The Role of Phosphorylation in Claudin 1 Mislocalization in Human Breast Cancer Cells

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Physiology and Pathophysiology

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ABSTRACT

Claudin 1, a major tight junction protein, is frequently deregulated and mislocalized to the cytoplasm in some human breast cancers. Mislocalization of claudin 1 has been recently shown to enhance the metastatic potential in other cancers, including melanoma, colon and liver cancers, and thus, may also promote metastasis in breast cancer. Moreover, the C-terminus of the claudin 1 protein has been shown to direct its membrane localization in normal epithelial cells. Furthermore, protein kinase activity (PKA/PKC) is important in regulating claudin 1 expression and localization in several cancers. Therefore, in breast cancer, it is possible that the phosphorylation of the PKA/PKC sites within the C-terminus may direct the localization of claudin 1. Thus, the hypothesis of the study is that mislocalization of claudin 1 in human breast cancer cells is regulated by phosphorylation.

First, to demonstrate whether the C-terminus of claudin 1 regulates its membrane localization in human breast cancer cells, GFP-tagged claudin 1 constructs lacking 24 amino acids of the C-terminus were generated. When these constructs were transfected into breast cancer cell lines (T47D and MCF-7), decreased membrane staining but increased cytoplasmic staining was observed compared to the full-length constructs that were observed primarily in the cell membrane. Next, in order to identify whether predicted phosphorylation sites within the C-terminal domain of claudin 1 protein were responsible for its mislocalization in human breast cancer cells, GFP-claudin 1 constructs were generated using site-directed mutagenesis that mimicked both constitutive phosphorylation and non-phosphorylation at PKC/PKA predicted target sites on the C-terminus. Following transfection in MCF-7 cells, constructs mimicking constitutive phosphorylation showed less membrane staining than their non-phosphorylatable counterparts.

Taken together, in human breast cancer cells, specific phosphorylation sites within the C-terminus of claudin 1 play a role in its subcellular localization. Thus, these results suggest that phosphorylation may be a mechanism regulating the mislocalization of claudin 1 to the cytoplasm in human breast cancer.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to all the people who encouraged, inspired, supported, and assisted me to the pursuit of the M.Sc degree. First of all, I thank my supervisor Dr. Yvonne Myal, for giving me the opportunity to conduct my study in her research group. During the three years, you have contributed to a rewarding graduate school by encouraging me to attend and present at seminars, supporting my attendance at various conference, offering me the opportunity to guide summer students in our laboratory and instructing me with my research work. I appreciate all your contribution of time, ideas, guidance, patience and funds to make my graduate school experience productive and stimulating.

I would also like to thank my committee members, Dr. Sabine Hombach-Klonisch, Dr. Suresh Mishra, and Dr. Jiuyong Xie, for their guidance, generous contribution of knowledge and experience, valuable comments and encouragement from the start until the end of my study.

My deepest gratitude also goes to our technician Ms. Anne Blanchard for the invaluable help from the start until the end of my study, as well as the corrections and critical comments on my thesis. I am also thankful to our technician Mr.Xiuli Ma for all technical assistance and help in life. I want to thank Olivia, Jamie, Vivake, Jihyun, Natasha and Amanda for coloring the research life in our laboratory.

I also would like to thank the Canadian Breast Cancer Foundation for the funds, Dr. Spencer Gibson's laboratory, and also the The Genomic Centre for Cancer Research and Diagnosis (GCCRD) for the use of their facility.

Finally, I would like to acknowledge my friends and family who support me during the entire process. I would like to thank my parents for the unconditional and constant love and support. Also, I am thankful to my Uncle Wei, Aunt Ling, Uncle Chunjie, Aunt Hongming and their families for the help and advices of my life and my career. Thanks my friend and role model Yi, you always make time to help me no matter how busy you are. The most

special thank goes to my best friend and life partner, Marius, for the continuous love and support regardless of what decision I have made.

ABBREVIATIONS

α	Alpha
β	Beta
µg	microgram
µl	microliter
°C	degree Celsius
1X	one time
10X	ten times
APS	ammonium persulphate
BCA	bicinchoninic acid
bp	base pair
BSA	bovine serum albumine
CK	cytokeratin
CLDN	claudin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ddH_2O	double-distilled water
E.coli	*Escherichia coli*
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FL	full length
GFP	green fluorescent protein
HBC	human breast cancer
HER2	human epidermal growth factor receptor 2
IF	immunofluorescence
IgG	immunoglobulin G
IPTG	isopropyl β-D-1-thiogalactopyranoside
JAM	junctional adhesion molecule
kDa	kilodalton
LB	Luria Berthani
mM	millimolar
M	molar
MAPK	mitogen-activated protein kinases
MCS	multiple cloning site
MDCK	Madin-Darby canine kidney
nM	nanometre
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C

PR	progesterone receptor
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TBST	tris buffered saline with Tween 20
TJ	tight junction
WT	wild type

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I. INTRODUCTION

1. Breast Cancer

1.1 Breast Cancer Statistics

Breast cancer is one of the most common malignancies among women worldwide (Cardoso et al. 2012). In Canada, about 1 in 9 Canadian women is expected to develop breast cancer during her lifetime and 1 in 30 will die from it (Statistics 2015).

In 2015, approximately 25,000 Canadian women will be diagnosed with this disease, representing 26% of all newly diagnosed cancer cases. Breast cancer is also responsible for the second leading death among Canadian women after lung cancer, it contributes to an estimated 5,000 Canadian women deaths in 2015, representing 14% of all cancer deaths in women (Statistics 2015). Breast cancer can also occur in men, however, the incidence and death rate were both much lower than women.

Breast cancer incidence rates in women are generally the same across Canada, and have remained relatively stable since the late 1980s (Statistics 2015). The actual number of cases each year has increased as the population of Canada has increased.

However, the risk of being diagnosed with breast cancer increases with age. In 2015, it is estimated that 82% of new breast cancers would occur in Canadian women over the age of 50 (Statistics 2015). While breast cancer is less common at a young age (age under 40), younger women tend to have more aggressive breast cancers than older women.

1.2 Histological and Molecular Subtypes of Breast Cancer

Breast cancer is now considered to be a heterogeneous disease with different molecular subtypes. Since 2000, five major molecular subtypes have been identified using gene expression profiling, which are luminal A, luminal B, Her2-enriched, basal-like and

normal-like subtype (Finak et al. 2006, Kwan et al. 2009, Nielsen et al. 2004, Perou, Sørlie, et al. 2000, Sørlie et al. 2001, Yu, Shen, and Shao 2009). Each subtype demonstrates significant variation in gene expression, clinical features, response to treatment and prognosis (Bild et al. 2009, Hugh et al. 2009, Kwan et al. 2009). These five subtypes are differentiated based on estrogen receptor/ progesterone receptor (ER/PR), epidermal growth factor receptor 2 (Her2), cytokeratin 5/6 (CK5/6), Ki-67, a nuclear marker of cell proliferation, and human epidermal growth factor receptor (EGFR) expression (Sabatier et al. 2014) see Table 1). It should be noted that the majority of basal-like breast cancers are characterized as triple-negative (absence of ER, PR and HER2 expression; (Bertucci et al. 2008); however, triple negative and basal-like are not equivalent term and 70% of triple negative are basal-like. Besides the major five molecular subtypes of breast cancer, a claudin-low subtype has also been identified by gene expression profiling studies (Prat et al. 2010).

Breast cancer has also been characterized by different histological subtypes (See Table 2). Broadly, breast cancer can be categorized into *in situ* carcinoma and invasive (infiltrating) carcinoma. Breast carcinoma *in situ* can be further sub-classified into ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). DCIS is more common than LCIS. Based on the architectural features of the tumor, DCIS was sub-classified into five subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid. Similarly, invasive carcinomas are also heterogeneous. The major types include infiltrating ductal, invasive lobular (ILC), ductal/lobular, medullary and mucinous (colloid) carcinomas. Based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index, ILC can be further sub-classified into well-differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3).

1.3 Hereditary Breast Cancer

Approximately, 5% to 10% of the total breast cancers are considered to be hereditary, caused by the autosomal dominant inheritance of mutated genes (Ellisen and Haber 1998,

Lux, Fasching, and Beckmann 2006). Most hereditary breast cancer cases are associated with two abnormal genes: breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2). BRCA1 and BRCA2 mutations contribute to two thirds of hereditary breast cancer and approximately 3-8% of the total breast cancer cases (Easton 2002, Lux, Fasching, and Beckmann 2006).

BRCA1 and BRCA2 are tumor suppressor genes located on chromosome 17q21 and chromosome 13q12-13 respectively (Bougie and Weberpals 2011). BRCA 1 and 2 proteins bear little resemblance in their structures; however, both of these two proteins share a key role in the DNA damage the repair of interstrand cross-links by RAD51-mediated homologous recombination (Shahid et al. 2014). Individuals who carry mutations in these two genes have a significant risk of developing breast cancer.

The majority of BRCA1-deficient breast cancers are triple negative (absence of ER, PR and HER2), whereas most BRAC2-deficient breast cancers are characterized as ER-positive, PR-positive and HER2-negative (Crown, O'Shaughnessy, and Gullo 2012, Severson et al. 2015) BRCA1 tumors have shown a similar gene expression profile with basal-like subtype, including strong expression of basal cytokeratin and high p53 mutation rates (Lakhani 2003, Lakhani et al. 1998). More recently, some studies have revealed that BRCA1-deficient breast cancers also share some characteristics with claudin-low tumors, such as triple negative, frequent medullary (a rare breast carcinoma with a syncitial growth pattern and high grade cytology with a good prognosis) and metaplastic (a rare subtype of invasive breast cancer that tends to have an aggressive clinical presentation as well as a variety of distinct histologic designations) histology, high expression of the stem cell marker ALDH1 and a high frequency of lymphocytic infiltrate (Bougie and Weberpals 2011, Lehmann et al. 2011, Prat et al. 2010). Notably, claudin proteins, such as claudin 1, 3, 4, 6 and 7, have been reported to be frequently overexpressed in BRAC1-related breast cancer, especially claudin 1 and 6 (Heerma van Voss et al. 2014).

1.4 Treatment of Breast Cancer

Tumor heterogeneity means that distinct morphological and phenotypic profiles exist between different tumor cells. These distinct morphological and phenotypic profiles include cellular morphology, gene expression, metabolism, motility, proliferation, and metastatic potential. Because of the heterogeneous nature of breast cancer, many aggressive forms are not well characterized, making it difficult to treat.

Currently, treatment of breast cancer consists of two main therapies: local therapy and systemic therapy (Cardoso et al. 2012, Sledge et al. 2014). Treatment options are depend on several factors including tumor stage, hormone receptors (ER/PR) and HER2 status, as well as patient age and general health (Cardoso et al. 2012). Surgery and radiotherapy are the treatment modalities used for local control of the disease intended to treat a tumor at the site without affecting the rest of the body. The most common types of breast surgery include lumpectomy and mastectomy. Lumpectomy, also called breast-conserving surgery, refers to the surgical removal of the tumor along with a small, cancer-free margin of normal tissue around the tumor (Fisher et al. 1998). Mastectomy is the removal of the entire breast and includes three types that are simple, modified radical and radical, which are progressively increasing the amount of tissue removed (Fisher et al. 1998). Chemotherapy, hormone therapy are used for systemic control of the disease that refers to drugs given by mouth or directly into the bloodstream (Rouzier et al. 2005). Adjuvant therapies including radiation therapy, chemotherapy or hormonal therapy are usually recommended after the surgery to lower the risk of recurrence and to get rid of any remaining cancer cells (Anampa, Makower, and Sparano 2015). Clinically, the best results are usually obtained when these treatment methods are applied in combination. With more insight into the complexity of this disease, there is now more emphasis on a personalized approach.

2. The Mammary Gland

2.1 Structure and Function of The Mammary Gland

The mammary gland is a complex secondary organ in female mammals that is responsible for the production of milk for the nourishment of the newborn (Daniel and Smith 1999). During growth and development, the mammary gland is regulated by several influences, including specific hormones (such as estrogens, progesterone and prolactin) and growth factors (such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β) and insulin-like growth factor (IGF-1)), as well as cell-cell interaction, extracellular matrix and neural inputs (Wysolmerski et al. 2001).

The human mammary gland is composed of 15 to 20 lobes, separated by adipose tissue. Each lobe can be divided into lobules consist of grape-like structures, referred as alveoli. Lobules drain into a serious of ducts that in turn drain into single lactiferous ducts opening at the apex of the nipple (Daniel and Smith 1999). The lactiferous ducts and lobules are involved in milk synthesis and secretion during lactation. The glandular epithelium is embedded in extracellular matrix and stroma composed of mesenchymal cells, such as fibroblasts, adipocytes and immune cells.

The main function of the mammary gland is milk production, which is regulated by the three types of epithelial cells: alveolar luminal epithelial cells, ductal luminal epithelial cells and myoepithelial cells. During lactation, milk is secreted by epithelial cells of the alveoli from the inner layer of lactiferous ducts and stored in the alveoli. Myoepithelial cells contract and push the milk from the alveoli through the lactiferous ducts toward the nipple. Therefore, the epithelial cells have to be sealed tightly from one to each other to prevent the interdiffusion of milk and interstitial fluid. This structure that seals the epithelial cells referred as tight junction (Nguyen and Neville 1998).

2.2 Development of The Mammary Gland

The development of the mammary gland can be divided into distinct stages: embryonic development, growth of the gland at puberty, development and differentiation of the gland during pregnancy and lactation, and finally the involution of the gland (Daniel and Silberstein 1987, Daniel and Smith 1999).

The mammary gland initiates to form during embryogenesis (Cowin, Rowlands, and Hatsell 2005). The early embryo constitutes three distinct cell layers: the ectoderm (outer layer), the mesoderm (middle layer) and the endoderm layer (inner layer). The mammary gland is derived from the ectoderm and the mesoderm. Each mammary gland begins as a budlike invagination of the surface ectoderm with the formation of mammary lines, which resolves into five pairs of mammary placodes. Each placode expands into the underlying mesenchyme to form an initial mammary bud, which then give rise to the secondary buds. The secondary buds then develop into lactiferous ducts and secondary branches, which are present at birth (Cowin, Rowlands, and Hatsell 2005).

In human, both males and females have glandular tissue within the breasts at birth, however, the development of glandular tissue only happens in females after puberty in response to estrogen release. At birth the gland is just a rudimentary ductal system, the immature ductal system enlarges and extends to create a ductal tree during puberty, filling the fat pad under control of the hormones and growth factors.

During pregnancy, the mammary gland undergoes extensive changes in preparation for lactation, including gland maturation and alveologenesis, under control of prolactin and progesterone. The ductal system expands vastly and differentiates into milk-secreting alveoli at the end of pregnancy. However, milk secretion is repressed by high concentration of circulating sex steroids, primarily progesterone. Colostrum, secreted by the mammary gland during late pregnancy and the first few days after giving birth, riches in antibodies and minerals and precedes milk secretion.

Lactation is induced by a decrease in estrogen and progesterone levels and the prolactin is not inhibited. During lactation, alveoli are fully matured and the luminal cells synthesize and secrete milk components (lactose, milk proteins and low concentrations of sodium and chloride) (Hennighausen and Robinson 2005). Milk is stored in alveoli until the suckling brings about the contraction of myoepithelial cells.

Involution of mammary gland occurs following cessation or significant reduction (weaning) of milk removal from the breast, during which mammary gland returns to non-lactating stage (Daniel and Silberstein 1987, Daniel and Smith 1999). Two phages of involution have been identified: apoptosis and remodeling (Watson 2006). The first phage is reversible, including apoptosis of epithelial cells, detachment of cells from the alveolar structures and shedding into the lumen, which is induced by systemic lactogenic hormones and observed within 12 hours involution. The second stage is irreversible that occurs at 48 hours with the beginning of alveoli collapse. During this phage, the milk supply is lost, breakdown of extracellular matrix and activation of proteases induces a second wave of apoptosis, resulting in a massive period of tissue remodeling. Morphologically, the remodeled gland is very similar to the virgin gland (Macias and Hinck 2012).

3. Tight Junctions

In epithelial tissue, cell–cell interactions are mediated by junctional complexes consisting of tight junctions, adherens junctions and gap junctions, each of which possesses unique morphological characteristics, composition, and functions.

Tight junctions are the most apical components of cell-cell interactions located between epithelial cells and constitute a continuous transepithelial barrier. Tight junctions regulate the transport of ions and small molecules through the paracellular pathway, which refers to the “barrier” function. Tight junctions are also associated with organization of cell polarity by separating the membrane into apical and basolateral domains as well as polarizing locations of ion channels, receptors and enzymes to the membrane domains, known as “fence” function

(Diamond, et al. 1977; Anderson, et al. 1993).

Tight junction components can be generally categorized into three groups: integral proteins that constitute tight junction strands; peripherally associated scaffolding proteins, such as Zonula Occludens (ZO-1, ZO-2 and ZO-3) that organize the integral proteins and connect them with actin cytoskeleton and other cytoplasmic proteins; and signaling proteins that are suggested to be involved in junction assembly, barrier regulation and gene transcription (Anderson 2001, Anderson, Balda, and Fanning 1993, Inoko et al. 2003, Itoh and Bissell 2003, Itoh et al. 1999).

3.1 The Integral Tight Junction Proteins

Tight junctions consist of three types of integral membrane proteins: occludins, claudins and the JAM (junctional adhesion molecules) (See Table 4).

Occludins

Occludins, the first identified integral membrane protein, regulate tight junction barrier function and tight junction proteins interaction. It contains four transmembrane domains, two extracellular loops enriched in tyrosine residues, an intracellular C-terminus and an intracellular N-terminus. Occludins have been shown to have little effect on tight junction strands formations, demonstrated by occludin knockout mice (Saitou et al. 1998). Its recruitment in tight junction strands has been shown to be associated with claudins co-expression (Van Itallie and Anderson 2013).

Claudins

The claudin family of proteins, discovered several years after occludin by the same research group, constitutes the backbone of tight junction (Furuse 1998). To date, twenty-seven members have been identified (Milatz et al. 2015). The expression pattern of claudins is tissue specific; however, most tissues express multiple claudins that can interact in

either a homotypic or heterotypic fashion to form tight junction. The exact combination of claudin proteins within a given tissue determines the selectibity, strength and tightness of the tight junction (Morin 2005). The claudins encode 20-27 kDa proteins and share a common transmembrane topology: four transmembrane domains, two extracellular loops where the first one is significantly longer than the second one, an amino intracellular tail and a carboxyl intracellular tail which contains a PDZ domain (with the exception of claudin 12). Aside from maintaining cell polarity and paracellular functions, claudins are also associated with regulating various cellular processes including cell growth, differentiation and ultimately tumorigenesis. PDZ domain is important for the interaction with cytoskeleton proteins and is also involved in several signaling transduction pathways by interacting with signaling molecules [such as protein kinase A (PKA) and C (PKC), mitogen-activated protein kinase (MAPK) and other PDZ domain containing proteins] (Heiskala, Peterson, and Yang 2001).

JAMs (Junctional Adhesion Molecules)

The JAM family, including JAM-A, B, C, L and JAM-4, are immunoglobulin (Ig)-like single-span transmembrane molecules and mediate Ca2+-independent adhesion. JAMs is suggested that to be associated with tight junction function and integrity, as well as the interaction with several tight junction-associated proteins, such as ZO-1 (Gonzales-Mariscal 2001, Gonzalez-Mariscal, Tapia, and Chamorro 2008). However, it has been reported that JAM proteins are not necessary for tight junction formation.

3.2 Tight Junctions In Mammary Gland

Tight junctions are highly dynamic during the mammary gland development and are under the control of several factors. The permeability of tight junction is closely related to milk secretion, resulting in significant varieties of tight junction components between pregnant and lactating animals (Morgan and Wooding 1982, Nguyen and Neville 1998). In non-lactating breast, tight junction strands are less organized with smaller numbers and fewer

branched networks, which allow them to function as barriers for diffusion to and from the pericellular environment in the mammary gland. During lactation, when the ducts and alveoli are filled with milk, tight junctions form highly impermeable organization in order to seal the epithelial cells tightly to prevent the leakiness of milk components from the lumen.

Hormones such as progesterone, prolactin and glucocorticoid, as well as growth factors such as TGF beta also appear to be involved in the regulation of tight junction permeability (Itoh and Bissell 2003, Nguyen and Neville 1998). Progesterone is one of the key hormones that is essential for establishment and maintenance of pregnancy. Studies have shown that withdrawn of progesterone triggers the closure of tight junction in pregnant mice mammary epithelia (Nguyen 2001). Prolactin, another key hormone that induces alveolar development and regulates the synthesis and secretion of the milk, may play a role in modulation of tight junction barrier (Stelwagen, McFadden, and Demmer 1999, Stelwagen et al. 1998). Furthermore, it also has been reported that glucocorticoid, which is essential for the maintenance of lactation, plays a role in enhancing TJ barrier function in the lactating mammary epithelia and preventing the associated reduction in milk secretion (Stelwagen, McFadden, and Demmer 1999, Stelwagen et al. 1998). In addition, the growth factor TGF beta also has been shown to alter tight junction permeability through its regulation of glucocorticoid (Nguyen and Neville 1998).

3.3 Tight Junctions In Breast Cancer

Breast cancer arises from the epithelial cells lining the breast ducts and lobules. Increasing evidences suggested that the breakdown of cell-cell interactions as well as the deregulated expression of junctional proteins are key steps during metastatic development and cancer progression (Mareel and Leroy 2003, Gonzalez-Mariscal, Lechuga, and Garay 2007). This complex process entails local invasion, followed by dissemination of malignant cells and finally re-establishment of cancer cells at distant sites. As a result, proteins that constitute the cell-cell junctional complexes, such as tight junction proteins and the

associated proteins at tight junction are suspected to directly contribute to invasion and metastasis (Brennan et al. 2010).

