirectly involve in breast cancer progression and metastasis.
Overall we have demonstrated a novel role for CBFβ in the control metastatic phenotypes in breast cancer and it is clear though functional assays and gene expression analysis that CBFβ is acting to regulate cell fate decisions in breast cancer.
5. CBFβ is a key regulator of tumourigenesis and metastasis in the PyMT breast cancer model

Introduction

The data produced by this thesis has demonstrated exciting roles for CBFβ as a novel regulator of mammary gland development and breast cancer cell fate.

Over the last decade, a role for the Runx genes, specifically Runx2, has been established in mammary gland development (Blyth et al., 2005; Blyth et al., 2010; McDonald et al., 2014; Owens et al., 2014; Shore, 2005) and there is now mounting evidence suggesting that Runx2 is essential for the development and progression of metastatic breast cancer (McDonald et al., 2014; Mendoza-Villanueva et al., 2010; Owens et al., 2014) Runx2 has been found to be upregulated in all breast cancer subtypes but to a higher extent in the aggressive basal subtype, specifically the ER/PR/HER2 triple negative cancers (McDonald et al., 2014) indicating it may be crucial for regulating the metastatic potential of breast cancer cells.

Our lab has shown that overexpressing Runx2 in the MCF10a normal breast cells results in the induction of epithelial to mesenchymal transition (EMT) whereby these cells undergo morphological and genetic modifications consistent with breast cancer. Similarly, overexpression of Runx2 in the HC11 mouse mammary epithelial cells leads to an increase in both the proliferative and migrational capacity of the cells, which are classic hallmarks of cancer (Owens et al., 2014). We and others have also shown that reducing Runx2 expression in the aggressive MDA-MB-231 breast cancer cell line leads to decreased ability of the cells to migrate and invade (Mendoza-Villanueva et al., 2010; Mendoza-Villanueva et al., 2011; Owens et al., 2014).

We have confirmed this role for Runx2 in breast cancer cell fate and progression in vivo by crossing the Runx2−/− mouse on to the luminal breast cancer PyMT mouse and performing mammary transplants. This resulted in a clear increase in both the tumour-free survival and the overall survival rates of the Runx2 knock out mice as well as a significant decrease in the proliferation levels of hyperplastic regions of the Runx2−/− glands, as shown by reduced Cyclin D1 expression (Owens et al., 2014). This was the first in vivo study to show a clear role for Runx2 in breast tumourigenesis. These data are complemented by results from a separate study that demonstrated atypical pre-
neoplastic and hyperplastic regions of the mammary epithelium in aged Runx2 transgenic mice (McDonald et al., 2014). This study suggests that prolonged ectopic expression of Runx2 in the mammary gland leads to a re-structuring of tissue reminiscent of EMT-like changes (McDonald et al., 2014).

CBFβ is the binding partner of all Runx genes and we have hypothesised that it plays an important role in mammary gland development, breast cancer and subsequent metastasis. We have now described a novel role for CBFβ in the regulation of mammary epithelial cell proliferation during ductal elongation gland *in vivo* and in addition, we have demonstrated that altering levels of CBFβ expression can either impair or accelerate migration and proliferation of human breast cancer cells, indicating a critical and novel role for CBFβ in mammopoiesis and breast cancer cell fate.

The CBF complex is known to be important for development, stem cell homeostasis and also in human disease, particularly in leukaemias (Davis et al., 2010; Kurosaka et al., 2011). In the last three years there has been growing interest in the role of CBFβ in solid tumours, a role that is yet to be elucidated. It has now been shown that CBFβ may be involved in the malignancy of both ovarian and prostate cancers (Davis et al., 2010) and that it is may be required to support the invasive capacity of breast cancer cells in vitro (Mendoza-Villanueva et al., 2011).

Breast cancer is one of the most common cancers amongst women and whilst the prognosis for primary breast cancer has improved markedly over the last 20 years, the survival rate for patients with distal metastasis is less than 10% and it is this metastatic disease that contributes the mortality and morbidity of breast cancer. Therefore it is imperative to determine key players that are involved in the process by which breast cancer is able to spread and CBFβ is becoming an increasingly plausible candidate.

In order to extend upon the *in vitro* data already described in this thesis and by others, we generated a tumourigenic mouse model in which CBFβ was deleted solely in the mammary gland, resulting in the first *in vivo* study investigating the role of CBFβ in breast cancer progression.

This chapter aims to explore the potential role CBFβ in breast cancer cell fate in the context of tumour latency, overall survival and distal metastasis through the use of an *in vivo* breast cancer model.
**Results**

**CBFβ deletion in the mammary gland leads to increase in breast tumour latency**

We have now demonstrated that CBFβ may regulate breast cancer progression, as shown through altered migration and proliferation of human breast cancer cells in response to CBFβ expression. In order to build on this *in vitro* data and to further investigate the role CBFβ plays *in vivo*, we generated a tumourigenic mouse model with mammary-specific CBFβ deletion of CBFβ.

