CHAPTER 7:
GENERAL DISCUSSION

7.1 Summary

7.1.1 Expression patterns of *Bcl11* genes in embryonic and adult tissues

Bcl11a and *Bcl11b* exhibited specific spatial and temporal expression patterns during mouse embryogenesis. Using *lacZ*-tagged alleles, *Bcl11a* and *Bcl11b* expression was detected. At 10.5 dpc, both genes showed overlapping expression patterns in the pharyngeal arches. From 12.5 dpc, expression of both genes was detected in the craniofacial regions of the embryos. In addition, both genes were highly expressed in the CNS from 12.5 dpc and this overlapping expression pattern was maintained to adulthood. Taken together, the overlapping expression patterns of both genes in the craniofacial regions and CNS of the mouse suggest that these two genes may play similar and complementary roles in the development of these specific regions. Alternatively, they may also have cross-antagonistic roles in these regions and the relative levels of each Bcl11 transcription factor ultimately determine the phenotype of the region.

Interestingly, several regions of differential *Bcl11* expression were observed during embryonic development. *Bcl11a* was expressed in the heart and not in the lungs at 14.5 dpc while expression of *Bcl11b* was detected in the lung and not in the heart. Intriguingly, at 18.5 dpc, expression of *Bcl11a* was detected in the lungs whereas expression of *Bcl11b* was absent in the lungs. These dynamic and reciprocal expression patterns of *Bcl11* genes in the lungs suggest that they may be involved in different phases of lung development: while *Bcl11b* may be required at the early pseudoglandular stage (initial generation of lung structures), *Bcl11a* may then be required at the late terminal sac stage (terminal differentiation of lung alveolar). In addition, differential *Bcl11* expression was also detected in the fetal liver, thymus, developing bones, cartilage and mammary gland, suggesting that *Bcl11* genes might play different roles in development of these tissues.

Taken together, whole mount X-gal staining revealed that *Bcl11* genes are expressed in a plethora of tissues with regions of overlapping expressions (craniofacial

regions and CNS) and regions of differential expression (lungs, fetal liver, thymus and mammary gland).

7.1.2 Reciprocal expression of *Bcl11* genes in hematopoietic lineages

The generation of *Bcl11-lacZ*-tagged mice made it possible to determine *Bcl11* expression at a single cell level. By using $Bcl11^{lacZ/+}$ cells with Fluorescein di-β-D-galactopyranoside (FDG) in combination with other cell surface markers, expression of *Bcl11* genes in hematopoietic cells was determined. Expression of *Bcl11a* was detected in hematopoietic stem cells (HSCs) and in all other blood lineages such as myeloid, erythroid and lymphoid cells with the exception of T lymphocytes. In contrast, expression of *Bcl11b* was only detected in T lymphocytes and not in other blood lineages.

Maturation of T lymphocytes occurs in distinct stages characterized by expression of different cell surface markers (Rothenberg, 2007a). Examination of the immature CD4/CD8 double-negative (DN) thymocytes with additional markers (CD44 and CD25) revealed that *Bcl11a* was only expressed at the DN1 stage in ~16% of immature thymocytes but not in other T cells. In contrast, *Bcl11b* was expressed from the DN1 stage in ~24% of immature thymocytes and became highly expressed in all thymocytes from DN2 stage. The dynamic, reciprocal, and possibly mutually exclusive expression patterns of *Bcl11* genes during T cell development are summarised in Figure 4.19 (Chapter 4). These expression patterns are consistent with *Bcl11b*'s role in T cell development as demonstrated in the knockout mouse where T cell development was blocked at the DN3 stage (Wakabayashi et al., 2003b). Taken together, these results demonstrate that while *Bcl11a* is expressed in all blood lineages including HSCs, expression of *Bcl11b* is restricted only to T cell lineages. Importantly, expression patterns of *Bcl11b* in early T cell development imply that it may be the key transcription factor for T cell lineage commitment and maturation. Indeed, it has been suggested in a recent review that *Bcl11b* could be the primary regulator of T cell lineage commitment (Rothenberg, 2007a).

