Analyses of *Pax9* functions in the developing and adult mouse mammary gland

Liming Wang

2013

A Thesis Submitted for the Degree of Doctor of Philosophy at Newcastle University

Institute of Genetic Medicine

International Centre for Life

Newcastle University

DECLARATION

This is to certify that this thesis titled “Analyses of *Pax9* functions in the developing and adult mouse mammary gland” presented for the Degree of Doctor of Philosophy at Newcastle University, is my own work except where stated, and has not been presented at any other institutions.

Liming Wang

June 2013

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Heiko Peters, for giving me the opportunity to carry out my PhD in his laboratory, for his guidance and for always being very supportive. I am also grateful to Dr. Ralf Kist, for teaching me everything he knows in developmental biology, to Dr. Nakatomi Mitsushiro, for his kind help in the lab and also as a very nice neighbour to share Asian dinner, to all nice colleagues and friends within and beyond the institute, who made up my enjoyable three years in Newcastle, to my family, for their forever support and love.

ABSTRACT

The development of the mammary gland is strictly directed by hormones, local signaling, epithelial-mesenchymal cross talk, as well as participation of innate immune cells. On the other hand, the dysregulation of this orchestration may initiate breast cancer and facilitate its progression.

In our study *Pax9* was found, for the first time, significantly reversely correlated with breast cancer malignancy, being reduced or absent in human DCISs (96%) and invasive breast cancers (78%), as well as in *MMTV-Neu* and *MMTV-PyMT* induced mouse mammary tumours, while it is expressed in normal human and mouse mammary epithelium.

By a full-range expression investigation, using semi-quantitative RT PCR and immunohistochemistry, covering all developmental stages of mouse mammary gland, *Pax9* was found to be expressed in the ductal epithelium with a strict spatial-temporal pattern, with an expression peak at puberty. Reduction or deletion of *Pax9* expression, using *Pax9* hypomorphs and mammary gland-specific knockout mouse models, resulted in ductal branching delay during puberty, alveolar formation at the wrong position during pregnancy, disrupted epithelial cell apoptosis and engulfment of excess milk fat globulin during postlactational involution. Mammary ductal epithelial cell detachment, basement membrane disruption and tumour-like structure expansion have been found in the mammary glands of parous mice. Taken together, we found that *Pax9* functions in the process of mammary epithelial cell differentiation, basement membrane integrity, apoptosis and epithelial cell engulfment.

By immunohistochemistry and western blot analysis of the mammary gland during involution, we identified the *Stat* signaling pathway as a candidate downstream pathway affected by *Pax9* deficiency in the mammary gland, which may be responsible for apoptosis delay.

Expression microarray profiling of *Pax9* deficient and control mammary glands showed the increase of insulin growth factor binding protein 5 (*Igfbp5*, an essential regulator of mammary gland involution), monocyte to macrophage differentiation-associated (*Mmd*, immune and inflammation associated genes), and *MMP3* and *MMP12* (metalloproteinase) genes, and the decrease of inhibitor of DNA binding 2 (*Id-2*, functioning in mammary cells with low proliferation and invasiveness). Furthermore, myosin-related genes were strikingly up-regulated, which may be a cellular stress response to the milk stasis from impaired involution in the *Pax9* deficient mammary gland.

All these phenotypes we discovered in the mutants and molecular changes suggested by immunohistochemistry and gene expression profiling during involution, provided us with candidate networks regulated by *Pax9* in mammary gland development. Further elucidation of these clues may help us to understand the multiple pathways in which *Pax9* takes part in normal mammary differentiation, and the underlying mechanisms how its dysregulation may promote breast cancer formation and progression.

Abbreviation

ADAM	a disintegrin and metalloproteinase
AKT/PKB	*Akt/*protein kinase B
AP-1	activator protein 1
AR	androgen receptor
AREG	amphiregulin
BAX	bcl-2-like protein 4
BCL-2	B-cell lymphoma 2
BIM	bcl2-like 11
BMP	bone morphogenic protein
BSAP	B cell-specific activator protein
C/EBPß	CCAAT-enhancer-binding protein-*ß*
ChIP	chromatin immunoprecipitation
CSF-1	macrophages in colony stimulating factor-1
DCIS	ductal carcinoma in situ
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELF5	E74-like factor 5
EMT	epithelial to mesenchymal transition
ER	oestrogen receptor
ERα	oestrogen receptor α
ERβ	oestrogen receptor β
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGFRII	*Fgf* receptor II
FISH	fluorescent in situ hybridization
GATA3	GATA binding protein 3

GH	growth hormone
GR	growth hormone receptor
HANs	hyperplastic alveolar nodules
ID1	inhibitor of DNA binding 1
IGF1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IGF2	Insulin-like growth factor-2
IGFBP-5	insulin-like growth factor binding protein 5
JAK	Janus kinase
K14	cytokeratin 14
LEF-1	lymphoid enhancer binding factor 1
LIF	leukaemia inhibitory factor
MAPK	mitogen-activated protein kinase
MaSC	mammary gland stem cell
MASH1	mammalian achaete-scute complex homolog-1
MDF	MyoD family
MEC	mammary epithelial cell
MET	mesenchymal to epithelial transition
MFG-E8	milk fat globule epidermal growth factor (*EGF*) factor 8
MFGs	milk fat globulins
MMP	matrix metalloproteinases
MMTV	mouse mammary tumour virus
CRE	cre recombinase protein
MSX1	msh homeobox 1
MSX2	msh homeobox 2
NEO1	neogenin
NRG	neuregulin
NTN1	netrin-1

PAX	paired box protein
Pg	progesterone
PI3-K	phosphatidylinositol 3′-kinase
PR	progesterone receptor
Prd	Paired domain
PrlR	prolactin receptor
PTHrP	parathyroid hormone-related protein
PyMT	polyoma virus middle T antigen
RANKL	receptor activator of NF-κB-ligand
SHH	sonic hedgehog
SOCS1	suppressor of cytokine signaling 1
SPRY2	sprouty homolog 2
STAT	signal transducer and activator of transcription
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5
TAM	Tumour-associated macrophage
TBX3	T-box 3
TDLU	terminal ductal lobular units
TEB	terminal end bud
TGF	transforming growth factor
TGFβ	transforming growth factor beta
TIMP	tissue-inhibitor of metalloproteinase
TIMP1	tissue-inhibitor of metalloproteinase 1
TIMP3	tissue-inhibitor of metalloproteinase 3
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TWEAK	TNF-related weak inducer of apoptosis
WAP	whey acidic protein

WNT4	wingless-related MMTV integration site 4
WNT1	wingless-related MMTV integration site 1

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 5. Mammary gland-specific *PAX9*-deficiency severely impairs mammary gland involution

Chapter 6. *PAX9* is related to favourable prognostic factors of breast cancer

Chapter 7. Summary and discussion

LIST OF TABLES

Chapter 2. Materials and Methods

Chapter 5. Mammary gland-specific *Pax9*-deficiency severely impairs mammary gland involution

Chapter 6. *PAX9* is related to favourable prognostic factors of breast cancer

Chapter 1. Introduction

In evolutionary terms, the mammary gland is a rather young organ, which evolved 200 million years ago when mammals appeared on the earth. The mammary gland might be derived from apocrine-like skin glands of synapsids for the needs as a source of nutrients for the hatchlings (Capuco and Akers 2009). The development of mammary gland is strictly directed by hormones, local signaling and the epithelial-mesenchymal cross talk, while the deregulation of this orchestration is one characteristic of breast cancer.

1.1 Key stages in Mouse Mammary Gland Development

Mammary gland development is characterized by two distinct phases: the first phase is the linear development, starting from embryonic anlage formation, going through a postnatal quiescence, and then developing rapidly after the onset of puberty to fully fill in the fat pat at the end of puberty. The second phase is the cyclic development, starting with pregnancy, going through lactation and involution, comprising alveoli formation, milk secretion, postlactational cell apoptosis and tissue remodeling (Richert, Schwertfeger et al. 2000). See Figure 1.1

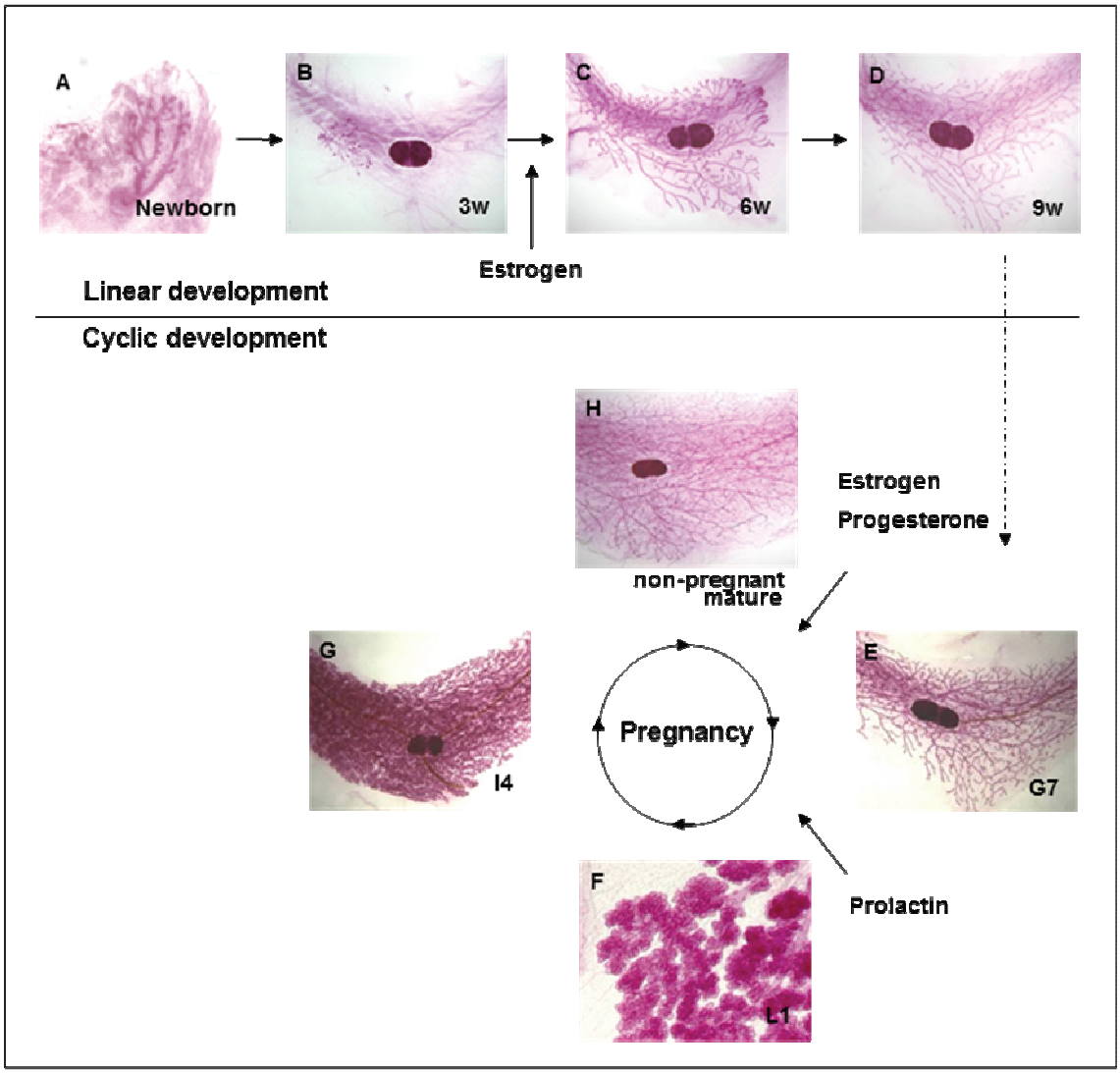

Figure1.1 Development of the mouse mammary gland

A brief mammary gland development atlas made by ourselves shows a linear development (A-D), mainly referred to ductal branching, from embryonic period to puberty, and a cyclic development (E-H), comprising alveologenesis during pregnancy, lactogenesis during lactation and involution after weaning. The development is regulated by hormones, such as ooestrogen, progesterone and prolactin, independently or synergistically. The mammary glands were taken from wild type C57BL6 female mice at indicated ages, whole-mount stained with Carmine-alum. Abbreviations: G7: genstation day 7; L4: lactation day1; I4: involution day 4.

1.1.1 Morphological change during embryonic development

Mammary gland development starts during embryonic period. Firstly, a ridge of the gland is elevated as milk line, then fragments into individual buds in specific regions lateral of the dorsal midline. In mice, at embryonic day 11, lens-like

placodes form and protrude slightly from the body wall. Within 1 day, bulb-shaped buds form from these placodes and invaginate the underlying mesenchyme. On embryonic day 15, epithelial cells at the tip of the bud undergo rapid proliferation, which leads the outgrowth of primary sprouts. The sprout elongates and bifurcates, finally creates a small ductal tree at birth, which is connected to the skin and the outside through the nipple to provide milk for pups during lactation. In males, around gestational day 14, the mammary rudiment regresses in response to androgen (Hens and Wysolmerski 2005).

1.1.2 Morphological change during puberty

Postnatal development forms the majority of the development of the mammary gland. Rapid and complex ductal development takes place during puberty. Mouse mammary epithelial structure remains quiescent until approximately 3 weeks of age when the ovaries begin secreting hormones. At this time, the terminal end buds (TEBs) form and ducts start to grow. TEB is composed of two main cell populations: the cap cells and the body cells, which are distinguishable by their morphology and expression of specific markers. The cap cells, located in the outer layer of the TEB, directly in contact with the stroma, are believed to be progenitors of the myoepithelial cells located in the out layer of the ducts. The body cells, organized in multiple layers within TEBs, are thought to be precursors of the luminal epithelial cells. Within the TEB, constantly high proliferation is conducted to fulfill the requirement of the mammary duct rapid growth, while apoptosis is also detected in some body cells, by which mechanism to form the lumen (Hinck and Silberstein 2005, Sternlicht, Kouros-Mehr et al. 2006). The ducts penetrate further into the fat pad to elongate, and new primary ducts develop by bifurcation. This development continues until 10–12 weeks of age, when the TEBs reach the edge of the fat pad and regress. By branching and bifurcation, the mammary gland forms an extensive ductal

network at the end of puberty.

1.1.3 Morphological change during pregnancy

With the repeated estrous cycle, alveolar buds form at tertiary branches. When entering the pregnant cycle, alveoli extensively develop and differentiate completely to be ready to secrete milk. At parturition, the alveoli begin copious milk secretion, which continues for about 3 weeks until pups stop suckling.

During the alveolar morphogenesis, the epithelial cell population increases rapidly by active proliferation to form abundant alveoli. From mid-pregnancy, epithelial cells undergo differentiation to prepare for milk secretion. Each individual alveolus is surrounded discontinuously by contractile myoepithelial cells. The discontinuous myoepithelium outside the alveolar epithelium allows the direct contact of luminal cells with the underlying basement membrane. Contact is required for complete lobuloalveolar differentiation guided by signals through extra cellular matrix (Fata, Werb et al. 2004). Around parturition, alveolar tight junctions close so that the colostrum moves into the alveolar lumen. After activation by parturition, the mammary gland goes into lactation stage and keeps synthesizing and secre*t*ing milk until weaning (Anderson, Rudolph et al. 2007). Along with the epithelial expansion, the adipocyte area reduces and the mammary gland is populated with alveoli and milk (Skopichev, Balakina et al. 1983).

1.1.4 Morphological change during involution

At weaning, the mammary gland begins a process of tissue remodeling, which involves apoptosis of mammary epithelial cells, clearance of dying cells and extra milk fat globulins (MFGs), and fat cell repopulation. This process consists

of two phases, i.e. a reversible phase, within the first 2 days of weaning, lactation is able to be reinitiated; and an irreversible phase, after 2 days of weaning, lactation is not able to be reinitiated and the gland regresses thoroughly. The process of involution takes approximately 2 weeks to complete. After that, the gland turns back to a pre-pregnancy state and is ready to serve another cycle of pregnancy, lactation, and involution.

During the first phase of mammary gland involution, apoptosis responds to the milk stasis immediately after weaning, apoptotic cells can be seen in the lumen of the alveoli, but remodeling is inhibited. In the second phase, apoptosis is accompanied by alveolar structure breakdown, stroma remodeling and adipocyte re-differentiation (Watson 2006). In the remodeling process, due to the large number of cells and debris that have to be removed, phagocytosis plays an important role, both by professional and non-professional phagocytes (Monks, Rosner et al. 2005).

1.2 Regulations in mammary gland development

1. 2.1 Regulations during embryonic stage

The development of the mammary gland during embryonic stage is hormone-independent, which occurs in mice lacking oestrogen receptor-α (*ER-α*), oestrogen receptor-β (*ER-β*), progesterone receptor (*PR*) and and prolactin receptor (*PrlR*). The embryonic mammary gland development is mainly guided by epithelial-mesenchymal interactions. Since derived from the ectoderm, it shares several common pathways with other skin-derived appendage, such as the tooth and hair follicle. Signaling factors, such as Wnt (Chu, Hens et al. 2004), fibroblast growth factor (*FGF*) (Mailleux, Spencer-Dene et al. 2002), parathyroid hormone-related protein (*PTHrP*) (Foley, Dann et al. 2001), and transcription

factors, such as *LEF-1* (van Genderen, Okamura et al. 1994), *TBX3* (Davenport, Jerome-Majewska et al. 2003), *MSX2* (Satokata, Ma et al. 2000), were found to take part in this process (Salomon and Lewis 2004).

Interestingly, *Hedgehog* signaling is not essential for mammary gland development and loss of *Hedgehog* signal in hair follicle induces hair follicle to mammary gland epithelial transition (Lewis and Veltmaat 2004). Also, *PTHrP* signaling is important to introduce the overlaying epidermis to form the nipple, in *PTHrP* or its receptor *PTH1R* knockout embryo, the nipple is not formed, and with its overexpression, the entire ventral surface of the embryo is transformed into nipple skin (Foley, Dann et al. 2001). In addition to development, because the embryonic mammary gland development is independent of hormones, the mechanisms at play here might illuminate the mechanism of hormone independent growth of breast cancers.

1.2.2 Regulations during pubertal stage

After birth, the mammary gland keeps quiescent until puberty when activated by oestrogen. Pubertal development is driven by a complex network of hormones that affects the expression of a variety of growth factors, signaling pathways, epithelial–mesenchymal interactions, extracellular matrix (ECM) remodellings, as well as innate immune system participation (Sternlicht, Kouros-Mehr et al. 2006).

Homonal and local regulation

Mammary branching requires growth hormone (GH), Insulin-like growth factor (*IGF-1*) and oestrogen, and their respective receptors. GH is the pivotal pituitary hormone. In the stroma, GH binds to GH receptor and induces *ER* and *IGF-1*, the latter binds to its receptor *IGF-1R* to stimulate proliferation and cell survival in

the epithelium. In the epithelium, *ER* induces amphiregulin (*Areg)*, an agonist of epidermal growth receptor (*EGFR*). *Areg* is cleaved by transmembrane metalloproteinase *Adam17*, and then binds to *EGFR* in the stroma. Activated *EGFR* induces multiple groups of downstream genes to promote duct growth and branching, for instance, *Fgf*, which promotes proliferation in the epithelium through *Fgf* receptor II (*FgfrII*), and metalloproteinase *MMP14* and *MMP2*, which degrade collagen in front of the TEB to facilitate the duct invading into the stroma. Negative mechanisms are also important for mammary gland morphorgenesis. *Tgf-β* limits epithelial proliferation, and may interplay with *Fgf* to form the branching pattern. *Timp1*, the inhibitor of metalloproteinase, limits the rate of cell division. In addition, Sprouty2 (*Spry2*) is also proposed to be an antagonist of *Fgf* to inhibit growth (Sternlicht, Kouros-Mehr et al. 2006).

Within the TEB: integrity, proliferation and apoptosis

The end bud integrity is required for ductal outgrowth and the maintenance of tissue architecture. *E-cadherin*, expressed by luminal cells that constitute the body of the end bud, and *P-cadherin*, expressed by cap cells that form the outer layer, mediate interactions between luminal cells and cap cells, and the cadherin-mediated cell-cell contact is responsible for cell proliferation. *Netrin-1*(*Ntn1*), expressed by luminal cells and then immobilized in association with ECM, maintains the integrity of the end bud by mediating contacts between cap and luminal epithelial cells through the *NTN1* receptor, *Neogenin* (*Neo1*) , which is expressed in a complementary pattern by overlying cap cells. The disruption to the contact leads to dissociation of cap cells and apoptosis. These contacts are required for robust forward growth of the mammary tree (Srinivasan, Strickland et al. 2003). *ErbB2* is another example for the regulation of the integrity of TEBs and normal ductal growth. *ErbB2*$^{-/-}$ mammary buds transplanted to a wild-type mammary fat pad result in less body cells, migration

of cap-like cells into the prelumenal compartment, and large luminal spaces, thus delay duct growth (Jackson-Fisher, Bellinger et al. 2004).

Fgf and *PTHrP* signalings are also known for their roles in duct branching through regulating TEB development. *Fgfr2* functions in the proliferation and invading of TEBs, but is not required in the mature ducts of the pubertal mammary gland. By *Fgfr2* inactivation in mosaic mammary epithelial cells tansplanted into cleared fat pad, *Fgfr2* null cells were found out-competed by neighboring *Fgfr2* heterozygous cells in the TEBs (Lu, Ewald et al. 2008). The impairment of ductal elongation caused by *PTHrP* is also associated with the TEB abnormality, where epithelial cell apoptosis increases with *PTHrP* overexpression and proliferation fails to respond to oestrogen and progesterone (Dunbar, Dann et al. 2001).

Lumen formation

Development of the luminal space is generated by three processes: apoptosis of centrally located cells, autophagy of centrally located cells, and epithelial remodelling (Reginato and Muthuswamy 2006). *Bim* regulates apoptosis in TEBs to form the lumen during mammary duct morphogenesis, the reduction of which results in lumen filling (Debnath, Mills et al. 2002, Mailleux, Overholtzer et al. 2007). Conversely, overexpression of either the receptor tyrosine kinase *ErbB2* induces ductal abnormalities by promoting development of multilayered epithelium that fills the luminal space (Debnath, Mills et al. 2002). Luminal filling may be the initial step of tumour formation in the mammary gland.

Maintenance of the lumen

The maintenance of luminal cell differentiation is important. *GATA3* plays an important role for luminal epithelial cell fate specification and maintenance. Loss

of *GATA3*, as a short-term effect, induces epithelial cell detachment from the basal membrane, as a long-term effect, induces epithelial cell apoptosis and degenerates ducts (Kouros-Mehr, Slorach et al. 2006, Asselin-Labat, Sutherland et al. 2007). Meanwhile, *GATA3* frequently decreases in invasive breast cancer (Albergaria, Paredes et al. 2009). Loss of *GATA3* is marked related to the progression from adenoma to early carcinoma and onset of tumour dissemination. Malignant progression occurred with an expanding *GATA3*-negative tumour cell population. Tumour was induced to differentiate and its dissemination was suppressed with the restoration of *GATA3* in late carcinomas. However, loss of *GATA3* is not sufficient for malignant conversion, since targeted deletion of *GATA3* in early tumours led to apoptosis of differentiated cells. Thus, *GATA3* maintains the epithelial organization and characteristics in the lumen, as well as regulates tumour differentiation and suppresses tumour dissemination in breast cancer (Kouros-Mehr, Bechis et al. 2008, Kouros-Mehr, Kim et al. 2008).

1.2.3 Regulations during pregnancy

Mammary alveologenesis and lactogenesis are characteristic events of pregnant mammary glands. The initial proliferative phase of alveolar morphogenesis is triggered by an increase in the level of serum prolactin (Prl) and progesterone (Pg). Prl and Pg synergistically regulate cell proliferation during early pregnancy (Neville, McFadden et al. 2002).

Progesterone receptor (*PR*) is essential for side branching and alveologenesis during pregnancy, with a heterogeneous expression pattern in the mammary gland. *PR* in the stroma is required essentially for tertiary side branching, and *PR* in the epithelium is for alveologenesis (Brisken, Park et al. 1998). Pg elicits proliferation by two different mechanisms, one is a small wave, by a cell-intrinsic

mechanism, through *Cyclin D1*, the other one is a large wave, by a paracrine mechanism, through the tumour necrosis factor (*TNF*) family member, receptor activator of NF-κB-ligand (*RANKL*) (Beleut, Rajaram et al. 2010).

Over-expression of *Wnt1* can rescue pregnancy-induced ductal side branching in *PR* knockout mice. *Wnt4* also stimulates epithelial ductal side branching downstream of *PR* during early pregnancy, in a paracrine fashion, but lobuloalveolar proliferation was not affected during the latter half of pregnancy with *Wnt4* absence. Thus there are other factors mediating proliferation in late pregnancy (Brisken, Heineman et al. 2000).

Prolactin signaling pathway plays a key role in promoting lactogenesis and milk secretion (Kelly, Bachelot et al. 2002, Hennighausen and Robinson 2005). Upon activation by prolactin, Janus kinase (*Jak*) activates signal transducer and activator of transcription 5 (*Stat5*, including *Stat5*a and *Stat5*b), then activated *Stat5* translocates into the nucleus to activate a series of target genes, including *RANKL, cyclin D,* caseins, *cyclin D*, *connexin 26*, *connexin 32, Socs1, Socs2, Socs3, Id1, Id2, and Igf2,* etc. These factors are known to be crucial for alveolar proliferation, milk protein production, secre*t*ory activation or the establishment of tight junctions. *Stat5* deficiency in the mammary gland profoundly leads to lobulo-alveolar development and lactation failure (Liu, Robinson et al. 1997). *Elf5* is a key regulator in alveolar differentiation upstream of *Stat5*. In *Elf5* conditional knockout mice, the mammary gland failed to develop alveoli and *Stat5* could not be activated (Choi, Chakrabarti et al. 2009). On the other hand, progesterone and *EGF* can inhibit lactogenesis in mammary epithelial terminal differentiation (Tanos, Rojo et al. 2012).

1.2.4 Regulations during postlactational involution and tissue remodeling

The first phase of involution: apoptosis

The first phase of involution is characterized by epithelial cell apoptosis but without tissue remodeling. Signaling pathways, which either promote, or delay involution and apoptosis, have been revealed by the use of genetically modified mice, particularly with tissue-specific gene deletion. For instance, deletion of the anti-apoptotic *Bcl-2* gene accelerates apoptosis, on the other hand, loss of the pro-apoptotic bcl-2-like protein 4 (*Bax*) protein delays involution. However, many of these factors are redundant or not essential, thus don't affect involution significantly (Schorr, Li et al. 1999) .

One essential pathway regulating involution is the *Jak/Stat3* pathway. Firstly, Jak is activated in response to cytokines and growth factors, *Stat3* is then activated by phosphorylation downstream *Jak*, specific *Stat* dimers form and translocate to the nuclei where they activate transcription of their target genes. While *Stat5* is important for lobuloalvoelar development and lactation (Liu, Robinson et al. 1997, Barash 2006, Vafaizadeh, Klemmt et al. 2012), *Stat3* is critical for the initiation of apoptosis and involution (Chapman, Lourenco et al. 1999). Inactivation of *Stat3* resulted in dramatic apoptosis repression and the first phase involution impairment. The cytokine leukaemia inhibitory factor (*LIF*) was found to be the activator of *Stat3* (Kritikou, Sharkey et al. 2003), while insulin-like growth factor binding protein 5 (*IGFBP-5*) and CCAAT-enhancer-binding protein-ß (*C/EBPß)* were found to be *Stat3* targets in the mammary gland (Thangaraju, Rudelius et al. 2005, Flint, Boutinaud et al. 2006). Prolactin could delay mammary gland involution by inhibiting cell loss and decreasing matrix metalloproteinase expression, but is not able to prevent cell loss in the mammary gland with constitutively activated *IGFBP-5* (Flint, Boutinaud et al. 2006).

Akt also plays a critical role during mammary gland involution, which acts as a survival/death signal node in mammary epithelium. Apoptosis can be suppressed in the presence of *Akt/protein kinase B* (*Akt/PKB*) (Schwertfeger, Richert et al. 2001, Wickenden and Watson 2010), while *Stat3* diminishes *pAkt* level by inducing the expression of its negative regulatory subunits of phosphatidylinositide 3-kinase (*PI(3) kinase*) (Abell, Bilancio et al. 2005).

The second phase of involution: tissue remodeling

Post 48 hours of weaning, the mammary gland transforms into the second phase of involution, accompanied by alveoli collapse and adipocytes refilling. Extra-cellular matrix (ECM) breakdown and plasminogen activation result in a second wave of apoptosis and tissue remodeling. *MMP*s are key regulators of these processes. *MMP2, MMP3* and *MMP9*, which are primarily expressed by the stromal cells, are up-regulated during involution to remodel the matrix. Removal of the matrix also induces apoptosis of the epithelial cells. When *MMP*s are activated at the beginning of the second phase of involution, epithelial cells that have gone through the first phase apoptotic wave will be induced to die by detachment-induced apoptosis. Thus the *MMP*s function in mammary gland involution both by initiating apoptosis and remodeling mammary gland architecture. The inhibition of *MMP*s is essentially demanded until 72 hours to ensure the reversibility of the first phase involution, which is provided by tissue inhibitor of metalloproteinases (*TIMP*s), the cognate inhibitors of *MMP*s. *TIMP3* is shown to fulfill this function by inhibiting *MMP2*. Involution is accelerated and the first phase reversibility is lost in *TIMP3*-deficient mammary glands (Hojilla, Jackson et al. 2011). In addition to the epithelial cell death in the involuting mammary gland, the surrounding adipocytes start to differentiate and repopulate the mammary gland. Plasmin and *MMP3* participates this process (Lund, Romer et al. 1996, Lund, Bjorn et al. 2000, Alexander, Selvarajan et al. 2001), however,

the role of adipogenesis and its regulation mechanism during mammary gland remodeling is poorly understood.

Phagocytosis takes an important part in the mammary gland remodelling process. With the large number of apoptotic cells and milk fat globules (MFGs) to be removed, autophagy and phagocytosis are carried out, both by professional and non-professional phagocytes (Monks, Rosner et al. 2005). The secreted glycoprotein milk fat globule epidermal growth factor (*EGF*) factor 8 (*MFG-E8*) is essential for the clearance of apoptotic cells and MFGs during the second phase involution. *MFG-E8* binds to apoptotic cells and MFGs by recognizing phosphatidylserine, a characteristic of apoptotic cells, thus bridges phagocytes and apoptotic cells or MFGs to be engulfed. *MFG-E8* deficiency severely impairs mouse mammary gland involution, where the mammary gland fills with excessive MFGs and the mammary ducts were dilated (Atabai, Fernandez et al. 2005, Hanayama and Nagata 2005).

Inflammatory mediators are activated very early during involution, while the influx of macrophages is not obviously seen until involution day 4. A balance between pro- and anti-inflammatory mediators is important to prevent overt inflammation (Clarkson, Wayland et al. 2004, Stein, Morris et al. 2004). Mastitis and inflammatory cells were seen in *Stat3* deficient mammary gland (Chapman, Lourenco et al. 1999), indicating that *Stat3* may not only function to moderate the death and survival balance but also control the pro- and anti-inflammatory response.

1.3 *PAX* Gene Family

1.3.1 PAX gene structure

The *PAX* family encodes a set of transcription factors, which is characterized by the possession of a Paired domain (*Prd*) and consists of nine family members. In addition to Paired domains that recognize specific DNA sequences, some of the family members have a whole or partial homeodomain that also recognize specific DNA sequences (Lang, Powell et al. 2007). Both domains have protein interaction activities. In addition, some *PAX* genes contain an octapeptide domain as well. According to whether containing an octapeptide region, and whether containing a complete or truncated a homeodomain, *PAX* genes are classified into four subgroups (*PAX1/PAX9, PAX2/PAX5/PAX8, PAX3/PAX7, PAX4/PAX6*). The structure of the combination of common and unique domains contributes to their particular regulation activities (Figure 1.2). *PAX9* lacks the homeodomain, but contains the octapeptide domain instead, which may confer its unique regulation properties different from other *PAX* genes containing homeodomains.

The importance of *PAX* genes for tissue development and cellular differentiation in embryos are well known. *PAX* genes regulate cell proliferation, survival, migration and cell-lineage specification, which attenuate when development is complete in most cases. However, in a few tissues, *PAX* genes either persist into adult life or are reactivated temporally, when these genes are needed for organ-specific regeneration or protection against stress-induced cell death. *PAX* genes are not only well demonstrated with their importance in regulating embryonic development, the roles in cancer also are emerging in these years (Robson, He et al. 2006, Lang, Powell et al. 2007, Wang, Fang et al. 2008, Li and Eccles 2012).

PAX family Group	Protein structure/domains Paired octapeptide homeodomain	Protein family member	Embryonic Expression Domain	Expression/Mutation in human disease
I		PAX1	Skeleton, thymus, 3rd/4th pharyngeal pouch	Jarcho-Levin Syndrome
		PAX9	Skeleton, thymus, teeth	Jarcho-Levin Syndrome, oligodontia, esophagus carcinomas
II		PAX2	Kidney, CNS	Hyperproliferative dysplastic kidney, renal hyperplasia, bladder and renal cancer, Coloboma Syndrome
		PAX5	B-cells, CNS	Lymphomas
		PAX8	Kidney, thyroid, CNS	Congenital hypothyroidism, thyroid carcinomas/adenomas
III		PAX3	Neural crest, CNS, somites/ muscle	Waardenburg Syndrome types I/III, melanoma, rhabdomyosarcoma
		PAX7	Neural crest, CNS, somites/ muscle	Rhabdomyosarcoma
IV		PAX4	Pancreas, gut	Diabetes
		PAX6	Pancreas, gut, CNS, eye	Aniridia, GI tumors, cataracts/ Peter's Anomaly

Figure1.2 *PAX* protein family and its correlation with embryonic development and human diseases

Adapted from Lang, Powell et al. 2007. *PAX* proteins are divided into four groups based on possession or absence of a Paired domain (blue rectangle), an octapeptide (green cylinder), and/or a homeodomain (red rectangle). Embryonic expression domains and expression/mutation related to human diseases are listed; CNS: central nervous system.

1.3.2 PAX genes in cancer

PAX genes in subgroups II *(PAX2, PAX5 and PAX8)* and III *(PAX3 and PAX7)* are frequently expressed in a wide range of cancer types, whereas subgroups I *(PAX1 and PAX9)* and IV *(PAX4 and PAX6)* are generally absent (Muratovska, Zhou et al. 2003, Robson, He et al. 2006). Tumour-associated expression of *PAX2* and/or *PAX8* have been observed in a variety of different tumour cell lines, including kidney, prostate, breast and ovary, and in Wilms tumour (Winyard, Risdon et al. 1996, Robson, He et al. 2006). High levels of *PAX5* expression have been reported in almost all non-Hodgkin lymphomas, forebrain-derived astrocytomas, neuroblastoma malignancy and medulloblastoma (Robson, He et al. 2006). *PAX3* and/or *PAX7* have been reported constitutively expressed in

embryonal rhabdomyosarcoma tumours, alveolar rhabdomyosarcoma, Ewing sarcomas, neuroblastomas, melanomas, and lung carcinomas (Bernasconi, Remppis et al. 1996, Robson, He et al. 2006). Rearrangements of *PAX5, PAX8, PAX3* and *PAX7* are associated with characteristic chromosomal translocations in specific cancers (Davis, Bennicelli et al. 1995, Barr 1997, Frascella, Toffolatti et al. 1998, Kroll, Sarraf et al. 2000, Poppe, De Paepe et al. 2005, Mullighan, Goorha et al. 2007, Eberhardt, Grebe et al. 2010). *PAX* genes function in promoting cell proliferation and survival, while knockdown of *PAX2, PAX3* and *PAX7* in cancer cells leads to cell apoptosis (Gnarra and Dressler 1995, Bernasconi, Remppis et al. 1996, Buttiglieri, Deregibus et al. 2004, He, Stevens et al. 2005). Transcriptional regulation studies revealed that *PAX8* can transcriptionally activate B-cell lymphoma 2 (*BCL-2*) proto-oncogene promoter in vitro, and *PAX2, PAX5* and *PAX8* are capable of inhibiting the tumor protein *p53* promoter in cell culture (Stuart, Haffner et al. 1995, Hewitt, Hamada et al. 1997), which proposed a mechanism of *PAX* gene regulation in tumour initiation or progression. However, there are no evidences showing that overexpression of *Pax* genes in vivo directly initiates cancer, except *Pax6* (Tremblay, Pituello et al. 1996, Wada, Holland et al. 1997, Yamaoka, Yano et al. 2000). Conversely, *PAX* genes in subgroups I and IV (*PAX6* and *PAX9*) normally indicate favourable outcomes, and *PAX6* even functions as a tumour-suppressor (Gerber, Richter et al. 2002, Ballestar, Paz et al. 2003, Zhou, Tan et al. 2003, Zhou, Wu et al. 2005). The different roles playing in cancer by *PAX* subgroup II/III and I/IV may be conferred by the structural basis of the four subgroups: the former two subgroups, each of which contains an octapeptide region and at least a partial homeodomain, are associated with malignant cancers, whereas the latter two subgroups, each of which possesses only one of these domains, predict good prognostics.

1.3.3 PAX genes in development

PAX genes play important roles during organogenesis, the expression of which is tightly regulated with their specific temporal and spatial *pattern* (Lang, Powell et al. 2007). *PAX* gene expression is activated primarily during embryonic development, but is switched off during latter phases of terminal differentiation. During organogenesis *PAX* proteins are expressed in target organs and tissues, for instance, *PAX1 and 9* in skeleton (Peters, Wilm et al. 1999), *PAX2, 3, 5, 6 and 7* in central nervous system (Jostes, Walther et al. 1990, Epstein, Vekemans et al. 1991, Walther and Gruss 1991, Adams, Dorfler et al. 1992, Gruss and Walther 1992, Tremblay, Kessel et al. 1995, Conway, Henderson et al. 1997, Schwarz, Alvarez-Bolado et al. 1999), *PAX2 and 8* in kidney(Poleev, Fickenscher et al. 1992, Dressler, Wilkinson et al. 1993, Narlis, Grote et al. 2007), *PAX5* in B-cells (Urbanek, Wang et al. 1994, Nutt, Morrison et al. 1998, Nutt, Heavey et al. 1999), *PAX8* in thyroid (Plachov, Chowdhury et al. 1990, Mansouri, Chowdhury et al. 1998), *PAX4 and 6* in pancreas(Sosa-Pineda, Chowdhury et al. 1997, St-Onge, Sosa-Pineda et al. 1997) and *PAX3 and 7* in skeletal muscle (Goulding, Lumsden et al. 1994, Relaix, Rocancourt et al. 2004, Relaix, Rocancourt et al. 2005). Mutations of *PAX* genes cause significant developmental abnormalities. Deregulation of *Pax2* in transgenic mice generates severe abnormalities in kidney, eye, ear and mammary gland, and *PAX2* mutations have been reported in patients with renal-coloboma syndrome (Dressler, Wilkinson et al. 1993, Eccles and Schimmenti 1999, Silberstein, Dressler et al. 2002, Burton, Cole et al. 2004). Compared to *Pax2* heterozygotes, compound heterozygous for *Pax2* and for *Pax8* showed impairment in nephron differentiation and branching morphogenesis of the metanephros (Narlis, Grote et al. 2007). Homozygous *PAX8* mutations lead to congenital hypothyroidism and thyroid aplasia (Macchia, Lapi et al. 1998, Mansouri, Chowdhury et al. 1998). *Pax3* mutation in mice results in hypopigmentation, defects of neurons, glial cells,

Schwann cells, inner ear, heart and trunk muscle (Tremblay, Kessel et al. 1995, Conway, Henderson et al. 1997, Buckiova and Syka 2004, Relaix, Rocancourt et al. 2004), and heterozygous *PAX3* mutation in human leads to Waardenburg syndrome (Tassabehji, Newton et al. 1994). *Pax7*-mutant mice die shortly after weaning, having face abnormality and impaired muscle regeneration (Mansouri, Stoykova et al. 1996, Seale, Sabourin et al. 2000). Homozygous *Pax5*-mutant mice show complete arrest of B-cell development, altered midbrain patterning and severe bone loss (Urbanek, Wang et al. 1994, Horowitz, Xi et al. 2004). Heterozygous *Pax6* mutations in mice give rise to the small-eye phenotype, while homozygous *Pax6*-mutant mice fail to develop eyes or nasal structures, and display abnormal pancreatic islet-cell morphology and aberrant forebrain patterning (Hill, Favor et al. 1991, Stoykova, Fritsch et al. 1996, Sander, Neubuser et al. 1997). In humans, heterozygous *PAX6* mutations are associated with a neurodevelopmental pathology, aniridia (van Heyningen and Williamson 2002). *Pax1* and *Pax9* double mutant fail in the formation of sclerotome derivatives (Peters, Wilm et al. 1999) , and *PAX9* haploinsufficiency in human is associated with oligodontia (Stockton, Das et al. 2000).

Though the importance of *PAX* genes in devemopment has been revealed profoundly, little is known regarding the specific mechanisms by which *PAX* proteins influence organogenesis. Main functions of *PAX* genes in development revealed by previous studies are anti-apoptosis, inhibiting terminal differentiation and cell lineage commitment, etc.

***PAX* genes suppress apoptosis**

An important role PAX genes playing in development is anti-apoptosis (Borycki, Li et al. 1999, Ostrom, Tang et al. 2000, Porteous, Torban et al. 2000, van Raamsdonk and Tilghman 2000). *PAX3* and P*AX3/FKHR* are able to directly

transcriptionally modulate the anti-apoptotic protein B-cell lymphoma-extra large (*BCL-XL*) in cells (Margue, Bernasconi et al. 2000), while *p53* loss of function can rescue neural tube defects in *Pax3*-deficient embryos (Pani, Horal et al. 2002). Increased apoptosis was observed in developing kidneys with reduced *Pax2* gene dosage (Dressler, Wilkinson et al. 1993, Porteous, Torban et al. 2000) and a significant inhibition of renal cyst growth due to increased cell death was also seen in *Pax2* heterozygotes. The human *p53* gene harbours a *PAX* binding site within its untranslated first exon, by which *p53* is directly transcriptionally regulated by *PAX* genes. *PAX2, PAX5 and PAX8* were shown able to inhibit p53 promoter and transactivation of a *p53*-responsive reporter in vitro (Stuart, Haffner et al. 1995).