This model was generated by first crossing the previously described CBFβ$^{f/f}$; BLG-Cre$^{tg/+}$ mice (Chapter 3) on to the PyMT$^{19/+}$ luminal breast cancer model (Lin et al., 2003) resulting in CBFβ$^{f/+}$;BLG-Cre$^{19/+}$;PyMT mice. The CBFβ$^{f/+}$;BLG-Cre$^{19/+}$;PyMT mice were then backcrossed on to the CBFβ$^{f/f}$ model in order to generate both CBFβ$^{f/f}$Cre$^{+/+}$PyMT and CBFβ$^{f/f}$Cre$^{-/}$PyMT mice.

A longitudinal study was undertaken to compare the tumour-free survival time of CBFβ$^{f/f}$Cre$^{+/+}$PyMT mice compared to the CBFβ$^{f/f}$Cre$^{+/}$PyMT mice. The mice were examined three times a week for signs of mammary tumour development. A mouse was considered as having undergone tumourigenesis at the first incidence of a palpable tumour. The results of this study showed a significant increase ($p = 0.0002$) in tumour latency in the CBFβ$^{f/f}$Cre$^{+/+}$PyMT mice (n=22), with an average tumour-free survival of 83.8 days compared to only 68.7 days in the CBFβ$^{f/f}$Cre$^{+/}$PyMT control mice (n=23) (Fig. 5.1.).

This data demonstrates a novel role for CBFβ in the regulation of breast tumourigenesis *in vivo*. 
Fig. 5.1. Deletion of CBFβ in the mammary gland increases tumour latency in the tumourigenic CBFβ PyMT mouse model.

There is a significant increase in tumour latency in the CBFβ^{f/f}Cre^{+/+}PyMT mice when compared to the CBFβ^{f/f}Cre^{+/+}PyMT mice (p = 0.0002, Hazard Ratio = 0.25, Log-rank (Mantel Cox) test)
Deletion of CBFβ in the mammary gland leads to increased overall survival within a tumourigenic population

Deletion of CBFβ in the mammary gland leads to a significant increase in tumour latency in mice, indicating a role for CBFβ in the progression of breast tumourigenesis \textit{in vivo}. We next sought to ascertain whether CBFβ deletion in the mammary gland has an effect on the overall survival in the CBFβ\textsuperscript{f/f}Cre\textsuperscript{+/+}PyMT tumourigenic population.

Similar to the previous study; mice were examined for tumours and overall health three times a week. In addition, all palpable tumours were measured using digital calipers and these readings were used to calculate the total tumour burden of each mouse. Once a mouse had reached the ethical endpoint (a total tumour burden of 10% by weight) it was sacrificed and all tumours and organs of interest were harvested and processed for histological analysis.

The data demonstrated that CBFβ deletion resulted in significantly increased overall survival in the CBFβ\textsuperscript{f/f}Cre\textsuperscript{+/+}PyMT population when compared to the CBFβ\textsuperscript{f/f}Cre\textsuperscript{+/+}PyMT control mice ($p < 0.0001$). CBFβ\textsuperscript{f/f}Cre\textsuperscript{+/+}PyMT mice (N=22) survived for an average of 162.9 days compared to the CBFβ\textsuperscript{f/f}Cre\textsuperscript{+/+}PyMT mice (N=34) that only lived to an average of 123.6 days. This was an increased survival of almost 5.5 weeks in the CBFβ\textsuperscript{f/f}Cre\textsuperscript{+/+}PyMT animals (Fig. 5.2.).

We have now shown a novel role for CBFβ as a regulator of tumour progression and as a critical factor in the overall survival in mice with breast tumours.
Fig. 5.2. Deletion of CBFβ in the mammary gland increases overall survival in the tumourigenic CBFβPyMT mouse model.

CBFβ deletion leads to a significant increase in overall survival in the CBFβ^{+/+}Cre^{+/+}PyMT mice when compared to the CBFβ^{+/−}Cre^{+−}PyMT mice. (p < 0.0001, Hazard Ratio = 0.16, Log-rank (Mantel Cox) test).
Characterisation of the $\text{CBF}\beta^{\text{fl}}\text{Cre}^\text{tg/+} \text{PyMT}$ tumourigenic mouse model

Through the use of the $\text{CBF}\beta^{\text{fl}}\text{Cre}^{\text{tg/+}} \text{PyMT}$ mouse model, we have demonstrated a new role for CBF$\beta$ in the regulation of tumour latency and breast cancer progression in a luminal breast cancer context. To confirm that the phenotypes described were due to deletion of CBF$\beta$ in the mammary gland, western blotting was performed for CBF$\beta$ on mammary glands harvested from CBF$\beta^{\text{fl}}\text{Cre}^{\text{tg/+}} \text{PyMT}$ and CBF$\beta^{\text{fl}}\text{Cre}^{+/+} \text{PyMT}$ mice. The ensuing results show a clear deletion of CBF$\beta$ and activation of Cre in the CBF$\beta^{\text{fl}}\text{Cre}^{\text{tg/+}} \text{PyMT}$ animals (Fig.5.3.A). Furthermore, we have shown that Runx2 expression is also down regulated in conjunction with CBF$\beta$ in these mice.