The claudins are the most widely studied integral protein in breast cancer. Cancer-specific phenotypic changes have been shown to be associated with modulations in claudin expression in various cancer types. The gene expression of members of claudin family can be either increased or decreased in breast cancer. Claudin 1 and 7 have been reported to be downregulated in breast cancer (Hewitt, Agarwal, and Morin 2006, Tabaries et al. 2012), which might associate with cell dissociation and an increase ability of cells to disseminate. In contrast, expression of claudin 3 and 4 has been observed significantly increased in breast cancers (Blanchard, Iwasiow, et al. 2009, Morin 2005). Consistent with this, knockout claudin 3 and 4 have resulted in a downregulation of E-cadherin mRNA and protein and b-catenin pathway signaling, which promotes tumor growth *in vivo*, cell migration and invasion *in vitro* (Shang et al. 2012). In addition, one study has shown that the decreased expression of claudin 6 enhances anchorage-indepandent growth and promotes cellular invasiveness of breast cancer (Osanai et al. 2007). Consistent with this, overexpression of claudin 6 has been reported to be associated with decreased anchorage-independent growth and tumor invasion, suggesting that claudin 6 may function as a tumor suppressor of breast cancer (Wu et al. 2010). Furthermore, claudin 2 has been shown that plays a role in promoting breast cancer metastasis to liver by facilitating tumor cell interaction with hepatocytes and is suggested as a prognostic biomarker (Kimbung et al. 2014, Tabaries et al. 2012). In addition, the overexpression of claudin 16 in human breast cancer cells was also shown to reduce aggressiveness and motility (Martin et al. 2008). The exact roles of the claudin family in tumorgenesis still remain unclear; however, it has been suggested that they represent promising targets for cancer detection, diagnosis and therapy.

In addition, tight junction-associated proteins have also been shown to play a role in breast cancer. ZO-1 has been discovered with a potential function as a tumor suppressor gene as it has been observed in several breast cancer cell lines with a decreases expression (Hoover, Liao, and Bryant 1998). It has also been demonstrated that the downregulation of ZO-1 could

be directly involved in progression of malignancy in breast cancer. In a human breast cancer cell line MCF7, the activation of insulin-like growth factor I receptor (IGF-IR) upregulate ZO-1 expression, which reduce invasive ability of tumorigenic cells (Mauro et al. 2001). Furthermore, ZO-2 has also been shown that posses a tumor suppressor potential in breast epithelial cells (Glaunsinger et al. 2001, Itoh and Bissell 2003).

4. Claudin 1

The importance of claudin 1 in normal cell function has been clearly established through knockout mice experiences that showed claudin 1 forms the backbone of tight junction strands and contributes to the epithelial barrier function (Furuse 1998, Furuse et al. 2002, Furuse et al. 1998). The deletion of claudin 1 in mice impairs the epidermal barrier and increased the permeability of the epidermis, followed by the death of the claudin 1-deficient mice within one day due to excessive water loss (Furuse et al. 2002). Claudin 1 also plays a role in the polarized location of ion channels, receptors, and enzymes to the different membrane domains in the epithelial cells, referred as its "fence" function (Diamond, et al. 1977; Anderson, et al. 1993). Furthermore, claudin 1 gene expression was found to be tightly regulated during different stages of normal mouse gland development. The gene was increased during pregnancy but was observed a sharp decrease by day 10 of lactation and once again was significantly up regulated by the first day of involution (Blanchard et al. 2007, Blanchard et al. 2006).

4.1 Claudin 1 Structure

Similar to the structure of other claudin family members (as mentioned in Section 1.3), claudin 1 (~21 kDa) contains four transmembrane domains with amino- and carboxyl-termini in the cytoplasm and two extracellular loops. The first loop is significantly longer than the second loop. The extracellular loop 1 (Claudin $1_{53\text{-}80}$) has been demonstrated to play a critical role in epithelial barrier function (Mrsny et al. 2008). A highly conserved motif

W_{30}-GLW_{51}-C_{54}-C_{64}, located in the crown of claudin 1 extracellular loop 1, has been reported to be required for Hepatitic C Virus (HCV) entry, as well as mediates cell-cell interaction formation (Cukierman et al. 2009, Evans et al. 2007). The shorter extracellular loop 2 (Claudin $1_{146-160}$) is suggested to be required for holding function and oligomerization of the protein (Krause et al. 2008).

Consistent with other claudins, the C-terminus of claudin 1 (Claudin $1_{187-211}$) also contains a PDZ-binding motif that interacts claudin 1 with several tight junction-associated proteins. These interactions provide a link to the actin cytoskeleton, which is suspected to anchor claudin 1 at the apical localization along cell membrane. However, it has been demonstrated that the C-terminal cytoplasmic tail of claudin 1 not the PDZ binding-motif is required for its apical localization at epithelial junctions (Rüffer and Gerke 2004). Indeed, deletion of C-terminus inhibited its localization to the cell membrane and led to its retention in cytoplasm, while claudin derivatives lacking only PDZ-binding motif continue to localize to the tight junction (Rüffer and Gerke 2004).

4.2 Deregulation of Claudin 1 in Specific Cancers

Both down regulation and overexpression of claudin 1 have been shown in several cancers (See Table 6), which is associated with cancer progression and invasion, suggesting that claudin 1 may play a dual role as a tumor promoter and as a tumor suppressor. The tumor-promoting role of claudin 1 is via its effect on invasion or motility of cancer cells. Overexpression of claudin 1 has been reported in several cancers, such as papillary thyroid tumors, oral squamous cell carcinoma, melanoma, ovarian, colon and gastric cancer, and has been shown to be associated with aggressiveness and increased malignant phenotype (Dhawan et al. 2005, Kleinberg et al. 2008, Leotlela et al. 2007, Nemeth et al. 2009, Oku et al. 2006, Resnick et al. 2005, Wu et al. 2008). For example, in colon cancer, study has shown that the expression of claudin 1 was up regulated and significantly increased xenograft tumor growth and metastatic behavior in athymic mice (Dhawan et al. 2005). Overexpression of

claudin 1 has been shown to be associated with tumor invasion and metastasis in gastric cancer (Huang et al. 2015). Functional studies have also shown that claudin 1 could recruit and promote the matrix metalloproteinase (MMP) and lead to a more aggressive phenotype in oral and ovarian cancer (Miyamori et al. 2001, Oku et al. 2006). Conversely, decreased claudin 1 expression was reported in breast, esophageal, prostate, liver and lung cancer. Loss of claudin 1 has been demonstrated to be correlated with cancer progression, invasion, metastasis, and shorter disease-free survival (Chao et al. 2009, Miyamoto et al. 2008, Sheehan et al. 2007, Swisshelm, Macek, and Kubbies 2005). For example, it has been shown that down regulation of claudin 1 expression correlated with malignancy of hepatocellular carcinoma and overexpression of claudin 1 protein suppresses metastasis, cell migration and invasion of lung cancer cells (Higashi Y 2007; Chao YC 2009).

4.3 Deregulation of Claudin 1 in Breast Cancer

The exact role played by claudin 1 in breast cancer is not well delineated. Several studies have demonstrated a low or absence of claudin 1 expression in most invasive breast cancers, which is associated with disease recurrence, progression, metastasis, and reduce survival, suggesting a tumor suppressor role for this protein (Morohashi et al. 2007, Swisshelm, Macek, and Kubbies 2005, Swisshelm et al. 1999, Tokés et al. 2005, Kramer et al. 2000). Further, *in vitro* experiments show that the down regulation of claudin 1 gene expression leads to the neoplastic transformation of breast epithelial cells and that the re-expression of claudin 1 alone in a human breast cancer cell line was sufficient to induce apoptosis (Hoevel et al. 2004, Kulawiec et al. 2008). Claudin 1 down regulation appears to be more prominent in ER+ or ER+/HER2+ luminal breast cancers, but has also been identified in some basal-like breast cancers as well as “claudin-low” subtype. Recently, it has been demonstrated that DNA promoter methylation is associated with claudin 1 downregulation in ER+ breast cancer but claudin 1 promoter was not methylated in ER- breast cancer (Di Cello et al. 2013). Moreover, slug and snail, the transcriptional factors during EMT

(epithelial-mesenchymal transition), have also been reported that could bind to claudin 1 promoter resulting in the repression of claudin 1 activation (Martinez-Estrada et al. 2006). Overexpression of claudin 1 has shown to contribute to an anti-apoptotic role in tamoxifen-treated MCF 7 cells (Liu et al. 2012). However, claudin 1 expression may be distinct within specific subtypes of breast cancer. In our laboratory, we have shown that in some ER- basal-like subtype of breast cancers, claudin 1 expression was observed to be overexpressed by using tissue microarray strategies, suggesting that the role of claudin 1 in human breast cancer may be more complicated than originally thought (Blanchard, Skliris, et al. 2009). Claudin 1 knockdown in basal-like breast cancer cells decreases cell migration by affecting the expression of genes involved in EMT, a process of converting adherent polarized epithelial cells into individual migratory mesenchymal cells able to invade the extracellular matrix (Blanchard et al. 2013). In addition, it has been shown that, claudin 1 alone was enough to exert tight junction mediated barrier function (paracellular sealing) in metastatic breast cancer cells in the absence of other tight junction proteins (Hoevel et al. 2004).

4.4 Mislocalization of Claudin 1 in Cancers

A mislocalization of claudin 1 from the plasma membrane to other cell compartments, such as cytoplasm and nucleus, has been reported in several cancers and may contribute to its role in tumorigenesis (Dhawan et al. 2005, French et al. 2009, Jakab et al. 2010, Leotlela et al. 2007, Oku et al. 2006). For example, in colon cancer, mislocalization of claudin 1 to cytoplasm and nucleus has been found in particular in metastatic tissues (Dhawan et al. 2005). Mislocalization of claudin 1 from tight junctions has also been reported in bladder tumors (Boireau et al. 2007). Cytoplasmic expression of claudin 1 in metastatic melanoma cells was shown to correlate to increased migration of tumor cells, controlled by phosphorylation (French et al. 2009). Additionally, subcellular localization of claudin 1 has also been shown to be disrupted in human invasive breast cancer, leading to a detection of this protein in the

cytoplasm (Blanchard, Skliris, et al. 2009, Soini 2005, Tokés et al. 2005). In some of these specific examples, mislocalization of claudin 1 was associated with enhances metastatic potential of the cancer (Dhawan et al. 2005, French et al. 2009, Leotlela et al. 2007). Such mislocalization of claudin 1 in cancers has been suggested to be regulated by posttranslational modifications such as phosphorylation (Koizumi et al. 2008, Leotlela et al. 2007, Lippoldt et al. 2000, French et al. 2009). The detailed function of phosphorylation of claudin 1, as well as other claudin family members, in various cancers will be described in the next section.

5. Posttranslational Modifications

5.1 Phosphorylation

Phosphorylation, the most well studied post-translational modification, plays critical roles in the regulation of cellular processes, as well as protein functions and activities. In eukaryotic cells, phosphorylation only occurs in three amino acids: serine, threonine and tyrosine, which contain a nucleophilic (-OH) group that attracts the terminal phosphate group (γ-PO_3^{2-}) on the universal phosphoryl donor adenosine triphosphate (ATP), resulting in the transfer of the phosphate group to the amino acid side chain. Protein phosphorylation is mediated by kinases, which are enzymes that facilitate phosphate group transfer to substrate. Protein kinases can be divided into serine/threonine-specific protein kinases that phosphorylate the OH group of serine or threonine and tyrosine-specific protein kinases that phosphorylate OH group of tyrosine.

The protein kinase A (PKA), also known as cyclic AMP-dependent protein kinase, is a serine/threonine kinase and has been shown to play a role in the maintenance of epithelial tight junction integrity (Klingler et al. 2000).

Protein kinase C (PKC) also belongs to the family of serine-threonine kinases and is

known to regulate epithelial barrier function. To date, at least twelve different isoforms of PKC have been identified, which can be divided into three subtypes: classic or conventional, novel and atypical PKC isozymes (See Table 6).

A number of studies suggest that phosphorylation may play a major role in regulating tight junction and tight junction-associating proteins both in normal cells and in cancer cells. Claudins are the most extensively studied tight junction protein with regards to regulation by phosphorylation. For example, phosphorylation of claudin 2 has been shown to promote its membrane retention and phosphorylation of claudin 4 is required for tight junction formation (Aono and Hirai 2008, Van Itallie and Anderson 2013). Moreover, the C-terminal tail of claudin 3 could be phosphorylated by PKA and contributes to the disruption of tight junction in the ovarian cancer (D'Souza, Agarwal, and Morin 2005). Also, phosphorylation of claudin 1 on a PKC site within the C-terminus is essential for mediating the heart looping (Simard, Di Pietro, and Ryan 2005). Another study has shown that phosphorylation on threonine 203 of claudin 1 is required for barrier function in a rat lung cell endothelial cell line with induced claudin 1 expression through a putative mitogen-activated protein kinase (MAPK) activation (Fujibe et al. 2004). Additionally, phosphorylation of claudin 5 by PKA could be involved in promotion of tight junction function in endothelial cells (Ishizaki et al. 2003).

Notably, analysis of claudin 1 revealed that a number of putative PKA and PKC sites located within the C-terminal tail (Table 7). These putative protein kinase sites were identified as important players in regulating claudin 1 expression and localization in several caners, including breast, oral, colon, liver cancers and melanoma (D'Souza, Agarwal, and Morin 2005, Koizumi et al. 2008, Leotlela et al. 2007, Lippoldt et al. 2000). Specifically, in melanomas, protein kinase activity increases both the transcription and phosphorylation of claudin 1 (French et al. 2009). In this later study, mutations of specific PKA/PKC phosphorylation sites resulting in the constitutive phosphorylation of claudin 1 led to its retention in the cytoplasm (French et al. 2009). Furthermore, in hepatoma cells, a decrease in PKA resulted in subcellular localization of claudin 1 to the cytoplasm (Farquhar et al. 2008). In addition, mislocalization of claudin 1 protein regulated by protein kinases was observed to

be associated with enhanced metastatic capacity in oral and colon cancers (Dhawan et al. 2005, Oku et al. 2006).

II RATIONALE AND HYPOTHESIS

RATIONALE

Claudin 1, a major tight junction protein, constitutes the backbone of the tight junction in epithelial cells, including the epithelial cells in the mammary gland (Figure 2) (Blanchard et al. 2006, Furuse 1998). Claudin 1 protein was frequently down regulated in human invasive breast cancers and this down regulation has been associated with poor prognosis (Kramer et al. 2000, Morohashi et al. 2007, Swisshelm et al. 1999, Tokés et al. 2005). Therefore, claudin 1 is suggested to be a tumor suppressor.

However, breast cancer is a heterogeneous disease with several different molecular subtypes (Table 1) (Perou, Sorlie, et al. 2000, Prat et al. 2010). Recently, our laboratory has shown that in some basal-like breast cancers, claudin 1 expression is significantly up regulated and mislocalized to the cytoplasm (Blanchard, Skliris, et al. 2009). Moreover, mislocalization of claudin 1 has been recently shown to enhance the metastatic potential in several other cancers, including melanoma, colon and liver cancers, and thus, may also promote metastasis in breast cancer (Dhawan et al. 2005, French et al. 2009, Leotlela et al. 2007, Oku et al. 2006).

As well, the C-terminus of the claudin 1 protein has been shown to direct its membrane localization in normal epithelial cells (Rüffer and Gerke 2004), and protein kinase activity (PKA/PKC) was important in regulating claudin 1 expression and localization in several cancers (French et al. 2009, Rüffer and Gerke 2004). Therefore, it is possible that phosphorylation of the PKA/PKC sites within the C-terminus may direct the localization of claudin 1 in breast cancer.

However, in breast cancer, the role of the C-terminus and whether phosphorylation affects localization of claudin 1 has not been addressed.

HYPOTHESIS

Mislocalization of claudin 1 in human breast cancer cells is regulated by phosphorylation.

III RESEARCH OBJECTIVES

Objective 1: To establish the endogenous level and localization of claudin 1 in human breast cancer cell lines.

The endogenous claudin 1 expression levels were analyzed by western blot analysis. Immunofluorescence and subcellular fractionation strategies were used to analyze the localization of endogenous claudin 1 in cells.

Objective 2: To determine whether the C-terminal of claudin 1 protein is required for its localization to the membrane.

GFP-tagged claudin 1 mutant constructs lacking the entire C-terminal domain (Figure 7) were generated in our lab and then were transfected into MCF7 (luminal-like human breast cancer cell lines with low claudin 1 expression) and T47D (luminal-like human breast cancer cell lines with high claudin 1 expression) cell lines. After the transfection, the localization of the mutant constructs and the full-length constructs were investigated by confocal microscopy.

Objective 3: To examine whether phosphorylation regulates the localization of claudin 1 in human breast cancer.

For this objective, site-directed mutagenesis was used to generate GFP-tagged claudin 1 constructs to mimic both constitutive phosphorylation and non-phosphorylation at PKC/PKA predicted target sites on claudin 1 protein C-terminus. I have then transfected these mutants into MCF7 breast cancer cells and investigate claudin 1 localization by using confocal microscopy strategy.

IV. MATERIALS AND METHODS

1. Cell Culture

1.1 Cell Lines

The Madin-Darby Canine Kidney (MDCK) cell line is a canine kidney cell line originally isolated from the kidney tissue of an adult female cocker spaniel by S. H. Madin and N. B. Darby in 1958 (Gaush, Hard, and Smith 1966). The MDCK cell line is commonly used as a general model for studying protein trafficking, polarity and junctions (tight, adherens, desmosome and gap) in epithelial cells (Dukes et al. 2011). MDCKII cells were obtained from higher passage MDCK epithelial cells. The MDCKII cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

The $MCF10A_1$ cell line is an immortal human mammary epithelial cell line originally isolated from the mammary gland of a 36-year-old female (Soule et al. 1990). This cell line was received from the laboratory of Dr. Leigh Murphy (Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Canada).

The T47D cell line is a human breast cancer cell line originally isolated from a ductal carcinoma of a 54-year-old female (Keydar et al. 1979). This cell line was ordered from ATCC.

The MCF-7 cell line is a human breast cancer cell line originally derived from a pleural effusion of a patient with adenocarcinoma (Soule et al. 1973). MCF-7 cell line was gifted from Dr. Robert Shiu (Department of Physiology and Pathophysology, University of Manitoba, Winnipeg, MB, Canada).

The BT-20 cell line is a human breast cancer cell line originally isolated from a carcinoma dissected from a 75-year-old female (Lasfargues and Ozzello 1958). The BT-20 cell line was obtained from the ATCC.

The MDA-MB-231 cell line is a human breast cancer cell line originally isolated from an adenocarcinoma obtained from a 51-year-old female (Cailleau et al. 1974). The MDA-MB-231 cell line was purchased from ATCC.

1.2 Culture Conditions

The MDCKII, T47D, MCF-7 and MDA-MB231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone Laboratories Inc., Logan UT, USA) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2mM glutamine (all from Hyclone Laboratories Inc.) and 10μg/ml insulin (Sigma-Aldrich Co., St. Louis, MO, USA).

BT-20 cells were cultured in Eagle's Minimum Essential Medium (EMEM, Hyclone Laboratories Inc.) supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/ml streptomycin, and 1mM pyruvate.

$MCF10A_1$ cells were cultured in DMEM with the addition of 5% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM glutamine and 10 μg/ml insulin. Additionally, the DMEM was supplemented with 1μM hydrocortisone, 0.02 μg/ml human epidermal growth factor (hEGF) and 0.1 μg/ml choleratoxin (all from Sigma-Aldrich Co.).

All cells were grown in 75 cm^2 polystyrene culture flasks (Thermo Fisher Scientific, NY, USA) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced every two days.

1.3 Propagation/ Freezing of Cell Lines

For routine cell passage, the culture flask containing the cells was moved to a biosafety cabinet and the culture medium was aspirated. The cells were gently rinsed with 3 ml of trypsin (0.05%, 1 X; Hyclone Laboratories Inc.) and then aspirated immediately. Two ml of fresh trypsin was added and the flask was returned to the incubator for 2-5minutes. After cells were detached from the flask, 8 ml of complete medium was added. The cell suspension was then pipetted 5-8 times to completely disperse cells. Two ml of cell suspension was added to a new culture flask with 10 ml fresh complete medium to continue growth of the cell culture.

For the storage of cells, the remaining 8 ml of cell suspension was transferred into a 15

ml conical centrifuge tube (Thermo Fisher Scientific) and the cell suspension was centrifuged at 700 RPM (IEC Centra-GP8R, International Equipment Company, Needham heights, MA, USA) for 5 minutes at room temperature. The supernatant was aspirated and the cell pellet was re-suspended in the freezing medium (10% DMSO, 20% FBS in complete medium). The cell suspension was aliquoted into cryogenic vials (Nalge Company, Rochester, NY, USA) and transferred to liquid nitrogen after storage at -80°C for 2-3 days.

1.4 Cell Counting

To determine cell numbers, cells were detached from the culture flasks (as described in Section 1.3) and transferred to a 50 ml conical centrifuge tube (Thermo Fisher Scientific) and then passaged 5 times through a 18G x 1 1/2 needle (Becton, Dickinson and Company, New Jersey, USA) to disperse to a single cell suspension. Ten µl of the cell suspension was loaded into each chamber of a counting slide, the slide was inserted into a TC20 cell counter (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the cell numbers were determined by the cell counter. The total cell numbers per ml were recorded for both chambers of the counting slide and were averaged.

2. Detection of Claudin 1 Protein by Western Blot Analysis

2.1 Protein Extraction

After culturing cells in a 6-well plate to 90% confluency, the cells were lysed in 200 µl of sodium-dodecyl-sulfate-isolation-buffer (SDS-isolation Buffer: 50 mM Tris-HCl, pH 6.8, 5% SDS, 20 mM EDTA, 5mM β-glycerophosphate) in the presence of a complete mini Protease Inhibitor Cocktail, containing serine, cysteine, metalloproteases and calpains (1 Complete Mini tablet/ 10 ml of extraction solution, Roche Diagnostics, Laval, QC, Canada). The lysed cells were then sonicated two times for 15 seconds in ice-cold SDS-isolation buffer using a 550 Sonic Dismembrator (Thermo Fisher Scientific). Following sonication, samples were centrifuged at 13,000 g for 5 minutes at 4°C. The supernatants were collected and stored

at -20°C.

2.2 Determination of Protein Concentration

The protein concentrations of the cell lysate were determined by using a mini BCA (Bicinchoninic Acid) Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions. Briefly, BSA (bovine serum albumin) stock, provided in the kit, was diluted to 40 µg/ml in double distilled H_2O (ddH_2O) and then diluted to obtain protein concentration standards: 1, 2, 5, 8, 10, 15 and 20 µg/ml (See Table 9). Protein samples were diluted 1:500 in ddH_2O in duplicates. To each sample, 500 µl of the mix solution containing 0.25ml of solution A, 0.24ml of solution B and 0.01ml of solution C (solutions are provided in the kit) were added. The tubes were gently vortexed and incubated at 60 °C for 1 hour, along with the standards. Following the incubation, samples were cooled to room temperature, and then 200 µl of each reaction was transferred to a 96-well plate. The absorbance was measured at a wavelength of 562 nm and a standard curve was plotted using the Softmax Pro 4.8 software (Molecular Devices LLC., Sunnyvale, CA, USA). The slope (m) and y-intercept (b) was obtained and used to calculate the unknown concentration of the protein samples according to the following equation with a dilution factor of 1000.