7.1.3 Dynamic expression patterns of *Bcl11* genes during mammary development

Bcl11a and *Bcl11b* exhibited unique and dynamic expression patterns in the mammary gland. Expression of both genes was detected in early embryonic mammary development. *Bcl11b* was expressed in the milk line at 10.5 dpc and from 12.5 dpc, its expression became localized in the mammary placodes and mammary buds. In contrast, expression of *Bcl11a* was detected in the mammary buds and mesenchyme only from 13.5 dpc. In the virgin gland, *Bcl11a* was expressed in terminal end buds (TEBs) and histological analysis revealed that this expression was specifically located within both the cap and body cells of TEBs. Conversely, *Bcl11b* was only detected in the neck region of TEBs within the cap cell layer which goes on to form the basal/myoepithelial layer of mature ducts. In the mature virgin glands, *Bcl11a* was expressed in both the luminal and basal layers, while expression of *Bcl11b* was detected predominantly in the basal layer. In the luminal compartments of the virgin gland, *Bcl11a* expression was found primarily in the luminal progenitors ($CD24^{hi}CD49b^{+}$) and majority of these *Bcl11a*-expressing cells were the $Sca1^{-}$ ($ER\alpha^{-}$) luminal progenitors. On the other hand, only a small number of $Sca1^{-}$ ($ER\alpha^{-}$) luminal progenitors expressed *Bcl11b*.

During gestation, both *Bcl11* genes were up-regulated and *Bcl11a* was detected in all luminal lineages while *Bcl11b* was restricted to the basal layer of ducts. During lactation, only expression of *Bcl11a* was detected in luminal secretory cells. These results suggest that *Bcl11a* may be important for the luminal lineages while *Bcl11b* may be important for maintaining basal cell identity and/or suppressing luminal cell fate. In addition, the differential expression patterns of both genes during involution suggest that the *Bcl11* genes might play different roles during the involution phase. Collectively, these unique and dynamic expression patterns of *Bcl11* genes during mammary gland development suggest the possible important roles of these genes at different stages of mammary gland development.

7.1.4 *Bcl11* genes are critical regulators of lineage commitment in the mammary epithelium

Loss of *Bcl11a* and *Bcl11b* in the mouse embryos resulted in embryonic mammary defects. Deletion of *Bcl11a* led to defective embryonic bud formation and failure of regression of mammary buds in the male embryo while loss of *Bcl11b* caused the absence of the third pair of mammary buds and significantly affected formation of the other buds. These data demonstrated that *Bcl11* genes play important roles in normal embryonic mammary development. Conditional deletion of *Bcl11a* in the virgin glands led to profound defects in the bi-layer ductal epithelium where luminal and basal cells appeared to form a thin cellular layer within many areas of the ducts. Flow cytometric analysis showed that the *Bcl11a*-deficient epithelium exhibited a $CD24^{hi}CD49f^{+}$ profile (luminal fraction in the wild-type virgin gland). At the cellular level, loss of *Bcl11a* also led to a relative increase in $ER\alpha^{+}$ luminal cells and a dramatic decrease in Gata-3^{+} luminal cells. RT-PCR also demonstrated that loss of *Bcl11a* resulted in a decrease in luminal and basal markers. Furthermore, *Bcl11a* was shown to be essential for the maintenance of terminally differentiated luminal secretory cells. Conditional deletion of *Bcl11a* in the lactation gland led to severe lactational defects and the premature onset of involution. Taken together, these results show that *Bcl11a* is a key gene in mammary lineage specification and function.

Conditional deletion of *Bcl11b* caused precocious alveologenesis in the virgin gland, resulting in the formation of alveolar-like structures that produced β-casein milk protein. Interestingly, flow cytometric analysis of the *Bcl11b*-deficient mammary epithelium showed that these epithelial cells exhibited a $CD24^{+}CD49f^{hi}$ profile (basal fraction in the wild-type virgin gland). However, further analysis of these sorted *Bcl11b*-deficient $CD24^{+}CD49f^{hi}$ cells showed that they expressed luminal markers such as *β-casein*, *NKCC1*, *Notch1*, *Notch3* and *Gata-3*, indicating that there was a basal to luminal lineage switch in these basal cells. These data demonstrate that *Bcl11b* maintains basal identity and suppresses the luminal lineage. *Bcl11b* also promotes the basal lineage as over-expression of *Bcl11b* was sufficient to induce the expression of basal cell specific genes such as *CK14*, *p63* and *SMA* in KIM2 mammary epithelial cells. Additionally, increased levels of *Bcl11b* in luminal progenitors were associated with a dramatic

