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## The Role of Connexins and Pannexins in Mammary Gland Development and Tumorigenesis

Michael K. G. Stewart

*The University of Western Ontario*

Supervisor

Dr. Dale W. Laird

*The University of Western Ontario*

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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THE ROLE OF CONNEXINS AND PANNEXINS IN MAMMARY GLAND  
DEVELOPMENT AND TUMORIGENESIS

(Thesis format: Integrated Article)

By

Michael Kenneth George Stewart

Graduate Program in Physiology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO  
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

## Abstract

The identification of key regulators of breast cancer onset and progression is critical for the development of targeted therapies. Connexins and pannexins are characterized by their ability to form large-pore channels and are frequently dysregulated in cancer. However, their role in breast cancer progression remains poorly understood due to a lack of *in vivo* models capable of assessing the proposed and opposing roles of connexins and pannexins as both tumor suppressors and/or facilitators in multiple stages of the disease. Using two previously uncharacterized genetically-modified mice, connexin43 (Cx43) and connexin26 (Cx26) were evaluated for their role in normal mammary gland development and function prior to using the mice to assess their linkage to breast cancer onset and progression. In addition, pannexin1 (Panx1) was evaluated for the first time in the context of mammary gland development and correlated to clinical outcomes in patients with breast cancer using *in silico* arrays. Using a mouse model expressing a loss-of-function Cx43 mutant it was revealed that the severity of milk ejection defects associated with Cx43 are linked to its functional status. Using a similar mouse model induced to develop primary breast cancer lesions, we identified that low functional levels of Cx43 resulted in mainly hyperplastic mammary glands that greatly increased the frequency of developing metastases to the lungs. Our assessment of mice with conditional knockout of Cx26 during pregnancy revealed that basal levels of Cx26 were sufficient for normal alveolar development and proper lactation, but increased the susceptibility of mammary tumor onset in a chemically induced mouse model of breast cancer. Finally, genetically modified mice with systemic knockout of Panx1 identified a role for Panx1 in timely alveolar development during early lactation. In addition, PANX1 mRNA expression was correlated with worse clinical outcomes in breast cancer. Collectively, our results redefine our view of Cx43, Cx26 and Panx1 in mammary gland development; supporting a tumor suppressive role for Cx43 and Cx26, and a tumor facilitating role for Panx1 in breast cancer progression which may have implications for extending to their use as therapeutic targets.

## **Keywords**

Gap junction, Connexin, Pannexin, Connexin43, Connexin26, Pannexin1, Mammary Gland, Breast, Mouse Models, Mammary Gland Development, Puberty, Lactation, Involution, Breast Cancer, 7,12-Dimethylbenz[a]anthracene

## Abbreviations

2D - Two-Dimensional

3D - Three-Dimensional

ATP- Adenosine Triphosphate

BLG-  $\beta$ -Lactoglobulin

BSA - Bovine Serum Albumin

cAMP - Cyclic Adenosine Monophosphate

cGMP - Cyclic Guanosine Monophosphate

CS – Cell Surface

Cx26 - Connexin26

Cx30 - Connexin30

Cx32 - Connexin32

Cx43 - Connexin43

ddH<sub>2</sub>O – Double Distilled Water

DMBA - 7, 12-Dimethylbenz[a]anthracene

DMEM – Dulbecco's Modified Eagle Medium

DMFS – Distant Metastasis Free Survival

DNA – Deoxyribonucleic Acid

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-linked Immunosorbent Assay

EMT- Epithelial to Mesenchymal Transition

ENU - *N*-ethyl-*N*-nitrosourea

ER – Estrogen Receptor

*ERαKO* – Mouse Estrogen Receptor Alpha Knockout

ErbB2 – erb-b2 Receptor Tyrosine Kinase 2

ERK1/2 – Extracellular Signal-regulated Kinase 1/2

ER $\beta$  – Estrogen Receptor Beta

EtOH - Ethanol

FGFR3 – Fibroblast Growth Factor Receptor 3

Fl - Floxed

Fra-2 – Fos-related Antigen 2

GFP – Green Fluorescent Protein

*GHRKO* – Mouse Growth Hormone Receptor Knockout

Gj – Junctional Conductance

*Gja1* – Mouse Gene encoding Connexin43

*GJA1* – Human Gene encoding Connexin43

*Gja5* – Mouse Gene encoding Connexin40

*Gjb1* – Mouse Gene encoding Connexin32

*Gjb2* – Mouse Gene encoding Connexin26

*GJB2* – Human Gene encoding Connexin26

*Gjb6* – Mouse Gene encoding Connexin30

GJIC – Gap Junctional Intercellular Communication

Gly – Glycosylation State

H&E – Hematoxylin and Eosin

H<sup>3</sup> - Tritium

hCG – human Chorionic Gonadotropin

HEK – Human Embryonic Kidney Cells

HER2 – Human Epidermal Growth Factor Receptor 2

Hsc70 – Heat Shock Protein 70

I - Intracellular

I3 – Involution Day 3

IP<sub>3</sub> – Inositol Triphosphate

JNK – c-Jun N-terminal Kinase

kDa - KiloDalton

KID – Keratitis-ichthyosis-deafness Syndrome

L0 – Lactation Day 0

L2 – Lactation Day 2

Lif – Leukemia Inhibitory Factor

MAPK – Mitogen-activated Protein Kinase

MEGM – Mammary Epithelial Growth Media

MMP – Matrix Metalloproteinase

MMTV – Mouse Mammary Tumor Virus

mRNA – Messenger Ribonucleic Acid

NCBI – National Center for Biotechnology Information

NF $\kappa$ B – Nuclear Factor Kappa B

NP-40 – Nonidet P-40

ODDD – Oculodentodigital Dysplasia

OS – Overall Survival

Panx1 – Pannexin1

*Panx1* – Mouse Gene encoding Pannexin1

*PANXI* – Human Gene encoding Pannexin1

Panx2 – Pannexin2

Panx3 – Pannexin3

PBS – Phosphate Buffered Saline

PBST – Phosphate Buffered Saline with Tween

PCNA – Proliferation Cell Nuclear Antigen

PCR – Polymerase Chain Reaction

PQ – Substituted Quinoline

PR – Progesterone Receptor

PrlR – Prolactin Receptor

*PrlRKO* – Mouse Prolactin Receptor Knockout

PyVmt – Polyoma virus Middle T Antigen

RFS – Relapse-free Survival

SDS – Sodium Dodecyl Sulfate

SEM – Standard Error of the Mean

shRNA – Short Hairpin Ribonucleic Acid

siRNA – Short Interfering Ribonucleic Acid

Sox2 – Sex Determining Region Y Box 2

Stat3 – Signal Transducer and Activator of Transcription 3

Stat5 – Signal Transducer and Activator of Transcription 5

TEB – Terminal End Bud

TIMP – Tissue Inhibitor of Metalloproteinase

TSP-1 – Thrombospondin-1

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF – Vascular Endothelial Growth Factor

WAP – Whey Acidic Protein

WGA – Wheat Germ Agglutinin

Wnt5a - Wingless-related MMTV Integration site 5A

WT – Wild-type

# Co-Authorship statement

## **Chapter 1**

Jamie Simek assisted in the creation of 3D graphic images (Figure 1.2, 1.3)

## **Chapter 2**

Kevin Barr assisted in mouse breeding and isolated the primary granulosa cells.

Dr. Xiang-Qun Gong performed the patch-clamp recording experiments under the supervision of Dr. Donglin Bai (Figure 2.1).

Dr. Glenn I. Fishman and Dr. Janet Rossant engineered the Cx43<sup>I130T/+</sup> mice and Cx43<sup>G60S/+</sup> mice, respectively.

## **Chapter 3**

John Bechberger under the supervision of Dr. Christian Naus created the BLG-Cre; Cx26<sup>f/f</sup> mice and performed the dissections of tissues used in this study.

Dr. Isabelle Plante performed all real-time PCR assessment of connexins in mammary gland development (Figure 3.1A,B, 3.S1A). Dr. Isabelle Plante also contributed to Western blot and immunofluorescent analysis of connexins and WAP, respectively, in the mammary gland (Figure 3.1C, 3.2A, 3.7C).

## **Chapter 4**

John Bechberger under the supervision of Dr. Christian Naus performed pituitary transplants (Figure 4.S1A,B) and treated mice with DMBA or oil. In addition, he monitored mice for palpable tumors and dissected tissues used in the study.

Dr. Ian Welch performed histological subtyping of primary mammary tumors (Figure 4.4)

## **Chapter 5**

Dr. Isabelle Plante and Dr. Silvia Penuela performed real-time PCR assessment of Panx1 in the mammary gland (Figure 5.1C).

Kevin Barr assisted in mouse breeding and genotyping.

## **Appendix1**

Dr. Isabelle Plante was the lead author on this study in which she designed the experiments, set up the breeding and treatments with Kevin Barr, performed many of the experiments (Appendix 1.1, 1.3, 1.4, are entirely her work) and she was the primary writer of the manuscript. Dr. Alison Allan provided critical insight into the experimental design of some experiments.

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## Dedication

This thesis is dedicated in memory of my Father,

Kenneth John Bruce Stewart.

(1951-2014)

“When you die, it does not mean that you lost to cancer. You beat cancer by how you lived, why you lived, and in the manner in which you lived”

Stuart Scott

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# Chapter 1

## 1 Introduction

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## 1.1 Gap junctions and connexins

Gap junctions are formed when six connexins co-oligomerize to form a connexon, or hemichannel, which docks with a connexon from an adjacent cell to form a gap junction channel [1]. Gap junction channels directly link the cytoplasm of adjacent cells allowing for the exchange of molecules less than 1000 Daltons in size that includes ions and second messengers ( $\text{Ca}^{2+}$ , IP<sub>3</sub>, cAMP) in a process known as gap junctional intercellular communication (GJIC) [1]. During development, a genetic program is initiated as the zygote prepares itself to develop into a multicellular organism for later preimplantation development. In the 1980's, it was determined that the presence of gap junctions at the 8 cell stage was critical to maintain the compacted state, highlighting an essential requirement for gap junctions in the formation of the blastocyst and an essential role for gap junctions in developmental processes [2-4]. At the 8 cell stage, it was later identified that these gap junctions were composed of Cx43, and loss-of-function studies using anti-Cx43 antibodies to disrupt GJIC, led to decompaction and suggested that Cx43 was absolutely essential for maintaining the compaction of the blastomeres and the further development of the embryo [3,5,6]. Surprisingly, not until the development of a genetically modified mouse with systemic ablation of *Gja1* was it discovered that loss of Cx43 still resulted in the development of the embryo to term [7]. This study highlighted how the use of genetically-modified mice altered our view of connexins in development previously based solely on *in vitro* studies.

We now know that 21 connexins exist in humans, while 20 connexins are found in mouse, and multiple connexins are generally expressed within the same cell [1]. In fact, nine connexin transcripts are detected at the 2-8 cell stage of murine preimplantation development suggesting that other connexins likely compensated for the loss of Cx43 during preimplantation in the Cx43 null mouse model [8,9]. However, channels formed by one connexin cannot always compensate for the loss of channel function from another as various connexins possess distinct gating properties and conductance when forming homomeric connexons comprised exclusively of one connexin isoform [1,10]. In addition, some connexins, such as Cx26, Cx30 and Cx32, are able to intermix and form heteromeric connexons, composed of multiple connexin isoforms, which display altered permeability

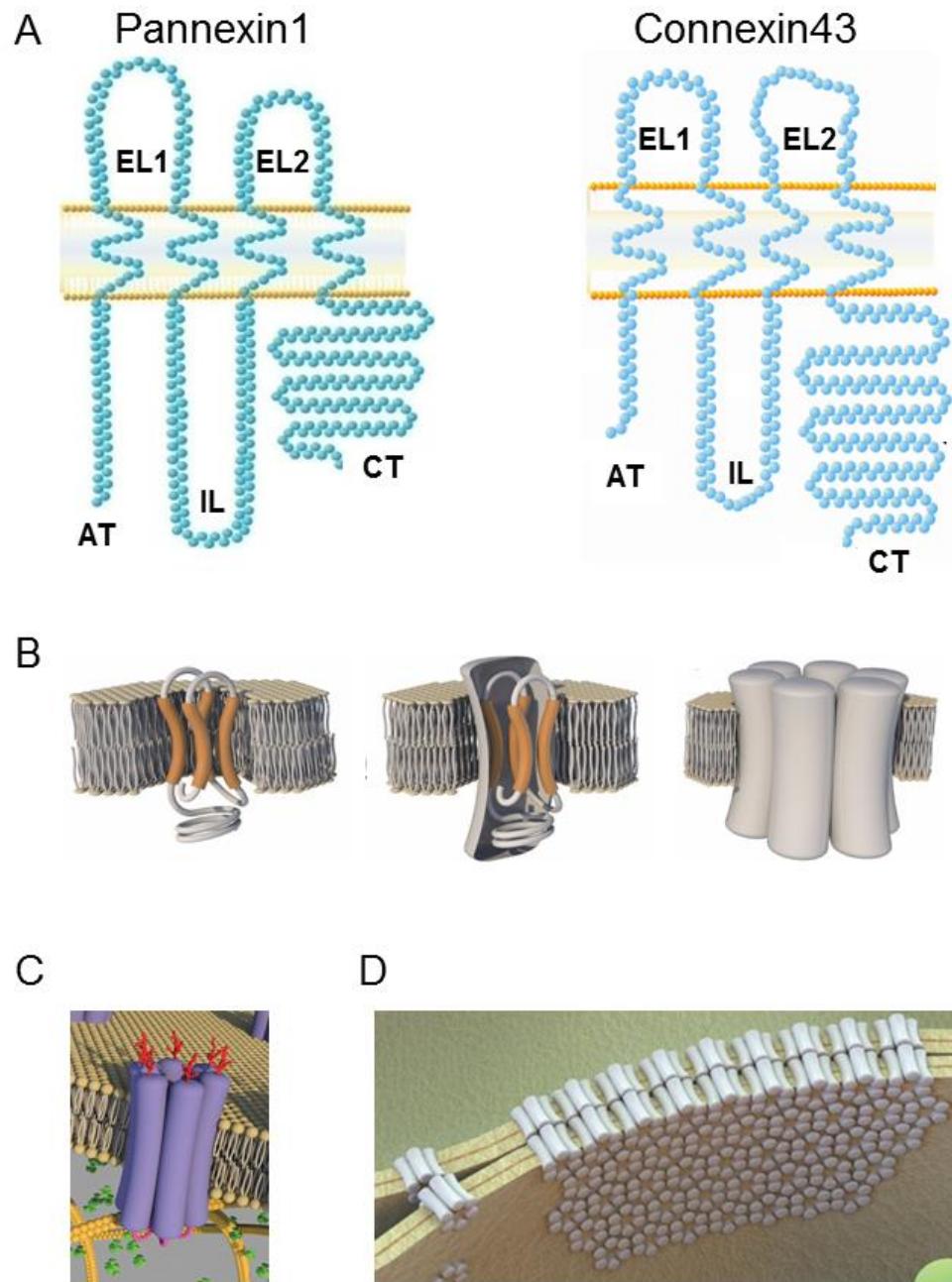
characteristics and gating sensitivities that likely reflect the biological need of the cell type *in vivo* [10,11]. In addition, apart from GJIC-dependent functions, connexins may also control cellular function independent of GJIC through the formation of gap junction hemichannels linking the intracellular and extracellular environment or through poorly understood regulatory protein-protein interactions [12,13]. Importantly, connexins are expressed in almost every cell type in the human body, including those of the breast, and are dynamically regulated throughout organ and tissue morphogenesis suggesting a key role in regulating developmental processes [1,14].

## 1.2 Single membrane pannexin channels

In 2000, pannexins, a new family of proteins with limited sequence homology to the invertebrate gap junction proteins innexins, were discovered [15]. Three members of the pannexin family were identified including pannexin1 (Panx1), pannexin2 (Panx2) and pannexin3 (Panx3) [15]. Each pannexin is predicted to be composed of four  $\alpha$ -helical transmembrane domains, two extracellular loops, an intracellular loop and cytoplasmic amino and carboxy termini, similar to the vertebrate gap junction proteins connexins [16]. Effectively, pannexin channels are not unlike connexin hemichannels as both are capable of transferring small ions and metabolites less than 1000 Daltons, which include ATP and  $\text{Ca}^{2+}$ , between the internal and external environment of cells [17]. Initially, the similar topology and the fact that pannexin subunits oligomerize to form a protein-lined pore, led to the proposal that pannexins share similar functional characteristics to connexins and possibly redundant roles within tissues, functioning as a new family of gap junction forming proteins (Figure 1.1) [15,16,18]. However, pannexins have only been proposed to form gap junction channels in a few select over-expressing *in vitro* systems but they have never been demonstrated to form intercellular channels *in vivo* [18,19].

As a result, evidence to date suggests that pannexins do not form gap junctions under physiological conditions which is supported by the fact that pannexins are glycosylated, which may act to prevent docking of adjacent channels. Moreover, pannexins do not cluster into semi-crystalline arrays as is well documented for gap junctions composed of connexins, adding further evidence that they are a very different type of channel [22,23].

**Figure 1.1. Connexins and pannexins are predicted to share similar membrane topology but only channels formed of connexin subunits form functional gap junctions *in vivo*.** (A) The prototypical connexin, Cx43, and pannexin, Panx1, share a similar predicted topology with four  $\alpha$ -helical transmembrane domains, two extracellular loops (EL), an intracellular loop (IL) and cytoplasmic amino (AT) and carboxy termini (CT). (B) Connexin and pannexin proteins oligomerize to form mainly hexameric channels known as connexons or pannexons, respectively (C) Upon arrival at the plasma membrane, pannexons remain undocked and function as single membrane channels while (D) connexons typically dock with connexons from an adjacent cell to form a gap junction channel. Hundreds of gap junction channels typically cluster forming a gap junction plaque. Adapted with permission (Appendix 2) from [20,21]. Figure 1.1B, D was reprinted from FEBS Letters, 588/8, Laird, Syndromic and non-syndromic disease-linked Cx43 mutations, 1339-1348, Copyright (2014), with permission from Elsevier. Figure 1.1C was reproduced with permission, from Penuela et al., 2014, Biochemical Journal, 461, 371-381. © the Biochemical Society.

**Figure 1.1**

While these observations do not rule out pannexins channels having the same function as connexin hemichannels, the fact that pannexin channels can be opened at normal resting membrane potentials due to their insensitivity to physiological levels of extracellular  $\text{Ca}^{2+}$  suggests distinct cellular roles. Moreover, pannexins typically have longer half-life than connexins, further arguing against the notion that they have redundant function [18,24,25]. Together, these observations suggest that pannexins have at least some distinct functions from connexins within tissues and therefore warrant further investigation.

Due to its expression in many cell types, Panx1 is the best characterized and has been shown to be activated by multiple stimuli, including mechanical stimulation, caspase cleavage, extracellular ATP and  $\text{K}^+$ , and membrane depolarization [26-29]. Panx1 has also been shown to regulate key cellular responses such as proliferation, differentiation and cell death mechanisms that are ultimately necessary for normal tissue development and function [27,30,31]. Consequently, it is no surprise that the role of Panx1 is beginning to be discovered, particularly with the generation of the Panx1<sup>-/-</sup> mouse [30,32,33]. However, the role of Panx1 in development, differentiation and function in many tissues remains unexplored, including that of the breast. While Panx1 has been detected in the mouse mammary gland and human breast as noted in NCBI's gene expression Omnibus database (ID 1416379, ID 49755742, [34]), no other studies have examined the role of Panx1 in the context of normal mammary gland development and function. Thus, given the lack of studies on pannexins in the mammary gland, this literature review will turn its attention to the current understanding of connexins in the mouse and human mammary glands.

### 1.3 The mouse mammary gland as a model of the human breast

The human breast, similar to that of the mouse mammary gland, functions to provide both nutrition and passive immunological protection against pathogens during nursing. Both are composed of a bilayered epithelial network consisting of a single luminal layer surrounded by a layer of myoepithelial cells separated from the mammary stroma by a basement membrane [35,36]. However, while the non-pregnant rodent mammary gland consists entirely of a single ductal tree consisting of blunt ended ducts that develop alveolar buds in response to the estrous cycle within a mainly adipose rich mammary fat pad, the human

mammary gland contains 15-20 independent branching epithelial networks that begin as terminal duct lobular units composed of alveoli and ducts surrounded by fibrous connective tissue [35,37]. Importantly, both human and mouse mammary glands undergo extensive proliferation and secretory differentiation of epithelium as the breast develops into a secretory gland during lactation [35,38-40]. This process requires tight regulation of hormonal and growth factor cues as well as epithelial-epithelial and epithelial-stromal interactions, and direct cell-cell communication through gap junctions [35,38-40]. Therefore, it is not surprising that the human and mouse epithelial stem cell hierarchies share many parallels (reviewed in [41]). Ultimately, due to these similarities between the mouse and human mammary gland, the mouse mammary gland has become a useful model to further our understanding of the mechanisms associated with gland development and disease, particularly through our ability to genetically manipulate molecular functions by mutation or ablation [42,43].

## 1.4 Mouse mammary gland development

The development of the murine mammary gland begins with the development of the milk lines at embryonic day 10 [44]. By embryonic day 11.5, the milk lines develop into 5 pairs of mammary gland placodes which gives rise to mammary gland buds and ultimately a rudimentary ductal structure [40]. Following birth, this rudimentary structure develops isometrically with body weight until the onset of puberty [42]. The hormonal secretion of estrogen and growth hormone then drives the first main phase of development by orchestrating ductal elongation from proliferative structures known as terminal end buds that invade the surrounding stroma and bifurcate creating new primary branches. Together with secondary lateral side branching, these branches loosely fill the mammary gland fat pad [40]. The adult mammary gland continues to undergo additional tertiary branching in response to cyclical ovarian estrus cycles, further developing a highly branched epithelial network with the development of alveolar-like structures that cyclically regress until the onset of pregnancy [37].

The second phase of mammary gland development occurs following the onset of pregnancy and is characterized by massive amounts of cell proliferation and tissue remodeling [38]. The gland undergoes extensive alveolar development to acquire a secretory lobuloalveolar

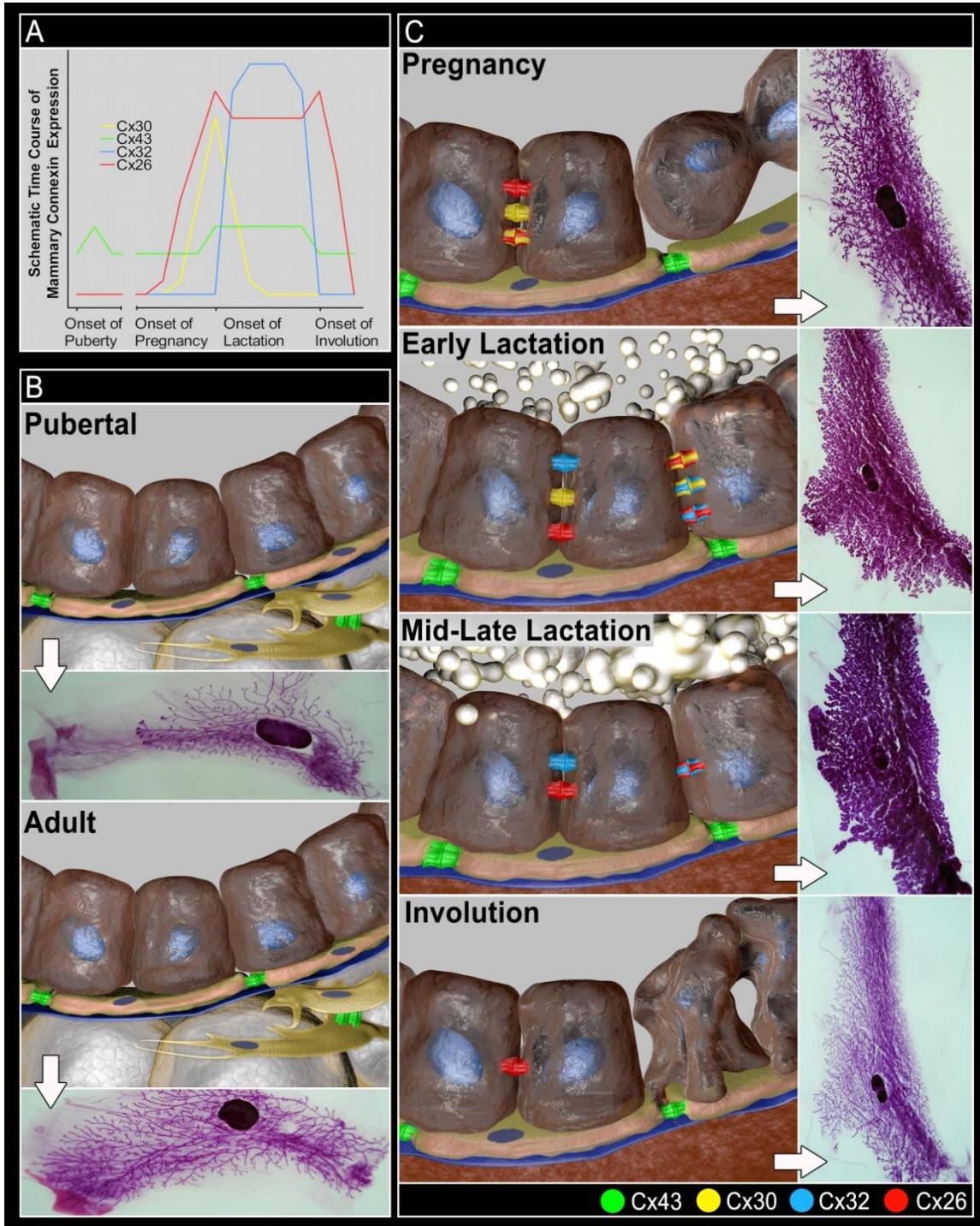
phenotype [45]. During early pregnancy, prolactin and progesterone are the main drivers of extensive branching of ducts and alveolar bud formation that continues during late pregnancy and into lactation [40]. As a result, the volume ratio of epithelial cells to adipocytes increases as the epithelial compartment expands and adipocytes undergo delipidation [46]. In parallel to the morphogenic changes in the pregnant mammary gland, luminal epithelial cells undergo differentiation as many genes involved in milk synthesis begin to be expressed during midpregnancy [46]. With the withdrawal of progesterone at parturition, the gland undergoes secretory activation as the mammary gland is set up as an exocrine gland to perform its main function to produce, secrete and deliver milk [47]. The gland functions to produce milk until weaning of the pups where the buildup of milk within alveoli acts as a trigger for the mammary gland to undergo involution. This two-step process is characterized by extensive cell death and remodeling that returns the mammary gland back to the adult gland state [48]. The first phase of involution is characterized by extensive cell death triggered by the activation of the LIF/Stat3 pathway while the second phase of involution is characterized by remodeling of the extracellular matrix, adipocyte differentiation and alveolar collapse [40].

## 1.5 Connexin expression in the rodent mammary gland

### 1.5.1 Virgin mammary gland

Up until about 2004, the majority of studies evaluating connexins in the rodent mammary gland focused on characterizing connexin expression, localization and regulation (Figure 1.2). Gap junctions were first detected joining ductal epithelial cells in 3-9 week old mammary glands of mice [49]. These likely represented gap junctions made from Cx43 as the vast majority of studies agree that Cx43 is expressed in the basal epithelium and stroma and further, represents the major connexin expressed in the non-pregnant rodent mammary gland [50-54]. Cx43 is also reported to be transcriptionally upregulated in 5-6 week old virgin mice suggesting a role in pubertal mammary gland development [53].

**Figure 1.2. Connexin expression throughout mouse mammary gland development.** (A) Schematic rendering of Cx43 (green), Cx26 (red), Cx32 (blue) and Cx30 (yellow) expression throughout mammary gland development based on the body of evidence discussed in this review. (B) The first main phase of mammary gland development occurs through puberty as a rudimentary duct structure extends and branches throughout the fat pad. The virgin mammary gland epithelium contains luminal cells (brown) expressing low levels of Cx26, Cx30 and Cx32 (not shown) throughout virgin gland development. Luminal cells surrounded by myoepithelial cells (peach) connected by gap junctions composed of Cx43 (green) that are upregulated during puberty. The epithelium is embedded in the stroma that contains mainly adipocytes (clear), as well as fibroblasts (yellow) that express Cx43, separated by a basement membrane (dark blue). (C) The second main phase of development occurs following the onset of pregnancy as extensive lobuloalveolar development prepares the mammary gland to produce and deliver milk during lactation before the gland undergoes involution where extensive cell death and remodeling returns the gland to a pre-pregnant state. Cx26 and Cx30 are dramatically upregulated in luminal alveolar cells during pregnancy while Cx32 is upregulated following parturition during early lactation. By mid-lactation, Cx30 expression reverts back to levels expressed in the virgin mammary gland while this occurs for Cx32 at the onset of involution. Cx26 remains elevated throughout lactation and into the early stages of involution in luminal alveolar cells. In some instances, these connexins may selectively intermix to form heteromeric and heterotypic gap junction channels. Myoepithelial cells continue to express Cx43 gap junctions throughout pregnancy, lactation and involution. Each development stage is paired with a representative epithelial architecture of the mouse mammary gland as revealed by whole mount analysis using carmine alum staining.

**Figure 1.2**

Other connexins expressed between luminal cells of the nonpregnant mammary gland are Cx26, Cx32 and Cx30 [52,54]. However, these connexins remain poorly characterized based on low connexin expression, poor sensitivity of anti-connexin antibodies, variations in tissue processing and differences in mouse strains being investigated [51,55-57].

### 1.5.2 Mammary gland during pregnancy

Following the onset of pregnancy, the connexin expression landscape changes as Cx26 and Cx30 are upregulated and become the dominant connexins expressed within the mammary gland, suggesting a role in regulating mammary gland alveogenesis and differentiation [52,55]. In the case of Cx26, the majority of studies in mice support a modest upregulation by day 5 of pregnancy [51,55], a dramatic upregulation at day 9-10 of pregnancy [51,52,56], before peaking near parturition [50,51,55-57]. Cx30 appears to increase between days 10.5-12.5 of pregnancy before peaking in late pregnancy prior to parturition [11,52]. When Cx26 and Cx30 are coexpressed in mammary epithelium, they colocalize into the same gap junction plaque and form heteromeric channels *in vitro* [11]. In contrast, Cx32 expression remains low throughout pregnancy prior to extensive upregulation following the onset of lactation, suggesting a role in regulating the secretory phenotype of the gland through GJIC-dependent regulation of signaling molecules as opposed to regulating alveogenesis [52,56,57]. In myoepithelial cells, Cx43 protein expression undergoes a slight increase during pregnancy associated with a shift to more highly phosphorylated forms of Cx43 which are commonly associated with greater GJIC [52,58,59]. Changes in Cx43 correlate with the expression of MAP Kinases ERK1/2 and JNK in primary rat mammary epithelial cells and may be indicative of more functionally active gap junctions although this has not been demonstrated directly *in vivo* [60].

### 1.5.3 Lactating mammary gland

Following parturition, gap junctions between luminal epithelial cells lining alveoli become larger and less numerous than during pregnancy [49] as elevated and intermixed luminal Cx26, Cx30 and Cx32 channels continue to be colocalized [11,56,57]. The different temporal pattern of Cx26, Cx30 and Cx32 expression as the gland transitions from pregnancy through lactation promotes discrete heteromeric and homomeric gap junctions.

These gap junctions have distinct permeabilities of regulatory molecules IP3, cAMP and cGMP as well as differences in channel insensitivity to closure by the osmolyte taurine present during milk protein synthesis [11,51]. The physiological importance of this remains unknown and less studied due largely to a lag in the development of assays to measure channel activity *in vivo*. Following day 7 of lactation, Cx30 levels decrease to a pre-pregnant state [11,52]. Most studies agree that both Cx26 and Cx32 remain high throughout lactation compared to the virgin gland, until the onset of involution [51,52,56,57,61]. In contrast, myoepithelial cells continue to readily express Cx43 in surrounding alveoli throughout lactation [49,58]. The majority of studies suggest that there is a lack of gap junctions between luminal and myoepithelial cells of alveoli [49,58]. However, more recent immunofluorescence based evidence suggests that Cx43 may be localized near myoepithelial-luminal cell contacts, suggesting possible heterocellular interactions [52]. However, as Cx43 has not been described to be expressed in luminal cells of mice it remains difficult to interpret which connexin expressed in luminal cells would be able to dock with Cx43 in myoepithelial cells. Electron microscopy studies may best answer whether gap junctions are present between the luminal and myoepithelial cells of alveoli.

#### 1.5.4 Involuting mammary gland

Unlike lactation, our knowledge of the expression of connexins during involution is limited. During the first 48 hours of involution, large gap junctions between luminal cells of alveoli, similar to those found in lactating mice, are present [49]. These gap junctions likely represent Cx26 as Cx32 levels drop dramatically following the onset of involution [51]. Cx43 also appears to be expressed and has been described to increase between 6-48 hours in the involuting mammary gland following weaning of the pups [53]. However, both Cx43 and Cx26 return to the pre-pregnant state of expression following 1 week of gland involution [50,52,55].

### 1.6 Expression of connexins in the human breast

Unlike that of the rodent gland, knowledge of connexin expression in the human breast is currently restricted to the nonpregnant adult (Figure 1.3) [50,62-66]. Spatial and temporal connexin expression in human breast development remains under-studied due to the

difficulty in obtaining pubertal, pregnant and lactating human samples. Similar to that of the rodent gland, Cx43 is readily detected in the non-pregnant breast localizing mainly with the myoepithelial marker keratin14 and forming gap junctions between basal cells of both the major ducts and lobules [50,62-66]. In addition, sparse Cx43 gap junction plaques between luminal cells have been reported [64,65]. This raises the potential for Cx43 gap junctions between myoepithelial and luminal cells although this is likely a rare event based on a lack of evidence for luminal/myoepithelial gap junctions at the ultrastructural level [64,65]. In addition, Cx26 has been localized between luminal epithelial cells [62,65]. Cx26 expression is likely low and its expression may reflect dynamic hormonal variation due to monthly cycles or interpatient variability as many studies have failed to detect Cx26 in ducts or lobules based on immunolabelling and electron microscopy [50,63,65,66].

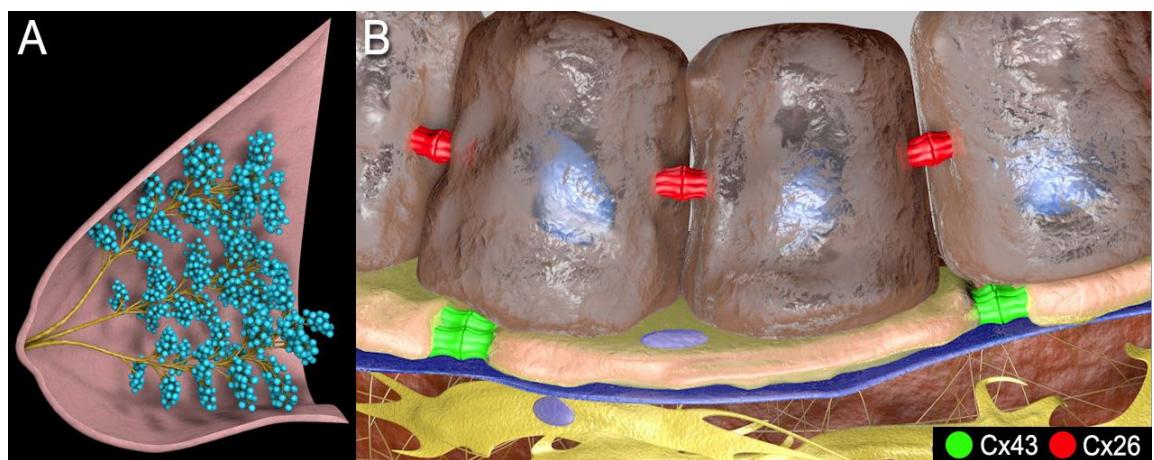
## 1.7 Connexins in mouse mammary gland development

With the development of genetically modified mice expressing either loss-of-function connexin mutants or lacking a specific connexin (Table 1.1), the depth and understanding of connexins in the mammary gland has increased but remains limited by the relatively few *in vivo* studies that have been reported. The sections below highlight the important stage-specific roles of connexins in gland development and function.

### 1.7.1 Embryonic mammary development

Embryonic mammary gland development is mediated by mostly local signaling through epithelial-stromal interactions suggesting a possible role for gap junctions in this process [67]. To date, no study has evaluated connexin expression in prenatal mammary gland development which is not surprising given that the majority of gland development occurs after birth [68]. However, a limited number of indirect studies suggest that connexins are

**Figure 1.3. Connexin expression in the non-pregnant human breast.** (A) Alveoli (blue) are connected to openings at the nipple by multiple converging duct systems (yellow). (B) The mammary epithelium consists of luminal cells (brown) connected by gap junctions composed of Cx26 (red), surrounded by myoepithelial cells (peach) connected by Cx43 (green) gap junctions. The epithelium is separated by a basement membrane (dark blue) from the connective tissue containing fibroblasts (yellow) linked by Cx43 gap junctions.

**Figure 1.3**

**Table 1.1 Genetically-modified mice used to evaluate the role of connexins in the mammary gland.**

Mouse Line	Background (DNA;ES cell;Blastocyst;Cross)	Modification	Mammary Gland Phenotype	Reference
<i>Gja1</i> <sup>+/−</sup>	129 SV; 129 R1; C57BL/6	Loss of one allele of Cx43	No defect reported	[7]
<i>Gja1</i> <sup>+/Gja5</sup>	129 SV; HM1; C57BL/6; C57BL/6	Cx43 allele replaced with Cx40	Normal development and function	[78]
<i>Gja1</i> <sup>+/Gjb2</sup>	129 SV; HM1; C57BL/6; C57BL/6 and Flp	Cx43 allele replaced with Cx26	Reduced branching of ductuli	[79]
<i>Gja1</i> <sup>+/Gjb1</sup>	129 SV; HM1; C57BL/6; C57BL/6 Mammary transplant into cleared fat pads of nude mice	Cx43 allele replaced with Cx32	Normal gland development	[80]
<i>Gja1</i> <sup>+/Gjb1</sup>	129 SV; HM1; C57BL/6; C57BL/6	Cx43 allele replaced with Cx32	Milk ejection defect	[78]
<i>Gja1</i> <sup>+/G60S</sup>	ENU mutagenized C3H/HeJ; FVB;C3; C57BL/6	Point mutation in the <i>Gja1</i> gene causes G60S substitution in one allele	Virgin – delayed ductal and stromal development Lactation – milk stasis and defect in milk ejection	[54,81]
<i>Gjb1</i> <sup>−/−</sup>	BALB/c; 129 SV J1; C57BL/6; C57BL/6	Loss of Cx32	Ductal elongation and branching normal	[80]
<i>Gjb6</i> <sup>−/−</sup>	129 SV; HM1; C57BL/6; C57BL/6	Loss of Cx30	Fertile, lactate; no rigorous assessment reported	[82]
<i>Gjb2</i> <sup>fl/fl</sup> <i>x</i> <i>MMTV-cre</i>	<i>Gjb2</i> <sup>fl/fl</sup> mice – BALB/c; HM1; C57BL/6; C57BL/6 MMTV-cre mice – Not Described	Conditional deletion of Cx26 prior to birth	Virgin – normal development Pregnancy - increased apoptosis and decreased alveologenesis	[80]
<i>Gjb2</i> <sup>fl/fl</sup> <i>x</i> <i>WAP-cre</i>	<i>Gjb2</i> <sup>fl/fl</sup> mice – BALB/c; HM1; C57BL/6; C57BL/6 WAP-cre mice – Not Described	Conditional deletion of Cx26 ~D17 of pregnancy	Normal development and function	[80]

not critical mediators of this process. The strongest argument for this stems from the fact that all Cx43, Cx26, Cx30 and Cx32 genetically-modified mice where the connexin is ablated or mutated proceed to develop either some kind of a rudimentary mammary gland, or exhibit no gland defects at all (Table 1.1). An obvious explanation for this may be that the impairment or loss of one connexin is compensated by another during this stage of development. Alternatively, another connexin family member may be expressed that is not typically expressed at a similar stage of development. No evidence exists for either of these two possibilities. In fact, adult stem/progenitor cells isolated from human mammoplasty [69,70] or from the bovine mammary gland [71] were found to lack GJIC, inferring that stem/progenitor cells in the mammary gland during embryogenesis may also lack GJIC although a more detailed assessment is needed.

### 1.7.2 Virgin mammary gland

Cx43 is the most highly expressed connexin in the virgin mammary gland and is reported to be upregulated during puberty [53]. Hormonal control of ductal elongation at puberty is driven by estrogen and growth hormone signaling. Estrogen receptor alpha null (*ER $\alpha$ KO*) and growth hormone receptor null (*GHRKO*) mice both exhibit absent or impaired ductal elongation, respectively, without affecting rudimentary gland growth prior to puberty [72,73]. Although estrogen, growth hormone, as well as hormone insulin like growth factor 1 have all been implicated in regulating the expression of Cx43 in other *in vitro* models, it still remains unclear whether these hormones regulate Cx43 in the pubertal mammary gland [74-76]. Importantly, genetically modified mice where Cx43 is expressed but not fully functional have revealed impaired ductal phenotypes similar to *ER $\alpha$ KO* or *GHRKO* mice (Table 1.1).

One mutant mouse line that has proven to be insightful in our understanding of the mammary gland is *Gja1*<sup>+/G60S</sup> (*Cx43*<sup>G60S/+</sup>) mutant mice. These mice mimic the rare, mainly autosomal dominant human developmental disease known as oculodentodigital dysplasia (ODDD). ODDD is linked with >70 mutations in *GJA1* gene that gives rise to mainly loss-of-function Cx43 mutants that yield pleiotropic phenotypes in humans including ocular impairments, craniofacial abnormalities, tooth defects and syndactyly of the digits [21,77]. These mice harbor a systemic autosomal dominant mutation in the gene encoding Cx43

that yields a glycine to serine substitution at position 60, decreasing total Cx43 function to only <30% of normal levels [83]. Evaluation of 4-10 week virgin mutant mice revealed a severe delay in ductal elongation as well as stromal impairment compared to control mice in prepubertal, pubertal and adult mice, highlighting a role for Cx43 in regulating epithelial morphogenesis and stromal development in the virgin gland [54]. However, much remains unanswered concerning Cx43 and virgin ductal development. As Cx43 is expressed in both the stromal and epithelial compartment in mice, it remains unknown as to which compartment, if not both, must maintain normal expression of Cx43. Moreover, further studies are needed to define the level of Cx43 required for normal ductal development and if this is dependent on a GJIC. Ultimately, additional *in vivo* studies are needed to clarify the role of Cx43 in virgin ductal development.

In the case of Cx26 and Cx32 which are normally expressed in mammary luminal cells, it is not surprising that mammary gland ablated *Gjb2* and global *Gjb1* null mice exhibit normal virgin gland architecture as their expression is low in the non-pregnant mammary gland [80]. Similarly, Cx30 appears dispensable both in the virgin gland and the developing gland, as *Gjb6* null mice are fertile and able to lactate suggesting that a ductal network is formed during adolescence, although a detailed evaluation of the virgin mammary gland in *Gjb6* null mice has not been performed [82]. Taken together, Cx43 appears to regulate ductal elongation and stromal development in the virgin mammary gland.

### 1.7.3 Mammary gland during pregnancy

Following the onset of pregnancy, Cx26 upregulation is likely driven by prolactin signaling as transcript profiling of mammary glands from prolactin receptor knockout mice (*PrlRKO*) revealed a downregulation of Cx26 compared to control mice during early pregnancy [84]. In support of this, a binding site for Stat5, a downstream mediator of prolactin signaling, has been reported in the promoter of *GJB2* [85]. As Cx30 is also upregulated during pregnancy and colocalizes with Cx26 it is likely that it is also regulated by prolactin. Indeed, Talhouk et al. observed that exogenous administration of prolactin and the corticosteroid dexamethasone were able to upregulate Cx30 expression in mouse mammary epithelial cells *in vitro* [52]. Thus, both Cx26 and Cx30 appear to be regulated by the hormone prolactin.

Apart from prolactin signaling, Cx26 has also been shown to be upregulated by the administration of exogenous human chorionic gonadotropin (hCG) in rat mammary glands and *ex vivo* organ culture, through downstream activation of Sp transcription factors [86,87]. However, as hCG treatment coincided with an increase in both 17 $\beta$  estradiol and progesterone, it remains unclear how each hormone individually affects the levels of Cx26 or how the presence of multiple hormones alters the expression of Cx26 [86]. Importantly, both estrogen and progesterone have been reported to regulate Cx26 expression in the endometrium, suggesting that ovarian steroid hormones may also regulate Cx26 in the mammary gland [88]. It remains unknown if Cx30 can also be hormonally regulated by hCG and ovarian steroids. Further studies are needed to understand hormonal regulation of Cx26 and Cx30, particularly in the more physiological context of multiple hormones.

Cx26 was first identified to be important in alveolar development during pregnancy following conditional deletion of Cx26 prior to birth by crossing *Gjb2*<sup>f/f</sup> mice with mice expressing Cre under the mouse mammary tumor virus promoter (*MMTV-cre;Gjb2*<sup>f/f</sup>) [80]. These mice exhibited impaired alveogenesis and reduced ability of the dams to feed their pups as a result of increased apoptosis [80]. As such, the working model suggested that, in response to prolactin and progesterone driven alveogenesis, Cx26 is upregulated to promote epithelial cell survival within the pregnant mammary gland [80]. However, in the same study another conditionally ablated Cx26 mouse model was created by crossing *Gjb2*<sup>f/f</sup> mice with mice expressing Cre under the whey acidic acid (WAP) promoter (*WAP-cre;Gjb2*<sup>f/f</sup>), yielding Cx26 silencing during late pregnancy. Unexpectedly, these mice revealed normal gland development and function, suggesting that Cx26 is important during early pregnancy [80]. While these results are intriguing, the fact that a large human population with syndromic and non-syndromic deafness express systemic loss of function mutations in the *GJB2* gene that encodes Cx26 but have no reports of breast feeding impairments argues against the translatability of these findings. In addition, *MMTV-cre* mice that have been used to generate the *MMTV-cre;Gjb2*<sup>f/f</sup> mice have been reported to have impaired alveogenesis in the mammary gland, further supporting a need for additional *in vivo* studies to clarify the role of Cx26 in the pregnant mammary gland. Similarly, the role of Cx30 is not clearly defined during pregnancy as a detailed evaluation of the mammary gland in *Gjb6*<sup>-/-</sup> mice is lacking.

Apart from extensive lobuloalveolar development that occurs in the mammary gland during pregnancy, the gland must also undergo differentiation as it prepares for secretory activation. To date, reduced or ablated Cx26, Cx32, Cx43 or Cx30 function suggests that loss of one connexin does not impair epithelial differentiation. For example in *MMTV-cre;Gjb2<sup>f/f</sup>* mutant mice, evaluation of numerous markers of epithelial differentiation including  $\beta$ -catenin, keratin 5, smooth muscle actin, E-cadherin and the NaKCl cotransporter 1 indicated that the gland differentiated normally, despite reduced lobuloalveolar development, suggesting that Cx26 is not required to maintain normal epithelial differentiation [80]. Similarly, loss of Cx43 function in *Cx43<sup>G60S/+</sup>* mice was not associated with changes in the expression of E-cadherin, P-cadherin,  $\beta$ -catenin, occludin or claudin 1, molecules involved in tight and adherens junctions [81]. Milk was produced in both of these mouse models as well as in *Gjb1<sup>-/-</sup>* and *Gjb6<sup>-/-</sup>* mutant mice, suggesting normal secretory differentiation of the gland and that no single connexin is essential for the production of milk, although a more rigorous assessment has not been performed [80-82]. Taken together, it is apparent that the loss of one connexin does not critically impede normal epithelial and secretory differentiation of the gland but that the loss of Cx26 does impair normal alveogenesis.

#### 1.7.4 Lactating mammary gland

Following parturition, Cx32 is dramatically upregulated and appears to be under the control of prolactin signaling, as both *PrlRKO* and *Stat5 null* mice failed to express Cx32 during lactation [89]. Importantly, as both Cx26 and Cx30 are also suggested to be upregulated by prolactin but exhibit distinct temporal regulation compared to that of Cx32, additional hormonal or local mechanisms likely also influence the expression of Cx32. The expression of Cx32 and Cx26 during lactation is believed to function through the diffusion of ionic and molecular gradients, allowing for coordinated secretion of adjacent cells [61]. Despite this, both *Gjb1* null and mammary gland ablated *Gjb2* mice show histological evidence of milk and lipid droplets in the lumen of alveoli, suggesting normal secretion of the gland [80]. However, the reduction of Cx43 function in the lactating gland of *Cx43<sup>G60S/+</sup>* mice led to the presence of large cytoplasmic lipid droplets in luminal cells that have previously been implicated in delayed secretory activation [46,81]. As a result, only myoepithelial

Cx43 and not luminal cell expressed Cx26 and Cx32 have been implicated in regulating the luminal secretory phenotype of the mammary gland, albeit only when Cx43 function is greatly impaired.

Apart from the production and secretion of milk, the lactating mammary gland must coordinate the contraction of myoepithelial cells to propel milk from the alveoli through the converging duct system towards the nipple. Cx43 was first suggested to contribute to the coupling of myoepithelial cells during milk ejection based on a shift in the phosphorylation state of Cx43 in the myoepithelial cells during lactation [52,58]. However, the hormonal control regulating the Cx43 phosphorylation shift and its increased expression is unknown. Likely candidates include estrogen and oxytocin as these have been shown to regulate the levels of Cx43 in the uterus and mouse embryonic stem cells, respectively [90,91].

It was not until the assessment of knockin Cx43 mouse models in which one allele of *Gja1* was replaced by *Gjb1* (*Gja1<sup>+/Gjb1</sup>*), *Gjb2* (*Gja1<sup>+/Gjb2</sup>*) or *Gja5* (*Gja1<sup>+/Gja5</sup>*) was evidence obtained of a role for Cx43 in milk ejection *in vivo*. For example, mammary glands of lactating *Gja1<sup>+/Gjb1</sup>* and *Gja1<sup>+/Gjb2</sup>* mice were unable to feed their pups despite evidence of milk production and secretion [78,79]. Surprisingly *Gja1<sup>+-</sup>* mice have no reported defect in mammary gland development or function, thus it may be the additional expression of Cx26 and Cx32 in myoepithelial cells, rather than the 50% loss of Cx43 function, that is ultimately responsible for the gland defects [7]. Although not directly demonstrated, myoepithelial cells engineered to express Cx26 and Cx32 could allow for gap junctions between luminal and myoepithelial cells, creating a gap junction channel mediated exchange that does not normally exist [92]. Conversely when Cx43 is replaced with Cx40 in *Gja1<sup>+/Gja5</sup>* mice, there is no luminal/myoepithelial exchange possibility as Cx40 in the myoepithelial cells is unable to form heterotypic channels with the Cx26, Cx30 or Cx32 found in luminal cells and these mice continue to lactate and feed their pups [78].

The role for Cx43 in milk ejection was established later as the reduction of Cx43 by 70% in *Cx43<sup>G60S/+</sup>* mice led to a defect in the ability of the mammary gland to respond to oxytocin, despite normal expression of the oxytocin receptor [54]. Thus, it was concluded

that Cx43 functions to coordinate synchronous contractions of myoepithelial cells for proper milk ejection [54]. This was corroborated by others where Wnt5a overexpression in human MCF10A cells led to an increase in Cx43 phosphorylation at serine 368 and a decrease in GJIC, suggesting that Wnt5a negatively regulates Cx43 function [93]. Indeed, Wnt5a overexpression in the mammary glands of lactating mice resulted in a similar inability of these mice to respond to exogenous oxytocin, further supporting a critical role of Cx43 in coordinating timely and proper milk ejection [93]. Oxytocin signaling is mediated through the oxytocin receptor coupled to  $G_{\alpha q}11$  and phospholipase C activation triggering the release of  $\text{Ca}^{2+}$  from intracellular stores [94].  $\text{Ca}^{2+}$  has previously been shown to pass through Cx43 gap junctions making it interesting to speculate that Cx43 functions via GJIC dependent mechanisms to coordinate synchronous contraction via transfer of  $\text{Ca}^{2+}$  [10]. However, further *in vivo* studies are needed better define GJIC-dependent versus independent role for Cx43 in lactating mice. In summary, the evidence to date points to a critical role for Cx43 in coordinating myoepithelial contraction and milk ejection.

### 1.7.5 Involuting mammary gland

Only Cx26 and Cx43 are described as being expressed during the first 48 hours of involution. To date, no evidence exists that would suggest connexins are critical for the mammary gland to undergo involution. However, evidence against a role for Cx26 in involution exists. *Gjb2* ablation did not initiate precocious involution of the gland despite an increase in the number of apoptotic cells during pregnancy as Stat3 phosphorylation, a marker and critical regulator of involution, did not increase [80].

## 1.8 Connexins and pannexins in cancer

Assessment of gap junctions in the mammary gland using genetically-modified mice has established a role for Cx43 in ductal elongation during puberty, a role for Cx26 in alveogenesis during pregnancy and a role for Cx43 in milk ejection during lactation. While additional *in vivo* studies are needed to clarify the roles of connexins in mammary gland development and function, there is an emerging foundation to advance our understanding of connexin dysregulation in breast pathologies, most notably breast cancer. Tumors frequently take advantage of proteins or signaling pathways critical in organ

morphogenesis during both embryonic and post-natal development, as recapped by the many reviews written linking the topics [95-97]. Therefore, it is no surprise that connexins are frequently dysregulated in the tumorigenic process of many cancers. This occurs early in the tumorigenic cascade in which connexins most frequently yield a tumor suppressive role in tumorigenesis as reviewed by many [98-100]. Tumor suppressors function to subdue cell proliferation, promote further cell differentiation and/or activate cell death mechanisms to prevent neoplastic cell transformation [101]. Ultimately, an increase in the likelihood of cancer occurs following the partial or complete inactivation of genes that govern these pathways [101].

While it currently remains unknown if connexins represent true tumor suppressors, four lines of evidence argue for this critical role in tumorigenesis as outlined by Naus and Laird [99]. First, connexins and/or gap junctions are typically downregulated in tumor cell lines, as well as in rodent and human tumors compared to normal healthy tissue/cell controls [64,98,102]. Secondly, chemicals and oncogenes that promote tumor onset and progression usually affect gap junction channels [103]. Thirdly, ectopic expression of connexins frequently suppresses cell and tumor growth and promotes a more differentiated phenotype [104,105]. Finally, and perhaps most convincingly, many gene knockout studies in mice observe an increase susceptibility to tumor onset mainly with the use of carcinogenic agents [106,107]. While these studies provide extensive evidence for connexins as tumor suppressors, this role is complicated by reports of connexin upregulation at later stages of tumor progression in many cancers, including breast cancer, suggesting a potential role to facilitate tumor progression [108-110].

Similarly, despite the relatively new field, Panx1 has also been described as both a tumor suppressor and a tumor facilitator. Panx1 was originally reported as a putative tumor suppressor based on reduced expression in C6 glioma cells compared to primary rat astrocytes. Furthermore, when overexpressed in C6 glioma cells Panx1 reduced cell proliferation, migration and anchorage independent growth while decreasing tumor size *in vivo* in a xenograft tumor model [111,112]. This role may extend to other cancers as Panx1 was found to be reduced in human basal and squamous carcinomas compared to normal skin controls [113]. Alternatively, knockdown of Panx1 in aggressive BL6 melanoma cells

led to a decrease in cell migration, conversion to a more melanocytic phenotype and reduced tumor size when injected into chick embryos [114]. Most interestingly, a *PANX1* nonsense mutation was recently identified in human metastatic breast cancer cell lines that yielded a truncated Panx1 mutant (Panx1<sup>1-89</sup>) at position 90 of the 426 amino acid polypeptide [115]. Panx1<sup>1-89</sup> increased Panx1 channel ATP release when co-expressed with wild-type Panx1, which was shown to promote metastatic cell survival during intravascular invasion of the lung [115]. This ultimately suggests that Panx1 may act as tumor facilitator in breast cancer metastasis, although the role of Panx1 in the primary tumor remains unknown. As the mechanisms and tumor stages associated with connexin and pannexin tumor suppressive and tumor facilitator function remain poorly understood, additional studies, particularly *in vivo*, are needed to help clarify the role of these large pore channels in tumor onset, progression and metastasis. As the role of Panx1 in breast cancer has only been investigated in one study, the remaining part of this chapter highlights our current understanding of the role of Cx43 and Cx26 in breast cancer.

## 1.9 Connexins in breast cancer

Breast cancer, like other cancers, is a disease characterized by the disruption of cellular homeostasis within the normal tissue environment that is caused by genetic mutations and/or epigenetic dysregulation [116]. Clinically, breast cancer progression is believed to develop from abnormal mammary hyperplasia into a primary tumor, which gains the potential to subsequently invade into the surrounding connective tissue before disseminating into the vasculature or lymphatics and ultimately developing metastases to secondary sites. The exact mechanisms underlying regulation of these processes are very complicated and can differ significantly between patients that appear to have near identical disease [117]. This heterogeneity suggests that no single pathway or histological subtype presents the same in all cases, leading to complications and difficulties in identifying the most effective therapeutics [117]. Conventional cancer chemotherapeutics have proven incompletely effective in treating the disease, frequently yielding significant side effects and highlighting a need for novel agents [118]. As a result, a push towards more targeted therapeutics has been made in recent years [116]. Connexins continue to remain on the radar as therapeutic targets in potentially new combination therapies.