Equation 1:

[(Optical Density-b)/m] X Dilution Factor = Protein (µg/ml)

2.3 Electrophoresis and Western Blot Analysis

For each sample, equal amounts of protein (50 µg of total protein) were mixed 3:1 with 4X SDS buffer [500 mM Tris, pH 6.8, 40% glycerol, 8% SDS, 0.04% (w/v) bromophenol blue and 0.4M dithiothreitol (DTT)]. Samples were boiled for 5 minutes at 100°C using a

PTC-100™ Programmable Thermal Controller (Bio-Rad Laboratories, Inc.). SDS-PAGE (PolyAcrylamide Gel Electrophoresis) was performed using the Bio-Rad Protean II system (Bio-Rad Laboratories, Inc) and protein samples were electrophoresed in a 4% polyacrylamide stacking gel and 16.5% polyacrylamide separating gel. Ten µl of the Precision Plus Protein ™ Dual Color Standards (Bio-Rad Laboratories, Inc.) was used as a molecular weight marker. Protein electrophoresis was performed with the 1 X Tricine-SDS-PAGE cathode buffer system (5 X Running Buffer=500 mM Tris, 500 mM Tricine, 0.5% SDS, pH8.25) and 1 X Tricine-SDS-PAGE anode buffer system (5 X Running Buffer=1 M Tris pH8.9). Protein was transferred from the gel onto a 0.2 µm nitrocellulose membrane (Bio-Rad Laboratories Inc.) in the transfer buffer [1 X transfer buffer =200 ml 10 X Tris/Glycine buffer (Bio-Rad Laboratories, Inc.), 400 ml methanol and 1400 ml ddH_2O] using Bio-Rad's Transblot Electrophoretic transfer cell at 100 V for 1 hour. After the transfer, the membrane was placed between two pieces of the Whatman paper and baked at 60°C for 15 minutes, followed by rinsing with 1X Tris-buffered saline with 0.05% Tween-20 (1X TBST= 20 mM Tris, 137 mM NaCl pH 8.25 and 0.05% Tween 20). The membrane was then used for immunoblotting.

Following 1 hour incubation in 1X TBST containing 5% non-fat milk powder, the membrane was then incubated overnight at 4°C in the rabbit polyclonal antibody against the C-terminal tail of claudin 1 protein (Life Technologies Inc.; dilution 1:500 in 5% Milk/TBST solution) with gentle shaking on a rocker (Hoefer Scientific Instruments, San Francisco, CA, USA). Subsequently, the membranes were washed with 1X TBST (three times for 10 minutes) and incubated with a goat anti-rabbit horseradish peroxidase conjugated IgG (Bio-Rad Laboratories Inc.; dilution 1:10000 in 5% Milk/TBST solution) for 1 hour at room temperature. Following washing in 1X TBST (three times for 10 minute), chemiluminesence detection was carried out using the Supersignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The membrane was then exposed to CL-X Posure ™ Film (Thermo Fisher Scientific).

Following the development, the membrane was then washed in 1X TBST (three times

for 10 minute), a mouse monoclonal antibody (Abcam Inc., Toronto, ON, Canada) against β-actin protein was also used on the membrane as a loading control in a dilution of 1:5000 in 5% Milk/TBST solution and incubated for 1 hour in the room temperature. The membrane was washed with 1X TBST (three times for 10 minutes) and incubated in a goat anti-mouse horseradish peroxidase conjugated IgG (1:5000 dilution; Bio-Rad Laboratories Inc.) for 1 hour at room temperature. Washing and detection steps were the same as for the claudin 1 antibody.

3. Immunofluorescence Microscopy

Glass coverslips (Thermo Fisher Scientific) were rinsed in 70% ethanol and placed in a 6-well plate. The ethanol was allowed to evaporate and then the slides were washed 3 times with 2 ml of 1 X Phosphate Buffer Saline (PBS; HyClone Laboratories Inc.). For each cell line, cells were counted (See Section 1.4) and the appropriate number of cells (Table 11) was plated and cultured for 48 hours on the glass coverslips. Coverslips were then rinsed twice with 1 X PBS and cells were fixed with 2 ml of 100% methanol (Thermo Fisher Scientific) for 10 min at -20°C. Fixed cells were then rinsed 3 times with 1 X PBS prior to the permeabilization with 0.2% Tween-20 in 1 X PBS for 5 minutes at room temperature. Following three 10 minutes washes with 1 X PBS, non-specific binding sites were blocked with 1% BSA diluted in 1 X PBS for 1 hour at room temperature. Cells were then incubated with the mouse polyclonal antibody specific for the cytoplasmic domain of E-Cadherin (Life Technologies Inc., dilution 1:50 in 1% BSA/PBS) for 2 hours at room temperature in a humid container. Following 3 times 10 minute washes in 1 X PBS with gentle shaking, cells were incubated in the rabbit polyclonal antibody against the C-terminal tail of claudin 1 protein (Life Technologies, Inc., dilution 1:50 in 1% BSA/PBS) overnight at 4°C in a humid chamber. The cells were washed three times for 10 minute with PBS and incubated with secondary antibodies, AlexaFluor® 488 goat anti-rabbit IgG (H+L) and with AlexaFluor® 555 goat anti-mouse IgG (H+L), (dilution 1:1000 each in 1% BSA/PBS; Life Technologies

Inc.) for one hour at room temperature, followed by three 10 minute washes with 1 X PBS. Cell nuclei were stained with 1µg/ml 4′, 6-diamidino-2-phenylindole-dihydrochloride (DAPI; gifted from the laboratory of Dr. Etienne Leygue, Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Canada) for 1 minute in the dark and coverslips were mounted on microscopy slides with FluorSave™Reagent (Calbiochem, La Jolla, CA, USA). Fluorescent images were captured and visualized with a Zeiss AxioObserver Z1 microscope (The Genomic Center for Cancer Research and Diagnosis, Manitoba Institute of Cell Biology, CancerCare, Winnipeg, MB, Canada) using AxioVision Rel. 4.8 software (Carl Zeiss, Jena, Germany).

4. Subcellular Fractionation

Cells were grown to 90% confluency in a 60 mm dish and subcellular fractions were isolated using the ProteoExtract® Subcellular Proteome Extraction Kit (S-PEK, Calbiochem, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, the growth medium in the dish was aspirated carefully without disturbing the cell monolayer. Following two 5 minute washes using the Washing Buffer (provided in the kit) at 4°C, 1 ml of ice-cold Extraction Buffer I containing 5 µl of protease inhibitor cocktail was added to the dish, followed by incubating at 4°C for 10 minutes with gentle agitation. The supernatant was then transferred to a clean tube without disturbing the cell monolayer, labeled as cytoplasmic fraction and stored on ice. One ml of ice-cold Extraction Buffer II containing 5 µl of protease inhibitor cocktail was then added into the dish prior to incubating at 4°C for 30 minutes with gentle agitation. The supernatant was then transferred to a clean tube, labeled as the membrane fraction, and stored on ice. Five hundred µl of ice-cold Extraction Buffer III containing 5 µl of protease inhibitor cocktail and 1.5 µl of Benzonase nuclease was mixed thoroughly and added to the dish. After the incubation at 4°C for 10 minutes with gentle agitation, the supernatant was transferred to a clean tube, labeled as the nuclear fraction, and stored on ice. A solution of 500 µl ice-cold Extraction Buffer III and 5 µl of protease inhibitor

cocktail was added to the dish by rotating the dish was rotated until the remaining cell structure was detached. The extract was then transferred to a clean tube, labeled as cytoskeleton fraction and stored on ice. The four subcellular fractions were then stored at -20°C.

The mini BCA assay (See Section 2.2) was used to determine the protein concentration of each fraction, prior to equal loading (MDCKII, MCF $10A_{1,}$ T47D, MCF-7 and BT-20 fractions: 20µg per fraction; MDA-MB231: 40µg per fraction) in a 15% SDS-polyacrylamide electrophoresis gel (Table 11), prior to Western Blotting analysis as described in Section 2.3. After the detection of the claudin 1 protein, the nitrocellulose membrane was then washed with 1X TBST (three times for 10 minutes) and incubated in a mouse polyclonal anti-E-Cadherin antibody (dilution 1:500 in 5% Milk/TBST solution) overnight at 4°C. The membrane was washed with 1X TBST (three times for 10 minutes) and incubated with a goat anti-mouse horseradish peroxidase conjugated IgG (dilution 1:5000) for 1 hour at room temperature prior to the chemiluminescence (See Section 4). Detection of E-Cadherin protein was used as a control for the membrane fraction.

The steroid receptor RNA activator protein (SRAP) has been demonstrated to localize in the cytoplasm of human cancer cells; therefore it was used as a control for the cytoplasmic fraction (Yan et al 2009). In order to blot for SRAP on the same Western blot membrane, stripping of the nitrocellulose membrane was performed to remove the primary and secondary antibodies of claudin 1 and E-Cadherin. The membrane was immersed in 10 ml of stripping solution (Thermo Fisher Scientific) at room temperature for 15 minutes, followed by three 10 minutes washes with 1 X TBST. The membrane was then re-blocked in 5% non-fat milk for 1 hour at room temperature and incubated with rabbit polyclonal anti-SRAP antibody 743A (dilution 1: 1000; Bethyl Laboratories, Montgomery, TX, USA). The following steps are as described in Section 3.

It should be noted that detection was carried out using Immobilon^{TM} Western Chemilumiescent HRP substrate (Millipore Corporation, Billerica, MA, USA) to obtain a

stronger signal for the claudin 1 protein for MCF-7 cells because MCF-7 cells contain very low level of the endogenous claudin 1 protein.

5. Generation of Claudin 1 Full Length and C-terminus Truncated Constructs

5.1 Plasmids

The GFP-Claudin 1 plasmid was obtained from Dr. Claas Ruffer's laboratory (Institute of Medical Biochemistry, Center for Molecular Biology of Inflammation, University of Munster, Munster, Germany). The full-length human wild type coding sequence of claudin 1 (GenBank Accessing Number: NM-021101.4) was cloned in frame with an N-terminally fused GFP-sequence into the pEGFP-C1 vector (GFP-Claudin 1-full length) using XholI/HindIII restriction sites.

The C-terminally truncated claudin 1 (GFP-Claudin 1-Δ24) plasmid was generated in our laboratory by Mr. Xiuli Ma (Figure 6), and the correct sequence was verified by DNA sequencing (Manitoba Institute of Cell Biology, CancerCare, Winnipeg, MB, Canada).

The pEGFP-C1 control vector was gifted from the laboratory of Dr. Xiaojian Yao (Department of Microbiology, University of Manitoba, Winnipeg, Canada).

5.2 Transformation

Two µl of plasmids DNA was added gently into a microcentrifuge tube containing 33 µl of *Escherichia coli (E. coli)* DH5α bacteria (Life Technologies Inc.) and incubated on ice for 20 minutes. After the incubation, the competent cells were heat shocked at 42 °C for 45 seconds and immediately placed on ice for 2 minutes. Two hundred µl of Luria-Bertani (LB; 1% Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl; Difco Laboratories, Detroit, MI, USA) broth was then added to the cells and the tubes were incubated in a shaking incubator (225 RPM) at 37°C for 45 minutes. The transformation culture (50µl and 100µl) was plated on LB agar plates containing 50 µg/ml kanamycin (Life Technologies Inc.) and incubated overnight

at 37°C. Bacterial colonies were picked for preparing plasmids DNA and glycerol stocks were prepared and stored at -80°C.

5.3 Plasmid Preparation

Plasmid DNA was isolated from the bacteria using the QIAquick Gel Extraction Kit (Qiagen Company, Toronto, ON, Canada), according to the manufacturer's instructions. Briefly, a bacterial culture was grown by placing a loopful of the glycerol stock into 5 ml of LB Broth containing 50 μg/ml kanamycin. The cultures were incubated in a shaking incubator (225 RPM) at 37°C overnight. The following day, 5 ml of the overnight culture was gently mixed and 1.8 ml was transferred to a 2 ml polypropylene flat top microcentrifuge tube (Thermo Fisher Scientific) and centrifuged at 8000 rpm for 3 minutes at room temperature. The supernatant was removed and another 1.5 ml of the overnight culture was then added to the tubes containing the bacterial pellet and centrifuged at 8000 rpm for 3 minute at room temperature. The supernatant was aspirated and the pelleted bacteria were re-suspended in 250 μl of Buffer P1 containing RNase A (provided in the QIAquick Gel Extraction Kit). Buffer P2 containing LyseBlue reagent (250 μl/tube) was then added into the suspended bacteria pellet and the tubes were mixed thoroughly by inverting 5~8 times with the observation that the cell suspension turned blue, meaning that the cell suspension is mixed thoroughly. Buffer N3 (350 μl/tube) was added and immediately the tubes were inverted 8~10 times until the cell suspension became colorless. After centrifuging for 10 minutes at 13,000 RMP, the supernatant was transferred into a fresh QIAprep spin column and centrifuged for 1 minute at 13,000 RMP. The flow-through was removed and the columns were washed with Buffer PB (500 μl/tube) and centrifuged for 1 minute. Buffer PE (750 μl/tube) was then added into the columns, followed by centrifuging 1 minute at 13,000 RMP. After aspirating the flow-through, an additional centrifuge step (13,000 RMP for 1 minute) was carried out to remove residual wash buffer. The final plasmid samples were eluted with 50 μl of elution buffer. The concentration of isolated plasmid was determined by spectrophotometric analysis using an ultraviolet wavelength of 260 nm. DNA concentration was determined by the following equation:

Equation 2:

$$\text{DNA } (\mu g/\mu l) = (\text{Optical Density})(50)(\text{Dilution Factor})/1000$$

5.4 Digestion

The extracted GFP-Claudin 1-full length plasmid was digested by the restriction enzymes: EcoRI (10U/µl), XholI (20U/µl), HindIII (15U/µl) and XholI (All from Life Technologies Inc.). Digestion was performed overnight at 37°C in a 20 µl reaction volume containing 1 µl of enzyme, 2 µl of 10 X REACT 2 (50mM Tris-HCl ph8.0, 10 mM $MgCl_2$, 50 mM NaCl; Life Technologies Inc.) buffer and the GFP-Claudin 1 plasmids. The sample was loaded into a 1% (w/v) agarose gel containing 1 X TBE (Tris-Borate-EDTA; 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA; pH 8.0) and run in 1 X TBE buffer. The products were electrophoresed at 150V for 45 minutes or until the bands were well separated.

6. Transfection

6.1 Transient Transfection of Breast Cancer Cells with Claudin 1 Constructs

MCF-7 and T47D cells were counted (See Section 1.4) and the appropriate number of cells (Table 11) was cultivated on glass coverslips in 12-well plate for 48 hours or until the cells reached 70-80% confluency for transfection. The DMEM culture medium was removed and fresh medium without antibiotics was added gently to the wells 1 hour prior to the transfection.

One µg of plasmid DNA was diluted in 100 µl of 1 X Opti-MEM I Reduced Serum Medium (Life Technologies Inc.) and was gently mixed. Four µl of Lipofectamine 2000 was then diluted in 100 µl of Opti-MEM Medium and mixed gently. After 5 minutes of

incubation at room temperature, 100 µl of the mixture containing Lipofectamine2000 (Invitrogen Corporation) was added into the 100 µl of diluted plasmid DNA mixture (total volume 200 µl) and mixed gently by pipetting up and down 5~8 times. The DNA-Lipofectamine2000 complex was then incubated at room temperature for 20 minutes, prior to adding to the cells. The solution was then mixed gently by rocking the plate back and forth 5~8 times. The plate was then returned to the incubator. The medium was replaced with fresh complete DMEM culture medium 6 hours post-transfection as Lipofectamine2000 reagent can be harmful to cells. After 48 hours incubation, the cells were fixed with 4% paraformaldehyde (Acros, New Jersey, USA) and stored at -20°C.

6.2 Stable Transfection of Breast Cancer Cells with Claudin 1 Constructs

The MCF-7 clonal cell lines stably transfected with the GFP tagged full-length claudin 1 (GFP-Claudin 1-FL) plasmid DNA and GFP tagged claudin 1 truncated constructs (GFP-Claudin 1-Δ24) plasma DNA, used for this study, were generated in our laboratory by Mr. Xiuli Ma. Briefly, transfection was carried out as described in section 6.1 and stable clones were selected using genetion (Life Technologies Inc.).

7. Claudin 1 Protein Phosphorylation Studies

7.1 Activation of PKC

For PKC activation studies, MCF-7 cells were cultivated in a 6-well plate to 80% confluency. The PKC activator 12-O-Teradecanoylphorbol-13-acetate (TPA, Cell Signaling Technology, Inc., Danvers, MA, USA) was diluted in dimethyl sulfoxide (DMSO), prior to adding to the wells containing MCF-7 cells. The final concentration was 200 nM. The cells were then returned to the incubator, lysed after 1, 4 and 18 hours incubation and protein was

extracted using SDS-isolation Buffer (See Section 2). For vehicle controls, cells were treated with equivalent amounts of DMSO. After measuring the protein concentrations, 50 µg of the total protein was used for Western blot analysis (See Section 2.3).

7.2 Site-Directed Mutagenesis

7.2.1 Synthesis of Mutant Strand

Site-directed mutagenesis was performed on the claudin 1 sequence using the Quikchange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) to mutate the sites indicated in Table 12.

The polymerase chain reaction (PCR) amplification was performed in a 50 µl reaction volume containing 5 µl of 10 X reaction buffer, 50 ng of full-length GFP-Claudin 1 DNA template, 125ng of each primer (See Table 13), 1 µl of dNTP mix and ddH_2O (to bring the final reaction volume to 50 µl). One µl of *PfuUltra* HF DNA polymerase (2.5 U/µl) was then added into the PCR reaction system. The PCR cycling system was performed in GeneAmp PCR System 9700 (Life Technologies Inc.) as indicated in Table 14.

A control reaction was also carried out in a 50 µl of reaction volume containing 5 µl of 10 X reaction buffer, 2 µl (10 ng) of *pWhitescript* 4.5-kb control plasmid, 1.25 µl of primer$^{\#}$1, 1.25 µl of primer$^{\#}$2, 1 µl of dNTP mix and 38.5 µl of ddH_2O to bring a final reaction volume of 50 µl. One µl of *PfuUltra* HF DNA polymerase (2.5 U/µl) was then added into the PCR reaction system. The PCR cycling system was performed as indicated in Table 14.

Following cycling, the reaction was placed on ice for 2 minutes to cool to 37°C.

7.2.2 *Dpn* I Digestion of Amplification Products

The PCR amplification products were then digested by *Dpn I* restriction enzyme. Two ul of Dpn I restriction enzyme was added directly to each control and sample amplification and the reaction mixture was mixed thoroughly by pipetting the solution up and down for 5~8 times. The reaction mixture was then incubated at 37°C for 5 minutes to digest the parental

supercoiled double strand (ds) DNA.

7.2.3 Transformation of XL1-Blue Supercompetent Cells

After digestion, 2 μl of *Dpn* I-treated DNA from each control and sample reaction were added to 45 μl of the XL10-Gold ultracompetent cell suspension, along with 2ul β-ME. The transformation reactions were swirled gently to mix and then incubated on ice for 30 minutes, prior to heating at 42°C for 40 seconds, followed by incubating on ice for another 2 minutes. The transformation reactions were then added into 0.5 ml pre-warmed LB broth (37°C) and then incubated at 37°C for 1 hour with shaking at 220 RMP. The transformation culture (100 μl) was placed on a 10 cm LB agar plate containing 50 μg/ml kanamycin and the plate was incubated overnight at 37°C. Single bacterial colonies were picked and glycerol stocks were prepared and stored at -80°C.

For the mutagenesis and transformation controls, cells were plated on LB ampicillin agar plates containing 80 μg/ml X-gal (also as abbreviated BCIG for 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) and 20 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and incubated overnight at 37°C. The colonies containing the mutation will be appeared as blue on agar plates containing IPTG and X-gal. The mutagenesis efficiency (ME) for the *pWhitescript* 4.5-kb control plasmid is calculated by the following equation:

Equation 3:

ME= (Number of blue colony forming units) X 100% / (Total number of colony forming units)

7.2.4 Nucleotide Sequence Analysis

Three clones were randomly chosen and verified by sequencing (Manitoba Institute of Cell Biology, CancerCare, Winnipeg, MB, Canada). The results were compared with the nucleotide sequence of the claudin 1 gene (Genbank accessing number: NM-021101.4) by using CLUSTAL W2 multiple sequence alignment online tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

V. RESULTS

1. Endogenous Claudin 1 Protein Levels in Different Human Breast Cancer Cell Lines

Claudin 1 protein level was frequently observed to be down regulated in human invasive breast cancer and this down regulation has been associated with poor prognosis. Claudin 1 is therefore suggested to be a tumor suppressor. However, breast cancer is a heterogeneous disease with several different molecular subtypes (Table 1). Recently, our laboratory has made a novel observation that in a particularly aggressive subtype, the basal-like subtype, claudin 1 expression is significantly up regulated (Blanchard, Skliris, et al. 2009, Swisshelm et al. 1999). Similarly, breast cancer cell lines also exhibit different phenotypes (Table 8). Therefore, I wanted to establish the levels of claudin 1 in different breast cancer cell lines in order to select specific cell lines that could be used to overexpress and inhibit claudin 1 protein.

Using Western blot analysis, an approximate 21kDa protein was detected by anti-claudin 1 antibody in a panel of human breast cancer cell lines, which corresponded to the size of claudin 1 protein (Figure 3). β-actin protein was used to evaluate the sample loading on the gel. The $MCF10A_1$, an immortal human mammary gland epithelial cell line, contained high level of endogenous claudin 1 and was used as a positive control.

Western blotting showed that the expression level of the endogenous claudin 1 protein was cell line specific. Both low and high endogenous claudin 1 protein levels were observed in the luminal-like as well as the basal-like subtypes of human breast cancer cell lines. T47D and MCF-7 are all luminal-like subtypes, claudin 1 was highly expressed in the T47D cells, but exhibited a low expression level in MCF-7 cells. A similar disparity with the level of endogenous claudin 1 protein was also observed in the basal-like subtype cell lines, BT20 and MDA-MB-231. BT20 cells contained high levels of claudin 1 protein, whereas MDA-MA-231 showed low levels of claudin 1 protein.