reduction in luminal mammary colony-forming-cell (Ma-CFCs) capabilities in *Stat6*$^{-/-}$ knockout mice. Taken together, *Bcl11b* is critical for the maintenance of basal identity in the mammary gland.

These results clearly demonstrate that both *Bcl11a* and *Bcl11b* are critical regulators of lineage commitment in the mammary gland and that levels of each of these transcription factors are crucial for ensuring proper mammary development and functionality.

7.1.5 *Bcl11* genes are connected to networks of mammary regulators

Mammary gland development is a highly co-ordinated series of events that is regulated by both systemic hormones and local growth factors (Hennighausen and Robinson, 1998, 2005). Recent studies have shown that transcription factors are also critical for lineage commitment in the mammary epithelium (Asselin-Labat et al., 2007; Khaled et al., 2007; Kouros-Mehr et al., 2006; Miyoshi et al., 2002). Developmentally important pathways such as Notch, Wnt and Hedgehog (Hh) have critical roles in the mammary gland. Our lab has previously shown that the canonical Notch signalling pathway was over-expressed in the *Bcl11a*-deficient T cell leukaemia (Liu et al., 2003b). Consistent with high levels of *Notch1* in the *Bcl11a* mutant T-cell leukemia, loss of *Bcl11a* in the virgin and lactation mammary glands resulted in increased *Notch1* expression. Activation of Notch signalling was confirmed by up-regulation of downstream targets of Notch signalling such as hairy-enhancer of split genes (*Hes*) in the *Bcl11a*-deficient lactation glands. In the *Bcl11a*-deficient lactation gland, Notch activation might be responsible for the activation of Stat3, and loss of phosphorylated Stat5 (p-Stat5) based on recent biochemical studies (Kamakura et al., 2004; Nie et al., 2008). Interestingly, in the *Bcl11a*-deficient mammary epithelium in both the virgin and lactation glands, *Bcl11b* expression was significantly increased. The increased level of *Bcl11b* could be partially responsible for the phenotypes in the *Bcl11a* mutant glands. This could be investigated using *Bcl11a*- and/or *Bcl11b*-over-expressing mice.

It has been suggested in a previous study that Notch1 and Notch3 function in a similar way as over-expression of Notch1 and Notch3 using the mouse mammary tumour virus (MMTV) provirus results in impaired ductal and lobulo-alveolar development

during pregnancy (Hu et al., 2006). However, the results herein clearly demonstrate that Notch1 and Notch3 do not have similar functions in the lactation gland. The *Bcl11a*–deficient lactation mammary epithelium did not have Notch3 expression. In addition, a dramatic reduction in the transcript levels of *Jagged1*, *Delta-like-ligand 4* (*Dll4*) and *Hes7* was found in the *Bcl11a*-deficient lactation gland. Whether gene expression changes in these Notch components are functionally related or directly caused the *Bcl11a* mutant phenotypes remained to be determined. Nevertheless, these data revealed that not all the Notch pathway components behave in a similar way, which is consistent with recent studies (Raouf et al., 2008; Stylianou et al., 2006; Yamaguchi et al., 2008).