$\text{CBF}\beta$ regulates breast cancer progression

We have now shown that deletion of CBF$\beta$ in the mammary gland increases both tumour latency and overall survival in mice with mammary tumours. To further characterise the effects of CBF$\beta$ deletion in a luminal breast cancer context, we performed cross-sectional analysis using a 14-week-old cohort of CBF$\beta^{\text{fl}}\text{Cre}^{\text{tg/+}} \text{PyMT}$ (N=8) and CBF$\beta^{\text{fl}}\text{Cre}^{+/+} \text{PyMT}$ (N=10) mice. Mice were euthanised at 14 weeks of age and mammary glands were harvested for whole mount and histological analysis.

Whole mount analysis of the mammary glands collected shows a clear reduction in the severity of tumourigenesis in the CBF$\beta^{\text{fl}}\text{Cre}^{\text{tg/+}} \text{PyMT}$ mammary glands to the CBF$\beta^{\text{fl}}\text{Cre}^{+/+} \text{PyMT}$ control mice. These glands were subsequently processed and sectioned for histological analysis where haemotoxylin and eosin staining highlights the decreased amounts of hyperplasia in the CBF$\beta^{\text{fl}}\text{Cre}^{\text{tg/+}} \text{PyMT}$ glands compared to the CBF$\beta^{\text{fl}}\text{Cre}^{+/+} \text{PyMT}$ glands (Fig. 5.3.C).
A)

<table>
<thead>
<tr>
<th>Protein</th>
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B)
Deletion of CBFβ in the mammary gland reduces the severity of breast tumourigenesis.

In a cross-sectional analysis, CBFβ^{f/f}Cre^{+/-}PyMT mice show less hyperplasticity compared to CBFβ^{f/f}Cre^{+/-}PyMT animals due to deletion of CBFβ in the mammary gland. A) Western blot showing a reduction in CBFβ levels, decreased Runx2 levels and Cre activation in CBFβ^{f/f}Cre^{+/-}PyMT mammary glands from 14-week-old mice. CBFβ deletion has been targeted only in epithelial cells therefore may still be present in other cells types, explaining the presence of CBFβ bands in western blotting B) Representative whole mount mammary gland images depicting the differences in tumourigenesis between CBFβ^{f/f}Cre^{+/-}PyMT and CBFβ^{f/f}Cre^{+/-}PyMT mice at 14 weeks of age. C) Haematoxylin and eosin staining on mammary glands depicting the differences in tumourigenesis between CBFβ^{f/f}Cre^{+/-}PyMT and CBFβ^{f/f}Cre^{+/-}PyMT mice at 14 weeks of age (Representative images of N=4 mice for each genotype).
**CBFβ regulates cancer cell proliferation in the PyMT mammary tumour model**

We have previously shown that CBFβ regulates proliferation of both mammary epithelial cells and human breast cancer cells. Given that tumourigenesis is a process mediated by the ability of cells to proliferate, we investigated the affect of CBFβ deletion on the proliferation of mammary tumours in CBFβ<sup>fl/fl</sup>Cre<sup>tg/+</sup>PyMT mice by IHC staining for the proliferative marker Ki-67 (Fig. 5.4.A).

We observed a significant difference (p <0.0001) in the number of Ki-67 positive cells, with 31.5% (±3.2) of Ki-67 positive cells in the CBFβ<sup>fl/fl</sup>Cre<sup>tg/+</sup>PyMT glands compared to only 4.4% (±1.7) of Ki67 positive cells in the CBFβ<sup>fl/fl</sup>Cre<sup>tg/+</sup>PyMT glands (Figure 5.4.B).

These results are similar to data observed in Chapter 4 where the rate of proliferation in human breast cancer cells was accelerated or reduced depending on CBFβ expression levels and indicates that CBFβ plays a key role in regulating breast cancer cell fate *in vivo* and *in vitro*.
Fig. 5.4. CBFβ deletion leads to a reduction in proliferating mammary cells in PyMT induced tumours *in vivo*.