In summary, the results have demonstrated that the breast cancer cell lines T47D and BT-20 expressed high endogenous claudin 1 level, whereas low endogenous claudin 1 level

was observed in the breast cancer cell lines MCF-7 and MDA-MB-231. Thus, MCF-7 and MDA-MB-231cells were selected for overexpressing claudin 1 in the following studies.

2. Determination of Subcellular Localization of Claudin 1 Protein in Human Breast Cancer Cell Lines by Immunofluorescence and Subcellular Fractionation

I first wanted to determine whether endogenous claudin 1 resided in any other cell compartments aside from the cell membrane and also wanted to determine whether this pattern of protein expression was different from one subtype to the other subtype and whether localization was associated with protein levels. Thus, both IF and subcellular fractionation strategies were carried out to identify the subcellular localization of claudin 1 in different breast cancer cell lines.

2.1 Immunofluorescence Analysis of Breast Cancer Cell Lines

MCDKII (a canine kidney epithelial cell line) cells, which have been widely used to study tight junction proteins including claudins in the literature, were used as a positive control for claudin 1 membrane localization (Dukes J 2011, Inai T 2009, Zheng W 2005, Furuse M 2001, Tokuda S 2015). $MCF10A_1$, reflection of normal mammary gland epithelial cells, was also used as control in the present study to determine the subcellular localization of claudin 1. Furthermore, E-Cadherin protein, a transmembrane protein localized in adherens junction of epithelial cells, was selected as a positive control for the co-localization with claudin 1 protein in the cell membrane (Schneeberger EE, 2004).

The immunofluorescence results revealed that the endogenous claudin 1 protein was primarily localized in the cell membrane in both cell lines, MCDKII and $MCF10A_1$, and the subcellular co-localization of claudin 1 and E-Cadherin was also observed (Figure 4). In $MCF10A_1$ cells, cytoplasmic localization of claudin 1 has been observed by

immunofluorescence, verified by subcellular fractionation (Figure 5).

Using IF and confocal microscopy strategies (See Section 3 in the Material and Methods), subcellular localization of claudin 1 was again observed to be cell line specific (Figure 4). In the two luminal-like subtype human breast cancer cell lines, T47D and MCF-7, claudin 1 protein was observed primarily in the cell membrane, and co-localization of claudin 1 and E-Cadherin was observed as well. However, claudin 1 protein was identified to be present in the cytoplasm of BT20 and MDA-MB231 cells, which are both basal-like subtype human breast cancer cell lines. Interestingly, claudin 1 expression was also observed in the cell nucleus in BT20 cells (Figure 4).

In summary, claudin 1 protein primarily localized in the cell membrane in the cell lines representing luminal-like subtype (T47D and MCF-7), whereas the protein were mainly in the cytoplasm and nucleus in the cell lines representing the basal-like subtype (BT-20 and MDA-MB-231). Therefore, it appears to be a difference in claudin 1 localization among the different subtypes. With regards to the level of claudin 1 had any effect on its localization, I observed some differences. Both T47D and BT-20 which express high level of endogenous claudin 1, the protein was mainly in the cell membrane in T47D cells but primarily in the cytoplasm and nucleus in BT-20 cells. Similarly, in both low endogenous claudin 1 expressing cell lines MCF-7 and MDA-MB-231, claudin 1 mainly localized in the cell membrane of MCF-7 cells but was observed primarily in cytoplasm in MDA-MB-231. Therefore, the results did not show a difference between the localization of claudin 1 in cells that express high endogenous claudin 1 and low endogenous claudin 1.

2.2 Detection of the Subcellular Localization of Claudin 1 Protein in Human Breast Cancer Cell Lines by Subcellular Fractionation

In addition to the immunofluorescence strategy, I also employed a subcellular fractionation assay as a second strategy (See Section 4 in the Material and Methods) to determine whether the endogenous claudin 1 protein within human breast cancer cells. The

cells were subjected to a subcellular fractionation assay, followed by Western blotting using claudin 1 specific antibody. The 21kDa claudin 1 protein was observed in all cell lines (Figure 5). E-Cadherin and SRAP (steroid receptor RNA activator protein) were used as membrane and cytosolic marker proteins, respectively. However, the expression of E-Cadherin protein was not detected in MDA-MB-231 cells (Figure 5, middle panel), which has also been shown previous by other laboratories (Lombaerts et al. 2006, Mbalaviele et al. 1999). Not surprisingly, the expression of SRAP was not detected in MDCKII cells as the antibody reacts with human but MDCKII is a dog kidney cell line. Furthermore, it was only weakly detected in $MCF10A_1$ cells (Figure 5, lower panel) as it has previously been shown that the expression level of SRAP appeared to be significant stronger in breast cancer cells than in the normal epithelial cells (Yan et al. 2009). The subcellular fractionation results also revealed that the membrane marker E-Cadherin protein is only in the membrane fraction. Similarly, the cytoplasmic marker SRAP was only detected in the cytoplasm fraction. These studies demonstrated that the subcellular fractionation was performed without any suggestion of cross-contamination between the subcellular fractionations (Figure 5).

Overall, claudin 1 protein expression was detected in both cytosol and membrane fractions in all other cell lines except for MDCKII which was detected only in the membrane fraction (Figure 5). In the control cell lines MDCKII and $MCF10A_1$, as well as the two luminal-like subtypes T47D and MCF-7, claudin 1 was primarily localized in the membrane fraction (Figure 5), which is consistent with the immunofluorescence result (Figure 4). In both basal-like subtypes BT-20 and MDA-MB-231, claudin 1 expression was also detected primarily in the membrane fraction with a small amount in the cytosolic fraction (Figure 5), which is contrary to the immunofluorescence results, which showed claudin 1 mainly localized in the cytoplasm (Figure 4). One explanation for this is that this could be attributed to the fact that besides the plasma membrane, the membrane fraction extracted by the subcellular fractionation kit also contained membranes from other organelles inside the cells, thus during subcellular fractionation, the membrane fraction may include not only the cell membrane but the membrane from within the cells as well.

In summary, based on the analysis of the subcellular localization of claudin 1 in the four breast cancer cell lines, using subcellular fractionation strategy, there appears to be no difference in the localization of claudin 1 between the luminal-like (T47D and MCF-7) and the basal-like subtypes (BT-20 and MDA-MB-231). One explanation could be attributed to the fact that although MCF-7 and T47D are both luminal subtype, as well as BT-20 and MDA-MB-231 are both basal-like subtype, there might be other molecular characteristics of each cell line which influence claudin 1 expression. As well, based on these results from subcellular fractionation strategy, it appears that there was no difference in claudin 1 localization between high endogenous claudin 1 expressing cell lines (T47D and BT-20) and low endogenous claudin 1 expressing cell lines (MCF-7 and MDA-MB-231).

3. The C-terminus of Claudin 1 Protein Plays a Role in its Membrane Localization in Human Breast Cancer Cells

3.1 Verification of GFP-tagged Claudin 1 Mutant Constructs

The C-terminus of the claudin 1 protein has been shown to be essential for its membrane localization in a canine epithelial cell line, MDCK-C11 (Ruffer and Gerke 2004). To determine whether the C-terminus is also necessary for claudin 1 protein to localize to the membrane in human breast cancer cells as well, GFP-tagged claudin 1 mutant constructs lacking the 24 amino acid of C-terminus (GFP-claudin 1-Δ24) were generated in our laboratory by our technician Mr. Xiuli Ma (Section 8.3 in the Material and Methods and Figure 6), along with the full-length GFP-tagged claudin 1 constructs (GFP-claudin 1-FL). It had been previously reported that C-terminal epitope tag could interfere with certain intracellular protein interactions (Ruffer and Gerke 2004); thus, in this study, the N-terminally tagged GFP-claudin 1 mutant constructs were used. To confirm that the claudin 1 coding sequence was successfully inserted into the pEGFP-C1 vector (Figure 2), restriction enzymes were used to digest the GFP-claudin 1 plasmids. Following digestion with HindIII

and Xho1, an approximately 700bp fragment was observed, which corresponded with the size of claudin 1 insert (Figure 7). A much higher band, was also observed in the same lane, which approximated the expected size of the pEGFP-C1 vector (4.7kb). Additionally, the GFP-claudin 1 constructs with and without the deletion of C-terminal tail were confirmed by DNA sequence analysis.

3.2 Visualization of the GFP Tag in Human Breast Cancer Cells: Methanol vs Paraformaldehyde

A number of studies have compared the fluorescence of GFP after fixation with methanol or 4% paraformaldehyde (PFA) and showed the fluorescence of GFP was retained in both conditions; however, the methanol fixation lead to a loss of soluble proteins (Kalejta, Shenk, and Beavis 1997, Wang et al. 1996). In the present study, we compared methanol and paraformaldehyde prior to visualizing GFP-claudin 1 constructs (pEGFP-C1) transfected into MCF-7 human breast cancer cells. The results (Figure 8) revealed that the GFP fluorescence signal could not be detected in the cells fixed with methanol; however, the cells fixed with 4% PFA showed that the cytoplasmic and nuclear localization of the GFP tag.

3.3 Transient Transfection of GFP-tagged Claudin 1 Mutant Constructs into Human Breast Cancer Cells

My previous study showed that the endogenous claudin 1 protein is primarily localized in cell membrane of the two luminal-like subtype human breast cancer cell lines, MCF7 and T47D (See Section 2 in Results). Therefore, guided by these results, these cell lines were selected to further investigate the C-terminus in claudin 1 membrane was essential for claudin 1 cytoplasmic localization.

Staining by the GFP vector alone was first evaluated in transfected human breast cancer cell lines MCF-7 and T47D cells by fluorescent microscopy. The results revealed that 48

hours after the transfection, the GFP localized in the cytoplasm and nuclei in both cell lines (Figure 9A and Figure 10A).

Next, MCF-7 and T47D cells were transiently transfected with either the GFP-claudin 1-FL or the GFP-claudin 1-Δ24 plasmids, and the protein distribution was recorded 48 hours after the transfection. The E-Cadherin protein, a membrane bound protein which was showed to be localized in the cell membrane in both MCF-7 and T47D cells, was used as control for showing the membrane co-localization of claudin 1 constructs. When these constructs were transfected into T47D and MCF-7 cells, the full-length GFP-claudin 1 construct showed a co-localization with E-Cadherin protein in the cell membrane in both MCF-7 and T47D cell lines (Figure 9B and 10B as red arrows indicated). However, GFP-claudin 1 mutant constructs lacking the entire C-terminus resulted in less membrane but more cytoplasmic localization and only partly co-localized with E-Cadherin protein in both cell lines (Figure 9C and 10C). The results indicate that the C-terminus of claudin 1 plays a role in its membrane localization in human breast cancer cells.

4. Expression and Localization of GFP-Claudin 1 Mutant Constructs in Stably Transfected MCF-7 Cells

4.1 Identification of GFP-Claudin 1 Stably Transfected Cells

Stably transfected MCF-7 cells with the GFP-claudin 1-Δ24 plasmid as well as the stable transfected GFP tagged full-length claudin 1 (GFP-claudin 1-FL) plasmids were then generated in our laboratory. To identify positive clones, twelve clones transfected with GFP-claudin 1-FL and another twelve clones transfected with GFP-claudin 1-Δ24 were randomly chosen and analyzed by Western Blotting, which was performed in our laboratory by Mr. Xiuli Ma. Using anti-GFP antibodies, a protein approximately 48kDa in size was detected in all twenty-four clones which corresponded with the expected the size of

GFP-claudin 1 protein (Figure 11A). Unfortunately, an anti-claudin 1 antibody specific for the C-terminus was used for these studies and therefore was unable to detect the GFP-claudin 1-Δ24 protein since it lacked the C-terminal tail. It was however able to detect GFP-claudin 1-FL. Western blot results revealed that the expression of GFP-claudin 1- FL was detected in the eight out of twelve clones with different expression levels (Figure 11A). This discrepancy between the results when using the anti-GFP antibody (12 positive clones) versus the anti-claudin 1 antibody (8 positive clones) suggested the possibility that the anti-GFP antibody may be binding non-specifically to a 48kDa protein. Aside from the anti-claudin 1 antibody specific for the C-terminus and anti-GFP antibody, I have also tried an anti-claudin 1 antibody specific for its first extracellular loop; unfortunately, the antibody was insufficient and non-specific binding was observed around the size 48kDa. Therefore, the antibody was not used for further studies.

To rule out that this was the case, wild type MCF-7 cells were used as a negative control and ran the Western blot together with a FL claudin 1 positive clone (clone $^{\#}$6) transfected with GFP-claudin 1-FL plasmids, and a C-terminal deleted positive clone (clone $^{\#}$3) transfected with GFP-claudin 1-Δ24 plasmids. The results showed the 48kDa band was detected in all samples strongly indicating non-specific binding of the anti-GFP antibody (Figure 11B). However, it was observed in the same gel, a band slightly lower than the non-specific band around size 48kDa was also detected in the stably transfected MCF-7 clones (clone $^{\#}$6 and clone $^{\#}$3) but not the wild type MCF-7 cells, suggesting that the lower band could be the GFP-claudin 1 protein.

4.2 Localization of GFP-Claudin 1 Constructs in Stably Transfected MCF-7 Cells

Based on the results of two stable clonal cell lines, GFP-Claudin 1-FL-clone$^{\#}$6 and GFP-claudin 1-Δ24-clone$^{\#}$3 were identified as high expressors and selected for further studies regarding the subcellular localization of claudin 1. IF study was carried out to visualize the GFP-tag proteins. DAPI (4,6-diamidino-2-phenylindole dihydrochloride)

staining was used to detect cell nuclei, while E-Cadherin protein was used as a membrane marker. The results showed that both GFP-Claudin 1-FL and GFP-Claudin 1-Δ24 proteins primarily localized in the cell membrane and showed a co-localization with E-Cadherin (Figure 12A).

Next, to determine whether level of expression is related to the localization, two stable clones with low expression level of GFP-claudin 1-FL and GFP-claudin 1-Δ24 proteins were selected and determined the localization. The GFP-claudin 1-FL -clone$^{\#}$3 showed both membrane and cytoplasmic localization of the protein; however, the GFP-claudin 1-Δ24-clone$^{\#}$8 was primarily located in the cytoplasm (Figure 12B).

In summary, GFP-Claudin 1-FL protein has observed to localize primarily in the cell membrane in both both low and high expression clones. However, when the C-terminus is absent, GFP-Claudin 1-Δ24 protein was localized mainly in the cytoplasm in the low expression clone, whereas in the clone with high expression of GFP-claudin 1-Δ24, the protein localized primarily in the cell membrane; indicating the localization of GFP-claudin 1-Δ24 is associated with its expression level.

5. Role of Phosphorylation

5.1 Activation of PKC Up-regulated the Expression Level of Claudin 1 in Human Breast Cancer Cells

Protein kinases has been shown to be associated with the abnormal expression of claudin 1 and had an effect on its subcellular localization in cancer cells, such as colon cancer cells and melanoma cells (Dhawan et al. 2005, French et al. 2009). The PKC appear to be the critical players (D'Souza, Agarwal, and Morin 2005, Koizumi et al. 2008, Leotlela et al. 2007, Lippoldt et al. 2000). Previously, I have identified that the human breast cancer cell line MCF-7 cells expressed a low level of the endogenous claudin 1 protein (See Section 1 in

Results). Our laboratory has previously observed that TPA (12-o-tetradecanoylphorbol 13-acetate), a protein kinase C activator, increased claudin 1 protein expression in MCF-7 cells. Therefore, I then wanted to optimize this by doing a time course experiment by treating MCF-7 cells with TPA for 1, 4 and 18 hours (See Section 2.10 in Material and Methods). DMSO was used as the vehicle control. After the treatment with TPA, Western blotting analysis was carried out to detect claudin 1 protein expression and β-actin was used as the loading control. Anti-claudin 1 antibody recognized a protein of approximately 21kDa, which corresponds to the size of claudin 1 protein. From the Western blotting results (Figure 13), we observed that the activation of PKC for 1 hour and 4 hours did not affect the level of claudin 1 protein in MCF-7 cells. However, upon the treatment with the PKC activator TPA for 18 hours, the activation of PKC resulted in a significant increase in claudin 1 protein level in MCF-7 cells.

5.2 Phosphorylation of the Claudin 1 Protein Plays a Role in its Cytoplasmic Localization

It has been demonstrated that a number of putative PKA and PKC sites are located within the C-terminal tail of claudin 1, which has been shown to be important in directing claudin 1 subcellular localization in several cancer, such as colon cancer and melanoma (Butt et al. 2012).

Thus, in the present study, I wanted to identify whether specific PKA and/or PKC phosphorylation sites within the claudin 1 C-terminus were also responsible for its subcellular localization in breast cancer cells. Site-directed mutagenesis of GFP-claudin 1 plasmids was used to make mutants that either mimicked a constitutively phosphorylated state (conversion of the phosphorylation site to an aspartic acid residue, referred to as “D” mutants) or mutants that are non-phosphorylatable (conversion of the phosphorylation site to an alanine reside, referred to as “A” mutants). The mutant constructs T190 (a PKA/PKC site), S192 (a PKA site) and T195 (a PKA/PKC site) were selected to be transfected into human

breast cancer cells, based on reports that demonstrated that these sites play a role in the localization of claudin 1 in melanoma cells (French et al. 2009). Following generation of the mutant construct, DNA sequence analysis was carried out to confirm the accuracy of the site-mutagenesis procedure. These cell lines, $MCF10A_1$, T47D and MCF-7, were used in these studies because the endogenous claudin 1 protein was observed to localize predominantly in the cell membrane. As well, these cells were previously used in the subcellular localization studies.

Both membrane and cytoplasmic localization were observed in $MCF10A_1$ cells 48 hours after transfection of the GFP-claudin 1-FL constructs (Figure 14A). Furthermore, after 48 hours transfection of claudin 1 mutants, no difference of subcellular localization between the full-length claudin 1 and claudin 1 mutants S192D and T195D has been observed (Figure 14A), suggesting the mutations to aspartic acid of these sites alone did not affect the subcellular localization of claudin 1 in $MCF10A_1$ cells.

In T47D cells, following 48 hours transfection of GFP-claudin 1-FL constructs, both membrane and cytoplasmic localization were detected (Figure 14B) which is consistent with the results shown previously (Figure 10B). Similarly, the mutants S192D and T195D also showed both membrane and cytoplasmic localization after 48 hours transfection, which indicated that mutation mimicking phosphorylation of the two sites within the C-terminus did not alter claudin 1 subcellular localization in T47D cells.

The GFP-claudin 1-FL constructs, used as the positive control and transfected into the MCF-7 cells, showed both membrane and cytoplasmic localization using fluorescence microscopy (Figure 15A), which is consistence with the localization of endogenous claudin 1 in MCF-7 cells (Figure 4). Similarly, after 48 hours transfection of the mutants S192A in MCF-7 cells, the membrane localization of the mutants was also observed, along with few cytoplasmic staining, indicating that the mutation to alanine in the PKA site did not affect the subcellular localization of claudin 1 (Figure 15A). However, the results of the “D” mutation on both S192 and T195 sites revealed increased cytoplasmic staining and decreased membrane staining. There was a generally diffused pattern in the membrane staining, that

was not observed with the full-length GFP-claudin 1 (Figure 15A). In contrast, the mutant T190D localized primarily in cell membrane, suggesting the site T190 is not responsible for subcellular localization of claudin 1 in MCF-7 cells. Confocal microscopy was also carried out to investigate the precise subcellular localization of the mutants (Figure 15B). The localization of GFP-claudin 1-FL constructs, as well as the mutants S192A, S192D and T195D were consistent with the results of fluorescence microscopy (Figure 15B). In summary, mutation mimicking phosphorylation of claudin 1 at the 192 and 195 sites within the C-terminus resulted in an increased cytoplasmic staining and deceased membrane staining in MCF-7 cells; however, mutation mimicking phosphorylation on site 190 did not affect the subcellular localization of claudin 1 in MCF-7 cells.

VI. DISCUSSION

1. Claudin 1 is Differentially Expressed in Different Human Breast Cancer Cell Lines

Breast cancer is a complex and heterogeneous disease with several molecular subtypes comprising luminal-like, basal-like, Her2-positive and normal-like subtypes. In breast cancer research, established human breast cancer cell lines are widely used as experimental models (Ceriani RL, 1992; Clarke R, 1996; Clarke R, 2001, Lacroix M, 2004). These cell lines share many phenotypic characteristics with human breast tumors. In the present study, four breast cancer cell lines, T47D, MCF-7, BT-20 and MDA-MB-231 were selected, representing luminal-like and basal-like subtypes of human breast cancer. T47D and MCF-7 cells are both ER+ and PR+ with low EGFR and represent the luminal-like subtype. BT-20 and MDA-MB-231 cells are both ER- and PR- with high EGFR and represent the basal-like subtype (Table 8).

Claudins are tight junction proteins that play a central role in tight junction cellular homeostasis, being important in the maintenance of cell polarity and regulation of paracellular permeability (Mineta et al. 2011). Claudin 1, a critical member of the claudin family of tight junction proteins, forms the backbone of tight junctions (Furuse 1998, Furuse et al. 2002, Furuse et al. 1998). The expression and function of claudin 1 protein varies in different cancers. In human invasive breast cancer, claudin 1 was suggested to play a dual role and function as either a putative tumor suppressor or a putative tumor promoter as its expression is distinct within specific subtypes of breast cancer (Morohashi et al. 2007, Swisshelm, Macek, and Kubbies 2005, Swisshelm et al. 1999, Tokés et al. 2005, Kramer et al. 2000, Blanchard, Skliris, et al. 2009). Claudin 1 was frequently down regulated in most human invasive breast cancer, indicative of a tumor suppressor (Kramer F, 2000; Swisshelm K 2005; Tokes AM, 2005; Morohashi S, 2007). Recently, our laboratory and others have shown that in a particularly aggressive subtype, the basal-like subtype, claudin 1 expression is significantly up regulated, and may act as a tumor promoter (Blanchard, Skliris, et al. 2009,

Lu et al. 2013). These studies were carried out on breast cancer samples of mixed pathologies and breast cancer cells also exhibit different phenotypes.