The JAK-Stat pathway plays essential roles during mammary gland development, particularly during gestation and lactation. Stat5 and Stat3 display reciprocal functions during gestation, lactation and involution. Stat5 is a key mediator of prolactin signalling and plays critical roles in the differentiation of lobulo-alveolar cells during gestation and lactation (Cui et al., 2004; Liu et al., 1997). Conversely, activation of Stat3 is a critical event during involution, resulting in the activation of the apoptotic pathway to facilitate the removal of redundant alveolar cells (Chapman et al., 1999). Hence, the physiological levels of phosphorylated Stat5 and Stat3 are critical determinants of the functional state of the mammary gland. As discussed in Chapter 6, deletion of *Bcl11a* resulted in premature onset of involution in the lactation gland where a loss of p-Stat5 positive cells and a dramatic increase in p-Stat3 positive cells were observed. As deletion of *Bcl11a* also led to dysregulation of Notch and JAK-Stat signalling pathways, and Hes binding Stat3 was shown to mediate crosstalk between Notch and JAK-Stat signalling (Kamakura et al., 2004), therefore it is possible that Bcl11a regulate the levels of Stat5 and Stat3 via the Notch signalling pathway that in turns determine the functional state of the mammary gland.

Gata-3 has recently been shown to be essential for the maintenance of luminal cell fate in the mammary gland (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Conditional loss of *Gata-3* in the mammary epithelium resulted in loss of luminal cells due to the block in differentiation of the luminal progenitors. However, the results herein indicate that loss of *Bcl11a* in the virgin gland resulted in an increase in $ER\alpha^+$ luminal cells concomitant with a loss of *Gata-3*$^+$ luminal cells, indicating a selective loss of $ER\alpha^-$

luminal cells. Additionally, conditional deletion of *Bcl11a* in the lactation glands did not affect levels of Gata-3, suggesting that Bcl11a and Gata-3 are likely to function in distinct epithelial populations. Taken together, these results suggest that Bcl11a but not Gata-3 is the key player in alveolar specification and maintenance.

7.1.6 *Bcl11* genes in mammary progenitors and lineage commitment

Bcl11a and *Bcl11b* were among the earliest genes that were specifically expressed in the mammary placodes, and were only expressed in small numbers of epithelial cells in the virgin gland. Loss of either gene in the virgin gland caused profound defects in the mammary epithelium. These results suggest that at least some of the *Bcl11a*- or *Bcl11b*-expressing cells might represent the multi-potent progenitors or even the mammary stem cells (Figure 7.1). Loss-of-function analysis in the virgin gland showed that *Bcl11a* was required for maintenance of both luminal and basal lineages, as deletion of *Bcl11a* resulted in down-regulated expression of both luminal and basal markers. In addition, *Bcl11a* was also required to maintain luminal progenitors; in particular, the alveolar progenitors as loss of *Bcl11a* in the virgin and lactation glands resulted in a dramatic reduction in *Elf5* levels (Figure 7.1). *Bcl11b*, on the other hand maintained basal identity, suppressed luminal cell differentiation, and also prevented precocious alveologenesis in the virgin gland. Loss- and gain-of-function analyses demonstrated that *Bcl11b* also promoted the basal lineage. *Bcl11a* continued to be necessary in the lactation gland where it maintained terminally differentiated luminal secretory cell fate. The phenotypes in the *Bcl11a*-deficient lactation gland led me to hypothesize that *Bcl11a* maintains secretory luminal cell fate by positively regulating Notch3 and negatively regulating Notch1 and Lif (Figure 6.14). Loss of *Bcl11a* would thus lead to Notch3 deficiency, *Lif* expression and activation of the canonical Notch signalling, resulting in Stat3-mediated apoptosis.