Both Cx26 and Cx43 were originally classified as a tumor suppressors in breast cancer following differential mRNA expression in cell lines derived from primary and metastatic tumors through subtractive hybridization [102,119]. Connexins are typically considered tumor suppressor genes in the context of breast cancer based on their downregulation in human breast cancer cell lines, mediated in part by methylation, as opposed to mutation or deletion [119,120]. Immunohistochemical analysis of human breast tumor samples typically reveals reduced connexin gap junctions, and presumably GJIC, in primary tumors compared to matched normal or benign breast lesions [64,66,121,122]. While these results do not appear to be specific to a defined histological subtype, loss of Cx26 appears to be more frequent in epithelial-derived breast lesions, while mesenchymal, fibroepithelial, myoepithelial and tumors of the nipple retain connexin expression more similar to the normal breast (Table 1.2).

However, several key findings challenge the tumor suppressive role of connexins in breast cancer. First, increased connexin expression has been reported in higher grade primary tumors, which in the case of Cx43 may represent altered Cx43 phosphorylation states [63,123]. Secondly, connexin expression has been reported to be increased in lymph node metastases compared to matched primary tumors suggesting a role for Cx43 in facilitating breast cancer metastasis [124]. Finally, correlation of connexin expression with breast cancer patient outcome has been associated with poor survival and an increase in recurrence [125,126], although this certainly is not always the case [127]. Therefore, the potential dual role of connexins as tumor suppressors and tumor facilitators represents a significant roadblock to the design and development of targeted therapeutics against these channels. The following sections represent current evidence of the tumor suppressive and tumor facilitating roles of connexins in regulating breast cancer as they relate to distinct tumor properties or hallmarks of cancer [128].

**Table 1.2. Connexin expression in human breast cancer primary tumors based on histological subtype.**

Primary Tumor Histological Classification	Cx26 Tumor Expression Intracellular (I) Cell Surface (CS)	Cx43 Tumor Expression Intracellular (I) Cell Surface (CS)	Ref.
<b>Epithelial Tumors</b>			
<i>Ductal Carcinoma In Situ</i>	3/4 (I) N/A N/A N/A N/A	0/4 1/4 (I) 4/11 (Mostly I) 17/17 - Phospho-Cx43 Ab (Weak/None;Pan-Cx43 Ab) 24/93	[63] [64] [122] [123] [129]
<i>Lobular Carcinoma In Situ</i>	0/1 N/A	0/1 5/5 -Phospho-Cx43 Ab (Weak/None;Pan-Cx43 Ab)	[63] [123]
<i>Tubular Carcinoma</i>	N/A	0/4	[123]
<i>Mucinous Carcinoma</i>	0/1 2/4 (I)	1/1 (CS) 5/5 -Phospho-Cx43 Ab N/A	[63] [123] [126]
<i>Apocrine Carcinoma</i>	0/1	N/A	[126]
<i>Invasive Breast Cancer No Special Type</i>	15/27 (Mostly I) 27/51 (I)	14/27 (Mostly I) 42/51 (Mostly I)	[63] [124]
<i>Invasive Ductal Carcinoma</i>	0/6 N/A N/A N/A 70/138 (I) N/A	0/6 0/21 26/29 (I) 21/21 -Phospho-Cx43 Ab (Weak/None;Pan-Cx43 Ab) N/A 62/182	[66] [64] [122] [123] [126] [129]
<i>Invasive Lobular Carcinoma</i>	0/1 N/A N/A 6/9 (I)	0/1 0/7 5/5 -Phospho-Cx43 Ab (Weak/None;Pan-Cx43 Ab) N/A	[66] [64] [123] [126]
<b>Mesenchymal Tumors</b>			
<i>Breast Sarcoma</i>	N/A	1/1	[123]
<b>Myoepithelial Tumors</b>			
<i>Adenomyoepithelioma</i>	1/1 (I)	1/1 (CS)	[63]
<b>Fibroepithelial Tumors</b>			
<i>Fibroadenoma</i>	0/3 1/9 (I)	3/3 (CS) 9/9 (CS)	[66] [63]
<i>Phyllodes Tumor</i>			
Benign	1/1 (I)	1/1 (CS)	[63]
Malignant	N/A	1/1	[123]
<b>Tumors of the Nipple</b>			
<i>Paget Disease</i>	N/A	3/3 -Phospho/Pan-Cx43 Ab	[123]

### 1.9.1 Proliferation and cell growth

Numerous *in vitro* studies have observed that ectopic Cx26 or Cx43 expression in breast cancer cell lines leads to a decrease in 2D and 3D cell growth rate compared to controls [130-135]. Knockdown strategies using siRNA and shRNA targeting Cx43 in benign breast cancer cell lines led to increased cell population growth compared to untransfected control cells, supporting Cx43 as a suppressor of cell proliferation [135,136]. Most convincingly, breast cancer cells ectopically expressing Cx43 or Cx26 had reduced mammary tumor volume when orthotopically injected into mice [130,131]. This is supported by a significant negative correlation between Cx43 and the proliferation marker Ki67 in human tissue microarrays, suggesting that connexins regulate proliferation of breast cancer cells *in vivo* [127]. Interestingly, connexin regulation of cell proliferation can be achieved by a GJIC-independent mechanism, as the overexpression of Cx43 or Cx26 in mainly GJIC-deficient breast cancer cell lines suppressed cell growth and proliferation without an increase in GJIC [131,135,137,138]. As a potential mechanism for GJIC-independent inhibition of cell growth, ectopic Cx43 expression reduced the levels of the growth factor receptor fibroblast growth factor receptor 3 (FGFR3), which may in turn reduce the effects of paracrine protumorigenic growth signals [131]. Importantly, the role of Cx43 in regulating cell proliferation may be superseded in the cellular environment by more oncogenic proteins, such as the epithelial-mesenchymal transition (EMT) marker Twist, as knockdown of Cx43 in human mammary epithelial cells over-expressing Twist did not affect proliferation in 2D culture [125].

When connexins are assessed for their ability to regulate breast cancer cell proliferation without attachment to a solid substrate, via a soft agar anchorage-independent growth assay, the role of connexins is not so clear. Ectopic expression of Cx43 or Cx26 in MDA-MB-231, MCF-7 and/or MDA-MB-435 human breast cancer cells had reduced colony forming ability in soft agar compared to control cells [132,133,138], while in a similar assay using Cx43 knockdown approach in metastatic 4T1 murine breast cancer cells, no difference was observed compared to controls [125]. Although it remains unclear whether these opposing effects are cell type specific, the vast majority of studies support a tumor

suppressive role for connexins in regulating cell proliferation and anchorage-independent cell growth.

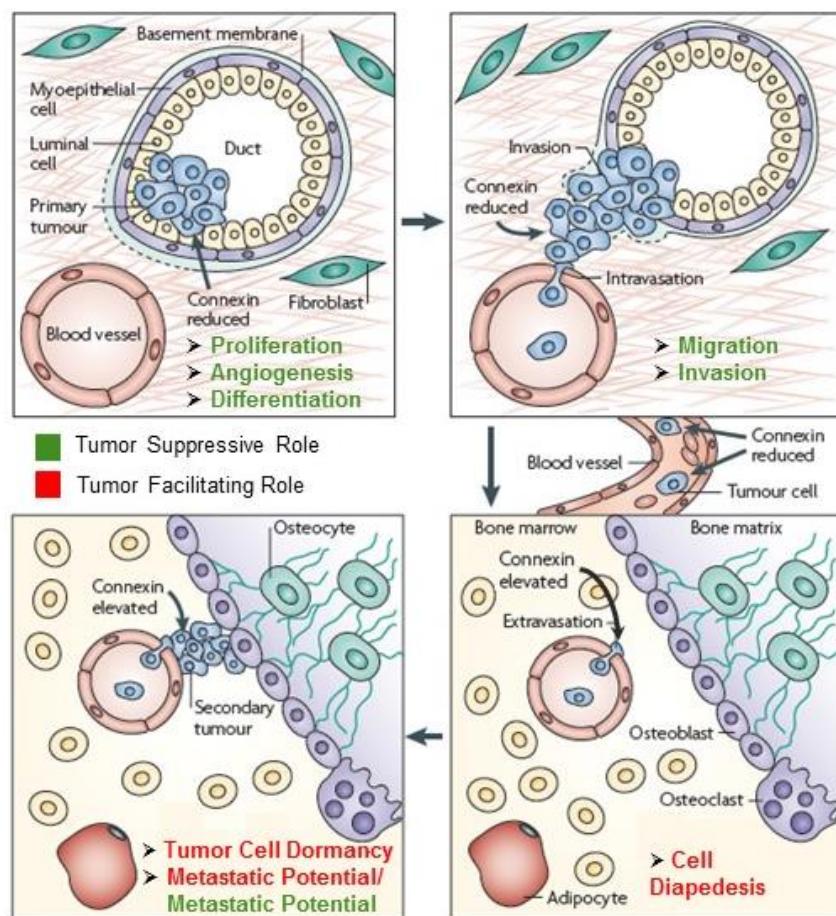
### 1.9.2 Apoptosis

While connexins appear to regulate cell proliferation in breast cancer, less is known about their role in regulating cell death. Connexins have been suggested to regulate apoptotic mechanisms in breast cancer. Intracellular Cx26 or Cx43 expression had a positive correlation with the expression of the pro-apoptotic protein Bak in human breast cancer biopsies, suggesting that connexins may be associating with apoptotic signaling pathways [121]. In support, exogenous Cx43 expression in breast cancer cells had increased caspase3 activity, a marker of apoptosis, upon retinoic acid treatment compared to controls [134]. However, as ectopic expression of connexins in breast cancer cell lines do not typically drive apoptosis, it appears that connexins are much more intimately associated with the regulation of cell proliferation than apoptosis [135].

### 1.9.3 Angiogenesis

Intimately associated with tumor growth is the ability of a tumor to feed itself by stimulating the growth of new blood vessels. Connexins have also been implicated in reducing angiogenesis that in turn may suppress the growth of primary breast tumors. Endothelial migration and tubule formation were significantly reduced when endothelial cells were grown in conditioned media collected from breast cancer cells ectopically expressing Cx26 or Cx43 in 2D and 3D cultures [133]. These results suggest that connxin expression in breast cancer cells may suppress either the release of angiogenic factors, such as vascular endothelial growth factor (VEGF), or promote the release of anti-angiogenic factors, such as thrombospondin1 (TSP-1). Indeed, this notion is supported by the observation that ectopic Cx26 expression in breast cancer cells increased the expression of TSP-1, while silencing of Cx43 resulted in decreased expression of the anti-angiogenic protein TSP-1 with a concomitant increase in pro-angiogenic VEGF [133,136,137].

**Figure 1.4. Current model of connexin expression and function in regulating breast cancer progression.** Connexin expression is differentially regulated throughout breast cancer progression to inhibit/acquire tumor suppressive (Green) or facilitating (Red) effects of gap junction channels. Following breast cancer onset, connexins are frequently downregulated or localized to the cytoplasm as tumors inhibit the proposed tumor suppressive role of connexins in proliferation, angiogenesis and differentiation. As breast cancer gains the ability to invade the surrounding connective tissue, loss of GJIC and connexin expression may be permissive to the acquisition of a more migratory and invasive phenotype. However, connexin expression may be upregulated at later stages of breast cancer progression facilitating breast cancer extravasation and metastasis. Adapted by permission (Appendix 2) from Macmillan Publishers Ltd: Nature Reviews Cancer [99], copyright 2010.

**Figure 1.4**

Mechanistically, Cx26 appears to regulate angiogenesis through both GJIC-dependent and -independent mechanisms as both wild-type Cx26 and GFP-Cx26, a GJIC-deficient chimeric mutant with GFP fused at the amino terminus, both regulated tumor angiogenesis genes as assessed through DNA array [137]. Importantly, these results were extended *in vivo* using a primary xenograft tumor model, revealing significantly reduced vascularization in tumors derived from breast cancer cells that ectopically expressed Cx43 compared to control cells [133]. To date, connexins appear to suppress a pro-angiogenic phenotype in breast cancer cell lines both *in vitro* and *in vivo*, however the translatability of these results to human breast tumors remains unknown.

#### 1.9.4 Differentiation and epithelial-mesenchymal transition

Typical of tumor suppressors, ectopic expression of Cx43 and/or Cx26 has been shown in numerous studies to induce partial re-differentiation of breast cancer cell lines to resemble more spherical duct-like acini in 3D cultures [130,133,135]. This mechanism may not be dependent on GJIC as over-expression of connexins in breast cancer cells, without a concomitant increase in GJIC, promoted proper organoid polarity as assessed by the localization of  $\beta 1$  integrin and collagen IV [133]. As a result, connexins appear to promote a more differentiated phenotype, a mechanism likely to protect carcinoma cells against the acquisition of a more invasive and migratory mesenchymal phenotype during the epithelial-mesenchymal transition (EMT) that often occurs as part of cancer metastasis. Indeed, connexin overexpression in breast cancer cell lines has been shown to induce the expression of the epithelial marker cytokeratin18 and reduce the expression of the mesenchymal markers vimentin and N-cadherin, suggesting that connexins play a role in regulating EMT processes by promoting a more epithelial phenotype [133,134]. Therefore, evidence to date suggests that connexins act as tumor suppressors to promote differentiation of a more epithelial phenotype.

#### 1.9.5 Migration and invasion

Following growth in the primary tumor, cancer cells ultimately develop the capacity to invade into surrounding tissues. Invasion in part requires tumor cells to tip the balance between secreted proteolytic enzymes, such as matrix metalloproteinases (MMPs), and

their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), as well as gain the ability to migrate [139]. Typically, ectopic expression of Cx26 and Cx43 reduces cell migration and invasion of human breast cancer cells through transwell chemotaxis assays with/without Matrigel coating [132,133,138]. Similarly, Cx43 knockdown in breast cancer cells, either through siRNA or overexpression of the transcription factor Fra-2, leads to increased cell migration and invasion compared to control cells [136,140]. Connexin regulation of cell migration and invasion is not GJIC-dependent, at least in the context of Cx26, as both ectopic expression of a Cx26 GJIC-incompetent variant or a Cx26 mutant that remained intracellular reduced cell migration similar to wild-type Cx26 [138]. Mechanistically, connexin regulation of invasion may be through control of extracellular matrix remodeling, as overexpression of Cx26 in breast cancer cells decreased the expression of MMP-9 while increasing the expression of TIMP-1 [133,138]. However, unlike *in vitro* models the role of connexins during invasion of human breast cancer cells in patients remains unclear. Cx43 expression was reduced in ductal carcinoma *in situ* with microinvasion compared with biopsies of pure ductal carcinoma *in situ*, suggesting a role for Cx43 in the initial stages of invasion [129]. In addition, since connexin expression has also been reported to be upregulated in invasive breast cancer lesions, it is clear that additional models are needed to establish whether connexins suppress invasive properties of tumor cells *in vivo* in a similar manner to that observed *in vitro* (Table 1.2) [63].

### 1.9.6 Extravasation

Following entry into the vasculature, breast cancer cells must bypass blood vessel endothelium in order to colonize and form metastases in secondary organ sites. Cx43 is likely more involved in this step than Cx26, as Cx43 more readily forms gap junction channels with Cx43, Cx40 and Cx37 typically expressed in endothelial cells [141,142]. Interestingly, unlike the mainly tumor suppressive roles for connexins in breast cancer cells described, ectopic Cx43 expression in HBL100 breast cancer cells increased the number of tumor cells undergoing transendothelial migration compared to control *in vitro* [143]. Importantly, this effect was reduced following treatment with GJIC inhibitors or when expressing GJIC-deficient GFP-Cx43, with GFP tagged to the amino terminus. Ultimately, these experiments suggest that Cx43 acts as a tumor facilitator during extravasation

through a GJIC-dependent mechanism [143]. In contrast, MDA-MB-231 cells overexpressing fully functional Cx43-GFP had reduced *in vitro* extravasation through endothelial cells grown to confluence on matrigel-coated invasion assays, suggesting cell-line specific differences [135]. Although further studies are needed to clarify the role of Cx43 in extravasation, two lines of evidence provide a potential mechanism for Cx43 as a tumor facilitator. First, Cx43 may increase cell adhesion between breast cancer cells and endothelial cells. This was observed in a tail vein injection metastasis model in which a greater number of breast cancer cell attachments to the pulmonary epithelium were seen in Cx43-overexpressing breast cancer cells compared to control cells [110]. Secondly, breast cancer cells may alter heterocellular GJIC between endothelial cells to facilitate movement through the vasculature. This was observed *in vitro*, where the co-culturing of breast cancer cells with endothelial cells resulted in a transient loss in GJIC between endothelial cells [144]. While further *in vivo* models are needed to more rigorously evaluate connexins in extravasation, evidence to date points to a role for Cx43 as a tumor facilitator at this stage of disease progression.

### 1.9.7 Metastatic potential

The potential role for connexins during extravasation suggests that Cx43 may help promote breast cancer cell metastasis. However, this is certainly not always the case as MDA-MB-435 human breast cancer cells stably expressing Cx43 exhibited a ~50% reduction in metastases to the lungs when injected into nude mice [134]. In support of this finding, a significant inverse correlation was observed between the metastatic potential of orthotopically injected mammary adenocarcinoma cells to the lungs, and their GJIC capacity *in vitro* [145]. Interestingly, when the breast metastasis suppressor 1 (BRMS1) protein was expressed in breast cancer cell lines one of the downstream effects observed was the re-establishment of GJIC through upregulation of Cx26, further supporting a tumor suppressive role for connexins in metastasis [146]. However, these studies are limited by their mostly correlative nature.

Alternatively, shRNA/siRNA knockdown of Cx43 in metastatic 4T1 murine breast cancer cells reduced micrometastasis formation in the brain when injected into mice, while an increase in Cx43 expression mediated through direct or indirect mechanisms following the over-

expression of the EMT-inducing transcription factor Twist increased the metastatic ability of these cells [125]. Collectively, these results suggest that Cx43 facilitates the formation of metastases, particularly to the brain [125]. This has been supported by others in which matched metastatic tumors from the brain had increased expression of Cx26 and Cx43 at the cell surface compared to primary tumors [147]. Interestingly, connexins may contribute to a tumor facilitating role in metastases in two ways. First, the tumor suppressive role of Cx43 in regulating cell growth in the primary tumor may be co-opted by breast cancer cells at later stages to promote tumor cell dormancy until appropriate conditions allow for metastatic growth. This is observed *in vitro* when breast cancer cells are co-cultured with bone marrow stroma yielding decreased proliferation that is GJIC-dependent [148]. In addition, this also was observed *in vivo*, where Cx43 knockdown reduced the number of breast cancer cells in the brain of an *in vivo* chicken embryo metastasis model [125]. Secondly, connexin expression may drive epithelial re-differentiation of metastatic tumor cells from the migratory/invasive mesenchymal phenotype to initiate the formation of metastases in secondary tissues [147]. Taken together, the role of connexins in the ability to form metastases in secondary sites remains unclear.

## 1.10 Mouse models used in this thesis

In order to further investigate the role of Cx43, Cx26 and Panx1 in mammary gland development *in vivo* in this thesis, we have obtained three previously uncharacterized genetically modified mouse models. First, *Gja1<sup>+/I130T</sup>* (*Cx43<sup>I130T/+</sup>*) mice were generated by Dr. Glen Fishman's laboratory through site-directed mutagenesis on a mixed C57BL/6 and CD1 background with systemic heterozygous expression of the I130T mutant similar to human patients with ODDD [149]. Importantly, *Cx43<sup>I130T/+</sup>* mice provide a means to evaluate the role of Cx43 in the mammary gland as these mice are viable and have reduced intercellular coupling of gap junction channels composed of Cx43 in primary cardiomyocytes [149] and likely all tissues and cell types that express Cx43. Secondly, Dr. Christian Naus' laboratory developed a novel conditionally ablated Cx26 mouse model on a mixed CBA and C57BL/6 background [150,151]. The role of Cx26 during pregnancy and lactation can be evaluated by crossing mice expressing the Cre-transgene under the mammary gland specific  $\beta$ -lactoglobulin (BLG) promoter with *Gjb2<sup>f/f</sup>* mice containing

flanked *loxP* sites on exon 2 of *Gjb2* to produce *BLG-cre;Gjb2<sup>f/f</sup>* (*BLG-Cre;Cx26<sup>f/f</sup>*) mice [151]. BLG activity is highly specific to the mammary gland and has been reported to become activated by ~D10 of lactation in mice with a Cre-mediated recombination efficiency of ~70-80% of lactating epithelial cells [150]. Finally, *Panx1<sup>-/-</sup>* mice were generated by Dr. Vishva Dixit's laboratory on a C57BL/6 background by crossing mice in which Exon 2 of *Panx1* were flanked by *loxP* sites with the *C57BL/6-Gt(ROSA)26Sor<sup>tm16(Cre)Arte</sup>* Cre deleter strain [152]. Ultimately, this cross generated mice with Cre-mediated deletion of exon2 of *Panx1* yielding a frameshift and premature stop codon in the Panx1 transcript [152]. Importantly, systemic deletion of Panx1 is not embryonic lethal allowing for evaluation of Panx1 in the context of the mammary gland.

## 1.11 Rationale and objectives

To date, the roles of Cx43 and Cx26 in mammary gland development and function are not fully understood while that of Panx1 remains completely unknown. The evaluation of new genetically-modified mice including the *Cx43<sup>II30T/+</sup>*, conditional *BLG-Cre;Cx26<sup>f/f</sup>* and *Panx1<sup>-/-</sup>* mice provides a means to evaluate all stages of mammary gland development. Importantly, as loss-of-function mutations in the *GJA1* and *GJB2* are associated with human diseases, results obtained from these studies may also have implications extending to patients with ODDD or hereditary deafness [77,153].

In addition, despite identification of a role for connexins in breast tumorigenesis over 20 years ago, it still remains unclear at which stages connexins support a tumor suppressive or facilitating role in the progression of breast cancer. In breast cancer, the tumor suppressive role of connexins is based mainly on the absence of connexin expression in malignant versus normal cell lines and the loss of connexin expression/GJIC observed in human breast cancer samples. Critics would argue that these studies do not conclusively provide evidence of connexins as tumor suppressors, as down-regulation of connexins may be a consequence of the tumorigenic process as opposed to a root cause. In addition, while overexpression of connexins reduces many tumor cell properties *in vitro*, it remains unclear if this is due to connexins being expressed at non-physiological levels. Ultimately, ectopic expression of connexins may yield growth suppressing effects that are tumor suppressor-like but not indicative of the true role of Cx43 and Cx26 in breast cancer. Similarly, the

role of Panx1 as a putative tumor suppressor or facilitator in cancer remains controversial. While Panx1 has been implicated to act as a tumor facilitator by promoting breast cancer cell survival during metastasis, the role of Panx1 in the primary tumor remains unknown. Taken together, the use of loss-of-function Cx43 mutant mice and conditionally ablated Cx26 knockout mice represent powerful tools to evaluate whether connexins act as putative tumor suppressors, while Panx1 knockout mice may further our understanding of Panx1 as a tumor facilitator. Importantly, these novel mutant mice also allow us to assess connexins and pannexins in the context of both the primary tumor and metastatic lesions in a unifying model system.

## 1.12 Hypothesis

It is therefore hypothesized that Cx43, Cx26 and Panx1 play critical roles in regulating normal mammary gland development and function. In addition, we hypothesize that Cx43 and Cx26 act as tumor suppressors, while Panx1 acts as a tumor facilitator, in breast cancer onset, progression and metastasis.

## 1.13 Objectives

The specific objectives of the project were to:

- 1) Determine if Cx43 affects mammary gland development in *Cx43<sup>II30T/+</sup>* mutant mice (Chapter 2) and tumorigenesis in *Cx43<sup>G60S/+</sup>* mutant mice (Appendix 1).
- 2) Examine if mice with Cx26 conditionally ablated from the mammary gland have altered mammary gland differentiation (Chapter 3) or sensitivity to tumorigenesis (Chapter 4).
- 3) Identify if Panx1 is involved in mammary gland development, differentiation and sensitivity to tumorigenesis using *Panx1<sup>-/-</sup>* mice (Chapter 5).

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## Chapter 2

- 2 The severity of mammary gland developmental defects is linked to the overall functional status of Cx43 as revealed by genetically modified mice.

Cx43 has previously been demonstrated to play a critical role in both mammary gland development and normal milk ejection during lactation. The purpose of this study was to further characterize the role of Cx43 in postnatal mammary gland development and function using a mouse model expressing a loss-of-function Cx43 mutant found in patients with ODDD. In addition, this study also aimed to improve our understanding of the implications of different loss-of-function Cx43 mutants to subclinical breast defects in patients with ODDD.

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## 2.1 Introduction

Oculodentodigital dysplasia (ODDD) is a rare pleiotropic disease characterized by developmental symptoms that include craniofacial defects, cornea and lens abnormalities, tooth defects and syndactyly of the digits [1]. This disease is linked to mainly autosomal dominant mutations in the *GJA1* gene that encodes connexin43 (Cx43) [1]. Cx43 is one of 21 connexins found in humans and is characterized by its ability to form hexameric gap junction channels that allow for the passage of molecules less than 1 kDa in size between the intracellular environments of adjacent cells [2]. This process, known as gap junctional intercellular communication (GJIC), is critical for the maintenance of key cellular processes including proliferation, differentiation and apoptosis in almost all cell types found in the human body, including the mammary gland [2,3].

The mammary gland of mice is comparable to that of humans in that it consists of a converging, branched epithelial ductal network embedded within a stromal mammary fat pad [4]. The epithelium of the mammary gland is very dynamic and undergoes two major phases of development, one during puberty and one following the onset of pregnancy [4]. At birth, a rudimentary ductal tree exists within the stroma of the mammary gland which begins significant branch elongation and amplification following the onset of hormones at puberty [5]. By 10 weeks, full extension of ducts throughout the fat pad is achieved and a series of branching and regression cycles begin in response to the estrous cycle [6]. At the onset of pregnancy, alveogenesis occurs in which secretory alveolar cells develop to produce milk for the pups throughout lactation [4]. Following weaning of the pups, the mammary gland undergoes extensive gland remodeling to revert back to a virgin gland state in a process known as involution [4]. Throughout these developmental changes, the gland requires exquisite regulation of proliferation, differentiation and apoptosis to allow for the proper growth and development of ducts throughout puberty, differentiation of secretory alveolar cells for milk production throughout pregnancy and extensive gland remodeling following cessation of lactation [4]. While hormonal regulation of these cell processes is well documented, less is known about locally acting factors such as gap junction proteins and intercellular communication [5,7].

The human mammary gland is known to express two connexins: Cx26, localized to the luminal cells of ducts and alveoli, and Cx43, which is mainly restricted to the surrounding contractile myoepithelial cells and stromal fibroblasts [8,9]. This expression is similar in mice, with the addition of Cx32 and Cx30 in luminal cells which are able to form heteromeric/heterotypic channels with Cx26 for additional luminal cell regulation that is not found in humans [10,11]. Cx26, Cx30 and Cx32 have all been detected at low levels at all stages of development and importantly are temporally up-regulated during pregnancy, lactation and/or involution, suggesting that these Cxs may regulate gland differentiation and function during these stages of development [12,13]. In contrast, Cx43 is constitutively expressed throughout mammary gland development, suggesting that Cx43 may have an important role in the maintenance of myoepithelial differentiation and coordinating function [14]. In addition, the importance of myoepithelial cells in the regulation of luminal cells through the induction of luminal cell polarity and through paracrine factors during branching morphogenesis suggests that dysregulation of myoepithelial cells may affect whole gland development and function [15,16].

Previously, to assess the role of Cx43 in mammary gland development and function, a mutant mouse model of ODDD (*Gja1<sup>Jrt/+</sup>* mice also called *Cx43<sup>G60S/+</sup>* mice) was evaluated as these mice express a dominant-negative mutant of Cx43 that reduces total Cx43 protein levels by far greater than 50% [17]. *Cx43<sup>G60S/+</sup>* mice express classical symptoms of ODDD including craniofacial abnormalities, loss of tooth enamel and syndactyly of the digits [17,18]; despite the fact that the glycine to serine substitution at position 60 of Cx43 has never been reported in ODDD patients [19]. Interestingly, severely decreased levels of Cx43 in virgin *Cx43<sup>G60S/+</sup>* mice resulted in impaired stromal development of the fat pad, smaller mammary glands and a delay in ductal development between 4-10 weeks, suggesting an important role of Cx43 in regulating gland maturation in virgin mice [20]. In addition, lactating *Cx43<sup>G60S/+</sup>* mice displayed impaired milk secretion and milk accumulation in the gland as a result of improper milk ejection [20, 21]. As a result, it was suggested that ODDD patients may also present with a defect in their ability to breast feed. However, despite over 65 identified mutations in *GJA1* resulting in ODDD, there have been no reports of lactation defects in humans with this rare disease [19]. Importantly, at

least a proportion of females with ODDD are fertile and there have been reports of mothers with ODDD in multi-generational families [22].

Functional characterization of electrical gap junction coupling in model cell systems that express only ODDD-linked mutants revealed two distinct populations of mutants that form gap junctions: mutants that have residual channel activity (such as the human mutants K134E, L90V and I130T), and mutants that are functionally dead (such as the human G21R, Y17S, and A40V, and mouse G60S mutants) [23,24]. It is unknown if the severe mammary gland phenotype observed in *Cx43<sup>G60S/+</sup>* mice is indicative of patients expressing ODDD mutants lacking residual channel function, or if a Cx43 mutant with residual channel function would rescue the functional and developmental defects in the mammary gland observed in *Cx43<sup>G60S/+</sup>* mice. In this pursuit, we obtained a mouse model of ODDD (*Cx43<sup>I130T/+</sup>*) expressing an I130T autosomal dominant mutant of Cx43 that is known to be expressed within the human population and maintains a junctional conductance of approximately 50% when co-expressed with wild-type (WT) Cx43 in the heart [25]. We hypothesized that these mutant mice would have fewer mammary gland defects as a result of a less severe reduction in Cx43-based levels of GJIC. Consequently, if this hypothesis was supported, it may help to explain why female patients with ODDD do not typically present with breast feeding problems.

## 2.2 Materials and methods

### 2.2.1 Constructs

The construct encoding the Cx43 G60S mutant was previously described [17]. The I130T-GFP cDNA construct was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the Cx43-GFP construct as a template and the following primers: forward 5'-CACTTGAAGCAGACTGAGATAAAGAAG-3' and reverse 5'-CTTCTTTATCTCAGTCTGCTTCAAGTG-3'. The I130T mutant was verified by sequencing.

## 2.2.2 Patch-Clamp electrophysiology

N2A (neuroblastoma) cells were transfected with GFP-tagged WT and mutant Cx43 respectively. Twenty four hours later, the isolated cell pairs with green fluorescence were selected to assess the intercellular coupling with dual whole-cell patch clamp recordings. Both cells were initially held at the same voltage potential (0 mV), an impulse of -20 mV was then applied to one cell and the junctional current was recorded from the other cell. Gap junctional conductance ( $G_j$ ) was calculated and presented as mean  $\pm$  S.E.M. Online series resistance compensation at 80% or off-line series resistance compensation were applied to improve the accuracy of the measured  $G_j$  [26].

## 2.2.3 Animals

Heterozygote mice expressing the I130T mutant were created as described by Fishman and colleagues [25] and were bred on a mixed background of CD1 and C57BL/6 (*Gja1tm3GFi*) mice. All *Cx43<sup>I130T/+</sup>* mice used were at generation 1-4 of backcrossing to C57BL/6 and compared to WT littermate controls. Both virgin and pregnant female mice at various ages were sacrificed using CO<sub>2</sub>. Inguinal mammary glands were used for weight measurement, whole mounts and paraffin embedding. Thoracic mammary glands were collected for western blot analysis and were stored at -80°C. Lactating mice were collected at day 21 of lactation. Pups from dams were weaned on day 21 of lactation and mammary glands from dams were collected 48 hours post-weaning which we denote as the involution time point. Lactating mice sacrificed for the oxytocin-induced milk ejection assay were used no more than 3 days following parturition. Blood was collected via cardiac puncture from pregnancy day 9.5 mutant and wild-type mice. In addition, heterozygote *Gja1<sup>Jrt/+</sup>* (*Gja1m1Jrt* also denoted as *Cx43<sup>G60S/+</sup>*) mice carrying the G60S mutant on a background of C3H/HeJ and C57BL/6J mice were used for experiments using primary myoepithelial and granulosa cultures. *Cx43<sup>G60S/+</sup>* mice used in the experiments were compared to littermate and non-littermate controls. All experiments were approved by the Animal Care Committee at Western University and conducted according to the guidelines of the Canadian Council on Animal Care (Appendix 3). For all experiments, N=6 unless specified otherwise.

## 2.2.4 Primary cultures

### 2.2.4.1 Myoepithelial cells

Primary myoepithelial cells were isolated from adult  $Cx43^{II30T/+}$  mice,  $Cx43^{G60S/+}$  mice and their respective controls, similar to that described in Plante and Laird (2008) [27]. Dissected inguinal and thoracic mammary glands were minced and digested in 12 ml of collagenase solution (0.2% trypsin, 0.2% collagenase A, 5% fetal calf serum, 5 µg/ml gentamycin in DMEM/F12 medium) at 37 °C for 30 min with gentle shaking (200 rpm). Cell suspensions were centrifuged for 10 min at 500 x g and both the supernatant (containing undigested tissue in the fat pad) and the pellets were pipetted up and down to further separate epithelial organoids from adipocytes and both were re-centrifuged as before. Pellets from both the 1st and 2nd centrifugation were combined into 4 ml of serum-free DMEM/F12 medium and 40 µl of DNase (2 U/ml) was added to the cell suspension. Cell suspensions were shaken by hand for 5 min at room temperature prior to the addition of 6 ml of serum-free DMEM/F12 medium and centrifugation at 500 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 10 ml of serum-free DMEM/F12 medium. The cell suspension was briefly centrifuged for 10 sec and the supernatant was discarded. The pellet was then resuspended in serum-free DMEM/F12 medium and this centrifugation process was repeated six times to remove fibroblasts. The final cell pellet was resuspended in 150 µl of MEGM and plated directly onto a coverslip. All pipettes and tubes used during the procedure were pre-coated with sterile PBS containing 5% BSA. Myoepithelial cells were grown for 1 week prior to microinjection. N=3.

### 2.2.4.2 Granulosa cells

Ovaries from adult  $Cx43^{II30T/+}$  and  $Cx43^{G60S/+}$  mice and their respective controls were digested in a collagenase and DNase solution (2 mg/ml type I collagenase (Sigma), 0.02% DNaseI (Sigma) in Waymouth Medium MB 752/1 complete medium (Sigma) at 37°C. Follicles were isolated through repeated aspiration and expulsion with a 1 ml pipetter. Follicles were washed twice and transferred to another dish in which the oocytes were separated from the granulosa cells by treatment with 0.05% trypsin-EDTA for 10 min

followed by repeated pipetting and centrifugation at  $600 \times g$  for 5 min. The supernatant was removed and granulosa cells were washed with Waymouth medium once and a second time in M199 medium containing Earle's salts and glutamine (Life Technologies). Granulosa cells were resuspended in M199 medium and cultured on 12-mm glass coverslips coated with collagen and incubated at  $37^{\circ}\text{C}$ , 5% CO<sub>2</sub>, 90% N<sub>2</sub> for 24 hours prior to microinjection.

### 2.2.5 Microinjection

One cell within patches of confluent myoepithelial or granulosa cells was microinjected with 0.5% Lucifer yellow (Molecular Probes) using an Eppendorf Femtojet automated pressure microinjector. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope and the percentage of cells that passed dye to at least one neighbour as well as the number of cells that received the dye after 1 min and the average number of cells receiving dye after one minute was recorded. Cells from three independent cell cultures from different mouse preparations were each injected 10-15 times for a total of ~43-50 injections per mouse model.

### 2.2.6 Western Blotting

Mammary gland tissue were homogenized using a tissue homogenizer in a lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% NP-40 and supplemented with protease inhibitor mixture (Roche-Applied Sciences) and phosphatase inhibitors (100 mM NaF and 100 mM Na<sub>3</sub>VO<sub>4</sub>). Protein lysate concentrations were measured using a BCA protein Determination kit (Pierce). Total protein lysates (60 µg) were resolved on a 10% or 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes using the iBlot Dry Blotting system (Invitrogen). Membranes were blocked using 3% BSA (Sigma) for 1 hour before being immunolabeled with primary antibodies: rabbit anti-Cx43 (1:5000, Sigma), goat anti-β-casein (1:1000, Santa Cruz) and goat anti-WAP (1:1000, Santa Cruz) and mouse anti-β-actin (1:200, Santa Cruz) at  $4^{\circ}\text{C}$ . Primary antibodies were detected using fluorescently-conjugated secondary antibodies: anti-mouse IRdye 800 (1:5000, Li-Cor) and anti-rabbit IRdye 680 (1:5000, Li-Cor), scanned and quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

### 2.2.7 Immunofluorescence and microscopy

Paraffin-embedded sections (6 µm) were deparaffinised in xylene, rehydrated in descending concentrations of ethanol (100%, 95%, 70% and 50%) and subsequently, microwaved for 5 min in antigen retrieval solution (1:50, Vector Labs). After 20 min of cool down, slides underwent a second antigen retrieval using 0.01 M Tris-1 mM EDTA buffer, pH 9.0 at 90-95°C for 30 min, followed by another 20 min of cool down. Cryosections (6 µm) were fixed in neutral buffered formalin for 15 min prior to blocking. Slides were blocked in 3% BSA, 0.1% Triton X-100 in PBS for 60 min. Slides were probed with the following primary antibodies: rabbit anti-Cx43 antibody (1:500, Sigma), rabbit anti-Cx30 (1:100, Invitrogen), rabbit anti-Cx26 (1:100, Invitrogen), rabbit anti-Cx32 (1:100, Sigma), mouse anti-keratin14 (1:100, Neomarkers), mouse anti-PCNA Clone PC10 (1:200, Dako) or rabbit anti-cleaved caspase 3 (1:400, Cell Signaling,) for 1 hour at 37°C, followed by anti-rabbit Alexa555 (1:400, Molecular Probe), anti-mouse Alexa488 (1:400, Molecular Probes) antibodies and nuclei were labelled with Hoechst 33342. Slides were mounted using Airvol. Images were captured using a Leica DM IRE2 inverted epifluorescence microscope and Openlab 5.5.3 imaging software. For quantification, 8-10 arbitrary images were taken for each sample and the numbers of positive cells or plaques were counted relative to the pixel area of nuclear staining per 0.18 mm<sup>2</sup> that was measured using ImageJ 1.46r (National Institutes of Health).

### 2.2.8 Evaluation of serum prolactin concentration

Four mutant and WT littermate mice were mated and sacrificed at day 9.5 of pregnancy. Blood was collected via cardiac puncture and allowed to clot overnight at 4 °C. Samples were centrifuged at 2000 x g for 5 min and serum was collected and stored at -80 °C. Prolactin concentrations were assessed by a prolactin mouse ELISA Kit (Abcam).

### 2.2.9 Whole mounting

Inguinal mammary glands were excised and processed as previously described in Plante et al. (2011) [28]. Briefly, mammary glands were spread on a glass slide and fixed in Carnoy's fixative (100% ethanol, chloroform, glacial acetic acid: 6:3:1) overnight at 4 °C. Mammary glands were washed in 70% ethanol for 15 min and gradually rehydrated in water. Glands

were stained overnight in carmine alum (2% carmine and 5% aluminum potassium sulfate in water) at room temperature. Mammary glands were then dehydrated in a series of ethanol baths and cleared in xylene overnight. Mammary glands were stored in methyl salicylate until pictures were taken using a numeric camera (Cybershot, Sony) or a SteREO Lumar V12 microscope (Zeiss).

### 2.2.10 Evaluation of ductal development

The distance of ductal migration was evaluated in virgin mice by measuring from the bottom of the lymph node to the end of the longest duct on the inguinal mammary gland using calipers. Ductal distance was recorded relative to the length of the mammary gland from the bottom of the lymph node to the edge of the fat pad in order to compensate for any mammary gland size differences between mutant and WT mice.

### 2.2.11 Hematoxylin & eosin staining

Mammary glands from *Cx43<sup>II30T/+</sup>* mice at parturition, lactation and involution were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin-embedded sections (6 µm) were deparaffinised in xylene and rehydrated in descending concentrations of ethanol (100%, 95%, 70% and 50%). Rehydrated tissues were stained in 1% Harris's hematoxylin for 4 minutes followed by 1% eosin for 2 min (Lerner Laboratories). Stained tissues were then rehydrated in ethanol baths (95% and 100%), followed by xylene baths and mounted using Cytoseal (Richard-Allan Scientific). Images were captured using 20X objective lens mounted on a Leica DM IRE2 inverted epifluorescence microscope equipped with a ProgRes C5 camera (Jenoptik) and ProgRes Mac CapturePro 2.7.6 imaging software (Jenoptik).

### 2.2.12 Oxytocin induced milk ejection assay

Pups were removed from the dam after feeding on parturition day. After 1 hour, dams were sacrificed and mammary glands were exposed to oxytocin as described in Plante et al. (2011) [28]. PBS, or 8 pg/ml or 80 µg/ml of oxytocin (Sigma) dissolved in PBS was applied directly to the mammary glands for 1 min and then removed. Milk entry into ducts was evaluated. Photographs were taken before and after PBS or oxytocin exposure with a

numeric camera (Cybershot Sony). Four mutant and WT littermate controls were used for this experiment.

### 2.2.13 Statistical analysis

Statistical analysis was done using Student's unpaired t-test or a one-way ANOVA test in which a p<0.05 was considered significant. Values are presented as mean ± S.E.M. All statistics were performed using GraphPad Prism version 4.03 for Windows.

## 2.3 Results

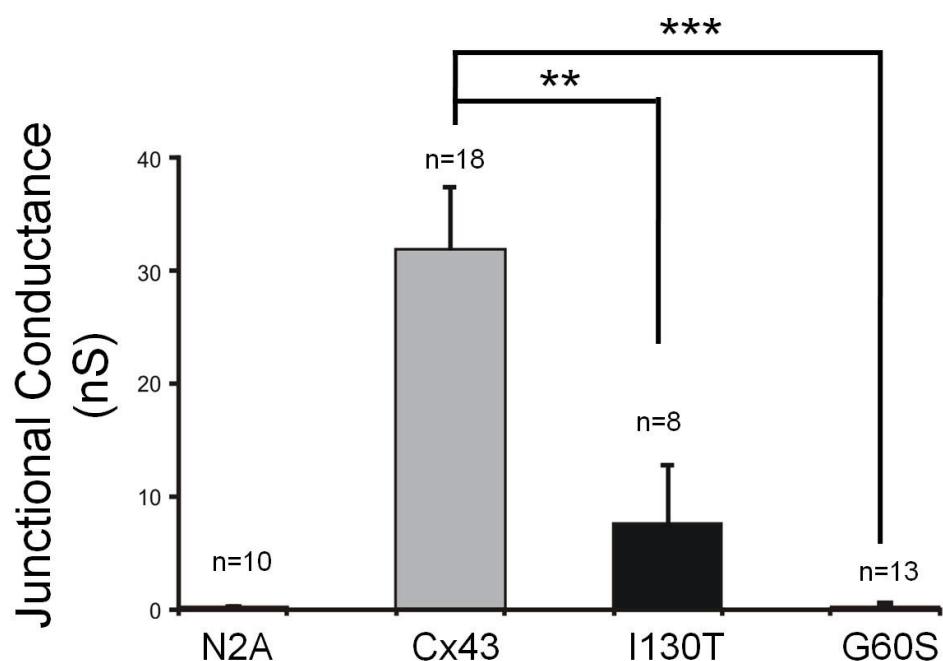
### 2.3.1 The Cx43 I130T mutant has partial junctional conductance in gap junction-deficient N2A cells

To determine if the I130T and G60S Cx43 mutants had similar capacities to form functional gap junction channels, gap junctional communication-deficient N2A cells were engineered to individually express these mutants. While N2A cells were observed to be free of functional gap junctional channels, cells expressing WT Cx43 were coupled. Consistent with previous reports, cells expressing the I130T mutant showed a highly reduced junctional conductance (G<sub>j</sub>) compared to control Cx43-expressing cells, while the G60S mutant was found to be functionally dead (Figure 2.1.) [17,23,30].

### 2.3.2 Primary myoepithelial and granulosa cells isolated from *Cx43*<sup>I130T/+</sup> mice are comparatively better coupled than cells from *Cx43*<sup>G60S/+</sup> mice

To determine if primary mammary cells from *Cx43*<sup>I130T/+</sup> and *Cx43*<sup>G60S/+</sup> mice were differentially coupled in relation to their respective controls, cultured cells were microinjected with Lucifer yellow and dye spread was assessed. Based on

**Figure 2.1. The Cx43 I130T mutant has reduced gap junctional coupling conductance.**  
Junctional conductance measurements of gap junctional intercellular communication-deficient control N2A cells engineered to express Cx43 or the G60S or I130T mutants. The I130T mutant had reduced channel conductance ( $G_j$ ) compared to wild type Cx43, while the G60S mutant channels were completely non-functionally. Bars represent mean levels of electrical conductance  $\pm$  S.E.M. \*\* $p$ <0.01. \*\*\* $p$ <0.001.

**Figure 2.1**

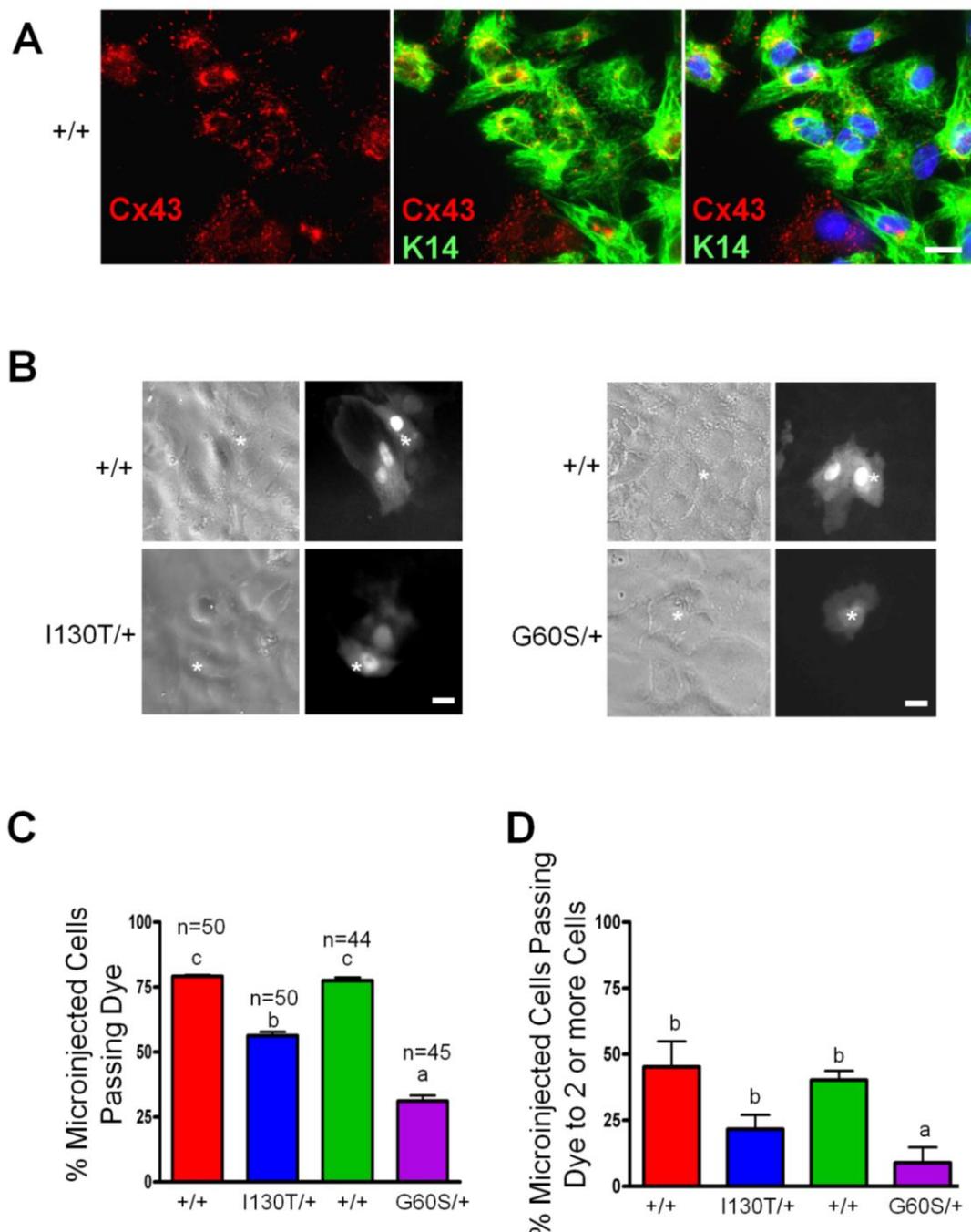
immunofluorescent labelling of keratin-14 and Cx43, mammary epithelium cultures were deemed highly enriched in myoepithelial cells and assembled gap junctions (Figure 2.2A). Similar to what we previously reported, dye transfer between myoepithelial cells isolated from *Cx43<sup>G60S/+</sup>* mice was severely reduced to ~39% of their WT control (Figure 2.2B, C) [27]. Interestingly, primary myoepithelial cell cultures from *Cx43<sup>I130T/+</sup>* mice showed a significant reduction in dye transfer but coupling remained at ~71% of the WT control (Figure 2.2B, C). In addition, the incidence of dye spreading to 2 or more cells was significantly reduced to ~22% of control in myoepithelial cells isolated from *Cx43<sup>G60S/+</sup>* mice but was non-significantly changed in myoepithelial cells isolated from *Cx43<sup>I130T/+</sup>* mice compared to its respective control (Figure 2.2D).

In support of the notion that the G60S mutant was a more potent inhibitor of dye transfer than the I130T mutant when normalized to their respective WT controls, granulosa cells of immature mouse ovarian follicles that are known to only express Cx43, were isolated from mutant mice and assessed for dye transfer [29] (Figure 2.3A, B). Similar to our findings from myoepithelial cells, the incidence of dye transfer between granulosa cells of *Cx43<sup>I130T/+</sup>* mice was 61% of control while coupling in *Cx43<sup>G60S/+</sup>* mouse granulosa cells was reduced to 33% of control. Collectively, the I130T mutant reduced GJIC to a lesser extent than the G60S mutant in primary cells known to express only Cx43.

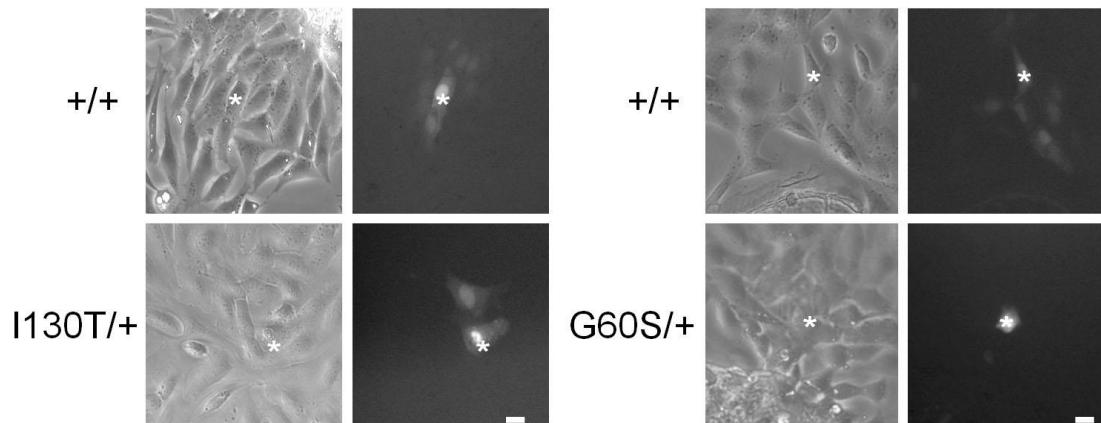
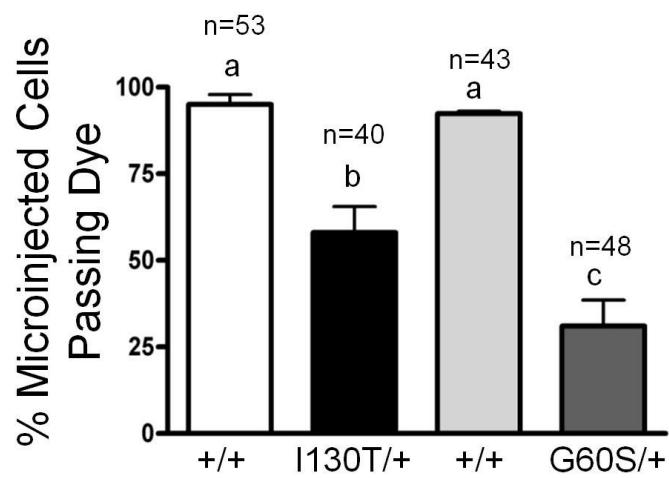
### 2.3.3 Highly phosphorylated species of Cx43 are reduced in the *Cx43<sup>I130T/+</sup>* mouse mammary gland

Western blot analysis of mammary glands from *Cx43<sup>I130T/+</sup>* mice at parturition revealed a non-significant decrease in total Cx43 compared to WT mice. However, as previously demonstrated in cardiac tissue [25], the highly phosphorylated species of Cx43 (P) were significantly reduced while the primarily unphosphorylated (P0) species of Cx43 remained unchanged in the *Cx43<sup>I130T/+</sup>* mutant mice (Figure 2.4A). Notably, the number of Cx43 gap junction plaques of mutant mice was significantly reduced compared to wild-type mice at parturition (Figure 2.4B, C). Consistent with previous reports, immunofluorescence revealed a similar distribution of Cx26, Cx32 and Cx30 gap junction plaques between luminal epithelial cells in *Cx43<sup>I130T/+</sup>* mice compared to controls

**Figure 2.2. Myoepithelial cells isolated from  $Cx43^{I130T/+}$  and  $Cx43^{G60S/+}$  exhibit differential gap junction coupling relative to their respective controls.** (A) Representative isolation of primary mammary epithelial cells from control mice are highly enriched for myoepithelial cells that form gap junctions based on keratin14 (green) and Cx43 (red) labeling, respectively. (B) Clusters of myoepithelial cells from  $Cx43^{I130T/+}$  mice,  $Cx43^{G60S/+}$  mice, and their respective controls, were microinjected with Lucifer yellow dye (asterisks). Scale bars = 10  $\mu$ m (C) Relative to controls, dye coupling was greater in cells expressing the I130T mutant than the G60S mutant. (D) Dye transfer to 2 or more cells was significantly reduced in myoepithelial cells from  $Cx43^{G60S/+}$  mice only. Bars represent mean percent incidence of dye transfer  $\pm$  S.E.M. N=3; n=number of injections; Letters on top of columns in 2.2C and 2.2D represent statistical differences using a one-way ANOVA.

**Figure 2.2**

**Figure 2.3. In comparison to controls, granulosa cells isolated from  $Cx43^{I130T/+}$  mice are better coupled than cells from  $Cx43^{G60S/+}$  mice.** (A) Isolated granulosa cells were microinjected with Lucifer yellow dye (asterisks). Scale bars = 10  $\mu\text{m}$  (B) Relative to controls, granulosa cells expressing the I130T mutant exhibited a higher incidence of dye coupling than those expressing the G60S mutant. Bars represent mean percent incidence of dye transfer  $\pm$  S.E.M. N=3; n=number of injections; Letters on top of columns in B represent statistical differences.