The first aim of this study was to investigate the expression of endogenous claudin 1 protein in different molecular subtypes of human breast cancer cell lines. Western blotting revealed both high and low claudin 1 protein levels in different cell lines representing the same molecular subtype (Figure 3). T47D and MCF-7 cell lines used were representative of the luminal-like subtype. However, T47D cells expressed high levels of endogenous claudin 1 protein, whereas MCF-7 cells display low levels of endogenous claudin 1 protein. This is consistent with other studies showing that claudin 1 mRNA was highly expressed in T47D but absent or greatly reduced in most breast cancer-derived cell lines compared to the normal human mammary epithelial cells (Swisshelm, Macek, and Kubbies 2005, Swisshelm et al. 1999). Similarly, when comparing protein levels in cell lines of the basal-like subtypes, claudin 1 protein was highly expressed in the BT-20 cells compared to the MDA-MB-231 cells. Our results therefore suggests that claudin 1 protein expression did not appear to be associated with the molecular subtype of human breast cancer cells.

However, when breast tumor tissue of mixed pathologies was analyzed in a large cohort of samples (314 tumor samples), claudin 1 was shown to be down regulated in the luminal-like subtype while up regulated in some of the basal-like subtype (Blanchard, et al. 2009). Although initially all human breast cancer cell lines are derived from tumors, one of the disadvantages of using cell lines for study is that over time clonality may develop. Therefore, there might be exceptional results by using a single cell line. For example, a small population of the luminal-like subtype tumors was identified to express high level of claudin 1 protein. This is a reasonable possibility since only 2 cell lines representing the luminal-like subtype and 2 cell lines representing the basal-like subtype were analyzed.

Based on the Western blotting data that showed high level of endogenous claudin 1 protein in T47D and BT-20 cells and low level of endogenous claudin 1 in MCF-7 and MDA-MB-231 cells, these cell lines were selected for overexpressing claudin 1 in the later

studies which aimed to investigate the subcellular localization of full-length claudin 1 and truncated claudin 1 in human breast cancer cells.

2. Subcellular Localization of Claudin 1 Protein in Human Breast Cancer Cell Lines

Besides the dysregulated gene expression of claudin 1 observed in several cancers, changes in the localization of claudin 1 protein has also been reported. Aside from the cell membrane, claudin 1 protein has been identified in other subcellular compartments, such as the cytoplasm and nucleus. This is true for melanomas, colon and oral squamous cancers (Dhawan et al. 2005, French et al. 2009, Leotlela et al. 2007, Morin 2005, Oku et al. 2006). In these cancers, changes of claudin 1 localization to cytoplasm and/or nucleus were shown to increase the invasiveness of the cancer cells. In human invasive breast cancer biopsies, the mislocalization of claudin 1 in cytoplasm was reported in our laboratory using immunohistochemistry (Blanchard, Skliris, et al. 2009, Blanchard et al. 2006). Such mislocalization was previously reported by other groups and shown to contribute to the enhanced metastatic potential of tumors (Soini 2005, Tokés et al. 2005).

In the present study, the localization of endogenous claudin 1 protein was assessed in different human breast cancer cell lines using immunofluorescence and subcellular fractionation strategies. An anti-claudin 1 antibody with high specificity was used in my study, which was widely used in the literature and previously used successfully in our laboratory (Blanchard, et al. 2009).

Immunofluorescence showed membrane localization of endogenous claudin 1 in T47D cells. This is consistent with previous results shown by others (Akasaka et al. 2010). In BT-20 cells, claudin 1 was observed to be mislocalized in the cytoplasm, which was consistent with the result shown in some of the basal-like breast cancer tumors (Blanchard, Skliris, et al. 2009). However, the localization of the endogenous claudin 1 in MCF-7 cells is not clear as there are different reports from different laboratories. My study showed that claudin 1 localized predominantly in the cell membrane in MCF-7 cells using

immunofluorescence analysis (Figure 4), whereas two other laboratories have shown cytoplasmic staining of the endogenous claudin 1 protein in the MCF-7 cells (Akasaka et al. 2010, Liu et al. 2012). These differences could be attributed to a difference in clonality of the MCF-7 cell line. Many different clones of MCF-7 cells exist worldwide and throughout the scientific community, and many of these display distinct phenotypes, indicating that they have undergone some genetic changes (Vargo-Gogola and Rosen 2007). There is evidence for this. Osborne *et al* demonstrated many discrepancies in the most commonly used breast cancer cell line MCF-7, obtained from different laboratories (Osborne, Hobbs, and Trent 1987). Furthermore, using 24-colour fluorescent *in situ* hybridization, it has been shown that MCF-7 cells from different laboratories showing markedly different karyotypes (Bahia et al. 2002, Burdall et al. 2003). Thus, disparities regarding the localization of claudin 1 in MCF-7 cells may be attributed to differences in the genetic drift of these cells in individual laboratories. Moreover, I have also observed that cell confluency could influence the expression and localization of claudin 1 protein and other junctional proteins. Claudin 1 mainly localized in the cell membrane when cells reach a high confluence; however, in the low confluence of cells, claudin 1 was mainly in the cytoplasm.

Subcellular fractionation studies showed that claudin 1 protein was primarily in the membrane fraction of the two luminal-like subtype cell lines T47D and MCF-7, consistent with the IF results. MDCKII and MCF10A$_1$ cells were used as controls and demonstrated membrane staining as well, validating that these results were not attributed to technical difficulties. However, results obtained from the two basal-like subtype cell lines, BT-20 and MDA-MB-231, using the two techniques were inconsistence. Claudin 1 was found to be localized primarily in the cytoplasm using immunofluorescence analysis (Figure 4) but the subcellular fractionation result showed a high claudin 1 protein expression level in the membrane fraction (Figure 5).

There are several plausible explanations for the inconsistency of claudin 1 localization in BT-20 and MDA-MB-231 using the two techniques. One, the disadvantage of immunofluorescence is that it requires suitable antibodies that recognize claudin 1 protein

with high specificity in fixed cells, and it necessitates fixation and cell permeabilization with associated artifacts. However, in the present study, an anti-claudin 1 antibody with high specificity was used, which was widely used in the literature and previously used successfully in our laboratory (Blanchard, et al. 2009). Two, it is well recognized that during subcellular fractionation, there is still some degree of cross-contamination between fractions since the separation and isolation of each compartment using subcellular fractionation is based on hydrophobicity (Michaelson and Philips 2006). However, in the present study, E-Cadherin and SRAP were used as controls to validate the purity of the membrane and cytoplasmic fractions. The Western blotting results indicated that there was no cross-contamination between the two fractions with each cell line (Figure 5); thus, cross-contamination is unlikely. Three, as described in the instructions provided with the fractionation kit, the membrane fraction consists of not only the cellular membrane but also membrane components from other subcellular compartments such as endoplasmic reticulum and Golgi complex. Therefore, if claudin 1 is localized in the membrane of organelles found within the cytoplasm, these organelles will contribute to the positive staining identified in the cytoplasm using fluorescence microscopy, while would be isolated with the membrane fraction during subcellular fractionation.

Taken together, I have shown that claudin 1 localization correlated with the subtypes of human breast cancer cells: in the luminal subtype, both membrane and cytoplasmic localization of the endogenous claudin 1 protein were identified with the primary site being in the membrane; whereas the majority of claudin 1 protein was located in the cytoplasm in the basal-like subtype cells (Figure 4).

3. The C-terminus of the Claudin 1 Protein Plays a Role in its Membrane Localization in Human Breast Cancer Cells

In MDCK cells, claudin 1 protein has been shown to be localized at the cell membrane

and contributes to the epithelial barrier function (Inai, Kobayashi, and Shibata 1999). Like other tight junction transmembrane proteins, such as occludins and JAMs, claudins are known to assemble through direct or indirect interactions with a number of proteins at their C-terminal cytosolic tails. In fact, the C-terminus of claudin 1 is required for its subcellular localization (Rüffer and Gerke 2004).

Mislocalization of claudin 1 protein from the plasma membrane to other compartments has been observed in several types of cancer cells including breast cancer cells. To investigate whether the C-terminus of claudin 1 is also required for its subcellular localization in human breast cancer cells, C-terminus deletion studies were carried out. The human breast cancer cell lines T47D and MCF-7 cells were selected for these studies because I previously showed that the endogenous claudin 1 protein was mainly localized in the cell membrane in the cell lines (Figure 4 and Figure 5). Transfecting N-terminally GFP-tagged claudin 1 mutants lacking the C-terminal domain (GFP-claudin 1-Δ24) into theses cells resulted in increased cytoplasmic staining and decreased membrane staining of the protein (Figure 9C and 10C), compared to the cells transfected with full-length protein (GFP-claudin 1-FL). It was visualized primarily in the cell membrane of both cell lines. GFP-claudin 1-FL was used in the present study as a control. The results suggest that the C-terminus of claudin 1 protein plays a role in directing its membrane localization in these human breast cancer cells (Figure 9B and 10B). This finding is in agreement with Ruffer's work which showed that the C-terminal tail of claudin 1 was responsible for its membrane localization in MDCK cells (Rüffer and Gerke 2004).

There are a number of possible mechanisms by which the C-terminus may direct the localization of claudin 1. The C-terminus constitutes the binding sites for other tight junction components and this interaction is required for association with the tight junctions. In the absence of such interaction, claudin 1 may be absorbed by and internalized into endocytic structures (Rüffer and Gerke 2004). Second, the C-terminal tail could harbor a domain required for the proper exocytotic transport from the endoplasmic reticulum to the plasma membrane (Rüffer and Gerke 2004). Third, several specific sites within the C-terminus of

claudins are suggested to be targeted for various post-translational modifications, such as phosphorylation, glycosylation and palmitoylation. Such modifications have been shown to be associated with its mislocalization in cancer cells (Butt et al. 2011, Singh, Sharma, and Dhawan 2010).

There are currently many tags available for protein studies. However, the GFP tag was used in my studies because GFP fusions offer distinct advantages over conventional immunofluorescence including lower background, higher resolution, robust dual color co-localization and avoidance of fixation artifacts (Michaelson and Philips 2006). Other studies have suggested to generate two constructs, one with GFP at the N-terminus and the other one with GFP at the C-terminus, as many proteins fold with their NH_2- and COOH-termini exposed on the surface, rather than buried in the protein core (Hovmoller and Zhou 2004). In the present study, the N-terminally GFP tag was selected based on the knowledge that the C-terminal epitope tag could interfere with certain intracellular protein interactions (Rüffer and Gerke 2004). However, there was also concern that the GFP tagged claudin 1 protein is relatively large and can affect the localization and functionality of protein (Snapp 2005). However, the full length GFP-claudin 1 localized mainly in the cell plasma membrane, which is consistent with the localization of endogenous claudin 1 protein, demonstrating that in these studies, it is unlikely that the GFP disrupted the normal localization of the protein.

To conduct functional studies with the mutant plasmids (GFP-tagged claudin 1 C-terminus deleted mutants, GFP-claudin1-Δ24), stably transfected cell lines were generated in the MCF-7 breast cancer cells. A GFP-tagged full-length claudin 1 (GFP-claudin 1-FL) was used as control. Using fluorescence microscopy, primarily membrane staining of GFP-claudin 1-FL was observed as well as some cytoplasmic localization (Figure 12A), results consistent with the observation from transient transfection (Figure 9B). However, different observations have been found in the two stable clonal lines selected for these studies with claudin 1 mutants (GFP-Claudin1-Δ24), GFP-claudin 1-Δ24-clone$^{\#}$3 and GFP-claudin 1-Δ24-clone$^{\#}$8. In one clone, clone$^{\#}$3, the C-tail deleted mutant protein GFP-Claudin1-Δ24 was found to be localize primarily in the cell membrane. The other clone, clone$^{\#}$8, however

exhibited more cytoplasmic staining and less membrane staining of GFP-Claudin1-Δ24 (Figure 12B).

In conclusion, these studies indicated that the localization of GFP-claudin 1-FL is not correlated with the protein expression level, both low and high expression clones showed localization mainly in the membrane. However, when the C-terminus is absent, it appears that the level of protein becomes a factor. The localization of GFP-Claudin 1-Δ24 was associated with the protein expression level. In the clone with high expression of GFP-claudin 1-Δ24, the protein localized primarily in the cell membrane, whereas in clone with low expression of GFP-claudin 1-Δ24, it localized mainly in the cytoplasm.

4. Claudin 1 Protein Phosphorylation Study

Mislocalization of claudins from the membrane to other cell compartments appears to be common among cancer cells (Dhawan, et al. 2005; French, et al. 2009; Jakab, et al. 2010; Leotlela, et al. 2007; Oku, et al. 2006(Dhawan et al. 2005, Zhang, Yao, and Wang 2008). Mislocalization of claudin 1 has been found in several cancers including colon cancer, melanoma as well as human invasive breast cancer (Dhawan et al. 2005, French et al. 2009, Jakab et al. 2010, Leotlela et al. 2007, Oku et al. 2006). A few studies have demonstrated that the subcellular localization of claudin 1 protein was influenced by kinase-dependent phosphorylation. In hepatoma cells, a decrease in PKA activity resulted in subcellular localization of claudin 1 to the cytoplasm (Farquhar et al. 2008). Mutations of PKA and PKA/PKC sites of claudin 1 were shown to be responsible for the retention of claudin 1 in cytoplasm, suggesting that these phosphorylation sites play a role in regulating claudin 1 subcellular localization in melanoma cells (French et al. 2009). A growing body of work has confirmed that the phosphorylation of other proteins in the claudin family, such as claudin 2, 3, 4 and 5 (CM Van Italli, et al, 2012; D'Souza, et al, 2005; D'Souza, et al, 2007; AonoS, et al, 2008; Yamamoto, et al, 2008). Recently, phosphoaminoacid analysis identified that

claudin 1 protein is phosphorylated on threonine and tyrosine residues in the intestinal mucosal cells (Li, Akhtar, and Choudhry 2012).

In melanoma cells, an up-regulation of claudin 1 protein has previously been reported after the treatment with PKC activator phorbol myristic acid (PMA), showing that expression level of claudin 1 could be regulated by PKC (Leotlela et al. 2007). It has also been reported that the up regulation of claudin 1 by PKC contributed to melanoma cell motility (Leotlela et al. 2007). Besides regulating the expression level of claudin 1 protein, PKC, as well as PKA has also been showed to affect the change of claudin 1 to nucleus in melanoma cells (French, et al. 2009).

Our laboratory as well as others reported the change of claudin 1 protein localization in the cytoplasm of human invasive breast cancer biopsies (Blanchard, Skliris, et al. 2009). However, the mechanisms affecting subcellular localization of claudin 1 in human invasive breast cancer have not been previously addressed. I showed that the C-terminus of claudin 1 has been identified to play a role in directing claudin 1 subcellular localization in breast cancer cells MCF-7 and T47D (Figure 9 and 10). I observed that, after 18 hours treatment of PKC activator TPA, the expression of endogenous claudin 1 in MCF-7 cells is up regulated. To address whether phosphorylation plays a role in directing the localization of claudin 1 in human breast cancer, site-directed mutagenesis of phosphorylated claudin 1 sites identified to play a role in melanoma cells was carried out. It was shown that in these cells the mutations of PKA and PKA/PKC sites 190, 192 and 195 within the C-terminus of claudin 1 resulted in the retention of claudin 1 in cytoplasm (French et al. 2009). Thus, a number of similar claudin 1 mutant constructs were generated in our laboratory; namely mutants that mimic non-phosphorylatable state ("A" mutation) and constitutive phosphorylation ("D" mutation) at 190, 192 and 195 sites.

The results revealed that, neither the "A" or the "D" mutants resulted in increased cytoplasmic retention of claudin 1 in the cell lines examined $MCF10A_1$ and T47D (Figure 14). The implication of these results is that it appears that these sites were not important for the cytoplasmic localization of claudin 1. Another plausible explanation could be

post-translational modifications, such as glycosylation and palmitoylation may play a role as well as these mutated sites were also glycosylation and palmitoylation sites. Similar to kinase-induced phosphorylation, palmitoylation was also observed in claudin 1 and other claudins including claudin 3, 4, 7 and 14, and altered their ability to maintain tight junction and cellular contacts (Butt et al. 2011, Van Itallie and Anderson 2006, Heiler et al. 2015). Although there is no study showing palmitoylation of claudin 1 is involved in regulating its membrane localization, palmitoylation of claudin 14 has been identified to be required for its efficient tight junction localization (Van Itallie and Anderson 2006). Additionally, it is suggested that claudin 1 also has high potential for O-linked glycosylation (Waqar Ahmad 2011). Further, the same group also identified that O-linked glycosylation and phosphorylation interplay at same Ser/Thr residues on Ser192, Ser205 Ser206 (Waqar Ahmad 2011).

However, I have demonstrated that, in MCF-7 cells, mutating Ser192 and Thr195 sites to an aspartic acid to mimic the phosphorylated state led to an increased cytoplasmic staining and a decrease in membrane staining, compared with the full length claudin 1 (Figure 15), suggesting a shift in claudin 1 localization from membrane to cytoplasm in MCF-7 cells. Similarly, in ovarian cancer cells, phosphorylation of claudin 4 at Ser194 by PKC was also identified that resulted in claudin 4 mislocalization from membrane to other compartments (Kyuno D et al., 2011). As well, phosphorylation of Thr192 in claudin 3 by PKA disrupts tight junction barriers and phosphorylation of claudins, such as claudin 2, 3 or 4, via myosin light chain kinase (MLCK) and rho kinase is more frequently associated with tight junction disassembly (D'Souza et al., 2005; Fernandez, A. L. 2007; Prasad S. 2005). Claudin phosphorylation associated with tight junction disassembly is also enhanced by EphA2, which is recruited to bind to claudin 4 by forming a complex with ephrin-B1 (Tanaka M, 2005).

In normal epithelial cells, phosphorylation of several claudins has been shown to be responsible for its membrane localization. The phosphorylation of claudin 4 at S194 in keratinocytes is required for its tight junction localization (Aono and Hirai, 2008).

Phosphorylation of transfected claudin 2 S208 as well as claudin 16 S217 was reported to be associated with plasma membrane localization in MDCKII cells (CM Van Itallie 2012; Ikari, 2006). In addition, a putative MAPK site of claudin 1, Thr203, is reported to promote tight junction barrier function, which might be via phosphorylation (Fujibe M, 2004). Conversely, de-phosphorylation of claudin 1 by protein phosphatase 2A is associated with increased paracellular permeability in epithelial cells, although in this case, ZO-1 phosphorylation was also reduced, so this may reflect altered interactions between claudin-1 and ZO-1 (Nubhakdi-Craig V, 2002).

Moreover, one study has shown that phosphorylation did not regulate the subcellular localization of claudins. It has been demonstrated that, in MDCK II cells, heterologous mutant WNK4 expression results in phosphorylation of claudins 1-4 and 7, with no change in localization of any claudins (Yamauchi et al. 2004). Taken together, these findings suggested that, in normal epithelial cells, the membrane localization of claudin 1 and other claudins might be due to its phosphorylation by protein kinases, but the phosphorylation might be the reason for its mislocalization from membrane to other compartments in cancer cells. Thus, a more comprehensive approach will be required to understand the consequences of claudins phosphorylation.

In conclusion, my results have demonstrated that, in human breast cancer cells, specific phosphorylation sites, S192 and T195, within the C-terminus of claudin 1 appears to play a role in its subcellular localization in MCF-7 cells. The effect of such mislocalization on the proliferation and/or metastasis of MCF-7 cells still remain to be determined.

VII. SUMMARY

1. The expression levels of endogenous claudin 1 protein are cell line specific and do not appear to correlate with the molecular subtype of the human breast cancer cells

2. T47D and MCF-7, which represent luminal-like subtype display membrane staining of claudin 1. BT-20 and MDA-MB-231, which represent basal-like subtype, display cytoplasmic staining.

3. Levels of claudin 1 do not affect its localization.

4. The C-terminus of claudin 1 plays an essential role in directing its membrane localization in the luminal subtype breast cancer cells.

5. Mutations of potential PKA/PKC target sites (S192 & T195) at the C-terminus which mimic constitutive phosphorylation, led to the retention of claudin 1 protein in the cytoplasm in MCF-7 cells.

6. Mutation of a PKA phosphorylation site (S192) to mimic the non-phosphorylated state, led to the membrane localization of the protein, as one would expect with native claudin 1.

VIII. SHORTCOMINGS IN THE PRESENT STUDY

1. *In vitro* studies, cell lines do not usually give the complete picture of what really happens *in vivo* but still provide a lot of information for studying the mechanisms underlying the development of diseases.

2. Immunofluorescence and subcellular fractionation strategies are two widely used methods to investigate the subcellular distribution of endogenous proteins in cells; however, each has its own limitations. The disadvantage of immunofluorescence is that it requires suitable antibodies that recognize claudin 1 protein with high specificity in fixed cells, and it necessitates fixation and cell permeabilization with associated artifacts. The disadvantage of subcellular fractionation is that a clean separation of cell plasma membranes from various compartments is difficult to achieve.

IX. FUTURE DIRECTIONS

1. Future functional studies, such as proliferation assays, invasion assays and anchorage independent growth assays, could be conducted to allow us to fully assess whether the retention of the protein in the cytoplasm alters the proliferation or invasive phenotype of the stably mutant clones.

2. Future studies will also focus on identifying other phosphorylation sites in the regulation of claudin 1 localization and function in T47D cells and basal-like subtype breast cancer cell lines.

3. Since some phosphorylation sites are also identified as potential glycosylation sites, future studies will also be aimed at whether glycosylation also plays a role in regulating claudin 1 localization in human breast cancer cells.

4. Future studies will also test the TPA effect on the subcellular localization of both MCF-7 WT and S/T mutants. As well, experiments using of kinase inhibitors will help confirm the validity of claudin 1 phosphorylation sites targeted in the study.