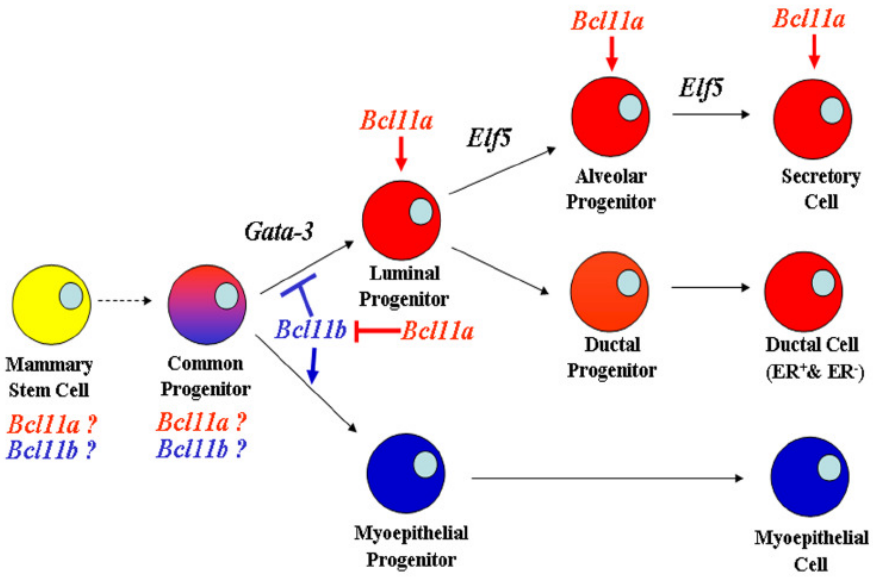

Figure 7.1. *Bcl11a* and *Bcl11b* are critical regulators for the development of mammary epithelial hierarchy. A working model of *Bcl11* genes in mammary epithelial lineage specification and maintenance. *Bcl11a* and *Bcl11b* could be expressed in the proposed multi-potent mammary progenitors or stem cells as deletion of these two genes in the virgin gland results in dramatic phenotypes. *Bcl11a* is also essential for the maintenance of the terminally differentiated lobulo-alveolar cells. *Bcl11b* promotes the basal lineage and maintains the basal identity by inhibiting the commitment of progenitors to the luminal lineage. Red – luminal cells; Blue – basal cells.

7.2 Significance

7.2.1 Novel roles of *Bcl11* genes in lineage commitment

The mammary phenotypes observed in the *Bcl11* knockout mice are striking and identify *Bcl11a* and *Bcl11b* as two new key transcription factors implicated in mammary development and lineage commitment. The data presented here indicate that Bcl11a and Bcl11b, together with Gata-3 and Stat6 are transcription factors that are required for mammary lineage commitment. Most importantly, *Bcl11a* and *Bcl11b* not only provide key functions in various epithelial progenitor compartments, they also serve as additional molecular markers for these progenitors. *Bcl11a* and *Bcl11b* are also required in lineage specification in lymphocyte development, suggesting that genetic control of mammary and lymphocyte development is conserved. Characterization of additional transcription factors implicated in lymphocyte development should uncover additional transcription factors that are critical to mammary lineage commitment.

7.2.2 Implications for tumour development

Human breast cancers have heterogeneous pathologies with diverse molecular profiles, therefore no single dominant pathway or histological presentation has emerged (Stingl and Caldas, 2007). Recent gene expression profiling using microarray analysis has offered an alternative to the classical histopathology for the classification of human breast cancers which clusters breast tumours into at least five reproducible subtypes: luminal A, luminal B, ERBB2, basal and normal-like (Perou et al., 2000; Sorlie et al., 2001). Comparing this molecular classification with the traditional histopathological analyses revealed some correlations and discrepancies between the two methods in the classification of breast tumours (Perou et al., 2000). In order to appreciate the new molecular classification, a thorough understanding of the cellular hierarchy of the normal mammary epithelium is required to establish the cellular origin of the tumours. Given the critical roles of *Bcl11a* and *Bcl11b* in the mammary gland, it is conceivable that they, or the pathways or networks that they are involved in, should have a causal role in breast cancer. Two *BCL11A* mutations have been identified in human breast cancer samples recently (Wood et al., 2007). However, no information on the histopathological or molecular subtype of these tumours is reported. The data presented in this thesis provide

evidence that *Bcl11a* is an important gene involved in breast cancer. In addition, loss of *Bcl11a* resulted in dysregulation of the Notch signalling pathway, a key signalling pathway that has been implicated in tumorigenesis, further linking *Bcl11a* and breast cancer (Efstratiadis et al., 2007).