PAX genes inhibit terminal differentiation

PAX genes have been found responsible for inhibiting terminal differentiation in some tissues. *PAX* gene expression is activated primarily during embryonic development and attenuate in adult tissues, though in some organs, for instance, oesophagus, tongue, muscle, pancreas, prostate and thyroid, *PAX* genes persist or reactivated with a restricted tissue-specific pattern (Peters, Schuster et al. 1997, St-Onge, Sosa-Pineda et al. 1997, Silberstein, Dressler et al. 2002, Jonker, Kist et al. 2004, Relaix, Montarras et al. 2006, Lang, Powell et al. 2007, Chen, DeGraff et al. 2010, Ozcan, Shen et al. 2011, Li and Eccles 2012). The continuous expression of *PAX* genes is not required at late stages of differentiation or terminal differentiation of cells. Persistent expression of *PAX* genes in terminally differentiated tissues is associated with a blockage in tissue differentiation and hyperplasia. Repression of *Pax2* is required for normal kidney development, whereas persistent expression of *Pax2* restricts the differentiation potential of renal epithelial cells. *PAX2* expression persists in the undifferentiated epithelium of human Wilms' tumour and deregulated *Pax2*

expression results in histologically abnormal and dysfunctional renal epithelium with properties similar to congenital nephrotic syndrome (Dressler, Wilkinson et al. 1993, Winyard, Risdon et al. 1996). *PAX5* codes for the transcription factor B cell-specific activator protein (*BSAP*), which is required for B-lineage commitment in the fetal liver and for progression beyond an early pro-B cell stage in adult bone marrow. Deregulated *PAX5* gene may interfere with the shut-down of *PAX5* transcription and with plasma cell differentiation, which contribute to tumourigenesis (Morrison, Nutt et al. 1998). *PAX3* expression is extinguished prior to activation of MyoD family (*MDF*) gene during myogenic differentiation in myogenic precursor cells (Williams and Ordahl 2000).

PAX genes are important for cell lineage commitment

PAX genes are important regulators in cell lineage commitment. *PAX2* and *PAX8* determine nephric lineage specification, and mouse embryos lacking both *Pax2* and *Pax8* are unable to form the pronephros or any later nephric structures (Bouchard, Souabni et al. 2002). *PAX3* is a nodal point in adult melanocyte stem cell differentiation, where *PAX3* functions to initiate a melanogenic cascade while acting downstream to prevent terminal differentiation (Lang, Lu et al. 2005). *PAX3* (Buckingham, Bajard et al. 2003) and *PAX7* (Seale, Sabourin et al. 2000) are both expressed by skeletal muscle stem cells, known as satellite cells, which are committed to the myogenic lineage yet remain undifferentiated at the periphery of adult muscle fibers. *PAX3* and *PAX7* are down-regulated in these cells following external stimuli, such as muscle injury, to initiate regeneration. *Pax6* is required for the multipotent state of retinal progenitor cells (Marquardt, Ashery-Padan et al. 2001). *Pax6* regulates the timing of retinal neurogenesis through repression of the neuron differentiation gene mammalian achaete-scute complex homolog-1 (*Mash1*) (Philips, Stair et al. 2005). *Pax5* is required for determining the B-cell lineage in immature lymphoid precursor cells.

In the absence of *Pax5* these cells differentiate to other cell types such as T-lymphocytes or natural killer cells (Nutt, Heavey et al. 1999).

The functions *PAX* genes play in development, such as anti-apoptosis, stem cell self-renewal and repressing terminal differentiation, may facilitate tumour progression in a similar mechanism. Thus understanding the role of *PAX* genes playing in development will help to illuminate the mechanism of tumour growth and malignancy and find theraputic targets.

1.4 *PAX9*

Paired box gene 9 (*Pax9*) belongs to the *PAX* gene family, and Homeobox gene super family. *Pax9* is located at chromosome 14q12 and is a transcription factor that regulates the expression of genes involved in mediating cell proliferation, apoptosis, and migration (Peters, Neubuser et al. 1998, Ogawa, Kapadia et al. 2006, Nakatomi, Wang et al. 2010). During embryonic development in the mouse, *Pax9* is required for the formation of thymus, parathyroids, limbs, secondary palate, teeth, and vertebral column (Neubuser, Koseki et al. 1995, Peters, Neubuser et al. 1998). In the adult mouse, *Pax9* expression is restricted to the tongue, oesophagus, salivary glands and thymus (Peters, Schuster et al. 1997). Dosage reduction of *Pax9* expression induces hypodontia and oligodontia in the mouse (Kist, Watson et al. 2005), and mutations of *PAX9* gene in human cause the autosomal dominant disorder of oligodontia (Peters and Balling 1999, Stockton, Das et al. 2000). *Pax1* and *Pax9* protein expression is significantly decreased in chondrocytes of the vertebral column of Jarcho-Levin Syndrome (JLS) and related disorders (Bannykh, Emery et al. 2003). In human oesophagus, *PAX9* has been reported as a marker of favourable prognosis in oesophagus cancer and decreased *PAX9* expression correlates with increased malignancy of the epithelial lesion (Gerber, Richter et al. 2002). In mouse tongue,

Pax9 was found to regulate morphogenesis of filiform papilla (FP) and suppress skin-specific differentiation of the mammalian tongue epithelium (Jonker, Kist et al. 2004).

1.4.1 Known function of Pax9 and interaction with other transcription factors and signaling pathways

Pax9 plays a pivotal role during mouse embryogenesis, which is expressed in a wide range of organs, for instance, somites, pharyngeal pouches, mesenchyme involved in craniofacial, tooth, and limb development. Homozygous *Pax9*-mutant mice die shortly after birth, lacking a thymus, parathyroid glands and ultimobranchial bodies, having aberrant limbs, disturbed craniofacial and visceral skeletogenesis, and the tooth development is arrested at the bud stage (Peters, Neubuser et al. 1998). The paralogous genes *Pax1* and *Pax9* are expressed in similar patterns during mouse embryogenesis, suggesting a functional redundancy between *Pax1* and *Pax9*. *Pax1/Pax9* double mutant mice show a much more profound phenotype than the single homozygous mutants, with the complete lack of the medial derivatives of the sclerotomes due to the inability of the sclerotome to undergo chondrogenesis. The rate of cell proliferation is reduced during early sclerotome development and the incidence of apoptosis increases at the later stages in the area normally forming vertebrae and intervertebral discs (Peters, Wilm et al. 1999). *Msx1* and *Pax9* synergistically regulate lower incisor and lip development by multiple signaling pathways, such as *BMP, Shh, Notch1and Fgf*, through the cross-talking between epithelium and mesenchyme. Cell proliferation is reduced in both the dental epithelium and mesenchyme of double heterozygous mutants (Nakatomi, Wang et al. 2010). *Pax9* is able to directly regulate *Msx1* expression at protein level. In addition, *Pax9* and *Msx1* can form a protein complex that interact with *Bmp4*, which determines the fate of the transition from bud to cap stage during tooth

development. *Pax9* regulates *Bmp4* expression through its paired domain rather than *Msx1* (Ogawa, Kapadia et al. 2006). These findings address the functions of *Pax9* in cell proliferation, apoptosis, epithelium-mesenchyme cross-talking and interations with pivotal signaling pathways during embryonic development.

1.4.2 New clues of Pax9 function

Our lab has focused on *Pax9* function in development for several years. The studies include the development of tooth, palate and tongue of the mouse. *Pax9* seems haplosufficient since $Pax9^{+/-}$ mice show no obvious difference with wild type, while complete knockout of *Pax9* results in cleft palate and $Pax9^{-/-}$ mice die shortly after birth. $Pax9^{neo/neo}$ mice, which carry a hypomorph of *Pax9*, often show small lower incisors and missing molars, and lower body weight. One phenomena observed recently is that the $Pax9^{neo/neo}$ dam could not feed the pups properly and the pup gain weight slowly (observed in our lab).

Ectodermal appendages, for instance, scales, teeth, feathers, hair, nails, and a variety of glands such as mammary, sweat, salivary, and lachrymal glands are formed through a series of interactions between epithelial cells derived from the surface ectoderm and the underlying mesenchyme. Thus they may utilize similar signaling pathways during morphological development (Pispa and Thesleff 2003). Therefore, we are curious to know if *Pax9* plays similar roles in the development of mammary gland, as it functions in other organs, such as tooth, palate, lip, tongue and oesophagus. Analyses of *Pax9* functions in the mammary gland biology were conducted in this thesis to answer this question.

1.5 Aims of the thesis

This study aims to investigate *Pax9*'s function on two aspects: the first, to

investigate the possible function of *Pax9* in normal mammary gland development; the second, to investigate whether *Pax9* is associated with breast cancer and postulate the possible mechanisms underlying it.

In the following chapters, we 1) look into the temporal and spatial expression pattern of *Pax9* during different developmental stages of mouse mammary gland, 2) make a survey of the phenotype of loss-of-function of *Pax9* in the mammary gland using *Pax9* hypomorph and *Pax9* mammary-specific knockout mouse models, 3) try to dissect the molecular regulation of *Pax9*'s function during mammary gland development, 4) investigate if *Pax9* is associated with breast cancer, 5) in the last chapter, summarize the functions of *Pax9* in mammary gland development and discuss the possible mechanisms, dysregulation of which might lead to breast cancer.

Chapter 2. Materials and Methods

2.1 Mouse Models

The wild type C57BL/6 (BL6) was used in *Pax9* spatial-temporal expression experiments. *Pax9*$^{LacZ/+}$ in BL6 strain (Peters, Neubuser et al. 1998) was used for X-Gal whole mount staining. *Pax9*$^{neo/neo}$ in C57Bl/6 and CD1 strain (Kist, Watson et al. 2005) were used for phenotype investigation of mammary gland development with *Pax9* hypomorph. The *Pax9 loxP*-flanked mouse in C57Bl/6 strain was generated in our lab and *K14-Cre* in C57Bl/6 was kept here as well (Asselin-Labat, Shackleton et al. 2006). The *MMTV-Cre* mouse in FVB background was kindly provided by Professor William Muller, McGill University, Canada. The brief description of mouse strains used in this study is summarized in Table 2.1. All animal work was carried out in line with Animal (Scientific Procedures) Act 1986.

Table 2.1 Mouse strain List

Mouse Line	Description	Gene Background
Pax9^{LacZ}	*Pax9* knockout mouse (*LacZ*) Heterozygous (+/-): normal Homozygous (-/-): die at birth (gasping), first digit duplication	BL6
Pax9^{neo} (BL6)	*Pax9* hypomorph mouse (neo) on BL6 Heterozygous (+/neo): normal Homozygous (*neo/neo*): hypoplastic lower incisors, small body size	BL6
Pax9^{neo} (CD1)	*Pax9* hypomorph mouse (neo) on CD1 Heterozygous (+/neo): normal Homozygous (*neo/neo*): hypoplastic lower incisors, small body size	CD1
Pax9^{flox}	*Pax9 flox*ed mouse Phenotype: normal	BL6
MMTV-Cre; *Pax9*$^{flox/flox}$	Mammary-specific *Pax9* knockout mouse under the control of MMTV promoter	FVB/BL6
K14-Cre; *Pax9*$^{flox/flox}$	*Pax9* knockout mouse under the control of K14 promoter	BL6
MMTV-Cre	*MMTV-Cre* transgenic mouse	FVB
K14-Cre	*K14-Cre*	BL6

2.2 Genotyping

Genotype of the mouse was determined by PCR routinely. Genomic DNA

samples were prepared either from the ear clipping, tail of adult mice or a small piece of embryos. The tissue for genotyping was dissolved in 100ul lysis buffer (100 mM Tris.Cl pH8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) with proteinase K (2µl of 20 mg/ml proteinase K stock was added into 100ul of lysis buffer), kept on vortex at 55°C for 30 minutes to overnight till the tissue is dissolved. 2ul DNA was applied in 25ul PCR reaction system. The specific primers used in PCR for genotyping are listed in Table 2.2. During the PCR reaction, DNA was denatured at 94°C for 30 seconds, annealled at 56°C for 40 seconds, extended at 72°C for 40 seconds. Usually 31 cycles of reaction were run to gain adequate PCR product. Water was used as negative control and *Pax9* plasmid as positive control.

Table 2.2 Genotyping Primers and PCR products

Moue Line	Primer Sequece	PCR product
Pax9^{LacZ}	P9-gen2-F1: ACT CAC CGG CCT GCA CCA ATT AC P9-gen2-R1: TTG TTC TCA CTG AGC CGG CCT GT (wt-R) P9-gen2-R2: GGA TGT GCT GCA AGG CGA TTA AG (mut-R)	wt= 268 bp mut=350 bp
Pax9^{neo}	*Pax9*lox1-F2: AGC GGA GAC AAG GAT GAA ACC A *Pax9*lox1-R2: AGA GGA ATC CCG ATG TTC ACC AG	wt=305 bp mut=351 bp
Pax9^{flox}	*Pax9*lox2-F: TTC GGC TGC TGT CTC TGG TT *Pax9*lox2-R: CCG GAC TGT ATG GTA CAG AA	wt=205 bp mut=313 bp
Cre	*Cre*5-F: TGCCACCAGCCAGCTATCAACT *Cre*5-R: AGCCACCAGCTTGCATGATCTC	*Cre*5=191 bp

2.3 RT-PCR

For RNA isolation, the 4th inguinal mammary gland was dissected carefully, the lymph node was excluded, and the mammary tissue was immediately frozen in liquid nitrogen, stored at -80°C. RNA was isolated using TRIzol REAGENT

(Invitrogen), the protocol strictly referred to Invitrogen TRIzol REAGENT product manual. Add 1ml Trizol in 50mg frozen tissue sample, homogenize for 1 minutes, place still at room temperature for 5 minutes, centrifuge at 12000g at 4°C for 10 minutes, add 0.2ml chloroform, shake vigorously for 15 seconds, place it at room temperature for 2 minutes, centrifuge at 12000g at 4°C for 15 minutes, keep aqueous layer (about 600ul), add 0.5ml isopropanol, mix and place it at room temperature for 10 minutes, centrifuge at 12000g at 4°C for 10 minutes, wash with 75% EtOH, vortex for 3 seconds, centrifuge at 7500g at 4°C for 5 minutes, collect the RNA pellet. Dry RNA pellet briefly at room temperature for 5 minutes. Dissolve the RNA in DEPC water at 55°C for 10 minutes. Store the RNA at -80°C.

For RT-PCR, reverse transcription was performed using 2 μg of total RNA, random primers and Superscript II reverse transcriptase (Invitrogen). Appropriate specific primers were used in RT-PCR, Gapdh was used as control. Primers used for RT-PCR were listed in Table 2.3. Annealing temperature for *Pax9* RT-PCR was 58°C, 35 cycles of PCR reaction were run; annealing temperature for *Gapdh* was 55°C, 22 cycles of reaction were run; annealing temperature for *Cre* is 56°C, 33 cycles of reaction were run. All the cDNA templates were denatured at 94°C for 30 seconds, annealed at their specific temperature for 40 seconds and extended at 72°C for 40 seconds.

Table 2.3 Primers used for RT-PCR

Primer	Primer Sequences	RT-PCR product
Pax9	P9-RT2-F: CTCCATCACCGACCAAGGAG P9-RT2-R: GAGTGCAGAAGCGGTCACAG	412bp
Gapdh	*Pax9*lox1-F2: AGC GGA GAC AAG GAT GAA ACC A *Pax9*lox1-R2: AGA GGA ATC CCG ATG TTC ACC AG	311bp
Cre	*Cre*5-F: TGCCACCAGCCAGCTATCAACT *Cre*5-R: AGCCACCAGCTTGCATGATCTC	191 bp

2.4 Carmine-Alum wholemount staining

Dissect the 4th mammary gland, place on a microscope slide, fix overnight in Carnoy's solution (EtOH : Chloroform : Glacial Acetic Acid = 6:3:1), wash in 70% EtOH for 2 hours, stain overnight in carmine-alum solution (2% carmine, 5% aluminum potassium sulfate), destaining the mammary gland in 70% EtOH with 2% HCl for 30 minutes, dehydrate in EtOH with increasing concentrations (70%, 80%, 95%, 100%), 2 hours for each grade, clear in methylsalicylate (Sigma) to take photographs.

2.5 X-Gal staining

the 4th mammary gland was dissected, placed on a piece of filter paper and fixed at 4°C for 1 hours in 2%PFA solution (2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP-40 in PBS). After fixation, the mammary gland was rinsed in PBS, removed from the filter paper, washed in X-Gal staining buffer (10 ml PBS with 2 mM MgCl2, 0.01% Na-deoxycholate, 0.02% NP-40) with slight shake for 30 minutes. After wash, the tissue was transfered into 10ml X-Gal staining buffer with 1 mg/ml X-gal (diluted from X-Gal stock solution: 40mg/ml in

DMF, stored at -20°C), incubated at 37 °C for 6-24 hours to allow the reaction to develop thoroughly. After X-Gal staining, the tissue was cleared in methylsalicylate to take photographs.

2.6 Pup growth measurement

Pax9^{neo} (CD1) mice were used in this experiment to investigate the capacity of milk production of the dams by measuring pup growth. In each litter, only 10 pups were kept with the dam at birth (extra pups were kept away), weighed every 24 hours and a pup growth curve was made according to their weight gaining. The pup growth curves reflect the lactation ability of the dam. Three groups were examined for each genotype, *Pax9*$^{+/neo}$ and *Pax9*$^{neo/neo}$.

2.7 Tissue processing

Mammary glands were fixed in 4% PFA in PBS at 4°C overnight, dehydrated with ethanol through a series of concentration, embedded in paraffin, sectioned at 5 μm thickness, stored at a cool place and ready for histochemistry experiment.

2.8 Histochemistry

H&E Staining

Dewax the section with xylene twice, 5 minutes each time, rehydrate in series of ethanol (100%, 100%, 80%, 70%, 50%, 30%, H_2O), stain sections in haematoxylin for 10 seconds, rinse and bluing in tap water, stain in eosin for 10 seconds, rinse in water, dehydrate, clear in xylene, mount with DPX.

Immunohistochemistry

Dewax the section and rehydrate as mentioned above. Antigen retrieval is

carried out by 8 minutes pressure cooker boiling (count time after steaming start) or microwave heating for 10 minutes in citric buffer (0.01 M citric acid, pH 6.0). Block unspecific binding by incubation with Diluent (DAKO) for 15 minutes at room tempreture prior to incubation with primary antibody. Incubate in primary antibody for 30 minutes, rinse in TBS (20 mM Tris and 150 mM NaCl , pH7.6) for 3 times, apply HRP labeled anti-rabbit polymer, develop color with AEC^{+} (Envision+ Kit, DakoCytomation, Dako K4008). For *Pax9* immunostaining, incubate the sections with secondary antibody for 30 minutes between applying anti-*Pax9* primary antibody and HRP labeled anti-rabbit polymer. Primary antibodies used were anti-*Pax9* (1:10, rat-anti mouse monoclonal, developed in Heiko's lab, followed with Rabbit Anti–Rat immunoglobulins (1:50, Dako, Z0455, Denmark), anti-*pStat5* (Tyr694, 1:200, Cell Signaling, #9359), anti-p*Stat3* (phospho Y705, 1:200, abcam, ab31370, England).

TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method widedly used to detect apoptosis. It identifies DNA fragmentation by DNA nicks in the apoptotic cells. The DNA nicks can be identified by terminal deoxynucleotidyl transferase, an enzyme that catalyzes the addition of dUTPs and are secondarily labeled with a marker. It may also label cells with severe DNA damage. ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, S7100) was used in apoptosis detection. Dewax and rehydrate the tissue sections as previously described, treat with proteinase K (20ug/ml) for 15 minutes at room temperature, wash in PBS, incubate with TdT at 37°C for 1 hour, stop the reaction, apply Digoxin-Peroxydase, wash the sections, develop color with AEC+ (Envision+ Kit, DakoCytomation, Dako K4008), complete the

staining with haematoxylin counterstaining and mount with DPX. Observe apoptotic bodies under the light microscope.

2.9 Western Blot

For western blot, the 4th inguinal mammary gland was dissected, immediately frozen in liquid nitrogen and stored at -80°C. Protein was isolated using TRIzol REAGENT (Invitrogen). The same samples were used for protein extraction and RNA isolation, protocols strictly referred to Invitrogen TRIzol REAGENT product manual. Thermo Scientific Pierce BCA Protein Assay Kit was used to measure total protein concentration. 40ug protein was loaded into each lane of SDS-PAGE gel (Ready Gel Tris-HCl Gel, BIO-RAD), electrophoresed in Running Buffer (Tris-glycine-SDS buffer: 0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.5) under 130V for 1hr. Nitrocellulose membrane was activated in absolute methanol for 30 seconds, rinsed in water, kept in TBST (TBS with 1% tween20). Proteins was transfered to activated nitrocellulose membrane from SDS-PAGE gel in Blotting Buffer (Tris-glycine: 0.025M Tris, 0.192M glycine, pH 8.5) under 100V for 1hr or 20V overnight at 4°C with stirring. For immunochemistry, block the membrane unspecific binding of the antibodies in 5% milk in TBST at room temperature for 1 hour, apply 5ml primary antibody (rabbit anti-mouse) at room temperature for 2 hours, wash in TBST three times, 10 minutes for each, apply 5ml secondary antibody (HRP-conjugated goat anti-rabbit IgG , Thermo Scientific Pierce, 1:5000) at room temperature for 1 hour, wash in TBST, three times, 15 minutes for each, apply ECL plus (Thermo Scientific Pierce) for 5 minutes. Primary antibodies were anti-K18 (1:500, abcam), pStat3 (phospho Y705, 1:500, abcam), Stat3 (1:500, abcam) and pStat5 (Tyr694, 1:200, Cell Signaling) as mentioned in the method of immunohistochemistry previously.

2.10 Affymetrix microarray Expression Profiling

GeneChip Mouse Genome 430 2.0 array (Affymetrix, CA, USA) was used in this experiment, which contains probes for detecting 45,000 transcripts with over 34,000 well-characterized genes. Mammary glands of *MMTV-Cre;Pax9*$^{flox/flox}$ and *Pax9*$^{flox/flox}$ control mice were dissected at involution day 1, day 2, day3 (n=1), frozen immediately in liquid nitrogen and stored at -80℃. RNA was extracted with Trizol Reagent. The concentration of RNA was measured by Nanodrop (Angilent) and the quality of RNA was estimated by RNA NanoLab chip on the 2100 Bioanalyser (Agilent, Palo Alto, CA). After RNA isolation, cDNA synthesis and biotin-labeling of cRNA, hybridization and scanning of the arrays were carried out as described in the Affymetrix guide manual. RNA was treated with deoxyribonuclease I (Invitrogen) to remove any residual genomic DNA. cDNA was then synthesized with 5 μg total RNA by SuperScript II reverse transcriptase (Invitrogen) and purified by phenol/chloroform extraction. Then cDNA was labeled using the RNA transcript labeling kit (Invitrogen) to generate biotinylated cRNA. Biotin-labeled cRNA was purified and fragmented according to Affymetrix's protocol. The fragmented cRNA was mixed with control oligonucleotide B2 (Affymetrix) and a hybridization control cRNA mixture (BioB, BioC, BioD, and Cre, Affymetrix). Chips were hybridized at 45°C for 16 hours. The arrays were subsequently washed and stained in a Fluidics Station (Affymetrix) and scanned by GeneScanner according the manufacturer's instructions (GeneChip Expression Analysis Technical Manual, Affymetrix). The data aquisition and gene expression analysis was conducted by Affymetrix GeneChip Operating Software (GCOS). The most highly changed genes in the mutants were determined by change log ratio above 2. For the first preliminary expression profiling purpose and the limited number of mammary gland-specific

Pax9-deficient animals, only one biological sample was applied on one chip at each time point.

2.11 Tumour microarray (TMA) design

CBCTR 2001 Tissue Microarrays (TMAs) were provided by the Cooperative Breast Cancer Tissue Resource (CBCTR, USA). The TMAs were constructed using tissue and associated pathological and clinical outcome data from CBCTR, designed to ensure high statistical power for evaluation and validation of breast cancer biomarkers. The TMA series we used was designed to permit comparisons of biomarker expression across three stages of disease (node-negative, node positive and metastatic) and other clinical and pathological factors.

Four identical tissue microarray paraffin blocks have been made of Design # 2 in 2001. The 4 blocks (for each design) are designated A, B, C and D. Histologic sections taken from these blocks are numbered sequentially. We received histologic sections from some of these paraffin blocks, and the slides were labeled in the following manner: sponsoring organization; year of manufacture; design number; block number; section number. The TMA sections applied in this study were CBCTR 2001 TMA#2: 2B.67, for PAX1 immunostaining as controls, 2A.68, 2B.68, 2C.70, for *PAX9* immunostaining, to evaluate the protein expression in a breast cancer cohort.

2.12 *PAX9* expression evaluation on TMA

The method of immunostaining of Pax9 on TMA refers to 2.8 “immunohistochemistry”. Primary antibodie was anti-*Pax9* (1:10, developed in Heiko’s lab), secondary anti body was Rabbit Anti–Rat immunoglobulins (1:50,

DAKO, Denmark), between the anti-*Pax9* rat primary antibody and an enzyme-conjugated antibody to rabbit immunoglobulins, the coupling reaction was developed with Fast Red (Sigma). According to the overall performance by the relative signal intensity of *Pax9* immunostaining and the percentage (whether above 50%) of positive cells on one section. The levels of *Pax9* protein expression *in situ* by immunohistochemistry on TMAs were aligned to 4 levels: negative, weak, moderate and strong, scored 0, 1, 2, 3 respectively. Negative and weark means low levels, moderate and strong mean high levels

2.13 TMA and statistical method

Each tumour microarray (TMA) represents 252 individual breast tissue specimens and 36 control specimens, in total a TMA slide containing a total of 288 cores. *Pax9* immunostaining was applied on TMA in triplicate. An average expression level of the triplicate specimens counts for statistics. Use Chi-square p-value to determine the significance (by using Mini-Tab software).

Chapter 3. *Pax9* is expressed in mouse mammary gland with a temporal-specific and spatial-specific pattern

3.1 Introduction

The mammary gland is an exceptional organ for its major development is completed in adult, while in most other organs it has been completed before birth. The mammary gland development is fulfilled through a linear path from embryonic to puberty stage, and a cyclic path during pregnancy, including gestation, lactation and involution. Thus it is a good model to study the molecular regulation of cell differentiation, organogenesis, tissue homeostasis and remodeling. The mammary gland comprises mainly epithelium and fat tissue. In addition, there are some other components of less quantity but of the same developmental importance, such as extracellular matrix (ECM), fibroblast and immune cells. The growth and function of mammary gland is concerted by endocrines, growth factors, intracellular signaling, crosstalk between the epithelium and stroma, and the participation of immune cells, for instance, macrophages and eosinophils.

We studied mammary gland development in mouse in this thesis. Mouse mammary morphogenesis comprises several key events: mammary bud and primary ductal tree formation during embryonic period, ductal branching during adolescent periods, successively, alveolar formation and differentiation during pregnancy and lactation, and apoptosis and tissue remodeling during post-lactation involution.

Mammary bud starts from the formation of bilateral milk lines on embryonic day

10.5, forms lens-shaped placodes by E11.5, then cells within the placode invaginate into the underlying mesenchyme to form the typical bulb-shaped mammary buds between E11.5 and E12.5. *Wnt, Fgf,* and parathyroid hormone-related protein signaling have been identified for their functional roles in mammary gland development during this stage (Hens and Wysolmerski 2005).

Mammary duct branching proceeds through 3 different phages: embryonic, adolescent and adult phases, each of which is differentially regulated. Adolescent branching requires oestrogen and oestrogen receptor-α (*ER-α*), growth hormone (GH) and growth hormone receptor (*GHR*), adult tertiary side branching requires progesterone and its receptor (*PR*), while embryonic branching is hormone independent (Hinck and Silberstein 2005). At the age of 3 weeks, the terminal end buds (TEBs) start to be active and invade the fat pad, leading to the growth of the mammary gland. Cells in TEB differentiate, proliferate and undergo apoptosis to form the elongated ducts. Through recurrent estrus cycles, the mouse ductal epithelial tree develops further with side branches where alveoli form from during pregnancy. Ductal branching is also coordinated by local cross-talk between the developing duct epithelium and nearby stroma, with multiple signaling pathways taking part, such as *Wnt, FGF, TGF-β* pathways, and metalloproteinase (Fata, Werb et al. 2004, Sternlicht, Kouros-Mehr et al. 2006).

Alveolar units expand and differentiate into milk-secre*t*ory cells during late pregnancy and lactation. During lactation, milk globules are secreted by the alveolar epithelial cells, and contracted into the ducts and delivered to outside when pups suckle. The pituitary prolactin is the trigger for lactation. It regulates lactation signaling through prolactin receptor (*PrlR*), *Jaks* and *Stat5*. After

weaning, the mammary gland undergoes a rapid involution and returns to the state of mature non-pregnant morphology.

The mammary gland is also an ideal organ to study molecular mechanisms of the origin and progression of cancer, as well as the regulation cues underlying cell fate, tissue homeostasis and organ regeneration. It greatly encouraged researchers when a single stem cell transplanted into cleared mammary fat pad was shown to be able to regenerate a complete and fully-functional mammary tree in 2006 (Shackleton, Vaillant et al. 2006).

The development and application of new techniques, such as primary mammary cell isolation, tissue recombination, mouse mammary gland humanization, ex-in-vivo organ culture, 3D matrix resembling in-vivo niche, have been widely used in mammary gland study. The advantage of modern techniques provides efficient and precisely-regulated tools for mammary gland study.

Pax9 was found to be expressed in normal mammary epithelial tissue and down-regulated in breast cancer, which will be presented in details in Chapter 6. In this Chapter, we carried out semi-quantitative RT-PCR to evaluate *Pax9* RNA expression levels during different developmental stages and used immunohistochemistry to demonstrate the spatial expression of *Pax9* protein in mouse mammary glands.

3.2 *Pax9* RNA expression is temporal-specific with a peak at puberty

Semi-quantitative RT-PCR was conducted to investigate *Pax9* mRNA expression throughout different stages of mammary gland development. Total RNA was extracted from mouse mammary glands at different age or developmental stages using Trizol reagent. In the mouse mammary gland, *Pax9* expression levels is far

less than in other tissues, such as tongue and oesophagus (data not shown), however, *Pax9* is indeed expressed throughout all stages of adult mammary gland development, and the expression dynamics follows a temporal-specific pattern (Figure 3.1).

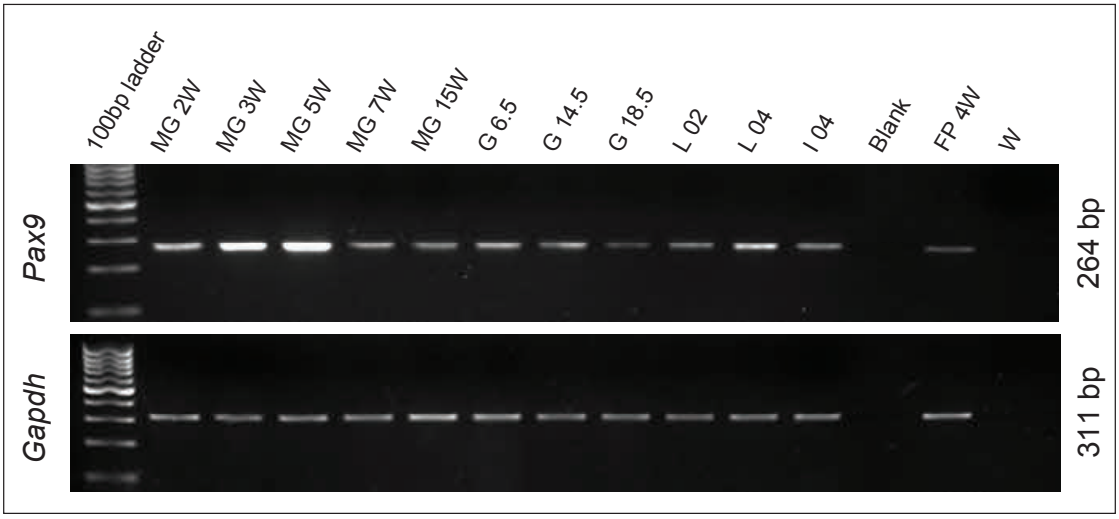

Figure 3.1 *Pax9* mRNA expression in the mammary gland at different developmental stages

Examined by semi-quantitative RT-PCR, expression of *Pax9* peaks at the age of 5 weeks. MG: mammary gland; G: gestation; L: lactation; I: involution; FP: cleared fat pad; W: water. n=2

Demonstrated by semi-quantitative RT-PCR, *Pax9* expression is weak before puberty (2 week old), significantly increases at the onset of puberty (3 week old), peaks during puberty (5 week old), decreases after puberty (7 week old) and maintains at a constant level in mature, non-pregnant mammary gland (15 week old). During pregnant cycle (gestation, lactation and involution), *Pax9* is slightly up-regulated during early gestation, drops down in late gestation and early lactation. In the cleared fat pad of 4 week old mammary gland, *Pax9* is slightly expressed as well.

3.3 X-gal whole mount staining of *Pax9*$^{+/LacZ}$ mammary glands

To conveniently monitor *Pax9* expression in the mammary gland, we tried to apply X-Gal whole mount staining to *Pax9*$^{+/LacZ}$ mouse line (Peters, Neubuser et al. 1998). The intact mammary gland was dissected, fixed slightly with 1% PFA and stained with X-Gal, using the tongue as positive control. Unfortunately, the *Pax9*$^{+/LacZ}$ mammary glands did not show any positive staining, even for 5 week old mice, at which stage *Pax9* mRNA expression is at its highest level, as revealed by semi-quantitative RT-PCR. We implemented X-Gal whole mount staining to *Pax9*$^{+/LacZ}$ mammary glands through different stages, from 1 week, 2 weeks, 3 weeks, 6 weeks, 8 weeks to 10 week old, and gestation day 6, day 10 and lactation day 2, however, none of which have shown positive X-gal staining (Figure. 3.2A), whereas the tongue, as a positive control, was strongly stained (data not shown).

The absence of X-Gal staining in *Pax9*$^{+/LacZ}$ mammary glands suggests *LacZ* not to be expressed under the control of all regulatory *Pax9* promoter/enhancer elements. To address this, *Pax9* and *LacZ* RT-PCR were carried out to detect their RNA expression in the mammary gland of 5 week old *Pax9*$^{+/LacZ}$ mice. As shown by RT-PCR, *Pax9* mRNA was expressed in *Pax9*$^{+/LacZ}$ mammary gland as expected, whereas *LacZ* mRNA was not detectable (Figure 3.2B). Since *LacZ* mRNA was not expressed in *Pax9*$^{+/LacZ}$ mammary gland, the negative X-Gal staining of *Pax9*$^{+/LacZ}$ mammary gland thus is not unexpected. The failure of *LacZ* mRNA expression in *Pax9*$^{+/LacZ}$ mammary gland might be due to the removal of a mammary gland-specific cis-regulatory element of *Pax9* within the sequence that is deleted in the *Pax9*^{LacZ} allele.

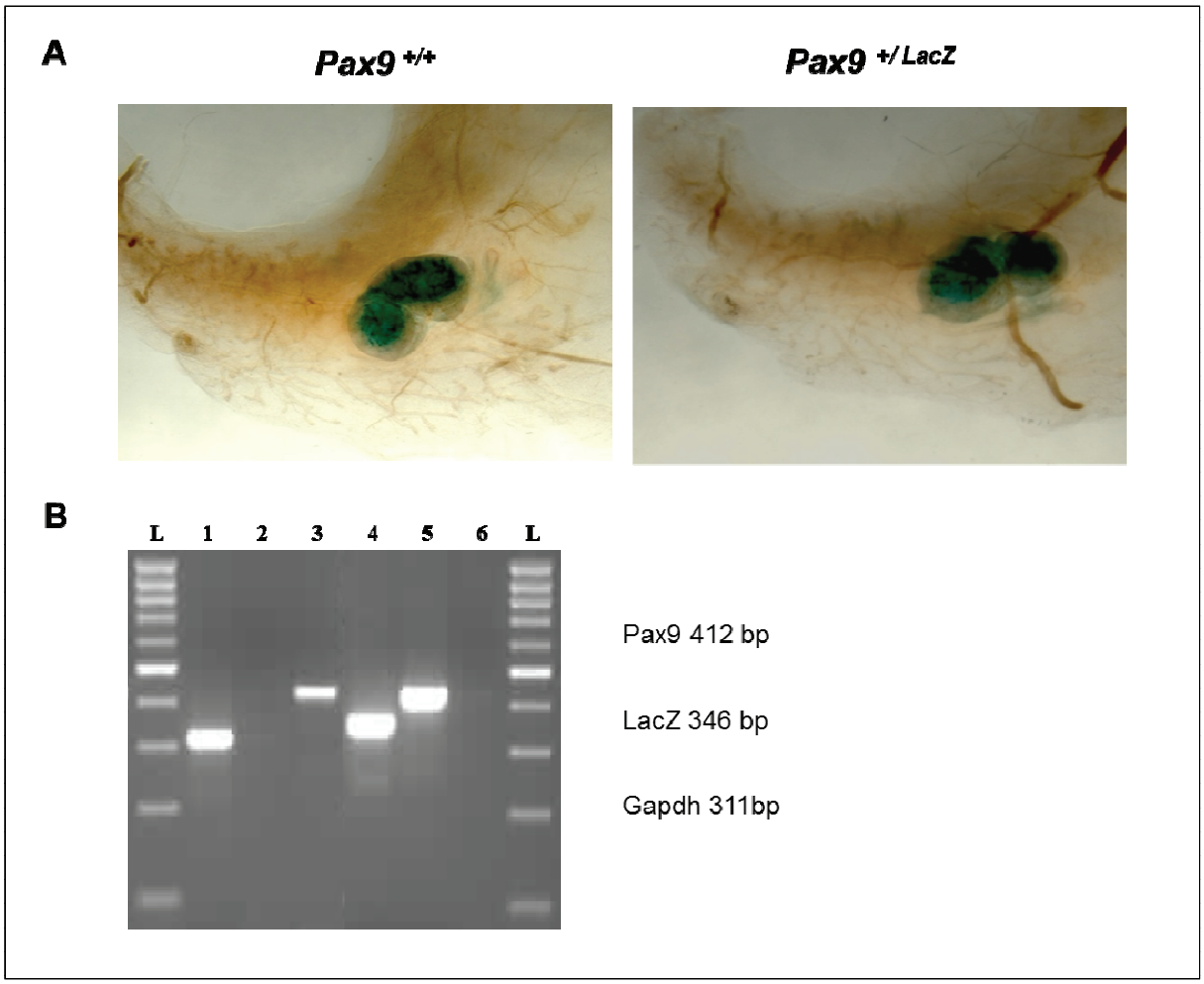

Figure 3.2 Whole mount X-gal staining of *Pax9*$^{+/LacZ}$ mammary glands
(A) X-gal staining of the *Pax9*$^{+/LacZ}$ mammary gland and a control at 5 weeks age. Magnification: 10x (B) RT-PCR to detect *Pax9* and *LacZ* expression in *Pax9*$^{+/LacZ}$ mammary glands at 5 week old. L: Ladder; 1: Gapdh; 2: *LacZ*; 3: *Pax9*; 4: *LacZ* positive control (*LacZ* plasmid); 5: *Pax9* positve control (*Pax9* plasmid); 6: Water control; L: ladder; n=2

3.4 *Pax9* protein expression is spatial-specific, with high levels in the terminal end buds and ducts during puberty

Since whole mount X-gal staining of *Pax9*$^{+/LacZ}$ mouse mammary gland was not able to provide the information about the spatial pattern of *Pax9* expression, *Pax9* immunostaining was used to detect *Pax9* protein expression instead. *Pax9* immunostaining was firstly applied to the mammary glands of 6 week old wild type C57BL6 mouse because puberty is found with the highest *Pax9* expression level by semi-quantitative RT-PCR. During this stage, ductal elongation and

bifurcation proceed rapidly, then at the end of puberty, primary ducts and secondary and tertiary branches form a complete ductal tree occupying the whole fat pad. During every estrous cycle, alveolar buds are formed and involute on tertiary branches.

Pax9 is strongly expressed in the ducts and TEBs during puberty (Figure 3.3), but absent in most areas of the stroma, only with weak expression in the mesenchyme around the neck of TEBs. Within the mammary ducts, *Pax9* is exclusively expressed in the lumimal epithelial cells, but absent in the myoepethelial cells. In TEBs, *Pax9* is expressed in cap cells, which are aligning along the outer layer at the top of TEB, where proliferation is active (Hinck and Silberstein 2005), and *Pax9* is expressed in body cells close to the TEB cavity, where apoptosis is active to form the cavity of TEBs and the lumen.