TABLES

Table 1. Molecular Subtypes of Breast Cancer

Spontaneous Breast Cancer	Luminal A	Luminal B	HER2-Enriched	Basal-like	Normal-like	Claudin-low
Frequency	40%	20%	10-15%	15-20%	5-10%	12-14%
Phenotype	ER+ PR+ HER2-	ER+ PR+/- HER2-/+	ER- PR- HER2+	ER- PR- HER2-	unclear	ER- PR- HER2-
Additional Markers		High Ki67		CK5/6 EGFR		Claudin 3,4,7 low E-Cadherin low EMT markers high
Proliferation	Low	High	High	High	Low	High
Histological Grade	Low	Intermediate	High	High	Low	High
Prognosis	Good	Intermediate	Poor	Poor	Intermediate	Poor

Table 2. Histologic Subtypes of Breast Cancer

Non-invasive Carcinomas of the Breast		
Histologic Type	**Frequency (%)**	**5-year Survival**
Intraductal Carcinoma	3.6	>99
Lobular Carcinoma in situ (LCIS)	1.6	>99
Intraductal & LCIS	0.2	>99
Papillary Carcinoma	0.4	>99
Comedecarcinoma	0.3	>99
Invasive Carcinomas of the Breast		
Histologic Type	**Frequency (%)**	**5-year Survival**
Infiltrating Ductal Carcinoma	63.6	79
Infiltrating Lobular Carcinoma	5.9	84
Infiltrating Ductal & Lobular Carcinoma	1.6	85
Medullary Carcinoma	2.8	82
Mucinous (colloid) Carcinoma	2.1	95
Comedocarcinoma	1.4	87
Paget's Disease	1.0	79
Papillary Carcinoma	0.8	96
Tubular Carcinoma	0.6	96
Adenocarcinoma, NOS	7.5	65
Carcinoma, NOS	3.5	62

Table 3. BRCA1 & BRCA 2

	BRCA1	BRCA2
Chromosome Site	17q21	13q12-13
Frequency	60%-80%	50%-70%
Phenotype	ER- PR- HER2-	ER+ PR+ HER2-
Proliferation	High	High
Histological Grade	High	High
Prognosis	Poor	Poor
Claudin 1 Expression Level	High	Intermediate

Table 4. The Integral Tight Junction Proteins

Protein	Size (kDa)	Structure	Associating Proteins at TJs	Possible Functions
Occludins	~60	4 transmembrane domains; 1 intracellular C-terminus; 1 intracellular N-terminus; 2 extracellular loops enriched in tyrosine residues	ZO-1, 2, 3	Regulation of TJ barrier
Claudins	20-27	4 transmembrane domains; 1 intracellular C-terminus with PDZ binding motifs (exception of claudin 12); 1 intracellular N-terminus; 2 extracellular loops	ZO-1, 2, 3; MUPP1	Formation of TJ strands; Regulation of selective permeability
JAMs		An extracellular region with 2 variable type Ig-like domains; 1 single transmembrane domain; 1 short cytoplasmic domain	ZO-1, PAR-3, MUPP1	Regulation of polarity

Table 5. Claudin 1 in Human Cancer

	Expression of Claudin 1	Mislocalization of Claudin 1	Role of Claudin 1
Breast Cancer	Down (Invasive HBC)	Cytoplasm	Tumor Suppressor
	Up (Basal-like subtype)	Cytoplasm	Tumor Promoter
Colon Cancer	Up	Cytoplasm & Nuclear	Tumor Promoter
Melanoma	Up	Cytoplasm & Nuclear	Tumor Promoter
Oral Cancer	Up	Cytoplasm	Tumor Promoter
Gastric Cancer	Up	Not Identified	Tumor Promoter
Lung Cancer	Down	Not Identified	Tumor Suppressor
Ovarian Cancer	Up	Not Identified	Tumor Promoter
Prostate Cancer	Down	Not Identified	Tumor Suppressor
Esophageal Cancer	Down	Not Identified	Tumor Suppressor
Liver Cancer	Down	Cytoplasm	Tumor Suppressor
Papillary Thyroid Cancer	Up	Not Identified	Tumor Promoter

Table 6. The Protein Kinase C (PKC) Isoforms

	PKC Isoforms
Conventional (cPKC)	α, βI, βII, γ
Novel (nPKC)	δ, ε, θ, η
Atypical (aPKC)	ξ, ι

Table 7. The Predicted PKA/PKC Sites on Claudin 1

Substrate	Position	PKA/PKC
Serine	34	PKA/PKC
Serine	69	PKA
Threonine	190	PKA/PKC
Threonine	191	PKA/PKC
Serine	192	PKA
Threonine	195	PKA/PKC
Serine	206	PKC

Table 8. Characteristics of Breast Cancer Cell Lines Used in This Study

Human Breast Cancer Cell Line	Endogenous Claudin 1 Expression	Estrogen Receptor Status	Breast Cancer Subtype	Molecular Morphology	Metastatic Potential
MCF7	low	+	luminal	epithelial	low
T47D	high	+	luminal	epithelial	low
MDA231	low	-	basal-like	mesenchymal	high
BT20	high	-	basal-like	mesenchymal	low

Table 9. BSA protein standard dilution used in the BCA protein assay

Final Concentration of BSA (µg/ml)	Stock BSA (40 µg/ml)	ddH_2O (µl)
0	-	500
1	25	475
2	50	450
5	125	375
8	200	300
10	250	250
15	375	125
20	500	-

Table 10. Protocol for Different Concentrations of Protein Electrophoresis Gels

Western Blot Tricine Gels				
	Separating (Resolving) Gel			Stacking Gel
	12%	15%	16.5%	4%
3X Gel Buffer	3 ml	3.33 ml	4.5 ml	1.5 ml
30% 29:1 Polyacrylamide	3.6 ml	5 ml	7.425 ml	600 µl
ddH_2O	1.45 ml	600 µl	150 µl	1.923 ml
1% SDS	0.9 ml	1 ml	1.35 ml	450 µl
10% APS	45 µl	70 µl	67.5 µl	22.5 µl
TEMED	5 µl	7 µl	7.5 µl	4.5 µl

Quantities are calculated for two 0.75mm gels

SDS= Sodium dodecyl sulfate

APS=ammonium persulphate (10% made fresh every time)

3 X Gel Buffer= 3.0M Tris, pH 8.45 and 0.3% SDS

Table 11. Cell numbers plated for immunofluorescence and transient transfection experiments

	Cell Numbers / ML					
	MDCKII	**MCF10A$_1$**	**T47D**	**MCF-7**	**BT20**	**MDA-MB-231**
6-well plate	2.5 X 10^6	2.5 X 10^5	4 X 10^5	3 X 10^5	3 X 10^5	2.5 X 10^5
12-well plate	1.25 X 10^6	1.5 X 10^5	2.5 X 10^5	2 X 10^5	2 X 10^5	1.5 X 10^5

Around 80% confluence after 48h of culturing in the appreciate medium (Section 3 and 6.1 in Material and Methods)

Table 12. Generation of Mutant Claudin 1 Constructs: Site-Directed Mutagenesis of PKA/PKC Phosphorylation Sites

	AMINO ACID SEQUENCE		PKA/PKC	DNA BASE CHANGE	PRIMER
192A	189-192	KTTS	PKA	A568T_A571G	F:5'-ctgttcctgtccccgaaaatcagcctcttacccaacac-3'
					R:5'-gtgttgggtaagaggctgattttcggggacaggaacag-3'
192D	189-192	KTTS	PKA	A568G_C569A_A570C_	F:5'-actttgctgttcctgtccccgaaaagacgactcttacccaacaccaaggccc-3'
				A571G_C572A	R:5'-gggccttggtgttgggtaagagtcgtcttttcggggacaggaacagcaaagt-3'
190D	188-190	TPR	PKA	A568T	F: 5'-aacaacctcttacccagcaccaaggccctatcc-3'
			PKC		R: 5'-ggatagggccttggtgctgggtaagaggttgtt-3’
195D	195-197	TPR	PKA	A589G_G590A_G591C	F: 5'-aaaacaacctcttacccaacaccagacccctatccaaaacctgca-3’
			PKC		R: 5'-tgcaggttttggataggggtctggtgttgggtaagaggttgtttt-3’

Table 13. PCR cycling profile for site-directed mutagenesis

Step	Temp (°C)	Time
Denaturation	95	2 min
Denaturation	95	20 sec
Annealing } **18 cycle**	60	10 sec
Elongation	68	2 min 40 sec
Elongation	68	5 min

Table 14. Expected band sizes of full-length GFP-Claudin 1 plasmid after digestion with restriction enzymes

Enzyme	**Expected band sizes** (approximate base pair)
EcoRI	5200, 200
EcoRI/XhoI	4700, 500, 200
HindIII/XhoI	4700, 700

FIGURES

Figure 1. Tight Junctions. Schematic representation of ductal epithelial cells, depicting the relative location of the tight junctions, tight junction proteins, and the junctional complex.

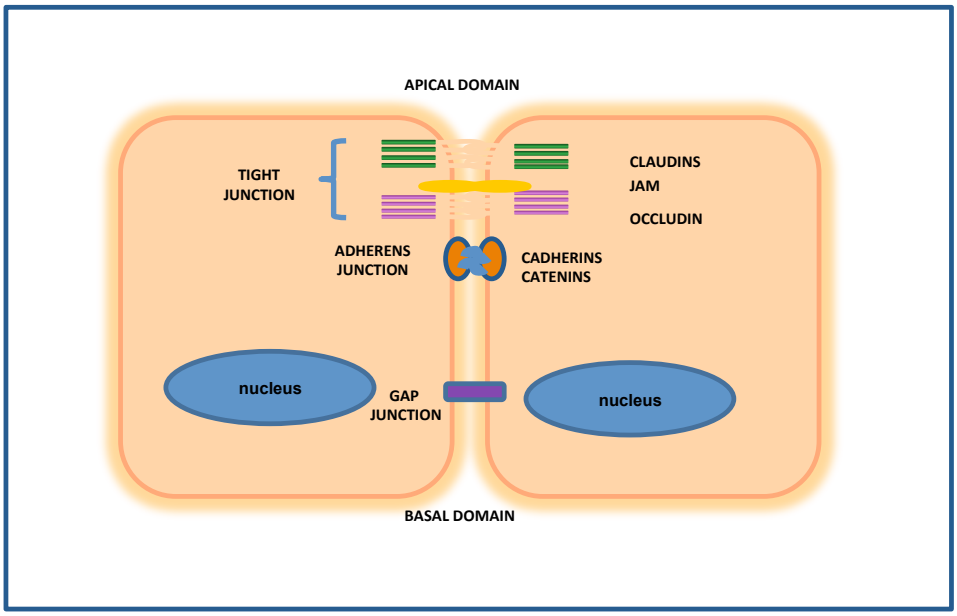


Figure 2. The restriction map and multiple cloning site (MCS) of pEGFP-C1. (A) The pEGFP-C1 vector used to generate GFP tagged claudin 1 constructs. (B) Claudin 1 was cloned into pEGFP-C1 vector using XholI and HindIII restriction sites. (Figures adapted from Clontech Laboratories, Inc.)

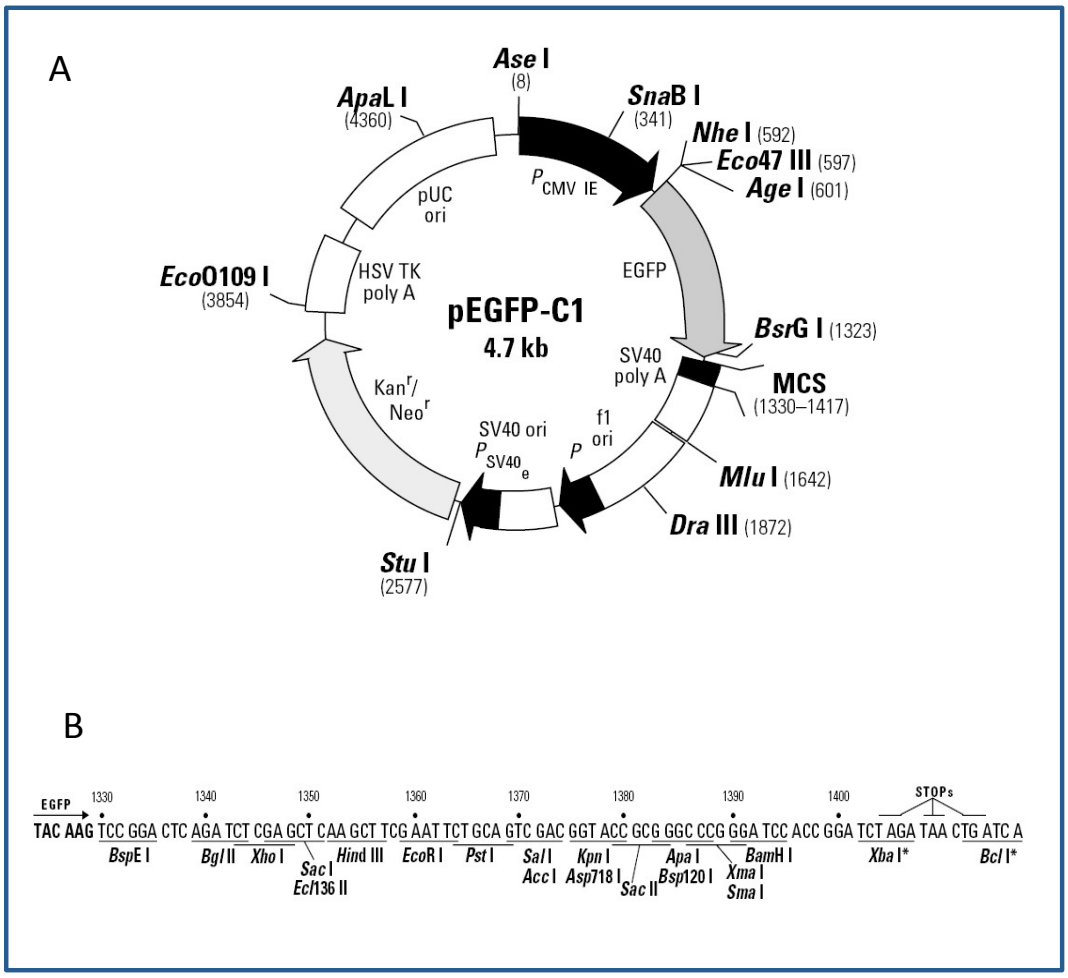


Figure 3. Differential expression of claudin 1 protein in breast cancer cell lines. Western blot analysis showing claudin 1 protein expression in a panel of human breast cancer cell lines. Claudin 1 protein was found to be most highly expressed in T47D, $MCF10A_1$ and BT20 cell lines whereas MDA-MB-231 and MCF-7 cells showed low levels of protein. 50 µg of the total protein of each cell line was loaded. β-actin was used as a control for protein loading. $MCF10A_1$, an immortal breast epithelial cell line, was used as a positive control for the expression level of claudin 1 protein. L: luminal subtype; B: Basal-like subtype. N=3 independent experiments.

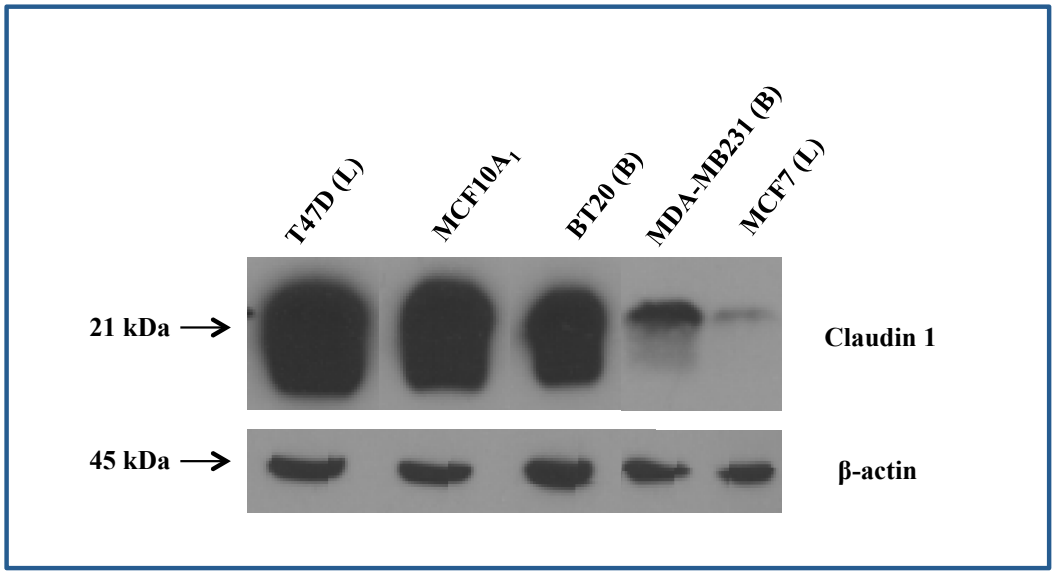


Figure 4. Localization of claudin 1 protein in human breast cancer cell lines by IF. Claudin 1 was localized primarily on the membrane of T47D and MCF7 cells (as indicated by the red arrows), whereas BT20 and MDA-MB 231 cells showed high levels of staining in the cytoplasm (as indicated by the white arrows). Madin-Darby canine kidney (MDCKII) cells and $MCF10A_1$ cells were used as positive controls for claudin 1 showing the predominant membrane localization of claudin 1 (as indicated by the red arrows). Anti-claudin 1 antibody was used to identify the localization for endogenous claudin 1 protein. Anti-E-Cadherin antibody was used as a marker for membrane staining (middle panel). The merge shows the co-localization of claudin 1 and E-cadherin. N=3 independent experiments. Magnification=20X.

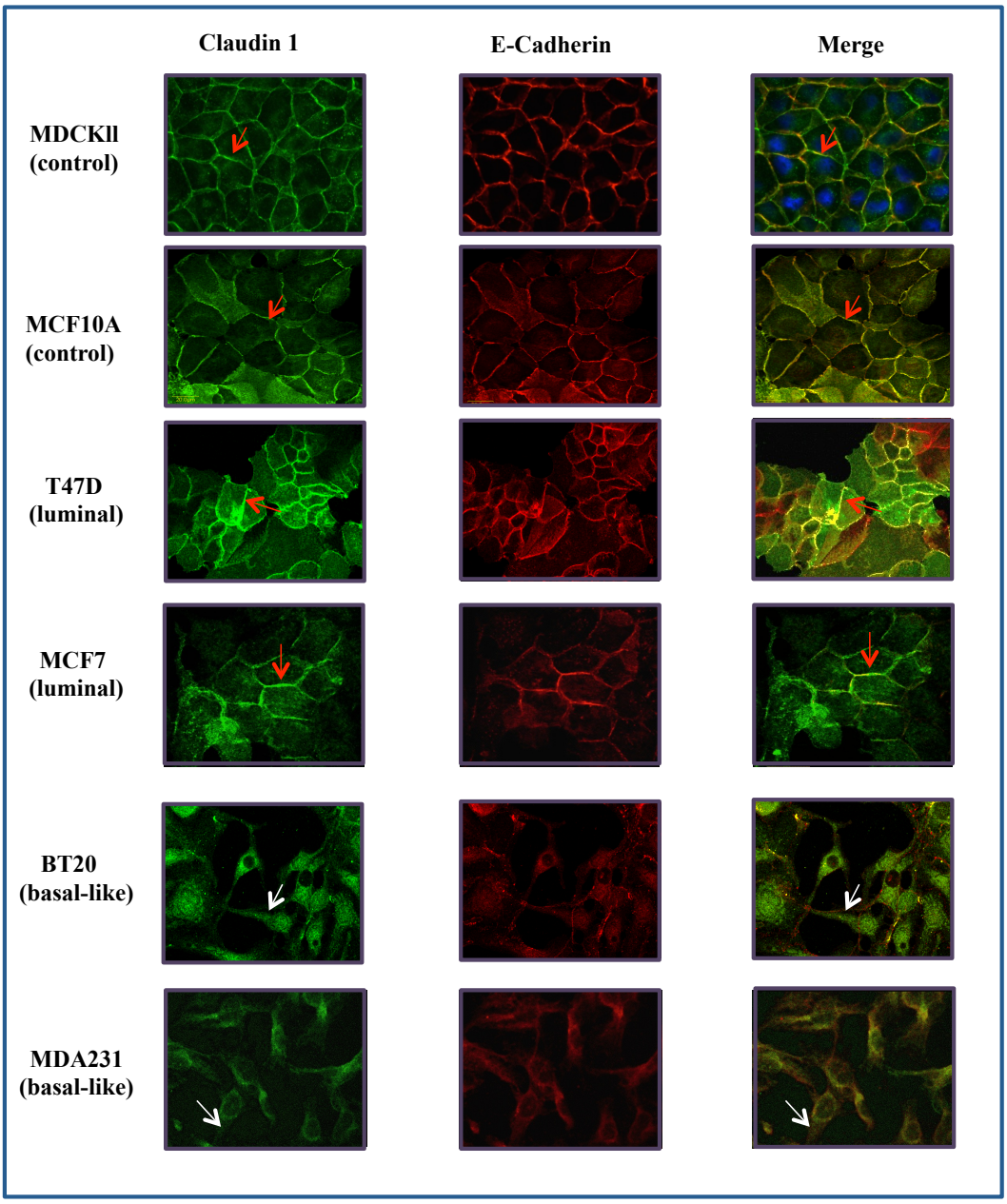

Claudin 1
E-Cadherin
Merge
MDCKll (control)
MCF10A (control)
T47D (luminal)
MCF7 (luminal)
BT20 (basal-like)
MDA231 (basal-like)

Figure 5. Analysis of claudin 1 protein subcellular localization in human breast cancer cell lines by subcellular fractionation. Western blot analysis showing relative levels of claudin 1 protein in the different subcellular fractions. Claudin 1 is primarily in the membrane fraction of MCF10A$_1$, T47D, MCF7, BT20 and MDA-MB231 cells. SRAP (steroid receptor activator protein) and E-cadherin were used as a cytoplasmic (C) and membrane (M) protein control, respectively. The Madin-Darby canine kidney (MDCKII) cell line was used as a positive control for the subcellular localization of claudin 1 in the membrane fraction. N=3 independent experiments.