Interestingly, luminal A tumours, which have higher expression of ERα and Gata-3 than luminal B tumours, have a better prognosis. This study has shown that loss of *Bcl11a* led to a relative increment in ERα$^+$ luminal cells concomitant with an absence of Gata-3$^+$ luminal cells. As *Bcl11a* is probably not expressed and functional in the Gata-3$^+$ and ERα$^+$ cells, it would be interesting to determine the expression status of *Bcl11a* in luminal A tumours. In addition, the molecular classification of human breast cancer samples with *BCL11A* mutations can be studied and the differences in the gene expression profiles compared to establish if this represents a distinct class of breast tumours from luminal A and luminal B. Additionally, using gene expression profiling to classify breast cancer tumours can theoretically provide insights into the ontological history of the tumour. Molecular profiling could also determine if the breast tumours arising from *BCL11A* mutations originated from mammary progenitor cells as expression of *Bcl11a* was detected in luminal progenitors. At present, no known *BCL11B* mutations are implicated in human breast tumorigenesis. As *Bcl11b* is predominantly expressed in basal cells, it would be interesting to investigate expression status of *BCL11B* in the basal-like breast cancer subtypes which are ERα$^-$ and PR$^-$ (progesterone receptor) and generally more aggressive and resistant to therapies.

7.3 Future experiments

The results presented in this thesis demonstrate that *Bcl11a* and *Bcl11b* are critical regulators of lineage commitment in the mammary epithelium. Both genes are required for normal mammary gland development and *Bcl11a* is also essential for maintenance of differentiated luminal secretory cell fate. Hence regulating the levels of both genes is crucial to the development of a functional mammary gland. The expression and phenotypic analyses also suggest that both of these genes are expressed in mammary progenitors and possibly in mammary stem cells (MaSCs). Future work is required to address the roles of *Bcl11* genes in the mammary stem and/or progenitor cells. In

addition, the molecular mechanisms underlying the phenotypes in the *Bcl11* knockout mice involved dysregulation of the Notch and JAK-Stat pathways. Future experiments are required to confirm if various components of the Notch and JAK-Stat pathways are direct targets of Bcl11 transcription factors and to find out whether other molecular pathways also play a role.

7.3.1 *Bcl11* genes and mammary stem/progenitor cells

One of the major findings in this thesis is that *Bcl11a* is expressed in luminal progenitors (both $Sca1^+$ putative ductal and $Sca1^-$ putative alveolar progenitors) and a small percentage of basal cells (where the MaSCs are believed to be located). To further confirm the expression of *Bcl11a* in mammary stem and/or progenitor cells, *Bcl11a*-expressing cells from $Bcl11a^{lacZ/+}$ mice can be sorted and transplanted in limiting dilutions into cleared mammary fat pads. The transplanted mammary gland can then be analysed to assess the contribution of *Bcl11a*-expressing cells to various mammary lineages. Expression of *Bcl11b* was first detected in the milk line of the embryos and predominantly in the basal fractions of the mature gland. These expression data suggest that *Bcl11b* could also be expressed in mammary stem/progenitor cells. To address this question, *Bcl11b*-expressing cells from $Bcl11b^{lacZ/+}$ mice can be sorted and transplanted in limiting dilutions into cleared mammary fat pads. The transplanted mammary gland can then be analysed to determine contribution of *Bcl11b*-expressing cells to various mammary lineages.

One hypothesis is that mammary progenitor cells that co-express both *Bcl11a* and *Bcl11b* have bi-potent capabilities (both luminal and basal). To test this hypothesis, each *Bcl11* gene can be tagged with a fluorescent protein [for example green fluorescent protein (GFP) and red fluorescent protein (RFP)]. Transgenic mice containing these tagged alleles can be crossed to obtain double heterozygotes. Finally, the *Bcl11a* and *Bcl11b* double positive mammary cells (GFP and RFP double positive cells) can be sorted using FACS and transplanted at limiting dilutions into cleared fat pad. Subsequently, the transplanted mammary gland can be analysed to determine the contribution of *Bcl11a* and *Bcl11b* double positive epithelial cells to the mammary lineages. Technically, the fluorescent proteins are much better than FDG for sorting out

live cells. These mice are therefore useful for detecting *Bcl11* expression in progenitor compartments.