This panel shows a significant reduction in proliferation in the mammary glands of the CBFβ^{f/f}Cre^{--}PyMT mice compared to the CBFβ^{f/f}Cre^{++}PyMT mice. A) Ki-67 IHC staining showing significantly less Ki-67 positive cells in the CBFβ^{f/f}Cre^{--}PyMT compared to the CBFβ^{f/f}Cre^{++}PyMT mammary glands from 14-week-old CBFβPyMT mice. B) A histogram quantitating the significant decrease of Ki-67 positive cells in the CBFβ^{f/f}Cre^{--}PyMT glands of 14-week-old CBFβPyMT mammary glands compared to the CBFβ^{f/f}Cre^{++}PyMT glands (p <0.0001, Student’s unpaired t-test). Error bars represent SEM (N=10).
Mammary specific CBFβ deletion reduces both the incidence and severity of distal metastasis in the PyMT breast cancer model

Proliferation and migration are two of the major hallmarks of cancer (Hanahan & Weinberg, 2000) and we have demonstrated that CBFβ regulates these two phenotypes in breast cancer cells in vivo.

In order to determine whether CBFβ deletion affected the metastatic potential of breast cancer in vivo, a post mortem was performed on the CBFβ^{loz}Cre^{tg/+}PyMT and CBFβ^{loz}Cre^{tg/+}PyMT mice in order to determine whether metastases had formed in any distal organs. The lungs are the most common sites of metastasis in the PyMT model but all major organs were examined including brain, liver and kidney. We found that only the lungs of these animals had been affected by metastatic disease and as such, were harvested for gross morphology and histological analysis.

We saw no difference in the incidence of lung metastasis, irrespective of size or severity, in response to CBFβ deletion with 78.8% of CBFβ^{loz}Cre^{tg/+}PyMT mice (N=33) presenting with lung metastases versus 76.2% of CBFβ^{loz}Cre^{tg/+}PyMT mice (N=21) (Fig. 5.5.A). Whilst there was no difference observed in the incidence of lung mets, there was a significant decrease in the severity of metastatic lung disease in CBFβ^{loz}Cre^{tg/+}PyMT mice compared to the control population (Figure 5.5.B-E).

Photographs of the affected lungs in Figure 5.5.B clearly show that in terms of gross morphology, the lungs of CBFβ^{loz}Cre^{tg/+}PyMT mice have substantially reduced metastatic disease than the CBFβ^{loz}Cre^{tg/+}PyMT mice. Both the size and number of the metastases are decreased in the CBFβ^{loz}Cre^{tg/+}PyMT mice. This phenotype was confirmed in Figure 5.5.C by haemotoxylin and eosin staining, confirming the decrease of the size and number of lung metastases in CBFβ^{loz}Cre^{tg/+}PyMT mice.

In addition to histological analysis, the number of lung metastases on each pair of lungs was counted before histological processing. These counts showed that lungs from a CBFβ^{loz}Cre^{tg/+}PyMT mouse had an average of 48 metastases, which is significantly lower than the CBFβ^{loz}Cre^{tg/+}PyMT mice with an average of 92 (p = 0.04) (Figure 5.5.E).

This data demonstrates a novel and critical role for CBFβ in the progression of breast cancer metastasis.
A)

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B)

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Fig. 5.5. **Mammary specific deletion of CBFβ results in decreased incidence and severity of lung metastasis in mice.**

CBFβ deletion in the mammary gland leads to decreased severity of metastatic lung disease in CBFβ<sup>Cre<sup>−/−</sup></sup>/PyMT animals. A) Table depicting the reduced incidence of secondary lung metastasis in CBFβ<sup>Cre<sup>−/−</sup></sup>/PyMT animals compared to CBFβ<sup>Cre<sup>−/+</sup></sup>/PyMT mice. B) Images showing the gross morphological differences between the lungs of CBFβ<sup>Cre<sup>−/+</sup></sup>/PyMT animals compared to the CBFβ<sup>Cre<sup>−/−</sup></sup>/PyMT animals. C) Haematoxylin and eosin staining of metastatic lungs show a decrease in the severity of metastasis in CBFβ<sup>Cre<sup>−/+</sup></sup>/PyMT mice compared to CBFβ<sup>Cre<sup>−/−</sup></sup>/PyMT mice. D) Histogram showing the severity of lung metastasis is significantly reduced in CBFβ<sup>Cre<sup>−/−</sup></sup>/PyMT animals compared to CBFβ<sup>Cre<sup>−/+</sup></sup>/PyMT animals (p = 0.04, Student’s unpaired t-test). Error bars represent SEM.
Discussion

CBFβ has a well-defined role in haematopoietic cancers, primarily leukaemias but recent studies have begun to explore the role of CBFβ in solid tumours, where it appears to have an oncogenic function (Davis et al., 2010). The data described in this chapter shows that deletion of CBFβ leads to increased tumour latency and overall survival in a breast cancer context, further supporting the hypothesis that CBFβ functions as an oncogene in solid tumours.