**Figure 2.3****A****B**

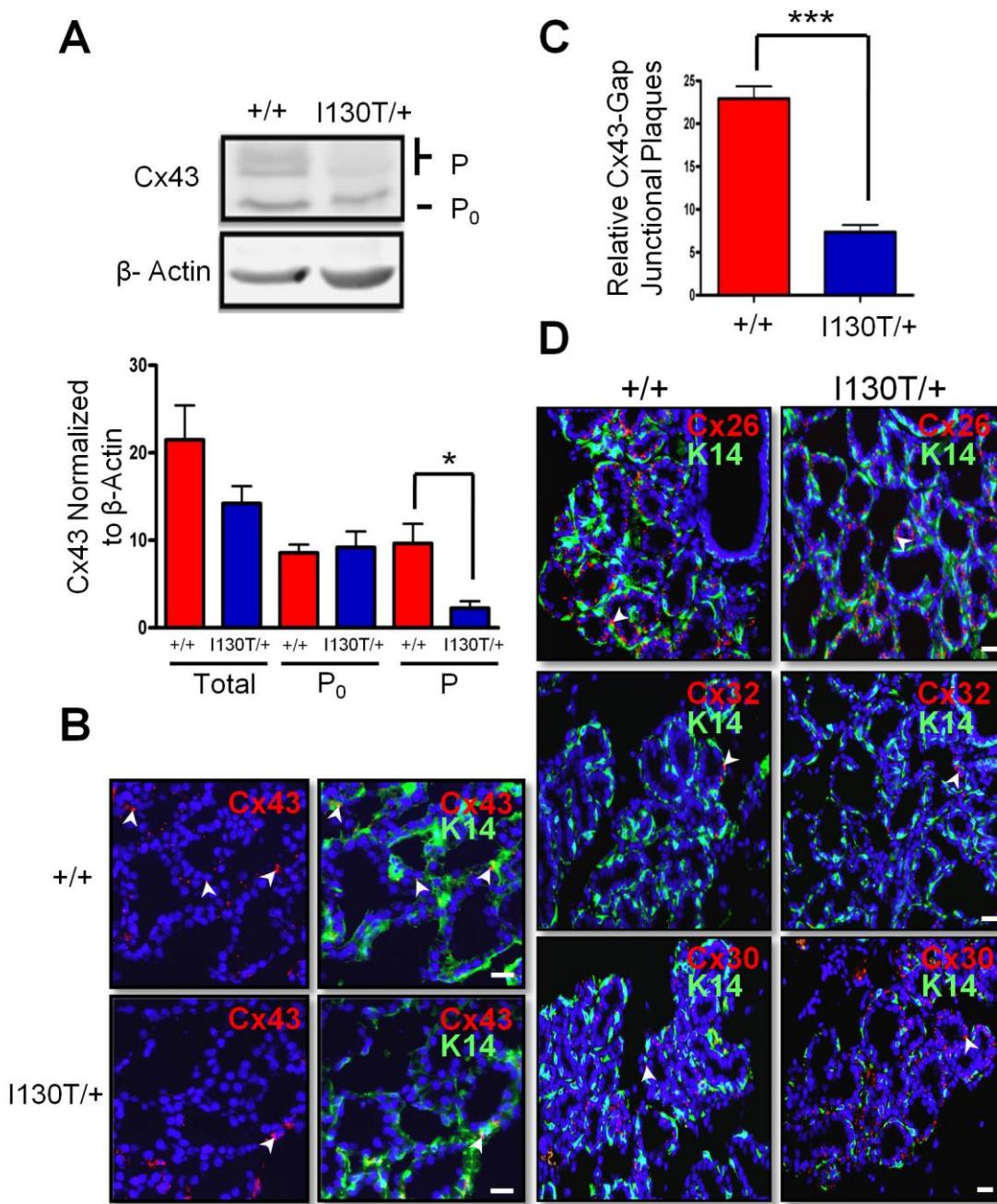
(Figure 2.4D) [20,21]. Collectively, the I130T mutant reduces the phosphorylation of total Cx43 and Cx43-gap junction plaques, with no obvious effect on other co-expressed mammary gland connexins.

### 2.3.4 Pre-pubertal $Cx43^{I130T/+}$ mice have smaller mammary glands with delayed ductal morphogenesis

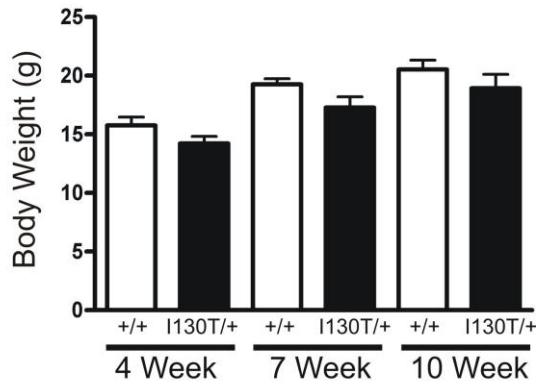
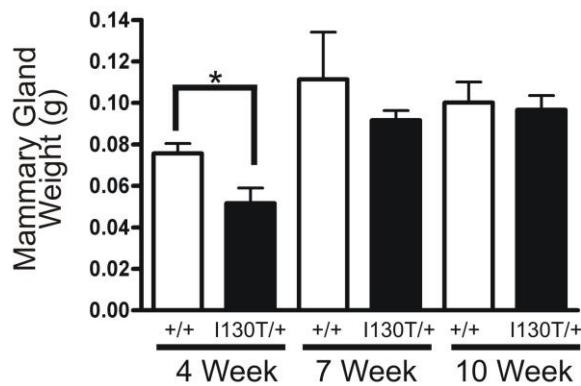
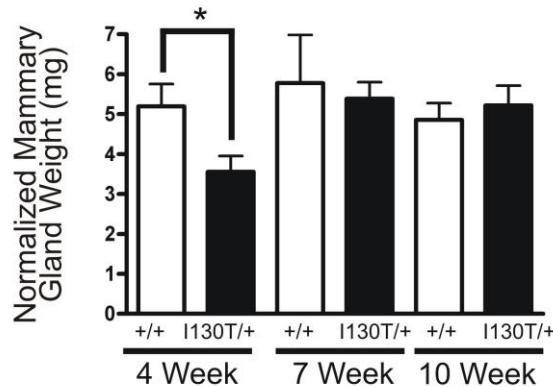
To assess mammary gland development in virgin mice, both body and mammary gland weights were measured and compared between  $Cx43^{I130T/+}$  and  $Cx43^{+/+}$  mice. Body weights were similar between virgin  $Cx43^{I130T/+}$  and  $Cx43^{+/+}$  mice at all time points assessed (Figure 2.5A), yet mammary glands of 4 week  $Cx43^{I130T/+}$  mice were significantly smaller than  $Cx43^{+/+}$  mice (Figure 2.5B). However, no difference was observed in mutant mice following the onset of puberty at 7 and 10 weeks (Figure 2.5B). When corrected for body weights, relative mammary glands of pre-pubertal mice were significantly smaller than control mice but there was no difference in relative gland weights after 7 weeks (Figure 2.5C).

In order to investigate the impact of the I130T Cx43 mutant on mammary gland development in virgin mice, mammary gland architecture was evaluated through whole mounting. Consistent with normal mammary gland development, virgin  $Cx43^{+/+}$  mice had numerous ducts with terminal end buds (TEBs) elongating within the stromal fat pad at 4 and 7 weeks, developing numerous side branches and filling the mammary fat pads by 10 weeks (Figure 2.6A-C). At 4 weeks,  $Cx43^{I130T/+}$  mice presented with many ducts having TEBs extending towards the lymph node within a well-developed stroma (Figure 2.6A), however, ductal length relative to the length of the gland was significantly delayed by ~45% in mutant mice (Figure 2.6D). Interestingly, both 7 and 10 week old mutant and control mice showed similar levels of ductal invasion, ductal lengths and side branches throughout the fat pad (Figure 2.6B-D). Together, pre-pubertal  $Cx43^{I130T/+}$  mice exhibit smaller mammary glands with delayed ductal development which was not observed following the onset of puberty.

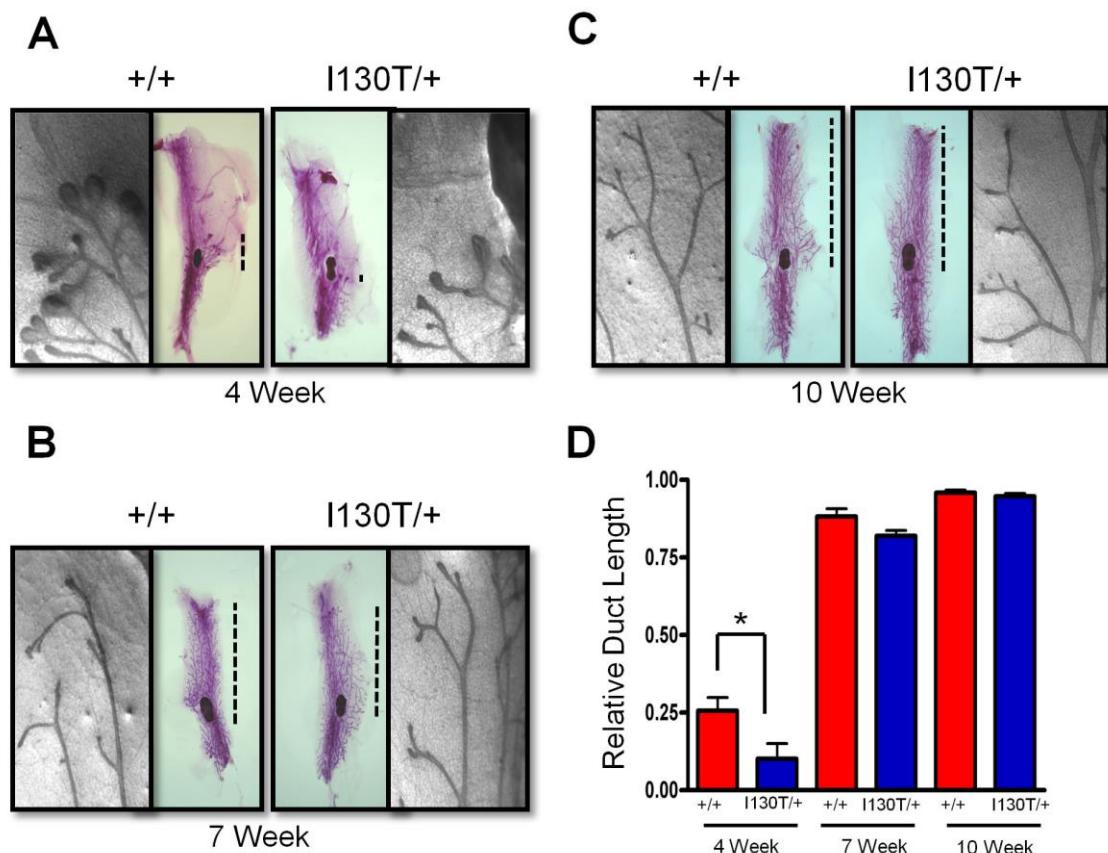
**Figure. 2.4. The highly phosphorylated species of Cx43 were reduced in the mammary gland of *Cx43<sup>II30T/+</sup>* mice at parturition while co-expressed mammary gland connexins remain unchanged.** (A) Western blot analysis of Cx43 revealed significantly reduced levels of the highly phosphorylated species (P) of Cx43 while the primarily unphosphorylated (P0) species remained similar to that found in littermate control mice. Bars represent mean levels of total, P0 and P relative to  $\beta$ -actin  $\pm$  S.E.M. (\*p<0.05) (N=6). (B) Mammary gland sections were immunolabeled for Cx43 (red) and keratin-14 (green), while nuclei were stained with Hoechst (blue). (C) Punctate Cx43-gap junction plaques (B, arrowheads) present at cell-cell interfaces of keratin-14 positive cells were significantly reduced in *Cx43<sup>II30T/+</sup>* mice compared to control mice (bars = 20  $\mu$ m). Bars represent mean number of Cx43-gap junction plaques relative to pixel area of nuclei per 0.18 mm<sup>2</sup>  $\pm$  S.E.M. (\*\*p<0.001) (N=5). (D) Paraffin-embedded mammary gland sections double immunolabeled for Cx26, Cx32 or Cx30 (red) and keratin-14 (green) revealed a similar profile of gap junctions between mutant and control mice. N=6.

**Figure 2.4**

**Figure 2.5. Pre-pubertal  $Cx43^{II30T/+}$  mice have smaller mammary glands.** (A) Body weights were recorded from virgin 4, 7 and 10 week old mice revealing similar body weights in  $Cx43^{II30T/+}$  mice compared to littermate controls. Bars represent mean body weights  $\pm$  S.E.M. (B) Inguinal mammary gland weights recorded in virgin mice revealed significantly reduced gland weight in mutant mice at 4 weeks. Bars represent mean mammary gland weight  $\pm$  S.E.M. (C) When normalized for body weight; mammary glands from 4 week old mutant mice were significantly smaller compared to controls. Bars represent mean normalized gland weight  $\pm$  S.E.M. \* $p<0.05$ . N=6.

**Figure 2.5****A****B****C**

**Figure 2.6. Pre-pubertal  $Cx43^{II30T/+}$  mice have delayed mammary gland development.**  
(A, B, C) Whole mount analysis of 4, 7 and 10 week mutant and control mice revealed similar gland architecture with ducts elongating from TEBs through a developed stromal mammary fat pad. (D) Duct elongation from 4 week old mutant mice was significantly delayed compared to controls. Bars represent the mean ductal extensions from the bottom of the lymph node to the furthest migrating duct (Dashed lines in A,B,C) relative to the length of the mammary gland from the bottom of the lymph node to the edge of the fat pad  $\pm$  S.E.M. \* $p<0.05$ . N=6.

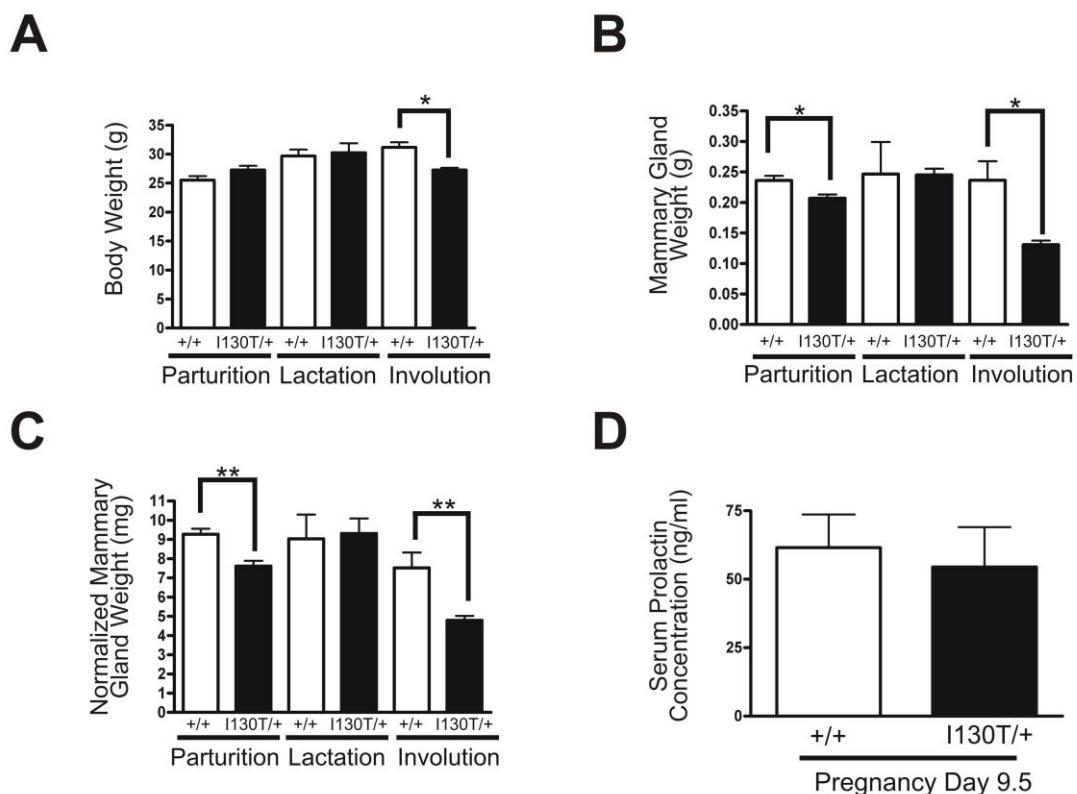
**Figure 2.6**

### 2.3.5 $Cx43^{I130T/+}$ mice have smaller mammary glands at parturition and involution

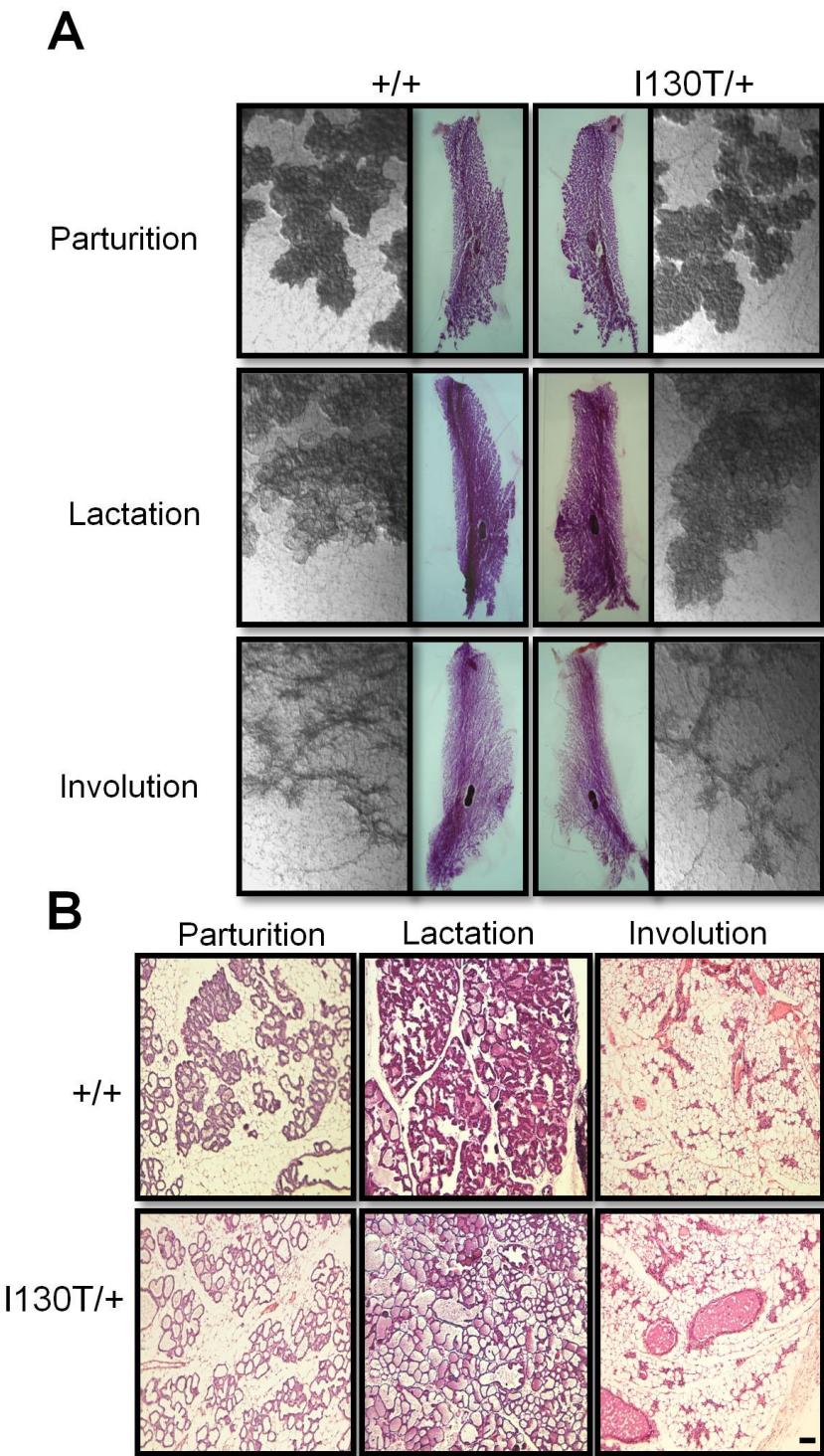
To assess if the I130T mutant affects the development of the mammary gland during pregnancy, lactation and involution, body and mammary gland weight were evaluated in mutant and  $Cx43^{+/+}$  mice. At parturition and lactation, body weight was similar in  $Cx43^{I130T/+}$  mice compared to control mice (Figure 2.7A). However, body weight was significantly reduced by ~12% in mutant mice compared to controls at involution (Figure 2.7A).  $Cx43^{I130T/+}$  mice showed a significant reduction in mammary gland weight at parturition and involution and when this was normalized to body weight, glands were ~20% smaller at parturition and ~33% smaller at involution compared to  $Cx43^{+/+}$  mice (Figure 2.7B, C). Normalized mammary glands of mutant mice were similar to controls during lactation (Figure 2.7C). To assess if changes in the weight of mutant and wild-type mice were the result of differences in prolactin hormone signaling, serum prolactin concentrations were assayed and found to be similar in  $Cx43^{I130T/+}$  and  $Cx43^{+/+}$  mice during mid-pregnancy (Figure 2.7D). Taken together, smaller mammary glands at parturition and involution suggests gland developmental defects at these time points were not a result of reduced prolactin in the blood.

To identify if the change in weight of mutant mouse mammary glands represented an acute indicator of structural changes within the gland, whole mount analysis revealed mammary glands filled with alveoli and ducts from  $Cx43^{I130T/+}$  mice at parturition and lactation consistent with normal mammary gland development in control mice (Figure 2.8A). Following the onset of involution, mammary glands from  $Cx43^{I130T/+}$  mice showed a reduction in the number of alveoli similar to  $Cx43^{+/+}$  mice, suggesting that the epithelial compartment within the gland was in a similar stage of gland remodelling compared to control (Figure 2.8A). Similarly, histological analysis revealed numerous alveoli and ducts with evidence of milk within the lumen of mutant and control mice at parturition and lactation (Figure 2.8B). In addition, mammary glands of both  $Cx43^{I130T/+}$  and  $Cx43^{+/+}$  mice had undergone extensive gland remodeling at involution, in which a reduction of alveoli was apparent within the stroma (Figure 2.8B).

**Figure 2.7. *Cx43<sup>II30T/+</sup>* mice have reduced mammary gland weights at parturition and involution.** (A) Evaluation of body weights during parturition, lactation and involution revealed that *Cx43<sup>II30T/+</sup>* mice were significantly smaller compared to control mice at involution. Bars represent mean body weights  $\pm$  S.E.M. N=6. (B, C) Mammary gland weight and normalized mammary gland weight were significantly reduced in *Cx43<sup>II30T/+</sup>* mice at parturition and involution compared to controls. N=6. (D) Serum isolated from mutant and wild-type mice at mid-pregnancy contained similar levels of prolactin. N=4. Bars represent means  $\pm$  S.E.M. \*p<0.05, \*\*p<0.01.

**Figure 2.7**

**Figure 2.8. Lactating and involuting  $Cx43^{II30T/+}$  mice have normal mammary gland architecture.** (A) Whole mount analysis of mice at parturition, lactation and involution revealed similar alveolar and ductal gland structure in mutant mice compared to controls. (B) Haematoxylin and eosin staining revealed similar histology in mutant and control mice at all time points. Scale bars= 100  $\mu\text{m}$ . N=6.

**Figure 2.8**

In another assessment of potential changes in the mutant mouse mammary glands at parturition, paraffin-embedded mammary glands were immunolabelled with the proliferation marker PCNA. These studies revealed a significant decrease in the number of epithelial cells undergoing proliferation in *Cx43<sup>I130T/+</sup>* mice (Figure 2.9A). In addition, cleaved caspase 3 immunolabelling of mammary glands 48h following weaning of pups showed minimal staining of cells undergoing apoptosis in *Cx43<sup>I130T/+</sup>* mice similar to controls (Figure 2.9B). Collectively, dysregulation of proliferation in mammary glands at parturition but not apoptotic mechanisms during involution may account for changes in mammary gland weight in the *Cx43<sup>I130T/+</sup>* mice.

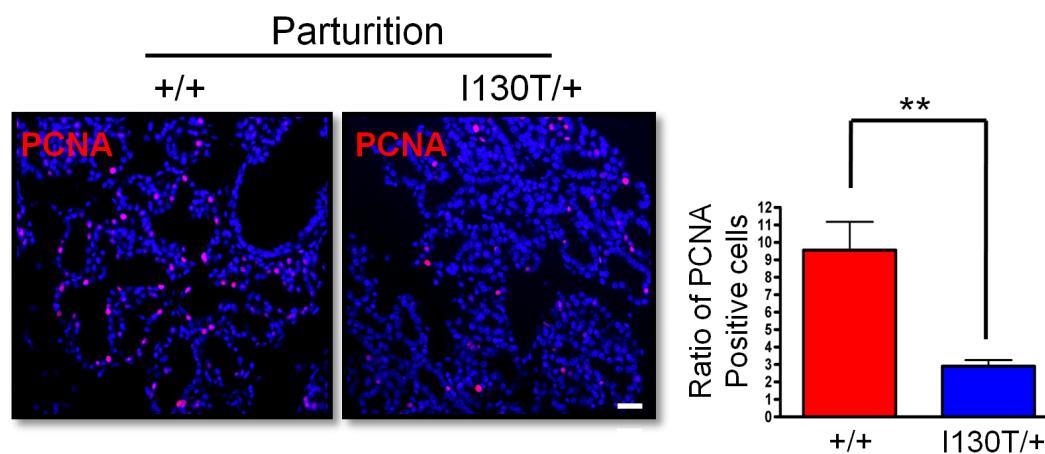
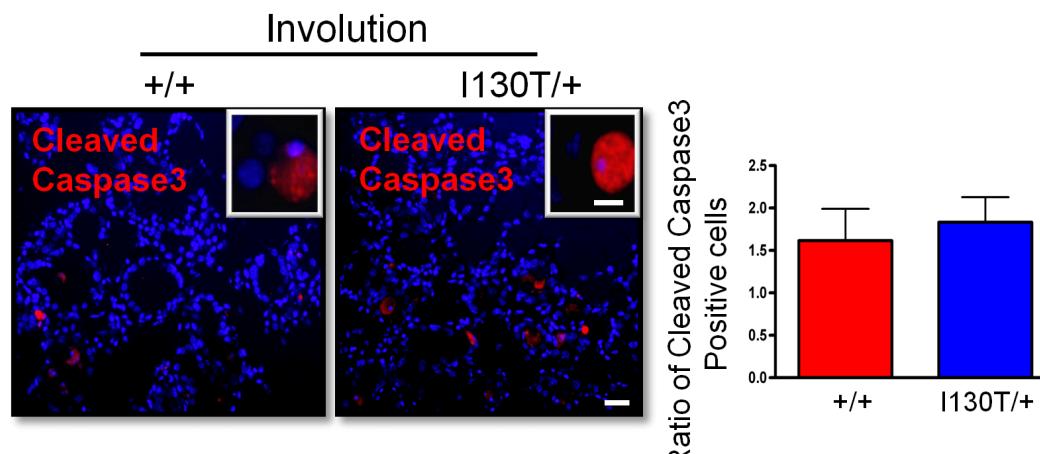
### 2.3.6 *Cx43<sup>I130T/+</sup>* mice show no signs of a lactational or milk ejection defect

In order to assess if the I130T mutant affected lactation in *Cx43<sup>I130T/+</sup>* mice, western blot analysis of two commonly produced milk proteins,  $\beta$ -casein and WAP, revealed similar protein levels in mutant and control mice at parturition (Figure 2.10A, B). In addition, to assess if *Cx43<sup>I130T/+</sup>* mice exhibited a defect in the contraction of myoepithelial cells, mammary glands of lactating mutant mice were assessed for milk entry into ducts following exposure to exogenous oxytocin. Similar to *Cx43<sup>+/+</sup>* mice, mutant mice showed the presence of low levels of milk within the ducts of the thoracic mammary glands prior to the addition of oxytocin (Figure 2.10C). Following treatment with 80  $\mu$ g/ml or 8 pg/ml oxytocin, milk rapidly filled the mammary gland ducts of *Cx43<sup>I130T/+</sup>* and control mice causing them to become easily observable (Figure 2.10D). These findings are consistent with the fact that *Cx43<sup>I130T/+</sup>* female mice can readily feed their pups. Together, mammary glands from mutant mice had no observable functional defects in milk production or ejection compared to control mice.

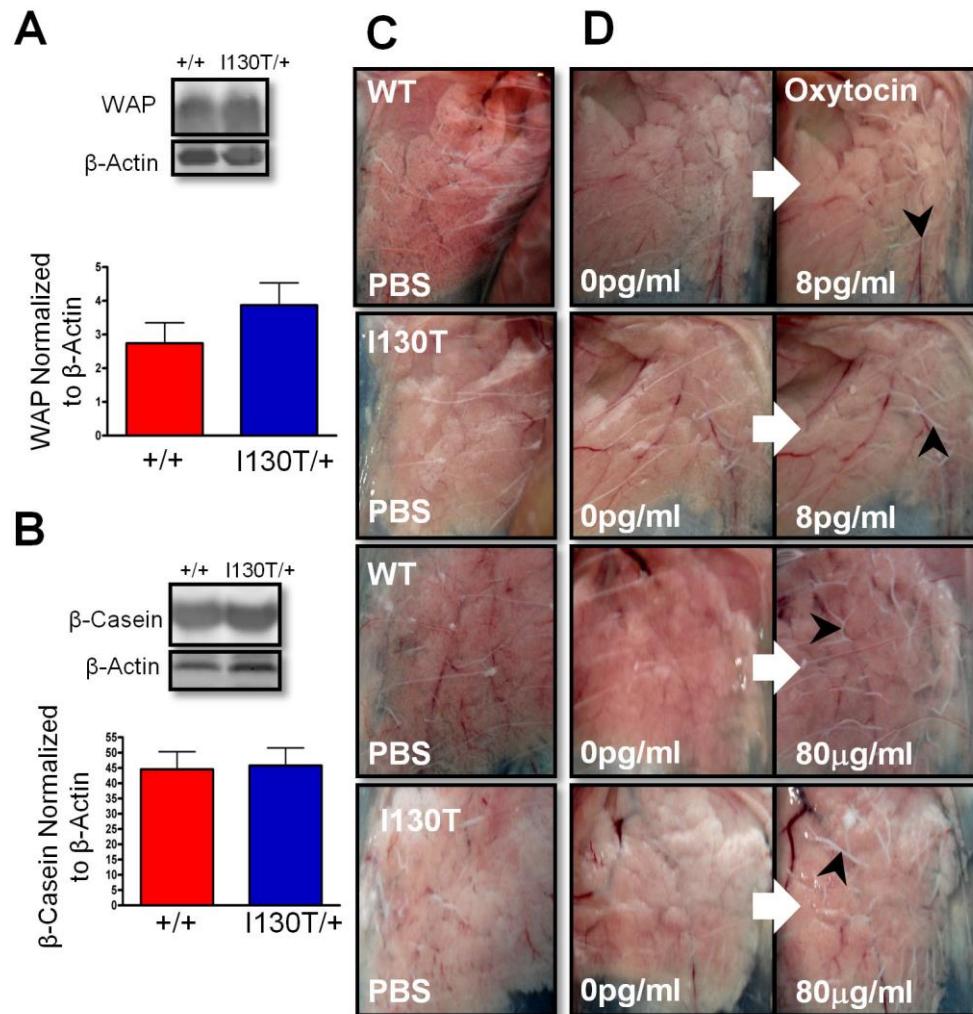
## 2.4 Discussion

The focus of this study was on two aspects: first, to evaluate the role of Cx43 throughout all stages of mammary gland development; and second, to determine if a Cx43 ODDD mutant with residual channel forming activity would be sufficient to prevent any functional or developmental

**Figure 2.9. Mammary glands from *Cx43<sup>II30T/+</sup>* mice exhibited a reduction in proliferation at parturition.** (A) Assessment of the proliferation marker PCNA in paraffin-embedded mammary glands at parturition revealed a significant decrease in cell proliferation in *Cx43<sup>II30T/+</sup>* mice compared to controls (\*\*p<0.01). (B) Evaluation of the apoptotic marker cleaved caspase3 at involution revealed no change between mutant and control mice. Bars represent mean positive cells relative to area of nuclei per 0.18 mm<sup>2</sup> ± S.E.M. Scale Bars = 50 µm (10 µm for insets). N=6.

**Figure 2.9****A****B**

**Figure 2.10. Lactating  $Cx43^{II30T/+}$  mice can produce and eject milk into ducts upon oxytocin stimulation.** (A, B) Western blot analysis revealed that  $Cx43^{II30T/+}$  mice express the common milk proteins, WAP and  $\beta$ -Casein, similar to controls. Bars represent mean expression  $\pm$  S.E.M. (C, D). An oxytocin-induced milk ejection assay revealed that lactating  $Cx43^{II30T/+}$  mice respond to exogenous oxytocin to deliver milk into ducts (arrowheads). Images in (D) represent the same gland field before and after (separated by arrows) the addition of oxytocin. N=4.

**Figure 2.10**

defects in the mammary gland. In this pursuit, we obtained a genetically-modified mouse model of ODDD that harbors a systemic autosomal dominant I130T mutation. First, we determined that the Cx43 I130T mutant has partial channel activity when expressed in the absence of other connexins and impairs Lucifer yellow dye transfer less than the G60S mutant when co-expressed with Cx43 in primary myoepithelial and granulosa cells. Second, we showed that, while they exhibited similar gland architecture, mammary glands from virgin pre-pubertal I130T mutant mice were smaller than matched controls and exhibited a delay in ductal elongation prior to the onset of puberty. Third, we found that mammary glands from *Cx43<sup>I130T/+</sup>* mice were smaller than controls at parturition due in part to impaired cell proliferation. Finally, we identified that mammary glands from I130T mutant mice exhibited no evidence of a defect in milk production or milk ejection. Thus, we clearly demonstrated that Cx43 is necessary for timely mammary gland development. In addition, we surmised that two distinct autosomal dominant mutations in the *GJA1* gene encoding Cx43 (I130T and G60S) can manifest as distinctly different phenotypes during gland development that appear to be dependent on the potency of the mutation in inhibiting GJIC.

#### 2.4.1 Differential coupling capacity of ODDD-linked mutants

ODDD mutants have previously been shown to present with differential abilities to traffic to cell-cell appositions and form plaques, to participate in GJIC and to act as dominant-negatives on the function of co-expressed wild-type Cx43 [23,30]. We evaluated the G60S and I130T mutants and found that only the I130T mutant retained partial junctional conductance when expressed alone in connexin-deficient N2A cells. This is consistent with others that also identified that the I130T mutant retained the ability to form gap junction plaques with residual coupling activity, while the G60S mutant was maintained mainly in an intracellular profile and functionally dead [17,23,30]. However, in ODDD disease these individual mutants are always co-expressed with wild-type Cx43, thus their impact on total Cx43 function needed to be assessed in primary cells from mutant mice that express only Cx43. To that end, in both primary myoepithelial and granulosa cells we discovered that the I130T mutant caused a smaller reduction in dye transfer compared to the G60S mutant when normalized to their appropriate control counterparts. Consistently, we and others

reported a more severe loss of GJIC in primary cells from G60S mutant mice [17,25], but our results presented here are the first to directly compare these two mutants in parallel experiments. Thus, we hypothesized that a higher basal level of total Cx43 function in I130T mutant mice might retain a more normal mammary gland function than we previously documented in the G60S mutant mice where the gland exhibits significant developmental delays and failed to eject milk [20].

Cx43 is known to be expressed in the mammary gland throughout development and differentiation and evidence suggests that at parturition Cx43 levels increase and the more highly phosphorylated species of Cx43 become more evident [14]. Similar to our findings in myoepithelial and granulosa cells, Kalcheva et al. (2007) showed that the highly phosphorylated species of Cx43 were less prevalent in cardiomyocytes of I130T mutant mice and this was accompanied by a reduction in Cx43 gap junctional plaques [25]. Thus, the reduction in GJIC observed in cells isolated from I130T mutant mice was not surprising as the highly phosphorylated species of Cx43 have been correlated with the level of Cx43 assembled into functional gap junction plaques [31]. Not unlike our observations in the *Cx43<sup>G60S/+</sup>* mice [20,21], changes in Cx43 expression and phosphorylation had no effects on Cx26, Cx30 or Cx32, suggesting no cross-talk between Cx43 changes and other mammary gland connexins.

#### **2.4.2 A full complement of Cx43 is necessary for the regulated development of mammary glands in virgin mice**

Prior to puberty, ductal elongation is largely hormone independent and is believed to be regulated by locally acting factors between the epithelium and the stroma [6,32]. At 4 weeks, *Cx43<sup>G60S/+</sup>* mice exhibited a delay in ductal elongation and severely impaired stromal development suggesting a role for Cx43 in ductal morphogenesis prior to puberty [20]. Although it is unknown if the delay in ductal development is a result of dysregulation of Cx43 in the stroma or in the epithelium, extracellular matrix components are able to bind and sequester signalling molecules that affect ductal/branching morphogenesis, suggesting that improper stromal development may contribute to the delay in ductal elongation observed in *Cx43<sup>G60S/+</sup>* mice [33]. Although slightly smaller mammary glands were observed in *Cx43<sup>I130T/+</sup>* mice at 4 weeks, these mice displayed a delay in ductal

differentiation despite exhibiting relatively normal stromal development suggesting that the poorly developed stroma in  $Cx43^{G60S/+}$  mice was not the only contributing factor underlying the delay in development. As such, our evidence provides support for a role of epithelium-localized Cx43 in regulating ductal growth prior to the onset of puberty.

At puberty, ductal growth becomes estrogen-dependent which is exemplified by the fact that estrogen receptor  $\alpha$  knockout mice have limited ductal elongation during puberty [34]. In  $Cx43^{G60S/+}$  mice, the onset of hormones at puberty was unable to overcome the delay in development observed in pre-pubertal mice, even after 10 weeks. Thus, we suggested that GJIC may in part mediate estrogen signaling between the epithelium and stroma [20]. Interestingly, ductal length from  $Cx43^{II30T/+}$  mice was similar to WT mice one week following the onset of puberty, suggesting three possible roles for Cx43 in pubertal ductal morphogenesis. First, that estrogen-driven signaling of ductal morphogenesis may not be dependent on GJIC and that the delay in development observed in  $Cx43^{G60S/+}$  following puberty was a result of improper stromal development [20]. Second, that the delay in ductal morphogenesis following puberty is a result of GJIC-independent mechanisms, which are further dependent on the mutant expressed. This concept is supported by the observation that Wnt5a overexpressing mice, in which Wnt5a overexpression was shown to decrease GJIC *in vitro*, had similar ductal morphogenesis compared to controls [35]. However, whether GJIC is reduced in the virgin mammary gland of Wnt5a overexpressing mice is unknown, thus limiting the impact of these results. Thirdly, the higher residual Cx43 function observed in mammary cells of  $Cx43^{II30T/+}$  mice, nearly double of that seen in  $Cx43^{G60S/+}$  mice, is sufficient to mediate estrogen or other hormonal signals during ductal morphogenesis. Although further mechanistic details remain to be elucidated, it is clear that Cx43 plays a role in regulating the development of the mammary gland in virgin mice.

#### **2.4.3 Cx43 regulates proliferation in the mammary gland following the onset of pregnancy**

Following the onset of pregnancy, the mammary gland enlarges as the epithelial to adipocyte ratio increases as a result of extensive proliferation [5]. Similar to what we have reported for the  $Cx43^{G60S/+}$  mice, mammary glands of  $Cx43^{II30T/+}$  mice were significantly smaller at parturition compared to WT controls but here we extend our understanding of

the process by showing that the defect was due to reduced cell proliferation in  $Cx43^{II130T/+}$  mice. Cx43 has previously been implicated in pathways that affect regulators of the cell cycle such as cyclin D1 [36]. Interestingly, *cyclin D1* null mice have poor alveolar development during pregnancy as a result of reduced proliferation, suggesting a possible regulatory pathway between Cx43 and cyclin D1 [37]. It is also important to note that both ODDD mutant mouse models showed similar gland architecture and histology suggesting that Cx43's role in proliferation of the mammary gland is not critical for development of the gland during pregnancy.

After weaning of the pups, the mammary gland undergoes involution which is characterized by both apoptotic cell death of epithelial cells and stromal activation [38]. Compared to their control counterparts,  $Cx43^{II130T/+}$  mice had significantly smaller mammary glands two days post weaning suggesting accelerated involution but this was not supported by the gross and histological evaluation of the epithelial ducts which revealed no observable difference. In addition, the level of apoptosis within the glands was similar to control. Therefore, the decrease in mammary gland weight observed in  $Cx43^{II130T/+}$  mice likely reflects changes in the stromal compartment of the gland, which is composed mainly of adipocytes. Importantly, the gap junction blocker 18-alpha-glycyrrhetic acid and siRNA knockdown of Cx43 have previously been shown to inhibit adipogenesis in pre-adipocytes, suggesting a role for Cx43 in regulating adipocyte differentiation [39].

Given the systemic reduction in Cx43 function in I130T mutant mice and the fact that this genetic modification causes a small reduction in the body weight of mutant mice, it is possible that hormonal signaling that regulates mammary gland development is altered due to the compromised role of Cx43 in endocrine glands such as the ovaries, adrenal gland or pituitary gland [40]. In this pursuit, we hypothesized that changes in Cx43 affected prolactin regulation which is supported by correlational evidence that an increase in Cx43 is associated with an increase in prolactin secretion [42]. Furthermore, prolactin receptor knockout mice exhibit reduced adipose tissue mass throughout the body including the mammary gland as a result of decreased adipocyte cell numbers, suggesting a role of prolactin in adipocyte proliferation and differentiation [41]. Our evaluation of serum prolactin levels at mid-pregnancy revealed no significant differences between mutant and

wild-type mice suggesting that reduced Cx43 function does not affect secretion of prolactin into the blood. However, it is unknown if changes in Cx43 alter prolactin signalling downstream in the mammary gland at the level of the prolactin receptor or whether this reflects changes in other hormones. Thus, we conclude that loss-of-function mutations in Cx43 may directly or indirectly affect the stromal component of the mammary gland during involution.

#### 2.4.4 Partial coupling by Cx43 gap junction channels is sufficient for normal mammary gland function

Together with our previous findings using *Cx43<sup>G60S/+</sup>* mutant mice [21], our results here strongly support the conclusion that a full complement of Cx43 is not necessary for the synthesis of constitutive proteins of milk. Due to Cx43 being mainly restricted to myoepithelial cells of mammary gland epithelium, previous studies suggested that Cx43 plays a role in myoepithelial differentiation, coordinated myoepithelial contraction and proper milk ejection [7,14]. However, it was not until our evaluation of *Cx43<sup>G60S/+</sup>* mice at parturition that we determined that Cx43 was essential for proper oxytocin-induced milk ejection which was later supported following examination of Wnt-5a over-expressing mice that presented with a similar ejection defect that the authors linked to severely impaired Cx43-mediated GJIC [35]. Surprisingly, *Cx43<sup>H30T/+</sup>* mice exhibited no apparent defect in milk ejection upon oxytocin stimulation and females could readily feed pups. Given that myoepithelial cells from *Cx43<sup>H30T/+</sup>* mice are approximately twice as well coupled by Cx43 gap junctions as cells from the *Cx43<sup>G60S/+</sup>* mice, we conclude that there was sufficient residual Cx43-based GJIC to rescue the milk ejection defect. The idea that different mutants of ODDD result in differential changes in the molecular properties of the Cx43 gap junctional channel has been suggested to contribute in part to the pleiotropic phenotype observed in ODDD patients [19]. However, it is also important to note that differences observed in myoepithelial GJIC may be due in part to the different mixed mouse strain backgrounds being studied, although both mutant mouse lines have been partially backcrossed to BL6. Importantly, the ODDD patient population includes all ethnic groups of mixed origin, suggesting that the variations observed in the mutant mice being studied

may in fact somewhat reflect the heterogeneity of the disease in the human patient population.

## 2.5 Conclusions

In summary, unlike the previously studied  $Cx43^{G60S/+}$  mice with severely compromised Cx43 function, we found that  $Cx43^{II30T/+}$  mice with partial Cx43 mutant channel function present with limited mammary gland developmental defects. In essence, severe defects in mammary gland function appear to require in excess of a 50% reduction in total Cx43 function which is consistent with the fact that  $Gja1^{+/-}$  knockout mice, expressing only a 50% complement of Cx43, are relatively normal and have no evidence of lactation or milk ejection defects [43]. In conclusion, we predict that ODDD patients that harbor mutations that maintain total Cx43 function in the breast at 50% or better will not suffer from milk production or ejection defects as appears to be the case for the vast majority of documented ODDD patients to date. Importantly, our study also highlights the fact that the use of multiple genetically-modified mouse models is beneficial for establishing results that translate to different populations of ODDD patients.

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## Chapter 3

### 3 Mammary gland specific knockout of the physiological surge in Cx26 during lactation retains normal mammary gland development and function

Cx26 has previously been suggested to be important in maintaining epithelial survival during alveogenesis following the onset of pregnancy. The purpose of this study was to further characterize the role of Cx26 during pregnancy and lactation in the mammary gland using a novel mouse model with mammary gland specific knockout of Cx26 during pregnancy. In addition, this study also aimed to address apparent discrepancies between the lack of breast feeding defects reported by hearing-loss female patients that harbor loss-of-function Cx26 mutants, and the severely impaired lactation defects previously described using conditionally ablated Cx26 mouse models.

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### 3.1 Introduction

Breast morphogenesis is unique to other organ systems in that the majority of its development occurs after birth. The progression towards a terminally differentiated gland requires the onset of pregnancy in which alterations in hormones and cell-cell signaling results in the growth and increased branching of ducts, along with the differentiation of alveolar cells to form the mature milk-secretory organ [1]. Weaning leads to the regression and remodeling of the breast to a virgin gland state in a process known as involution. The mammary gland of mice is comparable to that of humans and has frequently been used as a model to study mammary gland development [2]. Although much is known about the hormonal control of mammary gland throughout development, less is known about local communication such as the gap junction proteins connexins (Cxs) [3,4]. Importantly, gap junctions have been shown to play a role in coordinating cellular tissue responses downstream of hormonal/paracrine stimuli, suggesting that dysregulation of Cxs may alter hormonally controlled organ development and function [5].

Six connexin subunits oligomerize to form a hemichannel (or connexon) capable of permitting the exchange of small molecules between the intracellular and extracellular environment. More commonly, connexons from one cell dock with connexons from an adjacent cell to allow for direct gap junctional intercellular communication (GJIC) [6]. Two connexins, Cx26 and Cx43, were shown to be expressed within the breast of humans in which Cx26 is the only connexin localized to the luminal epithelial cells [7]. In mice, Cx26 is expressed at low levels at all stages of mammary gland development and is dramatically upregulated during pregnancy to become the most predominantly expressed connexin within the mammary gland [3,8]. As such, Cx26 has previously been suggested to play a role in coordinating gland development prior to secretory activation as well as having a possible role in maintaining normal tissue homeostasis and differentiation of the non-pregnant mammary gland [3]. In addition, luminal cells in the mouse mammary gland also express Cx32 and Cx30, which are able to form heteromeric/heterotypic channels with Cx26 during pregnancy and lactation. Heteromeric/heterotypic channels alter the properties and permeabilities of luminal gap junction channels, although the

transjunctional molecular exchange through these junctional networks remains unclear [9,10].

Homozygous knockout mice have been unsuccessful in determining the role of Cx26 in the mammary gland. *Gjb2*<sup>-/-</sup> mice die *in utero* as a result of placental defects rendering examination of the mammary gland impossible [11]. Similarly, the generation of *Gjb2*<sup>+/Gjb1</sup> mice die embryonically as a result of severe lymphedemas [12]. To overcome this, two conditionally ablated mice using the Cre-*loxP* system were developed to assess the role of Cx26 in the mammary gland [13]. *Gjb2*<sup>f/f</sup> (*Cx26*<sup>f/f</sup> mice) crossed with mice expressing the Cre transgene under a whey acidic protein (WAP) promoter (referred to as *Cx26*<sup>f/f</sup>; WC) or the mouse mammary gland tumor virus (MMTV) promoter (referred to as *Cx26*<sup>f/f</sup>; MC) were generated. The MMTV promoter expresses Cre recombinase in the mammary gland epithelium prior to birth while the WAP promoter initiates the expression of Cre in the later stages of pregnancy [14]. Interestingly, *Cx26*<sup>f/f</sup>; MC mice showed reduced lobuloalveolar development compared to controls due to an increase in apoptosis while no change was observed in *Cx26*<sup>f/f</sup>; WC mice. As a result, pups from *Cx26*<sup>f/f</sup>; MC dams were more likely to die before being weaned, likely from starvation [13]. It was suggested that Cx26 during early pregnancy plays a critical role in epithelial cell survival and that loss of Cx26 may result in abnormal lactation [13].

However, two discrepancies exist that highlight the need for further studies to evaluate the function of Cx26 in mammary gland development and function. First, Yuan et al. (2011) recently found that control *MMTV-Cre* mice developed by the Hennighausen laboratory (as the founding *MMTV-Cre* line A) had impaired mammary gland developmental defects, cautioning the use of this Cre mouse line in mammary gland development studies [15]. It is unknown which *MMTV-Cre* mouse line was used by Bry et al (2004) and whether such a concern existed in their study [13]. However pregnant *MMTV-Cre* line A mice were found to develop with reduced lobuloalveolar formation at the onset of lactation as a result of increased apoptosis, while virgin glands developed normally, results consistent with the findings in the *Cx26*<sup>f/f</sup>; MC mouse line [13,15]. As a result, additional studies are needed to further evaluate the role of Cx26 in mammary gland development and function. Secondly, a human population of patients exist with systemic dysregulated Cx26

expression and loss of Cx26 channel function that have never been reported to have altered breast function. Over 90 mutations in the *GJB2* gene that encodes Cx26 give rise to both syndromic and non-syndromic deafness that accounts for approximately half of congenital cases of hearing impairments [16]. Mutations in *GJB2* may result in both autosomal dominant and recessive loss of function mutations, of which the most frequent recessive mutations may lead to considerable, if not total, ablation of Cx26 channel function [17]. Interestingly, despite an incidence rate of this mutation similar to that of cystic fibrosis, there are no reports of breast feeding problems in the deaf community, suggesting that Cx26 may not be essential for normal gland function [16,18].

Thus, in order to further evaluate the role of Cx26 in the mammary gland, we developed a novel Cx26 conditional knockout mouse model in which *β-lactoglobulin (BLG)-Cre* mice were crossed with *Cx26<sup>f/f</sup>* mice. The resulting mice were found to have greatly reduced Cx26 expression in the mammary gland yet retained normal gland development, differentiation and function. These findings were even more remarkable given that cross-talk mechanisms cause a co-regulated reduction in Cx30. Taken together, these findings strongly suggest that mammary gland function can proceed normally in the absence of the physiological surge in Cx26 that occurs during pregnancy and in the presence of a substantial loss of gap junctional exchange of signalling molecules.

## 3.2 Methods

### 3.2.1 Animals

*BLG-Cre* mice [19] were crossed with *Cx26<sup>f/f</sup>* mice [20] to produce *BLG-Cre;Cx26<sup>f/f</sup>* mice (Cx26 knockout mice or *Cre+* mice). All mice were genotyped for the presence of the Cre transgene. In addition, *BLG-Cre* mice were crossed with WT mice showing no evidence of a lactation defect. Four virgin, pregnant d9.5 and pregnant d12.5, as well as eight Cx26 knockout mice at parturition, d4 of lactation and d2 of involution, along with *Cx26<sup>f/f</sup>* control mice (*Cre-* mice) at identical timepoints, were sacrificed using CO<sub>2</sub> and O<sub>2</sub>. Thoracic mammary glands were removed and either stored at -80°C (right side) or cryo-embedded (left sided glands), while inguinal mammary glands were either fixed in 10%

neutral buffered formalin and then embedded in paraffin (right sided glands) or processed for whole mounting (left sided glands). Mice were arbitrarily numbered to allow experiments to be performed by a process that was blinded to the investigator. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Western Ontario (2006-101-10) and the University of British Columbia (A11-0170) and the Canadian Council of Animal Care (Appendix 3).

### 3.2.2 Real-Time PCR

Total mRNA was isolated from the tissues using TRIzol (Invitrogen, Burlington, ON) and purified using RNeasy mini-kit (Qiagen, Mississauga, ON) following the manufacturers' instructions. cDNA were generated from isolated mRNA using RevertAid First Strand cDNA Synthesis Kits (Fermentas, Burlington, ON) and then subjected to specific amplification using SsoFast EvaGreen Supermix (Bio-rad, Mississauga, ON) using Cx26 (5' TCCGCATCATGATCCTCGTG 3'; 5' CCCAGAGCCGGATGTGA 3'), Cx30 (5' GCCGAGTTGTGTTACCTG 3'; 5' GCATTCTGGCCACTATCTGA 3'), Cx32 (5' CTTGCTCAGTGGCGTGAATC 3'; 5' CGGCTGGAGGGTGTACAG 3'), Cx43 (5'TATGACAAGTCCTCCCCAT 3'; 5' TGATTCAATCTGCTTCAGG 3') and  $\beta$ 2-microglobulin (5'CCCACTGAGACTGATAACATACGC3'; 5'GGTTCAAATGAATCTTCAGAGCAT3') primers, with a TM at 55°C (N=4).

### 3.2.3 Western blot analysis

Mammary gland tissues were homogenized in lysis buffer and subjected to western blot analysis, as described previously [21], [22]. Membranes were immunoblotted with the following primary antibodies: mouse anti-Cx26 (C14523, Lifespan, Seattle, WA, 1 $\mu$ g/ml), rabbit anti-Cx30 (71-2200, Invitrogen, Burlington, ON, 0.25  $\mu$ g/ml), rabbit anti-Cx32 (C3470, Sigma-Aldrich, Oakville, ON, 0.1  $\mu$ g/ml), rabbit anti-Cx43 (C6219, Sigma-Aldrich, Oakville, ON, 0.1  $\mu$ g/ml), goat anti-WAP and goat anti- $\beta$ -casein (sc-14832, sc-17971, Santa Cruz Biotechnology, Dallas, TX, 0.2  $\mu$ g/ml), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAB374, Millipore, Billerica, MA, 2  $\mu$ g/ml) and rabbit anti-GAPDH (14C10, Cell Signaling, Danvers, MA, 1:1000). Bound primary

antibody was detected using the following fluorescence-conjugated secondary antibodies; Alexa 680-conjugated goat anti-rabbit, Alexa 800-conjugated goat anti-mouse and Alexa 680-conjugated donkey anti-goat (Molecular Probes, Eugene, OR, 0.2 µg/ml) followed by visualization and quantification using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Protein levels were determined by normalization to the western blot band intensities of GAPDH ( $N \geq 6$ ).

### 3.2.4 Immunofluorescence

Paraffin-embedded sections (6 µm) were deparaffinized in xylene and rehydrated in descending concentrations of ethanol baths, microwaved for 5 min (80%) in antigen-retrieval solution (1:50, Vector Labs) and put into 0.01 M Tris/1 mM EDTA buffer (pH 9.0) at 90–95 °C for 30 min, as previously described [22]. Cryo-sections (7 µm) were fixed with 10% neutral buffered formalin. Paraffin and/or cryosections were blocked with 0.1% Triton X-100 and 3% BSA in PBS for 60 min at room temperature. Sections were then incubated with the following primary antibodies: rabbit anti-Cx26 (51-2800, Invitrogen, Burlington, ON, 2.5 µg/ml), rabbit anti-Cx30 (71-2200, Invitrogen, Burlington, ON, 2.5 µg/ml), rabbit anti-Cx32 (C3470, Sigma-Aldrich, Oakville, ON, 1.0 µg/ml), rabbit anti-Cx43 (C6219, Sigma-Aldrich, Oakville, ON, 1.0 µg/ml), mouse anti-pan-Cytokeratin (ab7753, Abcam, Cambridge, MA, 4 µg/ml), mouse anti-Proliferating Cell Nuclear Antigen (PCNA) (M-0879, Dako, Burlington, ON, 0.5 µg/ml), goat anti-WAP (sc-14832, Santa Cruz Biotechnology, Dallas, TX, 2µg/ml) and Wheat Germ Agglutinin-633 (WGA) conjugate (W-21404, Invitrogen, Burlington, ON, 1:400). Primary antibody was visualized by incubating sections with secondary antibodies: Alexa480-conjugated goat anti-rabbit, Alexa480-conjugated goat anti-mouse, Alexa555-conjugated anti-mouse secondary antibody, (Invitrogen, Burlington, ON, 0.5 µg/ml) or anti-goat Texas Red (Jackson ImmunoResearch Laboratories, Baltimore, PA, 1:100). Hoechst stain was used to visualize nuclei prior to mounting. Immunolabeled sections were imaged (5-10 images per sample) using a Leica DM IRE2 inverted epifluorescence microscope equipped with Openlab 5.5.3/Velocity 6.3.0 imaging software or a Zeiss LSM 510 inverted confocal microscope ( $N \geq 6$ ). For cytokeratin area quantification, green only and blue only fluorescent images at 20X magnification were converted to binary using ImageJ and the pixel area was measured.

Graphs represent the mean ratio of green fluorescent area (cytokeratin) to blue fluorescent area (nuclei). For PCNA quantification, the number of PCNA positive cells per 20X field was quantified. In addition, the blue only fluorescent images at 20X magnification were converted to binary using ImageJ and the pixel area was measured. Graphs represent the mean ratio of the number of PCNA positive cells to blue fluorescent pixel area (nuclei).

### 3.2.5 Whole mounting

As previously described [23], the left inguinal mammary glands were excised, spread on glass slides and fixed in Carnoy's fixative (100% EtOH, chloroform, glacial acetic acid; 6:3:1) for 4 h at room temperature. Mammary glands were washed in 70% EtOH for 15 min, gradually rehydrated in water, and stained in carmine alum (2% carmine and 5% aluminum potassium sulfate in water) overnight at room temperature. Tissues were then gradually dehydrated through serial ethanol baths and cleared in xylene overnight. Mammary glands were kept in methyl salicylate until images were captured with a numeric camera (Cybershot, Sony) and a SteREOLumar V12 microscope (Zeiss) ( $N \geq 4$ ).