To further address the roles of *Bcl11* genes in mammary stem/progenitor cells, the *Bcl11* conditional knockout mice can be crossed with the transgenic mice expressing Cre recombinase under the control of the keratin 5 (K5)-promoter that directs Cre expression to the basal cells of mammary epithelial (where mammary stem/progenitor cells reside) (Taddei et al., 2008). Mammary glands from *K5-Cre; Bcl11*$^{flox/flox}$ females can then be analysed for effects of Cre-mediated excision of *Bcl11* genes in the basal layer of mammary gland.

The *BLG-Cre; Bcl11*$^{flox/flox}$ females described herein, could also potentially be used to study the effects of *Bcl11* deletion in mammary luminal progenitors. I anticipate the BLG-Cre transgene to be expressed at sufficient levels in some non-secretory luminal cells, including the alveolar progenitors in the mice, to allow us to assay for the effects of *Bcl11a* deletion in the luminal progenitors. Therefore *BLG-Cre; Bcl11*$^{flox/flox}$ females that have undergone one round of pregnancy-lactation-involution development cycle would have *Bcl11* genes deleted in their luminal cells (differentiated and possibly progenitor cells). The effects of *Bcl11* deletion in luminal progenitors could then be assessed by *in vitro* mammary colony-forming cell assay.

Over-expression vectors of *Bcl11a* and *Bcl11b* described in this study could also be used to over-express *Bcl11* genes in mammary progenitors and these cells can then be transplanted into cleared mammary fat pad to assess the effects of over-expression.

7.3.2 Mammary fat pad transplantation of *Bcl11*-deficient mammary cells

The Cre recombinase in the *Cre-ERT2; Bcl11*$^{flox/flox}$ mice used in this study is driven by the *Rosa26* promoter, hence Cre recombinase is expressed in other cell types in addition to mammary epithelium (Hameyer et al., 2007). Following TAM injection to induce deletion of *Bcl11* genes, some *Cre-ERT2; Bcl11*$^{flox/flox}$ mice were found dead presumably because of the vital roles that *Bcl11* genes play in other tissues, hence hampering mammary gland analysis. Therefore, to minimise the effects of *Bcl11* deletion in other tissues, mammary epithelial cells from *Cre-ERT2; Bcl11*$^{flox/flox}$ mice could be

transplanted into cleared fat pads of wild-type mice. Deletion of *Bcl11* genes in the mammary epithelium of these transplanted mice can then be mediated by administration of tamoxifen and the mammary glands analysed for effects of loss of *Bcl11* genes. Alternatively, different populations of mammary cells such as $Sca1^+$ and $Sca1^-$ luminal progenitors can be sorted from *Cre-ERT2; Bcl11*$^{flox/flox}$ mice and Cre-mediated excision of *Bcl11* genes can be carried out *in vitro* by addition of tamoxifen. These *Bcl11*-deficient cells can then be transplanted into cleared mammary fat pads to assess the effects of deletion of *Bcl11* genes *in vivo*.

7.3.3 Mammary tumour formation in *Bcl11*-deficient glands

Mammary cells from *Cre-ERT2; Bcl11*$^{flox/flox}$ mice can be transplanted into wild-type mammary fat pad and Cre-mediated excision of *Bcl11* genes carried out before monitoring these transplanted mice for the development of mammary tumours. *Bcl11* heterozygous mice could also be crossed to other tumour-prone strains such as *p53* heterozygous mice. These double heterozygous mice can then be monitored to assess if loss of one copy of *Bcl11* gene could accelerate mammary tumour formation.

7.3.4 Microarray analysis of *Bcl11*-deficient mammary glands

The results from this thesis demonstrate that *Bcl11a* is a key transcription factor required to maintain terminal differentiated secretory luminal cell fate. Loss of *Bcl11a* in the lactation glands resulted in a lactation to involution transcriptional switch. The molecular pathways mediated by Bcl11a during lactation are still poorly understood. In order to gain insight into the effects of Bcl11a-mediated signalling on the lactation-involution switch, a genome-wide approach using microarray analysis could be performed. Gene expression profiles between lactation glands from *BLG-Cre; Bcl11a*$^{flox/flox}$, *BLG-Cre; Bcl11a*$^{flox/+}$ and wild-type mice can be compared and putative downstream targets verified by quantitative real-time PCR. Similarly, microarray analysis on sorted luminal and basal fractions from *Bcl11a* and *Bcl11b*-deficient virgin glands could also be carried out to ascertain the gene expression changes following *Bcl11* deletion and therefore identify putative targets that might be essential for maintenance of epithelial cell fate. These identified gene expression changes following deletion of *Bcl11*