We have also demonstrated that expression of CBFβ is required for breast cancer progression and metastasis, as indicated by the decreased hyperplasticity and severity of metastasis observed in the CBFβ^{f/f}Cre^{+/-}PyMT mice. This data complements results from a Runx2 study undertaken by our lab that observed an increase in tumour latency and overall survival of mice when Runx2 is deleted in the mammary glands of PyMT mice (Owens et al., 2014).

It was not unexpected to see that the phenotypes observed though deletion of Runx2 or CBFβ were somewhat similar as they are binding partners and it is known that CBFβ acts to primarily stabilise Runx2 and increase the transcription efficiency of downstream targets (Ito, 2008; Qin et al., 2014; Yoshida, 2002). There was however a greater increase in the tumour latency period in the CBFβ^{f/f}Cre^{+/-}PyMT mice than in the Runx2^{-/-} model. This may be due to the fact that deletion of CBFβ expression negates the functional redundancy that can exist between the Runx genes (Blyth et al., 2005), meaning that Runx1 and Runx3 are not able to compensate for the function of Runx2, which they are able to do in the Runx2^{-/-} model.

There is also the potential that CBFβ deletion is having a greater effect on tumour-free and overall survival due to a Runx2-independent function. Recent studies have shown evidence that the functions of Runx2 and CBFβ are not always mutually exclusive and it has been suggested that the interaction between these two co-factors may be a potential target for therapeutics (Mendoza-Villanueva et al., 2010). Furthermore, it has been shown in both prostate and ovarian cancer, that knocking down CBFβ expression leads to dysregulation of cell signalling pathways that play a role in malignancy (Davis et al., 2010). It is therefore evident that CBFβ is able to regulate of tumourigenesis and progression in solid cancers, including breast cancer.
Previous studies reported that CBFβ might be necessary for breast cancer invasion in an *in vitro* context, indicating a role for CBFβ in the breast cancer metastasis (Mendoza-Villanueva et al., 2010). We furthered this knowledge by generating the CBFβ^lox/lox^Cretg/+PyMT and observed a significant reduction in both the size and number of lung metastases in response to CBFβ deletion. This finding recapitulates the *in vitro* data described in Chapter 3 that demonstrated a novel role for CBFβ in breast cancer cell fate regulation and together, this study provides the first detailed description of the role of CBFβ in breast cancer.

The only other link between CBFβ and breast cancer comes from three independent studies sequenced a large array of human breast tumours that identified CBFβ as one of the most frequently mutated genes in luminal breast cancer (Banerji et al., 2012; CGAN, 2012; Ellis et al., 2012). The implication of this data is clear, that further investigation into CBFβ is required in regards to its involvement in breast carcinogenesis.

The data from this chapter describes a novel role for CBFβ in breast cancer tumourigenesis, progression and metastasis in an *in vivo* context. This is a pivotal discovery because up until this point there has been no *in vivo* data to recapitulate the limited data observed in breast cancer cell lines.
6. General Discussion

Mammary gland development occurs in precise and unique stages in response to series of regulatory hormones and genes that act on a smaller subset of transcription factors, essential in the control of mammary cell fate (Hennighausen & Robinson, 2005). Regulators of cell fate are crucial for normal developmental and offer potential targets for the management and treatment of breast cancer. The overarching aim of this thesis was to determine a novel regulator of mammary cell fate in the transcription factor CBFβ and to determine its implications in breast carcinogenesis and progression. We have now shown a definitive role for CBFβ in the regulation of mammary epithelial proliferation during ductal elongation and provided conclusive evidence that CBFβ controls breast cancer cell fate both in vitro and in vivo. Deletion of CBFβ in the mouse mammary gland leads to significant increase in tumour latency and overall survival whilst also leads to a reduction in the severity of metastasis. These results confirmed in vitro data that showed significant change in the ability of breast cancer cells to migrate and proliferate in response to CBFβ expression levels.

Together these data provide the first insight into the role of CBFβ as a novel regulator of mammary gland development and breast cancer.

**CBFβ regulates mammary epithelial cells during ductal elongation**

The development of the mammary gland, from in utero to adulthood, is dynamic and complex in regards to both structure and signalling pathways. Considerable advancement has been made in regards to our current knowledge of the signalling pathways involved in the mediation of the genes and hormones that control the cyclical changes in the mammary gland that occur during sexual development and reproduction. The role transcription factors play in mammopoiesis however, is still under investigation (Visvader & Lindeman, 2003).