### 3.2.6 Histology

Paraffin-embedded mammary gland sections (6  $\mu\text{m}$ ) were deparaffinized in xylene for 10 min, rehydrated in descending grades of ethanol baths, and stained with 1% Harris's haematoxylin and 1% eosin. Sections were dehydrated in ascending grades of ethanol and xylene baths and mounted with Cytoseal (Richard-Allan Scientific). Qualitative histological analysis was performed by imaging several arbitrary areas per 20 $\times$  field of view using a Leica DM IRE2 inverted epifluorescence microscope equipped with a ProgRes C5 camera (Jenoptik) and ProgRes Mac CapturePro 2.7.6 imaging software ( $N=8$ ).

### 3.2.7 Evaluation of apoptosis

Apoptotic cells were stained using a commercial kit for TUNEL assay (Apoptag, Chemicon International, Temecula, CA) following the manufacturer's instructions. Slides were treated with 0.5% TritonX-100 in PBS. 5-10 random fields per mouse were evaluated in which the

ratio of the number of apoptotic cells to the pixel area of nuclei were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) for each 20X field (N=5).

### 3.2.8 Statistical analysis

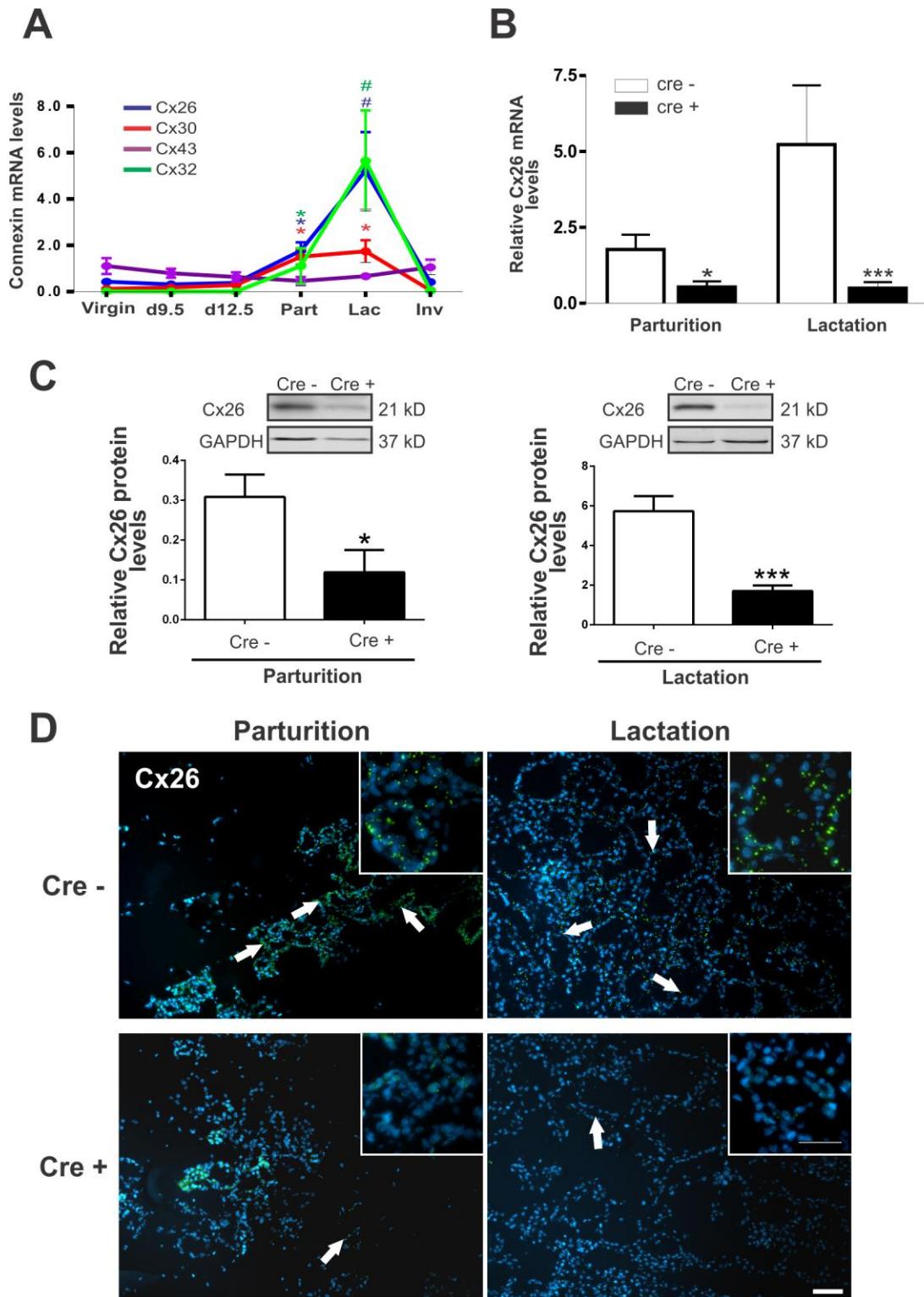
All statistical analyses were performed using GraphPad Prism version 4.03 for Windows. A Student's unpaired t-test or a one-way ANOVA test was used in which a p<0.05 was considered significant. Values are presented as means ± S.E.M.

## 3.3 Results

### 3.3.1 Conditionally ablated *Gjb2* in the mammary gland

In the mammary gland, Cx26, as well as other apical Cx30 and Cx32, expression is dramatically upregulated at parturition and remains elevated throughout lactation (Figure 3.1A, [10]). To assess the efficiency of the Cx26 knockout we performed real-time PCR analysis of Cx26 which revealed a significant decrease, but not complete ablation, of Cx26 mRNA at parturition and lactation (Figure 3.1B). Consistent with these results, western blot and immunofluorescent analysis revealed a significant ~65-70% decrease in Cx26 protein levels (Figure 3.1C) that correlated with a decrease in Cx26 gap junction plaques within the mammary epithelium of *Cre*+ mice at parturition and lactation (Figure 3.1D). As expected, since the BLG promoter is not activated before mid-pregnancy (day 10 of pregnancy), no differences were observed in other stages (Figure 3.S1). Together, residual Cx26 expression suggests that *Cre*+ mice maintain low basal expression of Cx26 throughout the pregnancy and lactation phases of mammary gland development while the typical physiological surge in Cx26 was eliminated.

Figure 3.1. ***BLG-Cre; Cx26<sup>fl/fl</sup>* mice exhibit a dramatic reduction in Cx26.** (A) Real-time PCR analysis of wild-type mice revealed that Cx26, Cx32 and Cx30 are upregulated at parturition and lactation. (B, C) Real-time PCR and western blot analysis of mammary glands from Cre- (open columns) and Cre+ (solid columns) mice revealed a dramatic reduction in Cx26 mRNA and protein levels at parturition and lactation. \*p<0.05, \*\*\*p<0.001. Values are mean levels  $\pm$  S.E.M. N $\geq$ 4. (D) Immunofluorescence for Cx26 revealed a decrease in Cx26 gap junctions (arrows) at parturition and lactation in *Cre+* mice compared to control mice. Hoechst staining denotes the nuclei. N=6. Scale Bar =50  $\mu$ m.

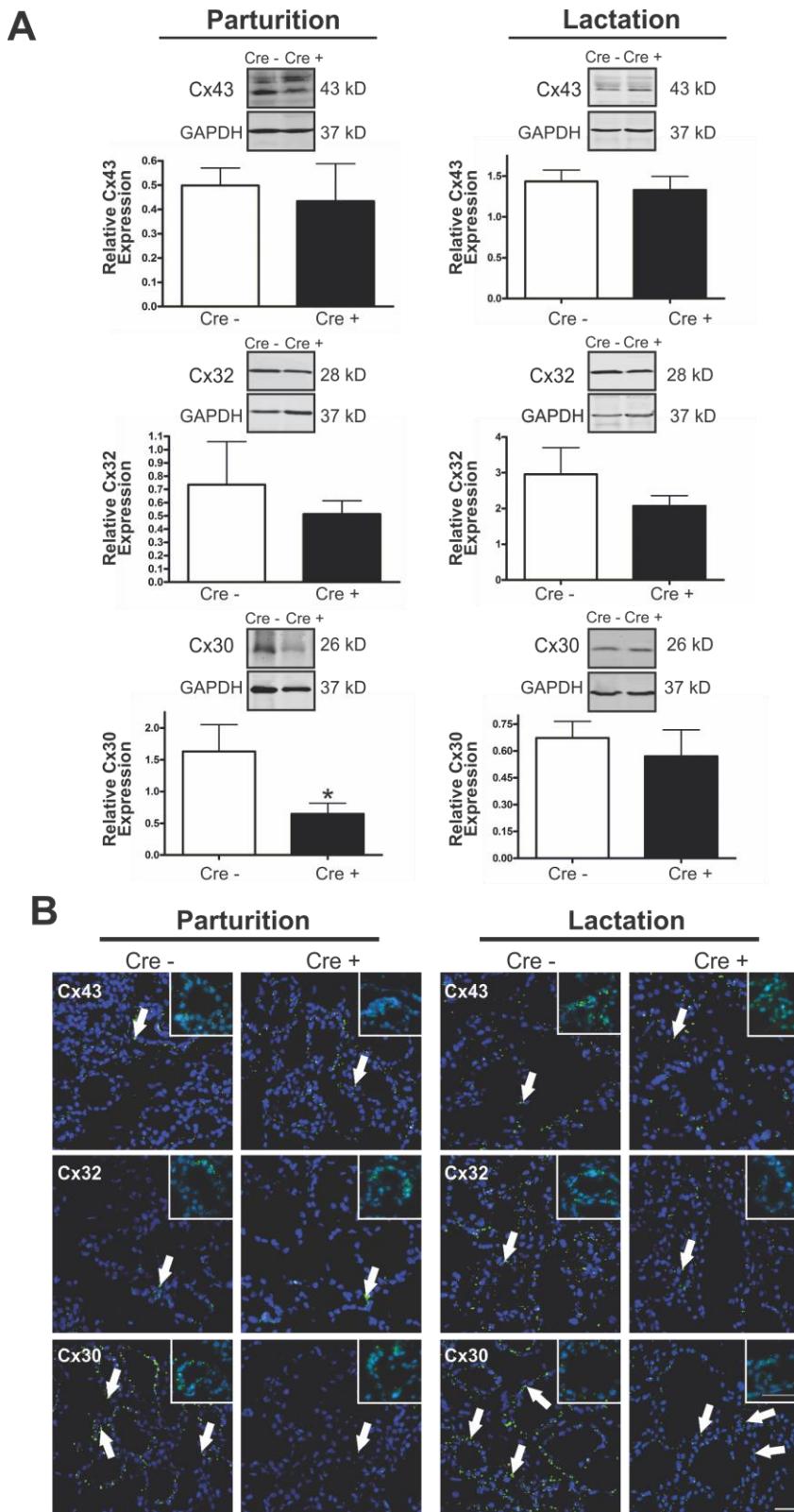
**Figure 3.1**

To assess the status of other connexins co-expressed within the mammary gland in response to a reduction in Cx26, Western blot and immunofluorescent analysis of Cx43, Cx32 and Cx30 were evaluated at parturition and lactation. Protein expression and localization of Cx43 and Cx32 were similar in the mammary glands of lactating *Cre*<sup>+</sup> mice compared to control mice (Figure 3.2A,B). Interestingly, our assays revealed a significant decrease in Cx30 expression at parturition to about ~40% of control and a significant reduction in Cx30 gap junction plaques in lactating *Cre*<sup>+</sup> mice compared to control (Figure 3.2A,B, 3.S2A). Qualitative immunofluorescent analysis of Cx30 gap junctions at lactation showed that Cx30 gap junctions appeared dramatically smaller in *Cre*<sup>+</sup> mice compared to control mice despite a similar number of overall Cx30 gap junction plaques (Figure 3.2B, 3.S2B). Therefore, the *Cre*<sup>+</sup> mice lacked the physiological surge in both Cx26 and Cx30 that are typically observed in the mammary gland during pregnancy.

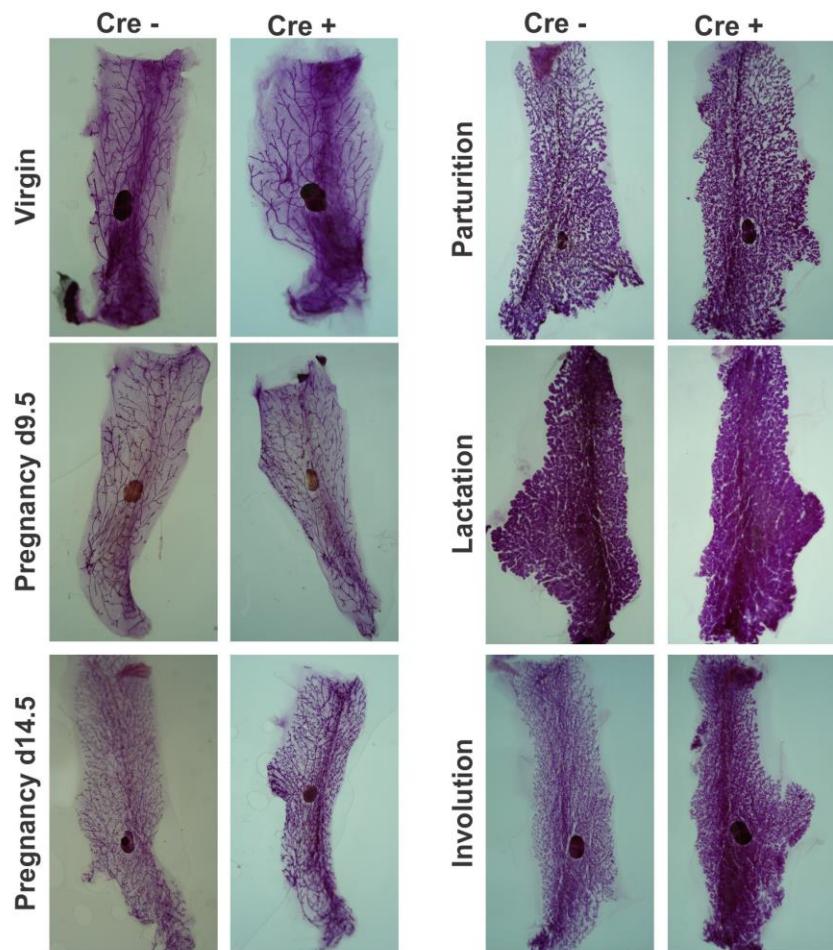
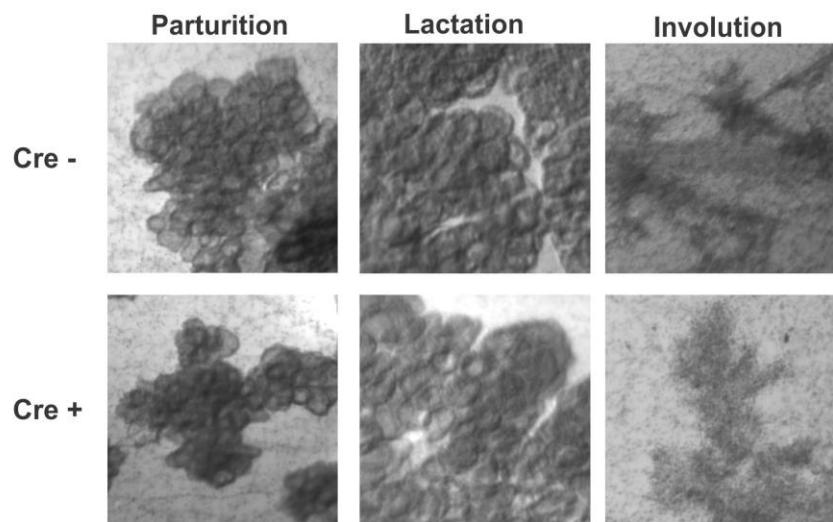
### 3.3.2 *BLG-Cre; Cx26<sup>f/f</sup>* mice retain normal mammary gland development throughout pregnancy

To assess whether a reduction in Cx26 gap junction plaques affects the development of the mammary gland during the pregnancy cycle, whole mount analysis was assessed in the mammary glands of virgin, pregnancy day 9.5, pregnancy day 12.5, at parturition, lactating and involuting mice. Adult virgin mammary glands of genetically-modified mice contained an expansive epithelial ductal network embedded within a developed mammary fat pad similar to control mice (Figure 3.3A). Following the onset of pregnancy, whole mount assessment revealed increased ductal branching and lobuloalveolar structures in the glands of *Cre*<sup>+</sup> mice similar to control mice, suggesting normal timing of alveolar development. In accordance, mammary glands of

**Figure 3.2. Lactating mammary glands of *Cre+* mice exhibited a reduction in Cx30 at parturition.** (A) Western blot and (B) immunofluorescent analysis of mammary glands from control and *Cre+* mice at parturition revealed a significant decrease in Cx30 expression and a reduction in Cx30 gap junction plaques, while no change was observed in either Cx43 or Cx32. Arrows denote connexin plaques. \* $p<0.05$ . Values are mean levels  $\pm$  SEM. N $\geq$ 6. Hoechst staining denotes the nuclei. Scale Bars = 50  $\mu$ m.

**Figure 3.2**

**Figure 3.3. Mammary glands from *BLG-Cre; Cx26<sup>f/f</sup>* mice at all stages of gland development have similar gross gland architecture.** (A) Whole mount analysis of developing and differentiating mammary glands from control and *Cre+* mice revealed similar ductal and alveolar structure as assessed with carmine alum staining. (B) High (25X) magnification of whole mounts revealed developed alveoli in lactating Cx26 knockout mice and alveolar turnover in involuting mice similar to controls. N≥4.

**Figure 3.3****A****B**

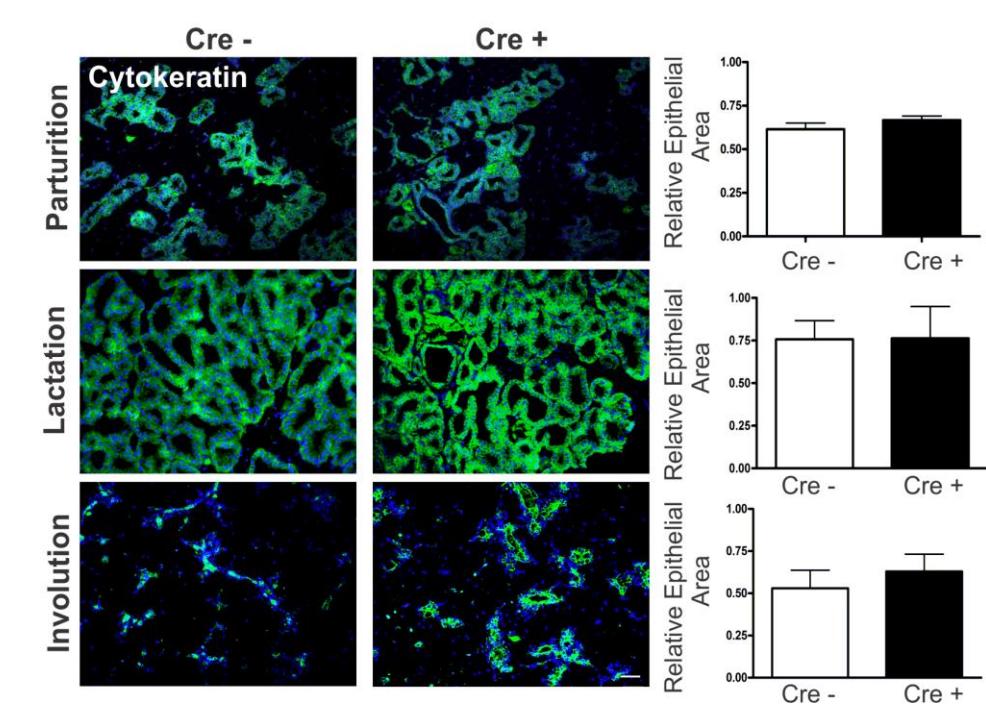
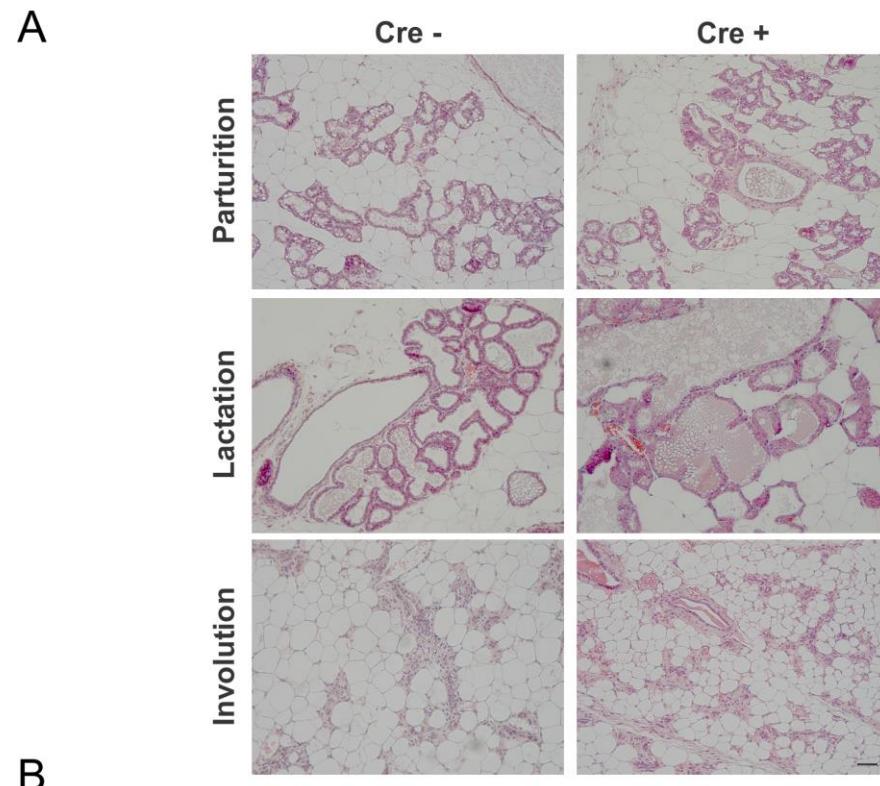
the genetically-modified mice at all stages of development following pregnancy were similar to *Cx26<sup>f/f</sup>* control mice with developed alveoli that fully encompassed the mammary gland by early lactation and comparable alveolar turnover during involution (Figure 3.3A,B).

Since whole mount analysis can conceal alveoli changes due to the density of alveoli, histological analysis of H&E stained sections were performed on control and genetically-modified mice. These studies revealed no qualitative differences in the density of alveoli and ducts throughout the mammary gland regardless of the stage of development after pregnancy (Figure 3.4A). In addition, to quantitatively evaluate whether conditionally reduced Cx26 led to defects in alveogenesis, mammary gland sections at parturition, lactation and involution were immunolabelled with the epithelial marker pan-cytokeratin. Ten arbitrary images were taken of each slide and the area of cytokeratin over the area of the nuclei was quantified. Quantification revealed no significant difference in cytokeratin labelling between *Cre+* mice and *Cre-* control mice at parturition, lactation and involution suggesting normal epithelial turnover throughout lactation and involution (Figure 3.4B).

Previously, Lee et al. (1992) demonstrated that Cx26 mRNA was upregulated in the late G1 and early S phase of normal mammary epithelial cells, suggesting that Cx26 may have a role in luminal cell proliferation [24]. To assess whether Cx26 regulates proliferation through gland development, *Cre+* mice at parturition, lactation and involution were immunolabelled with the proliferation marker PCNA and compared to *Cre-* mice. Quantification revealed no significant difference in the number of PCNA positive cells at all-time points in *Cre+* mice compared to controls suggesting that the physiological surge in Cx26 is not critical in regulating proliferative mechanisms of gland development during pregnancy (Figure 3.5).

As Cx26 has previously been implicated in regulating epithelial cell survival during the early phase of pregnancy, mammary glands of *Cre+* mice were assessed for changes in apoptosis using a TUNEL assay [13]. Quantification of TUNEL positive cells showed relatively low numbers of apoptotic cells at parturition and lactation and increased

**Figure 3.4. Mammary glands of *BLG-Cre; Cx26<sup>f/f</sup>* mice have similar epithelial development.** (A) Haematoxylin and eosin staining of lactating and involuting mammary glands revealed similar histology between genetically-modified and control mice. Scale bars=100µm. (B) Labeling for the epithelial marker, cytokeratin, using an anti-pan-cytokeratin antibody revealed similar levels of labeling in control and genetically-modified mice. Values represent the mean positive-staining pixel area (green) relative to the pixel area of the nuclei (blue) per  $0.18 \text{ mm}^2 \pm \text{S.E.M.}$  N=8. Hoechst staining denotes the nuclei. Scale Bars = 50 µm.

**Figure 3.4**

apoptosis during involution in *Cre*<sup>+</sup> mice similar to *Cre*<sup>-</sup> mice (Figure 3.6). These results suggest that conditional knockout of Cx26 during early pregnancy does not alter epithelial cell survival. Collectively, our results suggest that basal Cx26 expression is sufficient to retain normal mammary gland development.

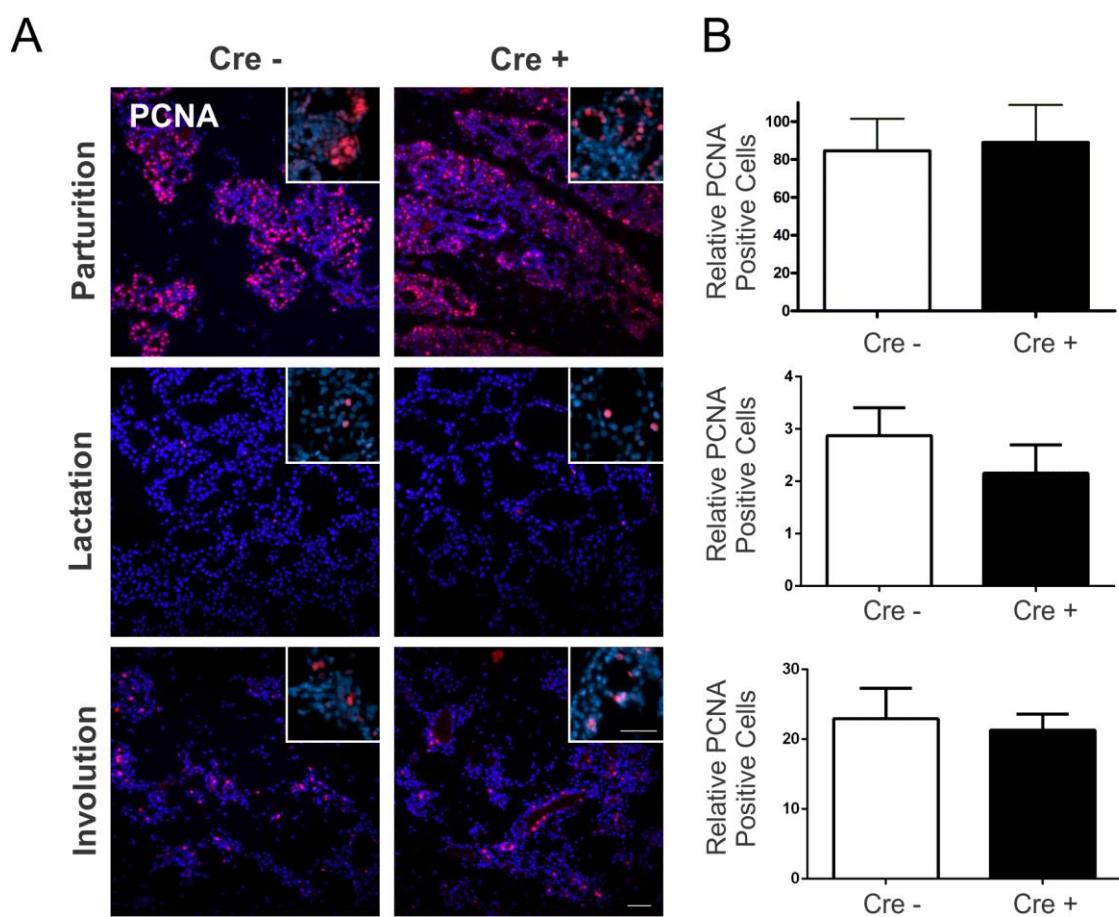
### 3.3.3 *BLG-Cre; Cx26<sup>f/f</sup>* mice have normal lactation

To determine the functional state of the gland, western blot analysis of 2 common milk proteins, WAP and β-casein, were performed. Our data revealed similar expression in lactating *Cre*<sup>+</sup> and *Cre*<sup>-</sup> mice (Figure 3.7A,B). As the western blot analysis is a measure of both secreted and non-secreted milk proteins, immunofluorescence of WAP within the ducts (apical epithelium marked with WGA) was used as a measure of milk secretion. Evidence of secreted milk within the alveoli of *Cre*<sup>+</sup> and control mice was observed (Figure 3.7C). In addition, postnatal day 18 pup weights from both *Cre*<sup>+</sup> and *Cre*<sup>-</sup> dams did not show any significant differences as well as similar litter sizes suggesting that pups survive to weaning age and do not die of starvation. During subsequent 2<sup>nd</sup> and 3<sup>rd</sup> pregnancies, no differences were seen in the litter weight sizes or health of the pups. Thus, a reduction in Cx26 did not affect later pregnancies as well. These findings are consistent with the whole mounting, histological and immunolabeling data further supporting the premise that the absence of the physiological surge in Cx26 during pregnancy does not result in an overt developmental or functional defect in the mammary gland in *BLG-Cre; Cx26<sup>f/f</sup>* mice.

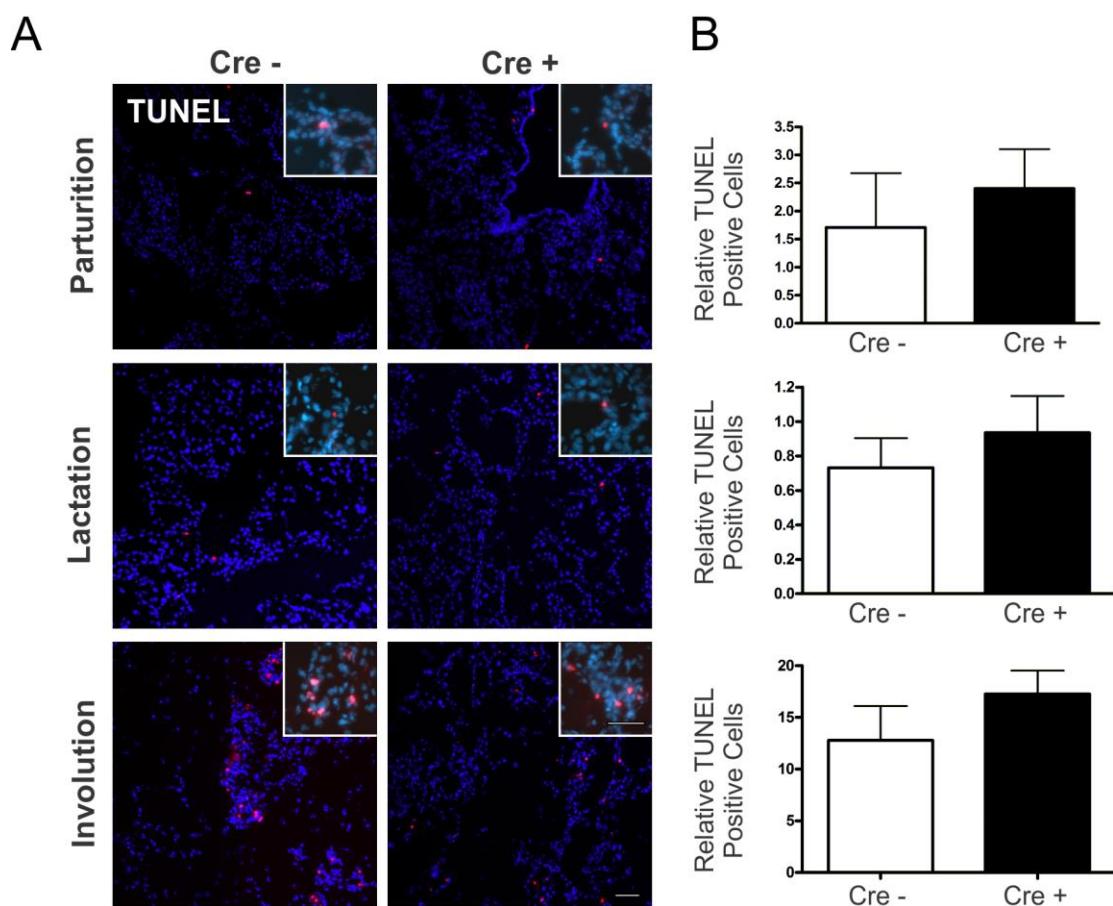
## 3.4 Discussion

In the present study we use a novel, conditional Cx26 knockout mouse model to evaluate the biological role of the Cx26 surge seen in the mammary gland as mice enter and proceed through pregnancy. In contrast to previous reports, we demonstrated that the absence in the physiological surge in Cx26 resulting in reduced Cx30 upregulation, during pregnancy is not necessary for normal mammary gland development. Furthermore, we showed that low basal levels of Cx26 in the mammary gland are sufficient for normal mammary gland function.

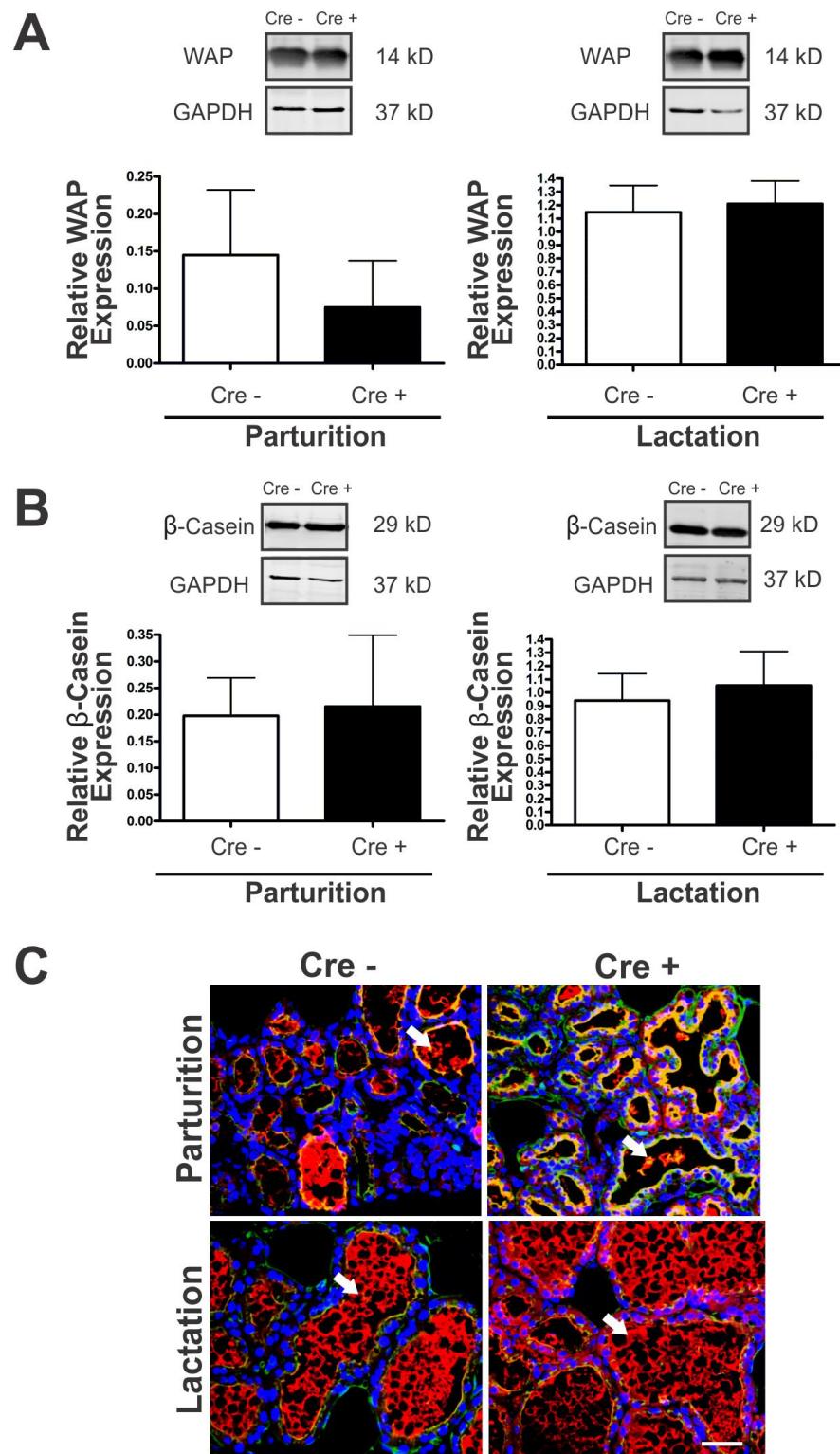
**Figure 3.5. The mammary gland of *BLG-Cre; Cx26<sup>fl/fl</sup>* mice has unaltered cell proliferation at parturition, lactation and involution.** (A) Assessment of PCNA labeling (red) revealed no change between *Cre*+ mice and control mice. (B) Values represent the mean number of positive cells (inserts, red) relative to the pixel area of the nuclei (blue), divided by a factor of  $1 \times 10^{-9}$ , per  $0.18 \text{ mm}^2 \pm \text{S.E.M.}$  N=8. Hoechst staining denotes the nuclei. Scale bars= 50  $\mu\text{m}$ .

**Figure 3.5**

**Figure 3.6. Programmed cell death is unaltered in lactating and involuting mammary glands form *BLG-Cre; Cx26<sup>f/f</sup>* mice.** (A) Evaluation of TUNEL positive (red) cells revealed no difference between genetically-modified mice and control. (B) Values are mean number of positive-cells (inserts) relative to the pixel area of the nuclei, divided by a factor of  $1 \times 10^{-9}$ , per  $0.18 \text{ mm}^2 \pm \text{S.E.M.}$  N=5. Hoechst staining denotes the nuclei. Scale bars=50  $\mu\text{m}$ .

**Figure 3.6**

**Figure 3.7. Similar to control mice, lactating *BLG-Cre; Cx26<sup>f/f</sup>* mice can produce and secrete milk.** (A, B) Western blot analysis revealed similar WAP and  $\beta$ -casein expression levels at parturition and lactation of control and genetically-modified mice. Values are mean levels  $\pm$  SEM. N=8. (C) Immunofluorescent analysis revealed secreted WAP (red, arrows) within the lumen of ducts and alveoli outlined with wheat germ agglutinin (green). N=4. Hoechst staining denotes the nuclei. Scale bars=50  $\mu$ m.

**Figure 3.7**

### 3.4.1 *BLG-Cre; Cx26<sup>f/f</sup>* mice as a model to study the role of Cx26 in mammary gland development and function

Conditionally ablated mice have previously been described to study the role of Cx26 in mammary gland development. These mice were engineered to express Cre under the MMTV or WAP promoters that become activated at an early embryonic stage or at the end stages of pregnancy, respectively [13,14]. However, both of these genetically-modified mice ablate Cx26 from birth or after pregnancy begins, but not at the onset of Cx26 upregulation at D8.5-9.5 of pregnancy [10]. Thus, in order to assess the role of Cx26 during pregnancy, we employed a similar Cre-*loxP* targeting strategy using Cre under the BLG promoter as previously described [19]. Cre-mediated recombination following upregulation under the BLG promoter has been reported to become activated at approximately day 10 of pregnancy causing recombination of the floxed allele in 70-80% of the lactating mammary gland [19,25]. In comparison to the MMTV or WAP promoters, BLG promoter activation is more restricted to the mammary gland with  $\leq 1\%$  recombination reported in other tissues while the MMTV promoter has also been reported in the salivary glands, seminal vesicles and lymphoid cells and the WAP promoter is expressed within the brain [14,26]. Thus, the BLG promoter driven loss of Cx26 more precisely mimics the physiological increase in Cx26 [19]. Here we demonstrated that *BLG-Cre; Cx26<sup>f/f</sup>* mice had a reduction in the physiological surge in Cx26 during mammary gland development but did not completely ablate Cx26 expression. This reduction in Cx26 was not unlike the *Cx26<sup>f/f</sup>;MC* mice reported by Bry et al (2004) where residual Cx26 may also have been present [13]. Importantly, the expression of Cx26 gap junctions is localized to only select luminal cells in our Cx26 knockout mice, suggesting that the majority of luminal cells may have complete ablation of Cx26.

Downregulation of untargeted connexins in response to specific connexin knockout targeting strategies has been observed in a variety of tissues, including the pancreas and liver of *Gjb1<sup>-/-</sup>* deficient mice in which Cx26 was also reduced [27,28]. In our study, we observed a reduction in Cx30 upregulation concordant with a knockout strategy targeting Cx26. Similarly, the conditional ablation of Cx26 in the inner ear of mice expressing Cre under the Sox2 promoter showed a delay in the expression of Cx30 till postnatal d14, unlike

in control mice where Cx30 expression was observed as early as postnatal d6 [29]. Our results suggest that cross talk regulation of Cx26 and Cx30 expression is not exclusively restricted to the inner ear. Although not tested, we speculate that the reduction in Cx30 occurs through the regulation of gene transcription similar to that described previously [29], although a post-translational role of Cx26 to stabilize Cx30 containing hemichannels through oligomerization cannot be ruled-out. Alternatively, Cx30 downregulation is also associated with a downregulation in Cx26 as mice generated to express the Cx30<sup>T5M</sup> mutant have a significant downregulation of Cx26 within the adult cochlea, suggesting that Cx26 and Cx30 share reciprocal cross talk mechanisms [30]. Indeed, this regulation between Cx26 and Cx30 was suggested to be mediated through calcium and NF-κB signaling, although it is unknown if a similar mechanism occurs within the mammary gland [31]. Importantly, both calcium and NFκB signaling occur during pregnancy and lactation within the mammary gland [32,33].

Although the down regulation of Cx26 or Cx32 has previously been reported to reciprocally down-regulate each other [27], this was not observed in the *Cre*<sup>+</sup> mice used in this study and may reflect the differential timing and possibly distinct mechanisms of Cx26 (upregulated during pregnancy) and Cx32 (upregulated during early lactation) expression within the mammary gland [10]. Similarly, Cx43 expression was not altered in *Cre*<sup>+</sup> mice compared to control mice which likely reflects their differential localization patterns as Cx26 is expressed mainly in luminal cells while Cx43 is found mainly in the myoepithelium and stromal fibroblasts [3]. Together, Cx26 knockout mice have acquired a phenotype where Cx26 is reduced in lactating mice with a delay in Cx30 upregulation.

### 3.4.2 Mammary gland development is maintained in Cx26 conditional knockout mice

All exocrine glands have previously been shown to express Cx26 and/or Cx32 gap junctions suggesting that these proteins may be critical in regulating gland development [34]. To date, systemic loss of Cx32 or Cx26 has yet to reveal impaired development of exocrine tissues [35]. However, as in the case of Cx26, this is difficult to assess due to the lethality of *Gjb2*<sup>-/-</sup> mice at embryonic D10.5 [11]. The question then arises as to why Cx26

appeared to play a key role in the exocrine mammary gland lobuloalveolar development which led to impaired lactation in *MMTV-Cre* mice [13]. Three possibilities exist to help explain this discrepancy. First, it is plausible that because mammary gland development is unique in that most of its development occurs after birth, Cx26 may have a unique role to guide its development [3]. Second, as the use of control *MMTV-Cre* mice in the Bry et al. study were not reported to assess whether the insertion of *MMTV-Cre* affected the phenotype of these mice, and given the new finding by Yuan et al (2011) that at least one mouse line of *MMTV-Cre* mice has mammary gland defects, we cannot rule out the possibility that conditional targeting strategies using *MMTV-Cre* mice are less than ideal to examine mammary gland development and function [15]. However, in support of the validity of the findings from *MMTV-Cre* driven Cx26 knockout mice, these mice showed a clear paucity of alveolar formation by pregnancy D15 compared to control mice, while decreased alveoli and ducts were not observed until parturition in *MMTV-Cre* mice suggesting a similar but distinct phenotype [13,15]. Finally, it is possible that Cx26 may play an important role in the development of other exocrine glands and that further evaluation of these glands using similar conditional targeting strategies is needed.

Whole mount, histological and immunofluorescent analysis of BLG-Cre driven Cx26 knockout mice revealed that the physiological surge in Cx26 is not required for mammary gland development or for controlling proliferative or apoptotic mechanisms associated with pregnancy, lactation and the involution cycle of the mammary gland. Our results may suggest one of two possibilities: first, low levels of Cx26 in the whole mammary gland during pregnancy and lactation are sufficient to mediate alveogenesis and gland function. This appears to be the case in terms of Cx43 within the mammary gland, in which the *Cx43<sup>H130T/+</sup>* mice that maintained GJIC above a certain threshold was able to retain proper mammary gland development and function not observed in *Cx43<sup>G60S/+</sup>* mice with low GJIC (Chapter 2, [22]). Our study did not evaluate GJIC therefore it is unknown whether a similar observation is observed in the BLG-Cre driven Cx26 knockout mice. Second, our results may also suggest that Cx26 does not regulate alveogenesis during pregnancy but instead is important earlier in development. As the MMTV promoter is activated embryonically and Cx26 has been found to be expressed at low levels in the virgin mammary gland, it is unknown whether Cx26 plays a role in regulating differentiation of

the stem/progenitor cells within the mammary gland prior to pregnancy [14,21]. Importantly, Cx26 has been reported to be expressed in stem/progenitor cells postnatal hippocampus but it is unknown if Cx26 is expressed in mammary stem/progenitor cells [36]. Regardless of which of these two possibilities is correct, the maintenance of basal levels of Cx26 during pregnancy does not appear to impair lobuloalveolar development of the mammary gland.

### 3.4.3 Mammary gland function is maintained in *BLG-Cre;Cx26<sup>f/f</sup>* mice

Both Cx26 and Cx32 have been suggested to regulate and fine tune the synthesis and release of factors in exocrine glands, of which Cx32 is thought to play the dominant role [34]. However, *Gjb1<sup>-/-</sup>* mice do not present with a lactation defect, suggesting that other connexins such as Cx26 or Cx30 channels are equally important in regulating mouse mammary gland secretion [3]. Our results suggest that the absence of the physiological surge of Cx26 does not appear to impair the secretory function of the gland in that two of the most common milk proteins had no change in their expression or release into the lumen of acini or ducts. Consistent with this, sufficient milk was produced from lactating dams as no difference in pup death was observed in Cx26 knockout mice, which may be the result of normal Cx32 expression fulfilling the need for the loss of Cx26 within the mammary gland. Importantly, both Cx32 and Cx26 channels are able to pass similar molecules through GJIC including ATP and IP3 [37]. In support of this, Cx32 is upregulated at the onset of lactation and has previously been shown to be insensitive to gating by the osmolyte taurine that is implicated in milk protein synthesis, unlike that of Cx26 homomeric and heteromeric Cx26/32 channels [10]. As a result, loss of the physiological surge in Cx26 does not have an overt impairment on milk production or delivery to pups.

## 3.5 Conclusions

In humans, Cx26 is the only consistently reported connexin to date to be expressed within the luminal epithelium, suggesting that Cx26 is the dominant connexin regulating human

mammary gland function [3]. As such, loss of this connexin would presumably result in breast feeding defects as previously reported in MMTV driven Cx26-ablated mice. Interestingly, a population of patients exist with mutations in the *GJB2* gene that encodes Cx26 representing a large population base with systemic and impaired Cx26 channels that result in deafness and skin diseases [17]. Although no study to date has specifically evaluated a relationship between loss of function mutations in *GJB2* patients and breast feeding, it is interesting that in the face of such a high prevalence of people with these mutations resulting in deafness, there are no reports of lactation defects within the deaf community [18]. Two possibilities arise to explain this discrepancy. First, that breast feeding defects occur within hearing impaired mothers expressing Cx26 mutants but are not reported. Second, Cx26 is not a critical regulator of epithelial cell survival and that maintenance of low levels of Cx26 is sufficient for breast feeding. Our mouse model supports the latter and suggests that mothers with *GJB2* mutations that maintain Cx26 levels above 30% will not develop breast feeding defects.

In summary, our novel mouse model suggests that in the absence of the physiological surge in Cx26, mammary gland development and function are retained within the mammary gland of mice. Our results suggest that as long as basal levels of Cx26 expression are maintained within the human population expressing mutations in the *GJB2* gene, mammary gland development and function may be unaffected.

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### 3.7 Supplementary figures

Figure 3.S1. ***BLG-Cre;Cx26<sup>fl/fl</sup>* mice exhibit a dramatic reduction in Cx26 at parturition and lactation only.** (A) Real-time PCR analysis of mammary glands from control (open columns) and *Cre+* (solid columns) mice revealed a dramatic reduction in Cx26 mRNA levels in lactating mice while no change was observed at other timepoints. Values are mean levels  $\pm$  S.E.M. \*p<0.05, \*\*\*p<0.001. Western blot analysis of Cx26 during pregnancy revealed no significant difference in Cx26 expression at D9.5 and d12.5. Values are mean levels  $\pm$  S.E.M. Immunofluorescent analysis of Cx26 (green, arrows) revealed a qualitative reduction in Cx26 at D12.5 of pregnancy in Cx26 knockout while no change was observed at D9.5 compared to control mice. Hoechst staining denotes the nuclei. Scale bars=50  $\mu$ m. N=4.

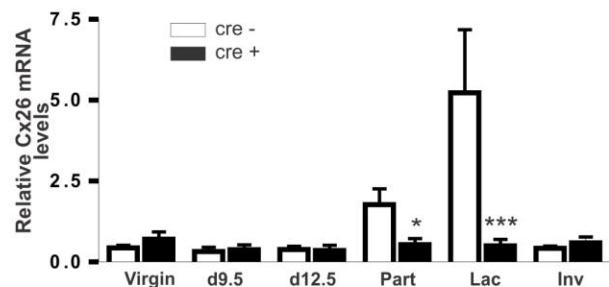
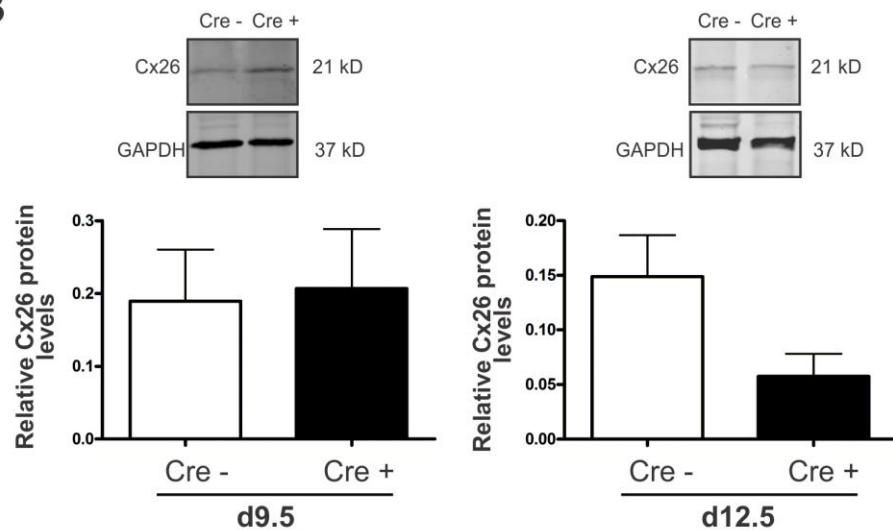
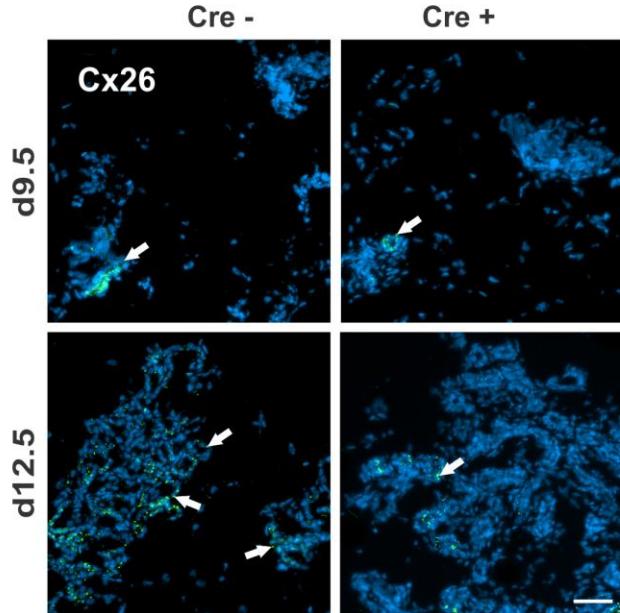
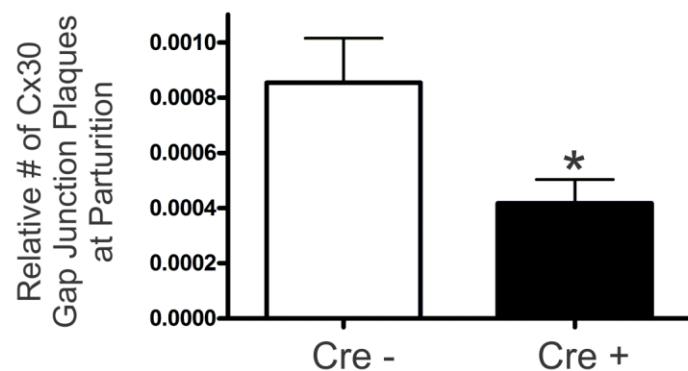
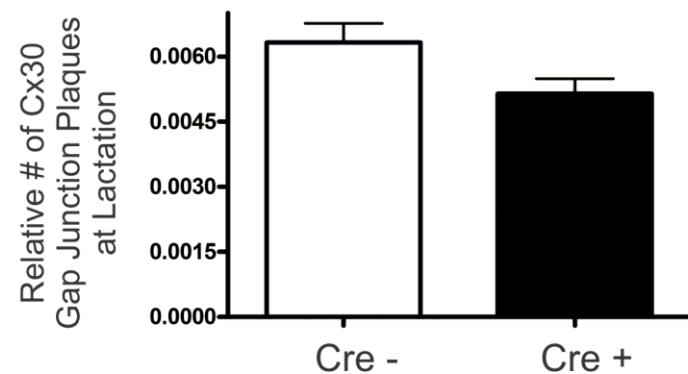
**Figure 3.S1****A****B****C**

Figure 3.S2. ***BLG-Cre;Cx26<sup>f/f</sup>* mice have significantly reduced number of Cx30 gap junctions at parturition.** Quantification of 10 arbitrary Cx30-labelled immunofluorescent images (Figure 3.2) per nuclear pixel area per sample revealed a significant decrease in Cx30 gap junctions compared to control mice at parturition (A) but not at lactation (B). Values are mean levels  $\pm$  SEM. N=8.

**Figure 3.S2****A****B**

## Chapter 4

### 4 Cx26 knockout predisposes the mammary gland to primary mammary tumors in a DMBA-induced mouse model of breast cancer

Similar to Cx43, Cx26 has been described to act as both a tumor suppressor and tumor facilitator in the context of breast cancer. The purpose of this study was to further clarify the role of Cx26 in breast tumourigenesis using our previously characterized *BLG-Cre; Cx26<sup>f/f</sup> mice* chemically treated to induce breast tumors. Importantly, these studies represented the first evaluation of the role of Cx26 in breast cancer using genetically-modified mice. In addition, this study aimed to indirectly determine if female patients with hereditary deafness due to loss-of function mutations in the *GJB2* that encodes Cx26 might potentially be at greater risk of developing breast cancer.

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A version of this chapter was submitted to Oncotarget:

[Stewart MKG](#), Bechberger JF, Welch I, Naus CC, Laird DW. Cx26 knockout predisposes the mammary gland to primary mammary tumors in a DMBA-induced mouse model of breast cancer (submitted, reviewed and now in revision).

## 4.1 Introduction

Breast cancer is the most frequently diagnosed cancer-affecting women in the world [1]. Early detection remains a key factor in patient survivability as the 5 year survival rate for stage 1 breast cancer is over 90% compared to <30% for stage 4 breast cancer [2-4]. Therefore, the identification of at risk populations may be important for early detection of the disease in order to improve patient survivability [5]. In addition, although much progress is being made in unraveling signaling pathways in breast cancer, key regulators of breast cancer progression and metastasis remains poorly understood. Due to the complexity of the disease, the identification of key proteins is critical for the development of new targeted therapies and biomarkers [6]. Indeed, gap junction proteins are interesting candidates as down-regulation of gap junctions remains one of the earliest events in tumor progression [7].