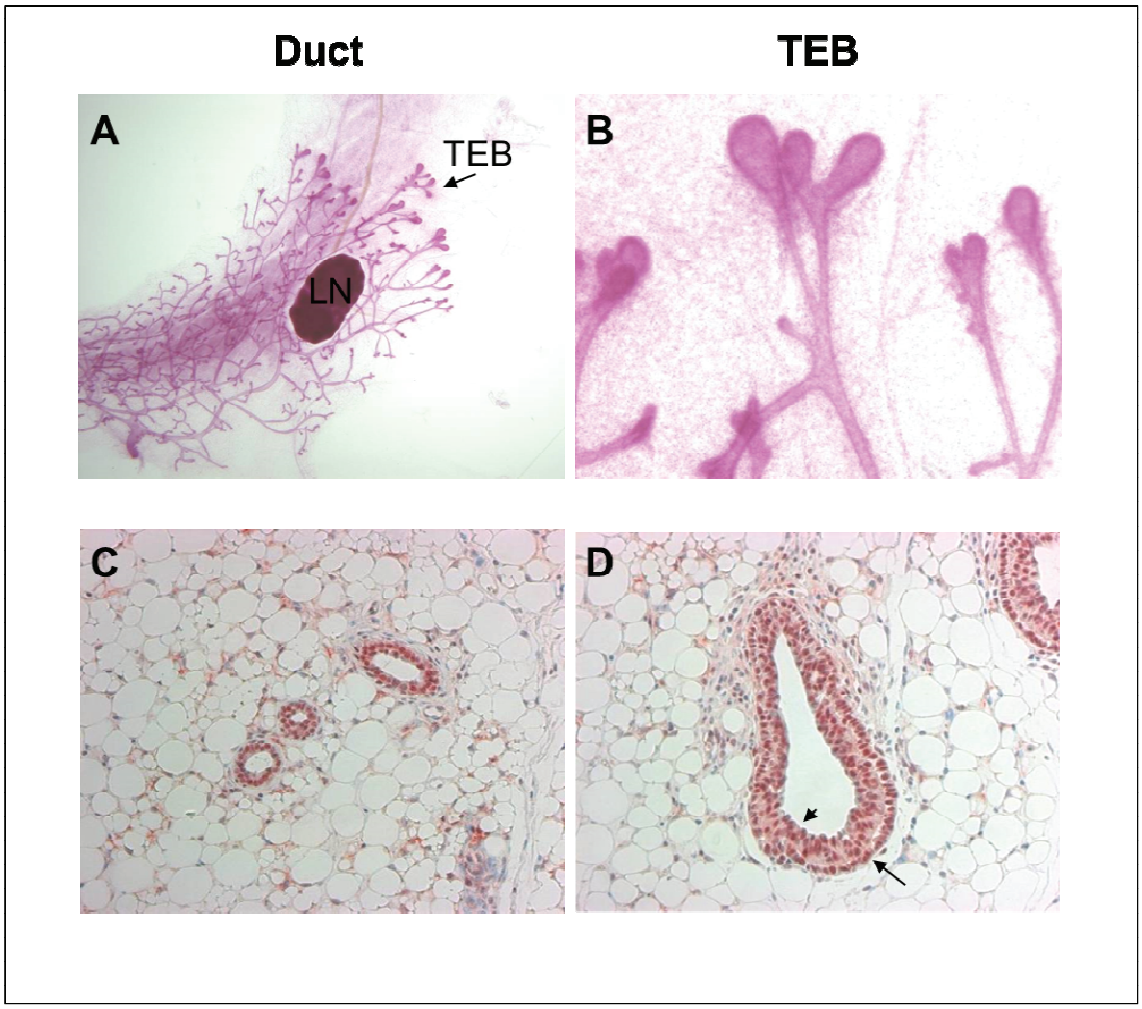

Figure 3.3 *Pax9* is expressed in the ducts and TEBs in the mammary gland in 6 week old **female mice**

(A, B) Whole mount Carmine-alum staining. LN: lymph node; TEB: terminal end bud; (C, D) *Pax9* immunostaining in the ducts (C) and TEBs (D) Cap cells are *Pax9* positive (indicated by arrow), body cells near the TEB cavity are *Pax9* positive as well (indicated by arrow head). Magnificaiton: (A) 10x, (B) 40x, (C, D) 200x. n=5

An investigation of *Pax9* protein expression throughout various developmental stages of the mammary gland was then carried out in wild type C57BL6 mice (Figure 3.4). *Pax9* is expressed weakly in the mammary ducts before adolescence (2 week old), increased to high levels in the luminal epithelial cells of the mammary gland at the end of puberty (6 week old), and maintains a high level in the mammary gland of parous non-pregnant mice (6 months old). *Pax9* protein expression is basically absent in the mammary stroma. During

pregnancy, at gestation day 12, day 14 and day 17, the alveolar structure develops and the alveolar epithelial cells increase rapidly, whereas *Pax9* protein expression levels and the proportion of *Pax9*-positive cells are lower than that of mammary ducts during puberty and parous non-pregnant. During lactation, *Pax9* protein expression decreased in most of the alveolar epithelial cells (lactation day 0, day 1 and day 8), while a few *Pax9*-positive epithelial cells sustain in every alveoli. After weaning, an extensive postlactational involution and tissue remodeling occurs and alveoli collapse and 90%mammary epithelial cells are eliminated within a few days (the majority of epithelial cells disappears in only 4 days). *Pax9* is up-regulated when involution is initiated (involution day 1), increases during involution (involution day 3), then returns to constant levels in mature ducts (involution day 8, 6-month). See Figure 3.4

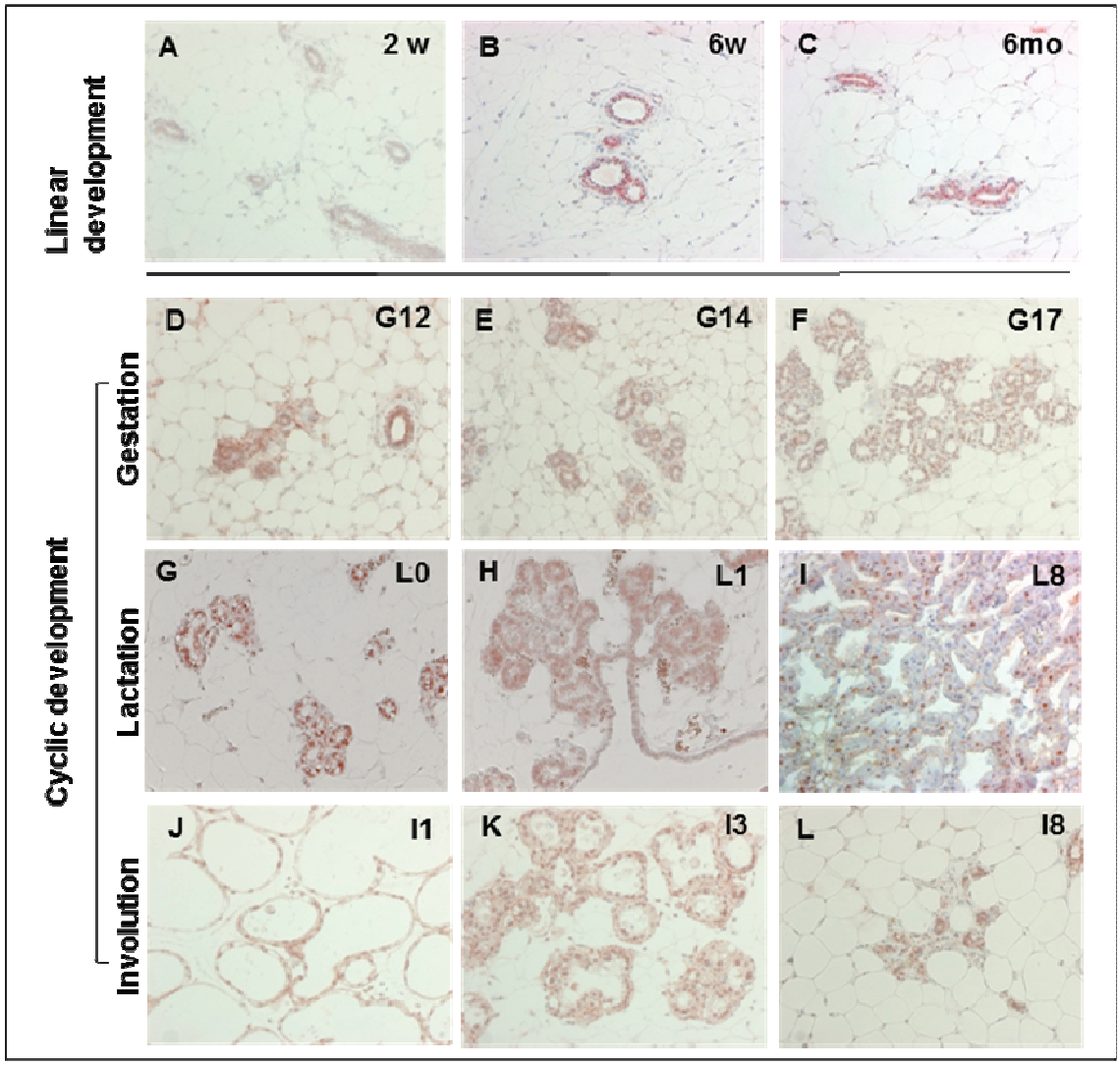

Figure 3.4 *Pax9* immunohistochemistry at different development stages

Pax9 in different structures in mammary gland, note that ductal epithelial cells line in the lumen and alveoli as a regular single layer. *Pax9* in ducts at pre-puberty (2-week old, A), puberty (6-week old, B) and mature parous duct (6-month old, C) and *Pax9* during gestation stages at indicated time(D,E,F), *Pax9* during lactationday 0, day1 and day8 (G, H, I) and involution day 1, day3 and day8 (J, K, L). Magnification: 200x

3.5 Discussion

The intrinsic low levels of *Pax9* expression in the mammary gland made it difficult to gain sensitive RT-PCR and intensive immunostaining signal. Many efforts have been made to optimize the experimental protocol to improve the quality of the results. For immunostaining, we tried various antigen retrieval

methods, blocking reagents, different antibody concentration and antibody incubation conditions. For RT-PCR, we were very cautious at every step during tissue dissection and RNA isolation to avoid RNase contamination. However, the signal was always moderate and the RT-PCR band was weak. Mouse tongue and oesophagus were used as positive control, the immunostaining signal and RTPCR product of which were intensive.

The results of *Pax9* expression in the mammary gland demonstrated by semi-quantitative RT-PCR and immunohistochemistry are basically consistent. Before puberty, *Pax9* expression is below detectable levels. During puberty, *Pax9* expression reaches the highest level, and *Pax9* protein was expressed in the luminal epithelial cells of the ducts, as well as in cap cells and body cells of inner layers of the TEBs. After puberty, *Pax9* is expressed at a constant level in mature ductal epithelial cells. During gestation, *Pax9* is expressed in the alveolar epithelial cells until parturition. When lactation starts, *Pax9* expression drops down significantly, but is detectable in a small part of alveolar epithelial cells when the lactating mammary gland maintains homeostasis. After weaning, the mammary gland undergoes involution rapidly and *Pax9* is up-regulated in the alveolar epithelial cells and maintains at a constant level in the ducts after alveoli collapse and the mammary tissue is remodeled (Figure 3.5).

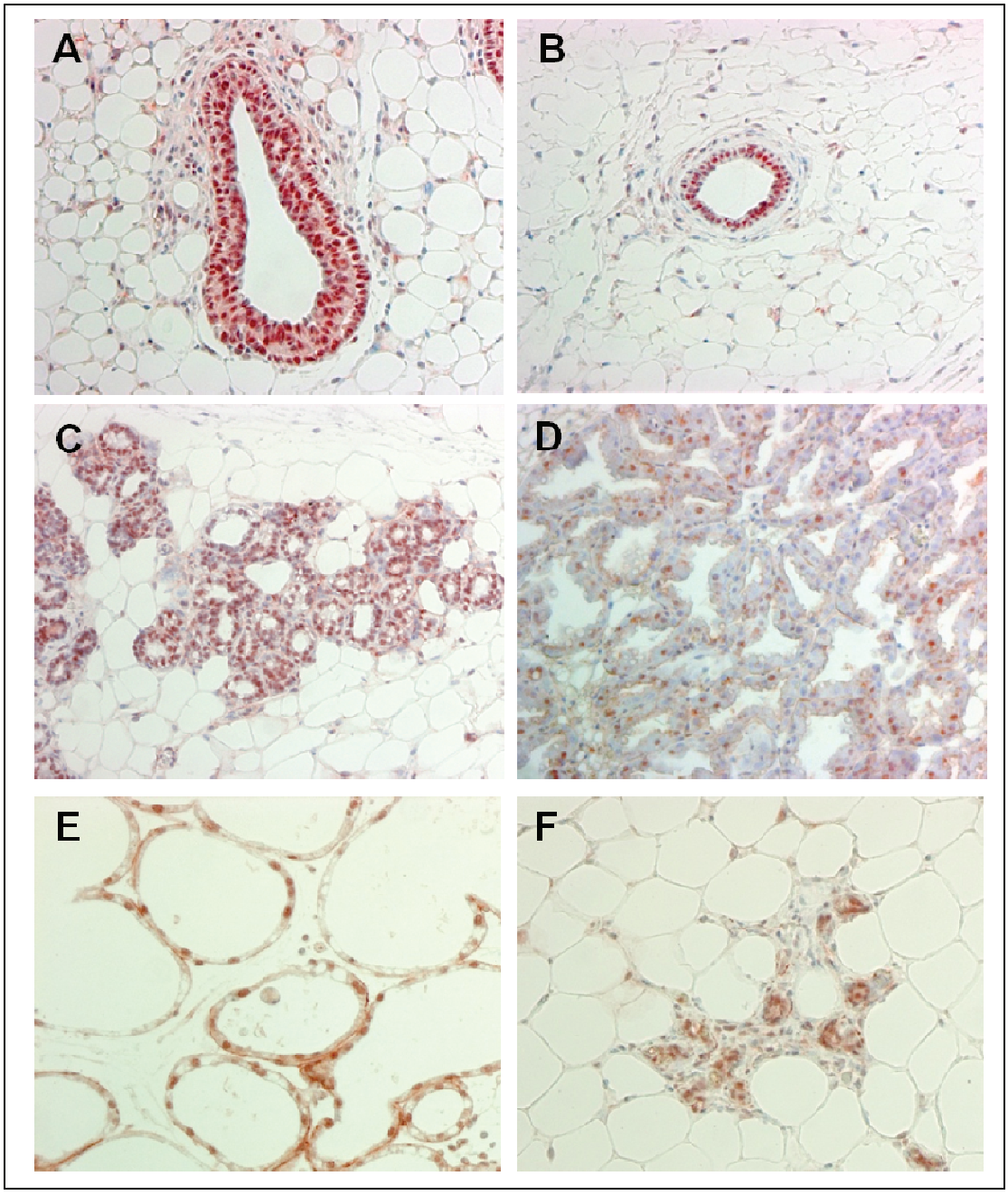

Figure 3.5 *Pax9* protein expression in different structures

(A) *Pax9* is strongly expressed in TEBs during puberty (note that not all body cells are *Pax9*-positive). (B) In the adult virgin female, *Pax9* is exclusively expressed in luminal epithelial cells of mature ducts. (C) During pregnancy, low levels of *Pax9* are expressed. (D) Expression is absent in the majority of epithelial cells when mammary gland is starting to secret milk. (E, F) *Pax9* is up-regulated at various stages during involution. Magnification: 200x

In the TEBs, *Pax9* is strongly expressed in the cap cells, which proliferate for duct elongation, and in inner body cells, which undergo apoptosis for lumen

formation. The mesenchyme adjacent to TEBs also expresses *Pax9* weakly (Figure 3.6). MMPs are supposed be active at the forefront of TEB to degrade the matrix and facilitate the TEB to invade the fat pad ahead. TEB bifurcation needs TGF-β accumulation ahead of the tip of the TEB, to inhibit cell proliferation at the bifurcating points of the TEB. Growth at the tip is hampered whereas the growth of its two sides continues, thus the TEB bifurcates at this point and forms two separate branches.

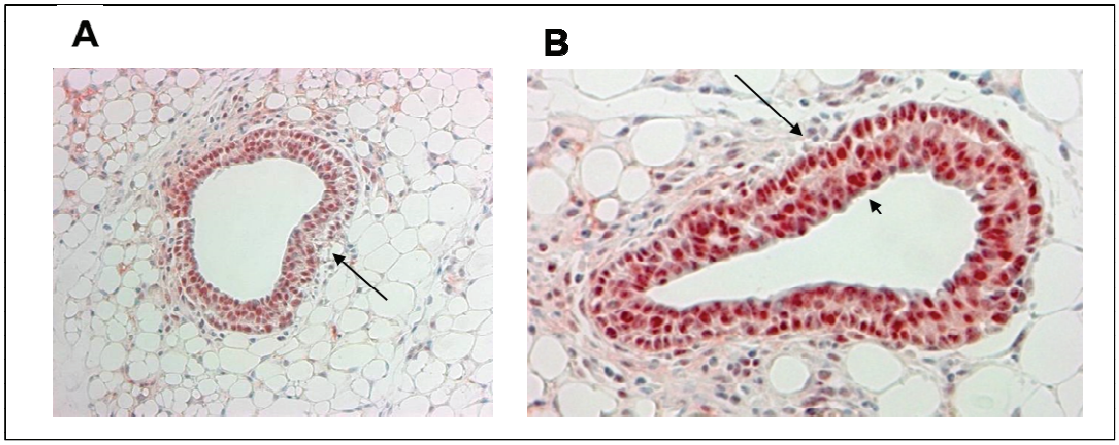

Figure 3.6 TEB growth, bifurcation and cavity formation

A) A bifurcation TEB with *Pax9* immunostaining, bifurcating point indicated by arrow. B) A representative complete TEB with *Pax9* immunostaining, inner body cells indicated by arrow head, cap cells indicated by arrow. Manification: (A)200x, (B) 200x, cropped

There is a complex hierarchy of mammary epithelial cells, including stem cell, progenitor cell, differentiated cell and terminally differentiated cell (Shackleton, Vaillant et al. 2006, Stingl, Raouf et al. 2006, Van Keymeulen, Rocha et al. 2011, Visvader and Lindeman 2011, Visvader and Smith (2011)). *Pax9* expression varies in different types of mammary epithelial cells (Figure 3.7). *Pax9* is increased in cap cells, body cells and ductal cells, when oestrogen triggers ductal branching during puberty, as well as in alveolar cells when progesterone initiates alveologenesis during pregnancy. It is decreased in the lactating alveolar cells, which have gone through further differentiation, when

progesterone withdraws and prolactin triggers lactogenesis. At weaning, *Pax9* is increased again. It seems that *Pax9* correlates with certain differentiation grades of the mammary epithelial cells.

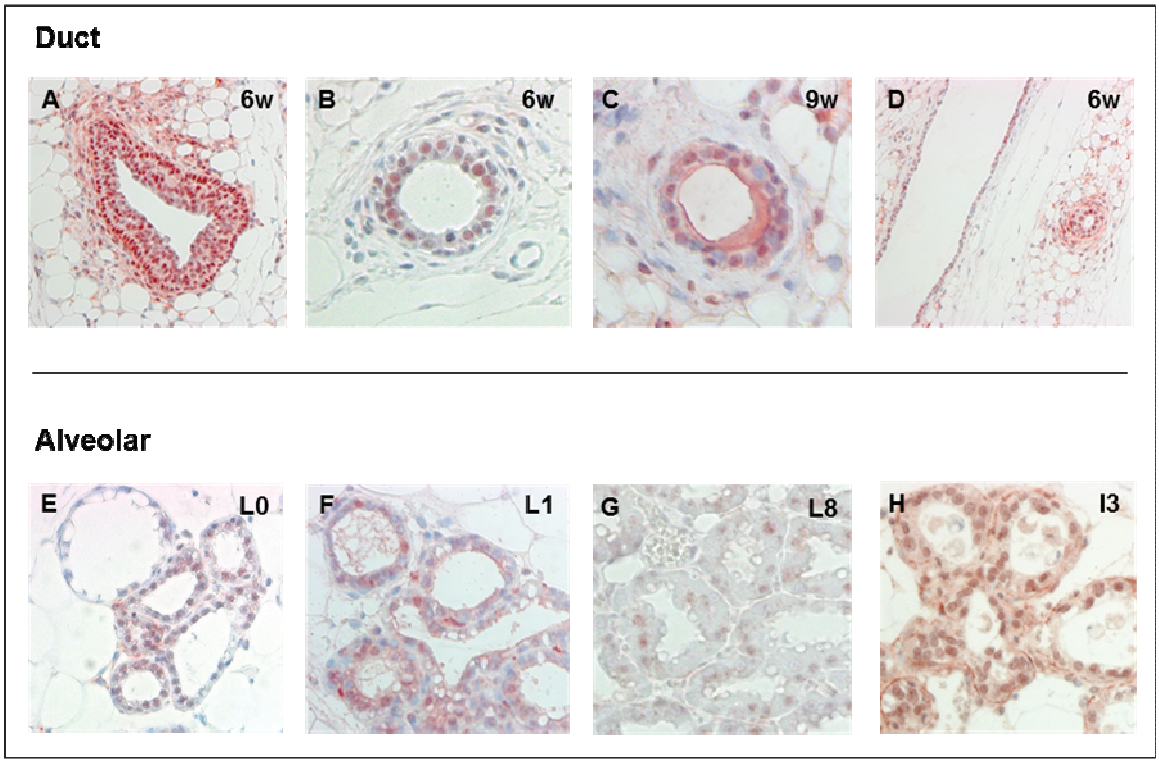

Figure 3.7 *Pax9* expression in mammary ducts and alveoli

Pax9 is expressed at various levels in different cell types (epithelial structures) in mouse mammary gland: strongly in most cap cells and a majority of body cells in TEBs (A), as well as ductal cell but in a heterogeneous pattern (B-D), decreased in alveoli (E), diminished to be undetectable in most of the lactating alveoli (F, G), increased back to a constant level in the epithelial cells going through involution (H). Development stages and time points were as indicated. Magnificaiton: 200x, cropped.

The *Pax9*-positive cell population in the alveolar cells at lactation (Figure 3.7 G) may have not gone through a terminal differentiation towards secretary cells, but instead, have maintained a specific pre-terminal-differentiation status in milk-secreting alveoli. The benefit of the maintenance of the pre-terminal-differentiation status of this cell population is suspected to reduce the presence of excessive milk globules (MGs), either by inhibiting milk secretion

or eliminating excessive MGs, thus maintain mammary homeostasis. One hypothesis is that *Pax9* plays a role in apoptotic cell and MG engulfment. MG engulfment described a mechanism by which epithelial cells can function as non-professional phagocytes, to remove apoptotic cells and excessive MGs during lactation, thereby helping to prevent mastitis, which might be induced by excessive production of milk (Monks, Rosner et al. 2005). The same mechanism also acts in involution, when large amount of apoptotic epithelial cells and MGs need to be cleared out rapidly for tissue remodeling (Hanayama and Nagata 2005)

After investigated *Pax9* expression in mouse mammary gland during different development stages, we started to explore the phenotypes of *Pax9* mutant mammary gland in the next chapter.

Chapter 4. Systemic *Pax9* gene dosage reduction delays pubertal ductal branching and facilitates formation of hyperplastic nodules

4.1 Introduction

Pax9 is known to play important roles during embryonic development, and abnormalities have been seen in tooth, palate, lip, and thymus development in *Pax9*-inactivated mice (Peters, Neubuser et al. 1998). In addition, it was found to be expressed in the adult oesophagus and progressive loss of *Pax9* was seen to correlate with esophageal cancer malignancy.

Three established *Pax9* mouse mutant lines with reduced *Pax9* levels were available in our lab, $Pax9^{LacZ}$, $Pax9^{neo}$ and $Pax9^{flox}$. Stragety for targeting and inactivation Pax9 in the mouse was schematically illustrated as Figure 4.1.

$Pax9^{LacZ}$ was established by Peters in 1998 (Fu, Ishii et al. 2007). In this mouse line, *Pax9* allele was inactivated by the exchange of exon 2 and 3 with *LacZ*, *Pax9* thus totally lost its function. $Pax9^{LacZ/LacZ}$ mice die shortly after birth, whereas $Pax9^{+/LacZ}$ mice are phenotypically normal.

$Pax9^{neo}$ was established by Kist in Peters' lab (Kist, Watson et al. 2005). A removable neomycin resistance cassette (*neo*) flanked by *FRT* sites was inserted into the intron between exons 2 and 3 of *Pax9*. The phosphoglycerate kinase 1 (*Pgk1*) promoter of the *neo*-cassette is known to contain cryptic splice sites thus alternatively spliced mRNAs were generated from the $Pax9^{neo}$ locus. RT-PCR analysis revealed that 44% and 32% of *Pax9* wild-type mRNA levels

were present in *Pax9*$^{+/neo}$ and *Pax9*$^{+/LacZ}$ embryos, respectively. The amount of *Pax9* wild-type transcripts was further reduced to 20% in *Pax9*$^{neo/neo}$ mutants and to 7% in *Pax9*$^{neo/LacZ}$ compound mutants. Tooth development is severely disturbed in *Pax9*$^{neo/neo}$, and the overall body growth of *Pax9*$^{neo/neo}$ is usually reduced compared to wild type and heterozygous controls.

Pax9^{flox} is generated with the insert of LoxP site-flanking *Pax9* exons, thus the functional part of *Pax9* gene could be excised by *Cre* enzyme. We used this mouse line to generate *Pax9*-conditional knockout mice, which will be analyzed in Chapter 5.

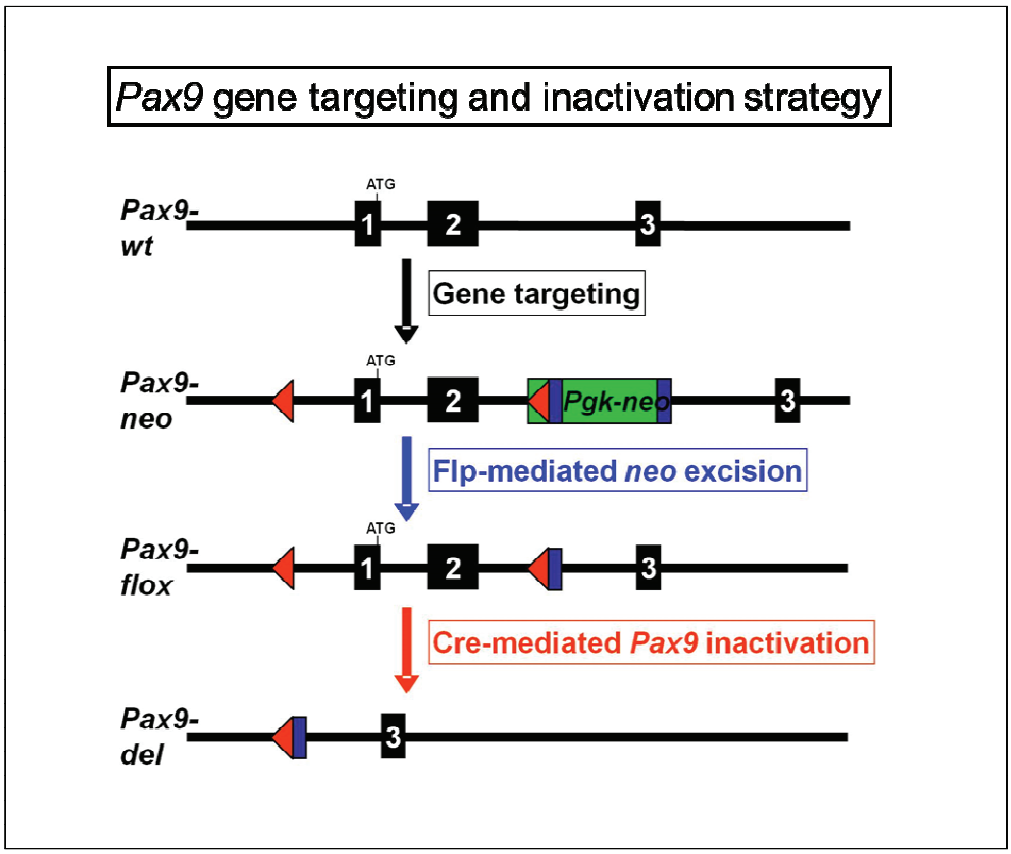

Figure 4.1 Schematic *Pax9* gene targeting and inactivation strategy.
Black rectangles: *Pax9* exons; red triangles: *loxP* sites; blue rectangles: *frt* sites. For detailed depiction, see decription in Chapter 4.1 and Chapter 5.2.1.

The established allelic *Pax9* mutant series in our lab provided mouse models to investigate phenotypes during mammary gland development. Though an intrinsic disadvantage was that systemic reduction of *Pax9* may bring in some unpredictable factors to mammary gland development, especially in terms of global hormone levels, but for a preliminary phenotype screening, these mouse models had easy access. Once phenotype was observed, more subtle models and techniques, for instance, mammary gland transplantation technique and *Pax9* conditional knockout mouse, would be carried out to avoid the interference by systemic *Pax9* reduction.

4.2 Mammary gland rudimental ductal tree appears normal at birth in *Pax9*$^{LacZ/LacZ}$ female mice

Pax9 is completely inactivated in *Pax9*$^{LacZ/LacZ}$. *Pax9*$^{LacZ/LacZ}$ mice die shortly after birth, while *Pax9*$^{+/LacZ}$ mice appear normal. Thus *Pax9*$^{LacZ/LacZ}$ is not able to be used after birth, but suitable to investigate embryonic mammary gland development.

Pax9$^{+/LacZ}$ mice were crossed to generate *Pax9*$^{LacZ/LacZ}$ offspring. Mammary glands were dissected from newborn mice, fixed in 4% PFA, whole-mount stained with Carmine-alum. Genotype and sex were identified by PCR.

The morphology of all five pairs of mammary glands in newborn *Pax9*$^{LacZ/LacZ}$ appeared basically normal, compared to *Pax9*$^{+/+}$ controls. The rudimental ductal trees were seen in the mutants, though occasionally less primary ducts were observed, however, the overall branching pattern were not disturbed. This observation demonstrated that *Pax9* was not an indispensable factor for

embryonic mammary gland development (Figure 4.2).

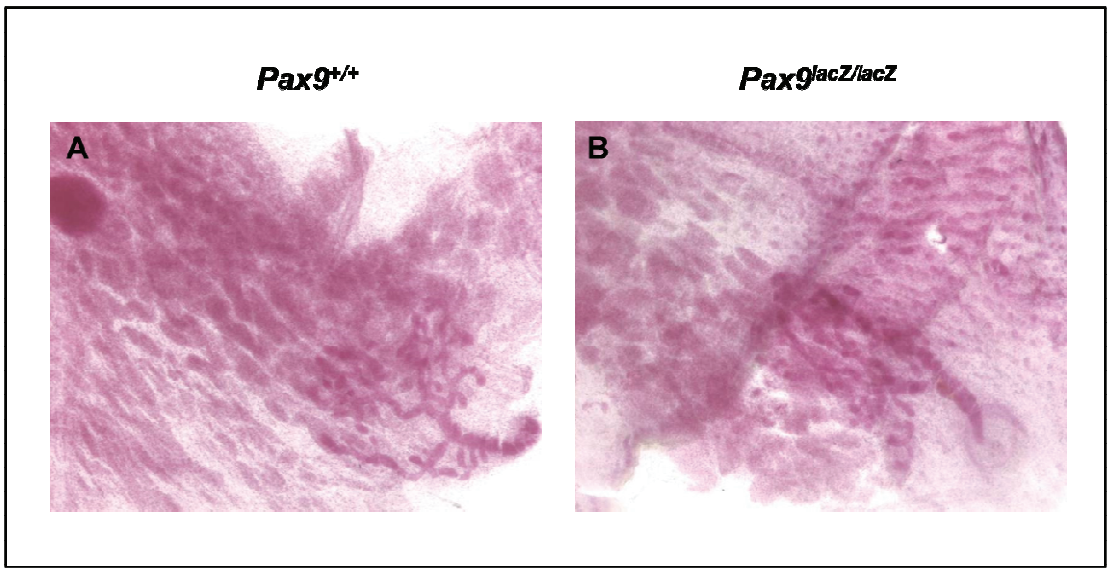

Figure 4.2 Mammary gland rudimental ductal trees showing similar branching pattern in wild type control and *Pax9* mutant mice

(A) A rudimental mammary ductal tree in *Pax9*$^{+/+}$ newborn. (B) A rudimental mammary ductal tree in *Pax9*$^{LacZ/LacZ}$ newborn. The overall branching *pattern* are similar. Magnification: 40x. n=2

4.3 Mammary gland ductal branching is significantly delayed at puberty in *Pax9*$^{neo/neo}$ females

To investigate the phenotype of linear development, mammary glands were dissected at 2 weeks, 3 weeks, 6 weeks and 9 weeks after birth, representing the stages of pre-puberty, onset of puberty, puberty and post-puberty, respectively.

Before puberty (2 weeks), the size and pattern of the primary ductal trees in *Pax9*$^{+/neo}$ and *Pax9*$^{neo/neo}$ mice appear similar, though moderately less branching in mutants was observed (Figure 4.3 A, B). At the onset of puberty (3 weeks), responding to oestrogen circulation, TEBs were promptly formed in *Pax9*$^{+/neo}$ controls, however, they were not formed yet in *Pax9*$^{neo/neo}$ mice. Since the TEB is

the typical organ for the mammary duct elongation and bifurcation, the onset of duct branching during puberty was apparently delayed in *Pax9*$^{neo/neo}$ (Figure 4.3 C, D). At puberty (6 weeks), the mammary gland duct elongation was severely impaired in *Pax9*$^{neo/neo}$ mice. Designating the lymph node as a landmarker, the mammary ducts in *Pax9*$^{neo/neo}$ mice had just reached the lymph node site, while the ducts in *Pax9*$^{+/neo}$ mice had grown past the lymph node and almost reached the distal end of the mammary gland. In addition, less side branching was seen in the *Pax9*$^{neo/neo}$ mammary gland. The number of TEBs at puberty stage in *Pax9*$^{neo/neo}$ mice is far less than in *Pax9*$^{+/neo}$ mice. The TEB is a motile structure of the mammary gland, leading mammary ducts invading into the fat pat until the ducts occupy the whole fat pad at the end of puberty. Cellular events in TEBs are very active, including cell differentiation, epithelial cell proliferation, apoptosis and ECM degradation and cell alignment. The inadequate development of TEBs might be an important causal event for the retarded ductal branching (Figure 4.3 E, F).

Though the ductal branching was delayed in the mutant mammary gland during puberty, it finally could catch up because the branching continued until the duct reaches the edge of the fat pad (Figure 4.3 G, H).

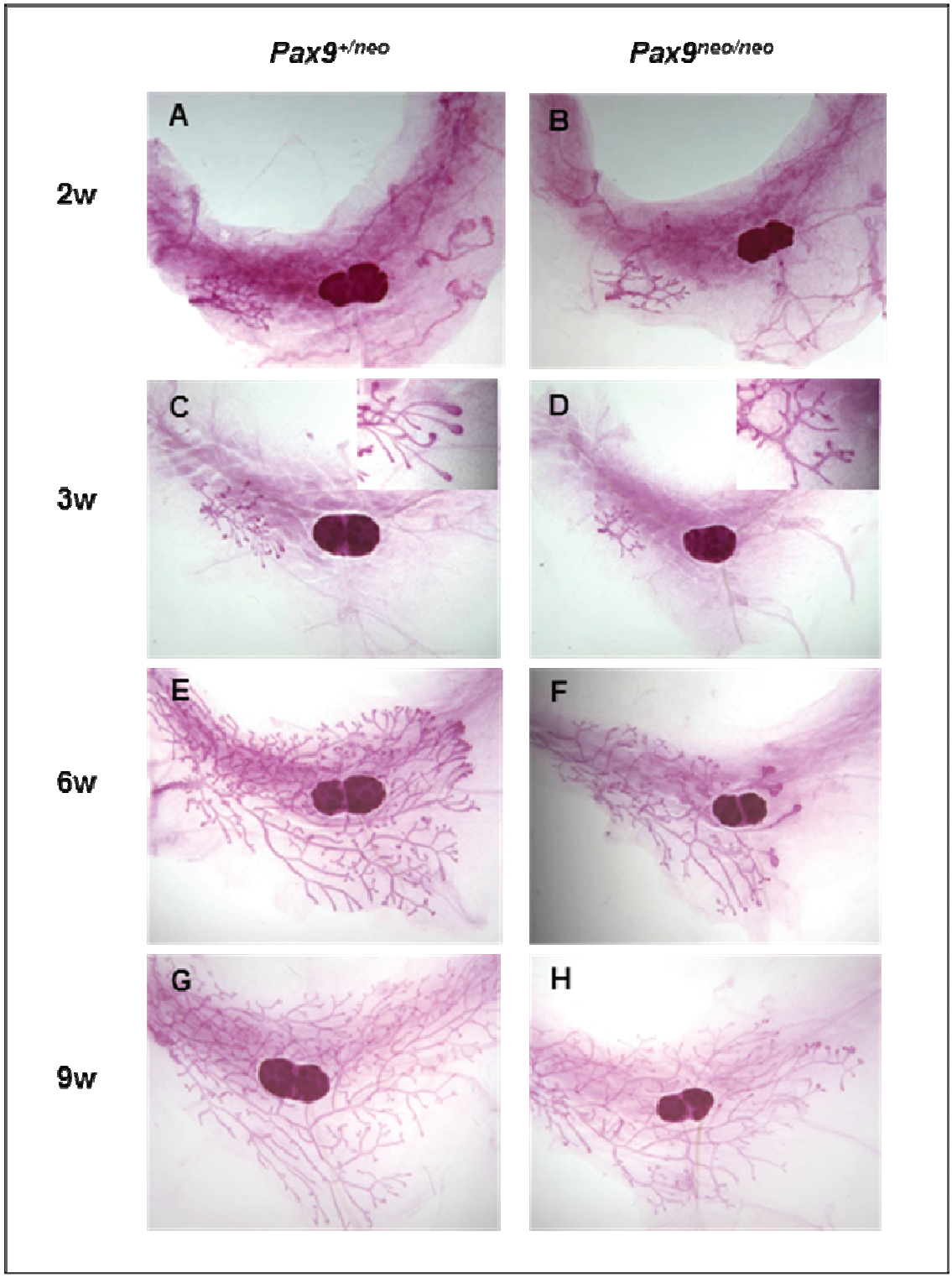

Figure 4.3 Linear mammary gland development in *Pax9* mutant mice and controls

Ductal tree patterns appear similar in *Pax9*$^{+/neo}$ and *Pax9*$^{neo/neo}$ mice (A, B) at 2 weeks age, TEBs formation is delayed at onset of puberty in *Pax9*$^{neo/neo}$ (C, D), ductal branching severely impaired in *Pax9*$^{neo/neo}$ at puberty, in terms of both elongation and side branching, with less TEBs formed at the leading end of ducts(E, F), the branching of the duct in *Pax9*$^{neo/neo}$ would finally catch up though delayed compared to control (G, H).n=3 for each time point. Magnification: 10x. n=3

However, the reduction by the overall body growth of *Pax9*$^{neo/neo}$ mice might need to be considered since *Pax9*$^{neo/neo}$ mice usually smaller than controls. To evaluate the overall growth of the mammary gland, mammary gland weight and body weight were taken into account. Mammary glands were dissected at 2 weeks, 6 weeks, and 9 weeks age respectively and weighted, mice were weighed before dissection. The weight of *Pax9*$^{neo/neo}$ mammary gland was usually less than the control, however, the body weight of the mutant was less than the control as well. Reduction of mammary gland weight in *Pax9*$^{neo/neo}$ female was found to correlate with reduction of the body weight (Figure 4.4).

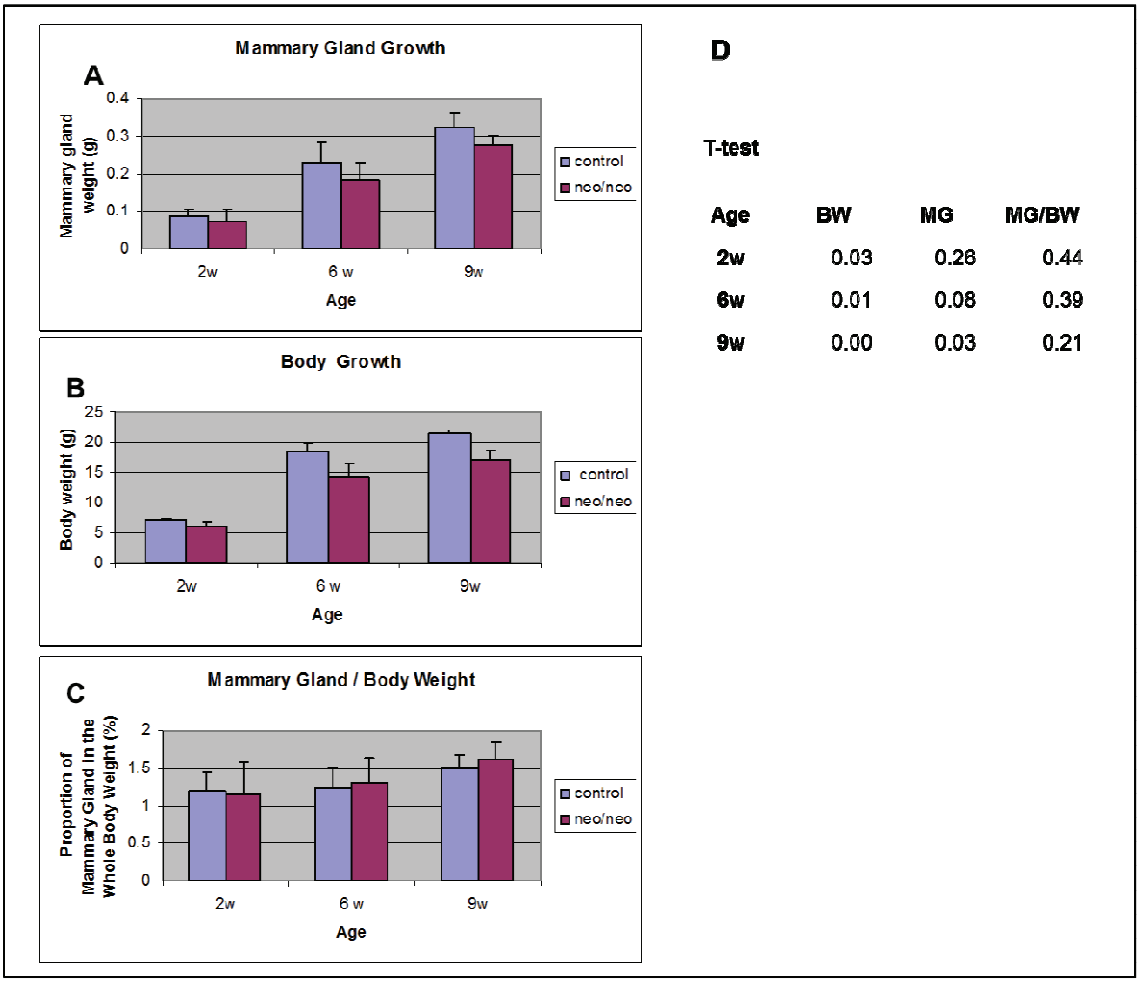

Figure 4.4 Mammary gland weight growth in *Pax9* mutant mice and controls

(A) Mammary gland weight measured at indicated ages. (B) Mouse body weight at indicated age. (B) Weight percentage of mammary gland in mouse body. (D) T-test showing significance between mammary gland and body weight. Three or four female mice were used for each time point. BW: body weight; MG: mammary gland weight. n=4 to 6 for each genotype.

4.4 Alveoli morphology is basically normal in *Pax9*$^{neo/neo}$ but the growth of pups nursed by *Pax9*$^{neo/neo}$ females is delayed during pregnancy

To investigate the impact of the reduction of *Pax9* on mammary gland development during pregnancy, we examined mammary gland morphology at lactation day 1, and traced the weight gain curve of pups from day 1 to day 13 after birth, which reflected the lactating ability of the mammary gland (Figure 4.5).

At lactation day 1, well-developed alveoli were seen in both of *Pax9*$^{neo/neo}$ and *Pax9*$^{+/neo}$ females, alveolar are known to develop from tertiary ducts, as seen in the control, but occasionally some alveoli were formed directly from the primary ducts and side branches of *Pax9*$^{neo/neo}$ females (Figure 4.5 A). This may indicate a misled differentiation of the mammary epithelial cell.

The weight gain curve of pups reflects the lactating capability of the dam. To normalize the lactating pressure, only 10 pups were kept with dam, while the extra pups were removed immediately after birth. The pups and dams were weighed at fixed time every day. Pups nursed by *Pax9*$^{neo/neo}$ dams were gaining weight behind those nursed by *Pax9*$^{+/neo}$ dams, which implicated that the lactating capacity of *Pax9*$^{neo/neo}$ dams was impaired to some extent. On the other hand, the body weight of *Pax9*$^{neo/neo}$ is usually smaller than *Pax9*$^{+/neo}$, thus the overall health and nutrition level possibly was below normal conditions, which could be one of the reasons that the lactating capacity of *Pax9*$^{neo/neo}$ females was weaker than controls (Figure 4.5 B, C).