The foundations for this thesis were formed as a result of recent studies from our lab describing a novel role for Runx2 in regulation of luminal mammary progenitors, specifically during lobuloalveolar development (Owens et al., 2014). Runx2 represents one half of the CBF complex, which is an essential activator or repressor of transcription in both developmental systems and cancer, depending on the context (Tanaka et al., 1995). CBFβ constitutes the non-DNA binding element of this complex.
and binding to CBFβ is compulsory for all three Runx proteins for efficient transcription of downstream targets (Ito, 2008). The original aim of this study was to exploit the common requirement for CBFβ by the Runx proteins in order to remove the element of redundancy between these transcription factors and therefore gain a better understanding of the role of Runx2 in the mammary gland. This thesis has instead elucidated an exciting and novel role for CBFβ in the mammary gland, independent of Runx2.

Whilst CBFβ expression is generally ubiquitous, Runx protein expression is tissue-specific (Adya et al., 2000; Hajra & Collins, 1995; Ogawa et al., 1993a; Wang et al., 1993). This is the first study to demonstrate that CBFβ is expressed in the mammary gland, specifically in the epithelial compartment (Chapter 3). Furthermore, it is now clear that CBFβ is developmentally regulated throughout mammopoiesis, exhibiting the same expression profile as Runx2 (Chapter 3), providing the first evidence that CBFβ is involved in mammary gland development. Expression levels of CBFβ are highest during virgin development and relatively low during pregnancy and lactation, indicating the importance of CBFβ during development of the virgin gland.

CBFβ is involved in controlling the expansion of the ductal network at the onset of post-natal mammopoiesis through regulation of mammary epithelial cell proliferation (Chapter 3). CBFβ has been shown to be an essential regulator of cell fate in other developmental systems, including haematopoiesis and osteogenesis (Coffman, 2003; Huang et al., 1999; Nagata et al., 1999). Interestingly, CBFβ also controls the proliferation and differentiation of stem cells within these systems (Kurosaka et al., 2011), and may therefore be regulating mammary epithelial cell fate through a similar mechanism. This hypothesis is supported firstly by expression of CBFβ in the myoepithelial compartment of the mammary epithelium (Chapter 3), which is where mammary stem cells are derived (Kendrick et al., 2008). The strongest evidence supporting a role for CBFβ in mammary stem cell regulation is the difference in the phenotypes observed between the two CBFβ knockout mouse models used in this study. When CBFβ deletion was mediated by the BLG-Cre transgene, deletion occurred in more differentiated cells and no developmental defects were observed. In the inducible transplantation model where CBFβ deletion was driven by CreERT2, every mammary cell was targeted, including the stem/progenitor populations and as such a delay in
ductal elongation was observed, indicating that CBFβ may regulate the specification of the stem/progenitor cell populations. Furthermore, our lab has recently shown that Runx2 is required for the regulation of luminal progenitor populations throughout lobuloalveolar development (Owens et al., 2014) so it is clear that there is mounting evidence to suggest that the CBF complex as a whole, is crucial for stem/progenitor cell fate regulation in the mammary gland.

Interestingly, microarray analysis of CBFβ perturbation in breast cancer cells showed that CBFβ (Chapter 4) regulates the expression of several gene sets that encompass crucial signalling pathways associated with several aspects of mammary gland development and the regulation of stem/progenitor cells. Some of the major signalling pathways identified as differentially expressed in the microarray analysis include Hedgehog, WNT and FGF pathways, all of which are essential in mammary gland development breast cancer formation and progression. It has been shown previously that CBFβ functions to maintain stem cell populations and drives cell proliferation in tooth development through regulation of Fgf9 and Shh (Kurosaka et al., 2011) and it may therefore have a similar function in the mammary gland.

In order to fully elucidate if CBFβ is regulating mammary gland development through a stem cell mechanism it would be interesting to perform additional transplants, and then use mammary epithelium from these recipients in serial transplantation experiments. Serial transplants would allow us to determine whether or not the proliferative defect we believe exists in the mammary stem cells of CBFβ−/− glands, becomes compounded over time and would finally result in complete failure of ductal elongation.

The identification of a role for CBFβ in the regulation of ductal extension of the mammary epithelium provides further insight into how this crucial phase of mammopoiesis is regulated. The importance of fully understanding the mechanisms behind ductal extension and branching morphogenesis is highlight by the statistics that show almost 90% of human breast cancers originating in the ductal cells of the mammary epithelium. CBFβ and its downstream targets may therefore provide potential therapeutic targets, which would ultimately increase the prognosis and outcome for breast cancer patients.
A role for CBFβ in the regulation of breast cancer cell fate

Breast cancer is highly prevalent in society and whilst the statistics and prognosis are relative positive for those diagnosed at the primary stage, there is still a very high mortality and morbidity rate for those patients who present with distal metastases, indicating that further investigation into the mechanisms behind how these tumour cells are able to escape the mammary gland and colonise areas such as the brain, lungs and the bone.