Gap junctions are clusters of intercellular channels formed by connexin subunits between adjacent cells allowing for metabolic and ionic signaling in a process known as gap junctional intercellular communication (GJIC) [8]. GJIC has been linked to critical cellular functions; such as proliferation, differentiation and apoptosis, which are frequently dysregulated in cancer [9]. In addition to GJIC, gap junction channel independent functions involving connexin protein-protein interactions and hemichannel function have been shown to be linked to cell growth [10-14]. The connexin family in humans consists of 21 genes but only Cx26 and Cx43 are typically expressed in the human breast [8,15]. Both Cx26 and Cx43 have been classically described as tumor suppressors in the breast based on loss of expression in many mammary tumor cell lines and the fact that ectopic re-expression of these connexins reverts some tumor cells into a more differentiated phenotype both *in vitro* and *in vivo* [11,12,16,17]. However, adding to the complexity of the role of connexins in breast cancer, both Cx43 and Cx26 have also been reported to be upregulated in human tumor biopsies at later stages of tumor progression and may even act as tumor facilitators [18-20]. These perplexing reports highlight the need for additional studies, particularly *in vivo*, to clarify the role of connexins throughout the progression of breast cancer from tumor onset to metastasis. In this pursuit, we recently demonstrated that Cx43 had a critical role in suppressing metastasis to the lungs in a genetically-modified

mouse model where Cx43 function was greatly reduced (Appendix 1) [21]. However, the role of Cx26 in the mammary gland has not been assessed in mice and this may be more important than examining Cx43 as mammary neoplasms typically express markers of luminal epithelial cells [22]. In addition, loss-of-function mutations in the *GJB2* gene that encodes Cx26 are common and responsible for over 40% of hereditary deafness and many skin diseases [23]. Importantly, the worldwide prevalence of biallelic *GJB2* related hearing loss accounts for 17.3 % of cases [23,24]. The 35delG mutation is by far the most common and results in the premature truncation of Cx26 and complete systemic loss of channel function, thereby acting like a knockout in the context of gap junction channel activity [23]. Thus, whether this patient cohort is more or less susceptible to breast tumor onset and progression could have profound clinical implications [23]. Therefore, using our previously described genetically-modified mice with conditional knockout of Cx26 expression in the mammary gland, we developed a DMBA-induced mouse model of breast cancer [25]. We hypothesized that low levels of Cx26 within the mammary gland would predispose the mammary gland to the onset of tumors and increase tumor progression and incidence of metastases.

## 4.2 Methods

### 4.2.1 Mice

All experimental procedures were approved by the Committee on the Ethics of Animal Experiments at the University of Western Ontario and the University of British Columbia following the guidelines of the Canadian Council on Animal Care (Appendix 3). To assess how loss of Cx26 would affect primary mammary tumor development, we utilized a conditional knockout mouse where the Cre transgene was under the control of the *β-lactoglobulin (BLG)* promoter [25]. In order to induce activation of the *BLG* promoter and subsequent Cx26 knockout, a pituitary isograft was surgically inserted into the renal capsule (Figure 4.S1A) of 6 week old *BLG-Cre; Cx26<sup>f/f</sup>* (*Cre+*) similar to that described by others [53, 31] in addition to *Cx26<sup>f/f</sup>* (*Cre-*) control mice lacking the Cre transgene. Prolactin and ovarian hormones derived from the pituitary transplant triggers epithelial proliferation and lobuloalveolar differentiation in the mammary gland independent of

pregnancy. The presence of milk proteins following pituitary isograft have been reported between 21-40 days following surgery, in which we observed the presence of milk in the mammary glands approximately 50 days after pituitary implantation [54,31]. This suggests that the activation of the *BLG* promoter occurs between 3-7 weeks in parallel with lobuloalveolar development (Figure 4.S1B). As such, in an attempt to induce mammary tumors in mice in which Cx26 knockout had occurred, *Cre+* and *Cre-* mice, in which pituitaries were implanted 12 weeks prior, were treated once a week with the carcinogen 7,12-dimethylbenz( $\alpha$ )anthracene (DMBA) or corn oil for 5 weeks (1 mg per 25 g) by gavage (Table 4.S1). In addition, a second experiment was performed to act as a control in which 6 week old *BLG-Cre; Cx26<sup>f/f</sup>* and *Cx26<sup>l/l</sup>* mice were treated with DMBA or corn oil by gavage only 1 week following pituitary transplant in order to induce mammary tumors in mice prior to Cx26 knockout (Table 4.S2). All mice were subsequently monitored weekly by palpation for evidence of mammary tumor formation. Mice were removed from the study if they presented with other health concerns that included developing lymphoma and/or stomach tumors that required them to be sacrificed (Table 4.S1, 2). Mice were sacrificed when the largest tumors reached a final volume of 1cm<sup>3</sup>. Finally, five *BLG-Cre; Cx26<sup>f/f</sup>* and *Cx26<sup>l/l</sup>* mice that had undergone at least 2 pregnancies were monitored for spontaneous mammary tumor formation for 1.5 years in which tissue was collected similar to that described above. All mice were genotyped for the expression of the *Cre* transgene and mammary gland cryosections were immunolabelled at the time of sacrifice for the presence of Cx26 revealing Cx26 knockout even after 1 year of the experiment (Figure 4.S1C).

#### 4.2.2 Whole mount

Whole mounts were performed as previously described [55]. Briefly, mammary glands were dissected and flattened onto a glass slide before being placed into Carnoy's fixative (100% EtOH, chloroform, glacial acetic acid; 6:3:1) overnight at 4 °C. Glands were immersed in 70% ethanol for 15 min and then transferred to descending concentrations of ethanol before being placed in carmine alum stain overnight at room temperature. Glands were put through increasing concentrations of ethanol and into xylene overnight. Glands

were then placed in methyl salicylate for long term storage. Images were captured using a numeric camera (Sony Cybershot).

#### 4.2.3 Hematoxylin and eosin staining

Dissected mammary tumors, mammary glands and lungs were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (6 µm) were deparaffinized in xylene (2x5 min) and rehydrated in descending concentrations of ethanol (2 x 100%, 95%, 70%; 5 min) before being placed in ddH<sub>2</sub>O (2 x 3 min). Tissues were then stained in 1% Harris's Hematoxylin for 1 min before being washed in tap water and differentiated in acid ethanol. Slides were dipped in 70% ethanol (30 sec) and put into alcoholic Eosin (2 min) and finally placed back into 70% ethanol (2 x 30 sec). Slides were then dehydrated and mounted using Cytoseal. Images were captured using a Brightfield microscope equipped with a ProgRes C5 camera (Jenoptik) and ProgRes Mac CapturePro 2.7.6 imaging software. Multiple rounds of sectioning and staining were used to evaluate lung histology for evidence of lung metastases. Clusters of nuclei in intimate proximity with bronchioles, typical of bronchus associated lymphatic tissue, were not included in the quantification of lung tumors. Lung tumor area was evaluated by measuring the length and width of lung tumors which were used to determine the area of an ellipse using ImageJ and the area of multiple lung tumors per mouse was averaged (National Institutes of Health, Bethesda, MD).

#### 4.2.4 Immunofluorescence microscopy

Paraffin-embedded sections (6 µm) were deparaffinized in xylene and dehydrated in descending concentrations of ethanol before being placed in ddH<sub>2</sub>O. Antigen retrieval was performed using either antigen unmasking solution (Vector Labs; microwaved for 5 min at 80% power) or by immersing slides in sub-boiling 1.8 mM citric acid and 8.2 mM of sodium citrate solution for 10 min before placing slides in an additional 0.01M Tris-0.001M EDTA antigen retrieval solution sub-boiling for 20 min. Cryosections were cut with a cryostat (8 µm) and stored at -80 °C and were fixed for 15 min in 10% neutral buffered formalin before use. All tissue sections were rinsed in PBS for 5 min and blocked

with 3% BSA in PBS with 0.02% Triton X-100. Sections were immunolabelled with the primary antibodies mouse anti-Cx26 (1:100, Invitrogen, 13-8100) or rabbit anti-Cx26 (Cryosections, 1:100, Invitrogen, 51-2800), rabbit anti-Cx43 (1:400 dilution, Sigma, C6219), rabbit anti-keratin8 (1:400, Abcam, ab53280), mouse anti-cytokeratin 14 (1:200, Thermo Scientific, Ms-115-P), and rabbit anti-keratin 10 (1:400, Thermo Scientific, MS 611-P1), mouse anti-E-cadherin (1:400, BD Transduction Laboratories, 610182), mouse anti- $\beta$ -catenin (1:400, BD Transduction Laboratories, 610154),  $\alpha$ -smooth muscle actin (1:400, Sigma, A5228) overnight at 4 °C. Sections were washed in PBS and then probed with Alexa Fluor® 555-conjugated anti-rabbit or anti-mouse (1:400 dilution, Molecular Probes, A21425 or A21429) and Alexa Fluor® 488-conjugated anti-rabbit or anti-mouse (1:400 dilution, Molecular Probes, A11008 or A11017) secondary antibodies for 1 hour at room temperature. Hoechst 33342 was used to label the nuclei and slides were mounted with Airvol. Images were captured using a Leica DM IRE2 inverted epifluorescence microscope and Velocity imaging software. Qualitative assessment of 5-10 images throughout mammary tumors was used to determine the percentage of cells expressing epithelial markers and connexins.

#### 4.2.5 Histological subtyping

Hematoxylin and eosin stained sections were classified according to histological subtype similar to that described by Dunn [56]. Mammary glands were classified into 4 groups; mammary alveolar carcinoma, mammary adenosquamous carcinoma, carcinosarcoma and miscellaneous tumors. Briefly, mammary alveolar carcinomas represented tumors with mainly uniform alveolar structure of glandular epithelial origin. Mammary adenosquamous carcinomas were characterized by tumors that acquired the capacity for epidermoid differentiation. Carcinosarcomas were represented by tumors classified as anaplastic and mainly devoid of distinct morphological differentiation or with significant amounts of spindle cells. Any tumors that presented outside of the previous three groups was considered a miscellaneous tumor. All tumors were evaluated in a blinded fashion.

#### 4.2.6 Statistical analysis

All statistical tests were performed using Graphpad Prism 4 (v. 4.02). For analysis of the percent of tumor free mice over time curves (Kaplan-Meier), a logrank test was applied. Palpable tumor onset, and average growth rate were evaluated using a two-tailed student's unpaired t-test. A p value less than 0.05 was considered significant.

### 4.3 Results

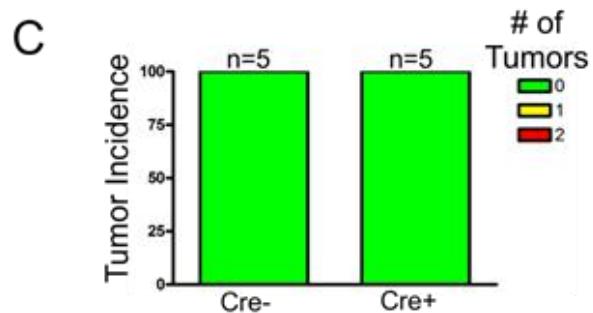
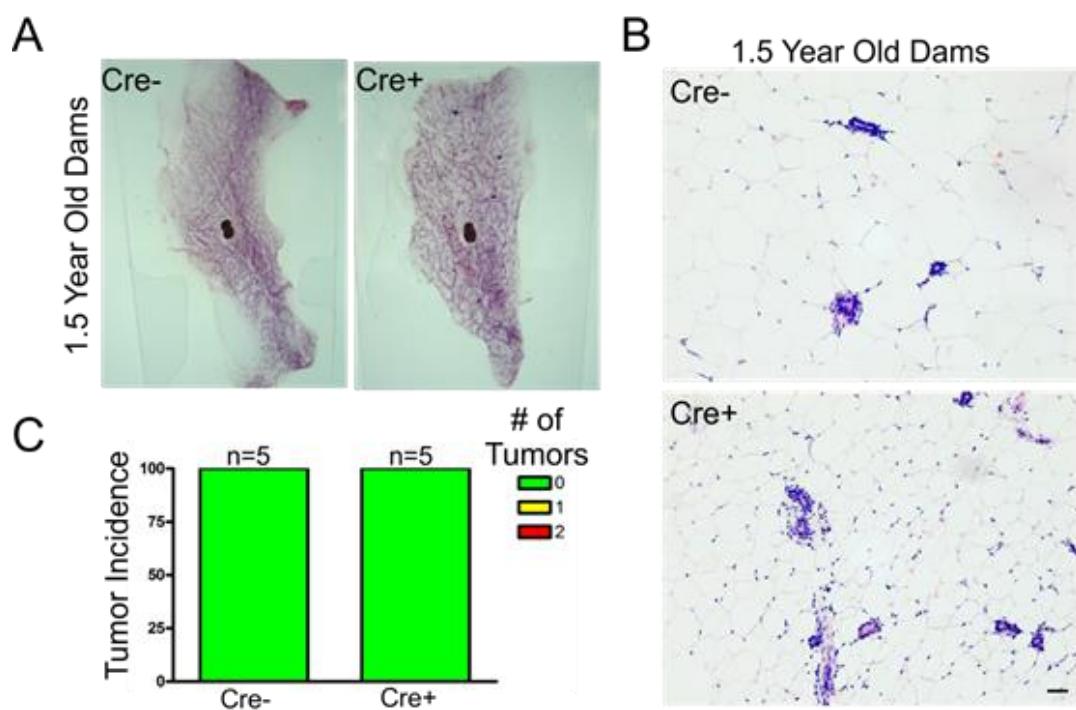
#### 4.3.1 Conditional Cx26 knockout does not result in spontaneous mammary tumors

To evaluate whether conditional Cx26 knockout mice spontaneously developed primary tumors in the mammary gland, five *Cre+* and *Cre-* mice were monitored for 1.5 years before being sacrificed and evaluated for evidence of tumor formation (Figure 4.1). All mice had at least two pregnancies, which acted to drive Cx26 knockout as we have previously described [25]. Whole mount and histological evaluation showed no evidence of primary tumors suggesting that a reduction in Cx26 alone is not sufficient to predispose mammary glands to tumor formation (Figure 4.1).

#### 4.3.2 DMBA-treated *Cre+* mice have greater primary tumor burden compared to control mice but develop mammary tumors with similar growth characteristics

As our Cx26 knockout mice do not develop spontaneous mammary tumors, we used a chemically-induced strategy where the carcinogen DMBA was used to treat mice where pituitary isografts were used to induce Cx26 knockout. Subsequently, *Cre-* and *Cre+* mice were treated with DMBA/oil either before (Group 2) or after Cx26 knockout (Group 1), and mice were evaluated for palpable tumor onset and number of tumors. No oil treated mice from either group developed mammary gland tumors (Figure 4.2A, C). Interestingly, when comparing *Cre-* and *Cre+* mice in which 3 months had

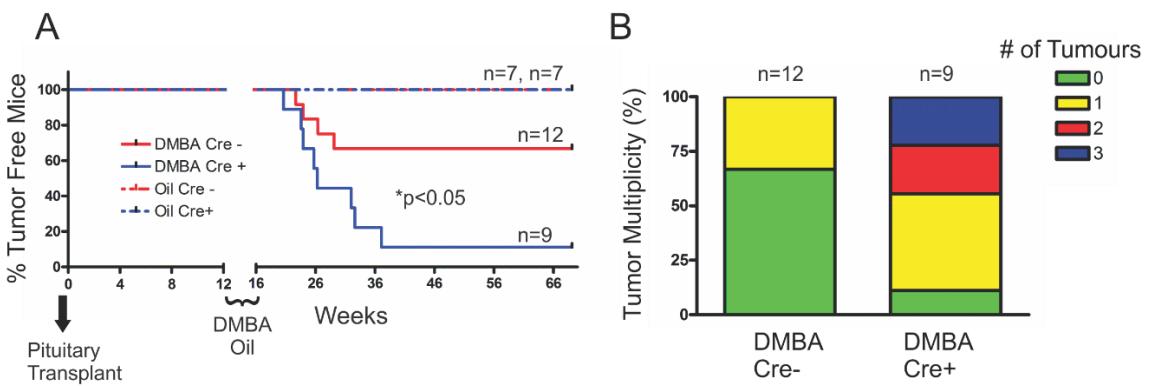
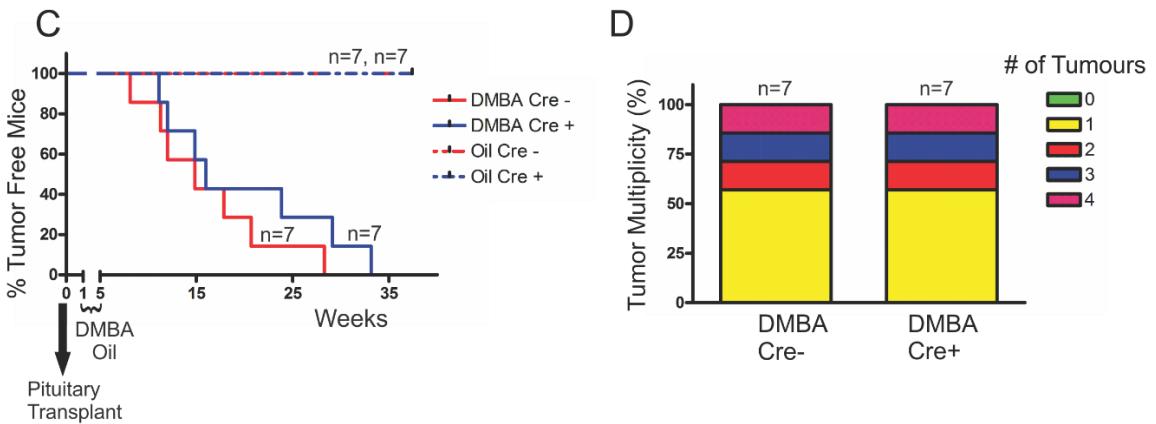
**Figure 4.1. Conditional Cx26 knockout does not result in spontaneous mammary tumors.** (A, B) Whole mount and hematoxylin and eosin staining revealed normal mammary gland architecture in 1.5 year old Cx26 knockout mice compared to control mice that have undergone at least 2 pregnancies. Scale bars = 50  $\mu$ m. (C) Quantification of primary tumor incidence revealed no macroscopic or microscopic tumors in Cx26 knockout mice compared to control mice. N=5.

**Figure 4.1**

passed before Cx26 knockout, DMBA-treated *Cre+* mice had a significantly lower number of mice that remained tumor free compared to *Cre-* mice (Figure 4.2A). This corresponded to 89% of *Cre+* mice developing tumors (8/9 *Cre+*) compared to only 33% of control mice (4/12 *Cre-*). In addition, half (4/8) of *Cre+* mice that developed tumors presented with multiple tumors while *Cre-* mice only ever developed one tumor over the course of the experiment (Figure 4.2B). Alternatively, when DMBA-treatment occurred 1 week following pituitary transplant (and thus no Cx26 knockout) no difference was observed in the number of mice that remained tumor free in which all mice of both groups developed palpable mammary tumors (Figure 4.2C). In addition, 43% (3/7) of both DMBA-treated *Cre+* and *Cre-* mice developed multiple mammary tumors suggesting similar tumor multiplicity (Figure 4.2C, D). Therefore, our results suggest that loss of Cx26 within the mammary gland prior to DMBA treatment predisposed the mammary gland for increased tumor burden compared to control mice. We next assessed whether the day of palpable tumor onset was earlier in *Cre+* mice following the end of DMBA treatment. Mammary tumors from *Cre+* mice treated with DMBA following Cx26 knockout had similar palpable tumor onset ( $75 \pm 14$  days) compared to *Cre-* mice ( $60 \pm 10$  days) suggesting that despite an increased frequency of developing mammary tumors, tumors arose at comparable times in *Cre+* and *Cre-* mice (Figure 4.3A). Similarly, palpable tumor onset in mice treated with DMBA 1 week following pituitary transplant was non-significantly different in *Cre+* ( $98 \pm 23$  days) compared to *Cre-* mice ( $71 \pm 18$  days) (Figure 4.3C). Taken together, knockout of Cx26 does not appear to increase the day of tumor onset in mice that developed chemically induced mammary tumors.

Once the largest mammary tumors reached  $\sim 1$  cm<sup>3</sup> or  $\sim 1$  year after DMBA treatment, mice were sacrificed and tissue was collected. Tumor volume of the largest tumor divided by the number of days since palpable tumor onset was used to calculate the average tumor growth rate. Comparing mice treated with DMBA 3 months after pituitary isografts, the average tumor growth rate were non-significantly different between *Cre-* ( $49 \pm 30$  mm<sup>3</sup>/day) and *Cre+* ( $81 \pm 39$  mm<sup>3</sup>/day) mice suggesting that Cx26 knockout prior to mammary tumor onset did not predispose the gland to primary tumors with increased growth rate (Figure 4.3B). Similarly, both *Cre-* ( $96 \pm 16$  mm<sup>3</sup>/day) and *Cre+* mice ( $76 \pm 17$  mm<sup>3</sup>/day)

**Figure 4.2. Cx26 knockout mice developed significantly greater tumor burden.** (A) Cx26 knockout mice had a significantly lower number of DMBA-treated *Cre*<sup>+</sup> mice that remained tumor free and a greater frequency of developing multiple tumors (B), compared to control mice. (C) DMBA-treated *Cre*<sup>+</sup> mice in which DMBA treatment occurred prior to Cx26 knockout had similar tumor burden and developed multiple tumors (D) compared to control mice.

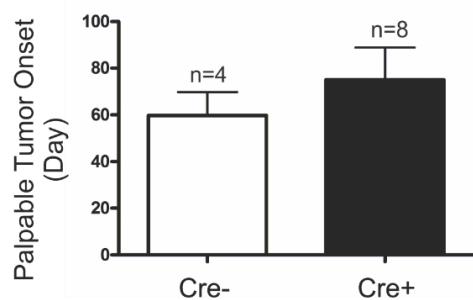
**Figure 4.2****Group 1****Group 2**

**Figure 4.3. Palpable mammary tumor onset and growth rate is similar in DMBA-treated Cx26 knockout and control mice.** Mammary tumors from *Cre+* mice, both DMBA-treated 3 months (A, B), or 1 week (C, D) after pituitary transplant, had similar primary tumor onset and average growth rate compared to those from *Cre-* mice. Bars represent means  $\pm$  S.E.M.

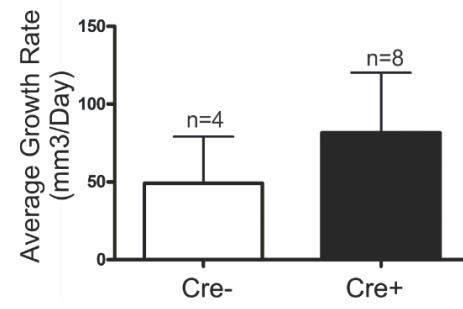
**Figure 4.3**

Group 1

A

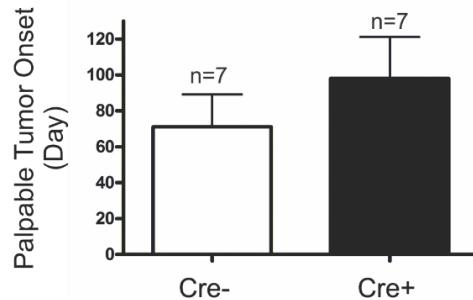


B

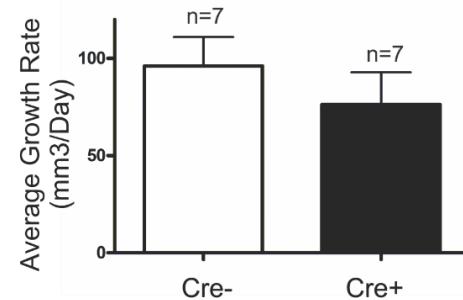


Group 2

C



D



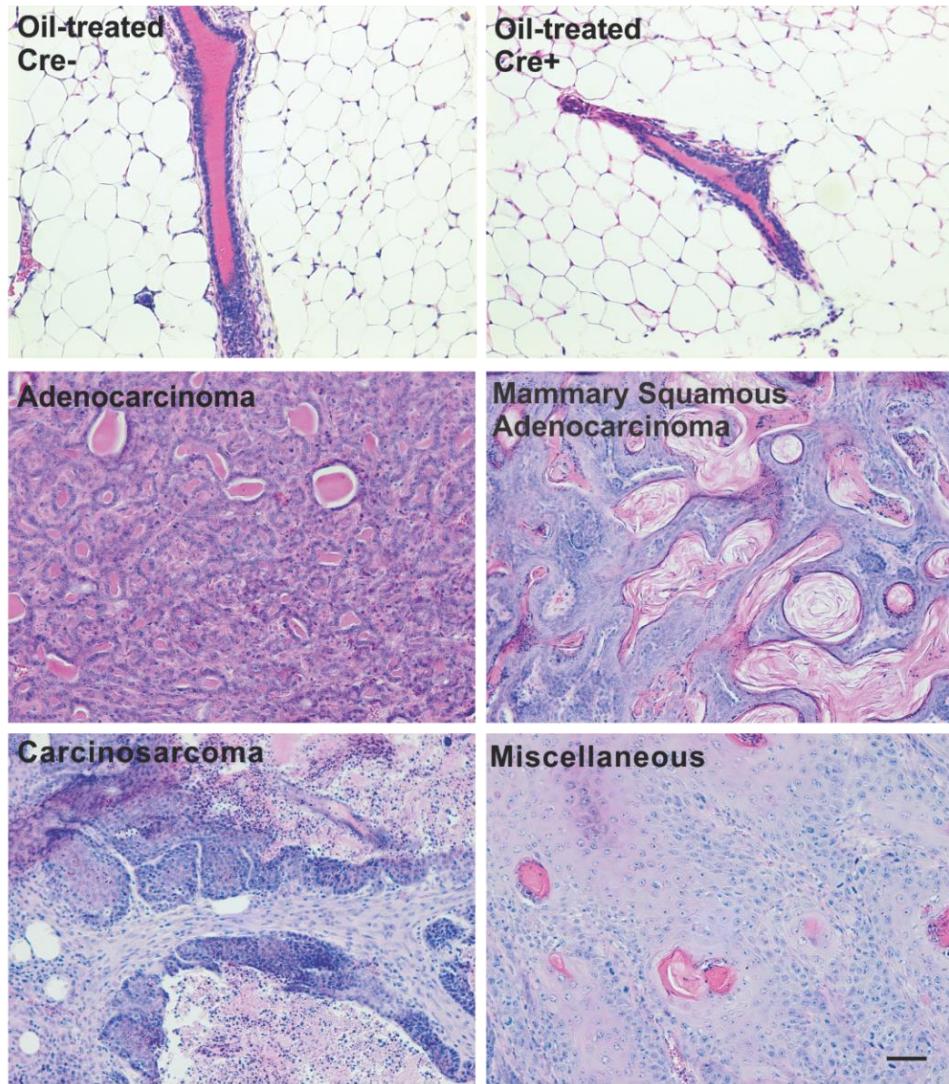
treated with DMBA 1 week after pituitary isografts developed tumors with non-significantly different average tumor growth rates (Figure 4.3D). Therefore, knockout of Cx26 does not appear to predispose the mammary gland to tumors with increased growth rate when Cx26 knockout occurs either before or after DMBA treatment.

#### 4.3.3 Cx26 knockout and control mice develop primary mammary tumors of multiple histological subtypes expressing markers of both luminal and myoepithelial cells

As differences in the frequency of mammary tumors arose only between *Cre-* and *Cre+* mice treated with DMBA after Cx26 knockout, we decided to further characterize samples from Group 1 to assess whether Cx26 knockout prior to DMBA treatment predisposed the mammary gland to develop into a specific mammary tumor histological subtype. H&E stained sections of mammary glands from oil-treated *Cre-* and *Cre+* mice revealed normal tissue histology in which typical epithelial ducts were found embedded within an adipose rich mammary fat pad (Figure 4.4A). Mammary tumor sections stained with H&E from *Cre-* and *Cre+* mice were characterized into either mammary adenocarcinoma, adenosquamous carcinoma, carcinosarcoma or miscellaneous subtypes (Figure 4.4A) revealing that mammary tumors from *Cre+* mice developed into tumors from multiple histological subtypes similar to mammary tumors from *Cre-* mice (Figure 4.4B). Therefore, our results suggest that knockout of Cx26 within the mammary gland prior to DMBA-treatment did not predispose the mammary gland to mammary tumors of a single histological subtype.

In order to further assess primary mammary tumors from mice in which Cx26 knockout occurred prior to DMBA treatment, mammary tumors were immunolabelled with a variety of connexin, luminal and myoepithelial markers (Figure 4.5A) and the percentage of cells expressing the markers were recorded (Figure 4.5B). Immunofluorescent analysis of Cx26 revealed little to no evidence of Cx26 labelling in tumors from *Cre+* mice unlike the lactating mammary gland which acted as a positive control (Figure 4.5Ai,iii, B). Interestingly, little to no evidence of Cx26 labelling was also observed in tumors from *Cre-* mice suggesting that Cx26 was also down-regulated in mammary tumors that did not express the Cre transgene (Figure 4.5Aii, B). In addition, mammary tumors

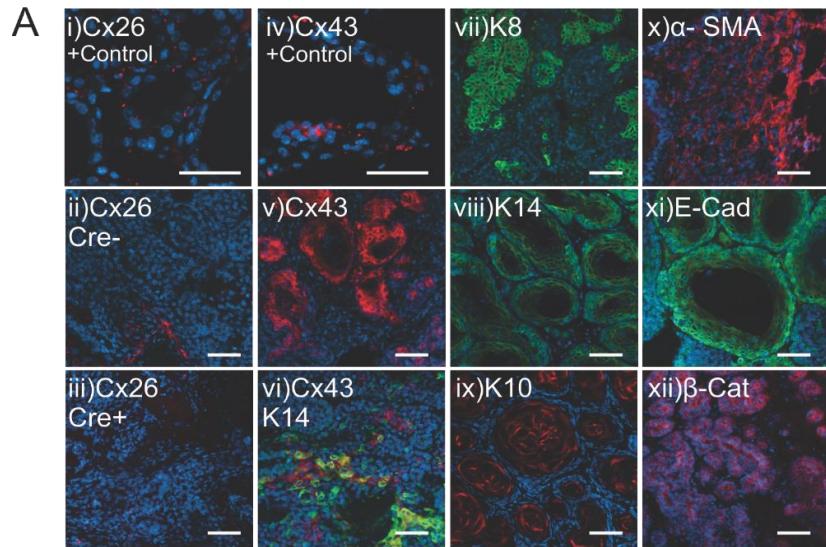
**Figure 4.4. Cx26 knockout mice develop tumours of multiple histological subtypes similar to control mice.** (A) Hematoxylin and eosin stained sections evaluated for histological subtypes of breast cancer revealed multiple tumour subtypes for both *Cre*<sup>+</sup> and *Cre*<sup>-</sup> mice treated with DMBA 12 weeks after pituitary transplant but no evidence of mammary tumors in oil-treated mice. Scale bar = 50  $\mu$ m. (B) Table lists the number of mammary tumours per mammary histological subtype for *Cre*<sup>-</sup> and *Cre*<sup>+</sup> mice.

**Figure 4.4****A****B**

Histological Subtypes of DMBA-treated Tumours

	Adenocarcinoma	Mammary Squamous Adenocarcinoma	Carcinosarcoma	Miscellaneous
Cre -	1	2	1	0
Cre +	2	2	3	1

**Figure 4.5. Mammary tumors from Cx26 knockout mice express similar epithelial protein markers as control mice.** (A) Representative images of paraffin-embedded primary mammary tumor sections immunolabelled with luminal and myoepithelial markers that included; Cx26 (i, ii, iii, red), Cx43 (iv, v, vi, red), keratin 14 (vi, viii green), keratin 8 (vii, green), keratin 10 (ix, red),  $\alpha$ -smooth muscle actin (x, red), E-cadherin (xi, green) and  $\beta$ -catenin (xii, red). Hoechst denotes nuclei. Scale bars = 50  $\mu$ m. (B) Table indicates relative number of cells that are positive for the luminal and myoepithelial markers based on immunofluorescent labelling. +++50-100%, ++11-49%, +1-10%, -0%. For keratin 10. + denotes presence of labelling.

**Figure 4.5**

**B**

	Adenocarcinoma		Mammary Squamous Adenocarcinoma		Carcinosarcoma		Misc. Tumour
	Cre - n=1	Cre + n=2	Cre - n=2	Cre + n=2	Cre - n=1	Cre + n=3	Cre + n=1
Cx26	+	- +	+	+	+	- - -	-
Cx43	++	+ +++	++ +++	++ +++	++	+++ - +++	++
Keratin8	+++	+++ +++	++ +	++ +++	++	+++ +++ +	+
Keratin14	++	+++ ++	+++ +++	+++ ++	+	+++ + -	++
Keratin10	-	-	+	+	-	- - -	-
$\alpha$ -Smooth Muscle Actin	+	++ +	+	++ ++	+	+++ +	++
E-Cadherin	+++	+++ +++	+++ +++	+++ +++	+++	++ - -	++
$\beta$ -Catenin	+++	+++ +++	+++ +++	+++ +++	+++	++ + +++	+++

immunolabelled for the expression of Cx43 revealed no overt difference in the level of Cx43 in the mammary tumors of both *Cre+* and *Cre-* mice although the expression appeared diffuse and intracellular compared to the more punctate pattern seen in the lactating mammary gland (Figure 4.5Aiv, v, B). Cx43 was also expressed in carcinoma cells that did not always co-localize with the myoepithelial marker keratin14 (Figure 4.5Avi, B). To assess for the expression of luminal and myoepithelial markers, when tumors were immunolabelled for the luminal markers keratin 8, E-cadherin and  $\beta$ -catenin and the myoepithelial markers keratin14 and  $\alpha$ -smooth muscle actin, no distinguishable differences were observed between *Cre-* and *Cre+* mice (Figure 4.5Avii, viii, x, xi, xii, B). Finally, only adenosquamous carcinoma tumors from both *Cre-* and *Cre+* mice labelled positively for the skin marker keratin 10 typical of the epidermoid differentiation of these tumors (Figure 4.5Aix). Taken together, mammary tumors from mice in which Cx26 was knocked down prior to DMBA treatment express similar luminal and myoepithelial markers to mammary tumors from control mice.

#### 4.3.4 Cx26 knockout and control mice exhibit similar levels of metastasis to the lungs

Following our evaluation of the primary tumors, we assessed the lung, a common site of metastasis in DMBA-induced mammary tumors, for signs of disseminated disease [21]. Hematoxylin and eosin stained sections of lung tissue revealed evidence of metastasis to the lungs (Figure 4.6A) in which a similar proportion of mice from *Cre+* (50%) and *Cre-* mice (63%) developed tumors in the lung (Figure 4.6B). Although only 2 DMBA-treated *Cre-* mice developed lung tumors, the average lung tumor area per mouse was calculated revealing a likelihood of larger average lung tumor area in *Cre-* mice ( $0.09 \text{ mm}^2$ ) compared to *Cre+* mice ( $0.02 \pm 0.009 \text{ mm}^2$ ) (Figure 4.6C). To evaluate whether lung tissue of *Cre-* and *Cre+* mice had greater evidence of cancer cell proliferation, lung tissue was immunolabelled with Ki67 and separated into high (>50%) and low (<50%) groups revealing a similar number of mice with high levels of Ki67 staining in *Cre-* (50%) and *Cre+* mice (38%) (Figure 4.6D, E). Lung tumors immunolabelled for Cx26 and Cx43 to evaluate if connexin expression changes between primary and metastatic tumors revealed mostly the absent expression of both Cx26 and Cx43 in *Cre-* (Cx26: 0/2, Cx43: 0/2) and

*Cre+* (Cx26: 0/5, Cx43: 1/5) mice suggesting that connexins are not upregulated during metastatic progression (Figure 4.6D, E).

## 4.4 Discussion

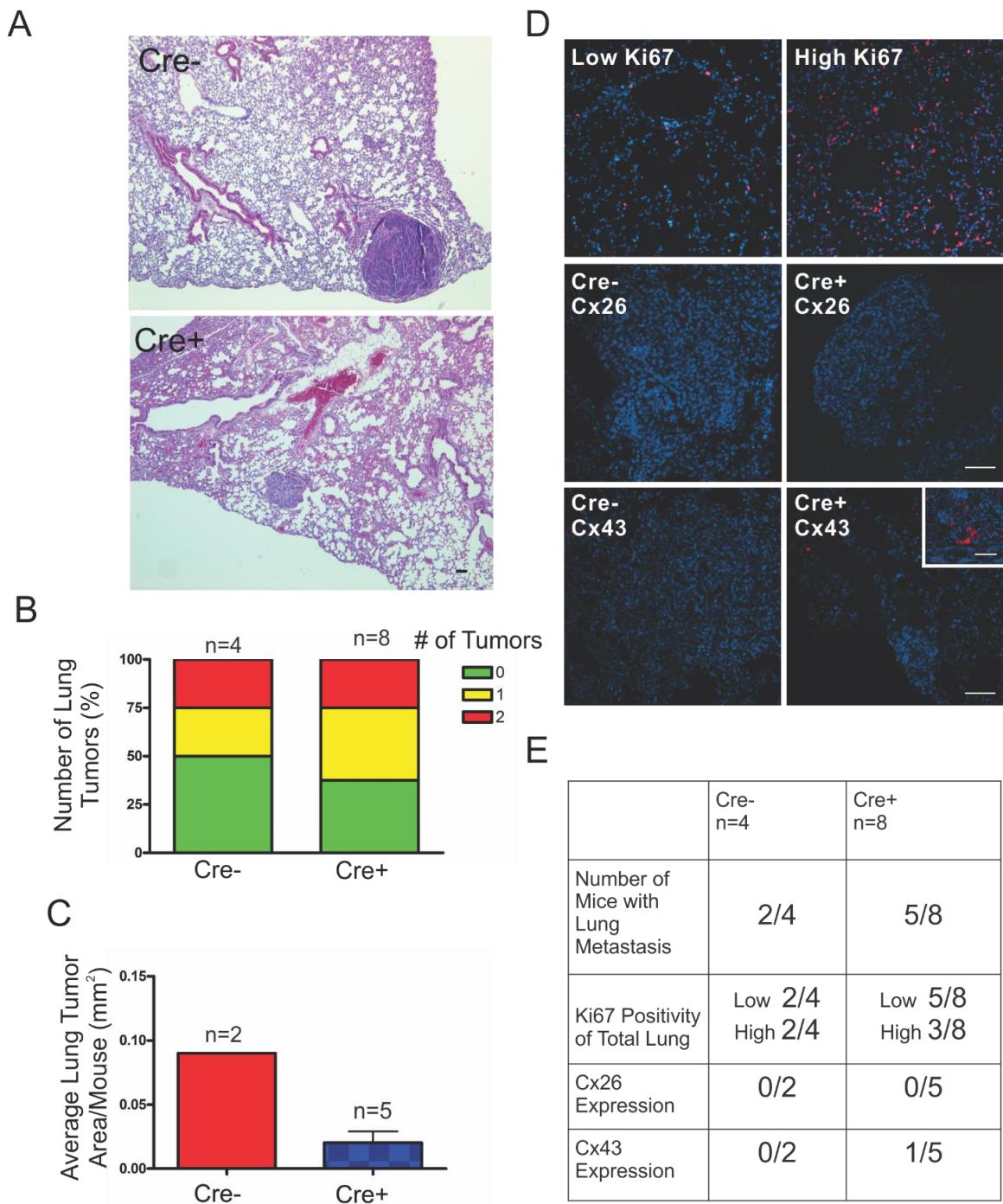
The aims of this study were two-fold; first, to evaluate whether the organ-specific loss of Cx26 predisposed the mammary gland to developing mammary tumors *in vivo*; and secondly, to evaluate if loss of Cx26 in primary mammary tumors led to altered progression and aggressiveness of the disease. In order to evaluate these aims, we developed the first chemically-induced conditionally ablated mouse model of breast cancer to assess the role of Cx26. We demonstrated that knockout of Cx26 prior to tumor induction by DMBA treatment increased the susceptibility of mice to primary mammary tumors but that this increase in the frequency of breast tumor onset was not associated with increased progression of the disease.

### 4.4.1 A model to investigate the role of Cx26 in mammary tumorigenesis *in vivo*

Evaluating the *in vivo* role of Cx26 in breast cancer is complicated by the fact that *Gjb2*<sup>-/-</sup> mice die embryonically due to defects in placenta, rendering them unusable for this kind of study [26]. As a result, we used our previously characterized mammary gland specific knockout mouse model of Cx26, in which ~70% knockout of Cx26 was observed in the mammary gland driven by Cre-mediated deletion under the *BLG* promoter following the onset of lactation [25]. We observed no evidence of spontaneous mammary tumors or abnormal histology in 1.5 year old dams that have undergone at least two pregnancies which is in agreement with results from a mammary gland specific deletion of Cx26 using similar Cre-loxP strategies under the *MMTV* and *WAP* promoters [27]. Therefore, it appears that the loss of Cx26 is not sufficient for initiating tumor onset, which requires additional genetic insults.

In order to promote additional genetic mutations in Cx26 knockout mice, we used the chemical carcinogen DMBA that preferentially promotes the induction of mammary tumors and paired this with the use of pituitary isografts to drive *BLG* promoter activity

**Figure 4.6. DMBA-treated Cx26 knockout mice exhibit similar incidence of metastases to the lungs.** (A, B) Hematoxylin and eosin stained lung sections were evaluated for evidence of lung tumors revealing a similar proportion of mice that developed lung tumors in *Cre*<sup>+</sup> and *Cre*<sup>-</sup> mice. (C) Evaluation of average lung tumor area per mouse between *Cre*<sup>+</sup> and *Cre*<sup>-</sup> mice revealed the likelihood of *Cre*<sup>-</sup> mice having larger lung tumor areas compared to *Cre*<sup>+</sup> mice. (D,) Representative images of paraffin-embedded lung sections immunolabelled for Ki67 (Red), Cx26 (Red) and Cx43 (Red) revealed a similar percentage of lung tissue expressing high levels of Ki67 positivity between *Cre*<sup>-</sup> and *Cre*<sup>+</sup> mice and tumors mostly negative, but not always (insert), for Cx26 and Cx43 expression. Hoechst denotes Nuclei. Scale bars = 50  $\mu$ m. (E) Quantification of Ki67, Cx26 and Cx43 immunofluorescent analysis.

**Figure 4.6**

[28]. The combination of pituitary isografts and DMBA has been frequently used to drive mammary tumor development through hormonal and chemical carcinogenesis [29,30]. This approach was used by Wang et al. where pituitary-driven activation of the BLG promoter was used to evaluate deregulated Pak1 activity in murine mammary tumorigenesis [31]. Importantly, we observed a clear reduction in the levels of Cx26 when paired non-tumorigenic mammary glands from all *Cre*+ mice were compared to *Cre*- mice at the time of sacrifice, indicating that the pituitary isografts were effectively driving the knockout of Cx26, and more notably, that Cx26 knockout persisted throughout the length of the experiment. Therefore, using this strategy we evaluated whether the loss of Cx26 prior to these additional genetic alterations would promote tumor incidence.

#### 4.4.2 Loss of Cx26 promotes tumor onset in chemically-induced mammary tumorigenesis

Most studies to date suggest a tumor suppressive role for Cx26 early in breast cancer progression based on evidence that Cx26 is frequently absent or down-regulated in human breast cancer cell lines or human primary tumors [17,19,32]. In agreement, we observed an increase in primary tumor incidence and tumor multiplicity when Cx26 was knocked out prior to DMBA treatments suggesting that expression of Cx26 acts in the context of a tumor suppressor and protects the mammary gland to primary mammary tumor onset. Unexpectedly, when DMBA treatment occurred only one week after the pituitary transplant, a similar tumor burden was observed in *Cre*+ and *Cre*- mice. While the pituitary isograft is necessary for Cx26 knockout, it serves a dual purpose as continual hormonal secretion of prolactin, which importantly acts on the ovary to induce synthesis and secretion of progesterone and estrogen, which together promote a greater frequency of chemical carcinogen induced mammary tumors [33]. As a result, the potential tumor suppressive effect of Cx26 may be masked by the pro-tumorigenic effects of hormones secreted from the surgically placed pituitary. Three lines of evidence support this. First, all mice treated with DMBA one week following pituitary transplant developed mammary tumors, suggesting hormones secreted or stimulated by pituitary isografts are promoting tumor onset. Secondly, we observed an increase in the frequency of tumor incidence in *Cre*- mice when the pituitary isografts occurred only one week before DMBA treatment

compared to *Cre-* mice in which the same procedure occurred 12 weeks prior to DMBA treatment. The pituitary-driven increase in chemically-induced mammary tumors is the result of increasing the proliferation of epithelial cells of the mammary gland, although this appears to be time dependent as the increase in the mitotic index begins to fall after 5 weeks as the levels of estrogen and progesterone drop [34]. Ultimately, differences in the frequency of mammary tumors between mice treated with DMBA one week or 12 weeks after pituitary transplant may be explained by increased ovarian hormonal stimulation by pituitaries transplanted closer to the time of DMBA treatment, limiting our assessment of Cx26 in mice treated with DMBA one week following pituitary isograft. Finally, a precedent exists that hormonal influence may override any tumor suppressive effects of Cx26 expression as stably transfected Cx26 expressing MCF7 cells, with strong growth suppressing effects *in vitro*, lacked growth suppressing effects *in vivo* potentially as a result of the pro-tumorigenic effects of 17 $\beta$  estradiol pellets [35]. Taken together, our data supports a role for Cx26 in protecting the mammary gland from DMBA-induced mammary tumor onset but this protective effect may be masked in hormonally-driven tumorigenesis.

#### 4.4.3 Cx26 knockout prior to DMBA treatment does not affect primary tumor growth or histological subtype

Cx26 overexpression studies have demonstrated a wide variety of tumor suppressive roles in which Cx26 may regulate primary tumor cell growth and proliferation, anchorage-independent and contact-dependent growth *in vitro*, as well as reduced tumor sizes *in vivo* when orthotopically injected into nude mice through gap junction dependent and independent mechanisms [10-12,17,35,36]. As a result, we hypothesized that knockout of Cx26 would contribute to increased tumor size but this was not the case as the average tumor growth rates were similar in *Cre-* and *Cre+* DMBA-treated mice both before and after Cx26 knockout. However, as Cx26 was absent or down-regulated even in the majority of *Cre-* tumors, it remains likely that Cx26 is down-regulated in tumors early in primary tumor progression. This is supported by studies suggesting that *Gjb2* may be, at least in part, methylated to induce down-regulation [16,37]. Ultimately, as the Cx26 status was similar in tumors from *Cre-* and *Cre+* mice, our assessment of the role of Cx26 in primary tumor growth is limited. However, Cx26 and tumor growth may not be as critical in the

human context as the majority, but not all [20], of studies agree that Cx26 mRNA or protein expression does not correlate with tumor size or Ki67 status [38-41].

DMBA-induced mammary tumors are often associated with multiple gene expression profiles giving rise to tumors with varying histological subtypes, particularly squamous carcinomas and adenocarcinomas, with many tumors expressing both luminal and basal cell markers [28,42,43]. Others have demonstrated a link between human histological subtypes correlating with specific genetic alterations, such as the inactivation of the *CDH1* gene that encodes E-cadherin and is frequently found in lobular carcinomas of the breast [44]. Importantly, there is some evidence that this correlation exists when modelling tumorigenesis in mice, as Derkson et al. showed that mammary specific deletion of E-cadherin and p53 resulted in mammary tumors similar to lobular carcinomas [45]. We aimed to test whether loss of Cx26 would promote a greater propensity of developing mammary tumors of a specific histological subtype. Interestingly, in tumors from both *Cre+* and *Cre-* mice we observed a wide variety of histological subtypes supported by varied expression of luminal and myoepithelial markers. Therefore, this suggests that the loss of Cx26 prior to DMBA treatment does not promote the development of mammary tumors of specific histological subtype, which is in agreement with a lack of correlation in human data of Cx26 expression with any histological subtypes [20]. In addition, a majority of studies document a lack of correlation between Cx26 and estrogen receptor, progesterone receptor and HER2 status in microarray or immunohistochemical analysis of human tumor samples [39,41,46], although a couple of exceptions have been reported in the case of progesterone receptor [20] and estrogen receptor status [39].

#### 4.4.4 Cx26 knockout does not promote metastatic dissemination to the lungs

The role of Cx26 in breast cancer metastasis remains much more controversial than that in the primary tumor particularly in studies using human samples. Some reports suggest that Cx26 expression does not correlate with lymph node positivity [20] or overall survival [39,41] while others have found that higher expression of Cx26 is associated with poor overall patient survival [47], particularly if Cx26 expression is elevated after chemotherapy

[40]. Still others have found that Cx26 is upregulated in lymph node metastases compared to matched primary tumors [19]. While our results are limited by the number of mice that developed potential lung metastases, we found no evidence of Cx26 expression in any lung tumors similar to the primary tumors. Thus, the knockout of Cx26 prior to DMBA treatment did not predispose the mammary gland to an increased frequency of metastases suggesting that Cx26 is not acting as a breast cancer metastasis suppressor. In addition, Cx43 was also reduced compared to primary tumors suggesting that Cx43 is down-regulated as tumors progress towards metastasis consistent with Cx43 as a breast cancer metastasis suppressor [21]. Of note, the average lung tumor area from *Cre-* mice appeared larger than those from *Cre+* mice, although the sample size was too low for a statistical assessment. While this finding hints that lung tumors without Cre-mediated deletion of Cx26 may grow larger or establish themselves earlier than those from *Cre+* mice, we observed no evidence that *Cre-* lung tumors upregulated their Cx26 expression. As a consequence, our results do not support either a tumor suppressive or facilitating role for Cx26 similar to finding by Chao et al. who found no correlation between upregulated Cx26 expression from primary tumors and metastasis to the lung in human breast cancer patient samples [46]. Taken together, our results support a tumor suppressive role for Cx26 in the context of primary tumor onset but this does not coincide with more aggressive tumors or more frequent metastases in our chemically-induced model of breast cancer.

#### 4.5 Implications to human disease

To date, *GJB2* gene mutations give rise most notably to syndromic and non-syndromic hearing loss with comparable carrier frequencies to other prevalent genetic diseases such as cystic fibrosis and sickle-cell anemia [23]. In addition to hearing loss, many patients will present with skin diseases including Bart-Pumphrey syndrome, Hystrix-like ichthyosis with deafness, Vohwinkel syndrome and Keratitis ichthyosis deafness (KID) [48,49]. Despite these skin diseases being relatively rare, patients presenting with Vohwinkel syndrome and KID syndrome have also been reported to develop skin tumors [50]. Most intriguing, a review of 61 patients with KID reported that ~10% of patients developed squamous cell carcinoma suggesting that loss of functional Cx26 in the skin predisposes KID syndrome patients to skin cancer [51]. Although it remains unknown whether other tissues or organs

that commonly express Cx26 are also susceptible to developing tumors, a single patient presenting with KID syndrome also presented with a primary invasive scirrhous ductal carcinoma of the breast suggesting that a loss-of-function mutant of Cx26 may also have contributed to the onset of a primary breast tumor in humans similar to our conditional Cx26 knockout mouse model of breast cancer [52]. However, although our results suggest an increased breast cancer risk to patients with loss-of-function *GJB2* mutations, it is important to note that our mouse model will not fully recapitulate all Cx26 mutants. Many are reported to have gain-of-function effects, particularly those associated with skin diseases, with increased Cx26 hemichannel function in addition to loss of gap junction channel function [24]. As a result, our findings may extend to only a subpopulation of patients with loss-of-function *GJB2* gene mutations. Therefore, we recommend a large epidemiologic study of breast cancer frequency in patients with loss-of-function *GJB2* mutations compared to familial healthy controls. In that over 1% of the general population worldwide is estimated to be carriers of mutant alleles of *GJB2* it remains critical to determine if this population as a whole, or in part, are at an increased risk of developing breast cancer [23].

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#### 4.7 Supplementary figures/tables

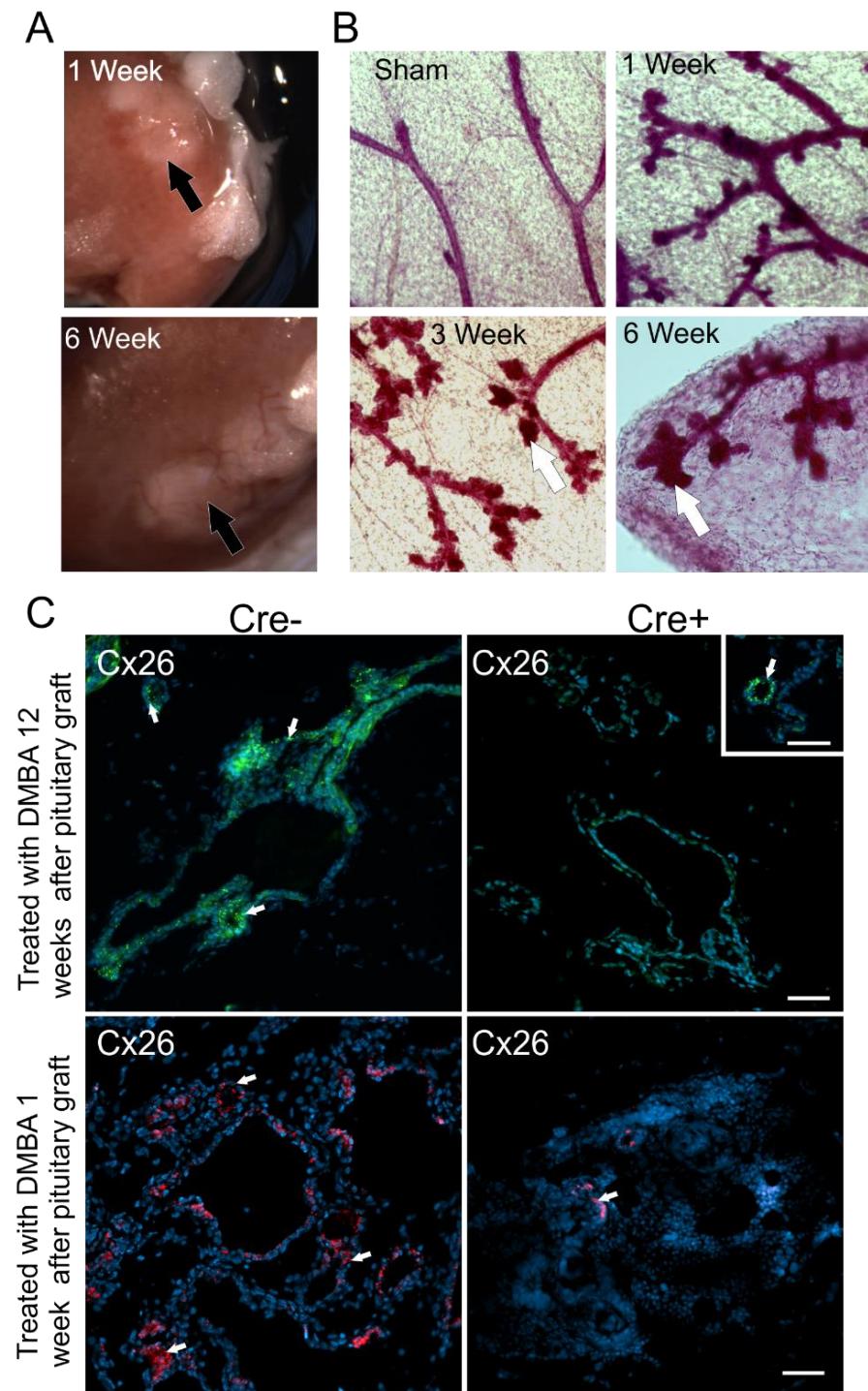
**Table 4.S1. List of Mice used in study for mice treated with DMBA or oil by gavage 1.5 months following pituitary transplant.**

	Oil		DMBA	
	Cre-	Cre+	Cre-	Cre+
Group 1 Following Cx26 Knockdown				
Initial N-Value For Study	7	7	16	12
# of Mice Removed from Study to date for other Health Concerns - Lymphoma - Intestinal Tumour - Blood Clot - Enlarged Lymph Node	0	0	4	3
Total # of Mice Included in Study	7	7	12	9
Total # of Mice with Mammary Tumours	0	0	4	8

**Table 4.S2. List of Mice used in study for mice treated with DMBA or oil by gavage 1 week following pituitary transplant.**

	Oil		DMBA	
	Cre-	Cre+	Cre-	Cre+
Group 2 Prior to Cx26 Knockdown				
Initial N-Value For Study	10	10	10	10
# of Mice Removed from Study to date for other Health Concerns - Lymphoma - Intestinal Tumour - Unknown	2	2	3	3
Total # of Mice Included in Study	8	8	7	7
Total # of Mice with Mammary Tumours	0	0	7	7

**Figure 4.S1. Pituitary implantation stimulates BLG that results in Cx26 knockout within the mammary gland.** (A, B) Pituitaries were implanted in the renal capsule leading to the development of alveoli in the mammary gland which began to be seen 3 weeks following pituitary transplant but was more evident at 6 weeks. (C) At the time of sacrifice, immunofluorescence revealed considerable but not complete (insert) knockout of Cx26 (green/red, arrows) in the mammary gland of *BLG-Cre; Cx26<sup>f/f</sup>* mice (*Cre+*), unlike *Cx26<sup>f/f</sup>* control mice (*Cre-*) of both mice treated with DMBA 1 and 12 weeks after pituitary transplant. Hoechst denotes nuclei. Scale bars = 50  $\mu$ m. N $\geq$ 6.

**Figure 4.S1**

## Chapter 5

### 5 Loss of Panx1 impairs mammary gland development at lactation: implications for breast tumorigenesis

The role of Panx1 in mammary gland development and function is unknown but some very recent data has implicated it in breast cancer. The purpose of this study was to characterize the role of Panx1 during pregnancy and lactation using a mouse model with systemic knockout of *Panx1* with the intent of understanding how this large pore channel may be important and dysregulated in breast cancer.