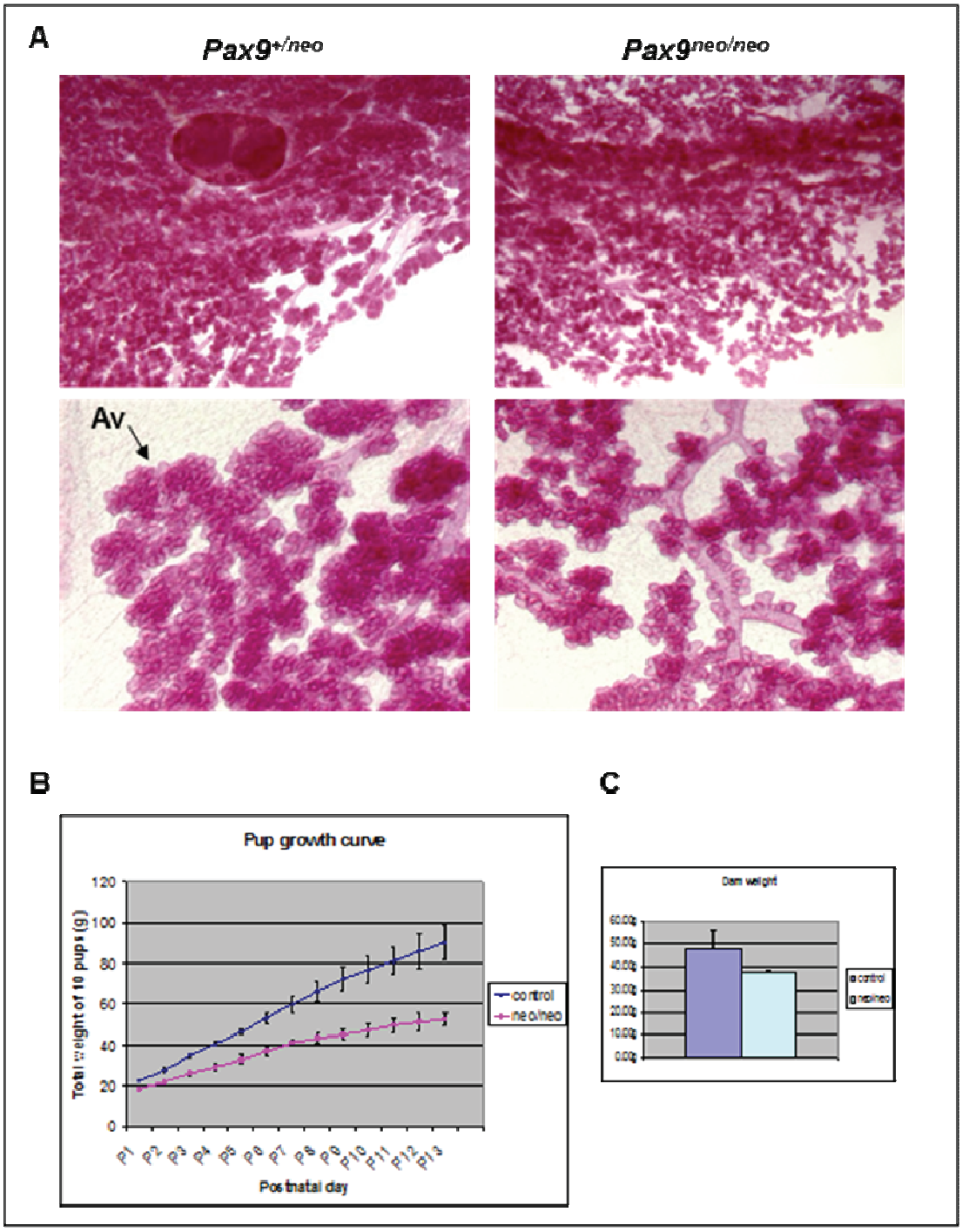

Figure 4.5 Alveolus formation and lactating capacity

(A) The mammary gland of *Pax9*$^{neo/neo}$ shows the morphology similar with the control at lactation day 1. (B) Litters from *Pax9*$^{neo/neo}$ females show 42% less body weight. (C) The body weight of *Pax9*$^{neo/neo}$ dams is reduced by 21%. n=3. Magnification: (A, B) 10x, (C, D) 40x. n=3

4.5 Ductal hyperplasia, hyperplastic alveolar nodules were sporadically seen in the mature mammary gland in *Pax9*$^{neo/neo}$ mice

After puberty with the most rapid ductal branching, and between every pregnant cycle with enormous alveolar development and tissue remodeling post-lactation, the mammary gland keeps a relative quiet state. Tertiary ducts and alveoli also moderately form and regress following every estrus cycle. Mechanisms to maintain the mammary epithelial cell differentiation state in the mature duct are crucial for the homeostasis of the mammary gland and to avoid epithelial cells transforming to breast cancer cells. Loss of some genes, for example, *GATA3* (Kouros-Mehr, Slorach et al. 2006, Kouros-Mehr, Kim et al. 2008, Raven, Williams et al. 2011), have been shown to change ductal epithelial cell characteristics and initiates cancer in some cases.

In order to see whether the reduction of *Pax9* expression disturbs the maintenance of the epithelium in the mature duct, or any other impact left by early events, we investigated the mammary glands of 3.5-month old virgin *Pax9*^{neoneo} females and 6 and 8-month old parous *Pax9*$^{neo/neo}$ females (Figure 4.6).

The ductal branching pattern of 3.5-month old *Pax9*$^{neo/neo}$ appeared more compact than controls, ductal walls were thicker, and structure resembling hyperplastic alveolar nodules (HANs) formed sporadically. In 6 and 8-month old parous non-pregnant *Pax9*$^{neo/neo}$, ductal hyperplasia was obviously shown and nodules were found at the distal ends.

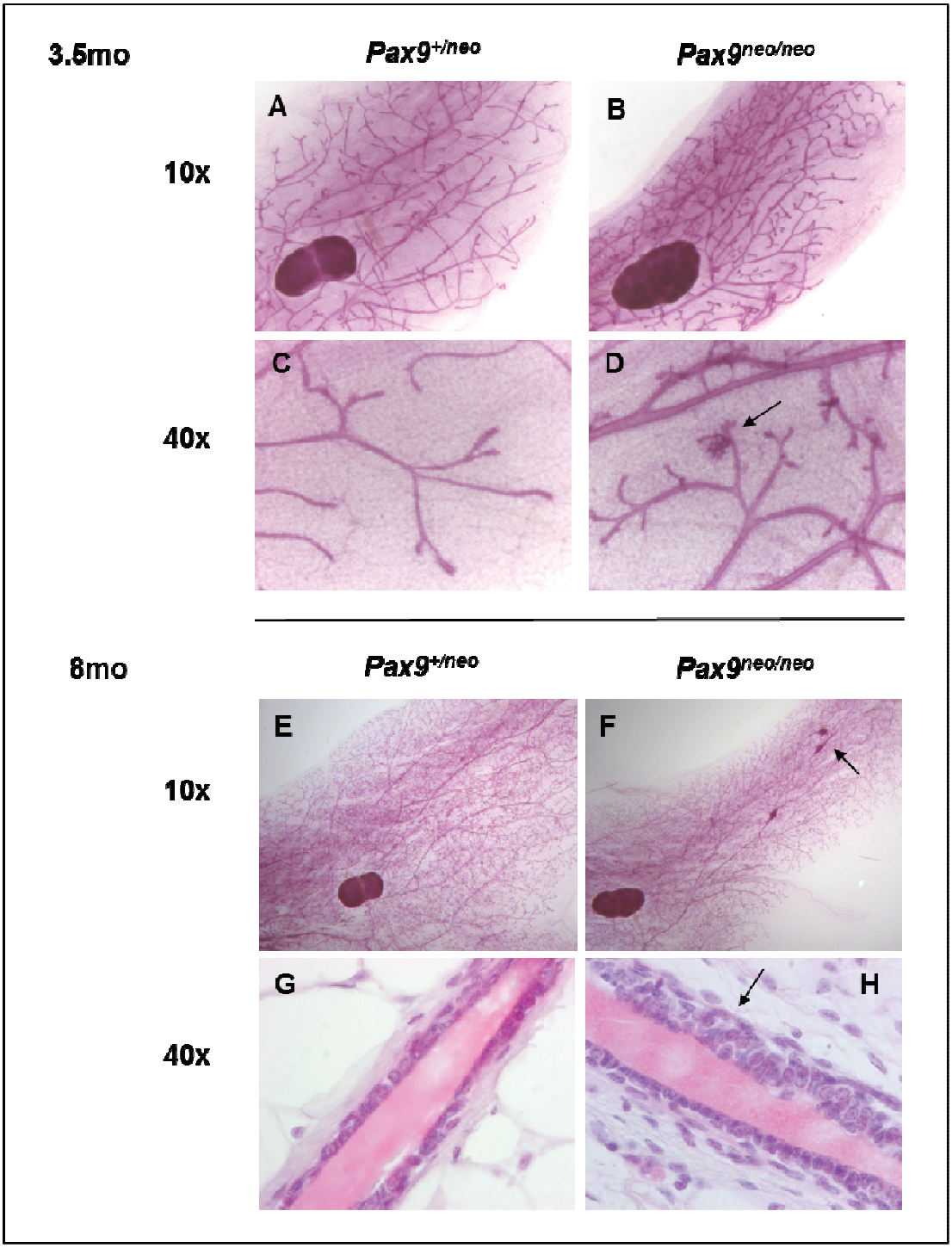

Figure 4.6 HANs and ductal hyperplasia in *Pax9*$^{neo/neo}$ females

Hyperplastic alveolar nodules (HANs, indicated by arrow in D) were seen in the mammary gland of 3.5 months old *Pax9*$^{neo/neo}$ virgin, by whole mount Carmine-alum staining (A, B, C, D) and hyperplasia in the duct (indicated by arrow in H) and small nodules (indicated by arrow in F) within mammary ducts in 8 months old *Pax9*$^{neo/neo}$ by H&E staining on sections (E, F, G, H). n=3

4.6 Loss of laminin in the basement membrane of the mammary duct in *Pax9*$^{neo/neo}$

The basement membrane is the outer layer of mammary duct epithelia, the important function of which is to keep the integrity of the duct and maintain polarity of the epithelial cells. Laminin is a characteristic component of basement membrane. We applied immunohistochemistry with anti-laminin1 antibody to investigate the integrity of the basement membrane of the mammary ducts. Parous non-pregnant wild type mice (WT) used as controls, both *Pax9*$^{+/neo}$ and *Pax9*$^{neo/neo}$ were examined.

In wild type controls, a single layer of ductal epithelial cells strictly aligns along the duct, with a single layer of myoepithelial cells aligning outside. *Pax9* is expressed in most dutal epithelial cells. In *Pax9* reduced mammary glands, the number of *Pax9* positive cells is much less than in wild type. The *Pax9* positive epithelial cells still align regularly along the duct, in a single layer pattern, mixed with *Pax9* negative ductal epithelial cells, but some of the *Pax9* negative cells expends and detaches from the single layer alignment (Figure 4.7 A, B, C).

Laminin1 is entirely lost outside the ducts in *Pax9*$^{+/neo}$ females, whereas intact in wild type. The duct filled with *Pax9* negative cells was also seen with the loss of *Laminin1* (Figure 4.7 D, E). These observations imply that *Pax9* may function to maintain ductal epithelial cell alignment in the duct and keep the integrity of the basement membrane.

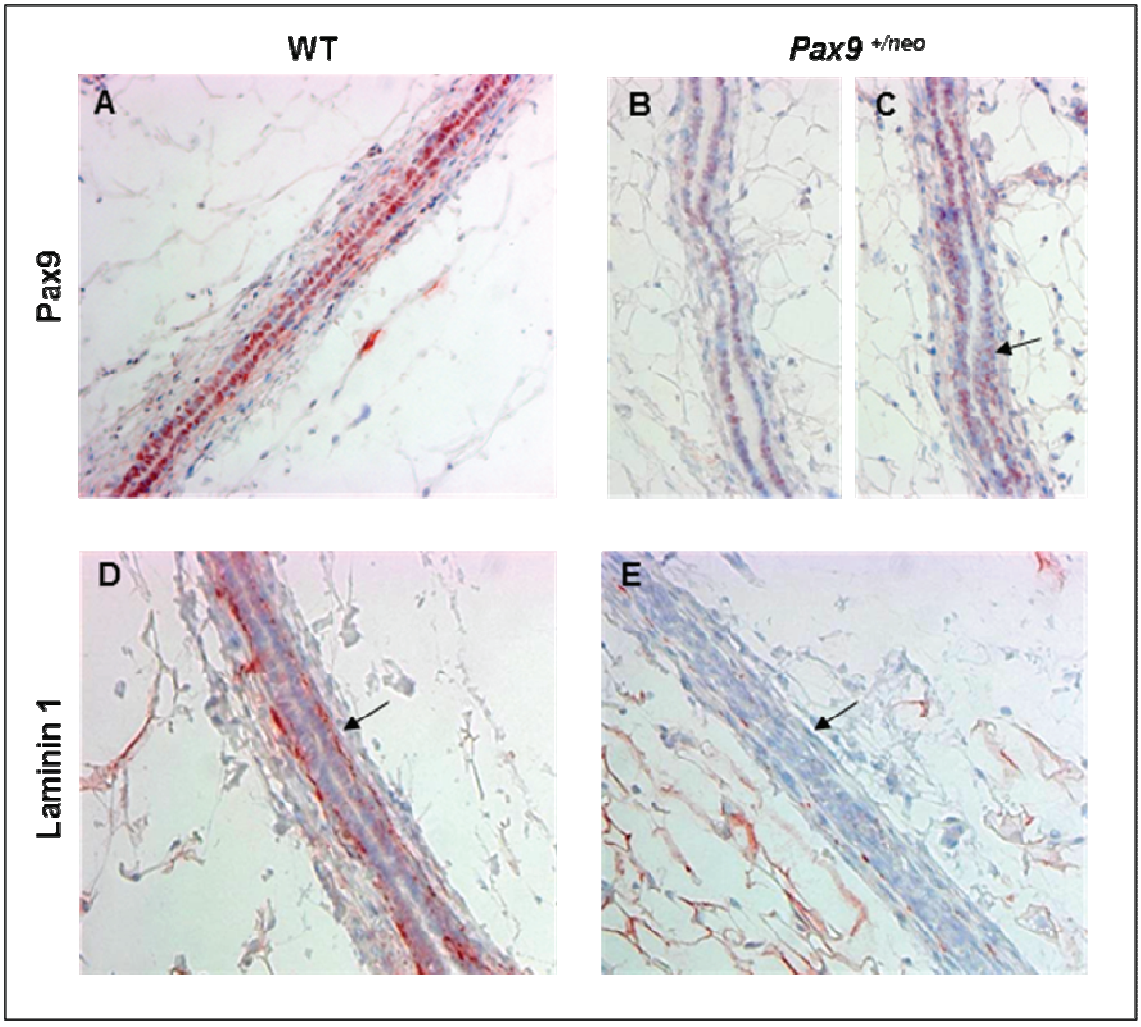

Figure 4.7 *Pax9* inactivated cells expand and basement membrane integrity is impaired in *Pax9*$^{+/neo}$ females

Pax9 positive cells align in a single layer pattern in wild type control (A). *Pax9* is reduced both at the average expression level and the number of positive cells (B), and *Pax9* negative cells expand (indicated by arrow) and detach from the single layer alignment (C). *Laminin1* is expressed in the basement membrane of the mammary duct in wild type (D, indicated by arrow), but lost in mutant and duct filled with expanded cells is seen in mutant (E, indicated by arrow). Magnification: (A, B, C) 200x, (D, E) 400x, cropped. n=3

4.7 The disorganized patterns of luminal epithelial cells in parous *Pax9*$^{neo/neo}$ are similar to the histology of human breast cancers

The whole mount staining of the mammary gland in *Pax9*$^{+/neo}$ and *Pax9*$^{neo/neo}$ mice did not show a profound phenotype towards mammary tumour formation, though it may need a longer latency or more replicates to make it statistically

meaningful, however, the histology and immunohistochemistry on sections provided more details about the cell alignment within ducts with *Pax9* expression.

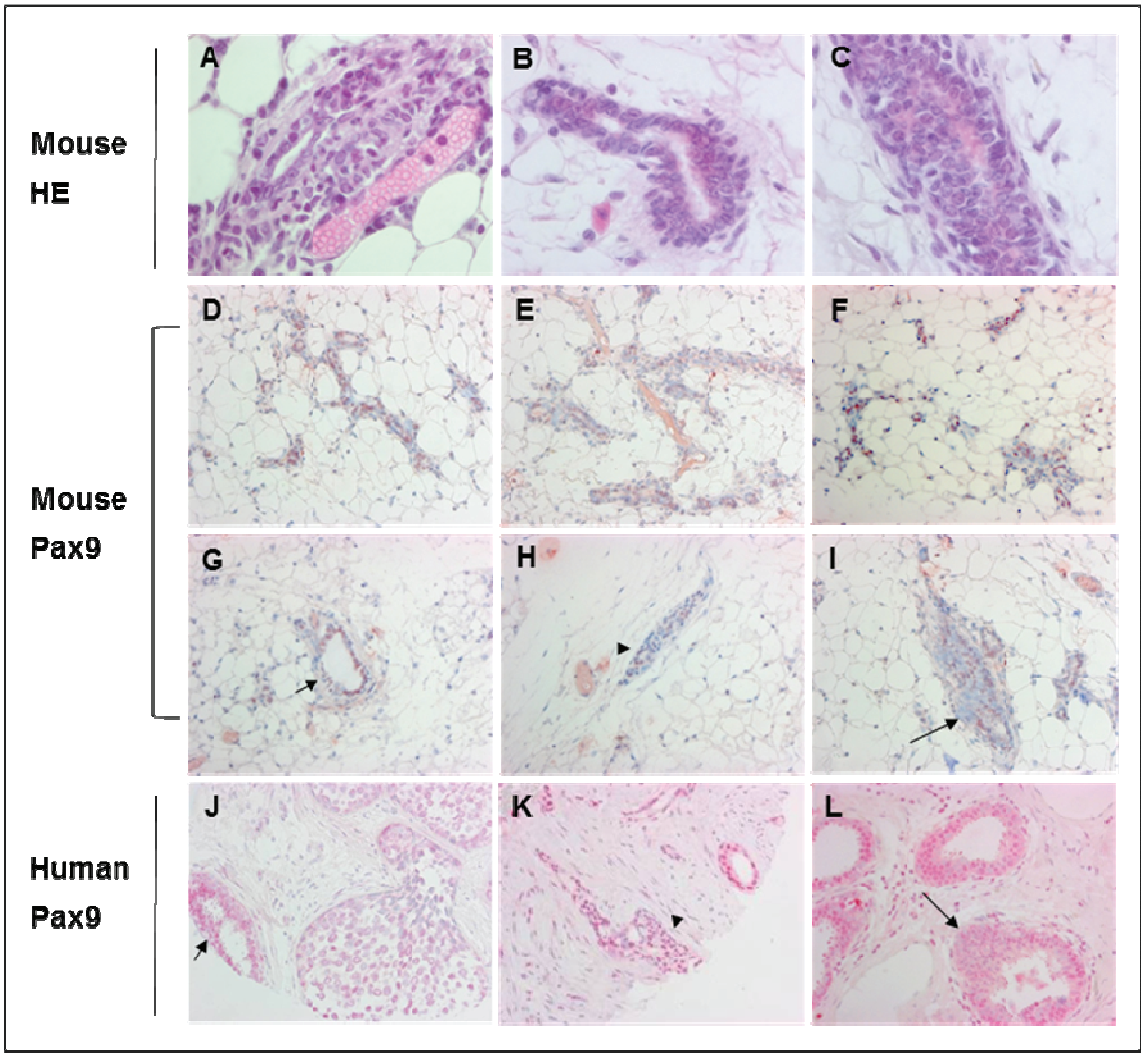

Figure 4.8 The patterns of disorganized luminal epithelial cells in *Pax9*$^{neo/neo}$ mammary gland mimic cancerous histology

In *Pax9*$^{neo/neo}$ mammary gland, some ductal epithelial cell expanded and filled the duct space (A-C, expanding cells indicated by arrows, a normal duct surrounded with unknown infiltrating cells indicated by arrowhead). *Pax9* positive cells lined in the duct (D-F), *Pax9* negative cells filled the duct (H) and form cell mass, some of *Pax9* negative cells invaded into the stroma (G, I, cell mass indicated by arrows), which were similar with the histology of breast cancer in human (J-L, duct filling indicated by arrowhead, cancer cell mass indicated by arrow). Magnificaiton: 200x. n=3.

The H&E staining of 3.5-month old *Pax9*$^{neo/neo}$ mammary gland showed disorganized ductal epithelial cells. Epithelial cells were expanded, escaped from the single layer alignment restriction and formed masses filling the duct (Figure 4.8 A, B, C). *Pax9*-immunohistochemistry showed that intact ducts were mainly composed with *Pax9* positive cells, though *Pax9*-reduced cells also mixed in, while the *Pax9*-negative luminal cells expanded, lost alignment and filled the ducts (Figure 4.8 D, E, F). *Pax9* negative luminal cells even formed cell mass migrating through the basement and invading into the matrix of mammary gland (Figure 4.8 G, H, I), which were similar with the histology of human breast cancer (Figure 4.8 J, K, L) (see Chapter 6 for details about *Pax9* expression in breast cancer). Thus, compared with human breast cancer, the progression to a disorganized pattern of luminal cells in *Pax9*$^{neo/neo}$ could, for some extent, mimic pre-cancerous stages.

4.8 Discussion

Though *Pax9*^{neo} mouse line was not an ideal mouse model to precisely study *Pax9*'s function in the mammary gland, the phenotype of *Pax9*$^{neo/neo}$ still provided us the first vision and a useful screen for possible roles of *Pax9* playing in the mammary gland at particular developmental stages.

The embryonic mammary gland development was not affected severely in *Pax9*$^{LacZ/LacZ}$ females, the rudimental tree basically developing normal. The significant phenotype was seen at puberty stage, when ductal branching delayed profoundly with less TEBs and slow ductal elongation. Previously we had found that *Pax9* transcript and protein expression peaks at puberty (see Chapter 3), and *Pax9* protein is strongly expressed in the TEB cap cells, a cell population with active proliferation. Therefore, less TEBs formation and delayed ductal elongation during puberty in *Pax9*$^{neo/neo}$ females further supported a function of

Pax9 in mammary gland development during puberty. This delay could also be secondary effect to delayed development of *Pax9*$^{neo/neo}$ mice.

Alveolar formation appeared normal, though some alveoli developed from the primary duct in the mutant. Lactating capacity was weaker in mutants than in controls, but considering the reduced body weight of the mutant dam, the reduced lactating capacity may not directly come from the reduction of *Pax9*.

Besides cell proliferation and differentiation, the maintenance of luminal epithelial cells differentiation state is important as well. The distortion of the ductal epithelial characteristics can initiate breast cancer. With the reduction of *Pax9*, ducts were occasionally filled with *Pax9* negative epithelial cells, and ductal epithelial cells lost their single layer alignment and formed cell mass. The integrity of the basement membrane appeared defective with the loss of Laminin1, while Laminin was reported to play an important role in keeping epithelial cell polarity (Deblois, Chahrour et al. 2010, Liu, Nugoli et al. 2011). When compared with human breast cancers, there is similar histology of the luminal cell disorganization in the mouse mutants and human breast cancer.

The reduction of *Pax9* using *Pax9*^{neo} mouse line is a systemic reduction, we did not know exactly whether it affects the hormone levels in the mouse. However, mammary development is a highly coordinated process involving global and local signaling, and hormone control is the most characteristic factor for mammary gland development. If *Pax9* is involved in the development and functions of other glands, for instance, ovary or pituitary, the mammary gland development will definitely be profoundly affected by the hormone changing. In fact, by RT-PCR, we found that *Pax9* was expressed in the ovary of a 5 week old wild type mouse (figure not shown here).

For further studies, we need to exclude the systemic effect of the *Pax9* inactivation. The mammary transplantation could not be used to investigate lactation because the transplanted mammary gland is not linked to the nipple, thus we decided to use a conditional knockout mouse model to study *Pax9*'s function specifically in the mammary gland.

Chapter 5. Mammary gland-specific *Pax9*-deficiency severely impairs mammary gland involution

5.1 Introduction

Demonstrated by previous studies in mice carrying a $Pax9^{neo}$ allele, *Pax9* dosage reduction delays mammary gland branching at adolescent stage, and forms ductal hyperplasia, hyperplastic alveolar nodes (HANs) and neoplasia in the mammary gland in parous and nulliparous non-pregnant mice, accompanied by loss of laminin1 in extracellular matrix (ECM). However, systemic reduction of *Pax9* using a $Pax9^{neo}$ allele had an inevitable disadvantage, by which hormone levels may have been affected by systemic *Pax9* reduction and hormonal signallings crucial to mammary gland development thus have been attenuated as well. To gain conclusive evidence for the function of *Pax9* playing in the mammary gland, and to provide reliable materials for successive molecular regulatory mechanism study, we needed to generate mammary gland-specific *Pax9*-deficient mouse models to exclude any other disturbance to mammary gland development rather than genetic and molecular changes from the mammary epithelium itself.

For this purpose, several lines of mammary gland-specific *Pax9*-deficient mouse model were built up, phenotypes were examined at adolescent and pregnant stages, immunohistochemistry, western blot and expression microarray were applied to identify candidate signaling pathways regulated by *Pax9*. Loss-of-function study of *Pax9* in the mammary gland was expected to provide clearer insight of the functions of *Pax9* in mammary gland biology.

5.2 Generation of mammary gland-specific *Pax9*-deficient mice and evaluation of *Cre* mediated recombination efficiency

5.2.1 Strategy to knockout Pax9 specifically in mouse mammary gland

Transgenic mouse models that use promoters active specifically in the mammary gland to selectively activate or remove particular genes from mammary epithelial cells have greatly facilitated the dissection of mammary gland developmental processes (Fantozzi and Christofori 2006). Many of these transgenic mouse models revealed unexpected connections between the processes of normal mammary gland development and mammary tumour growth and progression.

To determine whether *Pax9* is required for mammary gland development, our laboratory generated mice carrying a conditional *Pax9* allele (*Pax9*^{flox}), in which the first and second coding exons, containing the ATG translational start site of the *Pax9* gene, were flanked with LoxP recombination sites (Kist, Greally et al. 2007), Figure 5.1.

To facilitate excision of the Pax9flox allele in the epithelial compartment of the mouse mammary gland, *Cre* allele was introduced into the conditional *Pax9* strain (Figure 5.1). We had two mouse lines carrying *Cre* allele under the control of two different promoters: mouse mammary tumour virus (*MMTV*), expressing *Cre* in the mammary epithelium (*MMTV-Cre*) (Guy, Cardiff et al. 1992, White, Kurpios et al. 2004), and cytokeratin 14 (K14), expressing *Cre* in the mammary placode and the myoepithelium in mammary ducts (*K14-Cre*) (Fu, Ishii et al. 2007). *MMTV-Cre* strain was bred in FVB background, provided by Muller's lab in Canada. *K14-Cre* strain was bred in C57BL6 background, provided by Elaine' lab.

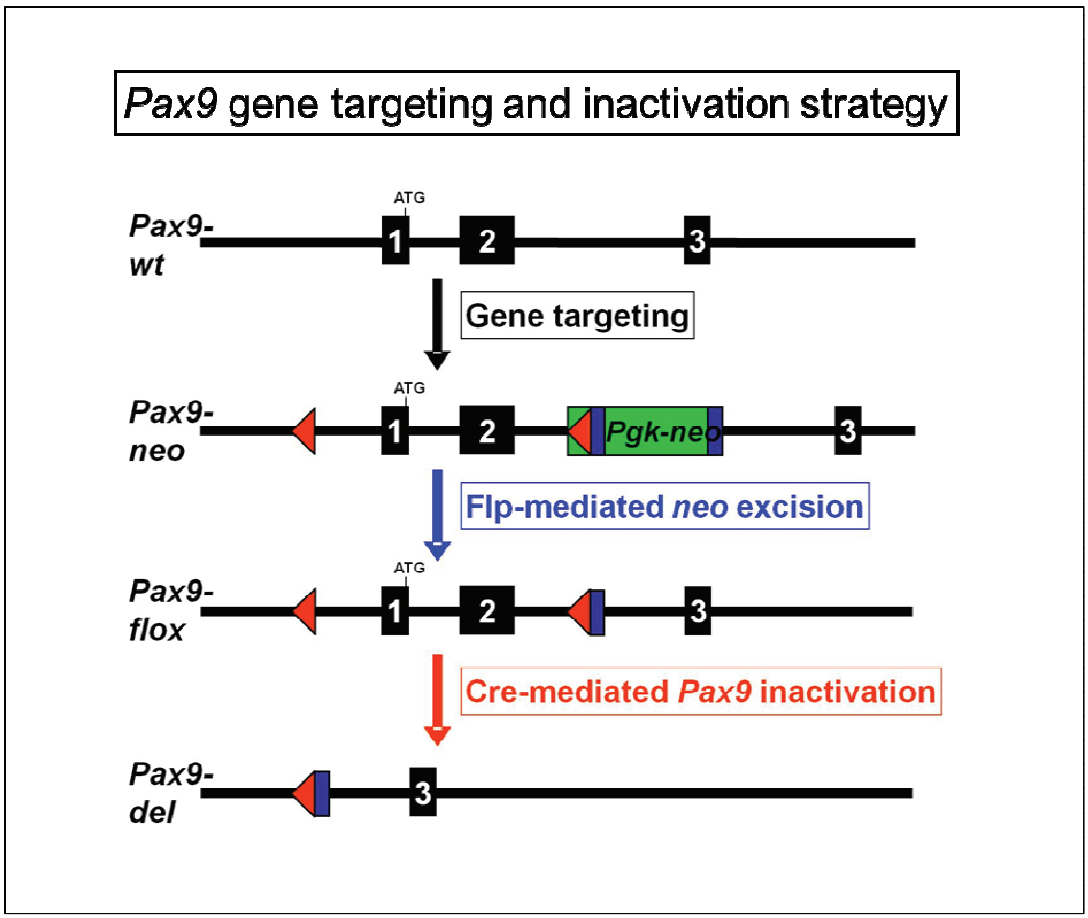

Figure 5.1 Schematic representation of the *Pax9* alleles used in this study

Black rectangles: *Pax9* exons; red triangles: *loxP* sites; blue rectangles: *frt* sites. For detailed depiction, see text in Chapter 5.2.1.

5.2.2 Cre-mediated recombination was heterogeneous and was up-regulated by pregnancy

To monitor *Cre*-mediated recombination, mice carrying *Cre* alleles could be bred to mice harbouring a *Cre*-responsive β-galactosidase reporter construct (GTRosa26 line) (Soriano 1999, White, Kurpios et al. 2004). The presence of the GTRosa26 allele in *Cre* mice enables us to detect *Cre*-mediated excision in situ (Figure 5.2 A). When the mammary gland from the bitransgenic mouse is stained in situ with the colorimetric β-galactosidase substrate X-gal, whole mount staining and sections of the stained glands reveals expression of the *Cre*

transgene.

The MMTV promoter has been shown to work efficiently in FVB mouse background in Muller's lab (Figure 5.2 B), but promoter activity may be different in a mixed genetic background. On the other hand, *K14* is known to be expressed as early as in the mammary placode at embryonic stage and in the myoepithelium of mammary ducts, and *K14-Cre* mediated recombination in mammary gland was seen in mammary gland (Figure 5.2 C), but the frequency of recombination has not yet been evaluated. Therefore, the efficiency of *Cre*-mediated recombination needed to be re-evaluated in the genetic background of our mouse strains.

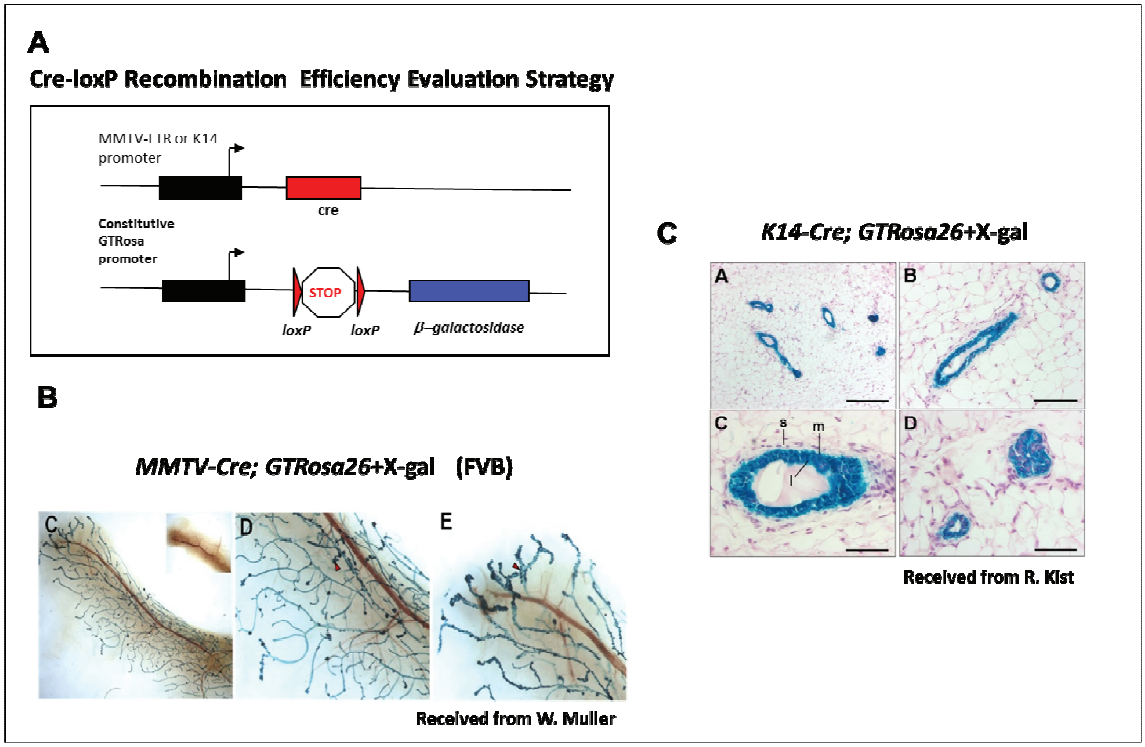

Figure 5.2 Efficiency of *Cre-loxP* recombination

A) Schematic representation of evaluating *Cre* recombination activity using *Cre*-responsive GTRosa26 β-galactosidase reporter construct. B) *MMTV-Cre* is active in mammary gland in FVB background provided by Muller's lab. C) *K14-Cre* is active in epithelial cells as well as myoepithelial cells in this tested mouse model, which may be due to K14 is active as early as in mammary placode during embryonic stage, though otherwise K14 is exclusively expressed in the myoepithelium after a primary ductal tree forms.

We bred mice carrying MMTV-Cre and K14-Cre, respectively, to mice carrying GTRosa26, and monitored the recombination by X-gal whole mount staining. The mammary ducts of K14-Cre;GTRosa26 mice showed heterogeneous pattern for X-gal staining (Figure 5.3 A), maybe due to heterogeneously Cre-mediated recombination in the mammary placode. In the mixed genetic background of the MMTV-Cre (FVB) and Pax9flox carriers (C57BL6), Cre-mediated recombination was found sporadically as well. The mammary glands in virgin MMTV-Cre;GTRosa26, X-gal were mostly negative for X-gal staining, but lactating mammary glands exhibited strong positive X-gal staining in some particular lobule alveoli (Figure 5.3 A). Based on above tests, we regarded MMTV-Cre mouse line as a better Cre allele carrier to generate mammary gland-specific Pax9-deficient mouse model.

We also confirmed targeted excision of the *Pax9*^{flox} allele in the mammary glands using molecular approach. When *MMTV-Cre* and *K14-Cre* mice were bred to *Pax9*$^{flox/flox}$ mice to generate mammary gland-specific *Pax9*-deficient mice, targeted recombination of the *Pax9*^{flox} allele could be demonstrated by PCR analysis of mammary gland DNA from *MMTV-Cre*;*Pax9*$^{flox/flox}$ and *K14-Cre*;*Pax9*$^{flox/flox}$ animals, using primers which amplify both intact and *Cre*-deleted forms of the *Pax9*^{flox} allele. Amplification of the intact form of the *Pax9*^{flox} allele likely reflects the presence of stromal cell DNA in the preparation.

As detected by PCR of *Pax9*^{flox} allele, *Pax9* deletion was weak in virgin *K14-Cre*;*Pax9*$^{flox/flox}$ and virgin *MMTV-Cre*;*Pax9*$^{flox/flox}$ (Figure 5.3 C, D). MMTV promoter is reported to be activated by pregnancy, so we set *MMTV-Cre*;*Pax9*$^{flox/flox}$ mice for pregnancy and examined *Pax9* deletion in the mammary gland at involution stage following pregnancy by PCR analysis as well. The deletion of *Pax9* was dramatically increased in the involuting mammary

gland of *MMTV-Cre*;*Pax9*$^{flox/flox}$ mice (Figure 5.3 D).

The activity of MMTV promoter is hormone-inducible, thus high levels of oestrogen and progesterone during pregnancy significantly increases *Cre*-mediated recombination. Compared to K14 promoter, MMTV promoter works more efficiently for *Cre*-mediated recombination following pregnancy in the mammary gland in FVB/C57BL6 mixed gene background. Weak recombination in virgin *MMTV-Cre*;*Pax9*$^{flox/flox}$ mice limits our study of *Pax9*'s function for mammary ductal elongation and branching during puberty, however, it endows us a tool to study the role of *Pax9* in mammary gland involution, with the late activation of *Pax9* deletion specifically in the mammary gland following pregnancy.

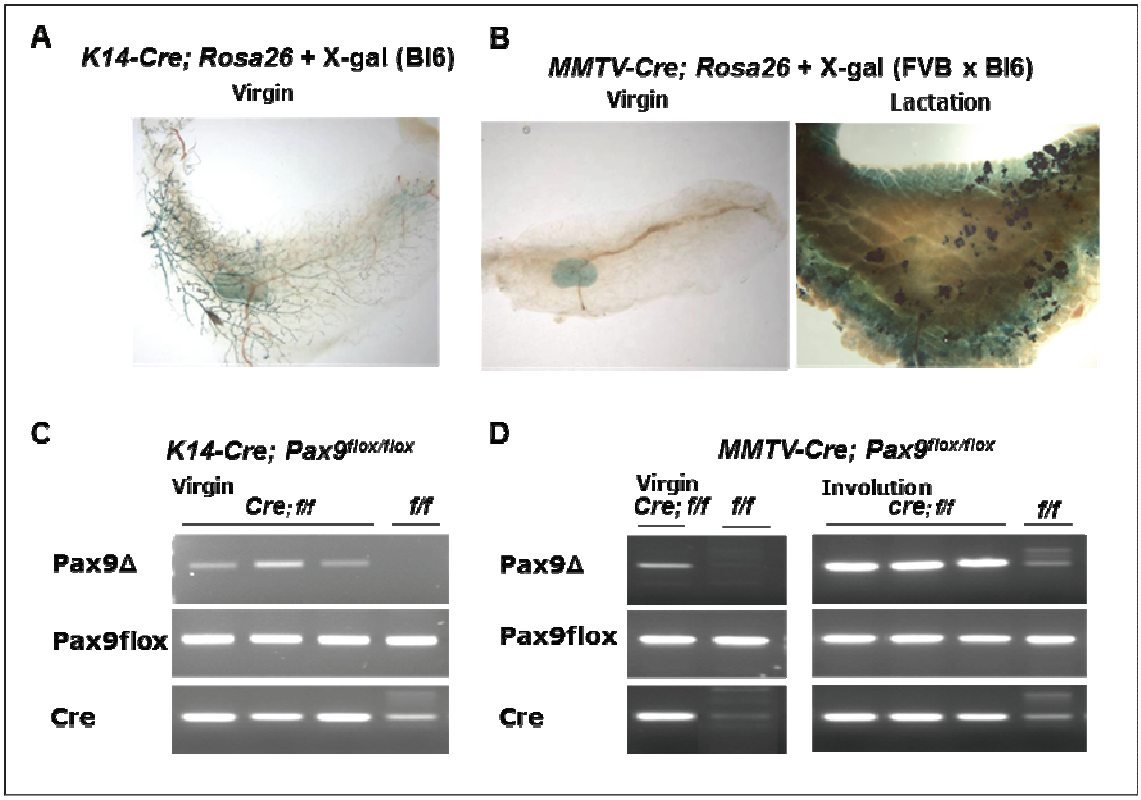

Figure 5.3 Evaluation of *Cre*-mediated recombination

(A, B) X-gal whole mount staining of the mammary glands in *MMTV-Cre;GTRosa26* and *K14-Cre; GTRosa26 mice*. (C, D) PCR detection of *Pax9* deletion in *MMTV-Cre*;*Pax9*$^{flox/flox}$ and *K14-Cre*; *Pax9*$^{flox/flox}$ mice. Magnification: 10x. n=3

5.3 Hyperplastic alveolar nodules are frequently seen in mammary glands in old virgin *K14-Cre*; *Pax9*$^{flox/flox}$ mice

Though *Cre*-mediated *Pax9* deletion in *K14-Cre*; *Pax9*$^{flox/flox}$ was heterogeneous, we s*cre*ened the mammary glands, by Carmine-alum whole mount staining, of a total of 8 mutant females, at the age between 10 month to 1 year. Hyperplastic alveolar nodules (HANs) were seen in 5 out of 8 mutants, but not in *Pax9*$^{flox/flox}$ controls. Thicken ductal wall was seen in the mutants as well. This phenotype is similar with the observation in 3.5 months *Pax9*$^{neo/neo}$ mutants.

HANs in the mouse mammary gland, is the compartment for terminal ductal lobular units (TDLUs) in human breast, small ductules at the end of mammary gland, where the majority of breast cancers arise. Reduction of *Pax9* in the mammary gland results in the HAN formation thus increased the risk of mammary gland oncogenesis.

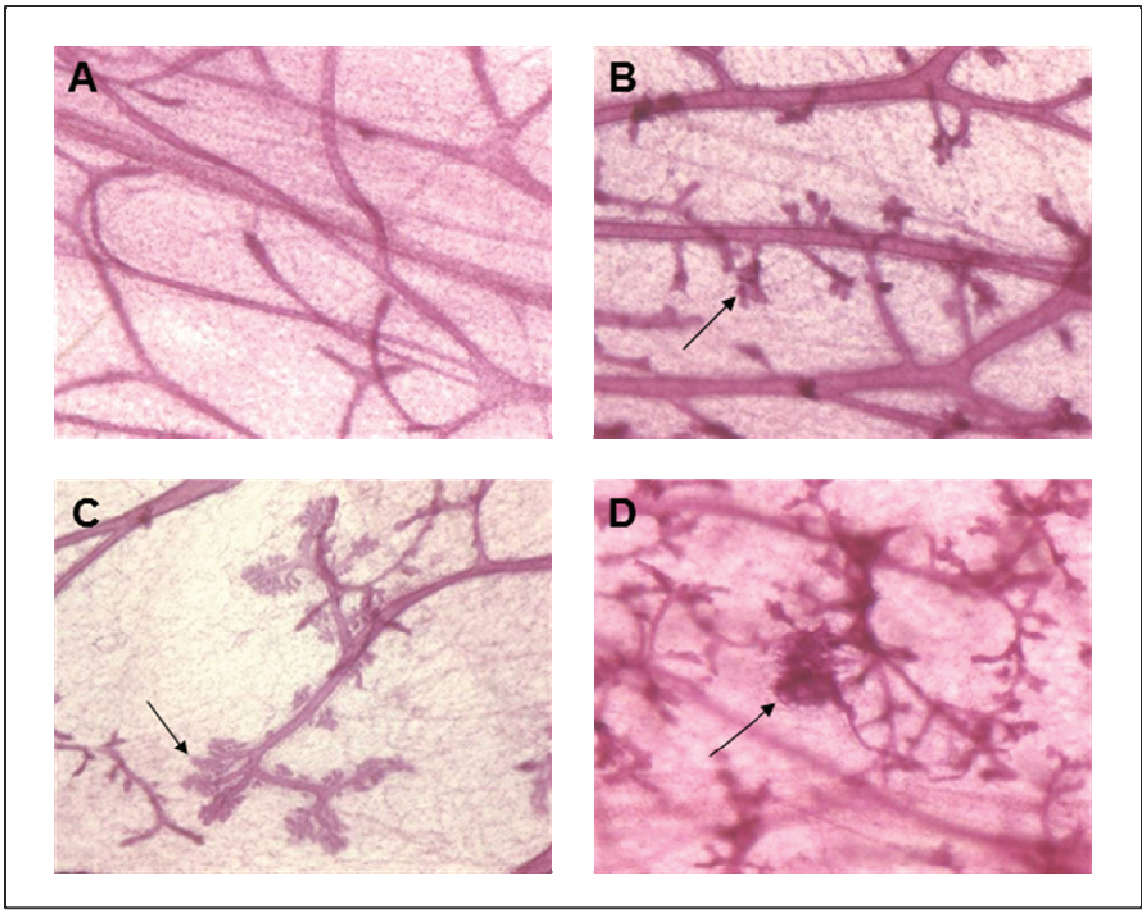

Figure 5.4 Hyperplastic alveolar nodules in *K14-Cre*;*Pax9*$^{flox/flox}$

The mammary glands of 10 month to 1 year old *Pax9*$^{flox/flox}$;*K14-Cre* virgin mice showed various morphology, 5 in 8 *Pax9*$^{flox/flox}$;*K14-Cre* mice developing Hyperplastic alveolar nodules (HANs, indicated by arrow). (A) *Pax9*$^{flox/flox}$ control. (B, C, D) HAN representatives show various morphology in mouse mammary glands. Magnification: 40x. n=8.