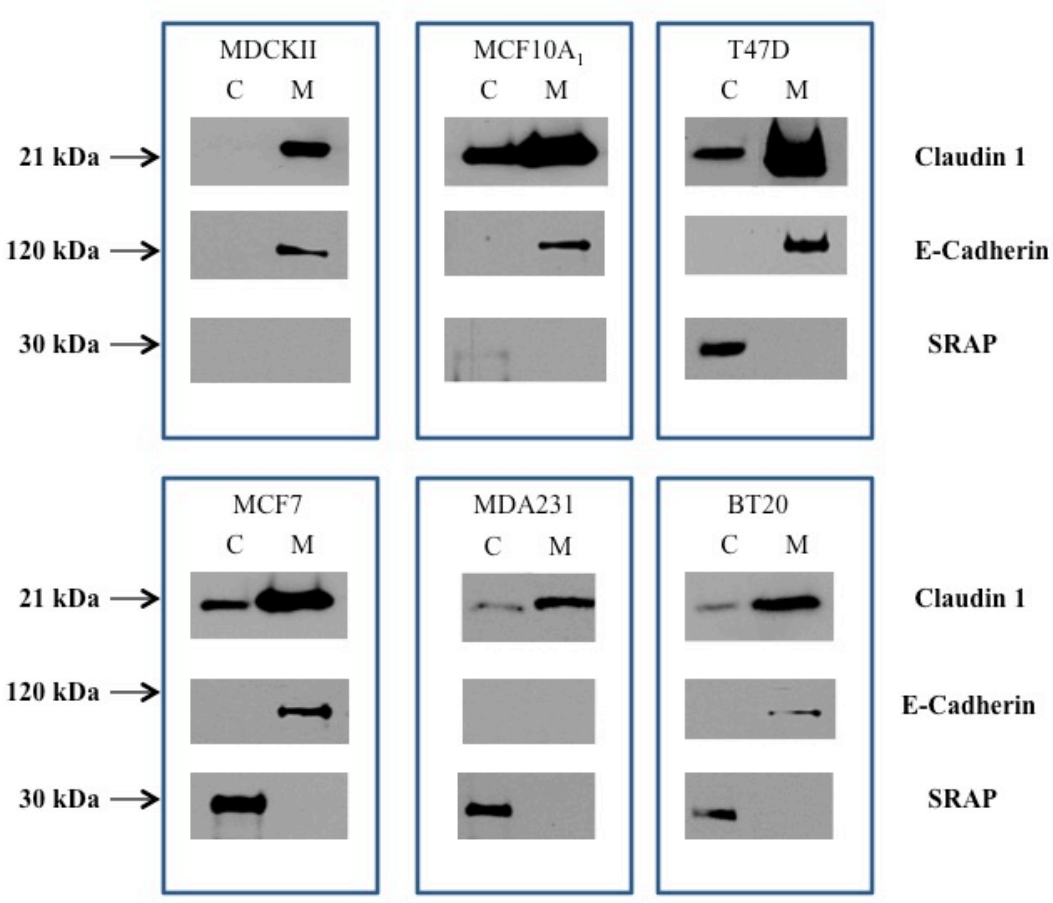


Figure 6. Claudin 1 protein structure and overview of the claudin constructs generated. (A) Topology of claudin 1**.** Claudin 1 consist of four transmembrane domains with two extracellular loops, a shorter intracellular N-terminus and a longer intracellular C-terminus which has been shown to be necessary for its membrane localization. (B) C-terminal claudin 1 deletion constructs (GFP-claudin1 Δ24). Amino acids 183-199 are representative of the sequences of claudin 1 C-terminal cytoplasmic tail. Residues (CCSCP) following the last amino acid (leucine) of the transmembrane were not removed in the mutant constructs in order to ensure correct insertion into the membrane.

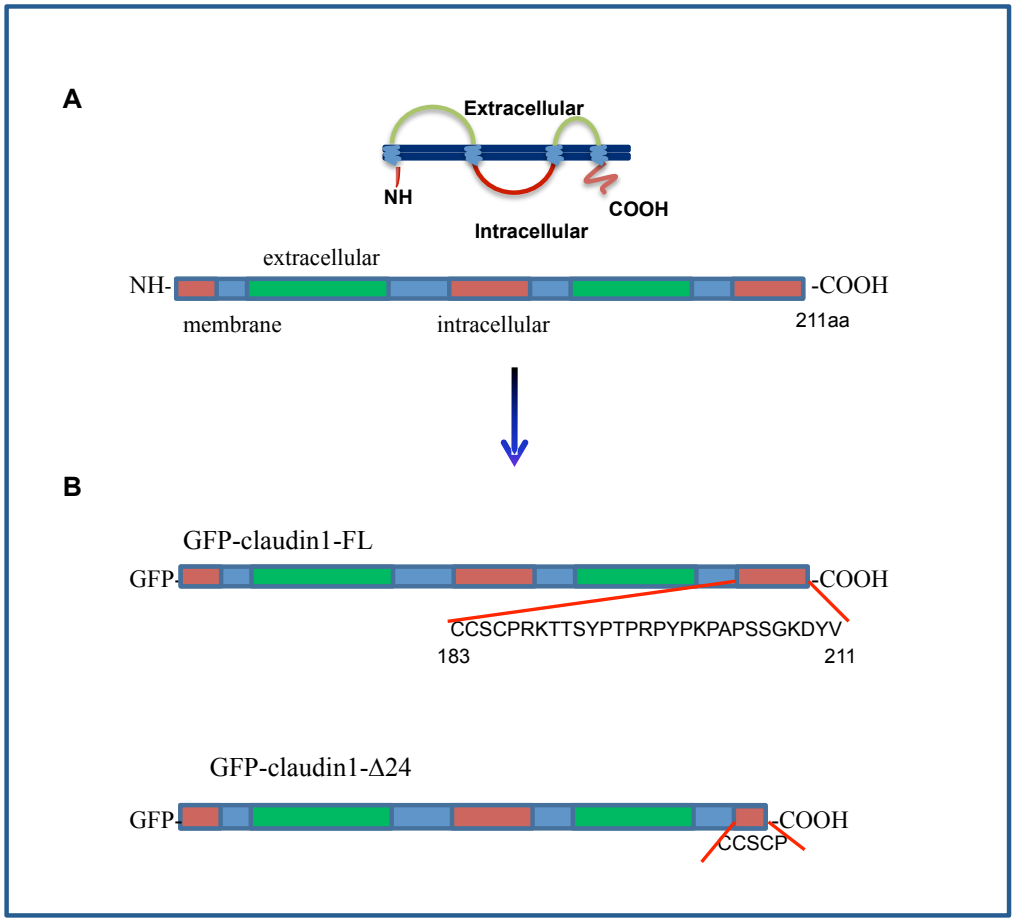


Figure 7. Verification of the full-length GFP-Claudin 1 (GFP-Claudin 1-FL) plasmid. The plasmids were digested with restriction enzymes EcoRI, EcoRI/XhoI, as well as HindIII/XhoI. Expected bands sizes after the digestion were observed as described in the Table 14. N=1 independent experiment. bp= base pair

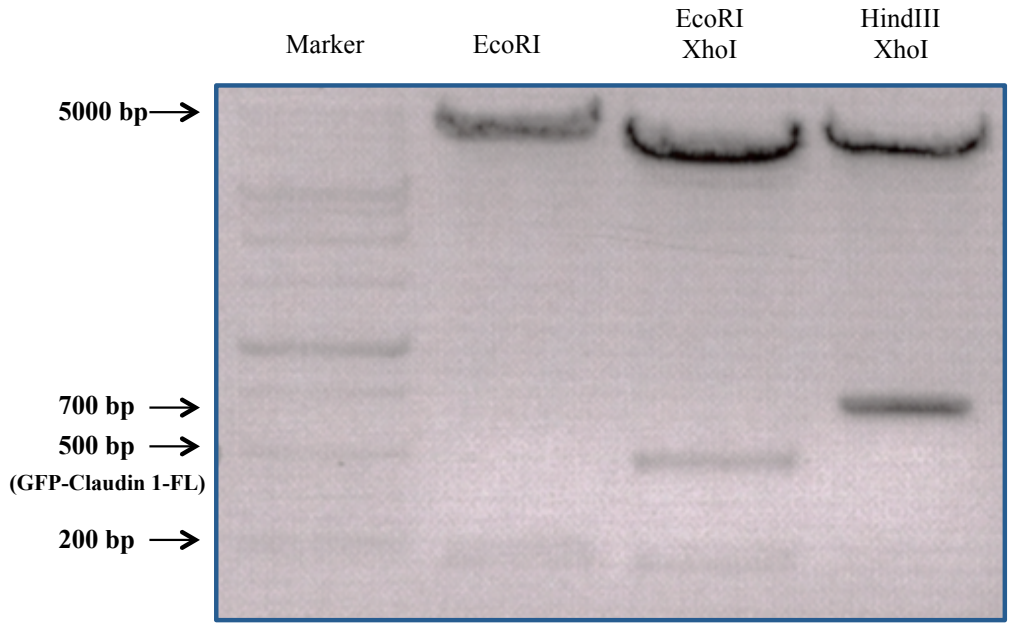


Figure 8. Subcellular localization of GFP in MCF-7 cells visualized by fluorescence microscopy. Images showing the subcellular localization of GFP in human breast cancer cell line MCF-7 after fixation with paraformaldehyde (10 min, room temperature; left panel) and methanol (10 min, -20 °C; right panel). Both cytoplasmic (white arrows) and nuclei localization (orange arrows) were observed in the cells fixed with 4% paraformaldehyde but no GFP was detected in the cells fixed with methanol. Nuclei were stained with DAPI (blue). N=3 independent experiments. Magnification=20X.

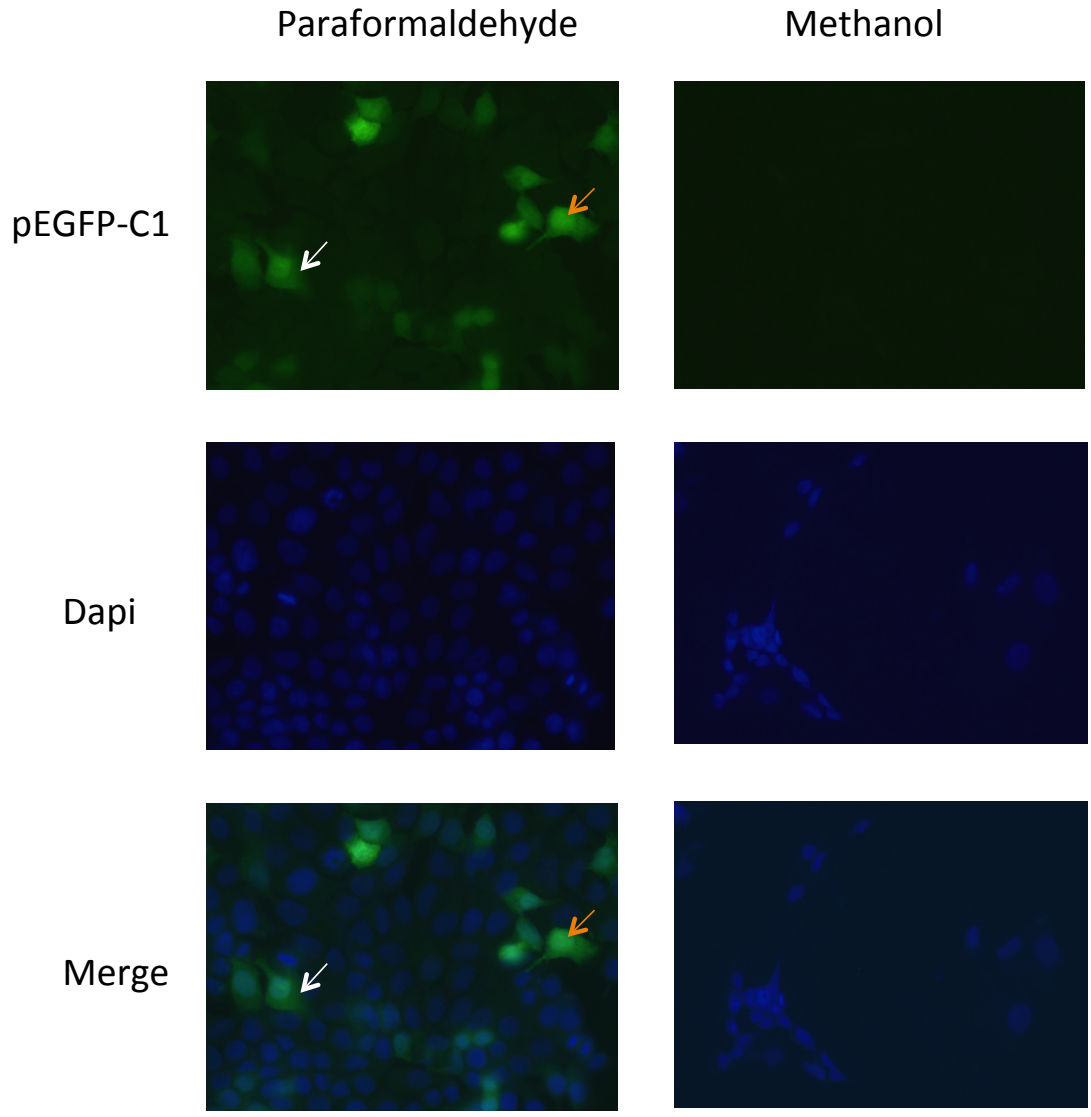
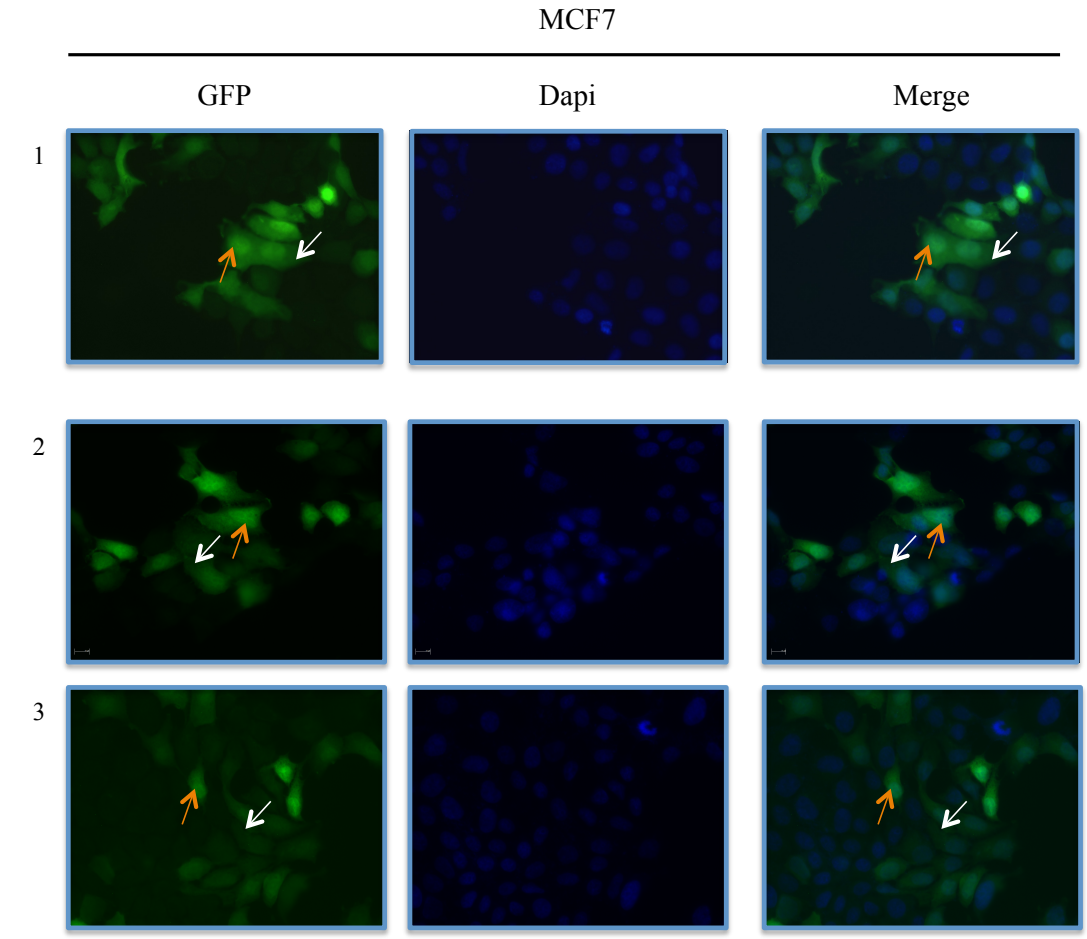


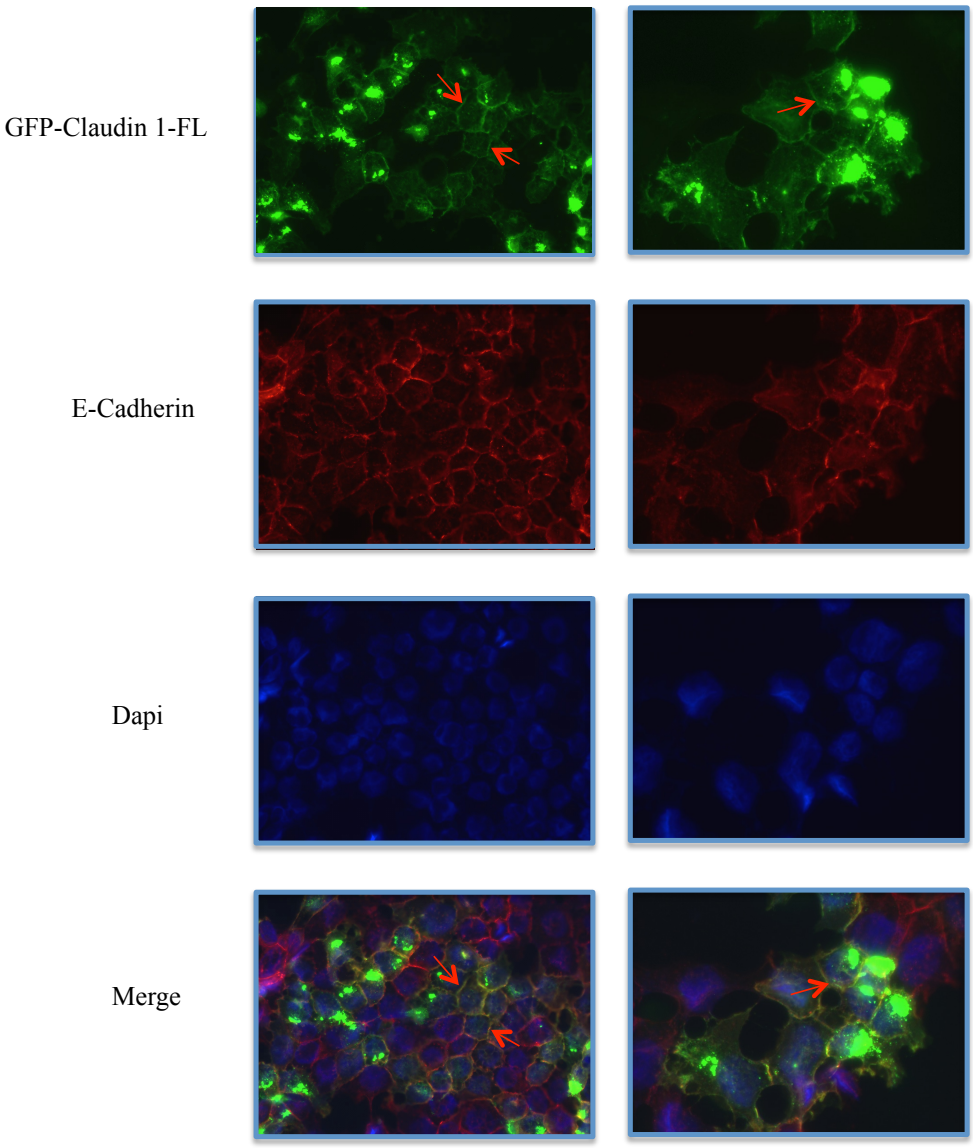
Figure 9. The C-Terminus plays a role in claudin 1 membrane localization in MCF-7 cells. (A) MCF-7 cells were transfected with pEGFP-C1 vector and localization (indicated by the green signal) was observed primarily in the cytoplasm (white arrows) as well as nuclei (orange arrows). Dapi was used for visualizing the nuclei. Three examples (1-3) are from 3 separate experiments. (B) Forty-eight hours after transfection of the GFP-Claudin 1-FL constructs, the protein was observed to be localized in the cell membrane (red arrows), and cytoplasmic localization was also observed (white arrows). E-Cadherin was used as the cell membrane marker and the merged images showed the co-localization of GFP-Claudin 1-FL and E-cadherin. Left and right panels are from 2 separate experiments. (C) Forty-eight hours after transfection of the GFP-claudin1-Δ24 constructs, the result revealed a decrease in claudin 1 membrane localization and an increase in the cytoplasmic localization (white arrows). Merged images showed that GFP-claudin1-Δ24 did not co-localize with E-Cadherin in the membrane. Left and right panels are from 2 separate experiments. N=3 independent experiments. Magnification=20X.