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A version of this chapter has been drafted for submission:

Stewart MKG, Plante I, Penuela S, Barr K, Laird DW. Loss of Panx1 impairs mammary gland development at lactation: implications in breast.

## 5.1 Introduction

Mammary gland development is a dynamic process occurring mostly after birth [1]. The mouse mammary gland undergoes extensive gland remodeling through two main phases of development following the onset of puberty and pregnancy [2]. During puberty, epithelial ductal elongation and branching loosely invades the adipocyte-rich mammary stroma [3]. The mammary gland undergoes terminal differentiation following the onset of pregnancy characterized by extensive proliferation and lobuloalveolar differentiation as numerous alveoli fill the mammary gland for secretory function during lactation [2]. Following weaning of pups, the mammary gland reverts back to a pre-pregnant state in a process known as involution [4]. These processes require extensive control of proliferation, differentiation, invasion, and cell death mechanisms mediated by hormonal signaling, local epithelial-stromal interactions and direct cell-cell communication mediated by gap junctions [1,5].

While the roles of the mammary gap junction proteins Cx43, Cx26, Cx30 and Cx32 are beginning to be defined within the mammary gland, particularly through the use of genetically-modified mice, less is known about the related large pore channels proteins pannexins in the context of the mammary gland [6]. Pannexins, similar to connexin hemichannels, oligomerize to form large protein-lined pores capable of transferring small ions and metabolites, such as ATP and  $\text{Ca}^{2+}$ , between the intracellular and extracellular milieu [7,8]. However, unlike connexin hemichannels, pannexin channels are glycosylated, insensitive to physiological levels of extracellular  $\text{Ca}^{2+}$  and can be opened at normal resting membrane potentials [9-11]. This suggests that pannexins have unique functions within tissues and warrant further investigation.

Three members of the pannexin family have been described in the mammalian genome, each predicted to have a similar topology to the vertebrate gap junction proteins connexins [7,12]. Due to its ubiquitous expression, pannexin1 (Panx1) is the best characterized and has been identified in both rodent and human tissue including the brain, muscle, and skin [13-16], as well as many other tissues including the mouse mammary gland and human breast as noted in NCBI's gene expression Omnibus database (1416379 ID, ID 49755742, [17]). Panx1 can be activated by multiple stimuli, including mechanical stimulation [18],

caspase cleavage [19], intracellular  $\text{Ca}^{2+}$  [20], and extracellular ATP [20], all of which may occur during the stages of mammary gland development that may trigger Panx1 opening [4,21-23].

Panx1 has also been shown to be dynamically regulated during brain, muscle and skin development [14-16]. Panx1 has been associated with changes in migration of primary keratinocytes, proliferation of dermal fibroblasts, neural stem cells and neural progenitor cells, as well as differentiation of skeletal muscle myoblasts [15,24,25]. Importantly, all of these cellular processes are necessary for normal mammary gland development and function suggesting a role for Panx1 in the highly regulated mammary gland [1]. In addition, Panx1 channels were shown to mediate the release of ATP from apoptotic cells which acts to recruit phagocytes for cell clearance following Panx1 C-terminal cleavage by caspases [19]. This is intriguing as macrophages have been shown to be important during mammary gland involution [26]. With the developmental and physiological roles of Panx1 are beginning to be elucidated, it is not surprising that Panx1 has been implicated in many pathologies, including tumorigenesis (as reviewed by Penuela et al. [27]).

Breast cancer is the most severe pathology associated with the breast and is the leading cause of cancer mortality in women worldwide [28]. Recently, Panx1 was shown to be mutated in metastatic breast cancer cell lines, leading to increased ATP-channel activity and promotion of breast cancer cell survival during extravasation [29]. This may suggest that Panx1 functions as a tumor facilitator in breast cancer similar to that described in melanoma, albeit through different reported mechanisms [29,30]. However, as Panx1 has also been suggested to act as a tumor suppressor in gliomas, and squamous and basal cell carcinomas, the role of Panx1 may be dependent on the type of cancer or stage of disease [31,32]. As tumors frequently exploit signaling pathways critical in organ morphogenesis, we set out to evaluate the role of Panx1 in normal mammary gland development to increase our understanding of how the role of Panx1 may be dysregulated in tumorigenesis. In addition, we explored potential implications of Panx1 as a biomarker in breast cancer.

## 5.2 Methods

### 5.2.1 Animals

All experiments were approved by the Animal Care Committee at Western University and conducted according to the guidelines of the Canadian Council on Animal Care (Appendix 3). *Panx1<sup>-/-</sup>* mice were generated as previously described [33]. *Panx1<sup>-/-</sup>* mice were developed on a homogeneous C57BL/6 background in which Panx1 was systemically ablated. C57BL/6N mice (*Panx1<sup>+/+</sup>*) acted as control mice for all experiments. Mice were collected at 4 weeks, 7 weeks, parturition (L0), early lactation (L2) and involution (Forced weaned at L15 and collected I3). Mice were genotyped as previously described [33]. Following sacrifice of mice using CO<sub>2</sub>, body weights were recorded prior to dissection of inguinal and thoracic mammary glands. Four mammary glands were dissected for subsequent paraffin processing, whole mount analysis, cryosections and for protein lysates. Right inguinal mammary gland weights were recorded. For all experiments, at least five different animals per group were evaluated. Panx1 pup weights were recorded from multiple lactating dams.

### 5.2.2 Western blot analysis

Following dissection, mammary gland tissue was frozen at -80°C. Mammary gland tissues were homogenized on ice in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% NP-40), supplemented with protease inhibitor mixture (Roche-Applied Sciences) and phosphatase inhibitors (100 mM NaF and 100 mM Na<sub>3</sub>VO<sub>4</sub>). Total protein lysates were quantified using the bicinchoninic acid assay similar to the manufacturer's instructions (Pierce). Sixty µg of protein was loaded and resolved using 10% SDS-PAGE and transferred to nitrocellulose membranes using the iBlot Dry Blotting system (Invitrogen). Membranes were blocked using 5% bovine serum albumin (BSA) and 0.05% Tween20 in PBS for 1 hour at room temperature. Membranes were incubated with rabbit anti-Panx1 antibodies targeting the C-terminal (0.4 µg/ml, [9]), goat anti-β-casein (1:1000, sc-17971, Santa Cruz Biotechnology) and rat anti-Hsc70 (1:5000, SPA-815, Stressgen Bioreagents) antibodies diluted in blocking solution at 4°C overnight. Membranes were washed in PBST and subsequently incubated with Alexa 800-conjugated

goat anti-rabbit, Alexa 800-conjugated donkey anti-mouse, Alexa 680-conjugated goat anti-rat and Alexa 680-conjugated donkey anti-goat (1:5000, Life Technologies) secondary antibodies followed by visualization and quantification using the Odyssey Infrared Imaging System (Li-Cor Biosciences). N=3.

### 5.2.3 Real-time PCR analysis

Total RNA was extracted using the Qiagen RNeasy kits (Qiagen) from the mammary gland of 7-week-old females Panx1<sup>-/-</sup> and Panx1<sup>+/+</sup> mice. cDNA was generated using the RevertAid H minus, first-strand cDNA synthesis kit (Fermentas). Panx1 transcript levels were determined using mouse Panx1-specific primers (5' ACAGGCTGCCTTGTGGATTCA3'; 5' GGGCAGGTACAGGAGTATG3') and the iQ SYBR green Supermix (Bio-Rad, Mississauga) in a Bio-Rad CFX96 real-time system. Results were normalized to β2 microglobulin (5'CCCACTGAGACTGATAACATACGC3'; 5' GGTTCAAATGAATCTTCAGAGCAT 3'). N=3.

### 5.2.4 Immunofluorescence microscopy

Paraffin embedded sections (6 μm) were deparaffinised in xylene and rehydrated in descending concentrations of ethanol before being washed in ddH<sub>2</sub>O. Sections underwent antigen retrieval using Vector Antigen Unmasking Solution (Vector Labs) by microwaving them for 5 minutes at 80% power. Sections were allowed to cool for 15 minutes prior to being rinsed in PBS and placed in a sub-boiling second antigen retrieval solution (10 mM Tris Base, 1mM EDTA (pH 9.0) for 30 min prior to being rinsed in PBS. Crysections were sectioned (7 μm), stored in -80 °C and subsequently fixed in 10% neutral buffered formalin and rinsed in PBS. Sections were then blocked (3% BSA and 0.2% Triton X-100 in PBS) for 1 hour at room temperature. The following primary antibodies were incubated on samples diluted in blocking solution overnight at 4 °C; rabbit anti-Panx1 (4 μg/ml, or with peptide pre-adsoption assays as previously described [9]), mouse anti-cytokeratin 14 (1:300, Neomarkers, CL002), mouse anti-pan-cytokeratin (1:400, Abcam, ab7753), rabbit anti-perilipin (1:400, Cell Signaling, 9349), rabbit anti-Ki67 (1:400, Abcam, ab66155), cleaved caspase 3 (1:400, Cell Signaling, D175), rabbit anti-keratin8 (1:400, Abcam, ab53280), E-cadherin (1:400, BD Transduction Laboratories, 610182), β-catenin (1:400,

BD Transduction Laboratories, 610154), Cx26 (Cryosections, 1:100, Invitrogen, 51-2800), Cx30 (Cryosections, 1:100, Invitrogen, 71-2200), Cx32 (Cryosections, 1:100, Sigma, C3470) and  $\beta$ -casein (1:400, Santa Cruz Biotechnology, sc-17971). Primary antibodies were visualized by incubating sections with Alexa Fluor® 555-conjugated anti-rabbit or anti-mouse or anti-goat (1:400, Molecular Probes, A21425, A21429 or A21431) and Alexa Fluor® 488-conjugated anti-rabbit or anti-mouse (1:400 dilution, Molecular Probes, A11008 or A11017) secondary antibodies for 1 hour at room temperature. Hoechst stain was used to visualize nuclei before being mounted using Airvol. Immunolabeled sections were imaged (5-10 images per sample) using a Leica DM IRE2 inverted epifluorescence microscope equipped with Velocity 6.3.0 imaging software. For cytokeratin area quantification, green only fluorescent images were converted to binary using ImageJ and the pixel area was measured per  $0.3\text{ mm}^3$ . For adipocyte quantification, perilipin positive cells were counted per  $0.3\text{ mm}^3$ . For Ki67 and connexin plaque quantification, the number of Ki67 positive cells/connexin plaques was quantified per  $0.3\text{ mm}^3$ . In addition, the blue only fluorescent images were converted to binary using ImageJ and the pixel area was measured per  $0.3\text{ mm}^3$ . Graphs represent the mean ratio of the number of Ki67 positive cells or connexin plaques to blue fluorescent pixel area (nuclei).  $N \geq 5$ .

### 5.2.5 Whole mount analysis

Whole mount analysis was performed similar to that described in Plante et al. 2011 [34]. Briefly, mammary glands were dissected, flattened out on slides, and submersed in Carnoy's fixative for 4 hours at room temperature or overnight at  $4^\circ\text{C}$ . Glands were then submersed in 70% ethanol and gradually rehydrated in ddH<sub>2</sub>O before being stained overnight in carmine alum stain at room temperature. Glands were then gradually dehydrated (ddH<sub>2</sub>O, 70%, 95%, 100%, xylene; 5 min) and stored in methyl salicylate. Whole mounts were captured using a stereoscopic Sony camera on a light board. Virgin ductal elongation was quantified using calipers as previously described [35,36] by measuring the ratio of the distance from the bottom of the lymph node to the end of the longest duct relative to the distance from the bottom of the lymph node to the edge of the fat pad.

### 5.2.6 Histology

Paraffin blocks were sectioned (6  $\mu$ m), deparaffinized in xylene (10 min) and gradually rehydrated in ethanol (100%, 95%, 70%; 5min) prior to submersion in Harris' hematoxylin for 2 min. Slides were washed, dipped in acid ethanol (4X) and placed in 70% ethanol (1 min) prior to submersion in eosin (1 ml of acetic acid in 250 ml of eosin; 2 min). Slides were gradually dehydrated (70%, 95%, 100%, xylene; 1 min) and mounted using cytoseal (Richard-Allan Scientific). Histological analysis was performed by imaging 5-10 arbitrary images using a 5X and 40X objective lens and a ProgRes C5 camera (Jenoptik) and ProgRes Mac CapturePro 2.7.6 imaging software. The average number of lumens was quantified, with the mutant and control mouse mammary glands blinded to the investigator, using ImageJ software. In addition, the average lumen area was quantified using ImageJ in which the length and width (pixels) of lumens was measured and the average area of the lumen was estimated by calculating the elliptical area ( $A = ((L/2)*(W/2)*\pi)$ ).  $N \geq 5$ .

### 5.2.7 Evaluation of Panx1 mRNA *in silico*

Using the publicly available Kaplan-Meier Plotter (<http://kmplot.com>) described by Gyorffy et al. 2010 [37], we compared high and low mRNA expression groups of PANX1 (Affy id 204715\_at) in human breast cancer samples to clinical endpoints using the HGU133A and HGU133 Plus 2.0 microarrays as previously described [38]. We set our parameters to remove redundant samples, exclude biased arrays, auto-select for best cutoff and to use only the JetSet best probe set. PANX1 was evaluated in relation to overall survival (OS), relapse-free survival (RFS), distant metastasis free survival (DMFS), as well as evaluating Panx1 in relation to OS and RFS in distinct subgroups such as by molecular subtype or lymph node positive patient samples.

### 5.2.8 Statistical analysis

All statistical analyses of mouse studies were performed using GraphPad Prism 4.03 software in which statistical analysis compared means using a two-tailed unpaired student t-test. A two-way ANOVA was performed on pup weights. Error bars represented  $\pm$  SEM. For assessment of Panx1 as a biomarker, a log-rank test was performed using the online

tool as described [39]. For all experiments, a p value of less than 0.05 was considered significant.

## 5.3 Results

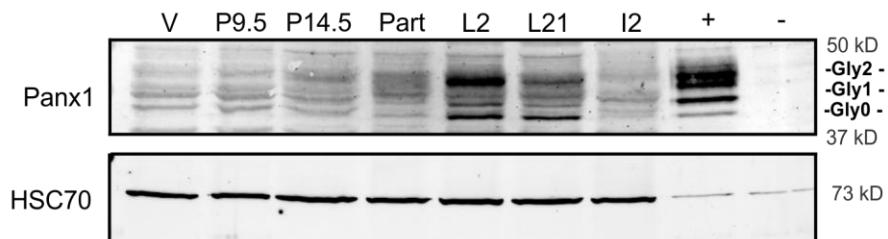
### 5.3.1 Panx1 is expressed in the mammary gland and dynamically regulated throughout mammary gland development

Mammary gland lysates from wild-type virgin, pregnant, lactating and involuting *Panx1*<sup>+/+</sup> mice were assessed by western blot for expression of Panx1 revealing expression at all stages of development and that Panx1 is upregulated in pregnant and lactating mice compared to the virgin gland (Figure 5.1A). As Panx1 appears to peak during early lactation, mammary gland sections of *Panx1*<sup>+/+</sup> mice at lactation D2 were immunolabelled to reveal the localization of the positive Panx1 expression in the mammary gland which was lost following adsorption of anti-Panx1 antibodies with immunizing peptides (Figure 5.1B). Panx1 expression appeared localized to the luminal epithelial cells, based on the absence of co-labelling with the myoepithelial marker keratin14 (Figure 5.1B). Mammary glands from *Panx1*<sup>-/-</sup> and *Panx1*<sup>+/+</sup> mice at lactation were evaluated for Panx1 protein and mRNA expression using western blot and real-time PCR analysis, respectively. Panx1 was found to be ablated from the mammary glands of Panx1 null mice (Figure 5.1C, D). Therefore, Panx1 is dynamically expressed in the pregnant mammary gland and ablated in *Panx1*<sup>-/-</sup> mouse mammary glands.

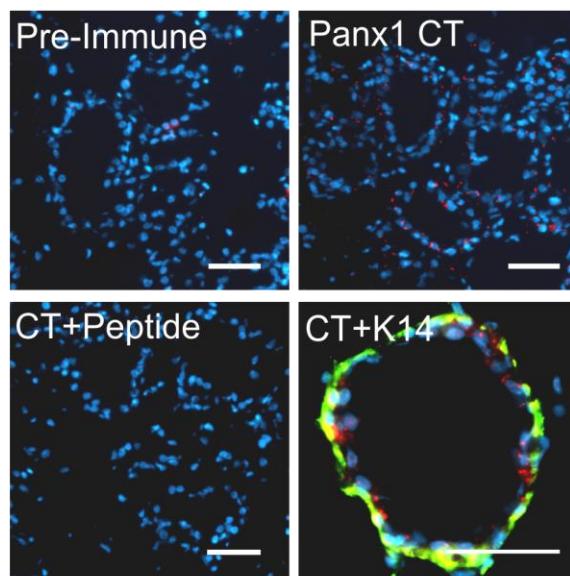
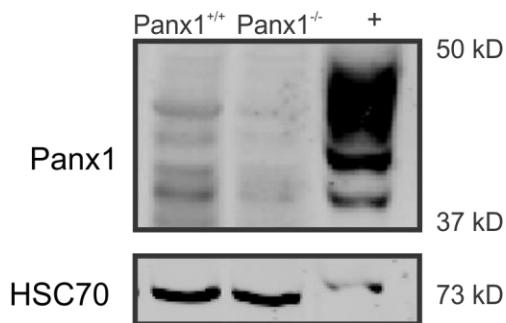
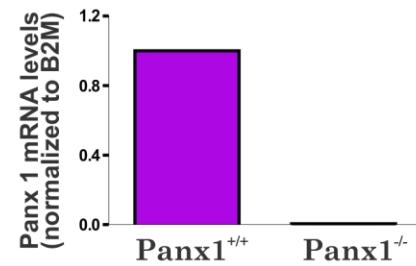
### 5.3.2 Virgin *Panx1*<sup>-/-</sup> mice retain normal mammary glands

Mammary glands from 4 and 7 week old mice were collected, weighed and subjected to whole mount and histological analysis. Although body weights were significantly elevated in 4 week old *Panx1*<sup>-/-</sup> mice (Figure 5.2A), this did not correspond to an increased mammary gland weight (Figure 5.2B, C). Body weight and normalized mammary gland weight were similar in 7 week old mice compared to controls (Figure 5.2A-C). Whole

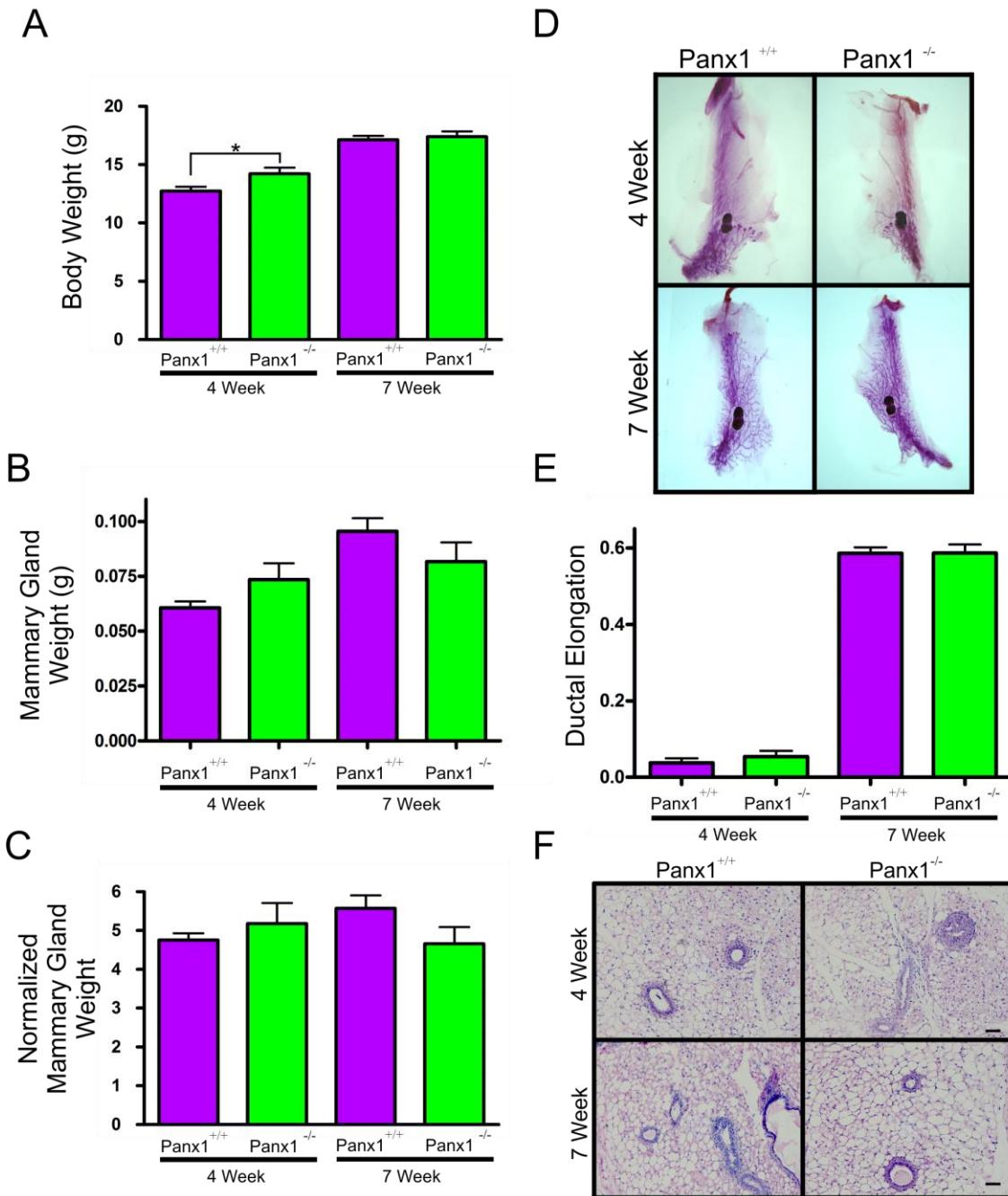
**Figure 5.1. Panx1 is upregulated during pregnancy in luminal epithelial cells and is not expressed in *Panx1*<sup>-/-</sup> mice.** (A) Western blot analysis revealed that Panx1 (red) is upregulated following the onset of pregnancy and remains elevated throughout lactation. Multiple bands on the western blot represent various Panx1 species due to changes in glycosylation (Gly0, Gly1 and Gly2). (B) Punctate staining observed for Panx1 (red) is not observed using pre-immune serum or following adsorption of the antibody with cognate peptide and does not colocalize with the myoepithelial marker Keratin14 (K14, green). (C, D) Western blot and real-time PCR analysis revealed that Panx1 is not expressed in *Panx1*<sup>-/-</sup> mice compared to *Panx1*<sup>+/+</sup> mice. + represents HEK 293T cells overexpressing Panx1. Hoescht (blue) denotes nuclei. Scale bar = 50  $\mu$ m. N=3.

**Figure 5.1****A****B**

WT Lactation D2

**C****D**

**Figure 5.2. Virgin *Panx1*<sup>-/-</sup> mice have normal mammary glands.** (A) Evaluation of body weights at 4 and 7 weeks revealed that pre-pubertal *Panx1*<sup>-/-</sup> mice were significantly larger compared to control mice. Values are mean body weights  $\pm$  S.E.M. \* $p<0.05$ . (B, C) Mammary gland weight and normalized mammary gland weight were not significantly different in *Panx1*<sup>-/-</sup> mice compared to *Panx1*<sup>+/+</sup> mice. Values are mean weights  $\pm$  S.E.M. (D) Whole mount analysis with carmine alum staining revealed normal epithelial ductal architecture embedded in a well-developed stroma. (E) Quantification of ductal elongation revealed no significant differences in knockout and control mice. Bars represent the mean ductal extensions from the bottom of the lymph node to the furthest migrating duct relative to the length of the mammary gland from the bottom of the lymph node to the edge of the fat pad  $\pm$  S.E.M. (F) Histological evaluation of hematoxylin and eosin stained glands revealed normal tissue architecture in virgin *Panx1*<sup>-/-</sup> mice compared with control mice. N=9. Scale bar = 50  $\mu$ m.

**Figure 5.2**

mount analysis of 4 week old mice revealed a rudimentary ductal structure in the mammary glands of *Panx1* knockout mice similar to control mice suggesting that loss of *Panx1* does not significantly impede embryonic mammary gland development (Figure 5.2D). In addition, the relative duct length of 4 and 7 week old whole mounts from *Panx1*<sup>-/-</sup> mice was similar to control mice suggesting that *Panx1* is not critical for ductal outgrowth during pubertal growth in virgin mice (Figure 5.2E). Similar to whole mount analysis, *Panx1* knockout mice had comparatively normal histology as epithelial ducts were embedded within a well-developed mammary fat pad in *Panx1* null and control mice (Figure 5.2F). Taken together, virgin mammary glands from *Panx1* knockout mice develop similar to wild-type mice.

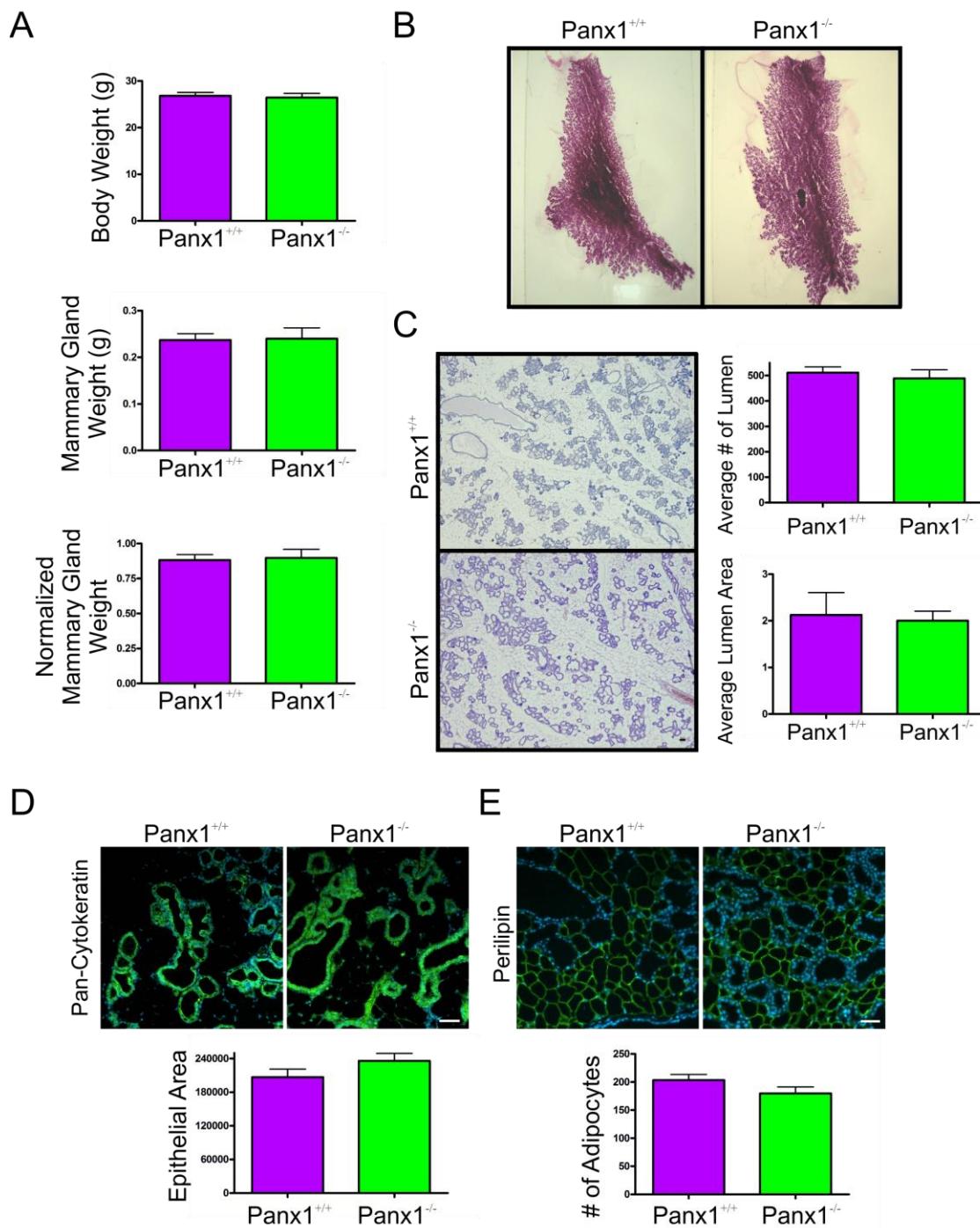
### 5.3.3 *Panx1*<sup>-/-</sup> mice at parturition have normal mammary glands

In order to assess the role of *Panx1* following pregnancy, mammary glands from *Panx1*<sup>-/-</sup> mice and wild-type control mice were collected at parturition, weighed, and assessed for changes in gland architecture using histological and whole mount approaches. Body weights, mammary gland weight and normalized mammary gland weight were similar between *Panx1*<sup>-/-</sup> and control mice (Figure 5.3A). Whole mount and histological analysis revealed similar tissue architecture of *Panx1*<sup>-/-</sup> mice compared to control mice (Figure 5.3C). Quantification of the average number of lumen and area of the lumen between *Panx1* knockout and wild-type H&E stained sections was similar suggesting comparable alveolar development at parturition (Figure 5.3C). Similarly, quantification of the pixel area of the epithelial marker pan-cytokeratin in mammary gland sections of *Panx1*<sup>-/-</sup> and *Panx1*<sup>+/+</sup> mice revealed similar epithelial area (Figure 5.3D). Finally, immunofluorescent labelling and quantification of the number of adipocytes revealed similar stromal development in the glands of *Panx1* knockout and wild-type mice (Figure 5.3E).

### 5.3.4 During Early Lactation *Panx1*<sup>-/-</sup> Mice have Impaired Alveolar Development but Relatively Normal Differentiation

To assess whether differences in gland development occurred following feeding of the pups, mammary glands of *Panx1*<sup>-/-</sup> were assessed 48 hours after parturition and compared

**Figure 5.3. *Panx1*<sup>-/-</sup> mice have normal mammary glands at parturition.** (A) Evaluation of body weight, mammary gland weight and normalized mammary gland weight revealed that *Panx1*<sup>-/-</sup> mice were similar to control mice at parturition. (B) Whole mount analysis revealed numerous alveoli filling the mammary fat pad. (C) Histological evaluation of haematoxylin and eosin stained glands revealed normal tissue architecture, a similar average number of alveoli/ducts (per 2.5 mm<sup>2</sup>) and a similar average alveolar/ductal lumen area (per 0.04 mm<sup>2</sup>) in *Panx1 null* mice compared to control mice. (D) Quantification of the average epithelial area (pixel) as assessed with immunofluorescent analysis using pan-cytokeratin (green) per 0.3 mm<sup>2</sup> revealed similar epithelial area in the mammary gland of *Panx1*<sup>-/-</sup> mice compared with control mice. (E) Quantification of the average number of adipocytes as assessed with immunofluorescent analysis using perilipin (green) per 0.3 mm<sup>2</sup> revealed a similar number of adipocytes in *Panx1*<sup>-/-</sup> mice compared with control mice. Hoescht (blue) denotes nuclei. Bars are means ± S.E.M. N=6. Scale bars = 50 µm.

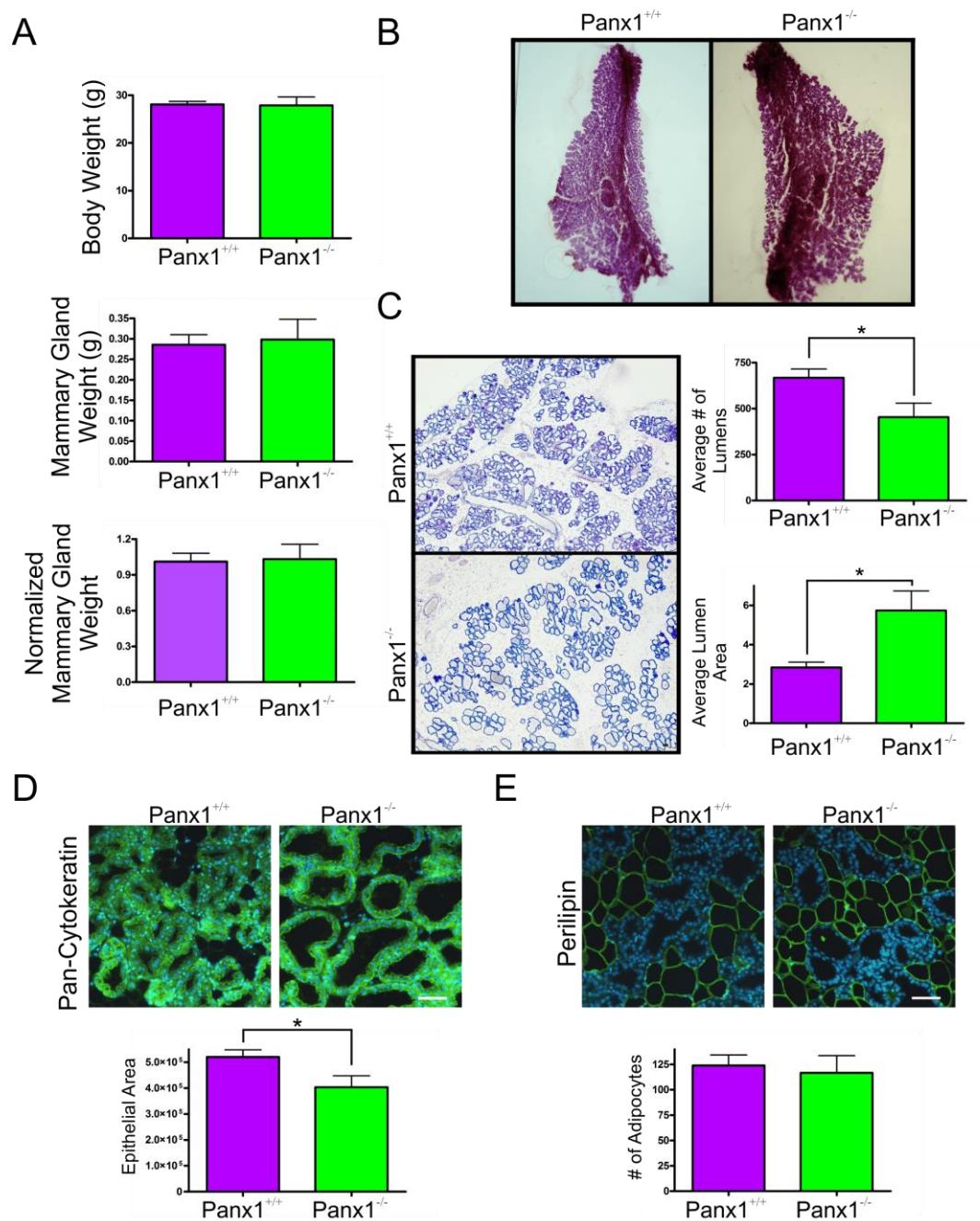
**Figure 5.3**

to *Panx1*<sup>+/+</sup> mice. Similar to mice on the day of parturition, body weights, mammary gland weights and normalized mammary gland weights were not significantly different during early lactation in *Panx1*<sup>-/-</sup> and control mice (Figure 5.4A). Interestingly, whole mount and histological analysis of mammary glands revealed a significant decrease in the average number of lumens of *Panx1* knockout mice with a concomitant significant increase in the average lumen area significant compared to wild-type mice suggesting reduced alveolar development in early lactation (Figure 5.4B,C). Furthermore, a reduction in the average epithelial pixel area, but not in the number of adipocytes glands, of *Panx1*<sup>-/-</sup> mice compared to *Panx1*<sup>+/+</sup> mice supported a role for reduced alveolar development of early lactating *Panx1* knockout mice (Figure 5.4D,F).

Comparison of the number of lumens in mammary glands at parturition and early lactation revealed a significant increase in the number of alveoli during the 48 hours following parturition in *Panx1*<sup>+/+</sup> mice but not in *Panx1*<sup>-/-</sup> mice (Figure 5.5A). To assess whether this difference was the result of impaired proliferation or increased apoptosis, mammary glands of *Panx1* knockout and wild-type mice were immunolabelled with the proliferation marker Ki67. This study revealed a significant decrease in the number of Ki67 positive cells during early lactation, but not parturition, between *Panx1*<sup>-/-</sup> and control mice (Figure 5.5B). Qualitative assessment of cleaved caspase-3 immunolabelling revealed relatively few apoptotic cells in the lactating glands of *Panx1*<sup>-/-</sup> mice compared to wild-type mice suggesting that the reduced alveolar development was the result of decreased proliferation as opposed to increased cell death (Figure 5.5D, F).

In order to assess whether the defect in alveogenesis affects the differentiation of the mammary glands, markers of differentiation including the luminal markers keratin8, E-cadherin and  $\beta$ -catenin as well as the myoepithelial marker keratin14, were assessed by immunofluorescent analysis (Figure 5.6). Both *Panx1* knockout and control mice had similar and well defined expression of all epithelial markers (Figure 5.6A-C). Furthermore, evaluation of the expression of luminal connexins revealed a significant decrease in the relative number of Cx32 gap junction plaques, but not Cx26 or Cx30, in *Panx1*<sup>-/-</sup> mice.

**Figure 5.4. *Panx1*<sup>-/-</sup> mice present with a reduction in the number of alveoli during early lactation.** (A) Evaluation of body weight, mammary gland weight and normalized mammary gland weight revealed that *Panx1*<sup>-/-</sup> mice were similar to control mice. (B) Whole mount analysis revealed numerous alveoli filling the mammary fat pad. (C) Histological evaluation of hematoxylin and eosin stained glands revealed a reduction in the number alveoli in the mammary glands (per 2.5 mm<sup>2</sup>) of *Panx1*<sup>-/-</sup> mice that were significantly larger than those from *Panx1*<sup>+/+</sup> mice (per 0.04 mm<sup>2</sup>). (D) Quantification of the average epithelial area (pixel) as assessed with pan-cytokeratin (green) per 0.3 mm<sup>2</sup> revealed a significant decrease in the amount of epithelium in the mammary gland of *Panx1*<sup>-/-</sup> mice compared with control mice. (E) Quantification of the average number of adipocytes, as assessed with perilipin (green) per 0.3 mm<sup>2</sup>, revealed similar cell numbers in the mammary gland of *Panx1*<sup>-/-</sup> mice compared with control mice. Hoescht (blue) denotes nuclei. \*p<0.05. Values are means ± S.E.M. N=6. Scale bars = 50 µm.

**Figure 5.4**

**Figure 5.5. *Panx1*<sup>-/-</sup> mice have reduced proliferation during early lactation.** (A) The average number of lumens was significantly increased in early lactation compared to parturition in *Panx1*<sup>+/+</sup> mice which was not observed in *Panx1*<sup>-/-</sup> mice. Values represent the mean number of lumen per  $2.5\text{mm}^2 \pm \text{S.E.M.}$  (B) Immunofluorescent analysis of the proliferation marker Ki67 (Red; parturition, green; lactation) revealed significantly reduced proliferation in mammary glands of *Panx1*<sup>-/-</sup> mice compared to controls during early lactation but not at parturition. Values represent the mean number of Ki67 positive cells relative to the pixel area of the nuclei, multiplied by a factor of  $1 \times 10^4$ , per  $0.3\text{ mm}^2 \pm \text{S.E.M.}$  (C) Immunofluorescent analysis of the apoptotic marker cleaved caspase3 revealed little apoptosis in the glands of knockout mice, similar to controls. Inserts represent positive controls in the involuting mammary gland. Hoescht (blue) denotes nuclei. \* $p<0.05$ . N=6. Scale bars = 50  $\mu\text{m}$ .

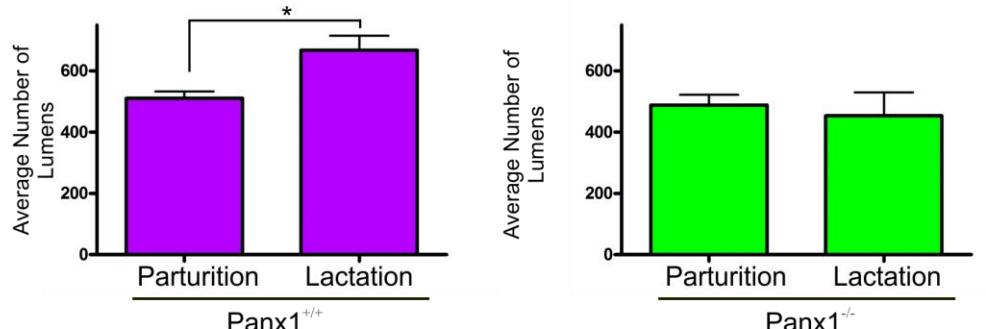
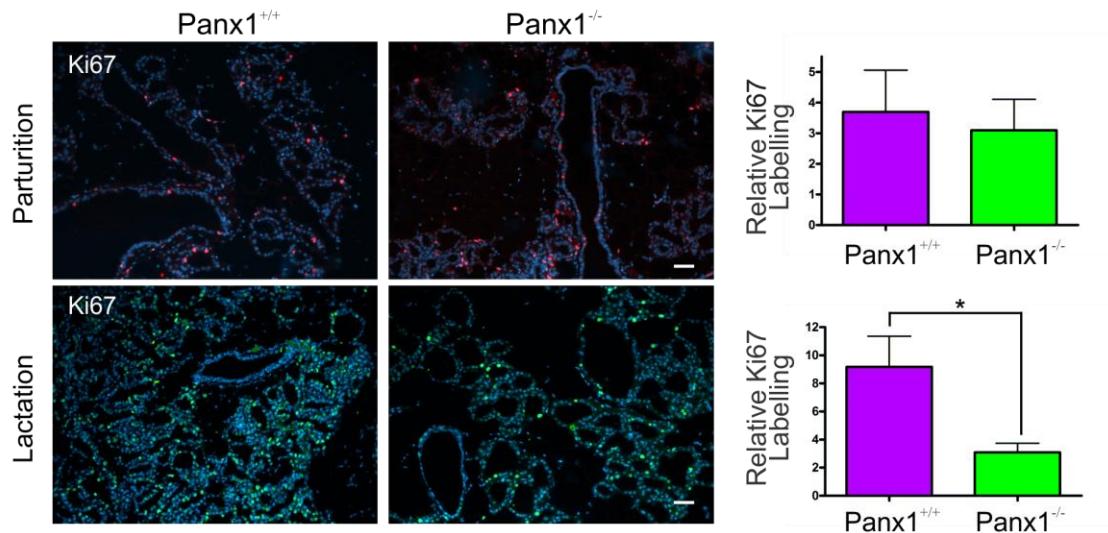
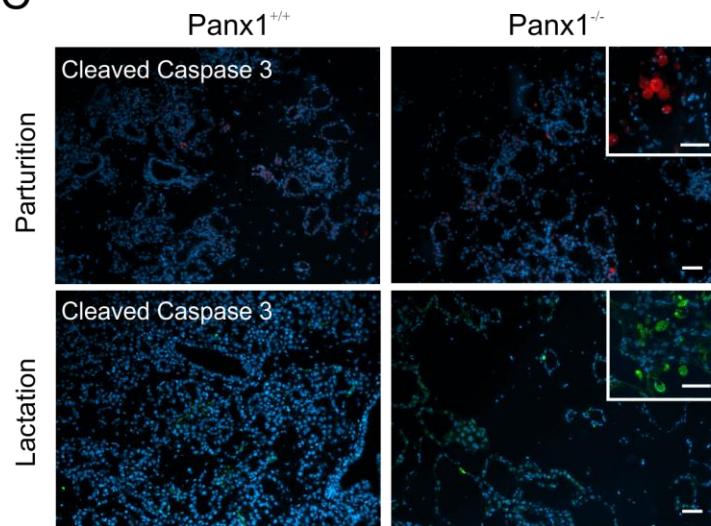
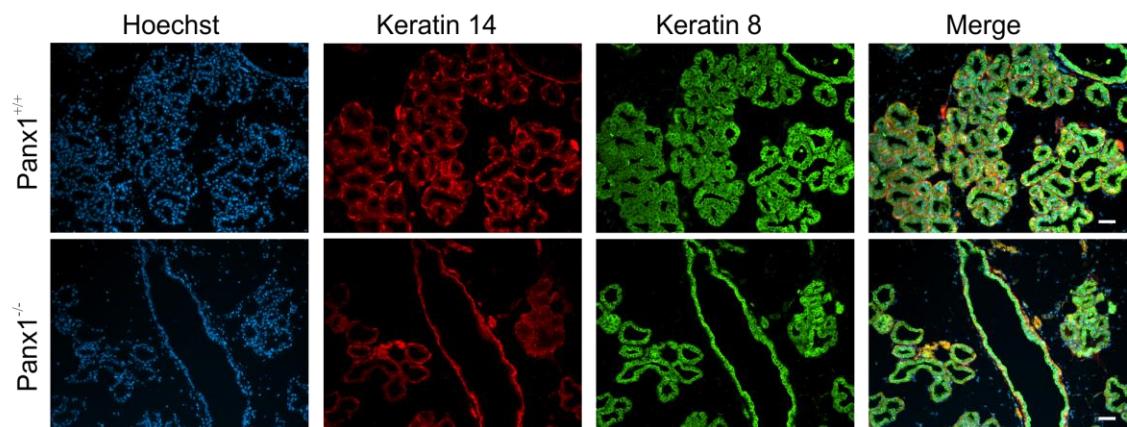
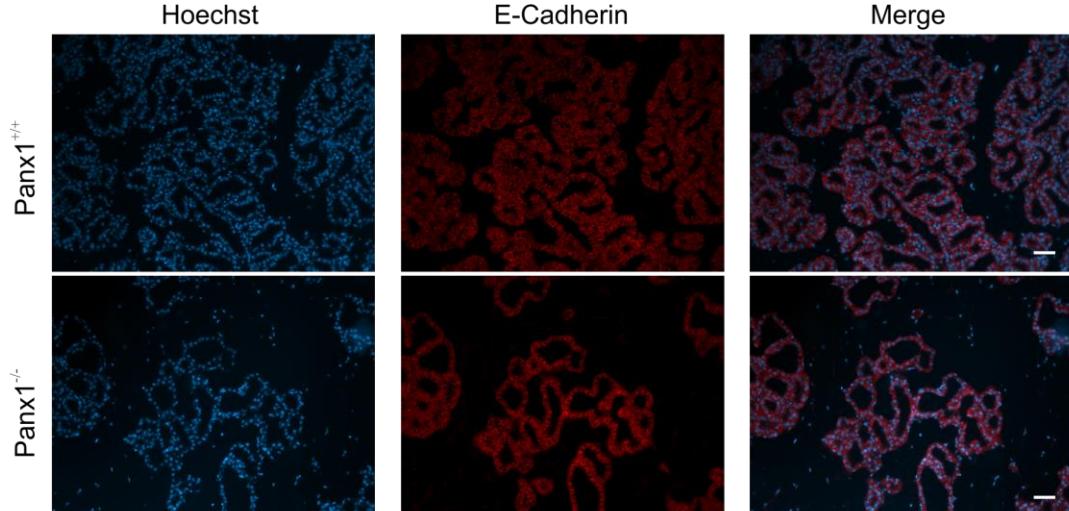
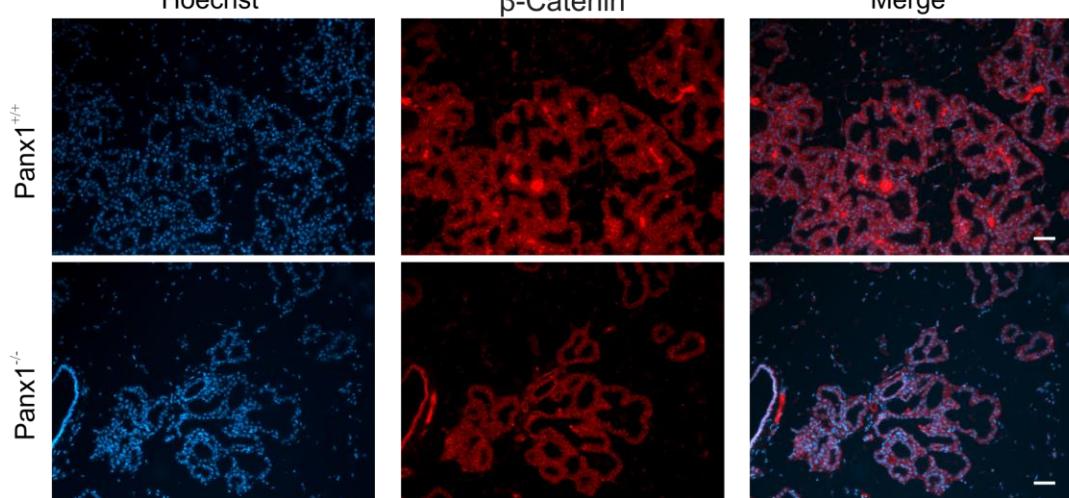
**Figure 5.5****A****B****C**

Figure 5.6. *Panx1<sup>-/-</sup>* mice have normal mammary gland epithelial differentiation at lactation. (A) Immunofluorescent analysis of luminal epithelial marker keratin 8 (green) and myoepithelial marker keratin14 (red) revealed a similar staining pattern in *Panx1<sup>-/-</sup>* mice compared to control mice during lactation. (B, C) Immunofluorescent analysis of mammary differentiation markers E-cadherin (B, red) and  $\beta$ -Catenin (C, red) revealed a similar staining profile in *Panx1<sup>-/-</sup>* mice and *Panx1<sup>+/+</sup>* mice. Hoescht (blue) denotes nuclei. N=6. Scale bars = 50  $\mu$ m.

**Figure 5.6****A****B****C**

As connexins have previously been used as markers of proper terminal differentiation [40], these results suggest that following parturition, *Panx1*<sup>-/-</sup> mice may not have completed the full differentiation of the mammary gland (Figure 5.7). Taken together, *Panx1*<sup>-/-</sup> mice have impaired mammary gland alveolar development during early lactation but relatively normal mammary gland differentiation.

### 5.3.5 Lactating *Panx1*<sup>-/-</sup> mice have normal mammary gland function

To determine whether the developmental defects associated with the mammary gland observed in lactating *Panx1*<sup>-/-</sup> dams resulted in impaired feeding of pups, 30-50 pups from multiple litters were weighed every other day revealing relatively normal pup weights between *Panx1*<sup>-/-</sup> dams and *Panx1*<sup>+/+</sup> dams (Figure 5.8A). However, significantly decreased pup weights were found at day 6 while significantly increased pup weights were recorded at day 18 and day 20 from Panx1 knockout dams compared to Panx1 wild-type dams (Figure 5.8A). Importantly, *Panx1*<sup>-/-</sup> mice had similar litter size to *Panx1*<sup>+/+</sup> mice in which pup death was uncommon (Figure 5.8B). Finally, western blot and immunofluorescent analysis of the common milk protein β-casein revealed no-significant difference between mammary glands of Panx1 knockout and control mice suggesting milk production is unaffected (Figure 5.8C,D). Taken together, loss of Panx1 does not severely impair mammary gland function.

### 5.3.6 *Panx1*<sup>-/-</sup> mice have normal mammary gland involution

In order to assess the role of Panx1 during involution, pups from *Panx1*<sup>-/-</sup> mice and wild-type dams were force weaned at day 15 of lactation and three days later mammary glands were collected. Body weights, mammary gland weight and normalized mammary gland weight were similar between *Panx1*<sup>-/-</sup> and control mice (Figure 5.9A). Whole mount and histological analysis revealed similar tissue architecture of *Panx1*<sup>-/-</sup> mice compared to control mice (Figure 5.9B, C). Qualitative assessment of immunolabelled mammary gland for the apoptotic marker cleaved caspase 3 revealed comparably similar amount of cell

**Figure 5.7. Lactating *Panx1*<sup>-/-</sup> mice have fewer Cx32 gap junctions in the mammary gland.** (A) Immunofluorescent analysis of mammary gland cryosections during early lactation for Cx26, Cx30 and Cx32 (red) and cytokeratin14 (green) revealed no change in Cx26 and Cx30 gap junctions in knockout mice, while fewer Cx32 gap junctions were observed compared to control mice. Hoescht (blue) denotes nuclei. Scale bar = 50  $\mu$ m. (B) Values represent the mean number of connexin plaques (red) relative to the pixel area of the nuclei (blue), multiplied by a factor of 1x10<sup>2</sup>, per 0.3 mm<sup>2</sup>  $\pm$  S.E.M. N=5.