5.4 Mammary gland involution following lactation was severely impaired in *MMTV-Cre*;*Pax9*$^{flox/flox}$ mice

Since *Cre*-mediated *Pax9* deletion in *MMTV-Cre*;*Pax9*$^{flox/flox}$ mice could not be activated until pregnancy, we decided to investigate the phenotype during involution, the last stage of pregnant cyclic development. 6 pups were kept with the dam at birth and the extra pups were disgarded, then the pups feeded by the dams were removed from the dams at certain timepoints to normalized the mammary gland involution of the dam, which is called forced involution.

Mammary gland involution is the last step of mammary gland development in the pregnant cycle and is characterized by extensive apoptosis and remodeling after the cessation of lactation. At this stage, the highly structured secre*t*ory gland returns into one that resembles the virgin state so that the differentiation program initiated by gestation may begin again. Mammary involution depends on epithelial apoptosis in which epithelial lumens collapse and lobulo-alveolar structures are deleted with rapid elimination of up to 90 % of the epithelium. Epithelial cell were recognized to take part in the engulfment of neighboring apoptotic cells as “non-professional” phagocyte (Monks, Rosner et al. 2005). This process occurs in parallel with adipogenesis. Microarray expression analyses have suggested the involvement of inflammatory processes in mammary gland involution (Clarkson, Wayland et al. 2004, Stein, Morris et al. 2004).

In our investigation, mammary gland involution was dramatically impaired in *MMTV-Cre*;*Pax9*$^{flox/flox}$ mice. Mammary glands were collected from *MMTV-Cre*;*Pax9*$^{flox/flox}$ mutants and from *Pax9*$^{flox/flox}$ controls at involution day 4, day 6 and day8 respectively. Rapid alveolar collapse was seen in controls from day 4, and the mammary gland regressed to an almost non-pregnant state at day 8, whereas, in mutants, alveolar structures were intact throughout day 4, day 6 and day 8 (Figure 5.5). However, the number of lobules in mutants appeared less than in controls, especially at involution day 8, which may be due to inadequate tertiary ducts or lobular development at early gestation, or over-compensation by other clearance mechanism during late involution. The secretory alveolar cells appeared still active for milk synthesis and secreting, because the duct were extremely dilated as if the milk fat globules (MFGs) kept increasing within it. The extent of the duct dilation is strikingly severe than the phenotypes previously reported in other genetically manipulated mouse models

for mammary gland involution (Yang, Spitzer et al. 1994, Atabai, Fernandez et al. 2005). Apparently *Pax9* plays important roles in mammary gland involution (Figure 5.5).

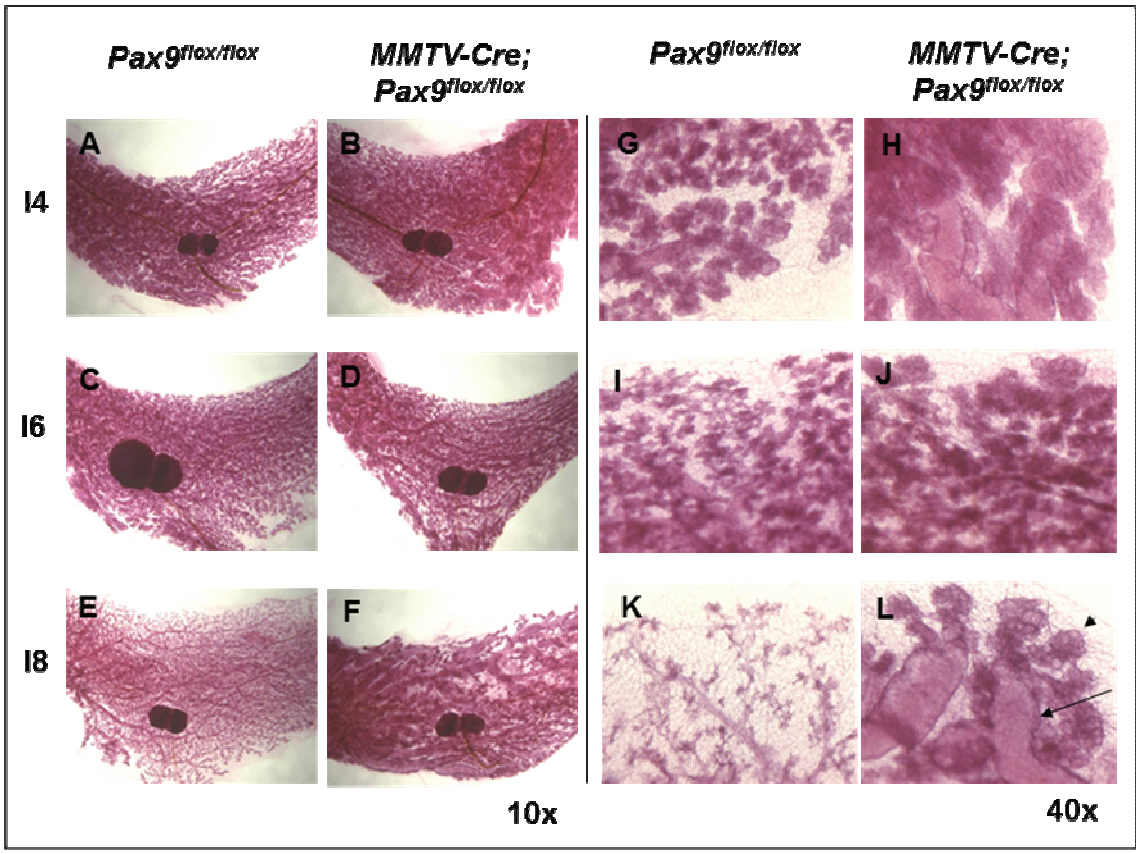

Figure 5.5 Phenotype of mammary gland-specific *Pax9* deletion during late stage of involution

Carmine-alum whole mount staining of mammary gland at involution day4, day 6 and day 8, shows strikingly intact alveolar structure (indicated by arrowhead in L) and extremely dilated ducts (indicated by arrow in L) due to persisting increasing milk fat globulins (MFGs) in the mutant mammary glands. n=2

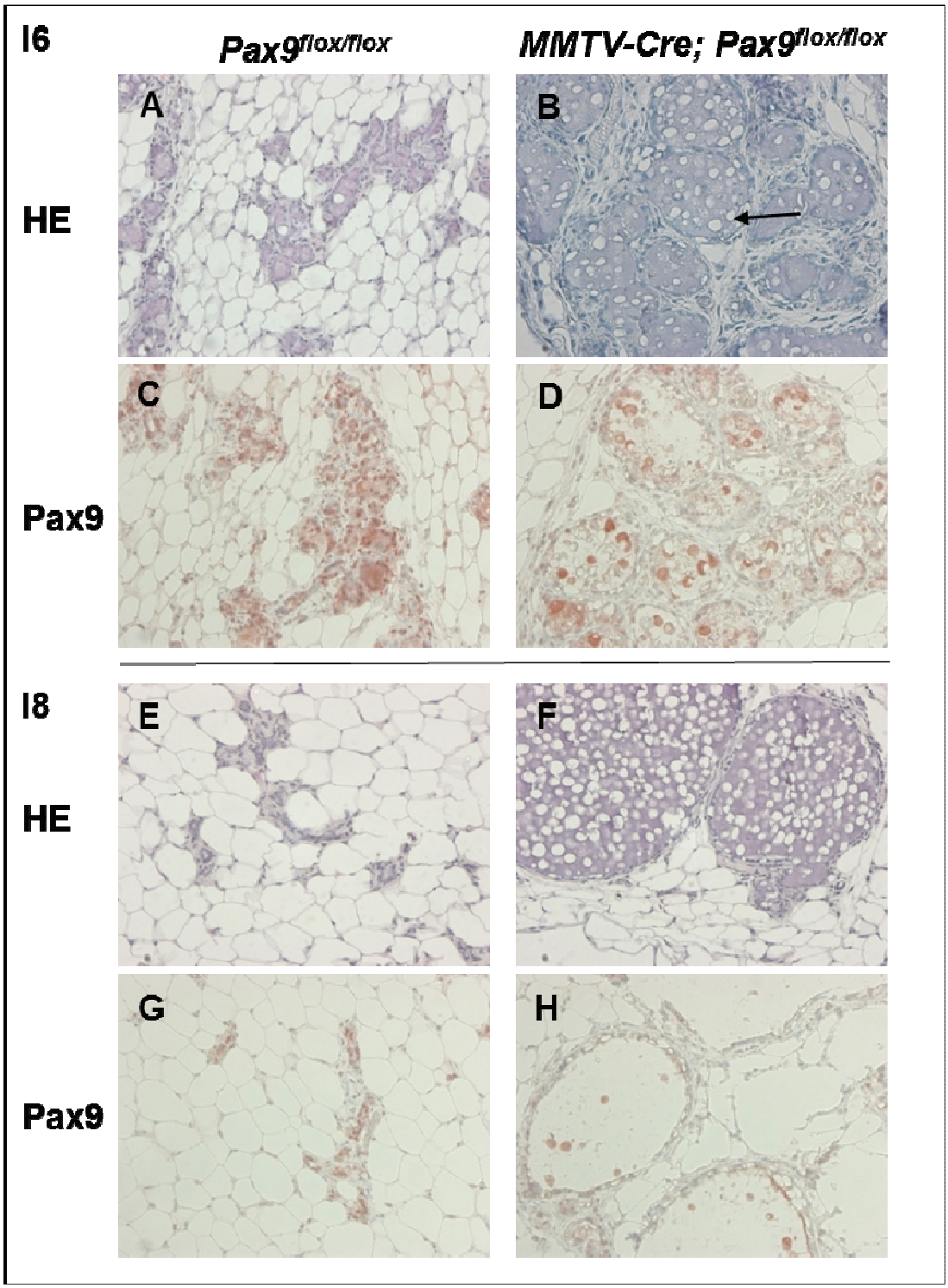

Figure 5.6 *Pax9* protein expression in mammary gland during the late phase of involution
Pax9 is expressed in control mammary epithelial cells at involution day 6 and day 8, with alveolae collapsed and tissue remodeled (A,C,E,G), whereas in *Pax9*-deficient mammary gland, alveolar sturcture is persisting, the duct is strikingly dilated, and the alveoli and ducts are full of MFGs (B,D,F,H, MFG indicated by arrow). Magnification: 200x. n=2

To confirm *Pax9*-deficiency in mutant mammary glands, we examined the deletion of *Pax9* in the mammary gland at involution day 6 and day8 by immunohistochemistry (Figure 5.6). *Pax9* protein is expressed in control mammary glands with normal involution, but absent in the persisting alveolar and ducts in the mutants. H&E staining showed that alveoli and ducts of the mutants are full of MFGs, which supported our postulation that alveolar cells in the mutants are still active to secrete milk and the *Pax9*-deficient mammary gland lacks proper mechanisms to clear the excessive MFGs.

5.5 *Stat*s activation and apoptosis evasion in *Pax9*-deficient mammary gland during postlactational involution

Pax9 was found to be fundamental in mammary gland involution (Chapter 5.4). During this stage, apoptosis of epithelial cells is an essential event, while *Stat3* is the key regulator for apoptosis during the first phase involution and *Stat5* is a cell survival signal and protects cell from death (Haricharan and Li 2013). To investigate if apoptosis is the reason for the involution impairment in *Pax9* deficient mice, and the possibility of a correlation between *Pax9* and *Stat*s, immunohistochemistry was applied to the involuting mammary glands.

Stat3 and *Stat5* activation in *Pax9* deficient mammary glands revealed by Immunohistochemistry

Firstly, we confirmed *Pax9* deletion in the mammary gland by *Pax9* immunostaining. Like the sporadic pattern of *Cre*-mediated recombination shown by X-gal whole mount staining of *MMTV-Cre;GTRosa26* mammary gland, *Pax9* deletion in *MMTV-Cre*;*Pax9*$^{flox/flox}$ mammary gland was heterogeneous as well. *Pax9* was not homogeneously deleted in the mammary glands of mutant mice on involution day 2 and day 3, however, it was deleted much more effectively in the mammary glands on involution day6 (Figure 5.7.1-3). *Pax9* protein was

absent in all persisting alveoli, in contrast, *Pax9* was presented in involuting alveoli of the controls.

Secondly, we examined apoptosis in mutant and control mammary glands using TUNEL assay, on involution day 2, day 3 and day 6, respectively. We found that apoptosis was significantly impaired in *Pax9*-deficient mammary gland during involution (Figure 5.7.1-3). In controls, apoptosis was seen from involution day 2, reached maximum on involution day 3, and gradually reduced till involution day 6. In contrast with the extensive apoptosis in controls, apoptosis rarely happened in *Pax9*-defincient mammary gland, throughout involution day 2 to day 6. Involution day 3 is the time point of maximum apoptosis happening (Clarkson and Watson 2003), the ratio of TUNEL positive cells in controls to mutants seen in one microscope field of vision was 30:1 (Figure 5.7.2 E, F). Apparently, *Pax9*-deficient alveolar cells escape apoptosis for mammary gland involution.

Thirdly, we analyzed the expression of *Stat3*/*Stat5* to study if these proteins could be involved in the reduced apoptosis of *Pax9*-deficient alveolar epithelial cell.

Stat3 is recognized as the most important transducer in mammary gland involution. It is activated by phosphorylation (p*Stat3*) and initiates apoptosis immediately when first stage involution starts. We performed p*Stat3* immunohistochemistry in mammary glands on involution day 2, day 3 and day 6, along with TUNEL assay. In controls, p*Stat3* was presented substantially throughout all three time points, in contrast, only weakly in mutants (Figure 5.7.1-3). Because *MMTV-Cre*-mediated *Pax9* deletion was sporadic in *MMTV-Cre*;*Pax9*$^{flox/flox}$ mammary glands, we compared neighboring lobules with and without successful *Pax9* deletion (Figure 5.7.2 B, D), and found that in the

lobule with successful *Pax9* deletion, p*Stat3* was absent, whereas in the lobule without *Pax9* deletion, p*Stat3* was present. The correlation between *Pax9* and p*Stat3* suggests that *Pax9*-deficiency induced apoptosis delay during mammary gland involution may act through a delay of *Stat3* activation.

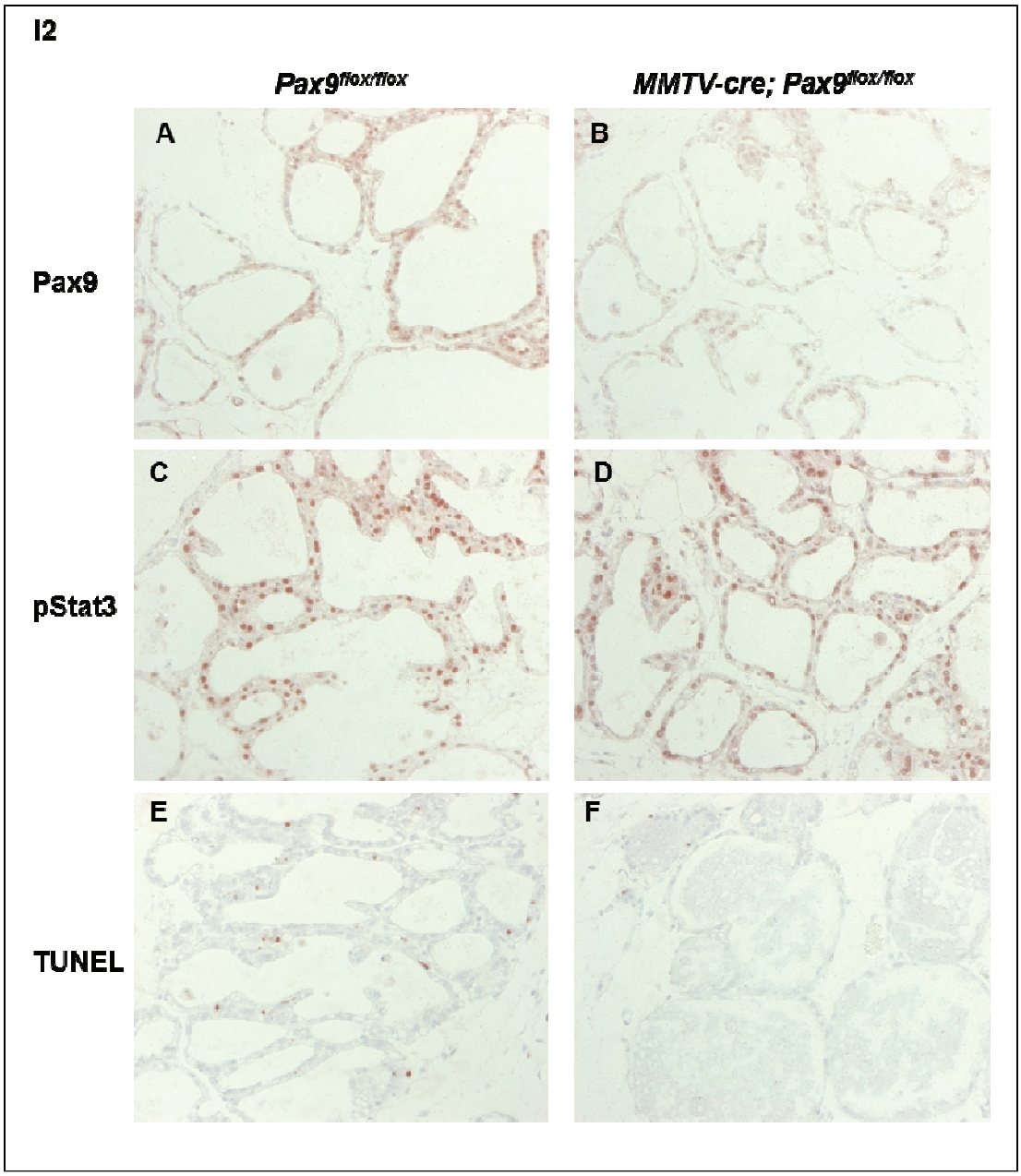

Figure 5.7-1 *Stat3* activation and apoptosis in the mammary gland on involution day 2

Pax9 deficiency was examined with Pax9 immunostaing (A, B). Stat3 was activated in control mammary glands and the non-recombined counterpart of mutant mammary glands (C,D). Apoptosis was severely impaired in the mutant mammary gland (E,F). Magnification: 200x. n=1

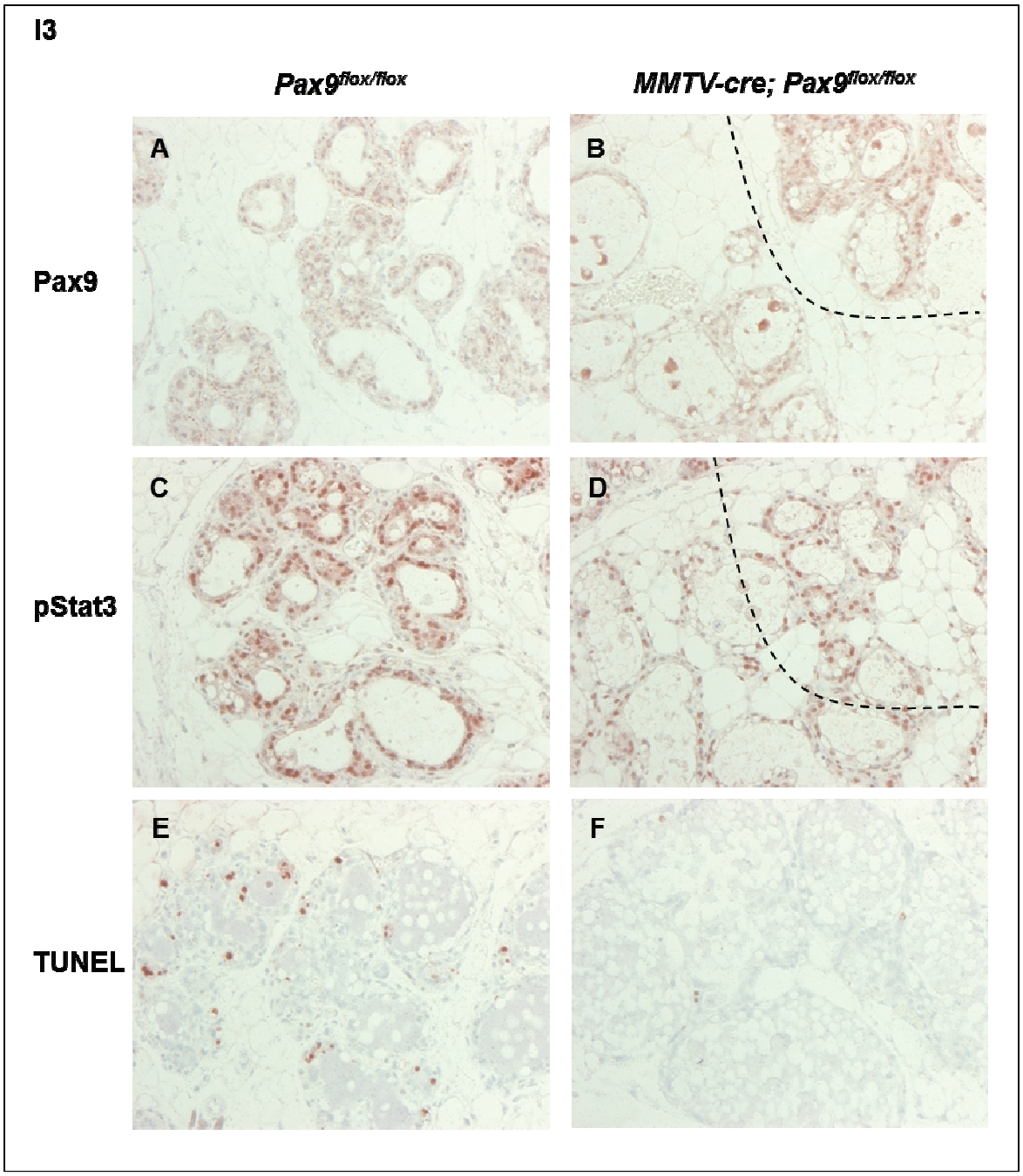

Figure 5.7-2 *Stat3* activation and apoptosis in the mammary gland on involution day 3
Pax9 deficiency was examined with *Pax9* immunostaing (A, B). *Stat3* was activated in control mammary glands and the non-recombined counterpart of mutant mammary glands (C, D). Apoptosis was severely impaired in the mutant mammary gland (E, F), and reach maximum level on involution day 3. Magnification: 200x. n=1

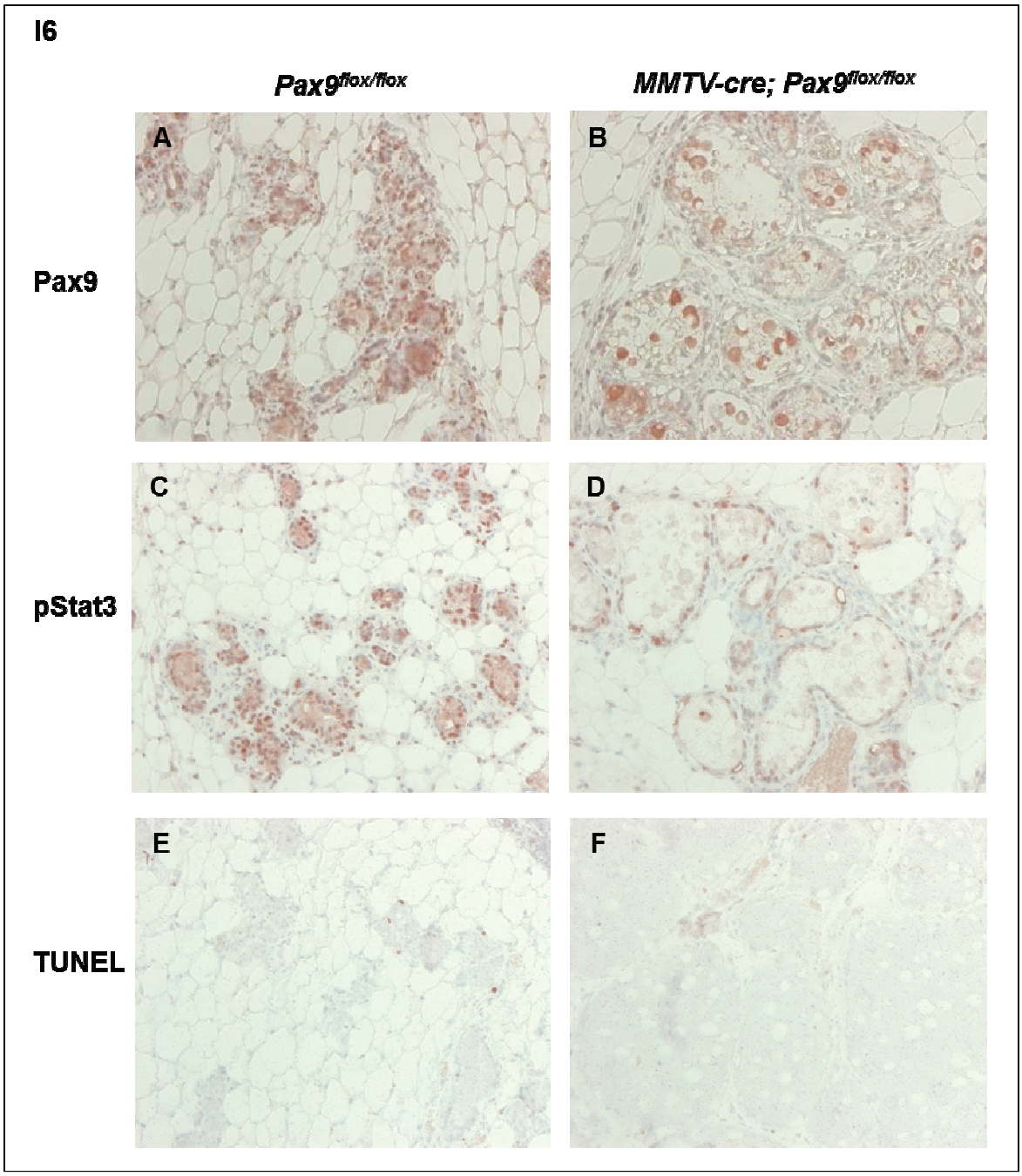

Figure 5.7-3 *Stat3* activation and apoptosis in the mammary gland on involution day 6

Pax9 deficiency was examined with *Pax9* immunostaing (A, B). *Stat3* was activated in control mammary glands and the non-recombined counterpart of mutant mammary glands (C, D). Apoptosis was severely impaired in the mutant mammary gland (E, F), and alveoli persisted even on involution day 6. Magnification: 200x. n=1

Moreover, *Stat3* and *Stat5* function in a pair during mammary gland involution. The activation of *Stat3* at involution stage is always accompanied by the inactivation of *Stat5*, which is active at lactation stage. *Stat3* functions as a death signal and *Stat5* functions as a cell survival signal (Chapman, Lourenco et al. 1999, Chapman, Lourenco et al. 2000, Iavnilovitch, Groner et al. 2002). In order to see whether the activation and inactivation dynamics of *Stat5* is also involved in *Pax9*-deficiency induced apoptosis delay, we examined the phosphorylated form of *Stat5* (p*Stat5*) by immunohistochemistry, paralleled with p*Stat3*, in mammary glands on involution day4 (Figure 5.8). It was shown that p*Stat5* already declined to very weak levels in the alveoli in the control mammary gland as reported in literature, but surprisingly, in *Pax9*-deficient mammary glands, *Stat5* was strongly activated in the nuclei of alveolar cells. At the same time, p*Stat3* partially diffused back to cytoplasm in the control mammary gland, but started to be activated in the nuclei of alveolar cells in the *Pax9*-deficient mammary gland, though the levels were still lower than in controls. As the translocation of p*Stat3* back to cytoplasm in controls means rapid apoptosis slowing down, p*Stat3* presented in the nuclei of the mutant alveolar epithelial cells demonstrated a later catch-up of *Stat3* activation after an initial delay at the early stage of involution in the *Pax9*-deficiency mammary gland.

Taken together, the correlation between *Pax9* expression and Stats activation indicated that *Pax9* may regulate mammary gland involution upstream of *Stat3* and *Stat5* (Iavnilovitch, Groner et al. 2002, Kritikou, Sharkey et al. 2003, Bertucci, Quaglino et al. 2010). Further studies need to be done to dissect subtle signaling pathways and find the direct downstream gene of *Pax9*.

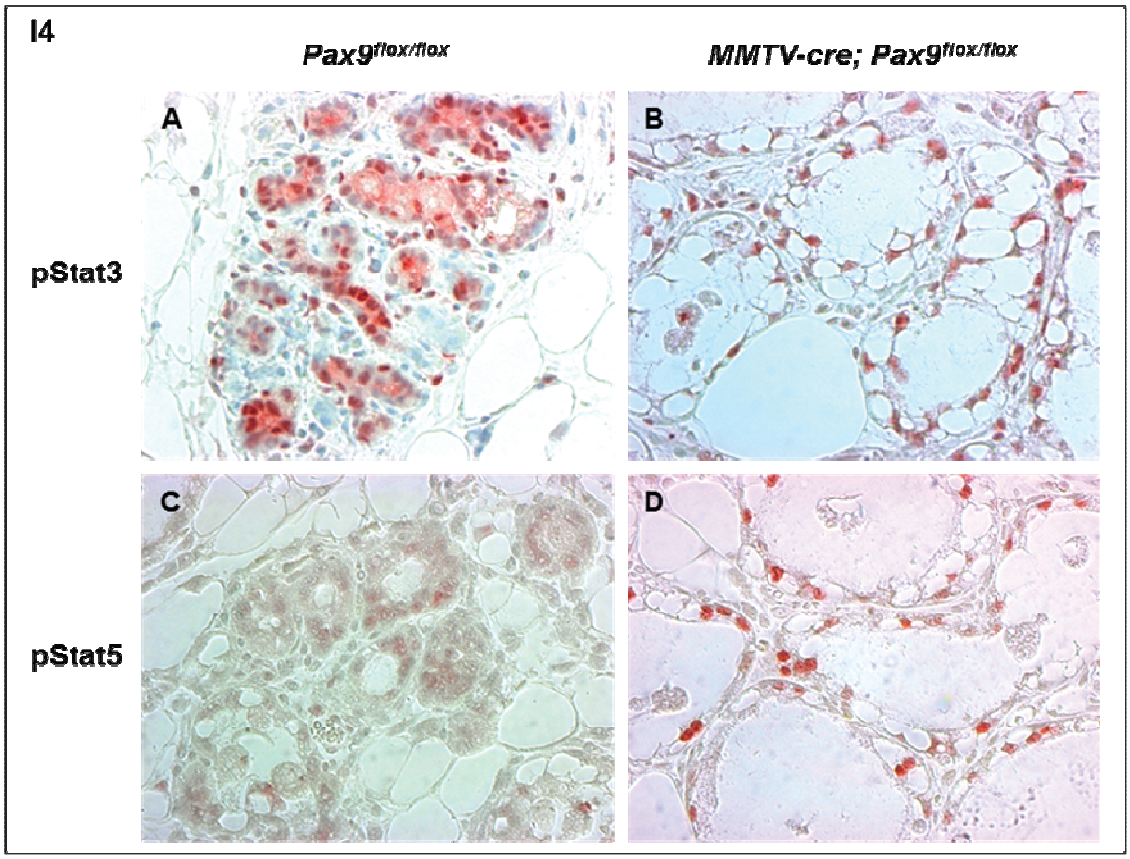

Figure 5.8 *Stat*s activation in the mammary gland on involution day 4
Stat3 is activated in control mammary gland, and already slightly diffuses (after the apoptosis peak on involution day 3), whereas presented in the nuclei of mutant at lower levels (A, B). *Stat5* is basically not activated in control mammary gland (C) but highly activated in *Pax9* mutant mammary gland (D). Magnification: 200x. n=1

Stat3 and *Stat5* activation in *Pax9* deficient mammary glands examined by Western blot

For a quantitative measurement of the dynamics change of p*Stat3* and p*Stat5*, we did western blot of the mammary glands through lactation day 8 (involution day 0) to involution day4 (Figure 5.9). In controls, p*Stat3* is absent at lactation, then increases immediately on involution day 1 and keeps activated on involution day2, day3, till day 4, to initiate apoptosis and maintain mammary gland involution; In mutants, p*Stat3* follows a similar dynamic curve but at lower levels than in controls. Keratin 18 (K18) was used as baseline control, on involution day 1 and day4.

p*Stat5*, in controls, is at an extensively activated level on lactation day8, maintaining the secret*o*ry differentiation state of epithelial cells to produce and secrete milk, decreases immediately on involution day1, decreasees further to almost absent on day2, to withdraw the cell survival signals, then slightly increases on day 3, and keeps at a moderate level on day4, balancing the cell death signals to avoid over-reacting to apoptosis. In mutants, surprisingly, p*Stat5* is absent on lactation day8, involution day1, day2, increases from day3 and reaches to an intensive level on day4.

Due to the heterogeneous deletion pattern of *Pax9* in the mammary gland, changes specifically in the *Pax9*-deleted alveoli might have been disguised by other non-recombined alveoli. On involution day 2 and day 3, the changes of expression levels of p*Stat3* and p*Stat5* were thus undistinguishable. Nevertheless, on involution day 4, using K18 as baseline, we found that p*Stat3* was decreased and *Stat5* was increased in *Pax9* deficient mammary gland, which is consistent with the results from immunohistochemistry of p*Stat3* and p*Stat5* (Figure 5.8, 5.9).

p*Stat5* is almost absent in *Pax9*-deficient mammary gland on lactation day 8, which absolutely contrasts to the extensive activation in *Pax9*-deficient mammary gland on involution day 4, nevertheless, p*Stat5* is the dictator signal for lactating differentiation of mammary epithelial cells. A possible explanation is that it is a leftover effect from an unpredictable early developmental failure by an early deletion of *Pax9*. The other reason might be that *Pax9*-*Stat5* regulatory mechanisms are distinct at lactation stage and at late phase of involution, for *Stat5* is reported playing distinct functions in mammary gland development and mammary tumour formation (Klinghoffer, Sachsenmaier et al. 1999, Barash 2006, Desrivieres, Kunz et al. 2006, Furth, Nakles et al. 2011, Wagner and Schmidt 2011).

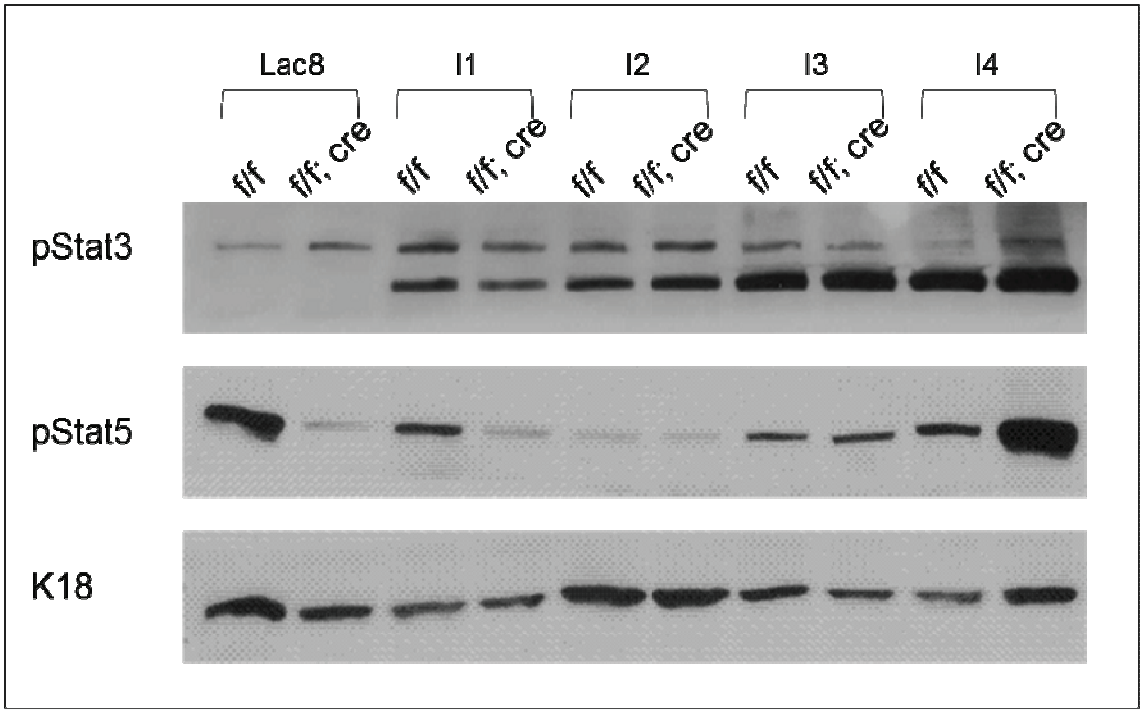

Figure 5.9 *Stat*s activation during lactation and involution exhibited by western blot. Activation of *Stat3* and deactivation of p*Stat5* determines the process of mammary gland involution. Using *K18* as a base line, in *Pax9*-mutant mammary gland, p*Stat3* was decreased on involution day1 and day4, p*Stat5* was first decreased on lactation day 8 and involution day1, and then increased on involution day 4. n=1.

5.6 *Pax9* may control epithelial cell lineage

The mammary gland is composed of a ductal epithelial tree that comprises an inner layer of luminal cells and an outer layer of myoepithelial cells. During pregnancy, alveolar cells arise and undergo terminal differentiation into milk-producing cells. Both lobule-limited and duct-limited pluripotent mammary epithelial cells exist in the mammary gland, and transplantation of limiting dilution of dispersed mammary epithelial cells into hosts could generate normal alveologenesis, given treated with hormone combinations (Kim, Oberley et al. 2000). Mammary gland stem cells (MaSCs) have been identified in recent years. A differentiation hierarchy in the adult mammary gland has been illustrated, as in hematopoietic compartment. A single MaSC has been shown to reconstitute a fully functional mammary gland in the mouse. These MaSCs are self-renewing and can differentiate into all epithelial cell types in the mature mammary gland,

which defines their characteristics of a stem cell (Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006). A second type of multipotent stem cell is identified during pregnancy and likely drives the significant expanding of alveolar epithelium in that period (Asselin-Labat, Shackleton et al. 2006).

In normal circumstances, alveoli develop from alveolar buds on tertiary ducts during pregnancy (Figure 5.10 A, C). However, in *Pax9*-deficient mammary gland, we found that alveoli also developed directly from primary and secondary ducts, in both of $Pax9^{neo}$ and *MMTV-Cre*;$Pax9^{flox/flox}$ mice (Figure 5.10 B, D). Combined with other phenotypes we previously noticed, a hypothesis thus has been raised that *Pax9* levels correlate to the mammary epithelial cell differentiation hierarchy: High level of *Pax9* defines ductal cell lineage and low level of *Pax9* defines alveolar lineage. *Pax9* is absent in further differentiated secre*t*ory alveolar cells, and increased back to a high level in epithelial cells to gain the capability of phagocytosis or apoptosis during involution. *Pax9* possibly is a switch of ductal lineage to alveolar lineage. When *Pax9* is depleted from the mammary epithelium, under the stimuli of pregnancy or lactation, for instance, by progesterone or prolactin, the ductal progenitor cells differentiate or transdifferentiate into alveolar progenitors, and respond to pregnancy stimuli.