Breast cancer is a heterogeneous disease and therefore individual tumours, even within the same patient, can be under the regulation of multiple hormones, oncogenes and transcription factors. The key to increasing the long-term survival rates of breast cancer lies in identifying these regulatory pathways and the factors that control them.

This thesis has aimed to identify a novel regulator of breast cancer cell fate by investigating the role of CBFβ, a transcription factor we have now shown to be essential for both the developing mammary gland and also breast tumourigenesis and progression both in vitro and in vivo. This is an exciting development as there has been little data linking CBFβ to breast cancer and only in an in vitro context.

CBFβ has been primarily known for its role in leukaemias, where it has been shown to be involved in translocation, dominant negatives events and formation of fusion proteins in conjunction primarily with Runx1 (Adya et al., 1998; Look, 1997; Lutterbach et al., 1999). There is also evidence that CBFβ is down regulated in gastric cancers along with Runx1 and 3 (Sakakura et al., 2005), indicating, a possible tumour-suppressor role for CBFβ in these cancers. However, one of the hallmarks of the CBF complex is that these transcription factors can act as both tumour-suppressors and oncogenes through the repression or activation of down stream targets depending on the context (Blyth et al., 2005).

Whilst there is almost a complete lack of data at present that describes a role for CBFβ in solid tumours, the evidence that does exist is indicative of an oncogenic function. It has been shown that knocking down CBFβ in prostate and ovarian cancer cell lines leads to inhibition of tumour growth when the cells are xenografted into mice (Davis et al., 2010). Furthermore, it has been shown through gene expression profiling, that several developmental and cell signaling pathways associated with malignancy are perturbed in these cells, indicating that CBFβ is potentially required for tumourigenesis in certain solid tumours (Davis et al., 2010). The data in this thesis also suggests an
oncogenic function for CBFβ as breast tumourigenesis was significantly delayed as a result of CBFβ deletion.

A previous study examining the role of Runx2 in breast cancer indicated that CBFβ might play a role in the invasive potential of breast cancer cells *in vitro* (Mendoza-Villanueva et al., 2010). We have extended upon this data and have now demonstrated that CBFβ is able to regulate the ability of breast cancer cells to proliferate and migrate *in vitro* (Chapter 4) and controls tumourigenesis and proliferation *in vivo* (Chapter 5). In addition, deletion of CBFβ leads to a significant reduction in the severity of lung metastasis in PyMT mice, demonstrating that CBFβ can also influence breast cancer progression (Chapter 5). This is the first study to investigate the role of CBFβ in an *in vivo* breast cancer model and clearly demonstrates that CBFβ regulates essential aspects of cell fate that drive aggressive and metastatic phenotypes that are attributed to poor prognosis in breast cancer.

In order to deduce the potential mechanism behind CBFβ mediated regulation of breast cancer cell fate, we performed microarrays and subsequent differential gene and GSEA analysis on breast cancer cells where CBFβ expression was perturbed (Chapter 4). These analyses showed that CBFβ significantly regulates genes involved in the essential WNT and Hedgehog signalling pathways, which have well-documented roles in both mammary gland development and also breast cancer (Howe & Brown, 2004; Kasper et al., 2009). GSEA analysis showed that perturbed CBFβ expression correlated with the expression of numerous breast cancer specific gene sets including Smid Breast Cancer Luminal A Up, Smid Breast Cancer Normal Like Up, Zhang Breast Cancer Progenitors, Biocarta ECM Pathway, Biocarta Met Pathway, Zhou Cell Cycle and Pujana BrCa Centered Network. Within these gene sets, there are multiple genes significantly regulated by CBFβ that are components of signalling pathways involved in mammary gland development and breast cancer, including; Brca1 and 2, Smad1, 2,4 and 5, Ihh and β-catenin interacting protein 1. The signalling pathways represented here are Hedgehog, WNT and TGFβ, which have proven roles in mammary gland development and have been implicated in breast cancer and suggest that CBFβ regulates breast cancer cell phenotype potentially through these pathways.

In regards to CBFβ and WNT signalling, a previous study has shown that CBFβ regulates expression on the WNT-antagonist, Sclerostin, which may play a role in the
ability of breast cancer cells to mediated osteoblast differentiation and ultimately the spread of breast cancer to the bone (Mendoza-Villanueva et al., 2011). Furthermore, there is evidence of Runx2 dependent cross talk between WNT and TGFβ signalling pathways in osteoblasts (McCarthy & Centrella, 2010). The fact that CBFβ has now been shown to regulate several Smad proteins, it is possible that CBFβ also plays a role in the cross talk occurring between these pathways.