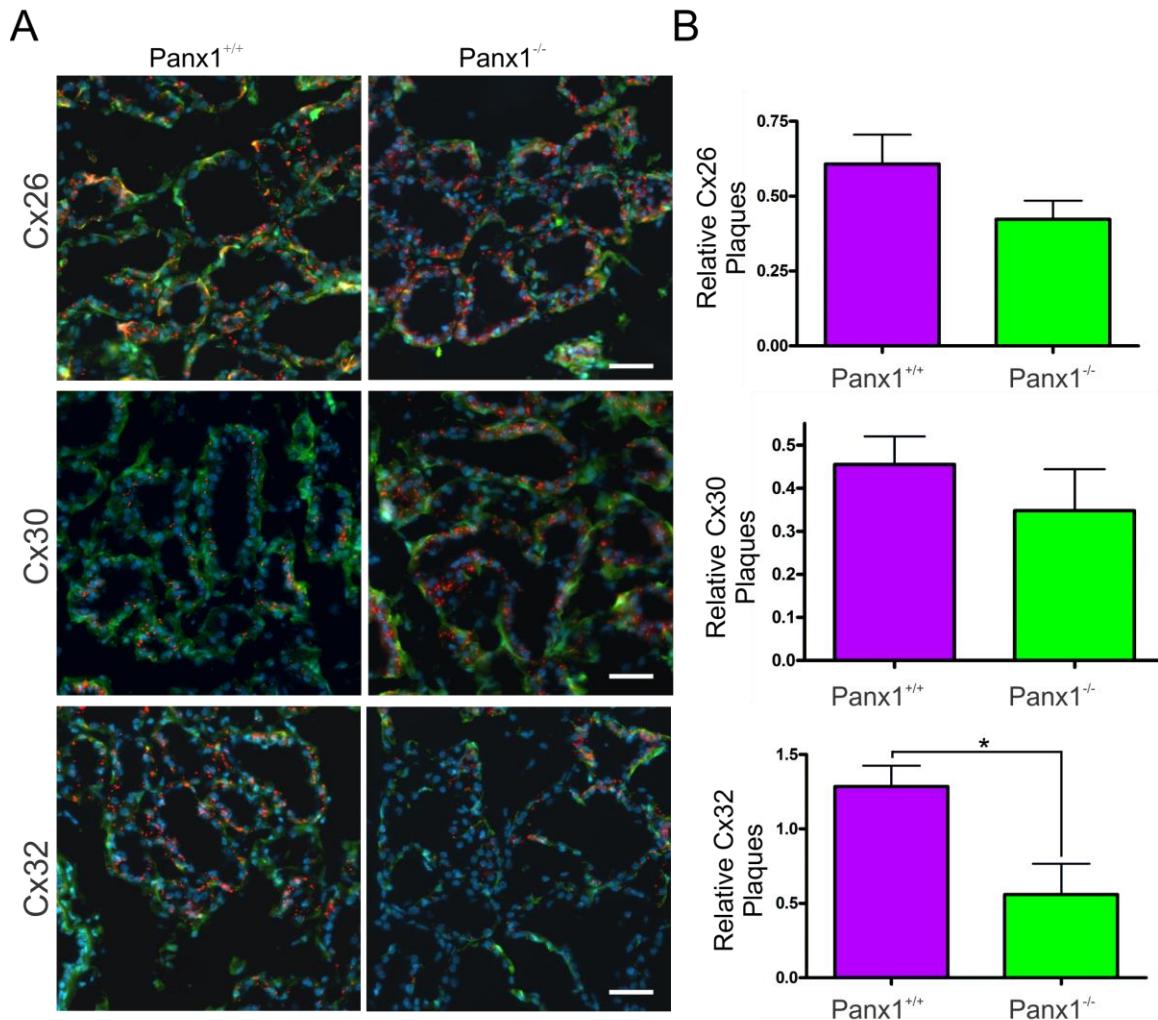
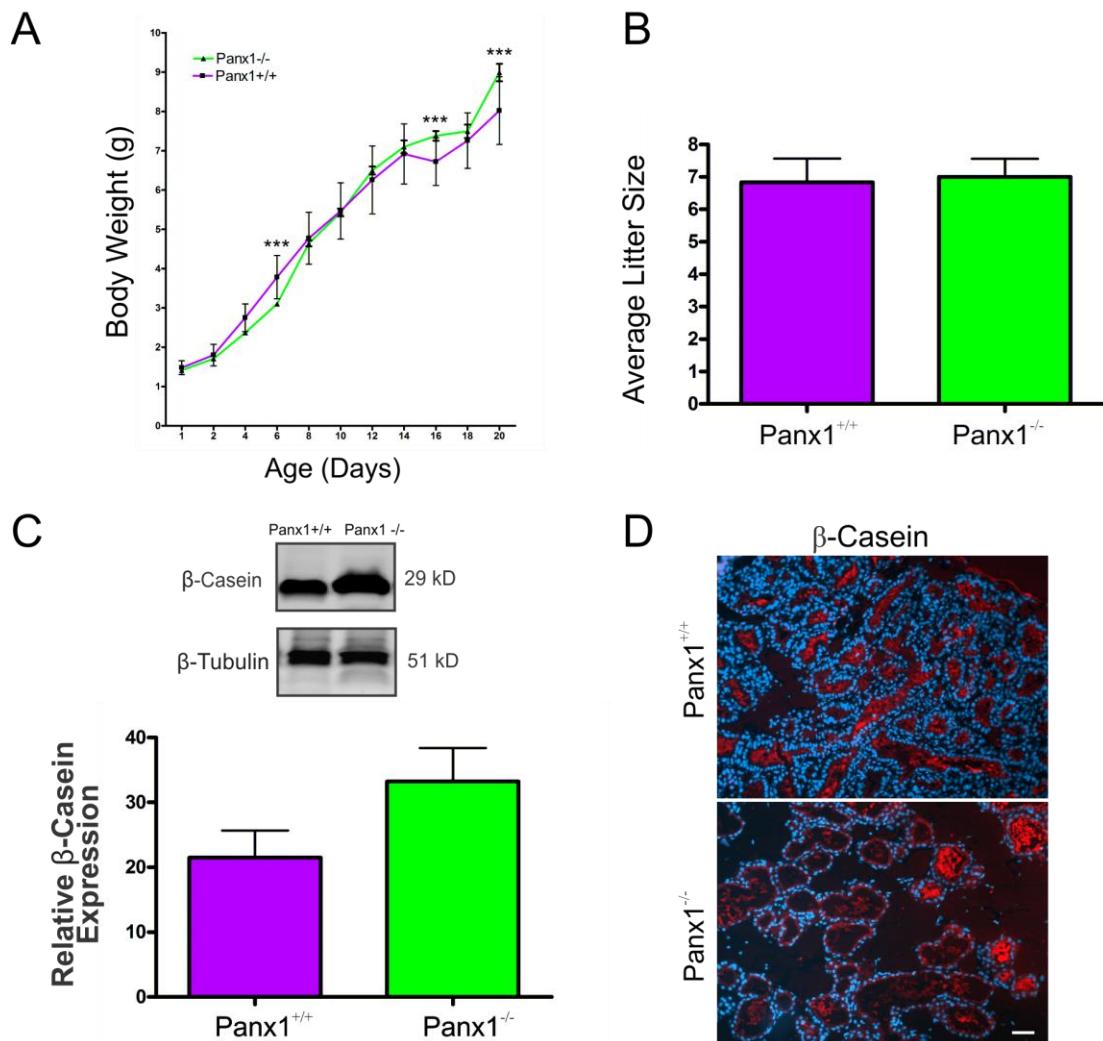
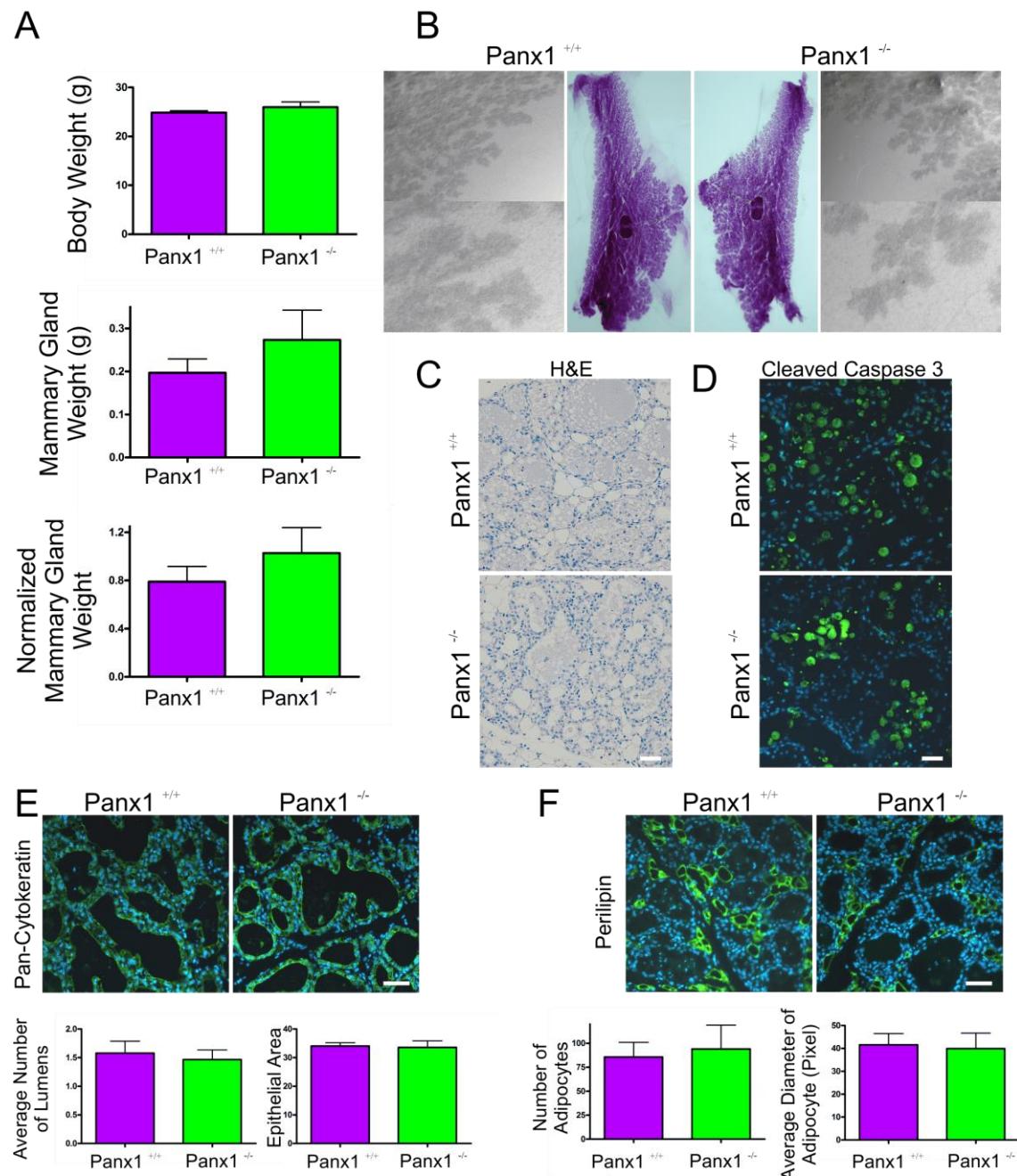
**Figure 5.7**

Figure 5.8. ***Panx1*<sup>-/-</sup> dams lactate and deliver milk to pups.** (A) Evaluation of pup body weights from 1-20 day old pups revealed significant differences between *Panx1*<sup>-/-</sup> mice near weaning age (D21) compared to *Panx1*<sup>+/+</sup> mice (N=30-50 pups). \*\*\*p≤0.001. (B) Litters from 10 *Panx1*<sup>-/-</sup> dams were evaluated for litter sizes revealing similar numbers compared to control mice. (C, D) Western blot and immunofluorescent analysis of lactating mammary glands revealed no significant difference in the milk protein β-casein (red) in knockout and control mice. β-casein normalized to β-tubulin. N=6. Hoescht (blue) denotes nuclei. Values are means ± S.E.M. Scale bars = 50 μm.

**Figure 5.8**

**Figure 5.9. Involuting *Panx1*<sup>-/-</sup> mouse mammary glands have normal gland regression.**

(A) Evaluation of body weight, mammary gland weight and normalized mammary gland weight revealed no significant difference between in *Panx1*<sup>-/-</sup> mice compared to control mice. (B, C) Whole Mount and histological evaluation with haematoxylin and eosin revealed similar regression of glands in *Panx1*<sup>-/-</sup> mice and *Panx1*<sup>+/+</sup> mice. (D) Immunofluorescent analysis of the apoptotic marker cleaved caspase-3 revealed qualitatively similar amounts of apoptosis in *Panx1* null mice and wild-type mice. (E) Quantification of the average epithelial area (pixel<sup>2</sup>) and lumen number as assessed with pan-cytokeratin (green) per 0.3 mm<sup>2</sup> were similar in *Panx1*<sup>-/-</sup> mice compared with control mice. (F) Quantification of the average number and diameter of adipocytes as assessed with perilipin (green) per 0.3 mm<sup>2</sup> revealed similar numbers in the mammary gland of *Panx1*<sup>-/-</sup> mice compared with control mice. Hoescht (blue) denotes nuclei. Values are means ± S.E.M. N=5. Scale bars = 50 µm.

**Figure 5.9**

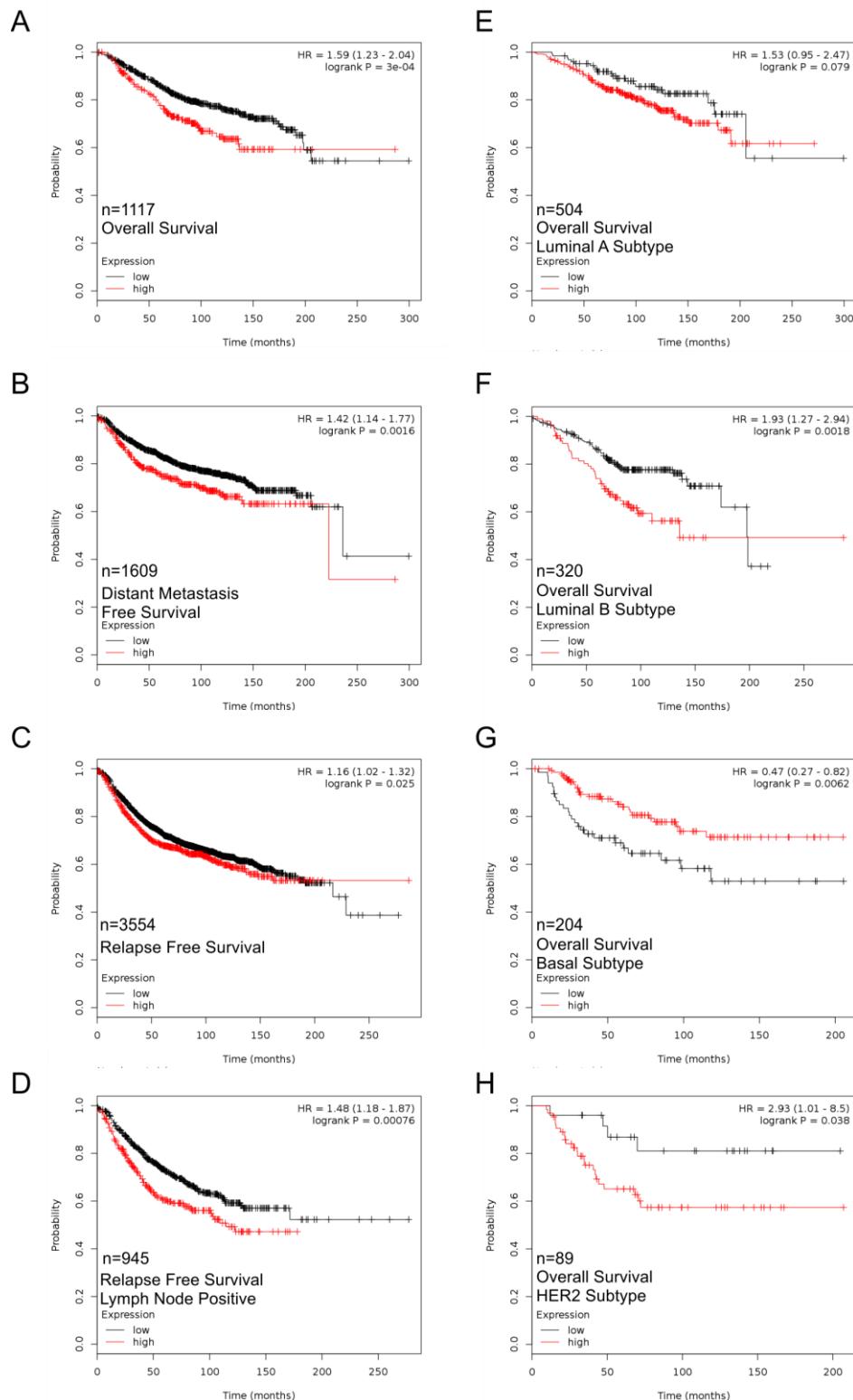
death between *Panx1*<sup>-/-</sup> mice and control mice (Figure 5.9D). Quantitative assessment of the number of lumen and relative epithelial area following labelling with pan-cytokeratin antibody also revealed a similar extent of epithelium in Panx1 knockout and wild-type involuting mammary glands suggesting comparable gland remodeling (Figure 5.9E). Similarly, evaluation of the number of adipocytes and the average diameter of adipocytes revealed no significant difference in the mammary glands of Panx1 knockout and control mice suggesting similar adipocyte repopulation of gland during involution (Figure 5.9F). Taken together, mammary glands of Panx1 null mice have comparable mammary gland involution to Panx1 wild-type mice.

### 5.3.7 Panx1 expression is correlated with poor overall survival in breast cancer

As Panx1 appeared to be a regulator of cell growth during early lactation, PANX1 was evaluated in the context of breast cancer by comparing human tumor samples with high or low mRNA expression of PANX1 with clinical outcomes. Interestingly, high expression of PANX1 in patient tumors was significantly correlated with worse OS, DMFS and RFS compared with those that had low PANX1 expression (Figure 5.10A- C). Importantly, when comparing high PANX1 expression to OS in lymph node positive patients with advanced disease, high Panx1 mRNA expression maintained a similar significant negative correlation with OS (Figure 5.10A- C). In addition, high PANX1 expression was compared with OS in the context of the Luminal A, Luminal B, Basal and Her2+ molecular subtypes. High PANX1 expression was not significantly associated with OS in the luminal A subgroup (Figure 5.10E). However, high PANX1 was significantly correlated with worse OS in luminal B and HER2+ samples compared to low PANX1 expressing tumours (Figure 5.10F,H). Interestingly, high PANX1 was associated with significantly better OS in tumors of the basal subtype suggesting potentially differential roles of PANX1 that are dependent on the molecular subtype (Figure 5.10G). Ultimately, PANX1 appears to be associated with worse clinical outcome, although this may be dependent on the molecular subtype of the tumor.

**Figure 5.10. High PANX1 mRNA expression is correlated with poor overall survival, particularly in the Luminal B and Her2+ subtypes, as revealed by *in silico* analysis.**

Breast cancer patient samples expressing high PANX1 mRNA expression were significantly correlated with worse overall survival (A), distant metastasis free survival (B), relapse free survival (C) and relapse free survival of lymph node positive patients (D) compared with low PANX1 expression patients. (E) Tumors of the luminal A subtype had a similar correlation to overall survival in high and low PANX1 expressing samples. High PANX1 expression in luminal B (F) and Her2+ (H) tumors was associated with significantly reduced overall survival compared to low PANX1 expression, unlike tumors of the basal subtype which was associated with improved overall survival. A p-value less than 0.05 was considered significant.

**Figure 5.100**

## 5.4 Discussion

The purpose of this study was threefold; first, to assess whether Panx1 was expressed and dynamically regulated in the mammary gland; second, to determine whether loss of Panx1 altered the development of pubertal and lactating mice while establishing if Panx1 affected normal mammary gland function; and finally, to assess whether Panx1 in the mammary gland may have implications extending to patients with breast cancer.

### 5.4.1 Panx1 is expressed in the lactating mammary gland

Panx1 has an ubiquitous expression profile and has been reported in the mouse mammary gland based on expression profiling arrays in NCBI's gene expression Omnibus database (ID 1416379, 78225667 [17]). In the murine mammary gland, Panx1 is expressed and upregulated during pregnancy where it remains elevated during lactation. Developmental regulation of Panx1 is associated with higher expression at earlier stages of development in many organs including the neonatal rat brain and murine newborn skin compared to aged counterparts [14,16,24]. Importantly, primary human muscle myoblasts induced to differentiate in culture upregulate the expression of Panx1 while ectopic expression of Panx1 in these cells induces differentiation *in vitro* [15]. Collectively, these results suggest a critical role for Panx1 in cell differentiation [15]. Unlike other organs, the mammary gland develops only a rudimentary ductal structure in prenatal mice and requires the onset of pregnancy to induce terminal differentiation of the gland [1]. Therefore, expression of Panx1 during pregnancy and lactation builds on the idea that Panx1 is upregulated in organs undergoing development and differentiation. As such, it might be expected that Panx1 is expressed in the embryonic mammary gland. While we cannot rule this out, loss of Panx1 does not significantly impair the ability of the gland to develop a rudimentary ductal structure and undergo normal ductal development during puberty in the virgin mammary gland. As a result, Panx1 may be more important in the pregnant than the embryonic mammary gland. This is similar to the gap junction, large-pore channel protein, Cx26, which has a critical role after the onset of pregnancy while being less important at earlier stages of mammary gland development [41]. Our results suggest that Panx1 is expressed

in luminal epithelial cells although many other cell types that can be found within the stromal compartment of the mammary gland have been reported to express Panx1 including fibroblasts [24], adipocytes [42], immune cells [43], erythrocytes [20], and cells of the vasculature [44]. While our staining was much more evident in the epithelium than in the stromal compartment of the gland, we have not ruled out that Panx1 upregulation may also occur in these other cell type residents of the mammary gland. Importantly, the *Panx1<sup>-/-</sup>* mice used in this study are null for Panx1 in all cell-types of the mammary gland.

#### 5.4.2 Panx1 is necessary for timely alveolar development and differentiation in the lactating mammary gland

Terminal development of the mammary gland is driven by hormonal signaling that regulates proliferation and differentiation in the mammary gland. *Panx1<sup>-/-</sup>* mice had reduced alveolar development in early lactating mice due to impaired proliferation of the mammary gland that was not apparent at parturition. Day 2 and 3 of lactation represents a major proliferative time point of epithelial expansion in the mammary gland as indicated by increased DNA synthesis measured through recordings of [ $H^3$ ] thymidine incorporation [45,46]. Importantly, hormonal regulation driving epithelial cell proliferation during early lactation is believed to be due to pituitary prolactin and ovarian estrogen secretion [45]. Prolactin and estrogen have previously been shown to be absolutely essential to normal lobuloalveolar proliferation and differentiation as evidence by impaired lobuloalveolar defect in knockout mouse models of their respective receptors [40,47,48]. Interestingly, when mammary tissue from prolactin receptor knockout (*PrlR<sup>-/-</sup>*) mice is transplanted into wild-type cleared fat pads, due to issues of infertility, mammary gland proliferation and differentiation are impaired [48]. Of note, this coincides with complete loss of expression of the gap junction protein, Cx32 [48].

Similarly, estrogen receptor beta knockout (*ER $\beta^{-/-}$* ) mice also develop with impaired alveolar development and altered differentiation and have been further assessed to have reduced numbers of Cx32 gap junction plaques [40]. Therefore, it is interesting to speculate that Panx1 may impair either prolactin or estrogen mediated signaling as *Panx1<sup>-/-</sup>* mice also present with mammary glands with fewer Cx32 gap junction plaques. It seems more likely that Panx1 is implicated in the ER $\beta$  pathway, as *ER $\beta^{-/-}$*  mice have reduced alveolar lumen

number and present with dilated alveolar lumen similar to *Panx1*<sup>-/-</sup> mice [40]. In addition, unlike *PrlR*<sup>-/-</sup> mammary glands, loss of Panx1 or *ER $\beta$* <sup>-/-</sup> mice is not associated with impaired milk production [48]. In fact, mammary function is relatively normal in *Panx1*<sup>-/-</sup> mice, as pup weights of lactating dams were relatively unaffected similar to what has been seen in *ER $\beta$* <sup>-/-</sup> mice. This suggests that impaired alveolar proliferation during early lactation in *Panx1*<sup>-/-</sup> mice likely represents only a delay in the onset of proliferation during early lactation. However, differences do exist between the *ER $\beta$* <sup>-/-</sup> and *Panx1*<sup>-/-</sup> mice as mammary glands of *ER $\beta$* <sup>-/-</sup> mice were associated with dysregulated epithelial markers E-cadherin and  $\beta$ -catenin which was not observed in *Panx1*<sup>-/-</sup> mice. This suggests suggests Panx1 is downstream of ER $\beta$  signaling, as *ER $\beta$* <sup>-/-</sup> mice have a more severe phenotype. Taken together, we propose that Panx1 ablation may impair ER $\beta$  rather than PrlR signaling. Alternatively, since Panx1 has been reported to be expressed in the pituitary gland and the ovary, we cannot fully rule-out that loss of Panx1 in these organs may be mediating alveolar defects in the mammary gland [13,49,50]. However, this seems unlikely as *Panx1*<sup>-/-</sup> mice have a relatively normal phenotype, which might be expected to be more severe if hormonal signaling was dramatically impaired.

While the mechanism of how Panx1 acts in the mammary gland is unknown, most studies assessing the role of Panx1 have found that Panx1 channel function involves ATP release that acts through purinergic receptors [51]. Interestingly, ATP release has been demonstrated to be important in Ca<sup>2+</sup> wave propagation in coordination with P2Y and P2X receptors [52]. Intriguingly, mechanical stimulation of mammary tumor cell leads to the release of nucleotides through an unknown mechanism that induces Ca<sup>2+</sup> signaling [53]. It remains interesting to speculate that Panx1 plays a role in this process. Importantly, Ca<sup>2+</sup> signaling is extremely important in the lactating mammary gland and contributes to proliferation, secretion, and myoepithelial contraction [54]. However, this remains to be verified by future studies.

#### 5.4.3 Loss of Panx1 does not impair involution

Recently, Panx1 was implicated as a critical channel during cell apoptosis, in which activation of caspases led to truncation of the C-terminal tail of Panx1 and the release of nucleotides that act as “find-me” signals for phagocytic cell-mediated clearance [19]. We

predicted that loss of Panx1 would impede normal mammary gland involution in which extensive apoptosis occurs requiring epithelial cell clearance from the mammary gland. However, loss of Panx1 did not affect mammary gland involution based on our assessment of epithelial cell area or adipocyte repopulation of the gland 72 hours following force weaning of the pups. Interestingly, Monk et al. has shown that apoptotic clearance in the mammary gland is mediated almost exclusively by alveolar epithelial cells, as opposed to macrophages [55,56]. This suggests that Panx1 is not the channel linked to cell clearance by which “find me” signals are released by apoptotic mammary epithelial cells, or that the loss of Panx1 channels is compensated by other nucleotide-releasing channels, or there is another mechanism involved [57]. Collectively, Panx1 appears dispensable for normal murine mammary gland involution at least in the first three days following forced weaning.

#### 5.4.4 Panx1 in breast cancer

High Panx1 expression was correlated with worse OS, RFS and DMFS in breast tumors from patients suggesting that Panx1 may act as a tumor facilitator in breast cancer. This is supported by Furlow et al. who demonstrated that Panx1 channel activity was critical in promoting breast cancer lung metastasis by increasing metastatic cell survival during extravasation [29]. Interestingly, the effect of Panx1 was similar in breast cancer cells of the basal (MDA-MB-468) and claudin-low molecular subtype (MDA-MB-231, BT-549), suggesting that this novel role for Panx1 may be seen in multiple breast cancer subtypes [29,58,59]. While we did not compare the correlation between Panx1 expression and clinical outcomes in the claudin-low subtype, we found that high Panx1 expression was correlated with better overall survival in the basal subtype group in contrast to those in MDA-MB-468 cells seen by Furlow et al [29]. Though these results do not make for the best comparison, these differential findings in the basal subgroup may speak to a dual role for Panx1 in tumorigenesis. Indeed, despite relatively few studies assessing the role of Panx1 in cancer, Panx1 has already been implicated as both a tumor suppressor and tumor facilitator in different types of cancer [30-32]. This suggests that the role of Panx1 in tumorigenesis may be complex and dependent on tumor type and stage of the disease. Alternatively, these results may also be influenced by the different chemotherapeutic treatments used by breast cancer patients of different histological subtypes. It remains to

be determined how Panx1 mRNA expression is correlated with clinical outcomes in distinct populations of breast cancer patients being treated with different chemotherapeutics. Nevertheless, the results to date suggest a tumor facilitating role of Panx1 in breast cancer. It remains interesting to speculate that Panx1's role in regulating cell proliferation in normal development may translate into dysregulated growth in the context of the primary tumor. Regardless, there is a need for further studies on the role of Panx1 in breast cancer.

In summary, through the use of a global *Panx1 knockout* mouse, it is clear that Panx1 is not critical for the normal function of the gland but is necessary for timely alveolar development and proliferation following the transition of the mammary gland from pregnancy into early lactation. Importantly, Panx1 expression within the mammary gland may have important implications to patients with breast cancer where increased expression of Panx1 is generally correlated with a worse clinical outcome.

## 5.5 References

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## Chapter 6

### 6 Overall Discussion

Genetically-modified mice have provided the tools to more rigorously assess previous predictions based on immunohistochemical and *in vitro* studies. This thesis used three genetically-modified mouse models to evaluate the role of Cx43, Cx26 and Panx1 in mammary gland development and to determine if these proteins act as tumor suppressor or tumor facilitators in breast cancer onset and metastasis *in vivo*. In Chapter 2 and Appendix 1, we demonstrated that milk ejection defects are linked to the functional status of Cx43 and, that low levels of Cx43 in the mammary gland of mice delayed palpable tumor onset and increased the frequency of metastases to the lungs, respectively. In Chapter 3, we identified that low levels of Cx26 expression in the mammary glands throughout pregnancy and lactation, and not the physiological surge in Cx26, is sufficient for normal gland development and function. In addition, despite little effect on normal gland development, Chapter 4 revealed that conditional knockout of Cx26 increased the susceptibility of mammary tumor onset in a chemically-induced mouse model of breast cancer. Finally, Chapter 5 identified a role for Panx1 in alveolar development following the transition from pregnancy to lactation, which may have implications extending to patients with breast cancer. The purpose of this section is to discuss how these studies may redefine our view of connexins and pannexins in the context of the mammary gland, focusing specifically on the broader implications and translatability of this work.

#### 6.1 Implications of connexins to breast feeding

The ability for mothers to adequately breast feed is underappreciated as many nutritional aspects of breast feeding may be substituted by formula. However, substantial evidence suggests that breast milk provides protection against bacterial infections, reducing the risk of developing obesity and ischemic heart disease, and promoting proper neural development [1,2]. Importantly, the mother also receives benefits from breast feeding by reducing the risk of developing breast and ovarian cancer [1]. As a result, it may be important for clinicians to understand the genetic factors, such as connxin proteins, that impair breast function in order to educate affected individuals to the risks associated with

**Table 6.1. Summary of key findings from thesis**

	<b>Breast Development</b>	<b>Breast Cancer</b>
<b>Cx43</b>	Normal, if Cx43 function is maintained above 50%	Loss of functional Cx43 increased metastasis to the lungs
<b>Cx26</b>	Low levels are sufficient for Gland Development	Cx26 knockdown increased susceptibility to primary mammary tumors
<b>Panx1</b>	Panx1 is necessary for timely alveolar development in the lactating mammary gland	High PANX1 mRNA expression in breast cancer is correlated with worse overall survival but this may be dependent on molecular subtype

not being able to breast feed. Importantly, our studies highlight that understanding the functional status of connexins may be critical in determining which patients may be affected.

Previous studies have highlighted the essential role of Cx43 and Cx26 in maintaining normal milk ejection and alveogenesis, respectively [3-5]. Surprisingly, both our evaluation of *Cx43<sup>H30T/+</sup>* and *BLG;Cx26<sup>f/f</sup>* mice revealed no impairment in mammary gland function with little evidence of impaired mammary gland development (Chapter 2 and Chapter 3, [6,7]). Ultimately, our results suggest that mammary connexins are made in excess and that even a 50% reduction in connexin function can be tolerated. This is supported by the relatively normal *Gja1<sup>+/−</sup>* and *Gjb2<sup>+/−</sup>* mice, in which one allele for Cx43 or Cx26 has been removed [8,9]. Interestingly, what may seem as excess levels of connexins is not restricted to the mammary gland as severely reduced Cx43 gap junctions in cardiac myocytes, or Cx36 gap junctions in pancreatic β-cells, is relatively well tolerated within the heart and pancreas, respectively [10-12]. These observations highlight the fact that many organs can tolerate a reduction in connexin levels and GJIC, at least in some unstressed and unchallenged circumstances, while the complete ablation of connexins can be catastrophic to the organ.

In humans, one might expect that impaired Cx43 and Cx26 function in the breast would lead to impaired breast feeding based on early studies using genetically-modified mice [4,5]. This is of particular interest in the context of Cx43 and Cx26-linked diseases such as ODDD or congenital syndromic and non-syndromic hearing loss, respectively [13,14]. No breast feeding defects have been reported in patients with hereditary hearing loss due to *GJB2* mutations despite a high worldwide prevalence of these mutations [15,16]. In contrast, the rarity of ODDD makes it difficult to assess if affected mothers have altered breast function [17]. However, the lack of reported breast defects despite multiple incidences of ODDD-inflicted mothers reported in clinical studies suggests the likelihood of normal lactation [18,19]. Two possibilities may help explain the discrepancies between mutant mice and humans. First, our mutant mouse models may not fully mimic the human

population. This is supported given that the Cx43 G60S mutant is not reported in the human population and differs in some phenotypic traits compared to patients with ODDD, such as being significantly smaller in size than control mice [5,20]. However, the Cx43 G60S mutant shares impaired channel function similar to the P59H mutant, found one residue removed from the G60S residue [21]. Therefore, it is more likely that our two mutant mouse lines represent distinct subgroups of ODDD patients based on mutant channel properties. With regards to Cx26, although our BLG Cx26 knockout model shares a similar reported phenotype to the human population [7], many human Cx26 mutants develop gain-of-function hemichannel activities [22]. This suggests that our knockout approach will not mimic all patients with *GJB2* related deafness. Consequently, it is likely that our BLG Cx26 knockout mouse model may best reflect a distinct subgroup of patients with Cx26-linked diseases expressing mutants that decrease Cx26 gap junction and hemichannel function.

## 6.2 Mechanism of connexins in the mammary gland

For Cx43, our results point to a GJIC-dependent mechanism in regulating normal mammary gland development and function as primary myoepithelial, and primary granulosa cells, isolated from *Cx43<sup>I130T/+</sup>* mice had significantly greater Lucifer yellow dye transfer compared those isolated from *Cx43<sup>G60S/+</sup>* mice [6]. While these experiments do not rule out GJIC-independent mechanisms, several lines of evidence argue against a role for dysregulated hemichannels and protein-protein interactions in this context. This is supported by Lai et al. who found that the Cx43 Y17S, G21R, A40V, F52FF and I130T mutants when ectopically expressed in C6 glioma cell all had similar impaired hemichannel function while differing greatly in their gap junction coupling capacity [23]. Although the effect of the G60S mutant on hemichannel function is unknown, the Y17S, G21R, A40V mutants share a similar dominant-negative action when expressed with wild-type Cx43 and functionally dead channel properties with expressed alone similar to the G60S mutant [23,24]. As a result, alteration in hemichannel function is unlikely. Interestingly, a small number of ODDD-linked mutants have been described as having increased hemichannel function *in vitro*, including the G138R mutant for which a *Cx43<sup>FloxG138R/+</sup>* mouse model of ODDD exists whose expression can be driven in a cell and tissue specific manner [25,26].

Therefore, the *Cx43*<sup>FlxG138R/+</sup> mouse may provide a potential model to better evaluate the role, if any, of Cx43 hemichannels in the context of the mammary gland [26]. In addition, differential effects of ODDD-linked mutants on the Cx43 proteome is an unlikely cause for the distinct mammary gland phenotypes observed in *Cx43*<sup>I130T/+</sup> and *Cx43*<sup>G60S/+</sup> mice. Transgenic expression of Cx26 in myoepithelial cells *in vivo* led to a trans-dominant negative effect on Cx43, revealing that loss of Cx43 had no effect on the expression of myoepithelial differentiation markers or intracellular signaling to myosin light chain [27]. In addition, *GJA1* mutations linked to ODDD do not typically arise in the protein-protein interacting domains within the C-terminal of Cx43, suggesting that these mutants may be embryonic lethal, highlighting their importance [28]. Taken together, evidence to date points to a GJIC-dependent function for Cx43 in the mammary gland.

Future studies may focus on the role of Cx43 in myoepithelial cell contraction. Oxytocin signaling is mediated through the oxytocin receptor coupled to G<sub>aq</sub>11 and phospholipase C activation triggering the release of Ca<sup>2+</sup> from intracellular stores [29]. Ca<sup>2+</sup> and inositol triphosphate have previously been shown to pass through Cx43 gap junctions making it interesting to speculate that Cx43 functions via GJIC-dependent mechanisms to coordinate synchronous contraction via transfer of Ca<sup>2+</sup> [30]. Isolation of primary myoepithelial cells from *Cx43*<sup>G60S/+</sup> or *Cx43*<sup>I130T/+</sup> mice and the direct Ca<sup>2+</sup> wave propagation comparison to littermate controls may yield further insight into the mechanism by which Cx43 functions in myoepithelial cells during lactation.

For Cx26, the use of conditionally ablated mice did not help us distinguish whether Cx26 functions through a GJIC-dependent or independent mechanism. However, as Cx26 possesses a very short C-terminal tail, it is likely that impaired alveogenesis associated with *MMTV-Cre;Cx26*<sup>fl/fl</sup> mice were the result of gap junction channel or hemichannel related mechanism and not related to the small Cx26 interactome [31-33]. Further comparative assessment of mice with mammary gland specific expression of the G45E or S17F Cx26 mutants, which differ in their gap junction channel and hemichannel function, may provide more clarity on the role of Cx26 hemichannels in mammary gland development and function [34,35].

Future studies should also focus on the potential role of Cx26 in secretory processes as Cx26 is commonly expressed in exocrine glands [36]. Therefore, expanding upon the role of Cx26 in the mammary gland and relating it to a GJIC-dependent or independent mechanism, quantitative evaluation of milk components following the milking of G45E or S17F mutant mice may be useful to reveal subtle changes in milk secretion that are not readily apparent using conditional knockout approaches [37]. Importantly, these studies must include an assessment of Cx32, due to its co-expression in many exocrine glands, to provide further insight into a potential role for connexins that may act in a compensatory manner [36].

### 6.3 Potential issues of connexin compensation

Until recently, only Cx43 and Cx26 were reported in the human mammary gland [38]. As these connexins are expressed in unique cell types, the idea of compensation between luminal Cx30 and Cx32 in mice would not be expected to translate to humans. However, Teleki et al. recently described both Cx32 and Cx30 in human breast luminal epithelial cells, similar to that found in the mammary glands of rodents, and Cx30 appeared to co-localize with Cx26 [39]. Intriguingly, these authors also identified Cx46 between luminal, myoepithelial and inflammatory cells despite a lack of expression in the rodent mammary gland, suggesting possible species-specific differences between human and rodent mammary glands [39,40]. While these results need to be taken with caution as they were derived from a low sample number and a lack of co-labelling with cell-type specific markers, the Teleki et al. study argues that a more rigorous re-evaluation of connexin expression at all stages of human breast development is needed. Importantly, these findings also suggest that connexin expression in the human breast may parallel more closely to that found in the mammary glands of rodents than previously thought.

Ultimately, questions concerning compensation in connexin mutant mouse models are difficult to entirely rule out. Our study demonstrated that conditional silencing of Cx26 also delayed the up-regulation of Cx30 during pregnancy suggesting Cx30 may not be compensating for the loss of Cx26 during this stage of development [7]. However, the idea of one connexin compensating for another will not be fully resolved without the development of double or triple mammary gland-specific connexin knockout mice.

Perhaps most importantly, a cross-breeding strategy where *Gjb6* and *Gjb1*-knockout mice are crossed with Dox-inducible mice for conditional knockout of Cx26 would be extremely beneficial to the field to establish a clear role for luminal connexins in the mammary gland during pregnancy [41]. However, these knockout approaches are limited in elucidating mechanistic insight. Therefore, similar strategies using connexin mutant mice may provide additional insight into the GJIC-dependent and independent roles of mammary connexins *in vivo*.

## 6.4 Connexins as tumor suppressors

A particularly powerful approach to validate candidate tumor suppressor genes *in vivo* involves the induction of cancer in genetically-modified mouse models in which the candidate gene has been mutated or lost. Through this approach, we provided the first *in vivo* support for a Cx26 and Cx43 tumor suppressive role in breast cancer using genetically-modified mice, bypassing the limitations of *in vitro* manipulation in previously evaluated xenograft models (Chapter 4 and Appendix 1, [42,43]). Interestingly, our results suggest that Cx26 has a tumor suppressive role in primary tumor onset, while Cx43 acts as a tumor suppressor during the later stages of the disease [44]. Therefore, Cx43 may best be described as a metastasis suppressor although these proteins are typically defined as having limited role in primary tumor onset or progression [45]. Cx43 does not totally fit this paradigm as the loss of functional Cx43 led to highly hyperplastic mammary glands suggesting a possible role in regulating growth in the primary tumor [44]. Ultimately, our evaluation of Cx26 and Cx43 using genetically-modified mice adds to a growing body of evidence that connexins are tumor suppressors [42,43,46,47].

In regards to Cx26, patients with *GJB2* mutations giving rise to keratitis-ichthyosis-deafness syndrome appear to be at an increased risk of developing squamous cell carcinomas, suggesting that Cx26 also plays a role as a tumor suppressor in the skin [48]. However, assessment of liver tumor incidence (as Cx26 is highly expressed in the liver) between chemically induced conditional Cx26 knockout mice and controls revealed no significant increase in overall tumor incidence [49]. Therefore, despite high expression of Cx26 in the mammary gland, skin and liver, the likelihood of increased susceptibility to neoplastic transformation appears to be organ-dependent and likely dependent on the

expression status of other connexins and/or the proteomic environment. In regards to Cx43, the use of genetically modified mice has only been previously used to evaluate the role of Cx43 in lung neoplasms. *Gja1<sup>+/−</sup>* mice had greater frequency and larger lung adenomas compared to wild-type mice treated with urethane or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, supporting a tumor suppressive role for Cx43 in cancer [50,51]. Other than Cx26 and Cx43, Cx32 has also been described as a tumor suppressor in hepatocellular carcinoma. Here, *Gjb1<sup>−/−</sup>* mice had increased susceptibility to chemically and radiation induced hepatocarcinogenesis [52,53]. These results were supported in mice expressing a liver specific dominant negative V139M mutant of Cx32, which impairs but does not fully ablate Cx32 function. Here again mutant mice had an increased susceptibility to chemically induced liver tumors but not spontaneous tumors [54]. A unifying theme of connexin-related susceptibility to carcinogenesis is the requirement of an additional use of a tumor initiating agent [55]. Therefore, it remains intriguing to determine whether connexins act as tumor suppressors in the context of different tumor initiators by crossing genetically-modified mice of connexins with mice that spontaneously develop mammary tumors including *ErbB2* or the *MMTV-PyVmt* mouse models [56,57]. In this regard, we used DMBA, ErbB2, or a combination to drive the formation of mammary tumors, providing evidence that the role of Cx43 in suppressing metastasis to the lungs may be a common feature of multiple tumor types [44]. As a result, these approaches may provide insight into how the role of connexins differs in tumors driven by different oncogenic signaling pathways.

Other studies have begun to assess how Cx43 expression differs in the molecular subtypes of breast cancer relative to traditional histological subtyping [58]. To overcome issues associated with poor clinical predictability of histological subtypes of breast cancer, breast cancer is now being characterized into six distinct molecular subtypes known as Luminal A (ER+/PgR+/HER2−), Luminal B (ER+/PgR+/HER2+/Ki67+), HER2 (ER-/PgR-/HER2+), Basal (ER-PgR-Her2−), Normal Breast-like (Adipose and fibroblast gene signature) and Claudin low (ER-/PgR-/HER2-/Claudin<sup>low</sup>) [59-61]. These molecular subtypes may better predict clinical prognosis but it remains unknown how connexin expression relates to this new framework or whether connexins have similar tumor suppressor or facilitating functions in relation to each molecular classification. Park et al.

revealed that invasive ductal carcinomas or pure ductal carcinomas *in situ* were more likely to be Cx43 positive in the luminal A subtype than those of the luminal B or Her2+ subtypes [58]. As luminal A tumors are correlated with the best predicted outcome, high Cx43 levels in these tumors fits with the tumor suppressor paradigm for Cx43 [60]. Unexpectedly, tumors histologically subtyped as invasive ductal carcinoma with evidence of ductal carcinoma *in situ* had high Cx43 levels in the basal subtype, known to have worse clinical prognosis, highlighting the complexity of understanding the role of connexins in breast cancer [58,60]. Taken together, our mouse models generally support a tumor suppressive role for Cx43 and Cx26 in breast cancer progression but further studies are needed to define the relationship between connexins and molecular subtypes. Ultimately, this may improve our understanding of the reported dual tumor suppressive and facilitating roles attributed to Cx43 and Cx26 in early and late stage disease.

## 6.5 Panx1 in the breast: pathological implications

Recently, a human developmental disease has been linked to a recessive germline loss-of-function mutation in *PANX1* in which the patient developed cognitive, hearing, skeletal and reproductive defects (Shao et al. 2015, submitted). While the ability to assess mammary gland function is hindered by primary ovarian failure of the patient, since pups from litters of *Panx1*<sup>-/-</sup> dams survive till weaning, we would predict that these patients would retain a relatively normal capacity to lactate (Shao et al. 2015, submitted). In mice lacking Panx1, we have observed relatively minor developmental and functional, defects in the mammary gland which may be due to compensation. Panx3 remains the most likely compensatory candidate as it has been reported to be expressed in the mammary gland [62]. However, unpublished evidence from our laboratories assessing colonies of *Panx3*<sup>-/-</sup> mice and double *Panx1*<sup>-/-</sup>*Panx3*<sup>-/-</sup> mice suggests that this is not likely the case. Both *Panx3*<sup>-/-</sup> mice and double *Panx1*<sup>-/-</sup>*Panx3*<sup>-/-</sup> mice have the capacity to reproduce and premature pup litter death is not readily apparent suggesting that Panx3 is not compensating for loss of Panx1 (unpublished personal observation). Importantly, a more detailed examination of these mice is necessary to confirm these observational findings as more subtle developmental defects may exist in these mice.

The documented defects in *Panx1*<sup>-/-</sup> mice suggests that Panx1 may regulate cell growth within the mammary gland consistent with a potential role in breast cancer. Indeed, Panx1 mRNA is detected in breast cancer cell lines [63]. Recently, Panx1 was shown to play a role in cell survival during metastatic progression by preventing mechanically-induced cell death following dissemination through the lung vasculature [64]. Our assessment of *in silico* databases is in line with this novel role for Panx1 in metastatic breast cancer as high Panx1 mRNA expression was correlated with worse predicted outcome. Taken together, this suggests that Panx1 may act as a tumor facilitator in breast cancer similar to the role of Panx1 in melanoma [64,65]. Future studies to assess the role of Panx1 in breast cancer using *Panx1*<sup>-/-</sup> mice through a chemical or oncogene induced approach may be useful in further establishing the role of Panx1 in primary tumor onset and progression. In addition, future studies correlating Panx1 protein levels with clinical outcome are needed to further investigate the role of Panx1 as a biomarker. Our data suggests that Panx1 may represent a potential biomarker to be used in conjunction with previously established markers, particularly with regards to Luminal B or Her2+ tumors.

## 6.6 Connexins and pannexins as therapeutic targets

Breast cancer remains the leading cause of cancer related death in women aged 20-59 and the second leading cause of cancer related death after age 60 [66]. Importantly, death rates for female breast cancer are falling as a result of early detection through screening and the development of targeted therapies [67]. Both our Cx43 and Cx26 mouse models support a tumor suppressive role in breast cancer. As a result, targeted strategies to either induce connexin expression or promote GJIC may be effective in suppressing the tumorigenic properties in the treatment of breast cancer [55]. Importantly, increased GJIC may also augment the effects of common therapeutics by mediating the transfer of cytotoxic molecules between cancer cells known as the bystander effect [68,69]. While in theory this seems a reasonable idea, the development of therapeutics targeted towards connexins is limited due in no small measure to the fact that it remains much easier to develop a drug to downregulate or inhibit an oncogenic protein than to upregulate a tumor suppressor.

One potential therapy involves a non-specific approach to target connexin in breast cancer. Demethylating drugs, such as 5-Azacytidine approved by the U.S. Food and Drug

Administration, can be used to alter the epigenetic profile of cancer cells, leading to the demethylation of tumor suppressed genes [70,71]. Cx26 and Cx43 are frequently downregulated in breast cancer, which is suggested to be, at least in the case of Cx26, due to methylation [46,72]. Early results appeared dependent on the breast cancer subtype as the related compound, 5-Aza-2'-deoxycytidine, showed variable benefits in different breast cancer cell lines [72,73]. Ultimately, this therapeutic strategy isn't well explored but warrants further investigation.

A second much more targeted approach uses first and second generation substituted quinoline compounds (PQ) that represent a group of GJIC enhancers currently being evaluated in breast cancer cells [56,74-77]. PQ1 and PQ7 were predicted through computer modelling to have high binding affinity to the structure of a gap junction hemichannel. Interestingly, PQs were shown to increase GJIC and even upregulate Cx43 protein expression in T47D breast cancer cells [74,78]. Importantly, PQ treated breast cancer cells had increased cell death and decreased cell proliferation *in vitro*, while tumor growth was significantly reduced in T47D xenografts and in *MMTV-PyVT* mice *in vivo* [56,74]. Intriguingly, combination therapy of PQ with tamoxifen or cisplatin generally had greater anti-proliferative and pro-apoptotic effects than either chemotherapeutic alone suggesting that PQs increase the effectiveness of common breast cancer chemotherapeutics through the bystander effect [75,79]. However, the anti-tumor effects of PQs have not conclusively been shown to be directly related to gap junction enhancement as there may be off-target effects that also contribute to anti-growth effect of tumor cells [78].

In a third example, the use of peptides to target Cx43 may be effective in breast cancer therapy. The ACT-1 peptide that mimics a carboxyl terminal domain of Cx43 (ACT1) was found to increase GJIC by stabilising the activity of gap junctions at the plasma membrane [80]. ACT-1 was effective in decreasing proliferation in MCF-7 and MDA-MB-231 cells when treated alone but more importantly improved the cytotoxicity of tamoxifen in ER+ MCF7 cells or lapatinib in HER2+ BT474 cells when used in combination [80]. Interestingly, ACT-1 peptides are currently being investigated in wound healing and have completed Phase2 clinical trials with positive outcomes in healing chronic venous leg ulcers [81]. Taken together, both PQ1/PQ7 and the ACT-1 peptide represent candidate

drugs that promote GJIC, which can be used in combination with existing chemotherapeutics to target breast cancer cells.

Drugs also exist to target Panx1, although the use of these in regards to breast cancer is relatively unexplored. Probenecid is approved by the U.S. Food and Drug Administration and has been shown to inhibit Panx1 channel function [82]. Interestingly, probenecid has been implicated as a chemosensitizer in T47D breast cancer cells as combination therapy of probenecid with cisplatin or paclitaxel increased cell cytotoxicity compared to either chemotherapy alone [83]. Ultimately, additional studies are needed to evaluate Panx1 as a viable therapeutic target in breast cancer.

## 6.7 Conclusions

In conclusion, further evaluation of genetically-modified mice targeting connexin or pannexin proteins are still needed to assess the roles of Cx43, Cx26 and Panx1 in the mammary gland. In particular, the future studies described above may provide further insight into the mechanism of action, issues of compensation and implications to patients with connexin-linked diseases. Importantly, new models of breast cancer are needed to evaluate connexins and pannexins, as well as potential therapies that target these large pore channels. We look forward to new discoveries that will further elucidate the mechanisms by which connexins act as tumor suppressors and/or facilitators in breast cancer as there remains considerable optimism that they will be of value in the clinic.

## 6.8 References

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## Appendix1

### Cx43 suppresses mammary tumor metastasis to the lung in a Cx43 mutant mouse model of human disease

The role of Cx43 in breast cancer remains controversial due to conflicting reports of Cx43 acting as both a tumor suppressor and tumor facilitator particularly in the later stages of the disease. To further evaluate the role of Cx43 in breast tumorigenesis, mice expressing the Cx43 G60S mutant were crossed with mice overexpressing the oncogene ErbB2 in order to evaluate spontaneous and DMBA-induced breast tumor development compared to controls. Dr. Isabelle Plante was the lead author on this study. My contributions were mainly to investigate if mice expressing the G60S mutant had altered mammary gland histology (Appendix 1.2) and to assess whether the increase in lung tumours observed in G60S mice originated from the mammary gland (Appendix 1.5). As I contributed to under 50% of this work, I have attached the manuscript as Appendix 1.

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A version of this chapter has been published:

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## Introduction

Metastatic breast cancer remains essentially incurable, highlighting the critical need for new therapeutic targets and treatment paradigms. For many years, several different experimental *in vivo* procedures have been used to investigate breast cancer progression. In many mouse models, tumorigenesis is initiated by exposing wild-type or genetically modified mice to a carcinogen. 7,12-Dimethylbenz[ $\alpha$ ]anthracene (DMBA) is one of the most common carcinogens used, and this treatment often causes cell transformation in the mammary gland that progresses to metastatic disease in distant tissues such as lung (Medina, 2007). In other mouse models of tumor onset and progression, genetically modified mice are engineered to overexpress an oncogene or to harbor a loss-of-function mutation in a key tumor suppressor gene, leading to increased incidence of tumorigenesis. For example, mice overexpressing a mouse mammary tumor virus (MMTV)-driven *ratneu* (*ErbB2*) gene (an oncogene overexpressed in ~30% of breast cancer patients) have been used extensively to mimic a subset of human breast cancers (Guy *et al.*, 1992). *ErbB2* overexpressing mice have been shown to develop spontaneous mammary gland tumors within ~30 weeks and these tumors readily metastasize to the lungs (Guy *et al.*, 1992). To further increase their versatility, *ErbB2* overexpressing mice can be cross-bred with other genetically modified mice to examine molecular mechanisms that may enhance or inhibit tumorigenesis (Hewitt *et al.*, 2002; Jacquemart *et al.*, 2009).

The mammary gland epithelium is composed of two layers of cells, an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells, surrounded by a basement membrane. The epithelium develops during puberty, under the influence of hormones, a process thought to require extensive interactions between myoepithelial and luminal cells, but also between the epithelium and the surrounding stroma (Pitelka *et al.*, 1973; Sternlicht, 2006). Myoepithelial cells are necessary for the development and function of the luminal cell layer, as paracrine regulation and junctional attachments allow for cross-talk within the epithelium (Pitelka *et al.*, 1973; Adriance *et al.*, 2005). As breast cancer typically arises from the luminal layer, with the rare exception of myoepithelioma (Foschini and Eusebi, 1998), the role of myoepithelial cells in tumorigenesis has not been acutely studied. Recently, myoepithelial cells have received more attention as they have been suggested as

a ‘natural tumor suppressor’ (Sternlicht and Barsky, 1997; Sternlicht *et al.*, 1997; Gudjonsson *et al.*, 2005). Indeed, through their strategic localization between the luminal cells and the surrounding stroma, it has been suggested that myoepithelial cells facilitate the polarity of the epithelium and function as a physical barrier against loss of growth control, tumor cell invasion and angiogenesis (Barsky and Karlin, 2005).

Gap junctions are intercellular channels assembled from a family of transmembrane protein called connexins (Cx). They allow the bidirectional passage of ions, metabolites and secondary messengers in a process known as gap junctional intercellular communication (GJIC) (Mese *et al.*, 2007). GJIC has been implicated in the regulation of homeostasis and a diverse array of cellular functions related to cell specialization, growth and differentiation (Herve *et al.*, 2007). In the mouse, gap junctions composed of Cx26, Cx30 or Cx32 have been identified between mammary luminal cells, whereas Cx43 gap junctions were found to be more restricted to myoepithelial cells and stromal fibroblasts (El-Sabban *et al.*, 2003). Similarly, in human breast epithelium, Cx26 has been localized to luminal cells, whereas Cx43 is mainly present between myoepithelial cells, but has been reported to be expressed in luminal cells as well (Monaghan *et al.*, 1996; Laird *et al.*, 1999). Although connexins were first proposed as tumor suppressors more than two decades ago (Lee *et al.*, 1991, 1992), their role in breast cancer is still poorly understood and somewhat controversial. Several studies examining primary breast tumor biopsy samples from patients have reported either a downregulation of Cx26 and Cx43 or mis-localization of these connexins to intracellular compartments (Lee *et al.*, 1991, 1992; Laird *et al.*, 1999; Kanczuga-Koda *et al.*, 2006). Consistently, it has been shown that re-expression of connexins in breast tumor cell lines can result in a partial cell re-differentiation to a more normal phenotype (Hirschi *et al.*, 1996; Kalra *et al.*, 2006; McLachlan *et al.*, 2007), whereas downregulation of connexins in breast cell lines render them more migratory and invasive (Shao *et al.*, 2005). However, a few studies have also reported an upregulation of connexins in breast cancer (Jamieson *et al.*, 1998; Kanczuga-Koda *et al.*, 2006; Naoi *et al.*, 2007). Finally, it has been suggested that the role of connexins may differ depending of the type of tumor or its stage of progression, even favouring metastasis (McLachlan *et al.*, 2007; Naus and Laird, 2010). Together, these studies reflect the need for an appropriate mouse model to study the role of connexins at all stages of mammary gland tumorigenesis.

In 2005, a mouse model of oculodentodigital dysplasia, a human disease linked to over 62 mutations in the *Cx43* gene, was generated through an *N*-ethyl-*N*-nitrosourea screening for dominant mutations (Flenniken *et al.*, 2005; Paznekas *et al.*, 2009). These mice, designated as *Gja1*<sup>Jrt/+</sup> or G60S mice, are autosomal dominant with the G60S mutant encoded on one allele. The mutant harboring mice were found to have a Cx43 trafficking defect and a substantial reduction in Cx43 protein levels in many tissues, including the mammary gland. These defects result in a decreased number of gap junction plaques and GJIC (Flenniken *et al.*, 2005; Manias *et al.*, 2008; Plante and Laird, 2008). When expressed in a variety of cell types, a population of the G60S mutant can reach the plasma membrane but assembles into gap junction-like structures with greatly impaired function (Flenniken *et al.*, 2005; McLachlan *et al.*, 2005). In essence, the Cx43 G60S mutant is functionally dead and furthermore has a dominant-negative effect on coexpressed wild-type Cx43 found in the G60S mice (Flenniken *et al.*, 2005). We previously showed that reduced Cx43 protein levels in G60S female mice are linked to delayed mammary gland development during puberty, as well as decreased milk secretion and ejection (Plante and Laird, 2008; Plante *et al.*, 2010). However, the consequences of mice harboring a functionally impaired form of Cx43 on breast cancer onset, progression and metastasis are still unknown.

This study aimed to determine whether the G60S mutant would render mice more susceptible to mammary gland tumor formation and whether tumors would be more aggressive. To this end, G60S mice were cross-bred with ErbB2 overexpressing mice to promote mammary gland-specific tumor formation, and both spontaneous and DMBA-induced mammary gland tumorigenesis and metastasis were assessed.

## Methods

### Animal treatment and tissue collection

Heterozygote *Gja1*<sup>Jrt/+</sup> mutant mice, carrying a G60S mutation in the *Gja1* gene encoding Cx43, have a mixed genetic background of C3 and C57BL/6J (Flenniken *et al.*, 2005). *Gja1*<sup>Jrt/+</sup> male mice were cross-bred with female FVB and female FVB/N-Tg(MMTVneu)202Mul/J, purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were randomly assigned to the different experimental groups and tumor onset was

evaluated every week by palpation. Mice were killed when the biggest tumor reached ~1 cm<sup>3</sup> or when mice showed signs of critical illness. For 7,12-dimethylbenz[α]anthracene (DMBA) studies, mice were exposed to DMBA or cotton oil by gavage. A total of 5 weekly doses (1 mg per 25 g of body weight) were administrated starting at 7 weeks of age. Female mice were killed using CO<sub>2</sub> and O<sub>2</sub> and collected tissues were stored at -80 °C or fixed in 10% neutral buffered formalin and then embedded in paraffin. All experimental procedures were approved by the Animal Care Committee at the University of Western Ontario following the guidelines of the Canadian Council on Animal Care.

## Histology

To perform a general histological analysis, paraffin-embedded sections (7 µm thick) were stained with haematoxylin and eosin and mounted with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI, USA). General histological analysis was performed by imaging several areas with 16 ×, 10 × and 5 × objective lenses mounted on a Zeiss (Thornwood, NY, USA) Axioscope microscope workstation equipped with a Sony (Tokyo, Japan) PowerHAD camera and Axiovision LE imaging software (Carl Zeiss Vision). At least five different animals per experimental group were evaluated.