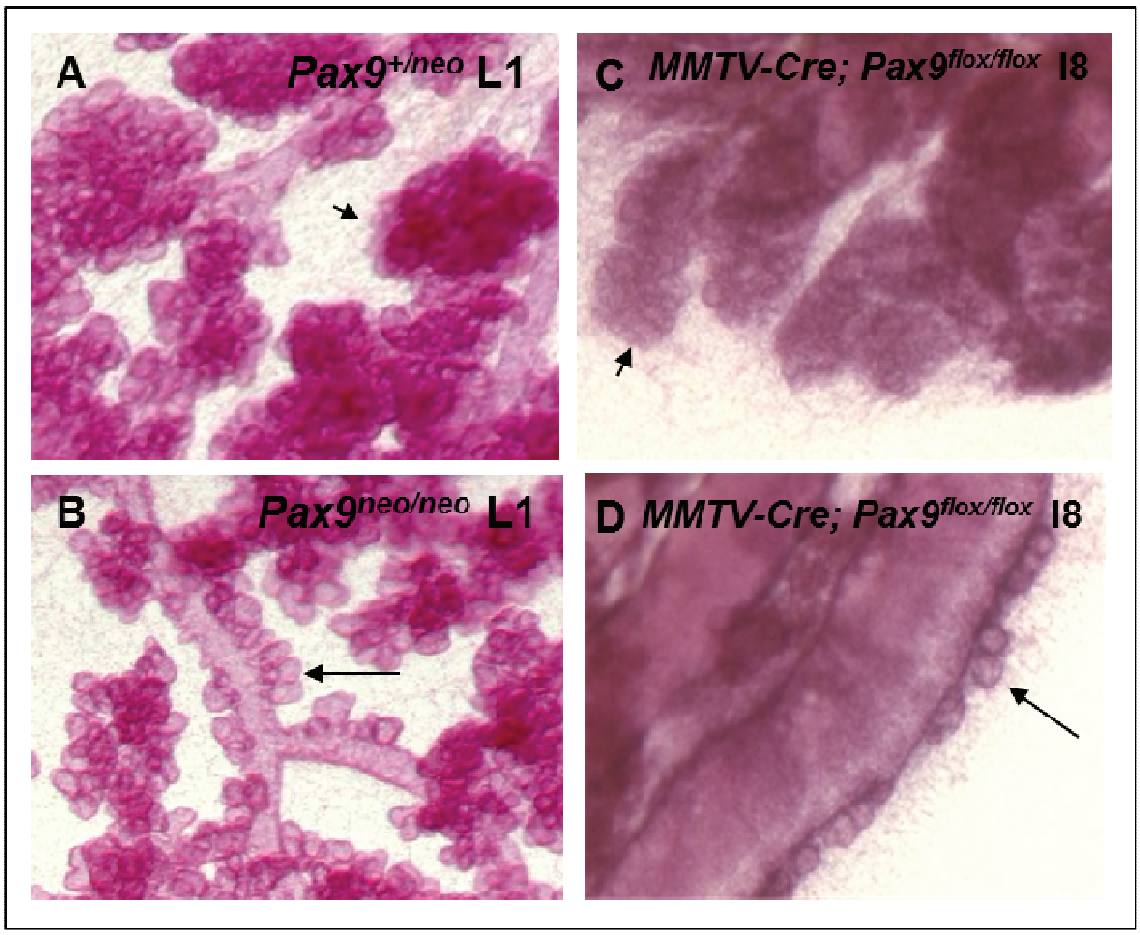

Figure 5. 10 *Pax9* may control mammary epithelial cell lineage

The alveoli develop from the alveolar bud at the tip of tertiary duct in normal mammary circumstance (A, C, short arrows indicated), but develop directly from the primary and secondary duct in *Pax9*$^{neo/neo}$ and *MMTV-Cre*; *Pax9*$^{flox/flox}$ mammary gland (B, D, arrows indicated). Magnification: 40x. n=2

5.7 Macrophage-like phagocytes massively appeared in the alveoli of *Pax9*-deficient mammary gland during the late phase of involution

Neutrophil and macrophage infiltration during the process of mammary gland involution have been described in early studies of dairy animals (cow, sheep and goat). Microarray studies in recent years also indicate an infiltration of inflammatory cells into involuting mammary glands of the mouse. Neutrophil influx occurs during involution and the maximal macrophage influx is on involution day 3 (O'Brien, Lyons et al. 2010, O'Brien, Martinson et al. 2012).

Recently a new concept proposed that milk secretion is not the final

differentiated state of the mammary epithelial cells (MECs), but that the secretory MECs have another function, i.e. final clearance and breakdown of their neighboring cells and milk fat globules (MFGs), to perform before they undergo cell death in the involuting mammary gland. They become non-professional phagocytes during that short term, and then likely to die and be cleared themselves (Monks, Rosner et al. 2005, Atabai, Sheppard et al. 2007, Nandrot, Anand et al. 2007, Monks, Smith-Steinhart et al. 2008).

Milk fat globule-EGF-factor (Mfge8) is secreted by macrophages and epithelial cells to bridge the apoptotic cells and phagocytes (Hanayama, Tanaka et al. 2002, Hanayama, Tanaka et al. 2004, Miyasaka, Hanayama et al. 2004, Atabai, Fernandez et al. 2005, Nandrot, Anand et al. 2007). Deletion of Mfge8 delays mammary gland involution with enlarged ducts filled with excess MFGs (Hanayama and Nagata 2005), which presents a similar phenotype as what we observed in *Pax9* deficiency-induced mammary gland involution impairment.

In the *Pax9*-deficient mammary gland on involution day 6 (Figure 5.11D), *Cre*-mediated *Pax9* deletion was confirmed by *Pax9* immunohistochemistry. The alveolar structure is intact and the alveolar epithelial cells continue to produce milk. In the mutated mammary gland, a tremendous amount of phagocytes have been seen engulfing excessive MFGs in the alveoli. In the normal mammary gland, MFGs clear-out is very quick, thus we haven't captured cell-cell engulfment image, but this phenomena was presented by Werb' lab (Atabai, Sheppard el al. 2007).

In the *Pax9*-deficient mammary gland on involution day 6, the extent of the phagocytes is striking. We assume that these phagocytes are macrophage-like cells, which infiltrate or residence in the mammary gland to remove the excessive MFGs. The definitive type of these cells should be identified by a further study with specific immune cell marker immunostaining, for instance,

F4/80. Interestingly, these phagocytes are *Pax9* positive, in contrast, *Pax9*-deleted MEC lost the capability of engulfment (Figure 5.11D), which implicates that *Pax9* might play a role in MEC and macrophage engulfment.

Pax9-positive infiltrators may also take part in developmental activities in the mammary gland. We therefore examined previous *Pax9* immunohistochemistry results in different mouse lines and at different developmental stages. Surprisingly, we found that these *Pax9*-positive infiltrators actually appeared around the ducts in most of the sections of the mammary glands we investigated, though at a low density. Moreover, the *Pax9* protein expression levels in these cells are apparently much higher than in mammary epithelial cells (Figure 5.11A, B, C).

If the *Pax9*-positive phagocytes in the mammary gland during involution is to clear out the excessive MFGs and apoptotic cells, while mammary epithelial cells from the epithelium fail to do so because of *Pax9* deletion, we can postulate that *Pax9* expression is necessary for alveolar epithelial cells to execute their function as phagocytes to clear out the MFGs and apoptotic cells during involution.

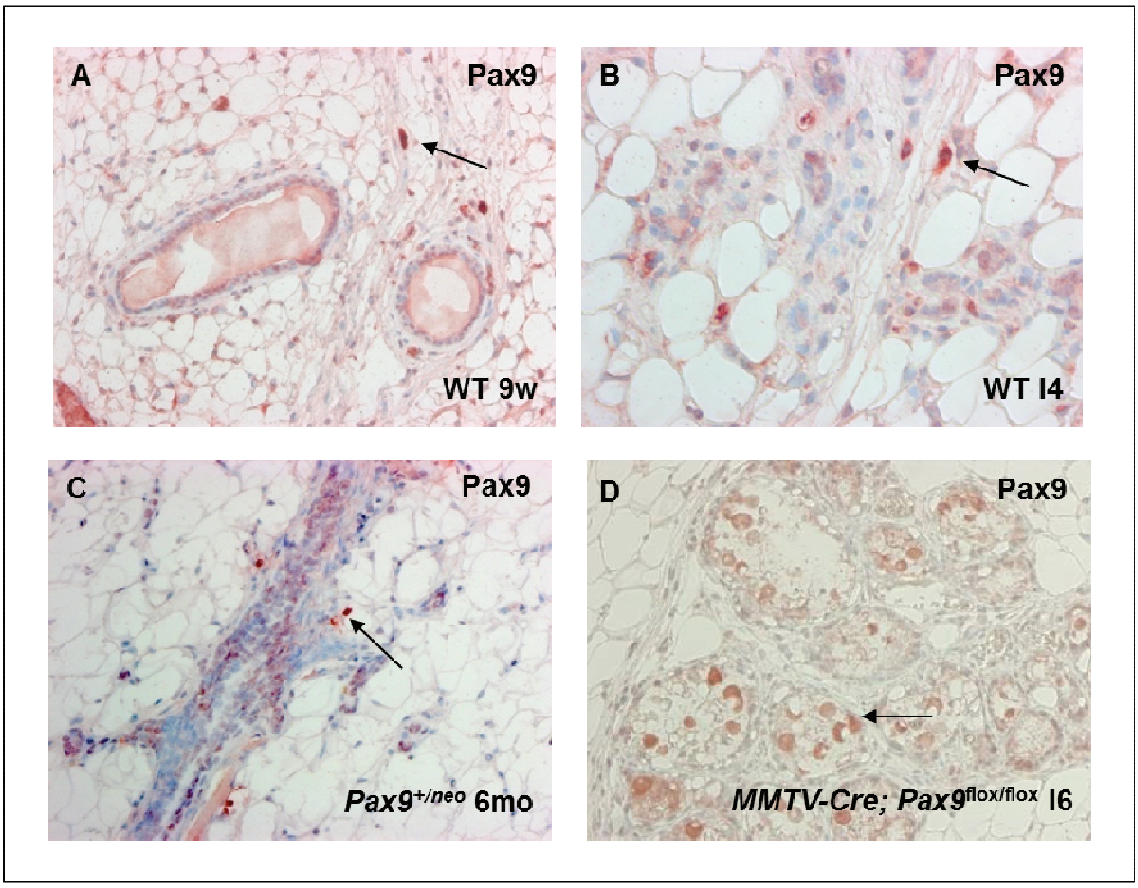

Figure 5.11 Macrophage-like cell and phagocytosis in the mammary gland

Pax9 immunostaining of mammary glands of 9 week old (A) and involution day 4 (B) wild type mice, and 6 months old *Pax9*$^{+/neo}$ (C) showed *Pax9* positive infiltrating cells (indicated by arrows). Massive phagocytes (indicated by arrow in D) appeared engulfing MFGs in *MMTV-Cre*; *Pax9*$^{flox/flox}$ on involution day 6 (D). Magnification: (A, C, D) 200x, (B)400x. n=2

In the normal mammary gland, by a general observation of our studies, compared to nulliparous, parous mammary ducts comprise more *Pax9* positive epithelial cells, which maybe a consequence by the competitive advantage of *Pax9* positive MECs, based on the postulation that differentiated *Pax9*-negative cells are engulfed and cleared out by *Pax9*-positive cells.

5.8 *Pax9* deficiency in the mammary gland leads to neoplasia and TEB filling

Though *Pax9* could not be effectively deleted by *MMTV-Cre* mediated recombination in the mammary gland of virgin mice (probably due to mixed gene

background of C57BL6 and FVB), we still observed some small *Pax9*-deficient fractions in the mammary gland of 5 week old mice (Figure 5.12). TEBs are filled with *Pax9*-negative epithelial cells (Figure 5.12 A) and *Pax9*-negative cells form neoplasia in the primary mammary duct (Figure 5.12 B). Apoptosis that is supposed to form the TEB cavity and luminal space is apparently impaired. Bim was reported to play a critical role in TEB development and luminal apoptosis during mammary ductal tree branching (Mailleux, Overholtzer et al. 2007). *Pax9* may execute a similar function of regulating apoptosis in the mammary gland TEBs.

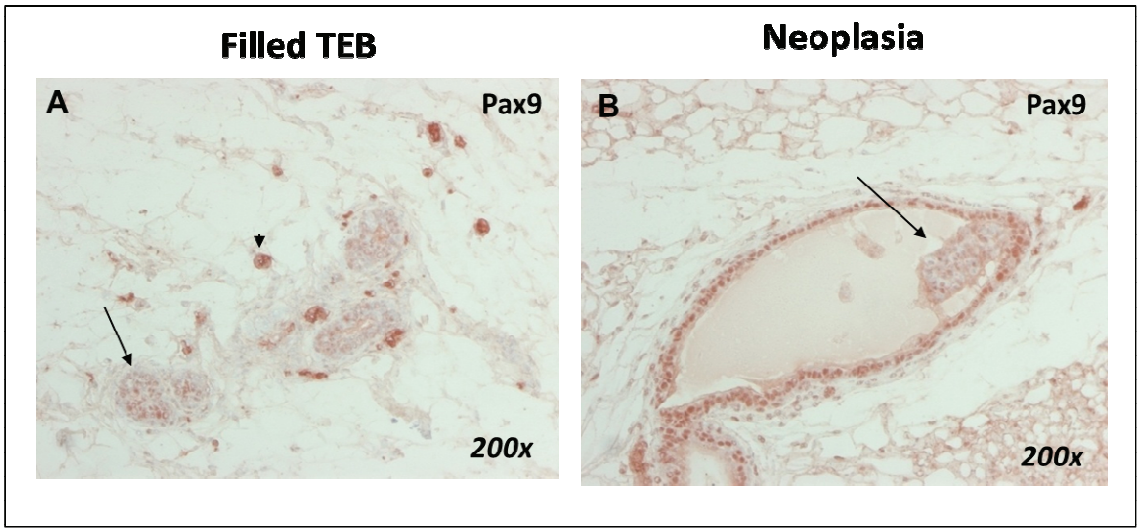

Figure 5.12 *Pax9* deletion in the mammary gland leads TEB filling and neoplasia in the duct

TEB filling (A, indicated with arrow) and Neoplasia in the duct (B, indicated by arrows) comprised by *Pax9* negative epithelial cells appeared in the mammary gland of 5 week old *MMTV-Cre*; *Pax9*$^{flox/flox}$ mouse, which possibly ascribe to *Pax9*'s function of regulating apoptosis. Macrophage-like cells were seen around TEBs (A, indicated by closed arrow head). Magnification: 200x. n=1

Strong *Pax9* positive cells, suspected as macrophages, were seen in mammary gland stroma around TEBs (Figure 5.12A, indicated with arrow head). Macrophage are found to help with duct branching (Gouon-Evans, Lin et al. 2002, Van Nguyen and Pollard 2002, Schwertfeger, Rosen et al. 2006), but also activate tumour growth and metastasis (Lin, Gouon-Evans et al. 2002). The

source and consequence of this macrophage-like cell is yet to be revealed.

5.9 Pregnancy-associated microenvironment in *Pax9*-deficient mammary gland during involution

To get a picture about what genetic signaling *Pax9* may regulate in mammary gland involution, we performed expression s*Cre*ening using Affymetrix Mouse 2.0 expression microarray to identify candidate genes downstream of *Pax9* in the mammary gland at different time points, i.e., involution day 1, 2, 3, from the early phase of involution to the late phase consecutively. *MMTV-Cre*; $Pax9^{flox/flox}$ mammary glands were used as experimental samples, and $Pax9^{flox/flox}$ mammary glands were used as controls. Genes with signal change log ratio above 1.0 were selected and annotated. Due to the heterogeneous recombination, the efficiency of *Pax9* knockout in the mammary gland was not ideal, actual gene expression change in the mutant may be masked by the large part of normal tissues with residue *Pax9* in the genotyped mutant. Thus the expression microarray data were used only to indicate signal cues instead of to provide pathway evidences. Significant gene expression changes related to mammary gland development and breast cancer, revealed by expression microarray, are presented in Figure 5.13 and related details of microarray data on involution day3 were listed in Table 5.1.

In *Pax9*-deficient mammary glands on involution day3, immune and inflammation associated genes, such as monocyte to macrophage differentiation-associated gene (*Mmd*) and complement component 3 (*C3*) increased significantly; metalloproteinase (*MMP*) genes, such as *MMP3* and *MMP12*, increase significantly, whereas inhibitor of DNA binding 2 (*Id-2*) was significantly decreased. Besides, myosin-related genes, such as myosin binding protein C, fast-type (*Mybpc2*), myosin light chain, phosphorylatable, fast skeletal muscle (*Mylpf)*, myosin, heavy polypeptide 4, skeletal muscle (*Myh4*) and

myosin, light polypeptide 1 (*Myl1*), etc., were strikingly up-regulated, which may indicate a cellular stress response to the milk stasis in the *Pax9* mutated mammary gland.

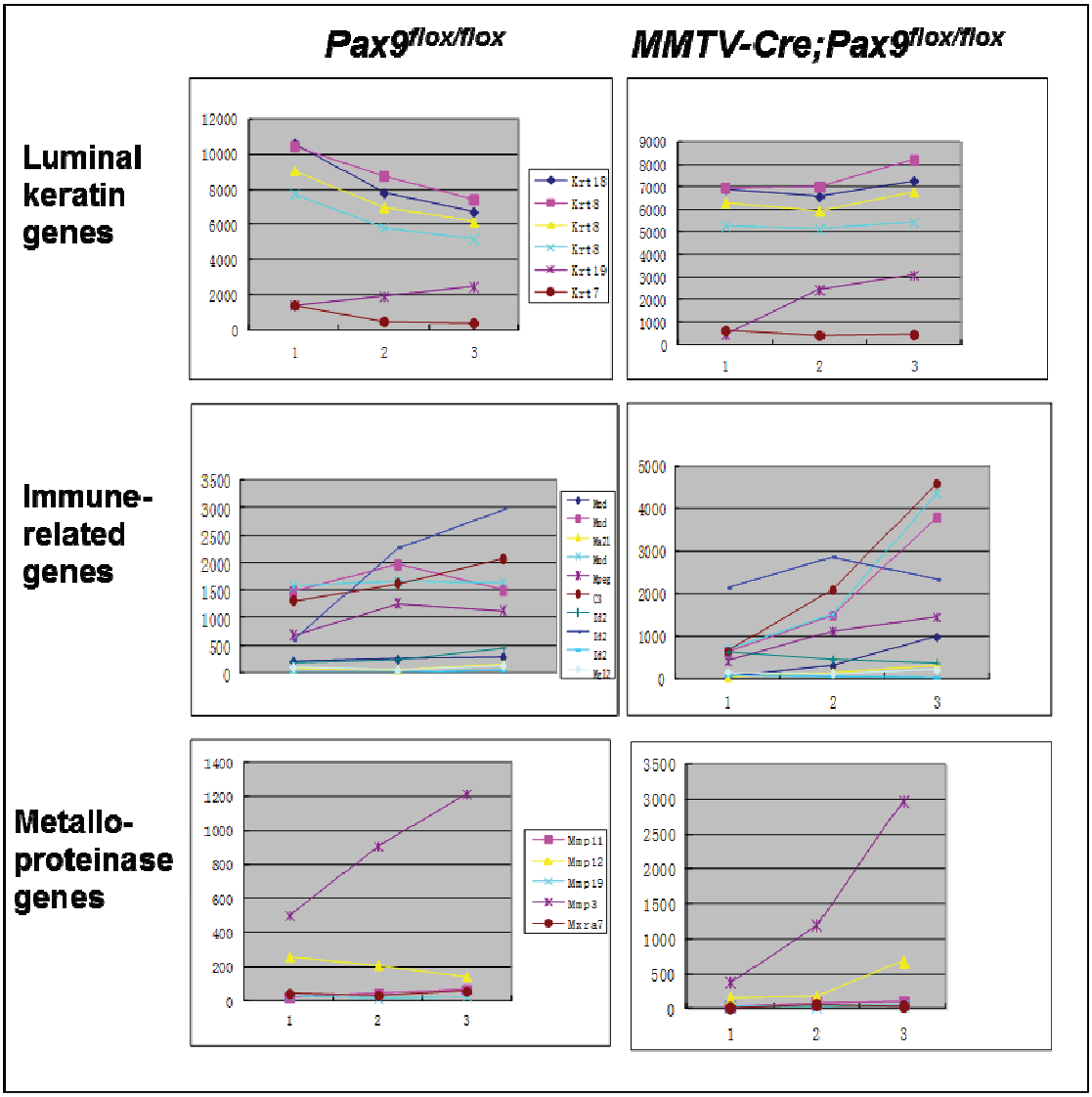

Figure 5. 13 Genes significantly changed in *Pax9* deficient mammary gland at involution
Identified by GeneChip Mouse Genome 430 2.0 array, at involution day1, day, and day3. Luminal keratins were used as internal control. In control mice, luminal keratins decreased following involution, whereas in mutants, luminal keratin was not decreased, may due to delayed apoptosis of epithelial cells. Some Immurelated-genes and *MMP*s increased significantly. Myosin-related genes were increased sharply, may be is a stress response from the alveoli to excessive MFGs. n=1.

Table 5.1 Expression profiling survey using Affymetrix microarray

Probe Set ID	Gene Symbol	Signal Log Ratio	Change	Change p-value	Conrol_ Signal	Control_ Detection	Control_ Detection p-value	Mutant_ Signal	Mutant_D etection	Mutant_ Detection p-value
1448169_at	*Krt18*	0.1	NC	0.124552	6714.6	P	0.000244	7276.8	P	0.000244
1420647_a_at	*Krt8*	0.2	NC	0.003041	7426.5	P	0.000244	8220.1	P	0.000244
1435989_x_at	*Krt8*	0.2	NC	0.061522	6159.7	P	0.000244	6780.6	P	0.000244
1423691_x_at	*Krt8*	0.1	NC	0.5	5179.1	P	0.000244	5429.8	P	0.000244
1417156_at	*Krt19*	0.3	I	0.00002	2469.6	P	0.000244	3081.3	P	0.000244
1423952_a_at	*Krt7*	0.2	NC	0.118009	398.6	P	0.000244	447	P	0.000244
1423488_at	*Mmd*	1.4	I	0.00002	1654.3	P	0.000244	4355.3	P	0.000244
1427076_at	*Mpeg1*	0.9	NC	0.105663	1131.5	P	0.000244	1442.9	P	0.001221
1423954_at	*C3*	1	I	0.00002	2083.9	P	0.000244	4584.5	P	0.000244
1422537_a_at	*Id2*	-0.6	D	0.99998	438.2	P	0.000244	365.8	P	0.001221
1435176_a_at	*Id2*	-0.3	D	0.999382	2971	P	0.000244	2330	P	0.000244
1453596_at	*Id2*	-0.5	NC	0.977068	48.6	P	0.001953	25.3	P	0.023926
1438467_at	*Mgl2*	0.5	I	0.000865	126.3	P	0.018555	208.7	P	0.00415
1417234_at	*Mmp11*	0.3	NC	0.035785	64.9	P	0.018555	91.4	P	0.018555

1449153_at	*Mmp12*	1.8	I	0.00002	140.4	P	0.000732	669.9	P	0.000732
1421977_at	*Mmp19*	1	I	0.000492	23.3	P	0.00415	48.3	P	0.000244
1418945_at	*Mmp3*	1.3	I	0.00002	1214.8	P	0.000732	2973.1	P	0.000244
1430539_at	*Mxra7*	-0.4	NC	0.984574	53.5	P	0.030273	37.3	A	0.129639
1420693_at	*Myom1*	3.2	I	0.00003	4.9	A	0.601074	77.9	M	0.056152
1457435_x_at	*Myom2*	3.3	I	0.00003	9.4	A	0.366211	99.4	P	0.000732
1435813_at	*Mypn*	2.9	I	0.001336	2.8	A	0.633789	29.5	P	0.046143
1455736_at	*Mybpc2*	3.7	I	0.00002	34.7	A	0.149658	420.3	P	0.000732
1449551_at	*Myo1c*	-1	NC	0.975245	53.2	P	0.030273	32	A	0.213135
1448371_at	*Mylpf*	4.5	I	0.00002	51.1	P	0.001221	1229.3	P	0.000244
1436051_at	*Myo5a*	1	I	0.000492	118.6	P	0.000244	232.5	P	0.000244
1427520_a_at	*Myh1*	5.5	I	0.00002	0.5	A	0.850342	35.8	P	0.001953
1427868_x_at	*Myh1*	2.8	I	0.001201	5.5	A	0.432373	23.9	P	0.008057
1427026_at	*Myh4*	5	I	0.00002	65.9	P	0.000732	2377.8	P	0.000244
1458368_at	*Myh4*	1.3	I	0.000307	30.4	A	0.095215	79.2	P	0.000732
1452651_a_at	*Myl1*	3.4	I	0.00002	142.1	P	0.001953	1729.5	P	0.000244
1447713_at	*Tpm1*	1	I	0.000492	30.4	A	0.246094	44.6	P	0.010742

1456623_at	*Tpm1*	-1.1	D	0.999965	264.6	P	0.000244	111	P	0.000244
1417464_at	*Tnnc2*	4.7	I	0.00002	92.8	A	0.067627	2202.3	P	0.000244
1416889_at	*Tnni2*	4.1	I	0.00002	58.4	A	0.080566	951.5	P	0.000244
1438608_at	*Tnni2*	4.2	I	0.001201	0.8	A	0.994141	9.6	A	0.466064
1438609_x_at	*Tnni2*	3.3	I	0.000346	23.1	A	0.334473	298.7	P	0.018555
1450118_a_at	*Tnnt3*	4.9	I	0.00002	33.5	P	0.046143	785.2	P	0.000244

5.9.1 Immune-related genes

Mmd increased in *Pax9* deficient mammary gland at involution day 3. However we could not distinguish the cell type expressing this gene (i.e. either expressed by mammary epithelial cells, or by the infiltrating leukocytes), phagocytes presented in the mutant mammary gland on involution day6, as shown in Figure 5.11D. The role macrophages play in cancers is ambiguous, which is generally known for their tumouricidal capacity, but it is emerging that regulatory networks within tumour tissues redirect their function into a tumour-promoting activity. For example, cocultivation of weakly invasive breast cancer cells with macrophages increases invasiveness of cancer cells (Hagemann, Robinson et al. 2004). Tumour-associated macrophages (TAMs) facilitate angiogenesis, ECM degradation, and tumour invasion through activation of epidermal growth factor receptor signaling, secretion of proteases and paracrine signaling between tumour cells (Schedin, O'Brien et al. 2007, DeNardo, Barreto et al. 2009). Loss of macrophages in colony stimulating factor 1 (*CSF-1*) deficient mice dramatically reduced malignant progression (Lin, Nguyen et al. 2001), while activation of *CSF-1* in xenografts derived from human MCF-7 cells in immune deficient mice suppresses mammary tumour growth by inhibiting macrophage infiltration, decreasing *MMP*s and vascular endothelial growth factor-A (*VEGF-A*), and repressing endothelial cell proliferation (Aharinejad, Paulus et al. 2004).

5.9.2 MMPs

MMP3 and *MMP12* were found to be increased in the *Pax9*-dificient mammary gland on involution day3. Involution not only involves apoptosis, but the mammary gland can also involute more quickly in a situation of reduced proteinase activity. *MMP3* is highly expressed during mammary gland involution and determines the rate of adipocyte differentiation during involutive mammary gland remodeling. In mice that overexpress the metalloproteinase inhibitor

TIMP-1, or mice that lack proteinase *MMP3*, involution is accelerated due to an increase in the re-differentiation of fat cells rather than an alteration in apoptosis (Alexander, Selvarajan et al. 2001). Prolactin inhibited gene expression of MMP3 and MMP12 during involution (Flint, Boutinaud et al. 2006).

MMP3 promotes epithelial-to-mesenchymal transitions (EMT) in cell culture and in vivo, thus promotes the development of premalignant and malignant mammary lesions in transgenic mice (Sternlicht, Lochter et al. 1999). In breast cancer brain metastasis, expression of *MMP2, MMP9* and *MMP3* were found increased, and this breast cancer brain metastasis could be decreased by treatment with PD 166793, a selective synthetic *MMP* inhibitor. This suggests that *MMP2, MMP3* and *MMP9* may be involved in the process of metastasis (Mendes, Kim et al. 2005).

Revealed by express profiling, the deletion of *Pax9* leads to the increase of *MMP3*, thus *MMP3* may be inhibited by *Pax9*, in increasing fat tissue remodeling and the susceptibility to breast cancer via EMT process.

5.9.3 Id-2

Id-2 is an important transcriptional regulator involved in the maintenance of a noninvasive, non-migratory, and low proliferative status of both normal mammary epithelial cells and nonaggressive breast cancer cells (Itahana, Singh et al. 2003). *Id-2* was up-regulated when mammary epithelial cells lost proliferative capacity and initiated differentiation, and when introduced in aggressive breast cancer cells *Id-2* could reduce the proliferation and invasiveness of cancer cells. *Pax9* is decreased in most invasive carcinoma, while *Id-2* expression is also rarely detected in human biopsies from aggressive and invasive carcinomas. Thus, the possibility of *Id-2* as a *Pax9*-regulated downstream target is worth to be investigated either by immunohistochemistry,

western blot or real-time PCR.

Though we do not know their exact roles, of genes identified by expression microarray analysis, the significant changes of these gene expressions at involution pointed to a pregnancy-associated cellular microenvironment that could facilitate breast cancer formation and metastasis. Further studies of molecular regulatory mechanism are required to identify the signaling pathways used by *Pax9* in the mammary gland biology and breast cancer formation.

Chapter 6. *PAX9* is related to favourable prognostic factors of breast cancer

6.1 Introduction

Breast cancer is the most frequent malignant tumour and the leading cause of cancer-related death in women worldwide. Although the overall outlook and quality of life for women with breast cancer has been improved, by curative strategies, 40% of patients still succumb to the disease.

Breast cancer arises from molecularly dysregulated mammary epithelium. The normal development of mammary gland is regulated by comprehensively strict orchestration involving hormones, growth factors, epithelial genetic and cellular regulatory machineries, epithelial-mesenchymal cross-talking, ECM signaling and the participation of immune system, under complex temporal-specific and spatial-specific controls. The genetic and epigenetic changes of genes that regulate mammary epithelial cell differentiation, proliferation, survival, apoptosis, polarity and adhesion, prone to initiate breast carcinogenesis, meanwhile, the stromal responses in premalignant mammary tissue may also promote progression to cancer.

Many transcription factors, previously known as important regulators in embryonic development, have been reported also to play important roles in promoting or inhibiting breast cancer, such as *GATA3, FOX1a, LEF1, MSX2, ER, PR, GHR, STAT5, STAT3, IGF1r, Fgfr2* etc (Nguyen, Rosner et al. 2005, Giulianelli, Cerliani et al. 2008, Kleinberg and Ruan 2008, Albergaria, Paredes et al. 2009, Klinakis, Szabolcs et al. 2009, Lanigan, Gremel et al. 2010, Raven, Williams et al. 2011, Tanos, Rojo et al. 2012, Haricharan and Li 2013). Precise

temporal and spatial activation or shutdown of these genes is critical for normal mammary epithelial cell differentiation, ductal branching, alveolar formation and function, mammary involution, stem cell hierarchy dictation and inflammatory responses, whereas uncontrolled expression of these genes may lead to cancer.

Approximate frequency of *PAX* genes expression in common human tumours had been evaluated by quantitative analysis of mRNA in 54 cancer cell lines (Muratovska, Zhou et al. 2003). 48 cancer cell lines (89%) expressed at least one *PAX* gene, while 35 cell lines (65%) expressed three PAX genes or more. The most frequently expressed *PAX* genes were *PAX3, PAX6, PAX8, and PAX9*. *PAX9* was expressed most frequently in breast, prostate and ovary cancer cell lines. Within breast cancer cell lines, *PAX9* was expressed in luminal (ER+) breast cancer cell lines (MCF7 and TD47) and HER2+ breast cancer cell line(SK-BR-3), in contrast, weak or undetectable in basal breast cancer cell lines (HS578T, BT-549 and MDA-MB-231/ATCC) (Muratovska, Zhou et al. 2003, Holliday and Speirs 2011).

Pax9 is important for embryonic development (Peters, Neubuser et al. 1998, Peters, Wilm et al. 1999), and also is expressed in a variety of organs in adult mice, including the epithelial cells of the thymus, parathyroid glands, salivary glands, and internal stratified squamous epithelia of the oesophagus, tongue, and oral cavity (Peters, Schuster et al. 1997, Gerber, Richter et al. 2002). The role of *Pax9* in adult epithelial differentiation was suggested in a study, which showed that the maintenance of *Pax9* protein expression in epithelial dysplasia and squamous cell carcinoma of the human oesophagus, appears to be associated with a favourable outcome (Gerber, Richter et al. 2002).

In addition, inactivation of *Pax9* in embryonic mouse tongue resulted in partial trans-differentiation to an epithelium with skin-specific characteristics. However, since *Pax9*-deficient mice die shortly after birth, the function of *Pax9* in adult

organ development and in tissue homeostasis is largely unknown.

Based on this knowledge, particularly, with *Pax9* expression levels in a wide range of breast cancer cell lines, and the loss of *Pax9* in ooesophagus carcinomas and transdifferentiation in tongue epithelium, it is conceivable that *Pax9* is differentially expressed in breast cancer with different malignancies. For this purpose, *PAX9* protein expression levels in breast cancer were estimated and the correlation between *PAX9* and breast cancer prognosis thus was investigated in this chapter.

6.2 *PAX9* protein expression in human breast cancer

In order to estimate *PAX9* protein expression levels in breast cancer and to decide whether *PAX9* correlated with breast cancer prognosis, we carried out *PAX9* immunohistochemistry of a wide range of breast cancers by using breast cancer tissue microarray (TMA), and applied comparison and statistical analysis of *Pax9* levels and breast cancer prognostics.

6.2.1 The arrangement of breast cancer tissue microarray (TMA)

CBCTR 2001 Tissue Microarrays (TMAs) were provided by the Cooperative Breast Cancer Tissue Resource (CBCTR). The TMAs were constructed using tissue and associated pathological and clinical outcome data from CBCTR, designed to ensure high statistical power for evaluation and validation of breast cancer biomarkers. The TMA series we used was designed to permit comparisons of biomarker expression across three stages of disease (node-negative, node positive and metastatic breast cancer).

Four identical tissue microarray paraffin blocks have been made of Design # 2 in 2001. The 4 blocks (for each design) are designated A, B, C and D. Histological sections taken from these blocks are numbered sequentially. We received histological sections from some of these paraffin blocks, and the slides were labeled in the following manner: sponsoring organization; year of manufacture; design number; block number; section number. The TMA sections applied in this study were CBCTR 2001 TMA#2: 2B.67, for *PAX1* immunostaining as controls, 2A.68, 2B.68, 2C.70, for *PAX9* immunostaining, to evaluate the protein expression in a breast cancer cohort.

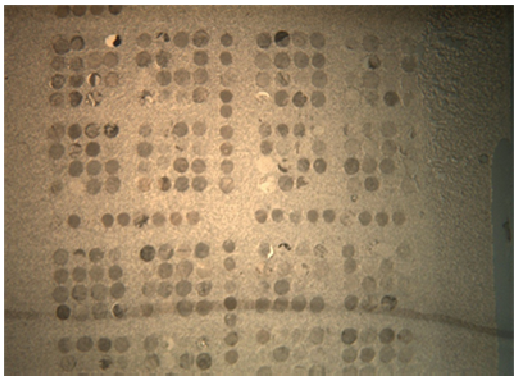

Figure 6.1 Example image of the TMA slide (CBCTR 2001 TMA#2)
TMAs applied in this study were bought from CBCTR, USA, each of which contains 192 invasive ductal carcinoma samples, 20 in-situ ductal carcinoma samples, 40 normal breast samples, 4 control tissues (i.e. appendix, endometrium, kidney and prostate), and 4 control cell lines (i.e. breast cancer cell line MCF-7 and T-47D, prostate cancer cell line PC-3 and colon cancer cell line HT-29). Magnification: 4x.

Immunohistochemistry estimation of *PAX9* expression in breast and other organ tissues was conducted with 3 sets of TMAs, each of which contains 192 invasive ductal carcinoma (IDC) samples, 20 ductal carcinoma *in situ* (DCIS) samples, 40 normal breast samples, 4 control tissues of other organs, i.e. appendix, endometrium, kidney and prostate, and 4 control cell lines, i.e. breast cancer cell lines MCF-7 and T-47D, prostate cancer cell line PC-3 and colon cancer cell line HT-29 (Figure 6.1).

The normal breast tissue was either from an individual without breast cancer, or a normal tissue adjacent to an invasive cancer. In the latter case, the location of the invasive specimen from the same patient was given by the coordinates. The DCIS sample was from a patient with no invasive cancer or the DCIS component of an invasive cancer. In the latter case, the location of the invasive specimen from the same patient was given by the coordinates as well.

Invasive breast cancer specimens were from primary invasive breast cancer patients. In some cases, DCIS or normal breast tissue from the same case was represented on the array.

6.2.2 PAX9 is specifically expressed in normal human breast epithelial tissue and frequently down-regulated in breast cancer

With the breast cancer TMAs from CBCTR and the specific anti-*Pax9* antibody developed in our lab, we evaluated *PAX9* protein expression in a wide range of breast cancers (192 invasive breast cancer and 20 DCIS specimens, 3 sets for each) in one batch of immunostaining.

Demonstrated by immunochemistry of the TMAs, *PAX9* is specifically expressed in the epithelial tissue in most of the normal breast samples, in both of the duct and the lobular, whereas expression was not seen in the mesenchymal tissue. Among control organs, *PAX9* is strongly expressed in prostate epithelial tissues, moderate in endometrium and kidney. In the appendix, due to little intact epithelial tissue is presented in the experimental sections, the expression of *PAX9* in appendix epithelium is not able to be evaluated and needs to be confirmed by more tests (Figure 6.2).

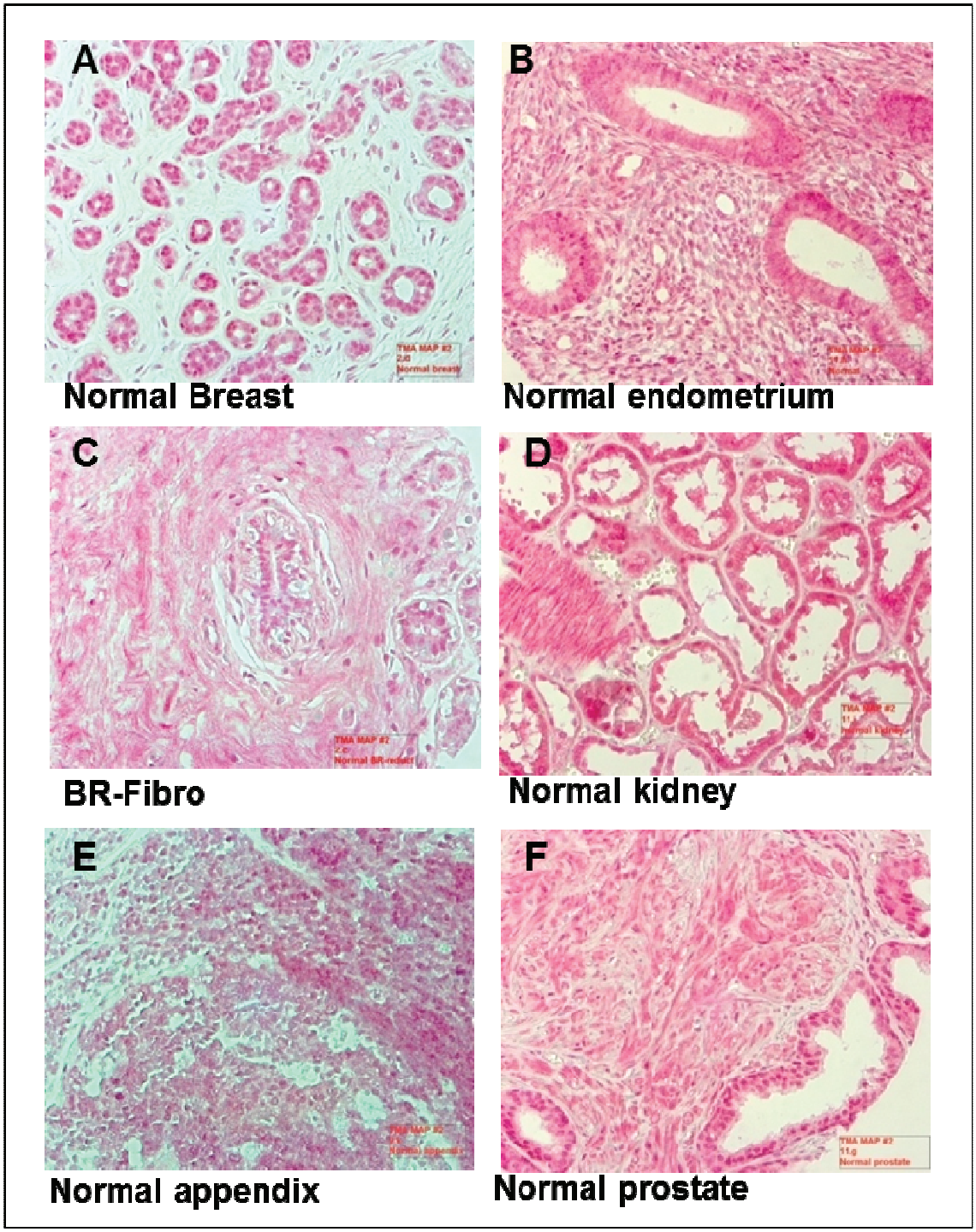

Figure 6.2 Protein expression evaluated in normal tissues with *PAX9* immunostaining
PAX9 is strongly expressed in the epithelium in normal breast (A) and prostate (F), moderately in endometrium and kidney (B,D), and absent in BR-fibro and appendix. Magnification: 200x.

In contrast to high level expression of *PAX9* protein in normal breast, *PAX9* expression is frequently seen at low levels in invasive cancer and DCIS samples (Figure 6.3). *PAX9* expression is specifically located in the nucleus, however, in

some rare cases, is diffused into cytoplasm as well (Figure 6.3 G). Interestingly, when normal breast tissues were seen in some breast carcinoma samples, *PAX9* protein expression was consistently high in the ductal and lobular epithelium, by contrast, low in the adjacent invasive breast cancer (Figure 6.3 B), and we have never seen the reverse, suggesting that loss of *PAX9* is possibly related to normal breast to cancer transformation.

Among cell lines, *PAX9* is expressed moderate to high in breast cancer cell line MCF-7, T-47D, high in prostate cancer cell line PC-3, but weak in colon cancer cell line HT-29 .

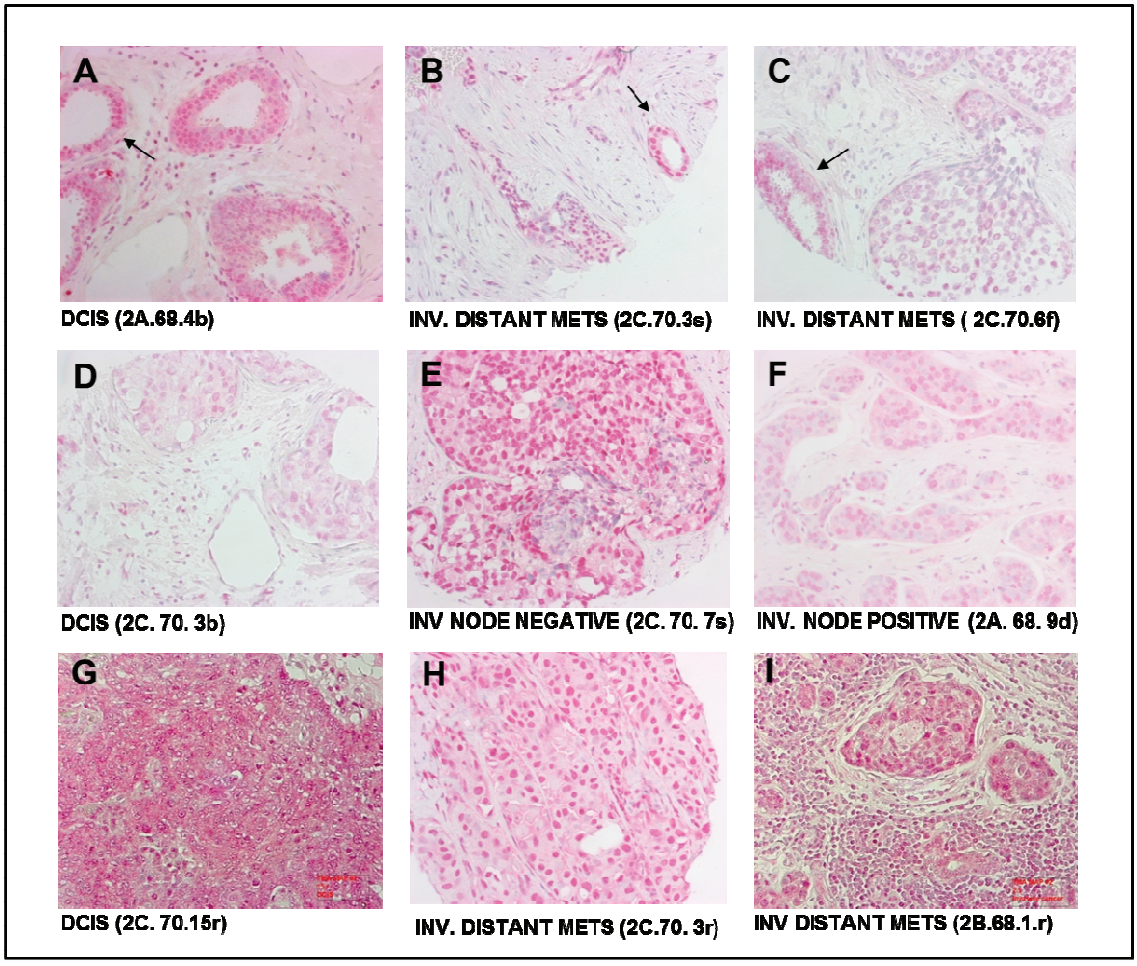

Figure 6.3 *PAX9* protein expression in representatives of DCIS and invasive breast cancer
Ducts and ducts with neoplasia are *Pax9*-positive (A, B, C, indicated by arrows); DCISs and invasive breast cancers express *Pax9* at various levels (other images in this figure). Magnification: 200x

Most normal breast tissues exhibited high *PAX9* expression by *PAX9* immunostaining on TMAs, whereas 3 of 10 normal breast epithelial tissues from non-breast cancer individuals, and 3 of 13 normal breast epithelial tissues from invasive breast cancer carrier patients, presented low levels of *PAX9* protein expression. Down-regulation of *PAX9* expression in these normal tissues may ascribe to the limitation of the sample sources, for the normal breast samples were not actually from absolutely healthy breast, but either from breast cancer carriers or mammary plastic surgery. Moreover, 2 of the 3 *PAX9*-weak normal breast samples from invasive breast cancer carriers were PR negative, which may suggest a correlation between *PAX9* expression and PR expression.