In addition, it has been shown that TGFβ is able to regulate expression of MMP9 in a Smad dependent manner in order to regulate breast cancer cell invasion (Wiercinska et al., 2011). The significance of this is that our lab has previously found MMP9 to be significantly regulated by Runx2 and although we did not observe any significant change in response to CBFβ expression, we do know that CBFβ increases the stability and DNA-binding affinity of Runx2. Therefore, it is possible that CBFβ is playing either a supportive role in this interaction through Runx2 binding or it is acting on the Smad proteins in a more direct manner.

As stated previously, the primary aim of this thesis was to identify novel regulators of mammary gland development and cell fate as it is a well-accepted paradigm that these factors are often perturbed in cancer. In order to fully understand and better treat breast cancer it is essential to understand its origins, a task made difficult due to the heterogeneity of this disease. We have now shown, for the first time that CBFβ transcriptionally regulates ductal extension in the mammary gland through controlling the proliferation of mammary epithelial cells and potentially, mammary stem cells. Furthermore, CBFβ has now been shown to control breast cancer cell fate both in vitro and in vivo, whilst differentially regulating several genes, gene sets and signalling pathways that have been implicated in breast cancer. Two of these pathways, WNT and Hedgehog, have been shown to regulate the existence of both multipotent mammary stem cells and luminal stem/progenitor cells respectively, the dysregulation of which has been shown to lead to breast cancer formation. It is therefore possible that CBFβ is acting to control mammary stem cell fate through cross talk between these pathways.

Whilst the exact mechanisms by which CBFβ regulates mammary gland development and breast cancer remains to be determined, the data produced in this thesis has conclusively shown a novel for CBFβ as a new regulator of mammary gland development and breast cancer cell phenotype.
Future Directions

This thesis has described several novel roles for CBFβ in the regulation of cell fate in both mammary gland development and breast cancer, however the mechanism behind this control is yet to be fully elucidated.

It is clear that CBFβ is acting to regulate the process of ductal extension in early mammary gland development through controlling the proliferation of mammary epithelial cells. The *in vivo* data and microarray data described in this thesis indicate that CBFβ may be involved in the regulation of mammary stem/progenitor cell populations and as mentioned previously, serial transplantation experiments would assist in confirming this hypothesis. Furthermore, it would be interesting to harvest cells from the CBFβ−/− transplants and assess the populations of stem and progenitor cell lineages using FACS sorting based on specific markers for these populations. This method would allow further interrogation of the mammary cell populations that are controlled by CBFβ.

In regards to the role of CBFβ in the direction of breast cancer cell fate, it would be informative to examine the affects of perturbing CBFβ levels in additional breast cancer cells lines, representative of all major subtypes, to confirm the phenotypes seen in this thesis. It would also be interesting to assess the ability of CBFβ to control the formation of tumour spheres in culture, an assay that would complement the proliferation and migration data described.

We have shown that CBFβ deletion is able to increase tumour latency and overall survival in mice with mammary tumours and that it also leads to a decrease in the severity of metastasis. In order to build on this data, it would be informative to use xenografting techniques to inject breast cancer cells with altered CBFβ levels into mice, either through the nipple or tail vein, in order to assess the ability of these cells to metastasise. Given the mounting evidence that the Runx2/CBFβ complex may be regulating the progression and colonisation of breast cancer cells to the bone, it would be advisable to use cell lines that are known to target the bone, such as MDA-MB-231 or 4T1.2 cells. These experiments would confirm that CBFβ is able to reprogramme breast cancer cell fate and regulate metastasis.

In Chapter 4 we show preliminary data showing the first evidence that CBFβ is expressed in human breast tumour. Further analysis should be performed using this
cohort of human breast tumors in order to ascertain whether CBFβ expression correlates with the size, stage and subtype of breast tumours as well as with diagnostic molecular markers such as ER, PR and HER2. This would allow insight into whether CBFβ can function as a diagnostic or prognostic marker for breast cancer patients and could potentially leads to the development of new treatments and targets.

Finally, additional analysis needs to be performed on the data produced by the microarrays performed on human breast cancer cell lines with perturbed CBFβ expression. The data so far confirms that CBFβ regulated numerous gene sets that are involved in many aspects of both mammary gland development and breast carcinogenesis and metastasis. There was minimal correlation at the gene level however, and further analysis is required to determine direct downstream targets of CBFβ, the validity of which could then be assessed by RT-qPCR and functional in vitro assays.

The principal paradigm behind this thesis is that many of the genes implicated in breast cancer have been shown to play important roles in the development of the mammary gland. In recent years, the transcriptional regulators of mammary cell fate have shown to play essential roles in the ability of breast cancer cells to proliferate, migrate, invade and colonise secondary sites, ultimately resulting in the poor prognosis for metastatic breast cancer. It is therefore essential that we continue to explore the factors that control cell fate decisions in mammary gland development in order to better treat and potentially find a cure for breast cancer.
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