## Evaluation of lung lesions

Once mice were killed, lungs were inflated and fixed by injecting 10% buffered formalin through the trachea. Inflated lungs were kept at 4 °C in buffered formalin for at least 48 h, and visible lesions present on the lung surface were counted. Lungs were then embedded in paraffin and processed for haematoxylin and eosin staining. For each mouse, 10 random pictures were taken with a 16 × objective (for a total of ~6 mm<sup>2</sup> of tissue area per mouse) and the area covered by lesions evaluated blindly using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Graphs represent the average lesion surface area (lesion area/total area evaluated), per group. A minimum of six different animals per experimental group were evaluated.

## Immunocytochemistry and confocal microscopy

Paraffin-embedded sections (7 µm thick) were subjected to rehydration with descending grades of ethanol baths followed by microwave antigen retrieval using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) for 5 min. After a 20 min of cool down, sections were subjected to a second antigen retrieval step, using 10 mM Tris Base, 1 mM EDTA solution (pH 9.0) at 95 °C for 30 min, followed by 20 min of cool down. Tissues were rinsed with water and blocked with 0.1% Triton X-100 and 0.15% goat serum in phosphate-buffered saline for 60 min at room temperature. Sections were then incubated with anti-mammaglobin (Abcam, Cambridge, MA, USA; 1.0 µg/ml, cat. # ab82203). Primary antibody was visualized by incubating sections with Alexa555-conjugated donkey anti-rabbit secondary antibody (Invitrogen, Burlington, ON, Canada; 0.5 µg/ml). Hoechst stain was used to visualize nuclei before mounting. Immunolabeled paraffin-embedded sections were imaged on a Zeiss LSM 510 inverted confocal microscope as previously described (Thomas *et al.*, 2005). At least five different animals per experimental group were evaluated.

## Statistical analysis and n-values

Values are presented as the mean±s.e.m. Statistics were analyzed using Student's *t*-test or ANOVA analysis followed by a Tukey's multiple comparison test (comparing all pairs) where *P*<0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA).

## Results

### Mice harboring the G60S mutant have a delayed onset of palpable tumors in the mammary gland

To study the effects of a Cx43 mutant on mammary gland tumorigenesis, we cross-bred male *Gja1<sup>Jrt/+</sup>* mice, harboring a G60S mutation within the *Cx43* gene, with female ErbB2 mice, overexpressing a rat ErbB2 (*Neu*) gene under an MMTV promoter (FVB/N-Tg(MMTVneu)202Mul/J), or with FVB control females. The resulting phenotypes were

designated WT-FVB (wild-type Cx43 and no ErbB2 overexpression), WT-ErbB2 (wild-type Cx43 and heterozygous for ErbB2 overexpression), G60S-FVB (harboring the G60S mutant and no ErbB2 overexpression) and G60S-ErbB2 (harboring the G60S mutant and heterozygous for ErbB2 overexpression). Surprisingly, a significant number of the resulting offspring, especially mice expressing both the mutant Cx43 and ErbB2, died before the end of the experiment. Although DMBA exposure didn't seem to affect the health of the mice based on body weight assessments during the treatment time, the percentage of mouse deaths was even higher when mice were exposed to DMBA. Mice killed or found dead before week 42 because of mammary gland cancer-unrelated diseases were removed from the experiment and data analysis. The remaining mice were assessed for mammary gland tumor formation and were killed either when the biggest tumors reached  $\sim 1 \text{ cm}^3$  or when they showed signs of critical illness.

To assess whether mice harboring the G60S mutant were more or less susceptible to tumor formation, two protocols were used. First, spontaneous tumor development was assessed in the two groups of mice overexpressing ErbB2 (designated as 'spontaneous'; WT-ErbB2 and G60S-ErbB2) by palpation. Second, mice (WT-FVB, WT-ErbB2, G60S-FVB and G60S-ErbB2) were treated with either oil or DMBA and then assessed for palpable tumors weekly. Tumors were first detected at  $\sim 40$  weeks in spontaneous WT-ErbB2 mice, with 50% of the mice exhibiting palpable tumors by  $\sim 72$  weeks (Appendix 1.1a). Surprisingly, only one G60S-ErbB2 mouse ever developed a palpable spontaneous tumor (Appendix 1.1a). In the DMBA-treated groups, the onset of palpable tumors in WT-ErbB2 and WT-FVB mice was  $\sim 20$ -weeks earlier with 50% of the mice exhibiting tumors at 32 and 36 weeks of age, respectively (Appendix 1.1b). In G60S mice exposed to DMBA, tumor formation was delayed compared with DMBA-treated WT groups, with the first tumors being detected between weeks 30 and 35 (Appendix 1.1b). However, G60S mice typically had to be killed before week 42 because of labored breathing, independent of the presence of a mammary gland tumor. Collectively, only one oil-treated WT-ErbB2 mouse formed a palpable tumor. Together, these results suggest that exposure to DMBA reduces tumor latency in all strains and that mice harboring the G60S mutant have delayed palpable mammary gland tumor onset.

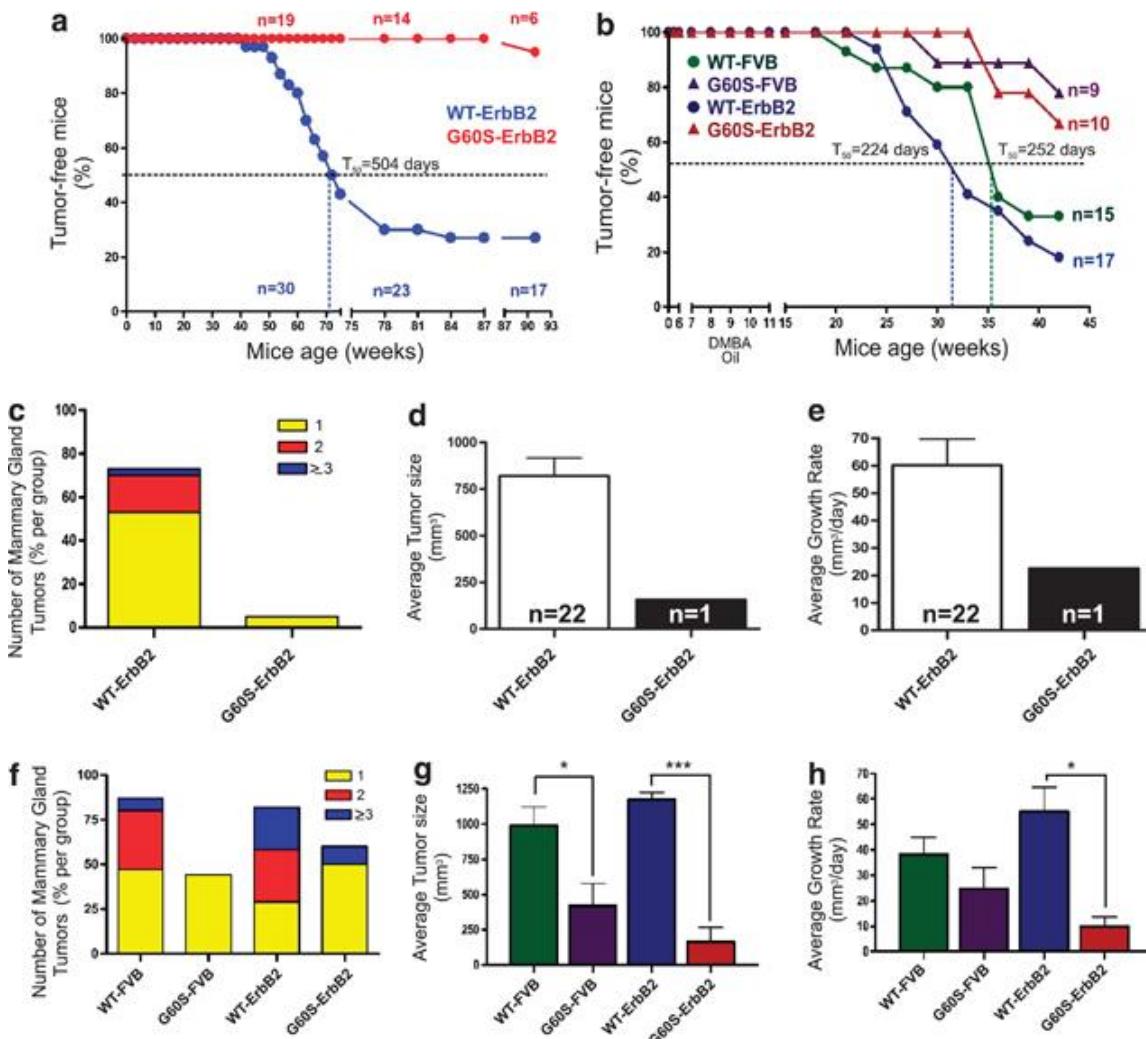
## WT mice have multiple and larger tumors compared with G60S mice

At the time of killing, all mouse mammary gland tumors were counted and measured. Approximately 70% of spontaneous WT-ErbB2 mice developed at least one tumor by the age of 87 weeks (Appendix 1.1c). Surprisingly, only one G60S-ErbB2 mouse ever formed a spontaneous tumor. In DMBA-treated animals, over 80% of WT-FVB and WT-ErbB2 mice formed at least one tumor, compared with ~45% of the G60S-FVB and ~60% of the G60S-ErbB2 mice (Appendix 1.1f). Although between ~30–53% of both spontaneous WT-ErbB2 mice and DMBA-treated WT mice had multiple tumors, only one DMBA-treated G60S-ErbB2 mouse developed three tumors (Figures A1c and f), indicating that not only the incidence of tumor formation is lower in Cx43 mutant mice, but also that these mice rarely have multiple tumors.

In keeping with our approved animal protocol, mice were killed when the largest tumor reached  $\sim 1 \text{ cm}^3$  or when they showed signs of critical illness. In all cases, both spontaneous and DMBA-treated WT mice could reach the maximum tumor burden (Appendix 1.1d and g). However, G60S mutant mice bearing a palpable tumor rapidly exhibited discomfort and/or labored breathing and thus had to be killed before reaching the maximum tumor burden, resulting in an average tumor size of  $<0.5 \text{ cm}^3$  (Appendix 1.1d and g). In all cases, the tumor growth rate was calculated by dividing the size of the tumors at death by the number of days since the tumors were first palpated. For WT mice,

**Appendix 1.1. Onset of palpable mammary gland tumors is delayed in Cx43 mutant mice.** (a) Spontaneous mammary gland tumor onset and growth were evaluated by palpation in WT-ErbB2 and G60S-ErbB2 mice. (b) 7-week-old mice were exposed weekly to DMBA (1 mg per 25 mg of body weight) for 5 weeks and tumor onset and growth was evaluated by palpation. Dotted lines in panels a and b represent the time when 50% of mice had at least one palpable tumor (T50). (c, f) Graph represents the percentage of mice that develop 1, 2 or greater than or equal to 3 spontaneous mammary gland tumors (c) or upon DMBA exposure (f). (d, e, g, h) Size and growth rate were evaluated from all groups. In all, 22 tumors in the spontaneous WT-ErbB2 mice group, but only 1 tumor in the G60S-ErbB2 mice group, were palpated and evaluated. For DMBA, N=15 for WT-FVB; N=9 for G60S-FVB; N=17 for WT-ErbB2; N=10 for G60S-ErbB2. (d, g) Graphs represent the average size of the biggest tumor at the time of killing mice (mean $\pm$ s.e.m.). \*P<0.05; \*\*\*P<0.001. (e, h) Graphs represent the average growth rate of the biggest tumors (mean $\pm$ s.e.m.). Growth rates were obtained by dividing the size of the biggest tumor at the time of killing by the number of days since the tumor was first palpated. \*P<0.05.

## Appendix 1.1



the average growth rate was similar for both spontaneous and DMBA-induced tumors, ranging from 38 to 60 mm<sup>3</sup> per day (Appendix 1.1e and h). Although the growth rate did not differ between G60S-FVB and G60S-ErbB2 mice exposed to DMBA, it was significantly lower in G60S-ErbB2 mice compared with WT-ErbB2 mice (Appendix 1.1h). Only one G60S-ErbB2 mouse developed a spontaneous palpable tumor (Appendix 1.1c–e), and both the size and the growth rate of that tumor was similar to those from DMBA-exposed G60S mice (Appendix 1.d, e, g and h). Together, these results suggest that Cx43 mutant mice are rapidly affected by the presence of tumors, and had to be killed before the tumors reached the maximum size threshold.

### G60S mice have hyperplastic mammary glands

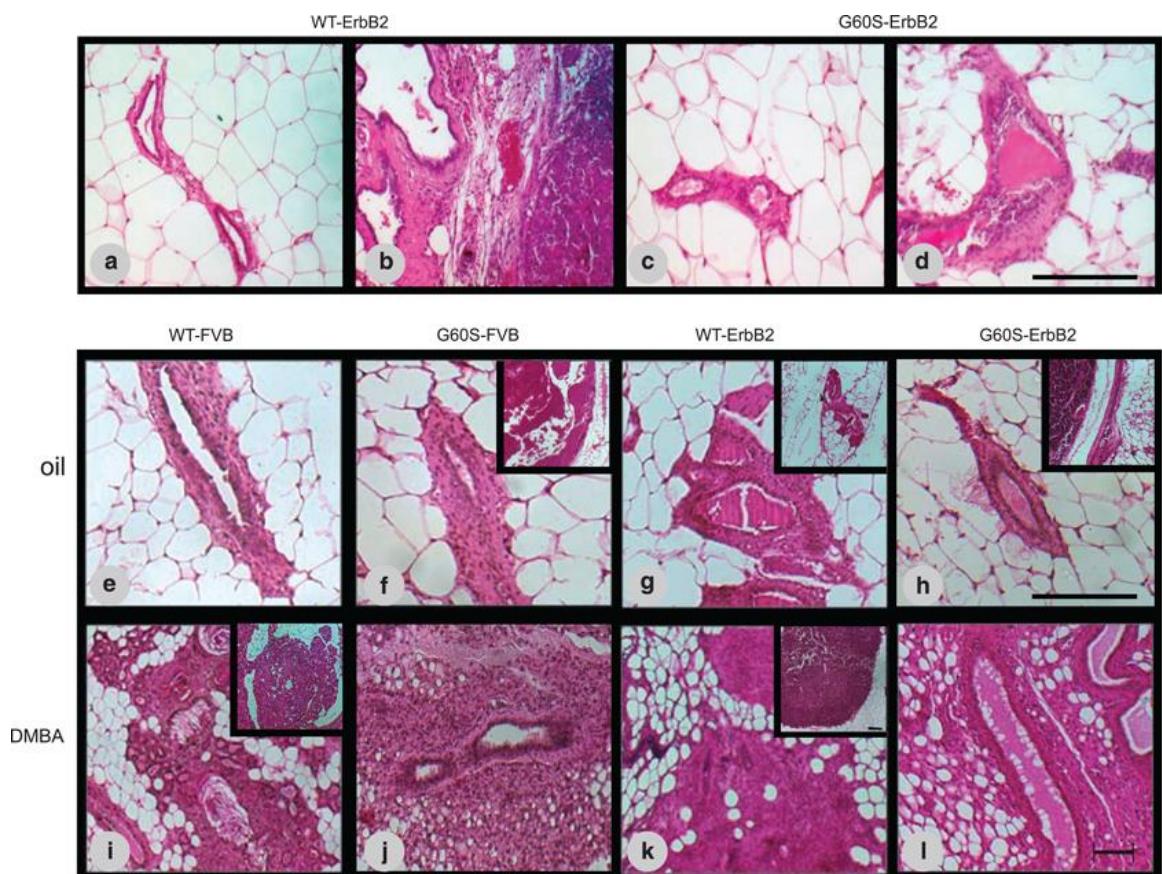
To assess the architecture and tumor status in the mammary gland, we performed histological analyses. In the spontaneous groups, most of the mammary glands were composed of adipocytes with sparse ducts (Appendix 1.2a and c). However, in all WT-ErbB2 mice analyzed, some areas of the glands showed either hyperplasia or solid tumor (Appendix 1.2b). Surprisingly, approximately 75% of G60S-ErbB2 mice also showed hyperplastic areas (Appendix 1.2d). Similarly, in oil-treated mice, histological analysis revealed primarily normal tissue (Appendix 1.2e–h); however, between 29–44% of WT-ErbB2, G60S-FVB and G60S-ErbB2 mice had hyperplastic tissue, as well as occasional solid tumors (Appendix 1.2f–h, inserts). Upon DMBA treatment, ≥63% of the mice in each group exhibited abnormal areas of tissue (Appendix 1.2i–l). Interestingly, although WT mice contained both hyperplastic and solid tumors (Appendix 1.2i and k, inserts), G60S mice contained almost exclusively hyperplastic mammary glands (Appendix 1.2j and l). These results suggest that mammary glands from Cx43 mutant mice become hyperplastic, independent of the overexpression of ErbB2 or exposure to DMBA.

### Levels of Cx43 are reduced in the mammary gland of G60S mice compared with WT mice, and remain unchanged upon DMBA exposure

As the parental *Gja1*<sup>Jrt/+</sup> mice are on a mixed genetic background of C3 and C57BL/6J and ErbB2 mice are on a FVB background, western blot analyses was conducted on mammary glands to determine the effect of the G60S mutant on Cx43 levels upon cross-

**Apéndice 1.2. Mammary glands from Cx43 mutant mice typically become hyperplastic.**  
Haematoxylin and eosin staining revealed that most of the mammary gland tissue from both spontaneous WT-ErbB2 (**a**) and G60S-ErbB2 mice (**c**) seem normal, with occasional areas revealing solid tumors and hyperplasia in WT-ErbB2 mice (**b**) or mainly hyperplasia in G60S-ErbB2 mice (**d**). Similar results were observed for oil-treated animals (**e–h**). In DMBA-treated mice, hyperplasia and solid tumors were observed in WT mice (**i, k**), whereas the mammary glands from G60S mice were mainly hyperplastic (**j, l**). Bars, 100 µm. For all groups, N≥5.

## Appendix 1.2



breeding. Typically, Cx43 resolves as multiple bands with the slower migrating bands representing highly phosphorylated species of Cx43 (P), and the faster migrating band, representing un-phosphorylated or a poorly phosphorylated species of Cx43 ( $P_0$ ) (as reviewed by Solan and Lampe, 2005 and by Solan and Lampe, 2007). Although we observed that Cx43 mRNA levels were the same in WT and mutant mice, total Cx43 protein levels were reduced by ~80% in G60S-ErbB2 mice compared with WT-ErbB2 mice, with the Cx43-P species being the most affected. Similar results were obtained when comparing oil-treated WT-ErbB2 and G60S-ErbB2 mice, or when comparing G60S-FVB with WT-FVB mice, confirming that the presence of the G60S mutant reduces the Cx43 content in all mice harboring this mutant. It has been reported that DMBA treatment can increase Cx43 levels in a normal human breast cell line (De Flora *et al.*, 2006), raising concerns that DMBA may compensate for the reduction in total Cx43 found in G60S mutant mice. In our studies, western blot analysis revealed that DMBA alone did not significantly change the levels of Cx43.

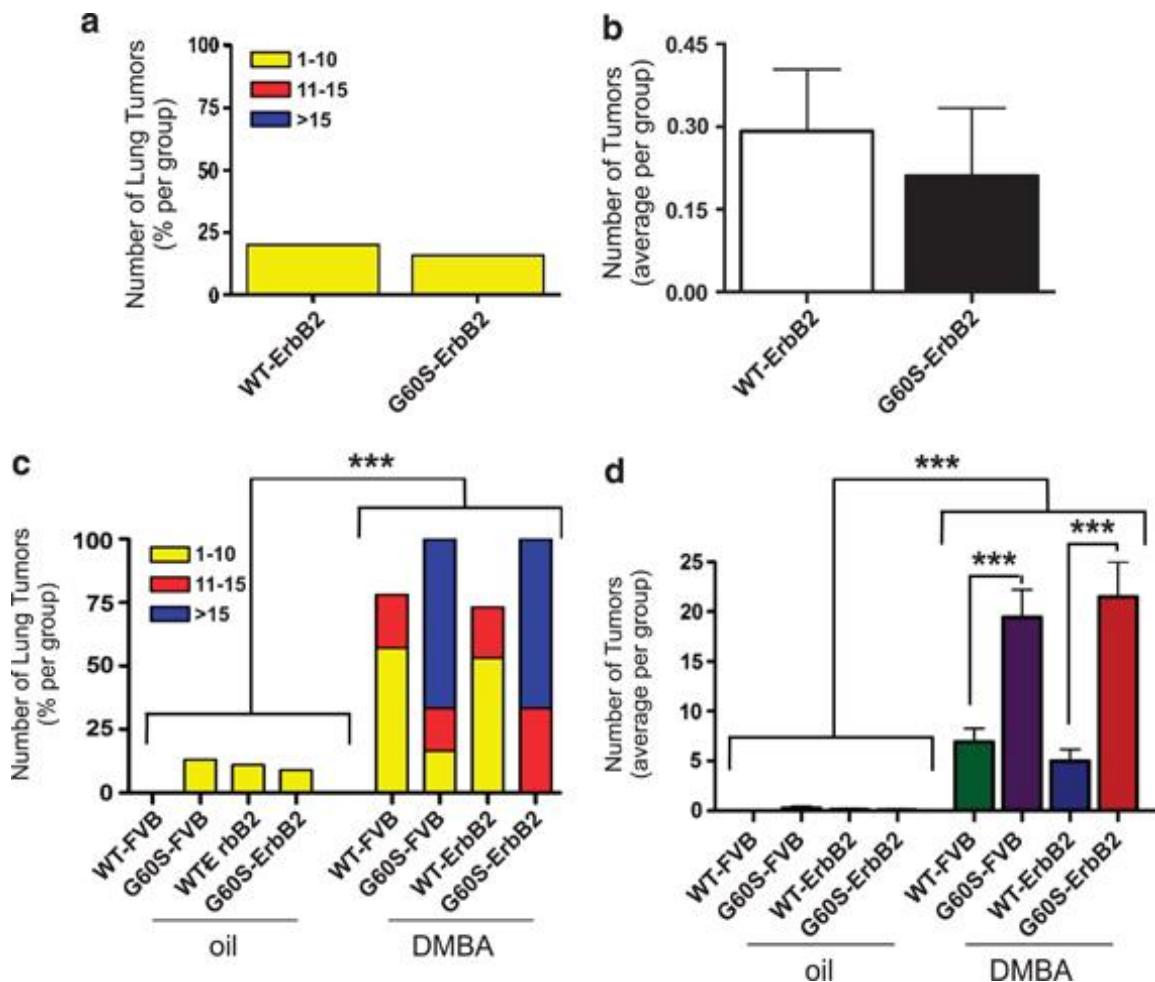
### **G60S mice are more susceptible to lung tumors than WT mice**

As the lungs are one of the primary organs for mammary gland metastasis and G60S mice typically exhibited labored breathing in later stages of the study, we examined the lungs for visible evidence of tumors upon intra-tracheal fixation. Lung tumors were infrequent in spontaneous groups, but ~20% of WT-ErbB2 and G60S-ErbB2 mice had between 1 and 10 visible tumors (Appendix 1.3a and b). Similarly, few tumors were observed in oil-treated mice, with less than 15% of mice having one or more tumors (Appendix 1.3c and d). In all DMBA-treated groups, lung tumors were common as ~75% of WT mice exhibited 1–10 tumors (Appendix 1.3c), resulting in an average of nearly 7 tumors per mouse (Appendix 1.3d). In G60S mutant mice, not only were 100% of the mice found to exhibit visible lungs tumors, but most mice had >15 visible lesions, (Appendix 1.3c) with an average of 18 tumors per mouse (Appendix 1.3d). These results indicate that DMBA-treated Cx43 mutant mice are more susceptible to form lung tumors than WT mice.

**Appendix 1.3. Cx43 mutant mice are more susceptible to lung tumors than WT mice.**

Visible lung tumors were counted for each spontaneous (**a**), oil-treated or DMBA-induced (**c**) mouse cohorts. (**a, c**) Graphs represent the percentage of mice having 1–10, 11–15 or >15 tumors. \*\*\* $P<0.001$ . (**b, d**) Graphs represent the mean number of lung tumors per group $\pm$ s.e.m. \*\*\* $P<0.001$ . For all groups,  $N\geq 7$ .

### Appendix 1.3



As G60S mutant mice are typically 50% smaller than WT mice (Plante and Laird, 2008), and microscopic tumors were not included in the initial assessment, we used histological evaluation to determine the lung tumor burden (percentage of lung tissue area occupied by tumor). In mice from spontaneous groups, a considerable area of the lung was normal for both WT-ErbB2 and G60S-ErbB2 mice (Appendix 1.4a). However, 57% of the WT-ErbB2 mice had sporadic lung tumors with approximately half of these tumors occupying >20% of the lung section area (Appendix 1.4b). In contrast, 100% of G60S-ErbB2 mice had lung tumors with ~60% of the mice having tumors occupying <20% of the lung and the remaining mice having tumors that occupied 20–50% of the lung section area (Appendix 1.4b). In comparing the spontaneous groups, the lungs from G60S-ErbB2 mutant mice had a similar tumor burden (that is, mean area of the lung occupied by tumor) as those taken from WT-ErbB2 mice (Appendix 1.4c), but mutant mice had increased incidences of lung metastases (number of mice in each group that developed lung tumors) compared with WT mice.

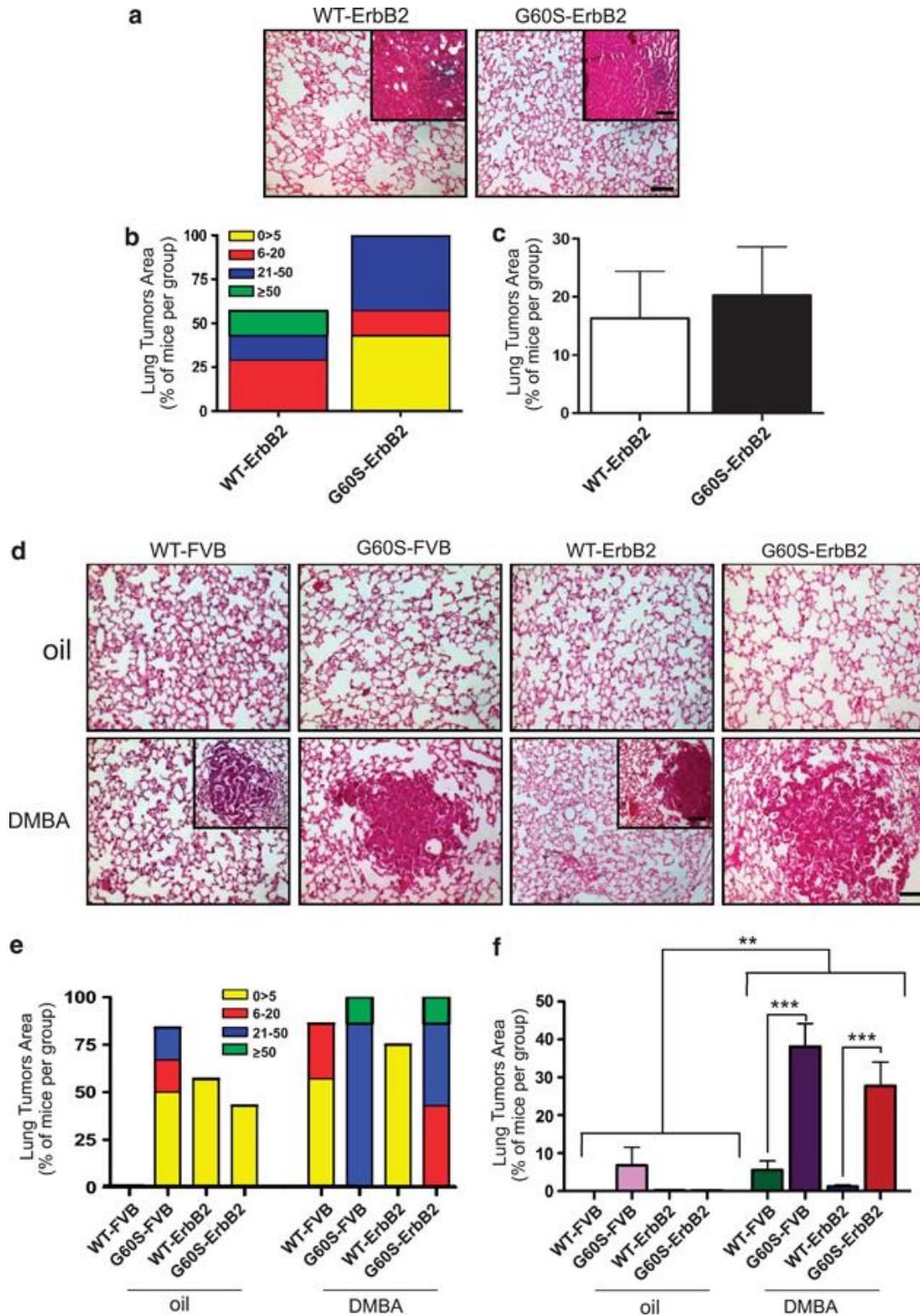
In oil-treated mice, over 80% of the lung tissues analyzed were tumor-free for the vast majority of genetically engineered mice (Appendix 1.4d and e), resulting in less than 6% lung tumor burden (Appendix 1.4f). Upon exposure to DMBA, more than 80% of the area of the lung tissue analyzed was still tumor-free for WT mice (Appendix 1.4d,e), resulting in less than 6% lung tumor burden (Appendix 1.4f). However, in G60S-mice, 100% of mice had at least 5% lung tumor burden, with more than half of them having greater than 20% lung burden (Appendix 1.4e). This resulted in an average of 30–40% lung tumor burden for both groups (Appendix 1.4f). Together, these results suggest that Cx43 G60S mutant mice have more numerous macroscopic and microscopic lung tumors than their control counterparts.

### Cx43 mutant mice have increased metastasis to the lung

To confirm that the lung tumors observed were truly metastases from the mammary gland, lung paraffin-embedded sections were immunolabeled with mammaglobin, a glycoprotein which is expressed in cells derived from the mammary gland (Yang and Nonaka, 2010). As expected, no mammaglobin was detected in normal lung tissues

**Appendix 1.4. Cx43 mutant mice have considerable lung tumor burden, which is further increased by DMBA treatment.** Haematoxylin and eosin staining revealed that lung tissue from WT-ErbB2 and G60S-ErbB2 mice was generally normal (**a**), with occasional areas harboring solid tumors (**a**, inserts). Similarly, lung tissue from oil-treated mouse cohorts was typically normal (**d**). In DMBA-treated mice, solid tumors were observed for all mouse groups, but were more frequently found in G60S mutant mice (**d**). Bars=150  $\mu$ m. For all mice, 10 random histological fields were imaged and the area of abnormal tissue versus normal tissue was blindly evaluated using ImageJ software. (**b, e**) Graphs represent the percentage of mice with <5%, 5–20%, 20–50% or  $\geq$ 50% of the total lung area occupied by abnormal tissue. (**c, f**) Graphs represent the percent mean tumor burden per total tissue area for each group $\pm$ s.e.m. \*\* $P$ <0.01; \*\*\* $P$ <0.001. For each group, N $\geq$ 6.

### Appendix 1. 3



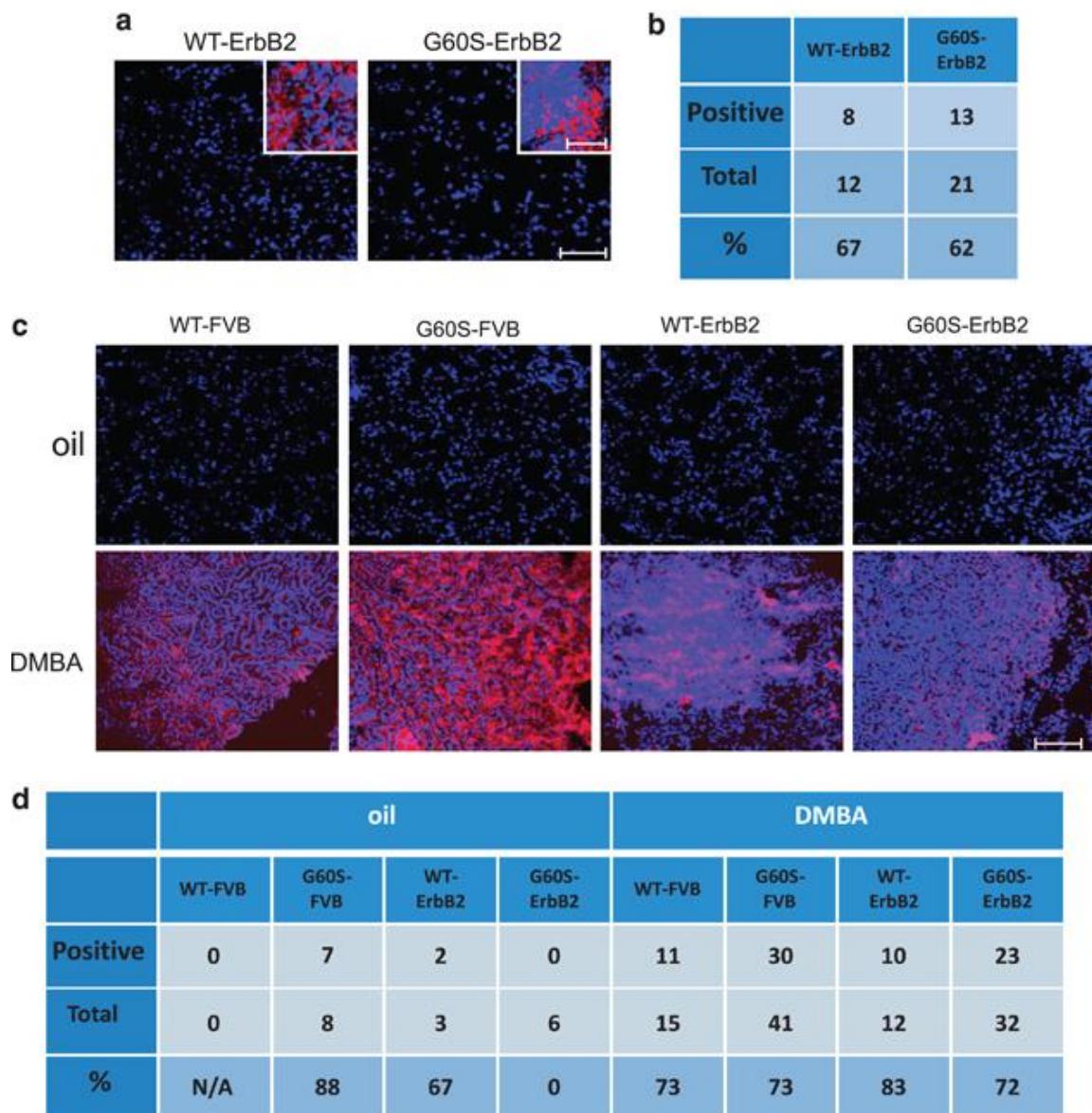
(Appendix 1.5a and c). In both spontaneous WT-ErbB2 and G60S-ErbB2 mice, over 62% of lung tumors stained positively for mammaglobin, confirming that they were from mammary gland origin (Appendix 1.5b). In oil- or DMBA-treated mice, 67–88% of all tumors were stained positively for mammaglobin in all groups, except oil-treated WT-FVB (no tumors found) and G60S-ErbB2 (no positive staining in six tumors analyzed) (Appendix 1.5c and d). Together, these results suggest that G60S mice are more prone to mammary gland tumor metastases to the lung.

## Discussion

The connexin family of gap junction proteins is essential for cell proliferation, differentiation and tissue homeostasis (Herve *et al.*, 2007). Recent evidence has challenged the paradigm that connexins are tumor suppressors, and further suggests that connexins may even facilitate late stage disease progression and metastasis (Naus and Laird, 2010). Thus, connexins may in fact best be considered as conditional tumor suppressors (Naus and Laird, 2010). Although connexins seem to be linked to tumorigenesis in the breast (Lee *et al.*, 1991, 1992; Laird *et al.*, 1999; Kanczuga-Koda *et al.*, 2006; McLachlan *et al.*, 2007), the current understanding of how these molecules are linked to cancer is relatively limited, as few studies have systematically examined the role of connexins in both early and late stages of disease progression in a unified model. We chose to assess the importance of Cx43 in breast cancer onset and progression using a mouse model that harbors an autosomal-dominant mutation in the gene encoding Cx43. As this loss-of-function mutation in a conditional tumor suppressor may not in itself be sufficient to initiate tumorigenesis and because Cx43 is also found in more than 35 cell types (Laird, 2006), we chose to focus our analysis on the mammary gland by using the *ErbB2* oncogene overexpressing mice under a mammary gland specific promoter. Not only did this new model circumvent limitations associated with *in vitro* and mouse xenograft models for studying the molecular mechanisms of breast cancer onset and progression, but it also allowed us to examine the ‘natural tumor suppressor’ role of Cx43-rich myoepithelial cells (Sternlicht and Barsky, 1997; Gudjonsson *et al.*, 2005). An added advantage of this model was the fact that both ErbB2 overexpression and DMBA

**Appendix 1.5. Cx43 mutant mice have more mammary gland metastases to the lung.**  
Paraffin-embedded normal and tumor-harboring lung tissue was immunolabeled with mammaglobin (red), a marker for cells of mammary gland origin in spontaneous (**a**) and oil/DMBA-treated mice (**c**). The number of mammaglobin-positive and negative lung tumors was counted for every mouse group (for all groups,  $N \geq 5$  mice except oil-treated WT-ErbB2 and WT-FVB mice where  $N=4$ ) (**b**, **d**). Tables denote the percent of mammaglobin-positive lung tumors and the total number of lung tumors evaluated per spontaneous (**b**) and oil/DMBA-treated group (**d**). Nuclei were stained with Hoechst (blue). Bars, 100  $\mu$ m.

### Appendix 1.4



disease progression and metastasis. Finally, this animal model was designed to examine the potential cross-talk mechanisms between the Cx43-rich myoepithelium and the ErbB2-positive luminal cell microenvironments as related to tumorigenesis.

Our study produced two major and somewhat unexpected key findings. First, low levels of Cx43 in the mammary gland resulted in a decreased frequency of palpable mammary gland tumors, as mice harboring the G60S mutant typically exhibited a hyperplastic mammary gland. Second, G60S mutant mice were far more prone to forming lung metastases. Together, our results suggest that a reduced complement of functional Cx43 renders mice more susceptible to hyperplastic mammary glands and that functional Cx43 is protective against lung metastases.

### Low levels of Cx43 result in delayed onset of palpable tumors

In mice overexpressing ErbB2 under the control of an MMTV promoter (Guy *et al.*, 1992), 50% of the mice developed spontaneous mammary gland tumors at ~30 weeks, and ~70% of tumor-bearing mice that live over 8 months exhibit lung metastasis (Guy *et al.*, 1992). In our hands, 50% of WT-ErbB2 mice developed spontaneous palpable mammary gland tumors by ~72 weeks of age, twice the time of the parental strain. A possible explanation for this delay in tumor onset may come from the fact that the parental strain is homozygous, having two copies of the *NeuI* rat gene, whereas our mice were heterozygous for ErbB2. However, a comparable cross-breeding approach between the same ErbB2-expressing mice and MMTV/*Rassf3* transgenic mice resulted in similar tumor onset latency as that found in the homozygous ErbB2 mice, suggesting that this is not a sufficient explanation (Jacquemart *et al.*, 2009). Another possibility is that this disparity may result from differences in mouse strains used. Although both FVB/N-Tg(MMTVneu)202Mul/J and MMTV/*Rassf3* transgenic mice were on the same FVB background, G60S mice are on a mixed background of C3 and the more tumor resistant strain, C57BL/6J (Rowse *et al.*, 1998; Flenniken *et al.*, 2005). Consistently, a cross-breeding study between ErbB2 mice on the FVB mouse background and ER $\alpha$ KO mice on the C57BL/6 strain resulted in a considerably longer latency in tumor onset (Hewitt *et al.*, 2002), similar to the findings in this study.

To our surprise, compared with appropriated controls, the onset of palpable mammary gland tumors was delayed in Cx43 mutant mice in both spontaneous and DMBA-treated experimental sets. However, histological analysis demonstrated that the mammary glands from mutant mice were subject to hyperplasia. Hyperplasia is one of the first steps in tumorigenesis, as further disease progression to solid tumors usually occurs within a few weeks (Boggio *et al.*, 1998; Hewitt *et al.*, 2002; Tsubura *et al.*, 2007; Allred and Medina, 2008). At least two possibilities could explain these results; either mutant Cx43 delays the onset of tumorigenesis, or mutant Cx43 inhibits hyperplastic cells from proceeding to solid tumors while resident in the mammary gland. The fact that 33% of oil-treated G60S-FVB mutant mice (compared with 0% of WT-FVB) exhibit hyperplasia by week 42 suggests that Cx43 mutant mice may actually be more prone to breast tumor initiation. However, the answer was not as clear when we compared G60S-ErbB2 and WT-ErbB2 mice, as 44 and 29% of these mice, (respectively), exhibited hyperplasia in the oil-treated group at 42 weeks, and 75% of mice harboring G60S-ErbB2 spontaneously became hyperplastic at week 50. Together, this suggests that Cx43 may only have a minimal effect on inhibiting the initial stages of tumorigenesis. Instead, it is more likely that mutant Cx43 is linked to inhibiting cells from forming solid tumors while resident in the mammary gland. Similar to human breast cancer, mouse mammary gland tumorigenesis is a multistep process involving epithelial hyperproliferation (hyperplasia), progression into *in situ* carcinoma, invasion and metastasis (Boggio *et al.*, 1998; Tsubura *et al.*, 2007; Allred and Medina, 2008). The major criteria to discriminate between *in situ* and invasive carcinoma is the presence of an intact myoepithelial layer and basement membrane, suggesting that myoepithelial cells can have a ‘fence’ role to inhibit invasiveness.

### Myoepithelial cell tumor-suppressive properties

Recently we showed that reduced GJIC in myoepithelial cells of *Gja1<sup>In/+</sup>* (G60S) mice results in a delay in mammary gland ductal development during puberty, suggesting a regulatory role of Cx43 (Plante and Laird, 2008). Similarly, it has been shown that interactions between myoepithelial and luminal cells are required for proper differentiation of luminal cells *in vitro*, and that this process is dependent on gap junctions (Talhouk *et al.*, 2008). Lately, myoepithelial cells have received more attention as they have been

suggested to be a ‘natural tumor suppressor’ by inhibiting various neoplastic events, including cell growth, invasion and angiogenesis (Sternlicht and Barsky, 1997; Sternlicht *et al.*, 1997; Barsky and Karlin, 2005; Gudjonsson *et al.*, 2005). In keeping with this concept, our results suggest that a full complement of Cx43 may be necessary to protect against tumor or hyperplastic cells from entering the stroma and eventually into the vasculature. Cx43 could be necessary for the proper differentiation of the myoepithelial layer. Less-differentiated myoepithelial cells would be expected to have altered expression of tumor suppressive molecules, such as maspin and TIMP-1, thereby facilitating luminal cell migration (Sternlicht and Barsky, 1997; Barsky and Karlin, 2005). Alternatively, Cx43 may be necessary to physically strengthen the epithelium barrier as we already know that Cx43 is critical to synchronous myoepithelial cell contraction (Plante and Laird, 2008). It is also possible that decreased levels of Cx43 in the myoepithelium interfere with other adhesion mechanisms or complexes present at the plasma membrane (Laird, 2006). However, our previous study suggests that a low level of Cx43 is sufficient to maintain the proper localization of protein constituents of tight and adherens junctions (Plante *et al.*, 2010). Finally, this study does not allow us to conclude whether the effects observed are due to deceased levels of Cx43, or to a decrease in GJIC, as the G60S mutant is functionally dead and also has a dominant-negative effect on coexpressed wild-type Cx43 (Flenniken *et al.*, 2005). Additional studies would be necessary to further dissect the full contribution of Cx43 to the myoepithelial cell layer.

### Cx43 suppresses mammary gland metastasis to lung

An intriguing finding from our study using this unifying model of breast tumor onset and disease progression was the fact that Cx43 protects against mammary tumor cell metastases to the lung. Confirmation that we were indeed assessing lung metastases and not serendipitous primary lung tumor formation was provided by positive identification of the tumors as being of mammary origin via the expression of mammaglobin. Given that it is known that not all mammary cell-derived tumors express mammaglobin (Yang and Nonaka, 2010), it is likely that we are even underestimating the number of tumors of mammary origin. Furthermore, it has previously been demonstrated that ErbB2-expressing

mice are known to be prone to mammary gland tumor metastases to the lung (Guy *et al.*, 1992).

As Cx43 mutant mice exhibit hyperplastic glands without much evidence for the presence of solid tumors in the mammary gland, we were somewhat surprised to observe a greater prevalence of metastatic lesions in the lungs. Moreover, the fact that only one G60S-ErbB2 mouse in the spontaneous experiment and less than 40% of the mutant mice in the DMBA experiment developed a palpable tumor but all mice showed metastatic lung tumors, suggests that lung seeding happened before a palpable tumor could be detected. Although not particularly common in the clinical setting, it has been reported that metastases can be found in the absence of a detectable primary tumor, or even 5–7 years before the detection of a primary tumor (Engel *et al.*, 2003; van de Wouw *et al.*, 2003). In a recent mouse mammary tumor study, tumor cells were found in both the lungs and bone marrow from two different mouse models before invasive tumor cells were ever detected in the mammary gland (Husemann *et al.*, 2008). Interestingly, the authors observed that the basement membrane underlying hyperplastic cells within the mammary gland seemed disrupted suggesting that the myoepithelium and basement membrane may have in fact been functioning as a natural tumor suppressor, as suggested by the current study. However, we cannot rule out the possibility that undetected invasive tumor cells were present in the mammary gland of Cx43 mutant mice and it is these cells that are metastasizing to the lungs. Furthermore, it is important to note that the ‘soil’ where lung tumor metastases flourish is a Cx43-enriched environment (Nagata *et al.*, 2009) and it is possible that the metastases thrive better in an environment where Cx43 function is reduced. Although it is hard to model a mechanism to support this conjecture, we can also not fully eliminate this possibility.

Although connexins do not necessarily fulfill the definition of a metastasis suppressor, it is interesting to see that in our mouse model, Cx43 seems to share several characteristics with members of the metastasis suppressor family. Metastasis suppressor genes are defined as molecules that can inhibit metastasis while having little or no effect on primary tumor growth (Bodenstine and Welch, 2008). Since the discovery of *Nm23* (non-metastatic clone 23) in the late 1980s (Steeg *et al.*, 1988), over 23 members of this new class of cancer-

related genes have been identified to have a role at various steps of the metastasis process. Among them, expression of the breast cancer metastasis suppressor 1 (BRMS1) not only inhibits metastasis of MDA-MB-435 cells to the lungs and lymph nodes when injected in the tail vein of athymic mice, but it can also restore GJIC and increase Cx43 expression *in vitro* (Saunders *et al.*, 2001), suggesting a link between those two molecules in preventing metastasis.

In summary, this novel unifying model investigating the role of Cx43 in mammary gland tumorigenesis suggests that Cx43 in the myoepithelium minimally inhibits the initial stage of disease leading to mammary cell hyperplasia. However, Cx43 was found to be more potent at inhibiting the progression of tumorigenesis as defined by metastasis to the lung. Given that Cx43 is expressed extensively in the myoepithelial layer of the mammary gland, these results allow us to propose a working model where a full complement of Cx43 in the myoepithelium may serve to enhance the ‘barrier’ function of this cell layer inhibiting transformed luminal cells from invading the stroma during breast cancer progression. We propose that if the integrity of the myoepithelium is compromised by reduced levels of functional Cx43, tumor cells can more readily escape the confines of the primary site and metastasize to the lung. Although we cannot determine the transformation state of the cells that are metastasizing to the lungs, we suspect that within the heterogeneity of the hyperplastic tissue in the mammary gland some cells are fully transformed and are aggressive at metastasizing to the lung. However, this working model will need further consideration as we attempt to fully understand the role of Cx43 and myoepithelial cells in breast cancer progression.

Taken together, the findings of our study suggest that a molecular mechanism encrypted into the myoepithelium is protective against breast cancer progression. As most breast cancer related deaths are linked to metastasis, understanding the mechanisms of how Cx43 contributes to this process is critical in order to determine whether connexins are potential targets for therapeutic intervention.

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## Appendix 3 Animal use protocol approval



AUP Number: 2006-101-10  
PI Name: Laird, Dale W  
AUP Title: The Role of Gap Junction in Diseases

**Official Notification of AUS Approval:** A MODIFICATION to Animal Use Protocol 2006-101-10 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee



**AUP Number:** 2015-030  
**PI Name:** Laird, Dale W  
**AUP Title:** The Role Of Gap Junction In Diseases

**Approval Date:** 05/19/2015

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) entitled "The Role Of Gap Junction In Diseases"

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2015-030::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura  
on behalf of the Animal Use Subcommittee  
University Council on Animal Care

## Curriculum vitae

<b>Name:</b>	Michael Kenneth George Stewart
<b>Post-secondary Education and Degrees:</b>	<p>University of Western Ontario            London, Ontario, Canada            2006-2010 B.M.Sc.            Honours Specialization Physiology and Pharmacology            Major Medical Cell Biology</p> <p>The University of Western Ontario            London, Ontario, Canada            2010-Present Ph.D.            Physiology</p>
<b>Honours and Awards:</b>	<p>Province of Ontario Graduate Scholarship            2010-2011, 2011-2012, 2012-2013, 2013-2014</p> <p>Canadian Institutes of Health Research Strategic Training Program in Cancer Research and Technology Transfer Studentship            2010-2012, 2012-2014, 2014-2015</p> <p>Translational Breast Cancer Studentship award funded in part by the Breast Cancer Society of Canada            2012-2014, 2014-2015</p> <p>PSAC 610 Scholarship for Outstanding Research Contributions            2015</p> <p>Graduate Thesis Research Award            2012-2013</p> <p>Schulich Graduate Scholarship            2011-2012, 2012-2013, 2013-2014</p> <p>Western Graduate Research Scholarship            2010-2011, 2014-2015</p> <p>Dean's Honor List            2006-2007, 2007-2008, 2008-2009, 2009-2010</p> <p>Western Scholarship of Excellence            2006</p>

<b>Related Work Experience</b>	Teaching Assistant The University of Western Ontario Medical Sciences 4900; Course Coordinator: Dr. Doug Jones 2011-2015
	Teaching Assistant The University of Western Ontario Physiology 1021; Course Coordinator: Dr. John Ciriello 2013-2014
	Teaching Assistant The University of Western Ontario Physiology 2130; Course Coordinator: Dr. Anita Woods 2010-2013
	Scholars' Elective 3303 Laboratory Supervisor 2011-2013
	CaRTT Undergraduate Summer Student Laboratory Supervisor 2011

### **Publications:**

Stewart MKG, Plante I, Penuela S, Barr K, Laird DW. *Loss of Panx1 impairs mammary gland development at lactation: implications to breast tumorigenesis.* (To be submitted)

Stewart MKG, Bechberger JF, Welch I, Naus CC, Laird DW. *Cx26 knockdown predisposes the mammary gland to primary mammary tumors in a DMBA-induced mouse model of breast cancer.* (In revision for Oncotarget)

Stewart MKG, Simek J, Laird DW. Insights into the role of Connexins in Mammary Gland Morphogenesis and Function as Revealed by Genetically-modified Mice. Reproduction. 149 (6); 279-290, 2015.

Stewart MKG, Plante I, Bechberger JF, Naus CC, Laird DW. Mammary gland specific knockdown of the physiological surge in Cx26 during lactation retains normal mammary gland development and function. PLoS ONE 9(7): e101546. doi:10.1371/journal.pone.0101546, 2014.

Stewart MKG, Gong X, Barr KJ, Bai D, Fishman GI, Laird DW. The severity of mammary gland developmental defects is linked to the overall functional status of Cx43 as revealed by genetically-modified mice. Biochem J. 449(2):401-13, 2013.

Plante I, Stewart MKG, Laird DW. Evaluation of Mammary Gland Development and Function in Mouse Models. J. Vis. Exp. (53), e2828, DOI: 10.3791/2828, 2011.

Plante I, Stewart MKG, Barr K, Allan AL, Laird DW. Cx43 suppresses mammary tumor metastasis to the lung in a Cx43 mutant mouse model of human disease. *Oncogene*. (30), 1681–1692, 2011.

### **Oral Presentations:**

Stewart MKG and Laird DW. Connexins in mammary gland development and tumorigenesis. Physiology and Pharmacology Seminar, February 9, 2014, London, ON, Canada.

Stewart MKG, Bechberger JF, Plante I, Naus CC, and Laird DW. Connexins in mammary gland development and tumorigenesis. Oncology Research and Education Day, June 20, 2014, London, ON, Canada.

Stewart MKG, Gong X, Barr KJ, Bai D, Fishman GI, Laird DW. Mammary gland defects as revealed by genetically-modified mice harboring an oculodentodigital dysplasia-linked Cx43 mutant. American Society of Cell Biology, December 15-19, 2012, San Francisco, CA, U.S.A.

Stewart MKG, Barr K, Fishman GI, Laird DW. The Cx43 I130T mutant delays mammary gland development while not affecting mammary gland function in a mouse model of oculodentodigital dysplasia. Gap Junction Research Forum, January 27, 2012, London, ON, Canada.

### **Poster Presentations:**

Stewart MKG, Bechberger JF, Welch I, Naus CC, and Laird DW. Cx26 knockdown predisposes the mammary gland to primary mammary tumors in a DMBA-induced mouse model of breast cancer. Oncology Research and Education Day, June 26, 2015, London, ON, Canada.

Stewart MKG, Bechberger JF, Welch I, Naus CC, and Laird DW. Cx26 knockdown predisposes the mammary gland to primary mammary tumors in a DMBA-induced mouse model of breast cancer. International Gap Junction Conference 2015, March 28-April 2, 2015, Valparaiso, Chile.

Stewart MKG, Bechberger JF, Plante I, Naus CC, and Laird DW. The physiological surge in Cx26 is not essential for mammary gland development during pregnancy, but has important tumor suppressive properties *in vivo*. Department of Physiology and Pharmacology Research Day, November 4, 2014, London, ON, Canada.

Stewart MKG, Plante I, Bechberger JF, Naus CC, and Laird DW. Mammary gland specific knockdown of the physiological surge in Cx26 during pregnancy retains normal mammary gland development and function. International Gap Junction Conference 2013, July 13-18, 2013, Charleston, South Carolina, U.S.A.

Stewart MKG, Plante I, Bechberger JF, Naus CC, and Laird DW. Mammary gland specific knockdown of the physiological surge in Cx26 during pregnancy retains normal mammary gland development and function: Implications in Tumorigenesis. Oncology Research and Education Day, June 21, 2013, London, ON, Canada.

Stewart MKG, Gong X, Barr KJ, Bai D, Fishman GI, and Laird DW. Mammary gland defects as revealed by genetically-modified mice harboring an oculodentodigital dysplasia-linked Cx43 mutant. American Society of Cell Biology, December 15-19, 2012, San Francisco, CA, U.S.A.

Podder S, Stewart MKG, Shao Q, and Laird DW. Pannexin 1 Expression Reduces Tumorigenic Properties in Human Breast Cancer Cells. The Canadian Undergraduate Conference on Healthcare, November 16-18, 2012, Kingston, ON, Canada.

Stewart MKG, Gong X, Barr KJ, Bai D, Fishman GI, and Laird DW. The severity of mammary gland developmental defects is linked to the overall functional status of Cx43 as revealed by genetically-modified mice. Department of Physiology and Pharmacology Research Day, November 6, 2012, London, ON, Canada.

Stewart MKG, Barr K, Fishman G, and Laird DW. Impaired Mammary Gland Development in a mouse model of oculodentodigital dysplasia. Oncology Research and Education Day, June 22, 2012, London, ON, Canada.

Laird DW, Stewart MKG, Barr K, Allan A, and Plante I. Cx43 Suppresses Mammary Tumor Metastasis to the Lung in a Cx43 Mutant Mouse Model of Human Disease. BIT 4th World Cancer Congress-Breast Cancer Conference, November 16-18, 2011, Guangzhou, China.

Stewart MKG, Plante I, Barr K, Bechberger JF, Fishman GI, Naus CC, and Laird DW. Impaired mammary gland differentiation in connexin mutant mice. International Gap Junction Conference 2011, Aug 6-11, 2011, Ghent, Belgium.

Stewart MKG, Plante I, Barr K, Bechberger JF, Fishman GI, Naus CC, and Laird DW. Impaired mammary gland differentiation in connexin mutant mice. Oncology Research and Education Day, June 17, 2011, London, ON, Canada.

Laird DW, Penuela S, and Stewart MKG. Cell-Cell Communication in Health and Disease. Canada Research Chair Conference, November 25, 2010, Toronto, ON, Canada.

Plante I, Stewart MKG, Barr K, Allan A, and Laird DW. Mammary Gland Tumor Onset is Delayed in Cx43 Mutant Mice, but Metastasis to the Lungs is Enhanced. The European Molecular Biology Organization Meeting 2010, September 4-7, 2010, Barcelona, Spain.

Plante I, Stewart MKG, Barr K, Allan A, and Laird DW. Cx43 Mutant Mice Show Delayed Onset of Palpable Mammary Gland Tumors but Are More Susceptible to Lung Metastasis. Oncology Research and Education Day, June 18, 2010, London, ON, Canada.