Contrasted to *PAX9*, *PAX1* protein expression is absent in normal breast tissues and breast carcimomas, revealed by *PAX1* immunostaining with TMAs, which is consistent with previous estimation of *PAX* gene expression in breast cancer cell lines using Real-time PCR (Muratovska, Zhou et al. 2003). Thus *PAX1* was used as a negative control for *PAX9* immunostaining in our experiment (Figure 6.4). However, the *PAX1* immunostaining of TMAs lacked positive controls, leaving the results uncertain to some extent.

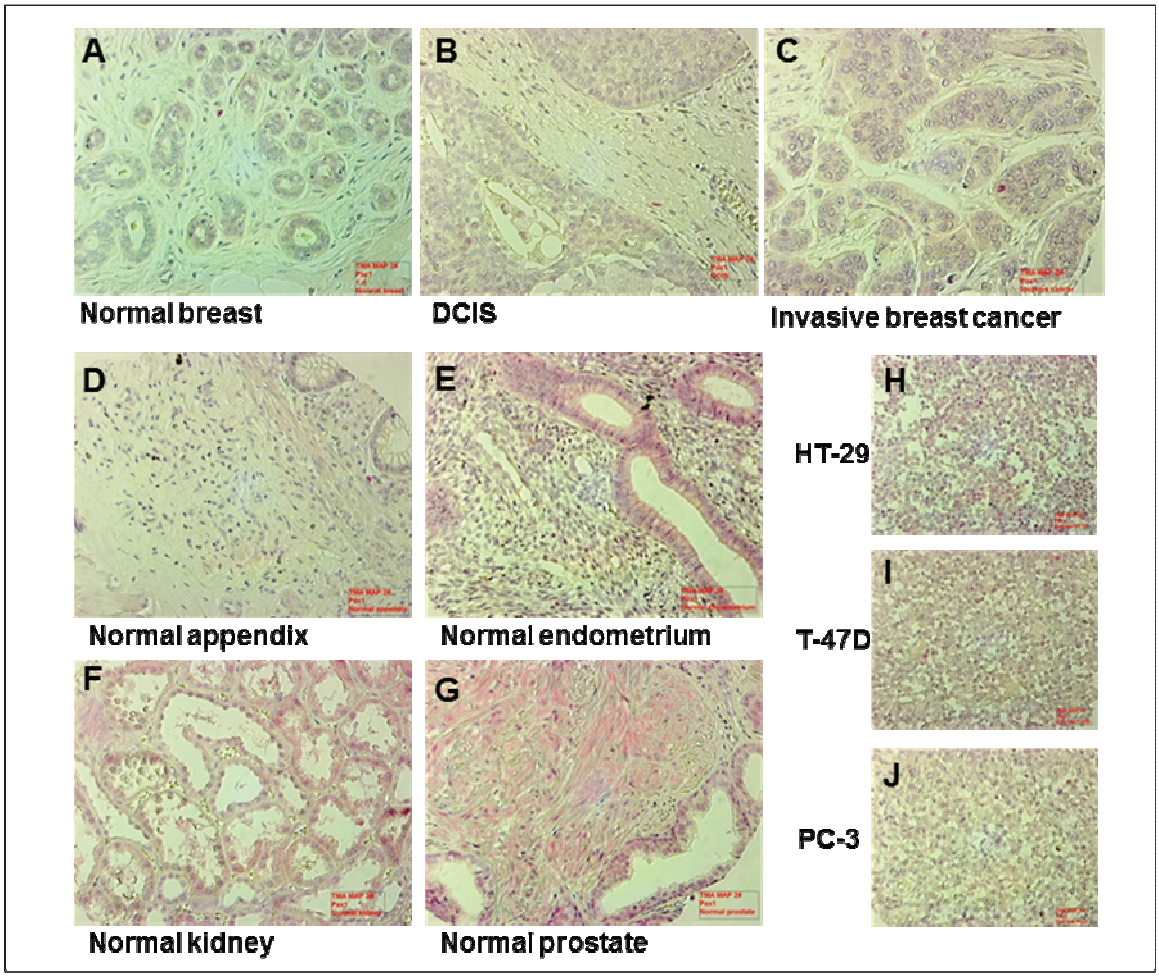

Figure 6.4 *PAX1* immunostaining of TMAs

PAX1 is absent in the normal breast tissue (A), breast cancer (B, C) and cancer cell lines (H, I, J), as well as in appendix, endometrium, kidney and prostate (D, E, F, G). Magnification:200x

6.2.3 PAX9 is related to favourable breast cancer prognostics

We evaluated *PAX9* protein expression in a wide range of breast cancers (192 invasive breast cancer and 20 DCIS specimens, 3 sets for each), and most of them have complete clinic follow-up information, thus we further investigated the associations of *PAX9* protein expression levels with clinical and pathological factors of breast cancer. Clinical and pathologic factors are listed and elucidated in Annex 1. Occasionally, some cores on the breast cancer TMAs were missing, damaged or there is no tumour presented, we thus finally obtained 292 invasive breast cancer and 25 DCIS cores to proceed with the statistical analysis. Furthermore, some cases lacked follow-up information of patients, so that *PAX9*

association with a particular breast cancer prognostic factor was analyzed only with a particular subset of the patient cohort with relative information (the clinical and pathological information of patients related to the TMAs used in this study was not shown.) The levels of *PAX9* protein expression of TMAs exhibited by immunohistochemistry were aligned to 4 levels: negative, weak, moderate and strong, scored 0, 1, 2, 3 respectively, according to the overall performance combined with the intensity of *Pax9* immunostaining and the ratio of positive and negative cells (Figure 6.5).

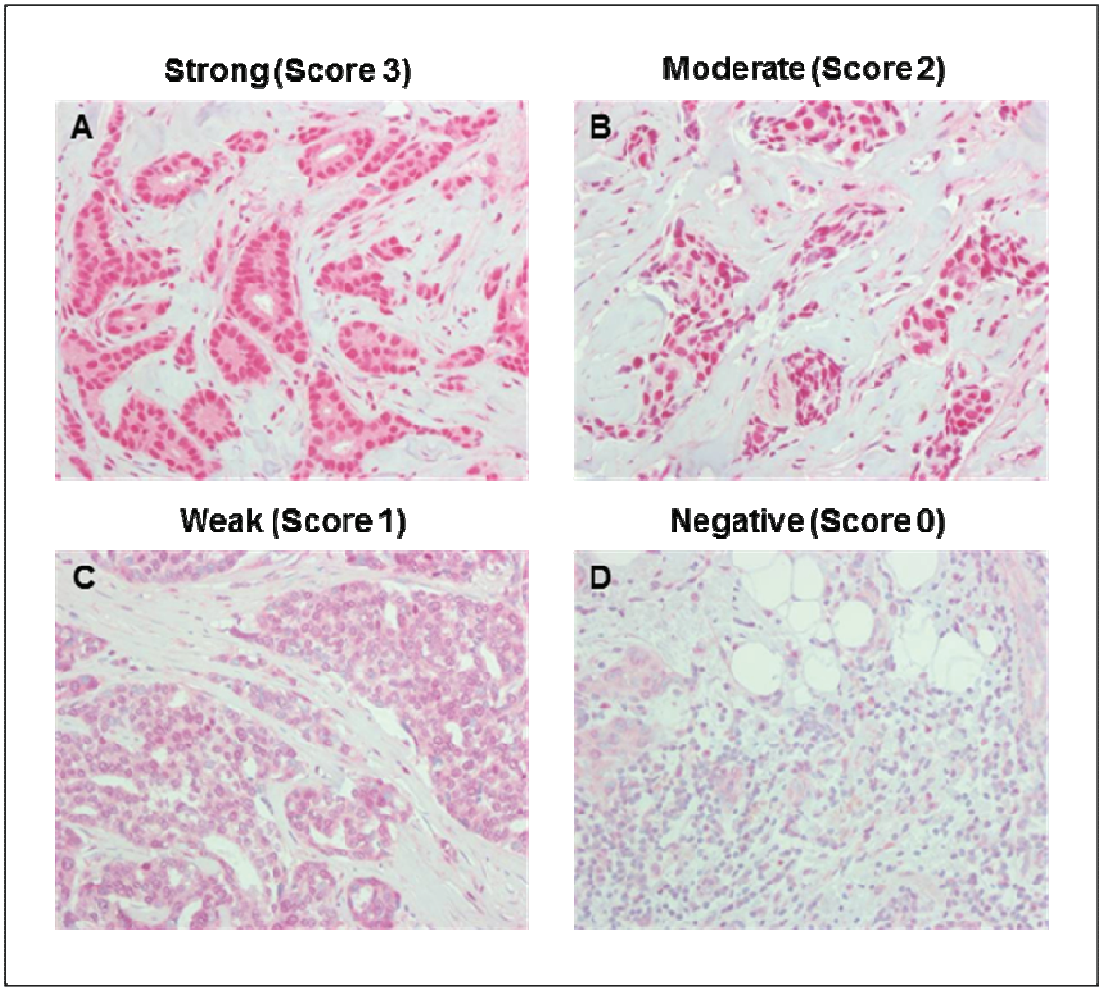

Figure 6.5 Representative breast cancers with *PAX9* expression at 4 levels
PAX9 protein expression are aligned to 4 levels: strong (A), moderate(B), weak(C), and negative(D), scored 3, 2, 1, 0 respectively, according to the overall performance combined with the intensity of *Pax9* immunostaining and the ratio of positive and negative cells. Magnification: 200x.

According to the statistical analysis of the distribution of clinicopathological variables in breast cancer patients by *PAX9* levels (Table 6.1), high *PAX9* expression was found presented in most normal breast tissues (74%), by contrast, presented only in a small part of DCISs (4%), and less in invasive breast cancers (22%). Low *PAX9* expression was observed frequently in breast cancer, both in DCIS and invasive breast cancer. Using Chi-square test, the correlation was statistically significant ($p< 0.0001$). *PAX9* expression is associated with favourable prognostic factors, such as lower histologic tumour grade (Chi-square test $p = 0.03$), less lymph node infiltration ($p = 0.01$) and positive PR ($p< 0.001$). *PAX9* was not associated with ER. Despite *Pax9* being associated with several favourable breast cancer prognostics identified above, higher *PAX9* levels were seen in breast cancers with distant metastasis than in those non-metastasis ($p< 0.001$), implying *PAX9* may play different roles in different stages and events of tumour initiation and progression.

Table 6.1 Distribution of Clinicopathological Variables in Breast Cancer Patients by *PAX9* Levels

Diagnosis factors		*PAX9* level				P-Value (Chisquare test)
		High		Low		
Case type	IDC	63	22%	229	78%	<0.001
	DCIS	1	4%	24	96%	
	Normal breast	17	74%	6	26%	
Node status	Node negative	23	24%	73	76%	0.01
	Node positive	11	10%	94	90%	
Metastasis	Non-metastasis	34	17%	167	83%	<0.001
	Distant metastasis	29	32%	62	68%	
Total score	Grade 1	18	32%	38	68%	0.03
	Grade 2	39	21%	144	79%	
	Grade 3	6	11%	47	89%	
Age	30-39	1	8%	12	92%	<0.001
	40-49	22	27%	60	73%	
	50-59	4	10%	35	90%	
	60-69	2	6%	29	94%	
	>=70	27	33%	55	67%	
PR	PR-	26	15%	142	85%	<0.001
	PR+	32	34%	61	66%	
ER	ER-	17	17%	83	83%	0.11
	ER+	41	25%	120	75%	
T-stage	T1	40	25%	117	75%	0.09
	T2	20	17%	98	83%	
	T3,T4	1	7%	14	93%	
N-stage	N0	25	25%	77	75%	0.03
	N1, N2, N3	19	13%	123	87%	
M-stage	M0	34	17%	167	83%	<0.001
	M1	29	32%	62	68%	
Number of nodes positive	0	25	25%	77	75%	<0.001
	1	4	10%	35	90%	
	2	2	9%	20	91%	
	[3,7]	0	0%	36	100%	
	[8,10]	8	47%	9	53%	
	[11,31]	5	21%	19	79%	
Size of invasive cancer in cm	<1	9	29%	22	71%	0.38
	[1,)	20	22%	71	78%	
	[2, 3)	16	22%	57	78%	
	[3, 4)	5	11%	41	89%	
	[4, 6]	11	22%	38	78%	

6.4 *Pax9* is expressed in mouse mammary gland and is down-regulated in MMTV-Neu and MMTV-PyMT induced mouse tumours

MMTV-Neu and *MMTV-PyMT* are well known transgenic tumour mouse models. Tumour formation and progression in these transgenic mouse models share similar developmental processes with human breast cancers: hyperplasia, adenoma/ mammary intra-epithelial neoplasia, and early and late carcinoma (Muller, Ho et al. 1998, Andrechek, Hardy et al. 2000, Lin, Jones et al. 2003). Paraffin sections of mammary tumour from MMTV-Neu and *MMTV-PyMT* mice were kindly provided by Muller's lab, McGill University, Canada. The same antibody was applied in the *Pax9* immunohistochemistry of mouse mammary tumours. 30 sections were tested in our lab and the others were tested in Muller's lab. Wild type CD1 mouse was used as normal mammary gland control. Different color developer regents were used in the two labs, but the results agreed with each other.

In normal mouse mammary gland, *Pax9* was expressed exclusively in the luminal epithelial cells lining the inner layer of the mammary duct, in contrast, *Pax9* was entirely absent in *MMTV-PyMT* induced tumours, and is down-regulated in most cells in *MMTV-Neu* induced tumours (Figure 6.6).

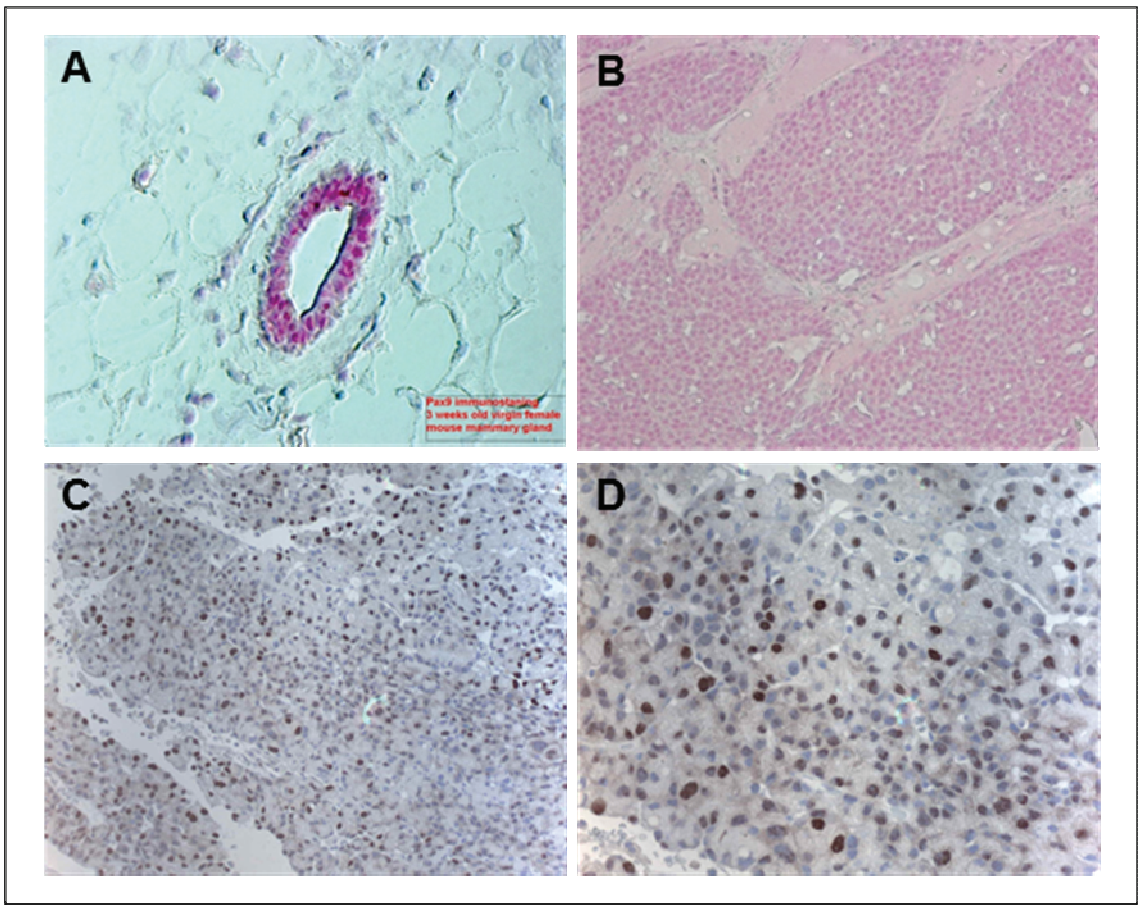

Figure 6.6 *Pax9* protein expression in mouse mammary gland and mouse mammary tumours

Pax9 is expressed in luminal cells in mouse mammary duct (A), down-regulated in most cells in MMTV-Neu induced tumours (C, D, provided by Muller's lab), and entirely absent in MMTV-PyMT induced tumours (B). Magnifiction: (A, D) 400x, (B, C) 200x. n=5

The mammary epithelium-specific expression of *Pax9* in normal mouse mammary gland and the gradual loss of *Pax9* in mouse mammary tumour strongly supported the similar phenomena seen in human breast cancer samples.

6.5 Discussion

In this study, we found, for the first time, that *PAX9* protein was expressed specifically in epithelial tissue in human breast and mouse mammary gland. Reduction or loss of *PAX9* was observed frequently in human DCISs (96%) and invasive breast cancers (78%), as well as in *MMTV-Neu* and *MMTV-PyMT*

induced mouse tumours.

By Immunohistochemistry and statistical analysis of human breast cancer TMAs, *PAX9* protein expression levels was found to correlate with favourable pathoclinical prognostic factors, such as lymph node negative status, low histological tumour grade, *PR* positive and small tumour size, which is similar with previously reported correlation between progressive loss of *PAX9* and increasing malignancy of ooesophagus carcinoma in ooesophagus epithelia (Gerber, Richter et al. 2002). However, on the other hand, high *PAX9* is associated with distant metastasis. High *PAX9* protein expression levels in favourable breast cancer prognostic factors raises a conjecture that *PAX9* possibly play roles in maintaining epithelial differentiation state and inhibiting epithelial-mesenchymal transition (EMT) in mammary tumours.

Gene expression profiling on cancer cell lines has shown that *PAX9* is expressed in luminal subtype cell lines (*ER*-positive) and *HER2* subtype cells (*HER2*-positive), but absent in all 3 basal subtypes, i.e., MDA-MB-231, BT549 and HS578T, according to previous report (Muratovska, Zhou et al. 2003)**.** When incorporated into xenograft models, *ER*-positive luminal cell lines only form tumours in the presence of oestrogen and the growth can be inhibited by anti-oestrogen therapy, *HER*2 subtype cells have poor tumourigenic potential, in contrast, the basal subtype is tumourigenic and invasive, the derivatives of MDA-MB-231 developed by Massague's group are able to metastasize to particular metastatic sites (Holliday and Speirs 2011). Basal subtype breast cancer cell lines lack expression of *ERα, PR* and *HER2* (triple-negative), and exhibit epithelial-mesenchymal transition (EMT). The consistent loss of *PAX9* expression in basal type breast cancer cell lines suggests that *PAX9* may maintain the epithelial–specific differentiation state of breast cell lines, and also agrees with *PAX9* as a marker for favourable outcome of breast cancer.

High *PAX9* was seen related to distant metastasis in breast cancer by TMAs analysis, on the other hand, basal type breast cell lines (*PAX9*-negative) have more potential for metastasis. The contrasting results about *PAX9* association with distant metastatic capacity in human breast cancer and human breast cancer cell lines, maybe due to the different mechanisms of particular stages of metastasis progress, such as EMT, cell proliferation and cell survival. At the beginning of metastasis, EMT is the most essential mechanism to allow tumour cell transformation to escape the tight alignment of epithelial cells and degrade the basement membrane to facilitate invasion, but after the tumour cells migrate to a new niche, other cellular events, for instance, cell proliferation and survival, would take a more important place to decide the new tumour growth. Thus, if *PAX9* were playing different roles in these different events, for instantce, EMT and cell proliferation and survival, the final results would be the balance of the overall effects.

Aberrant molecular events drive cells in breast and other tissues to transform cancers and progress to malignancy. Mammary gland development is strictly regulated by global and local signalings, as for cell differentiation, proliferation, survival, apoptosis, dead cell clearance, epithelium-stromal cross-talking, tissue remodeling, immune response, etc. Meanwhile, the same general processes that take place during normal mammary development also occur in malignant diseases, which initiate tumour cell proliferation, transformation, dissemination and metastasis. The molecular signaling networks could process information from outside or within the tissue. Some developmental pathways influencing mammary stem cell hierarchy maintenance, ductal branching and postnatal involution, have been found associated with the initiation and progression of breast cancer.

Chapter 7. Summary and discussion

7.1 *Pax9* expression pattern, phenotype of mutants and function summary

Demonstrated by semi-quantitative RT PCR and immunohistochemistry, crossing all developmental stages of mouse mammary gland, *Pax9* was found to be expressed exclusively in the ductal epithelium of mammary gland, with a strict spatial-temporal pattern, which peaks at puberty, decreases significantly in lactating epithelial cells, and increases again during involution.

Reduction or deletion of *Pax9* expression, by using， *Pax9* knockout, hypomorph and mammary gland-specific knockout mouse models, did not show obvious disturbance to mammary gland development at birth, but led to ductal branching delay during puberty, alveolar formation at wrong position during pregnancy, disrupted epithelial cell apoptosis and failed engulfment of excess milk fat globulin during involution (Figure 7.1). In addition, mammary TEB filing, ductal filing, hyperplasia, neoplasia and basement membrane breakdown were seen in *Pax9* mutants.

7.2 *Pax9* in ductal branching

In the TEBs, *Pax9* is strongly expressed in the cap cells, which actively proliferate for duct elongation, and inner body cells, which undergo apoptosis to form the luminal space. In the *MMTV-Cre*; *Pax9*$^{flox/flox}$ mouse, some TEBs are filled with *Pax9*-negative epithelial cells (Figure 5.12 A). Bim regulates apoptosis in the TEB, deletion of which results in TEB filling (Mailleux, Overholtzer et al. 2007). *Pax9* is expressed in the body cells, which is close to the TEB's cavity, whose expression pattern similar to *Bim*, thus it is possible that *Pax9* also regulates apoptosis to form the TEB cavity and the lumen space.

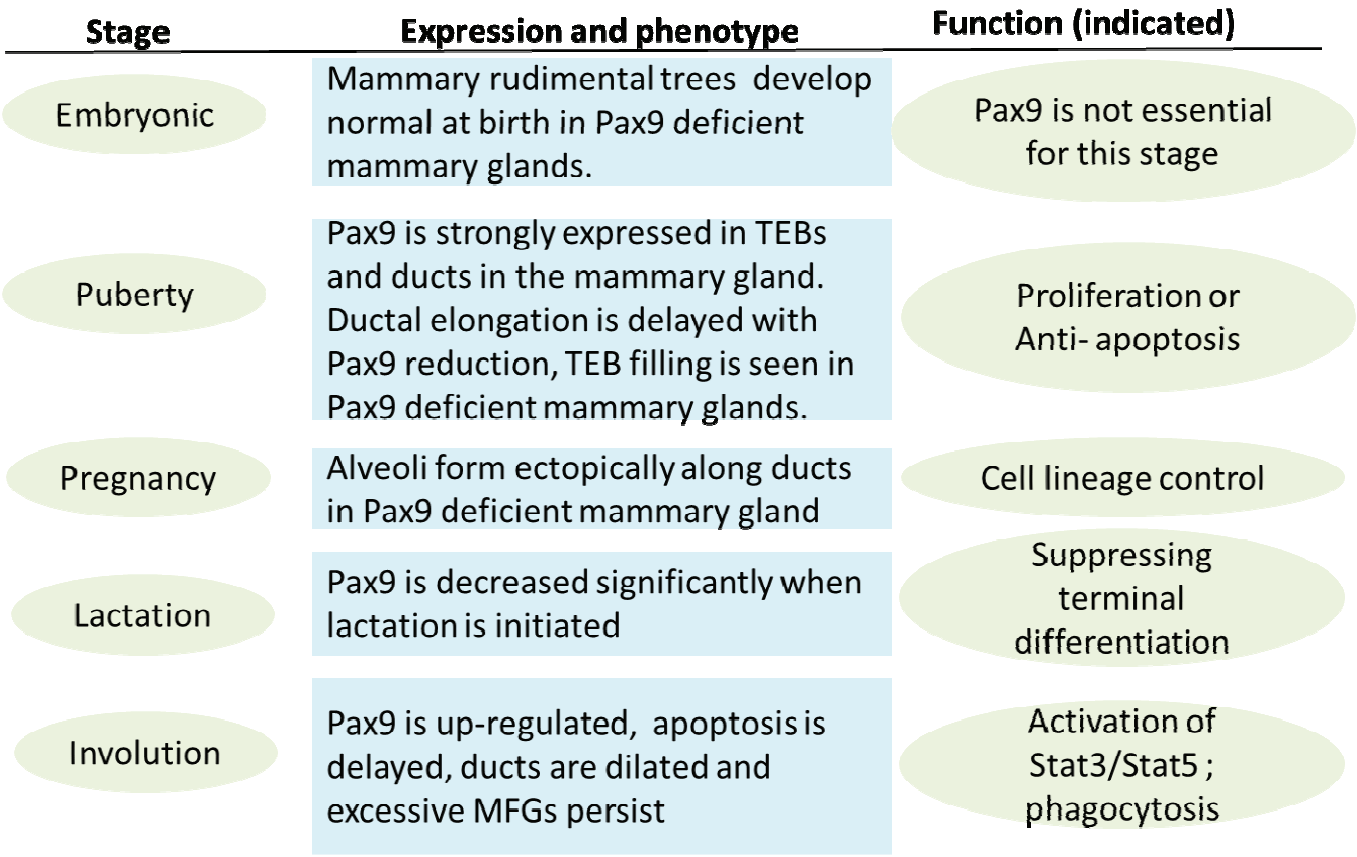

Figure 7.1 *Pax9* expression pattern, phenotype of mutants and function summary

7.3 *PAX9* in Breast cancer

In our study, *PAX9* was found to reversely correlate with breast cancer malignancy. While expressed in the epithelia of normal mammary gland of human and mouse, *PAX9* is decreased or absent in human DCISs (96%) and invasive breast cancers (78%), as well as in MMTV-Neu and MMTV-PyMT induced mouse mammary tumours. *PAX9* is also correlated with good prognosis in breast cancer revealed by our statistics analysis.

Hyperplastic alveolar nodules are frequently seen in mammary glands in 10 months to 1 year old virgin *K14-Cre*;*Pax9*$^{flox/flox}$ mice. *Pax9* deficiency in mammary gland leads to neoplasia in mature *Pax9*$^{neo/neo}$, TEB filling and neoplasia were seen in a 5-week old *MMTV-Cre*;*Pax9*$^{flox/flox}$. Besides cell proliferation and differentiation, the maintenance of luminal epithelial cells differentiation state is important as well. The distortion of the ductal epithelial characteristics can initiate breast cancer. With the reduction of *Pax9*, ducts were filled with *Pax9* negative epithelial cells, ductal epithelial cells lost their single layer alignment and formed cell mass. Laminin plays an important role in maintaining the integrity of the basement membrance and guiding the polarity of the epithelial cell, but *Laminin1* was lost in the mammary ducts of *Pax9* deficient mice, which also maybe a factor to facilitate breast cancer initiation.

7.4 *Pax9* in engulfment

Macrophage-like cells were seen with very strong *Pax9* expression at different stages of mammary gland, around the TEBs during puberty and massive during involution. Macrophage is reported to help duct branching during puberty (Gouon-Evans, Lin et al. 2002, Lin, Gouon-Evans et al. 2002, Van Nguyen and Pollard 2002, Schwertfeger, Rosen et al. 2006, Gyorki, Asselin-Labat et al. 2009), eliminate excess MFGs during involution, but also activate tumour growth and

metastasis (Lin, Gouon-Evans et al. 2002). The attractants and consequence of this macrophage-like cell infiltration is yet to be revealed.

Mammary epithelial cells (MECs) can be non-professional phagocytes, to help with the final clearance and breakdown of their neighboring apoptotic cells and likely MFGs as well (Monks, Smith-Steinhart et al. 2008, Monks, Rosner et al. 2005, Atabai, Sheppard et al. 2007). Milk fat globule-EGF-factor (*Mfge8*) is secreted by macrophage and epithelial cell to bridge the apoptotic cells and phagocytes (Hanayama, Tanaka et al. 2002, Hanayama, Tanaka et al. 2004, Atabai, Fernandez et al. 2005, Hanayama and Nagata 2005, Atabai, Sheppard et al. 2007, Nandrot, Anand et al. 2007). Deletion of *Mfge8* delays mammary gland involution with enlarged ducts filled with excess MFGs. Our observation in *Pax9* deficiency-induced mammary gland involution impairment presents a very similar phenotype, with extremely dilated ducts filled with excess MFGs. It thus implicates that *Pax9* might play a role in MEC and MFG engulfment, and possibly in macrophage as well.

7.5 *Pax9* in apoptosis

Mammary gland involution was delayed significantly in *Pax9*-deficient mammary glands. TUNEL essay confirmed the striking interference to MEC apoptosis. *Pax9* immunohistochemistry revealed delayed activation of *Stat3* and a persistent activation of *Stat5*, the former is responsible for acute response and rapid apoptosis during the first phase of involution, whereas the latter is a cell survival signal (Chapman, Lourenco et al. 1999, Chapman, Lourenco et al. 2000, Iavnilovitch, Groner et al. 2002). *Stat* family genes are recognized as important factors of breast cancer in recent years (Li, Rosen et al. 2013), the function of *Pax9* in involution and breast cancer initiation and progression may be partially through the interference to *Stat* signaling pathway.

7.6 *Pax9* in differentiation

Mammary gland has a complex hierarchy of MECs, including stem cell, progenitor cell, differentiated cell and terminally differentiated cell (Van Keymeulen, Rocha et al. 2011, Visvader and Lindeman, 2011, Visvader and Smith, 2011, Asselin-Labat, Shackleton et al. 2006, Shackleton, Vaillant et al. 2006; Stingl, Eirew et al. 2006). During pregnancy, alveoli develop from the tip of the tertiary duct, where the cells has a certain differentiation state. Surprisingly, in *Pax9*$^{neo/neo}$ and *MMTV-Cre*;*Pax9*$^{flox/flox}$ mammary gland, the alveoli were seen to develop directly from the primary and secondary duct. Thus, *Pax9* deficiency may lead to a change in epithelial cell lineage decision.

Pax9 may be able to guide pre-terminal differentiation, since its expression increases from puberty but not before, and drops when progesterone withdraws and prolactin increases. Most likely, it is correlated with *PR*, since progesterone need to be down-regulated to start lactation, and *Pax9* is decreased when alveoli initiate milk-producing.

7.7 Future work to illuminate the mechanism of the regulation downstream of *Pax9*

The importance of *Pax9* in regulating mammary gland development has been well addressed in our study. *Pax9* plays important roles in mammary ductal branching, alveolar differentiation, and postlactational involution. In addition, *Pax9* is proposed as a tumour suppressor gene, since *Pax9* is decreased in most breast cancers and reduction of *Pax9* in the mutants can form precancerous histology. However, the exact mechanisms by which *Pax9* regulates these biological events are yet to be known. Further investigation is required to better understand the roles that *Pax*9 play in there, for instance, organogenesis, cell lineage commitment, terminal differentiation, adult tissue maintenance, apoptosis, phagocytosis and cancer progression.

The disturbance by *Pax9*-deficiency to postlactational involution of the mammary gland is the most striking phenotype we observed in *Pax9*-deficient mammary glands. Apoptosis of epithelial cells were severely delayed and alveolar structures persisted till involution day 8 in the mutants. However, due to the lack of homogenous recombination in the existing mammary gland-specific conditional knockout mouse models, the study of the mechanism that *Pax9* regulates mammary gland involution was still relatively limited. By the evidence from the limited number of animals with validated *Pax9* deletion in the mammary gland, *Stat* signaling pathway, especially *Stat5,* is found to be affected by *Pax9* during involution, while persistent of *Stat5* and delayed *Stat3* activation (*pStat5*/*pStat3*) were seen in the *Pax9*-deficient mammary gland on involution day 4. *Stat* signaling is well known for its roles in oncogenesis, apoptosis, cell survival, stem cell differentiation, cancer progression and metastasis (Vafaizadeh, Klemmt et al. 2010, Hernandez-Vargas, Ouzounova et al. 2011). In addition to apoptosis and cell survival during involution stage, may *Pax9* also regulate ductal and alveolar cell lineage comitment through *Stat* signaling? In order to confirm *Pax9* regulation to *Stat5* and *Stat3,* in the future, we need to increase the Pax9-deficient biological replicates to 4 to 6, to get convincing results on *pStat5* and *pStat3* expression in the mammary gland, by immunostaining at lactation day 8, involution day 2, day 3, day 4, respectively. On the other hand, *Pax9* is decreased in most malignant cancers, may *Pax9* affect breast cancer also through *Stat* signaling? In the future, an correlation analysis of *Pax9, pStat3 and pStat5 expression by* immunostaining on tumour microarray is needed to investigate the correlation between *Pax9* and *Stat* signaling in breast cancer.

What roles *Pax9* playing in mammary gland ductal branching is not yet very clear due to the lack of suitable mouse models during puberty, for recombination has not been activated in virgin *MMTV-Cre;Pax9*$^{flox/flox}$ mice. In this case, mammary

gland transplantation technique is an accessible substitution of mammary gland-specific conditional knockout to study mammary ductal tree branching (puberty) and alveolar formation (pregnancy). Since *the* mammary gland exhibited normal ductal tree pattern in *Pax9*$^{LacZ/LacZ}$ null mice at birth, the *Pax9*$^{LacZ/LacZ}$ rudimental mammary gland can be transplanted into a cleared fat pad of control mice to observe *Pax9*-deficient mammary epithelial development, including ductal branching and alveolar formation, in normal systematic environment and normal stromal niche. Expression profiling by microarray could be applied to these mammary glands to screen candidate genes regulated by *Pax9* at these developmental stages. This also will expand our knowledge about the mechanism by which *Pax9* functions in ductal/alveolar cell lineage commitment.

Pax9 was found expressed in macrophage-like cells throughout adult developmental stages, from ductal branching to postlactational involution. Excessive milk fat globules persisted in *Pax9*-deficient mammary gland during early involution, due to impaired cell clearance either by epithelial cells or macrophage, while massive *Pax9*-positive phagocytes appeared to compensate that at late involution. Does *Pax9* directly regulate epithelial cells to exert engulfment function as non-professional phagocytes, or *Pax9* even also function in macrophage? To answer these questions, firstly it is worth to identify the type of these *Pax9*-positive macrophage-like cells by macrophage-specific cell markers, and compare their characteristics with mammary epithelial cells, which are reported function as innate non-professional phagocyte during involution. In-vitro function analysis can be done by an in-vitro Phagocytosis Assay, by which phagocytosis can be easily evaluated by observing the cells under a microscope after phagocytosis proceeds (Knockdown *Pax9* in a suitable mammary epithelial cell line and a macrophage cell line, allow phagocytosis by mix the *Pax9*-deficient cells with induced apoptotic cells, fix the cells and subject to the TUNEL reaction, observe phagocytosis by light microscopy. The number

of macrophages that carry TUNEL-positive apoptotic cells was counted.) Experiments of comparative expression profiling between macrophage and mammary epithelial cells can be applied to find out common mechanisms regulating engulfment in both cell types.

Tumour formation is believed as an aberrant form of organogenesis in adult tissues. Anti-apoptosis is an essential charactertistic of tumour cells to survive in its microenvironment during initiation and progression. *Pax9* deficiency in the mammary gland leads apoptosis inhibition and impaired apoptotic cell clearance, both of which add chance to cancer formation. Understanding the roles of *Pax9* playing in mammary gland development will also help to illuminate the mechanism of breast cancer initiation and progression, and provide new therapeutic targets in the future.

ANNEX

Annex 1: Descriptions of key clinicopathological variables in breast cancer

1. T Stage

T Stage at initial diagnosis. From Manual for Staging of Cancer, 5th Ed.

TIS=Carcinoma In-situ

T1=Tumour ≤ 20 mm

T1a=Tumour ≤ 5 mm

T1b=Tumour > 5 mm and ≤10 mm

T1c=Tumour > 10 mm and ≤ 20 mm

T2=Tumour > 20 mm and ≤ 50 mm

T3=Tumour > 50 mm

T4=Tumour any size + extension

T4a=Extension to chest wall

T4b=Edema or ulceration or satellite skin nodules

T4c=Both T4a and T4b

T4d=Inflammatory carcinoma

2. N Stage

N Stage at initial diagnosis (From Manual for Staging of Cancer, 5th Ed.)

NX=Minimum req. not met (unknown)

N0=No evidence of regional LN involvement

N1=Mets to movable, ipsilateral node

N1a=Only micromets (≤ 2 mm)

N1b=Any nodal mets > 2 mm

N1b1=Mets to 1-3 nodes, any > 2 mm and all <20 mm

N1b2=Mets to 4 or more nodes, any > 2 mm and all <

20 mm

N1b3=Extension of tumour beyond node capsule and < 20 mm

N1b4=Mets to nodes ≥ 20 mm

N2=Mets to ipsilateral nodes that are fixed to one another or to other structures

N3=Mets to ipsilateral internal mammary lymph node

3. M Stage

M Stage at initial diagnosis (From Manual for Staging of Cancer, 5th Ed.)

MX=Distant mets not assessed.

M0=No known distant mets

M1=Distant mets present

4. Number of Nodes Positive

The number of nodes positive at initial diagnosis.

5. Size of Invasive

Measurement (in centimeters) of the longest Cancer in cm. diameter of tumour. For multifocal tumours, size is taken from the largest grossly evident mass in the breast. Size may be unknown in cases where T stage is known OR M stage is M1.

6. Grade

The histologic grade applies only to the invasive component of the tumour. Grade is determined by the Elston and Ellis approach to the Scarff Bloom Richardson method. The grade is derived from the total score calculated from the extent of tubule formation, extent of nuclear pleomorphism,

and the mitotic count.

1=Grade 1

2=Grade II

3=Grade III

References

Abell, K., A. Bilancio, R. W. Clarkson, P. G. Tiffen, A. I. Altaparmakov, T. G. Burdon, T. Asano, B. Vanhaesebroeck and C. J. Watson (2005). "Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition." Nat Cell Biol **7**(4): 392-398.

Adams, B., P. Dorfler, A. Aguzzi, Z. Kozmik, P. Urbanek, I. Maurer-Fogy and M. Busslinger (1992). "Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis." Genes Dev **6**(9): 1589-1607.

Aharinejad, S., P. Paulus, M. Sioud, M. Hofmann, K. Zins, R. Schafer, E. R. Stanley and D. Abraham (2004). "Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice." Cancer Res **64**(15): 5378-5384.

Albergaria, A., J. Paredes, B. Sousa, F. Milanezi, V. Carneiro, J. Bastos, S. Costa, D. Vieira, N. Lopes, E. W. Lam, N. Lunet and F. Schmitt (2009). "Expression of FOXA1 and GATA-3 in breast cancer: the prognostic significance in hormone receptor-negative tumours." Breast Cancer Res **11**(3): R40.

Alexander, C. M., S. Selvarajan, J. Mudgett and Z. Werb (2001). "Stromelysin-1 regulates adipogenesis during mammary gland involution." J Cell Biol **152**(4): 693-703.

Anderson, S. M., M. C. Rudolph, J. L. McManaman and M. C. Neville (2007). "Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis!" Breast Cancer Res **9**(1): 204.

Andrechek, E. R., W. R. Hardy, P. M. Siegel, M. A. Rudnicki, R. D. Cardiff and W. J. Muller (2000). "Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis." Proc Natl Acad Sci U S A **97**(7): 3444-3449.

Asselin-Labat, M. L., M. Shackleton, J. Stingl, F. Vaillant, N. C. Forrest, C. J. Eaves, J. E. Visvader and G. J. Lindeman (2006). "Steroid hormone receptor status of mouse mammary stem cells." J Natl Cancer Inst **98**(14): 1011-1014.

Asselin-Labat, M. L., K. D. Sutherland, H. Barker, R. Thomas, M. Shackleton, N. C. Forrest, L. Hartley, L. Robb, F. G. Grosveld, J. van der Wees, G. J. Lindeman and J. E. Visvader (2007). "Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation." Nat Cell Biol **9**(2): 201-209.

Atabai, K., R. Fernandez, X. Huang, I. Ueki, A. Kline, Y. Li, S. Sadatmansoori, C. Smith-Steinhart, W. Zhu, R. Pytela, Z. Werb and D. Sheppard (2005). "Mfge8 is critical for mammary gland remodeling during involution." Mol Biol Cell **16**(12): 5528-5537.

Atabai, K., D. Sheppard and Z. Werb (2007). "Roles of the innate immune system in mammary gland remodeling during involution." J Mammary Gland Biol Neoplasia **12**(1): 37-45.