genes can then be compared to those identified in this thesis and validated using quantitative real-time PCR. Components of the Notch signalling pathway would be of particular interest as dysregulation of this pathway was identified following loss of *Bcl11a*. Luciferase reporter assays for various known downstream targets of Notch signalling pathway can be performed. In addition, immunohistochemistry of *Bcl11a*-deficient glands using specific antibodies to activated Notch1 ICN should be performed to confirm activation of the Notch signalling pathways.

7.3.5 Direct targets of Bcl11 transcription factors

Putative targets of Bcl11a transcription factor have been identified in this study, including Notch1, Notch3, Lif and Stat5. Validation of these targets could be carried out using chromatin immunoprecipitation (ChIP). Next-generation sequencing technology combined with ChIP provides an efficient method to identify transcription factor binding sites on a genome-wide scale (Robertson et al., 2007). Conventional ChIP protocol would first be performed on wild-type mammary tissues using Bcl11 antibodies and the DNA fragments released after ChIP would then be sequenced using the Solexa sequencing technology. The resulting sequences obtained would be mapped back to the reference genome, where the most frequently sequenced DNA fragments formed peaks at specific genomic regions, indicating an enrichment of these DNA sites bound by the transcription factors. These DNA sequences would therefore represent the target sites directly bound by Bcl11 transcription factors.

7.3.6 Human breast cancer cell lines and tissues

There are many human breast cancer cell lines that are widely used to study signalling pathways implicated in breast cancer. These different human breast cancer cell lines are derived from different sources and genomic approaches have demonstrated that the gene expression profiles of these cell lines can be clustered into luminal and basal subtypes of breast cancers (Sorlie et al., 2001). Given the results obtained in this study, it would be interesting to determine the expression of *BCL11* genes in these different cell lines and determine if different subtypes of breast cancer cell lines exhibit differential expression of *BCL11* genes. Correlation of *BCL11* expression with the various subtypes

of cell lines could then be made and this would provide an indication whether expression of *BCL11* genes could be used as prognostic markers for different subtypes of human breast cancers. Similarly, tumour and normal breast tissue arrays can be used to study tumour-specific cellular localization of BCL11 expression, allowing the comparison of BCL11 expression between normal and pathological samples.

7.3.7 Quantification and statistical analysis

Quantification of the reduction in secondary branches following loss of *Bcl11a* in the virgin glands should be performed using microscopic images of whole mount carmine alum stained glands. Glands from three independent *Bcl11a*-deficient glands should be assessed and the numbers analysed using the Mann-Whitney U test. This statistical analysis is a non-parametric test for assessing whether two samples of observations come from the same distribution. This statistical analysis is important so that a conclusion can be drawn on the effect of changes in the values. Quantification of the changes in levels of transcripts (RT-PCR) and proteins (Immunoblot) should also be quantified using available softwares such as Image J (http://rsb.info.nih.gov/ij/). In addition, independent appropriate controls should be carried out for each set of experiments and it is unacceptable to use the same control in separate experiments.

7.4 Conclusions

In this study, two *Bcl11-lacZ*-tagged mice were generated using gene targeting technologies. Characterization of the spatial and cellular expression patterns of *Bcl11* genes using these *lacZ*-tagged mice revealed that these two genes exhibited unique differential expression patterns in the mammary gland. Subsequently, novel roles of both Bcl11 transcription factors in mammary development were demonstrated using loss- and gain-of-function analyses. While *Bcl11a* is important for normal mammary development and maintenance of terminally differentiated luminal secretory cell fate, *Bcl11b* is essential for the maintenance of basal cell fate. This study has identified both Bcl11a and Bcl11b as critical regulators of lineage commitment in the mammary epithelium and demonstrated that transcription factors that specify lymphoid lineages play important roles in mammary cell fate decisions.