Ballestar, E., M. F. Paz, L. Valle, S. Wei, M. F. Fraga, J. Espada, J. C. Cigudosa, T. H. Huang and M. Esteller (2003). "Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer." EMBO J **22**(23): 6335-6345.

Bannykh, S. I., S. C. Emery, J. K. Gerber, K. L. Jones, K. Benirschke and E. Masliah (2003). "Aberrant Pax1 and Pax9 expression in Jarcho-Levin syndrome: report of two Caucasian siblings and literature review." Am J Med Genet A **120A**(2): 241-246.

Barash, I. (2006). "Stat5 in the mammary gland: controlling normal development and cancer." J Cell Physiol **209**(2): 305-313.

Barr, F. G. (1997). "Chromosomal translocations involving paired box transcription factors in human cancer." Int J Biochem Cell Biol **29**(12): 1449-1461.

Beleut, M., R. D. Rajaram, M. Caikovski, A. Ayyanan, D. Germano, Y. Choi, P. Schneider and C. Brisken (2010). "Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland." Proc Natl Acad Sci U S A **107**(7): 2989-2994.

Bernasconi, M., A. Remppis, W. J. Fredericks, F. J. Rauscher, 3rd and B. W. Schafer (1996). "Induction of apoptosis in rhabdomyosarcoma cells through down-regulation of PAX proteins." Proc Natl Acad Sci U S A **93**(23): 13164-13169.

Bertucci, P. Y., A. Quaglino, A. G. Pozzi, E. C. Kordon and A. Pecci (2010). "Glucocorticoid-induced impairment of mammary gland involution is associated with STAT5 and STAT3 signaling modulation." Endocrinology **151**(12):

5730-5740.

Borycki, A. G., J. Li, F. Jin, C. P. Emerson and J. A. Epstein (1999). "Pax3 functions in cell survival and in pax7 regulation." Development **126**(8): 1665-1674.

Bouchard, M., A. Souabni, M. Mandler, A. Neubuser and M. Busslinger (2002). "Nephric lineage specification by Pax2 and Pax8." Genes Dev **16**(22): 2958-2970.

Brisken, C., A. Heineman, T. Chavarria, B. Elenbaas, J. Tan, S. K. Dey, J. A. McMahon, A. P. McMahon and R. A. Weinberg (2000). "Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling." Genes Dev **14**(6): 650-654.

Brisken, C., S. Park, T. Vass, J. P. Lydon, B. W. O'Malley and R. A. Weinberg (1998). "A paracrine role for the epithelial progesterone receptor in mammary gland development." Proc Natl Acad Sci U S A **95**(9): 5076-5081.

Buckingham, M., L. Bajard, T. Chang, P. Daubas, J. Hadchouel, S. Meilhac, D. Montarras, D. Rocancourt and F. Relaix (2003). "The formation of skeletal muscle: from somite to limb." J Anat **202**(1): 59-68.

Buckiova, D. and J. Syka (2004). "Development of the inner ear in Splotch mutant mice." Neuroreport **15**(13): 2001-2005.

Burton, Q., L. K. Cole, M. Mulheisen, W. Chang and D. K. Wu (2004). "The role of Pax2 in mouse inner ear development." Dev Biol **272**(1): 161-175.

Buttiglieri, S., M. C. Deregibus, S. Bravo, P. Cassoni, R. Chiarle, B. Bussolati and G. Camussi (2004). "Role of Pax2 in apoptosis resistance and proinvasive phenotype of Kaposi's sarcoma cells." J Biol Chem **279**(6): 4136-4143.

Capuco, A. V. and R. M. Akers (2009). "The origin and evolution of lactation." J Biol **8**(4): 37.

Chapman, R. S., P. Lourenco, E. Tonner, D. Flint, S. Selbert, K. Takeda, S. Akira, A. R. Clarke and C. J. Watson (2000). "The role of Stat3 in apoptosis and mammary gland involution. Conditional deletion of Stat3." Adv Exp Med Biol **480**: 129-138.

Chapman, R. S., P. C. Lourenco, E. Tonner, D. J. Flint, S. Selbert, K. Takeda, S. Akira, A. R. Clarke and C. J. Watson (1999). "Suppression of epithelial apoptosis

and delayed mammary gland involution in mice with a conditional knockout of Stat3." Genes Dev **13**(19): 2604-2616.

Chen, Q., D. J. DeGraff and R. A. Sikes (2010). "The developmental expression profile of PAX2 in the murine prostate." Prostate **70**(6): 654-665.

Choi, Y. S., R. Chakrabarti, R. Escamilla-Hernandez and S. Sinha (2009). "Elf5 conditional knockout mice reveal its role as a master regulator in mammary alveolar development: failure of Stat5 activation and functional differentiation in the absence of Elf5." Dev Biol **329**(2): 227-241.

Chu, E. Y., J. Hens, T. Andl, A. Kairo, T. P. Yamaguchi, C. Brisken, A. Glick, J. J. Wysolmerski and S. E. Millar (2004). "Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis." Development **131**(19): 4819-4829.

Clarkson, R. W. and C. J. Watson (2003). "Microarray analysis of the involution switch." J Mammary Gland Biol Neoplasia **8**(3): 309-319.

Clarkson, R. W., M. T. Wayland, J. Lee, T. Freeman and C. J. Watson (2004). "Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression." Breast Cancer Res **6**(2): R92-109.

Conway, S. J., D. J. Henderson and A. J. Copp (1997). "Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant." Development **124**(2): 505-514.

Conway, S. J., D. J. Henderson, M. L. Kirby, R. H. Anderson and A. J. Copp (1997). "Development of a lethal congenital heart defect in the splotch (Pax3) mutant mouse." Cardiovasc Res **36**(2): 163-173.

Davenport, T. G., L. A. Jerome-Majewska and V. E. Papaioannou (2003). "Mammary gland, limb and yolk sac defects in mice lacking Tbx3, the gene mutated in human ulnar mammary syndrome." Development **130**(10): 2263-2273.

Davis, R. J., J. L. Bennicelli, R. A. Macina, L. M. Nycum, J. A. Biegel and F. G. Barr (1995). "Structural characterization of the FKHR gene and its rearrangement in alveolar rhabdomyosarcoma." Hum Mol Genet **4**(12): 2355-2362.

Deblois, G., G. Chahrour, M. C. Perry, G. Sylvain-Drolet, W. J. Muller and V.

Giguere (2010). "Transcriptional control of the ERBB2 amplicon by ERRalpha and PGC-1beta promotes mammary gland tumorigenesis." Cancer Res **70**(24): 10277-10287.

Debnath, J., K. R. Mills, N. L. Collins, M. J. Reginato, S. K. Muthuswamy and J. S. Brugge (2002). "The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini." Cell **111**(1): 29-40.

DeNardo, D. G., J. B. Barreto, P. Andreu, L. Vasquez, D. Tawfik, N. Kolhatkar and L. M. Coussens (2009). "CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages." Cancer Cell **16**(2): 91-102.

Desrivieres, S., C. Kunz, I. Barash, V. Vafaizadeh, C. Borghouts and B. Groner (2006). "The biological functions of the versatile transcription factors STAT3 and STAT5 and new strategies for their targeted inhibition." J Mammary Gland Biol Neoplasia **11**(1): 75-87.

Dressler, G. R., J. E. Wilkinson, U. W. Rothenpieler, L. T. Patterson, L. Williams-Simons and H. Westphal (1993). "Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities." Nature **362**(6415): 65-67.

Dunbar, M. E., P. Dann, C. W. Brown, J. Van Houton, B. Dreyer, W. P. Philbrick and J. J. Wysolmerski (2001). "Temporally regulated overexpression of parathyroid hormone-related protein in the mammary gland reveals distinct fetal and pubertal phenotypes." J Endocrinol **171**(3): 403-416.

Eberhardt, N. L., S. K. Grebe, B. McIver and H. V. Reddi (2010). "The role of the PAX8/PPARgamma fusion oncogene in the pathogenesis of follicular thyroid cancer." Mol Cell Endocrinol **321**(1): 50-56.

Eccles, M. R. and L. A. Schimmenti (1999). "Renal-coloboma syndrome: a multi-system developmental disorder caused by PAX2 mutations." Clin Genet **56**(1): 1-9.

Epstein, D. J., M. Vekemans and P. Gros (1991). "Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3." Cell **67**(4): 767-774.

Fantozzi, A. and G. Christofori (2006). "Mouse models of breast cancer metastasis." Breast Cancer Res **8**(4): 212.

Fata, J. E., Z. Werb and M. J. Bissell (2004). "Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes." Breast Cancer Res **6**(1): 1-11.

Flint, D. J., M. Boutinaud, C. B. Whitelaw, G. J. Allan and A. F. Kolb (2006). "Prolactin inhibits cell loss and decreases matrix metalloproteinase expression in the involuting mouse mammary gland but fails to prevent cell loss in the mammary glands of mice expressing IGFBP-5 as a mammary transgene." J Mol Endocrinol **36**(3): 435-448.

Foley, J., P. Dann, J. Hong, J. Cosgrove, B. Dreyer, D. Rimm, M. Dunbar, W. Philbrick and J. Wysolmerski (2001). "Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development." Development **128**(4): 513-525.

Frascella, E., L. Toffolatti and A. Rosolen (1998). "Normal and rearranged PAX3 expression in human rhabdomyosarcoma." Cancer Genet Cytogenet **102**(2): 104-109.

Fu, H., M. Ishii, Y. Gu and R. Maxson (2007). "Conditional alleles of Msx1 and Msx2." Genesis **45**(8): 477-481.

Furth, P. A., R. E. Nakles, S. Millman, E. S. Diaz-Cruz and M. C. Cabrera (2011). "Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer." Breast Cancer Res **13**(5): 220.

Gerber, J. K., T. Richter, E. Kremmer, J. Adamski, H. Hofler, R. Balling and H. Peters (2002). "Progressive loss of PAX9 expression correlates with increasing malignancy of dysplastic and cancerous epithelium of the human oesophagus." J Pathol **197**(3): 293-297.

Giulianelli, S., J. P. Cerliani, C. A. Lamb, V. T. Fabris, M. C. Bottino, M. A. Gorostiaga, V. Novaro, A. Gongora, A. Baldi, A. Molinolo and C. Lanari (2008). "Carcinoma-associated fibroblasts activate progesterone receptors and induce hormone independent mammary tumor growth: A role for the FGF-2/FGFR-2 axis." Int J Cancer **123**(11): 2518-2531.

Gnarra, J. R. and G. R. Dressler (1995). "Expression of Pax-2 in human renal cell carcinoma and growth inhibition by antisense oligonucleotides." Cancer Res **55**(18): 4092-4098.

Goulding, M., A. Lumsden and A. J. Paquette (1994). "Regulation of Pax-3

expression in the dermomyotome and its role in muscle development." Development **120**(4): 957-971.

Gouon-Evans, V., E. Y. Lin and J. W. Pollard (2002). "Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development." Breast Cancer Res **4**(4): 155-164.

Gruss, P. and C. Walther (1992). "Pax in development." Cell **69**(5): 719-722.

Guy, C. T., R. D. Cardiff and W. J. Muller (1992). "Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease." Mol Cell Biol **12**(3): 954-961.

Hagemann, T., S. C. Robinson, M. Schulz, L. Trumper, F. R. Balkwill and C. Binder (2004). "Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases." Carcinogenesis **25**(8): 1543-1549.

Hanayama, R. and S. Nagata (2005). "Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8." Proc Natl Acad Sci U S A **102**(46): 16886-16891.

Hanayama, R., M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu and S. Nagata (2002). "Identification of a factor that links apoptotic cells to phagocytes." Nature **417**(6885): 182-187.

Hanayama, R., M. Tanaka, K. Miyasaka, K. Aozasa, M. Koike, Y. Uchiyama and S. Nagata (2004). "Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice." Science **304**(5674): 1147-1150.

Haricharan, S. and Y. Li (2013). "STAT signaling in mammary gland differentiation, cell survival and tumorigenesis." Mol Cell Endocrinol.

He, S. J., G. Stevens, A. W. Braithwaite and M. R. Eccles (2005). "Transfection of melanoma cells with antisense PAX3 oligonucleotides additively complements cisplatin-induced cytotoxicity." Mol Cancer Ther **4**(6): 996-1003.

Hennighausen, L. and G. W. Robinson (2005). "Information networks in the mammary gland." Nat Rev Mol Cell Biol **6**(9): 715-725.

Hens, J. R. and J. J. Wysolmerski (2005). "Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland." Breast Cancer Res **7**(5): 220-224.

Hernandez-Vargas, H., M. Ouzounova, F. Le Calvez-Kelm, M. P. Lambert, S. McKay-Chopin, S. V. Tavtigian, A. Puisieux, C. Matar and Z. Herceg (2011). "Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells." Epigenetics **6**(4): 428-439.

Hewitt, S. M., S. Hamada, A. Monarres, L. V. Kottical, G. F. Saunders and T. J. McDonnell (1997). "Transcriptional activation of the bcl-2 apoptosis suppressor gene by the paired box transcription factor PAX8." Anticancer Res **17**(5A): 3211-3215.

Hill, R. E., J. Favor, B. L. Hogan, C. C. Ton, G. F. Saunders, I. M. Hanson, J. Prosser, T. Jordan, N. D. Hastie and V. van Heyningen (1991). "Mouse small eye results from mutations in a paired-like homeobox-containing gene." Nature **354**(6354): 522-525.

Hinck, L. and G. B. Silberstein (2005). "Key stages in mammary gland development: the mammary end bud as a motile organ." Breast Cancer Res **7**(6): 245-251.

Hojilla, C. V., H. W. Jackson and R. Khokha (2011). "TIMP3 regulates mammary epithelial apoptosis with immune cell recruitment through differential TNF dependence." PLoS One **6**(10): e26718.

Holliday, D. L. and V. Speirs (2011). "Choosing the right cell line for breast cancer research." Breast Cancer Res **13**(4): 215.

Horowitz, M. C., Y. Xi, D. L. Pflugh, D. G. Hesslein, D. G. Schatz, J. A. Lorenzo and A. L. Bothwell (2004). "Pax5-deficient mice exhibit early onset osteopenia with increased osteoclast progenitors." J Immunol **173**(11): 6583-6591.

Iavnilovitch, E., B. Groner and I. Barash (2002). "Overexpression and forced activation of stat5 in mammary gland of transgenic mice promotes cellular proliferation, enhances differentiation, and delays postlactational apoptosis." Mol Cancer Res **1**(1): 32-47.

Itahana, Y., J. Singh, T. Sumida, J. P. Coppe, S. Parrinello, J. L. Bennington and P. Y. Desprez (2003). "Role of Id-2 in the maintenance of a differentiated and noninvasive phenotype in breast cancer cells." Cancer Res **63**(21): 7098-7105.

Jackson-Fisher, A. J., G. Bellinger, R. Ramabhadran, J. K. Morris, K. F. Lee and D. F. Stern (2004). "ErbB2 is required for ductal morphogenesis of the mammary gland." Proc Natl Acad Sci U S A **101**(49): 17138-17143.

Jonker, L., R. Kist, A. Aw, I. Wappler and H. Peters (2004). "Pax9 is required for filiform papilla development and suppresses skin-specific differentiation of the mammalian tongue epithelium." Mech Dev **121**(11): 1313-1322.

Jostes, B., C. Walther and P. Gruss (1990). "The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system." Mech Dev **33**(1): 27-37.

Kelly, P. A., A. Bachelot, C. Kedzia, L. Hennighausen, C. J. Ormandy, J. J. Kopchick and N. Binart (2002). "The role of prolactin and growth hormone in mammary gland development." Mol Cell Endocrinol **197**(1-2): 127-131.

Kim, N. D., T. D. Oberley, J. Yasukawa-Barnes and K. H. Clifton (2000). "Stem cell characteristics of transplanted rat mammary clonogens." Exp Cell Res **260**(1): 146-159.

Kist, R., E. Greally and H. Peters (2007). "Derivation of a mouse model for conditional inactivation of Pax9." Genesis **45**(7): 460-464.

Kist, R., M. Watson, X. Wang, P. Cairns, C. Miles, D. J. Reid and H. Peters (2005). "Reduction of Pax9 gene dosage in an allelic series of mouse mutants causes hypodontia and oligodontia." Hum Mol Genet **14**(23): 3605-3617.

Kleinberg, D. L. and W. Ruan (2008). "IGF-I, GH, and sex steroid effects in normal mammary gland development." J Mammary Gland Biol Neoplasia **13**(4): 353-360.

Klinakis, A., M. Szabolcs, G. Chen, S. Xuan, H. Hibshoosh and A. Efstratiadis (2009). "Igf1r as a therapeutic target in a mouse model of basal-like breast cancer." Proc Natl Acad Sci U S A **106**(7): 2359-2364.

Klinghoffer, R. A., C. Sachsenmaier, J. A. Cooper and P. Soriano (1999). "Src family kinases are required for integrin but not PDGFR signal transduction." EMBO J **18**(9): 2459-2471.

Kouros-Mehr, H., S. K. Bechis, E. M. Slorach, L. E. Littlepage, M. Egeblad, A. J. Ewald, S. Y. Pai, I. C. Ho and Z. Werb (2008). "GATA-3 links tumor differentiation and dissemination in a luminal breast cancer model." Cancer Cell **13**(2): 141-152.

Kouros-Mehr, H., J. W. Kim, S. K. Bechis and Z. Werb (2008). "GATA-3 and the regulation of the mammary luminal cell fate." Curr Opin Cell Biol **20**(2): 164-170.

Kouros-Mehr, H., E. M. Slorach, M. D. Sternlicht and Z. Werb (2006). "GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland." Cell **127**(5): 1041-1055.

Kritikou, E. A., A. Sharkey, K. Abell, P. J. Came, E. Anderson, R. W. Clarkson and C. J. Watson (2003). "A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland." Development **130**(15): 3459-3468.

Kroll, T. G., P. Sarraf, L. Pecciarini, C. J. Chen, E. Mueller, B. M. Spiegelman and J. A. Fletcher (2000). "PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]." Science **289**(5483): 1357-1360.

Lang, D., M. M. Lu, L. Huang, K. A. Engleka, M. Zhang, E. Y. Chu, S. Lipner, A. Skoultchi, S. E. Millar and J. A. Epstein (2005). "Pax3 functions at a nodal point in melanocyte stem cell differentiation." Nature **433**(7028): 884-887.

Lang, D., S. K. Powell, R. S. Plummer, K. P. Young and B. A. Ruggeri (2007). "PAX genes: roles in development, pathophysiology, and cancer." Biochem Pharmacol **73**(1): 1-14.

Lanigan, F., G. Gremel, R. Hughes, D. J. Brennan, F. Martin, K. Jirstrom and W. M. Gallagher (2010). "Homeobox transcription factor muscle segment homeobox 2 (Msx2) correlates with good prognosis in breast cancer patients and induces apoptosis in vitro." Breast Cancer Res **12**(4): R59.

Lewis, M. T. and J. M. Veltmaat (2004). "Next stop, the twilight zone: hedgehog network regulation of mammary gland development." J Mammary Gland Biol Neoplasia **9**(2): 165-181.

Li, C. G. and M. R. Eccles (2012). "PAX Genes in Cancer; Friends or Foes?" Front Genet **3**: 6.

Lin, E. Y., V. Gouon-Evans, A. V. Nguyen and J. W. Pollard (2002). "The macrophage growth factor CSF-1 in mammary gland development and tumor progression." J Mammary Gland Biol Neoplasia **7**(2): 147-162.

Lin, E. Y., J. G. Jones, P. Li, L. Zhu, K. D. Whitney, W. J. Muller and J. W. Pollard (2003). "Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases." Am J Pathol **163**(5): 2113-2126.

Lin, E. Y., A. V. Nguyen, R. G. Russell and J. W. Pollard (2001).

"Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy." J Exp Med **193**(6): 727-740.

Liu, X., M. Nugoli, J. Laferriere, S. M. Saleh, I. G. Rodrigue-Gervais, M. Saleh, M. Park, M. T. Hallett, W. J. Muller and V. Giguere (2011). "Stromal retinoic acid receptor beta promotes mammary gland tumorigenesis." Proc Natl Acad Sci U S A **108**(2): 774-779.

Liu, X., G. W. Robinson, K. U. Wagner, L. Garrett, A. Wynshaw-Boris and L. Hennighausen (1997). "Stat5a is mandatory for adult mammary gland development and lactogenesis." Genes Dev **11**(2): 179-186.

Lu, P., A. J. Ewald, G. R. Martin and Z. Werb (2008). "Genetic mosaic analysis reveals FGF receptor 2 function in terminal end buds during mammary gland branching morphogenesis." Dev Biol **321**(1): 77-87.

Lund, L. R., S. F. Bjorn, M. D. Sternlicht, B. S. Nielsen, H. Solberg, P. A. Usher, R. Osterby, I. J. Christensen, R. W. Stephens, T. H. Bugge, K. Dano and Z. Werb (2000). "Lactational competence and involution of the mouse mammary gland require plasminogen." Development **127**(20): 4481-4492.

Lund, L. R., J. Romer, N. Thomasset, H. Solberg, C. Pyke, M. J. Bissell, K. Dano and Z. Werb (1996). "Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways." Development **122**(1): 181-193.

Macchia, P. E., P. Lapi, H. Krude, M. T. Pirro, C. Missero, L. Chiovato, A. Souabni, M. Baserga, V. Tassi, A. Pinchera, G. Fenzi, A. Gruters, M. Busslinger and R. Di Lauro (1998). "PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis." Nat Genet **19**(1): 83-86.

Mailleux, A. A., M. Overholtzer, T. Schmelzle, P. Bouillet, A. Strasser and J. S. Brugge (2007). "BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms." Dev Cell **12**(2): 221-234.

Mailleux, A. A., B. Spencer-Dene, C. Dillon, D. Ndiaye, C. Savona-Baron, N. Itoh, S. Kato, C. Dickson, J. P. Thiery and S. Bellusci (2002). "Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo." Development **129**(1): 53-60.

Mansouri, A., K. Chowdhury and P. Gruss (1998). "Follicular cells of the thyroid gland require Pax8 gene function." Nat Genet **19**(1): 87-90.

Mansouri, A., A. Stoykova, M. Torres and P. Gruss (1996). "Dysgenesis of cephalic neural crest derivatives in Pax7-/- mutant mice." Development **122**(3): 831-838.

Margue, C. M., M. Bernasconi, F. G. Barr and B. W. Schafer (2000). "Transcriptional modulation of the anti-apoptotic protein BCL-XL by the paired box transcription factors PAX3 and PAX3/FKHR." Oncogene **19**(25): 2921-2929.

Marquardt, T., R. Ashery-Padan, N. Andrejewski, R. Scardigli, F. Guillemot and P. Gruss (2001). "Pax6 is required for the multipotent state of retinal progenitor cells." Cell **105**(1): 43-55.

Mendes, O., H. T. Kim and G. Stoica (2005). "Expression of MMP2, MMP9 and MMP3 in breast cancer brain metastasis in a rat model." Clin Exp Metastasis **22**(3): 237-246.

Miyasaka, K., R. Hanayama, M. Tanaka and S. Nagata (2004). "Expression of milk fat globule epidermal growth factor 8 in immature dendritic cells for engulfment of apoptotic cells." Eur J Immunol **34**(5): 1414-1422.

Monks, J., D. Rosner, F. J. Geske, L. Lehman, L. Hanson, M. C. Neville and V. A. Fadok (2005). "Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release." Cell Death Differ **12**(2): 107-114.

Monks, J., C. Smith-Steinhart, E. R. Kruk, V. A. Fadok and P. M. Henson (2008). "Epithelial cells remove apoptotic epithelial cells during post-lactation involution of the mouse mammary gland." Biol Reprod **78**(4): 586-594.

Morrison, A. M., S. L. Nutt, C. Thevenin, A. Rolink and M. Busslinger (1998). "Loss- and gain-of-function mutations reveal an important role of BSAP (Pax-5) at the start and end of B cell differentiation." Semin Immunol **10**(2): 133-142.

Muller, W. J., J. Ho and P. M. Siegel (1998). "Oncogenic activation of Neu/ErbB-2 in a transgenic mouse model for breast cancer." Biochem Soc Symp **63**: 149-157.

Mullighan, C. G., S. Goorha, I. Radtke, C. B. Miller, E. Coustan-Smith, J. D. Dalton, K. Girtman, S. Mathew, J. Ma, S. B. Pounds, X. Su, C. H. Pui, M. V. Relling, W. E. Evans, S. A. Shurtleff and J. R. Downing (2007). "Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia." Nature **446**(7137): 758-764.

Muratovska, A., C. Zhou, S. He, P. Goodyer and M. R. Eccles (2003). "Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival." Oncogene **22**(39): 7989-7997.

Nakatomi, M., X. P. Wang, D. Key, J. J. Lund, A. Turbe-Doan, R. Kist, A. Aw, Y. Chen, R. L. Maas and H. Peters (2010). "Genetic interactions between Pax9 and Msx1 regulate lip development and several stages of tooth morphogenesis." Dev Biol **340**(2): 438-449.

Nandrot, E. F., M. Anand, D. Almeida, K. Atabai, D. Sheppard and S. C. Finnemann (2007). "Essential role for MFG-E8 as ligand for alphavbeta5 integrin in diurnal retinal phagocytosis." Proc Natl Acad Sci U S A **104**(29): 12005-12010.

Narlis, M., D. Grote, Y. Gaitan, S. K. Boualia and M. Bouchard (2007). "Pax2 and pax8 regulate branching morphogenesis and nephron differentiation in the developing kidney." J Am Soc Nephrol **18**(4): 1121-1129.

Neubuser, A., H. Koseki and R. Balling (1995). "Characterization and developmental expression of Pax9, a paired-box-containing gene related to Pax1." Dev Biol **170**(2): 701-716.

Neville, M. C., T. B. McFadden and I. Forsyth (2002). "Hormonal regulation of mammary differentiation and milk secretion." J Mammary Gland Biol Neoplasia **7**(1): 49-66.

Nguyen, A., A. Rosner, T. Milovanovic, C. Hope, K. Planutis, B. Saha, B. Chaiwun, F. Lin, S. A. Imam, J. L. Marsh and R. F. Holcombe (2005). "Wnt pathway component LEF1 mediates tumor cell invasion and is expressed in human and murine breast cancers lacking ErbB2 (her-2/neu) overexpression." Int J Oncol **27**(4): 949-956.

Nutt, S. L., B. Heavey, A. G. Rolink and M. Busslinger (1999). "Commitment to the B-lymphoid lineage depends on the transcription factor Pax5." Nature **401**(6753): 556-562.

Nutt, S. L., A. M. Morrison, P. Dorfler, A. Rolink and M. Busslinger (1998). "Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments." EMBO J **17**(8): 2319-2333.

O'Brien, J., T. Lyons, J. Monks, M. S. Lucia, R. S. Wilson, L. Hines, Y. G. Man, V. Borges and P. Schedin (2010). "Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species." Am J Pathol **176**(3): 1241-1255.

O'Brien, J., H. Martinson, C. Durand-Rougely and P. Schedin (2012). "Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution." Development **139**(2): 269-275.

Ogawa, T., H. Kapadia, J. Q. Feng, R. Raghow, H. Peters and R. N. D'Souza (2006). "Functional consequences of interactions between Pax9 and Msx1 genes in normal and abnormal tooth development." J Biol Chem **281**(27): 18363-18369.

Ostrom, L., M. J. Tang, P. Gruss and G. R. Dressler (2000). "Reduced Pax2 gene dosage increases apoptosis and slows the progression of renal cystic disease." Dev Biol **219**(2): 250-258.

Ozcan, A., S. S. Shen, C. Hamilton, K. Anjana, D. Coffey, B. Krishnan and L. D. Truong (2011). "PAX 8 expression in non-neoplastic tissues, primary tumors, and metastatic tumors: a comprehensive immunohistochemical study." Mod Pathol **24**(6): 751-764.

Pani, L., M. Horal and M. R. Loeken (2002). "Rescue of neural tube defects in Pax-3-deficient embryos by p53 loss of function: implications for Pax-3-dependent development and tumorigenesis." Genes Dev **16**(6): 676-680.

Peters, H. and R. Balling (1999). "Teeth. Where and how to make them." Trends Genet **15**(2): 59-65.

Peters, H., A. Neubuser, K. Kratochwil and R. Balling (1998). "Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities." Genes Dev **12**(17): 2735-2747.

Peters, H., G. Schuster, A. Neubuser, T. Richter, H. Hofler and R. Balling (1997). "Isolation of the Pax9 cDNA from adult human esophagus." Mamm Genome **8**(1): 62-64.

Peters, H., B. Wilm, N. Sakai, K. Imai, R. Maas and R. Balling (1999). "Pax1 and Pax9 synergistically regulate vertebral column development." Development **126**(23): 5399-5408.

Philips, G. T., C. N. Stair, H. Young Lee, E. Wroblewski, M. A. Berberoglu, N. L. Brown and G. S. Mastick (2005). "Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type." Dev Biol **279**(2): 308-321.

Pispa, J. and I. Thesleff (2003). "Mechanisms of ectodermal organogenesis." Dev Biol **262**(2): 195-205.

Plachov, D., K. Chowdhury, C. Walther, D. Simon, J. L. Guenet and P. Gruss (1990). "Pax8, a murine paired box gene expressed in the developing excretory system and thyroid gland." Development **110**(2): 643-651.

Poleev, A., H. Fickenscher, S. Mundlos, A. Winterpacht, B. Zabel, A. Fidler, P. Gruss and D. Plachov (1992). "PAX8, a human paired box gene: isolation and expression in developing thyroid, kidney and Wilms' tumors." Development **116**(3): 611-623.

Poppe, B., P. De Paepe, L. Michaux, N. Dastugue, C. Bastard, C. Herens, E. Moreau, F. Cavazzini, N. Yigit, H. Van Limbergen, A. De Paepe, M. Praet, C. De Wolf-Peeters, I. Wlodarska and F. Speleman (2005). "PAX5/IGH rearrangement is a recurrent finding in a subset of aggressive B-NHL with complex chromosomal rearrangements." Genes Chromosomes Cancer **44**(2): 218-223.

Porteous, S., E. Torban, N. P. Cho, H. Cunliffe, L. Chua, L. McNoe, T. Ward, C. Souza, P. Gus, R. Giugliani, T. Sato, K. Yun, J. Favor, M. Sicotte, P. Goodyer and M. Eccles (2000). "Primary renal hypoplasia in humans and mice with PAX2 mutations: evidence of increased apoptosis in fetal kidneys of Pax2(1Neu) +/- mutant mice." Hum Mol Genet **9**(1): 1-11.

Raven, J. F., V. Williams, S. Wang, M. L. Tremblay, W. J. Muller, J. E. Durbin and A. E. Koromilas (2011). "Stat1 is a suppressor of ErbB2/Neu-mediated cellular transformation and mouse mammary gland tumor formation." Cell Cycle **10**(5): 794-804.

Reginato, M. J. and S. K. Muthuswamy (2006). "Illuminating the center: mechanisms regulating lumen formation and maintenance in mammary morphogenesis." J Mammary Gland Biol Neoplasia **11**(3-4): 205-211.

Relaix, F., D. Montarras, S. Zaffran, B. Gayraud-Morel, D. Rocancourt, S. Tajbakhsh, A. Mansouri, A. Cumano and M. Buckingham (2006). "Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells." J Cell Biol **172**(1): 91-102.

Relaix, F., D. Rocancourt, A. Mansouri and M. Buckingham (2004). "Divergent functions of murine Pax3 and Pax7 in limb muscle development." Genes Dev **18**(9): 1088-1105.

Relaix, F., D. Rocancourt, A. Mansouri and M. Buckingham (2005). "A Pax3/Pax7-dependent population of skeletal muscle progenitor cells." Nature **435**(7044): 948-953.

Richert, M. M., K. L. Schwertfeger, J. W. Ryder and S. M. Anderson (2000). "An atlas of mouse mammary gland development." J Mammary Gland Biol Neoplasia **5**(2): 227-241.

Robson, E. J., S. J. He and M. R. Eccles (2006). "A PANorama of PAX genes in cancer and development." Nat Rev Cancer **6**(1): 52-62.

Salomon, D. S. and M. T. Lewis (2004). "Embryogenesis and oncogenesis: Dr Jekyll and Mr Hyde." J Mammary Gland Biol Neoplasia **9**(2): 105-107.

Sander, M., A. Neubuser, J. Kalamaras, H. C. Ee, G. R. Martin and M. S. German (1997). "Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development." Genes Dev **11**(13): 1662-1673.

Satokata, I., L. Ma, H. Ohshima, M. Bei, I. Woo, K. Nishizawa, T. Maeda, Y. Takano, M. Uchiyama, S. Heaney, H. Peters, Z. Tang, R. Maxson and R. Maas (2000). "Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation." Nat Genet **24**(4): 391-395.

Schedin, P., J. O'Brien, M. Rudolph, T. Stein and V. Borges (2007). "Microenvironment of the involuting mammary gland mediates mammary cancer progression." J Mammary Gland Biol Neoplasia **12**(1): 71-82.

Schorr, K., M. Li, S. Krajewski, J. C. Reed and P. A. Furth (1999). "Bcl-2 gene family and related proteins in mammary gland involution and breast cancer." J Mammary Gland Biol Neoplasia **4**(2): 153-164.

Schwarz, M., G. Alvarez-Bolado, G. Dressler, P. Urbanek, M. Busslinger and P. Gruss (1999). "Pax2/5 and Pax6 subdivide the early neural tube into three domains." Mech Dev **82**(1-2): 29-39.

Schwertfeger, K. L., M. M. Richert and S. M. Anderson (2001). "Mammary gland involution is delayed by activated Akt in transgenic mice." Mol Endocrinol **15**(6): 867-881.

Schwertfeger, K. L., J. M. Rosen and D. A. Cohen (2006). "Mammary gland macrophages: pleiotropic functions in mammary development." J Mammary Gland Biol Neoplasia **11**(3-4): 229-238.

Seale, P., L. A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss and M. A. Rudnicki (2000). "Pax7 is required for the specification of myogenic satellite cells." Cell **102**(6): 777-786.

Shackleton, M., F. Vaillant, K. J. Simpson, J. Stingl, G. K. Smyth, M. L. Asselin-Labat, L. Wu, G. J. Lindeman and J. E. Visvader (2006). "Generation of a functional mammary gland from a single stem cell." Nature **439**(7072): 84-88.

Silberstein, G. B., G. R. Dressler and K. Van Horn (2002). "Expression of the PAX2 oncogene in human breast cancer and its role in progesterone-dependent mammary growth." Oncogene **21**(7): 1009-1016.

Skopichev, V. G., G. B. Balakina and O. I. Turbaeva (1983). "[Morphofunctional organization of intercellular interaction in mammary gland alveoli]." Nerv Sist **24**: 59-66.

Soriano, P. (1999). "Generalized lacZ expression with the ROSA26 Cre reporter strain." Nat Genet **21**(1): 70-71.

Sosa-Pineda, B., K. Chowdhury, M. Torres, G. Oliver and P. Gruss (1997). "The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas." Nature **386**(6623): 399-402.

Srinivasan, K., P. Strickland, A. Valdes, G. C. Shin and L. Hinck (2003). "Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis." Dev Cell **4**(3): 371-382.

St-Onge, L., B. Sosa-Pineda, K. Chowdhury, A. Mansouri and P. Gruss (1997). "Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas." Nature **387**(6631): 406-409.

Stein, T., J. S. Morris, C. R. Davies, S. J. Weber-Hall, M. A. Duffy, V. J. Heath, A. K. Bell, R. K. Ferrier, G. P. Sandilands and B. A. Gusterson (2004). "Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3." Breast Cancer Res **6**(2): R75-91.

Sternlicht, M. D., H. Kouros-Mehr, P. Lu and Z. Werb (2006). "Hormonal and local control of mammary branching morphogenesis." Differentiation **74**(7): 365-381.

Sternlicht, M. D., A. Lochter, C. J. Sympson, B. Huey, J. P. Rougier, J. W. Gray, D. Pinkel, M. J. Bissell and Z. Werb (1999). "The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis." Cell **98**(2): 137-146.

Stingl, J., P. Eirew, I. Ricketson, M. Shackleton, F. Vaillant, D. Choi, H. I. Li and C. J. Eaves (2006). "Purification and unique properties of mammary epithelial stem

cells." Nature **439**(7079): 993-997.

Stingl, J., A. Raouf, P. Eirew and C. J. Eaves (2006). "Deciphering the mammary epithelial cell hierarchy." Cell Cycle **5**(14): 1519-1522.

Stockton, D. W., P. Das, M. Goldenberg, R. N. D'Souza and P. I. Patel (2000). "Mutation of PAX9 is associated with oligodontia." Nat Genet **24**(1): 18-19.

Stoykova, A., R. Fritsch, C. Walther and P. Gruss (1996). "Forebrain patterning defects in Small eye mutant mice." Development **122**(11): 3453-3465.

Stuart, E. T., R. Haffner, M. Oren and P. Gruss (1995). "Loss of p53 function through PAX-mediated transcriptional repression." EMBO J **14**(22): 5638-5645.

Tanos, T., L. J. Rojo, P. Echeverria and C. Brisken (2012). "ER and PR signaling nodes during mammary gland development." Breast Cancer Res **14**(4): 210.

Tassabehji, M., V. E. Newton, K. Leverton, K. Turnbull, E. Seemanova, J. Kunze, K. Sperling, T. Strachan and A. P. Read (1994). "PAX3 gene structure and mutations: close analogies between Waardenburg syndrome and the Splotch mouse." Hum Mol Genet **3**(7): 1069-1074.

Thangaraju, M., M. Rudelius, B. Bierie, M. Raffeld, S. Sharan, L. Hennighausen, A. M. Huang and E. Sterneck (2005). "C/EBPdelta is a crucial regulator of pro-apoptotic gene expression during mammary gland involution." Development **132**(21): 4675-4685.

Tremblay, P., M. Kessel and P. Gruss (1995). "A transgenic neuroanatomical marker identifies cranial neural crest deficiencies associated with the Pax3 mutant Splotch." Dev Biol **171**(2): 317-329.

Tremblay, P., F. Pituello and P. Gruss (1996). "Inhibition of floor plate differentiation by Pax3: evidence from ectopic expression in transgenic mice." Development **122**(8): 2555-2567.

Urbanek, P., Z. Q. Wang, I. Fetka, E. F. Wagner and M. Busslinger (1994). "Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP." Cell **79**(5): 901-912.

Vafaizadeh, V., P. Klemmt, C. Brendel, K. Weber, C. Doebele, K. Britt, M. Grez, B. Fehse, S. Desrivieres and B. Groner (2010). "Mammary epithelial reconstitution with gene-modified stem cells assigns roles to Stat5 in luminal alveolar cell fate decisions, differentiation, involution, and mammary tumor formation." Stem Cells

28(5): 928-938.

Vafaizadeh, V., P. A. Klemmt and B. Groner (2012). "Stat5 assumes distinct functions in mammary gland development and mammary tumor formation." Front Biosci **17**: 1232-1250.

van Genderen, C., R. M. Okamura, I. Farinas, R. G. Quo, T. G. Parslow, L. Bruhn and R. Grosschedl (1994). "Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice." Genes Dev **8**(22): 2691-2703.

van Heyningen, V. and K. A. Williamson (2002). "PAX6 in sensory development." Hum Mol Genet **11**(10): 1161-1167.

Van Keymeulen, A., A. S. Rocha, M. Ousset, B. Beck, G. Bouvencourt, J. Rock, N. Sharma, S. Dekoninck and C. Blanpain (2011). "Distinct stem cells contribute to mammary gland development and maintenance." Nature **479**(7372): 189-193.

Van Nguyen, A. and J. W. Pollard (2002). "Colony stimulating factor-1 is required to recruit macrophages into the mammary gland to facilitate mammary ductal outgrowth." Dev Biol **247**(1): 11-25.

van Raamsdonk, C. D. and S. M. Tilghman (2000). "Dosage requirement and allelic expression of PAX6 during lens placode formation." Development **127**(24): 5439-5448.

Visvader, J. E. and G. J. Lindeman (2011). "The unmasking of novel unipotent stem cells in the mammary gland." EMBO J **30**(24): 4858-4859.

Visvader, J. E. and G. H. Smith (2011). "Murine mammary epithelial stem cells: discovery, function, and current status." Cold Spring Harb Perspect Biol **3**(2).

Wada, H., P. W. Holland, S. Sato, H. Yamamoto and N. Satoh (1997). "Neural tube is partially dorsalized by overexpression of HrPax-37: the ascidian homologue of Pax-3 and Pax-7." Dev Biol **187**(2): 240-252.

Wagner, K. U. and J. W. Schmidt (2011). "The two faces of Janus kinases and their respective STATs in mammary gland development and cancer." J Carcinog **10**: 32.

Walther, C. and P. Gruss (1991). "Pax-6, a murine paired box gene, is expressed in the developing CNS." Development **113**(4): 1435-1449.

Wang, Q., W. H. Fang, J. Krupinski, S. Kumar, M. Slevin and P. Kumar (2008). "Pax genes in embryogenesis and oncogenesis." J Cell Mol Med **12**(6A): 2281-2294.

Watson, C. J. (2006). "Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ." Breast Cancer Res **8**(2): 203.

White, D. E., N. A. Kurpios, D. Zuo, J. A. Hassell, S. Blaess, U. Mueller and W. J. Muller (2004). "Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction." Cancer Cell **6**(2): 159-170.

Wickenden, J. A. and C. J. Watson (2010). "Key signalling nodes in mammary gland development and cancer. Signalling downstream of PI3 kinase in mammary epithelium: a play in 3 Akts." Breast Cancer Res **12**(2): 202.

Williams, B. A. and C. P. Ordahl (2000). "Fate restriction in limb muscle precursor cells precedes high-level expression of MyoD family member genes." Development **127**(12): 2523-2536.

Winyard, P. J., R. A. Risdon, V. R. Sams, G. R. Dressler and A. S. Woolf (1996). "The PAX2 tanscription factor is expressed in cystic and hyperproliferative dysplastic epithelia in human kidney malformations." J Clin Invest **98**(2): 451-459.

Yamaoka, T., M. Yano, T. Yamada, T. Matsushita, M. Moritani, S. Ii, K. Yoshimoto, J. Hata and M. Itakura (2000). "Diabetes and pancreatic tumours in transgenic mice expressing Pa x 6." Diabetologia **43**(3): 332-339.

Yang, Y., E. Spitzer, N. Kenney, W. Zschiesche, M. Li, A. Kromminga, T. Muller, F. Spener, A. Lezius, J. H. Veerkamp and et al. (1994). "Members of the fatty acid binding protein family are differentiation factors for the mammary gland." J Cell Biol **127**(4): 1097-1109.

Zhou, Y. H., F. Tan, K. R. Hess and W. K. Yung (2003). "The expression of PAX6, PTEN, vascular endothelial growth factor, and epidermal growth factor receptor in gliomas: relationship to tumor grade and survival." Clin Cancer Res **9**(9): 3369-3375.

Zhou, Y. H., X. Wu, F. Tan, Y. X. Shi, T. Glass, T. J. Liu, K. Wathen, K. R. Hess, J. Gumin, F. Lang and W. K. Yung (2005). "PAX6 suppresses growth of human glioblastoma cells." J Neurooncol **71**(3): 223-229.