9A



9B

MCF7



9C

MCF7

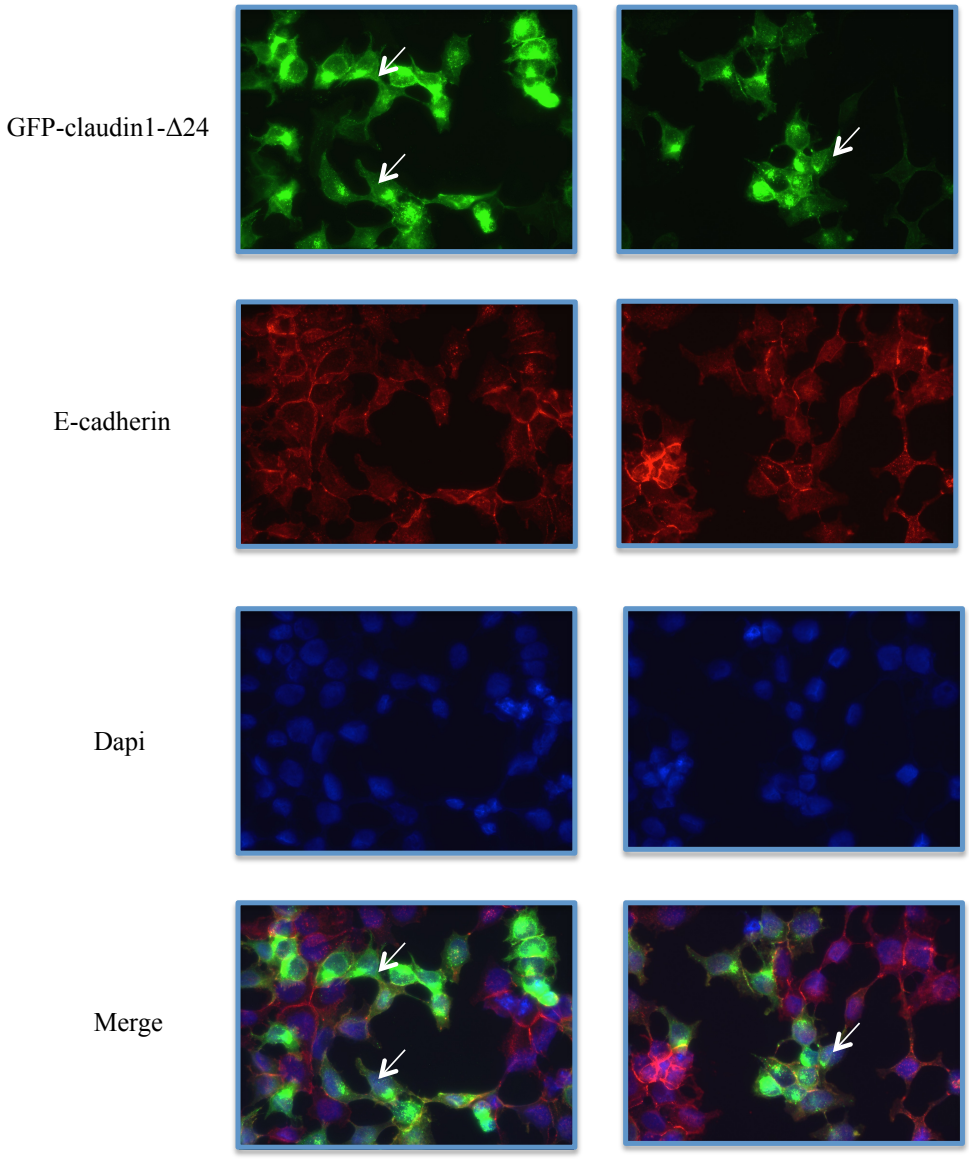
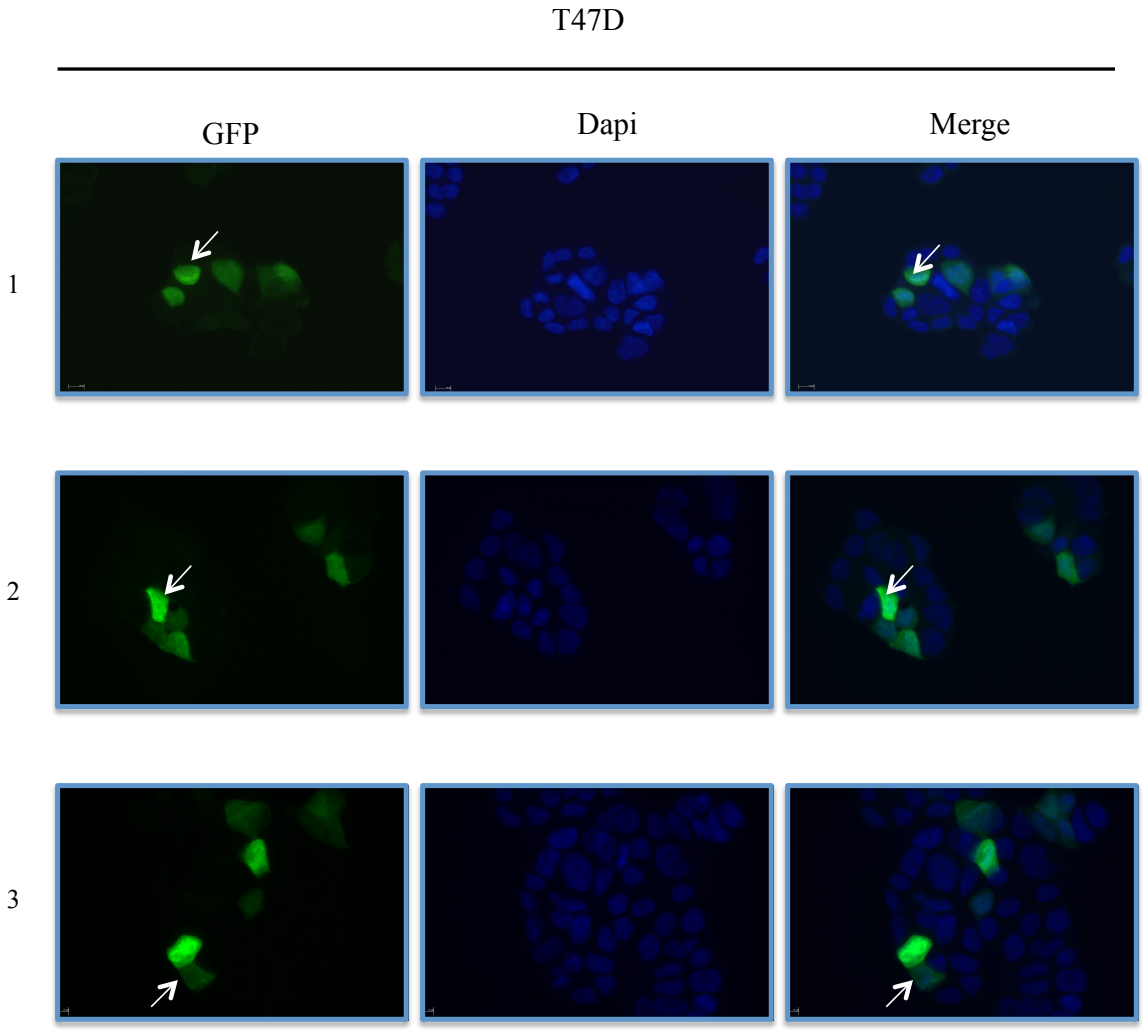
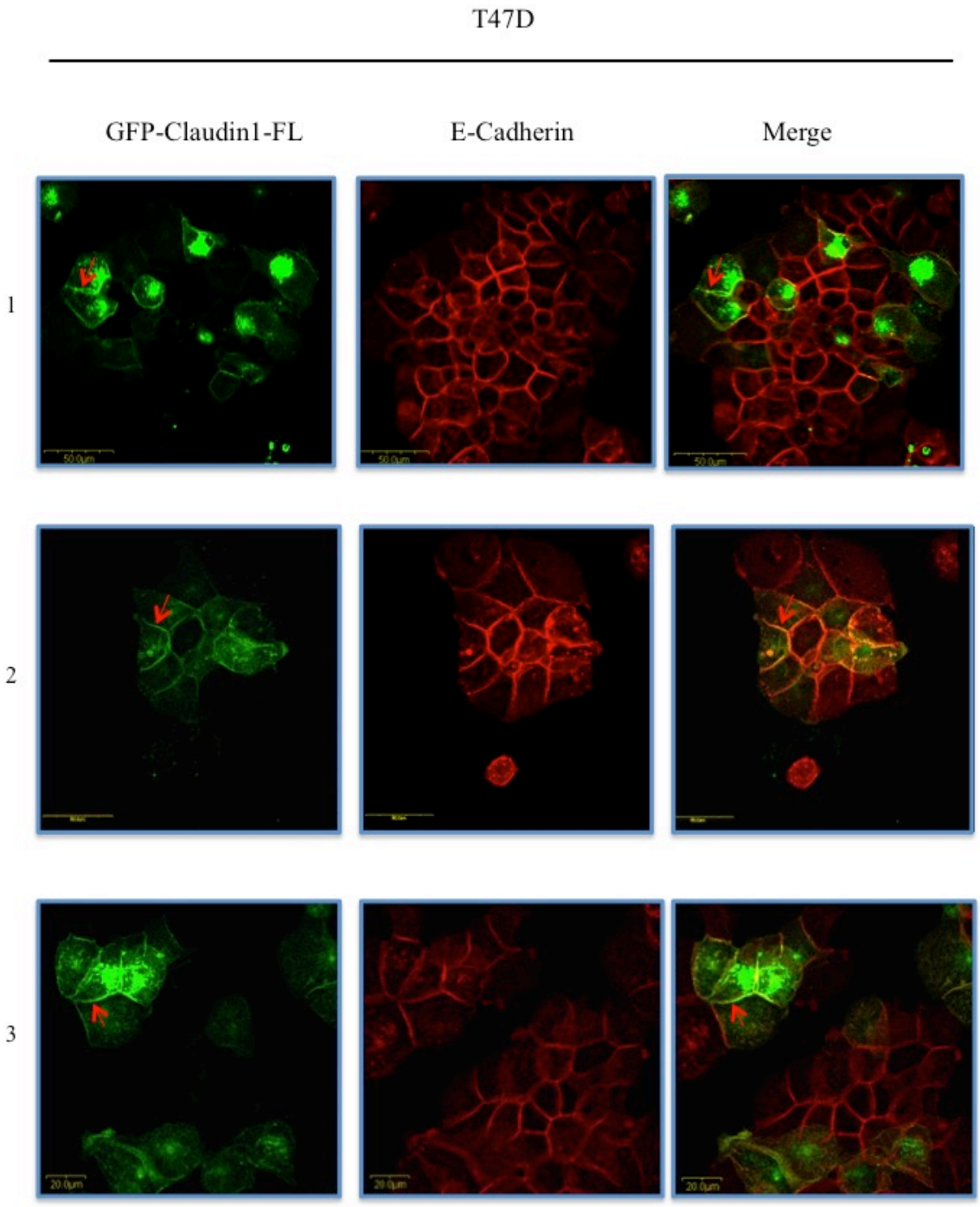


Figure 10. The C-Terminus plays a role in claudin 1 membrane localization in T47D cells. (A) T47D cells were transfected with pEGFP-C1 vector and the localization was observed primarily in the cytoplasm. Dapi is used for visualizing the nuclei. Three examples (1-3) are from 3 separate experiments. (B) Using confocal microscopy, the localization of GFP tagged full length claudin 1 (GFP-Claudin 1-FL) was observed in the cell membrane (red arrows), and some cytoplasmic localization was also observed after 48h transfection. E-Cadherin was used as the cell membrane marker and showed the co-localization with GFP-Claudin 1-FL in the merged images. Three examples (1-3) are from 3 separate experiments. (C) Transfected with the constructs bearing a deletion of the entire C-terminal cytoplasmic tail (GFP-claudin1-Δ24) resulted in a decrease in claudin 1 membrane localization compare to the full length constructs that showed the membrane localization in the cells. Three examples (1-3) are from 3 separate experiments. N=3 independent experiments. Magnification=20X.

10A



10B



10C

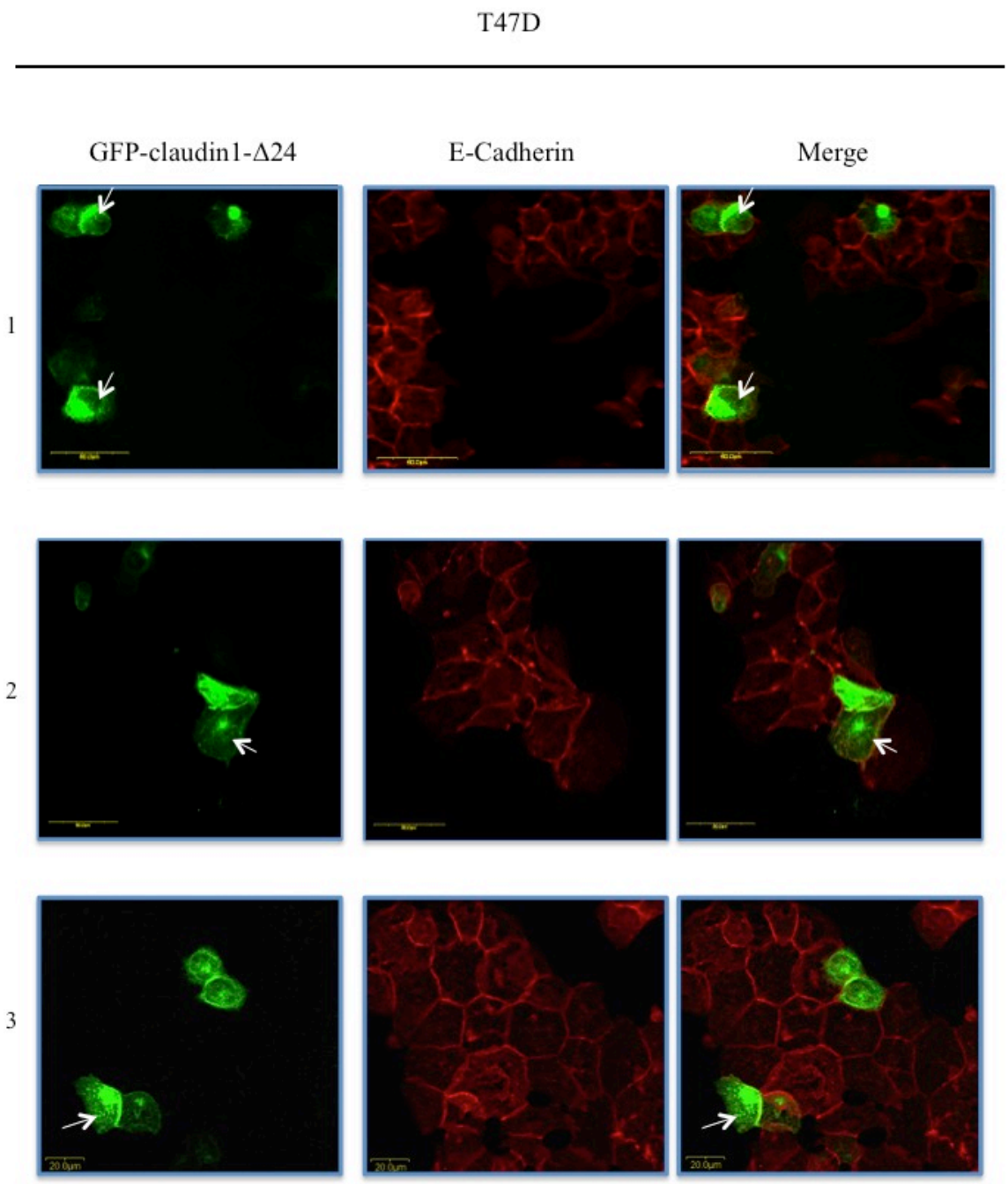
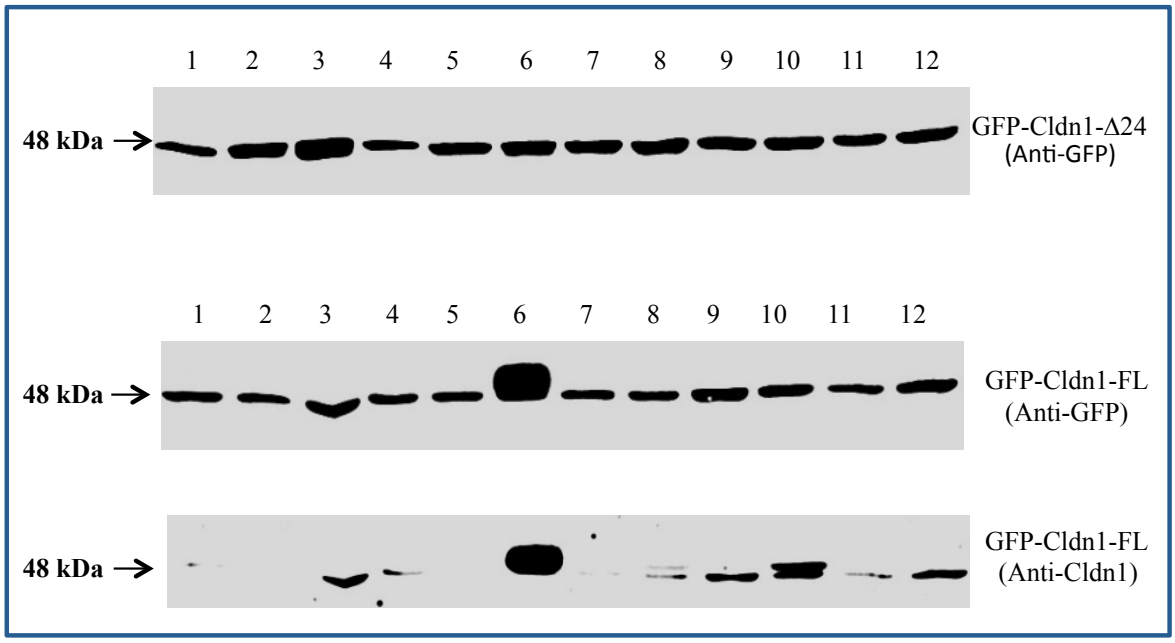


Figure 11. Identification of overexpressing claudin 1 protein and the specificity of anti-GFP antibody in stably transfected MCF-7 cells. (A) MCF-7 cells were stably transfected with the GFP-tagged full-length claudin 1 (GFP-Claudin 1-FL) and GFP-tagged claudin 1 with C-terminus deletion (GFP-Claudin1-Δ24). Verification of stably transfected clones was conducted using Western blot analysis. GFP-Claud1n 1-Δ24 was detected in all 12 clones using anti-GFP antibody (upper figure) and the 12 clones transfected with GFP-Claudin 1-FL also shows positive expression of the protein using anti-GFP antibody (middle figure). However, using anti-claudin 1 antibody, only 8 out of the 12 clones transfected with GFP-Claudin 1-FL was observed to be positive (bottom figure), indicative of the non-specificity of the anti-GFP antibody. (B) Identification of the specificity of anti-GFP antibody. Using the anti-GFP antibody, a non-specific band was detected in the wild type MCF-7 cells, as well as GFP-Claudin 1-FL-clone$^{\#}$6 and GFP-Claud1n 1-Δ24-clone$^{\#}$3. In the GFP-Claudin1-Δ24-clone$^{\#}$3, a band slightly lower than size 48kDa was also observed (45kDa), indicating that the lower band could be GFP-claudin 1-Δ24 protein. The molecular weight of GFP-claudin 1-FL was 48 kDa.

11A


1 2 3 4 5 6 7 8 9 10 11 12
48 kDa
GFP-Cldn1-Δ24
(Anti-GFP)
1 2 3 4 5 6 7 8 9 10 11 12
48 kDa
GFP-Cldn1-FL
(Anti-GFP)
48 kDa
GFP-Cldn1-FL
(Anti-Cldn1)

11B

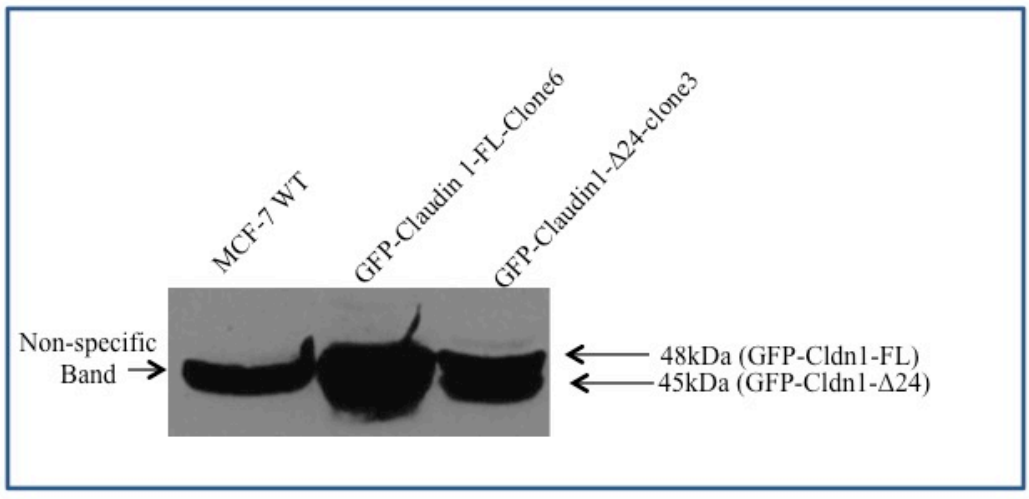
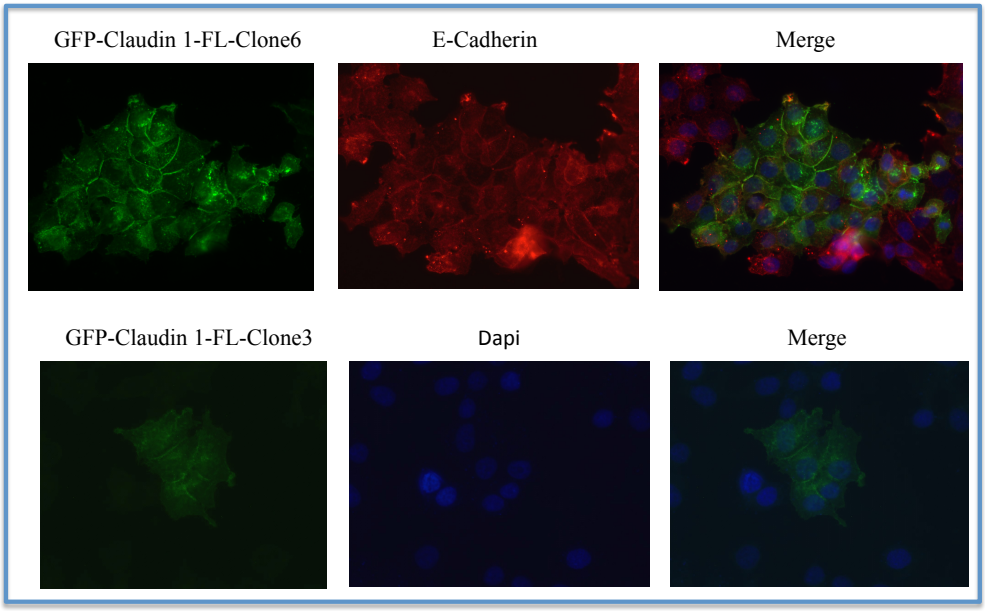

MCF-7 WT
GFP-Claudin 1-FL-Clone6
GFP-Claudin1-Δ24-clone3
Non-specific
Band
48kDa (GFP-Cldn1-FL)
45kDa (GFP-Cldn1-Δ24)

Figure 12. Subcellular localization of GFP-Claudin 1 protein in stably transfected MCF-7 cells. (A) MCF-7 cells were stably transfected with GFP-claudin 1-FL plasmids. Using fluorescent microscopy, the localization of GFP-claudin 1-FL was primarily in the membrane compartment in the two stably transfected clones, cytoplasmic staining was also observed. (B) MCF-7 cells were stably transfected with GFP-claudin 1-Δ24 plasmids. Using fluorescent microscopy, the localization of GFP-Claudin1-Δ24 was mainly in the membrane in the clone3; however, more cytoplasmic but less membrane staining of GFP-Claudin1-Δ24 was observed in clone8. Anti-E-Cadherin antibody was used for membrane staining. DAPI was used for visualizing the nuclei. N=3 independent experiments. Magnification=20X.

12A


GFP-Claudin 1-FL-Clone6
E-Cadherin
Merge
GFP-Claudin 1-FL-Clone3
Dapi
Merge

12B

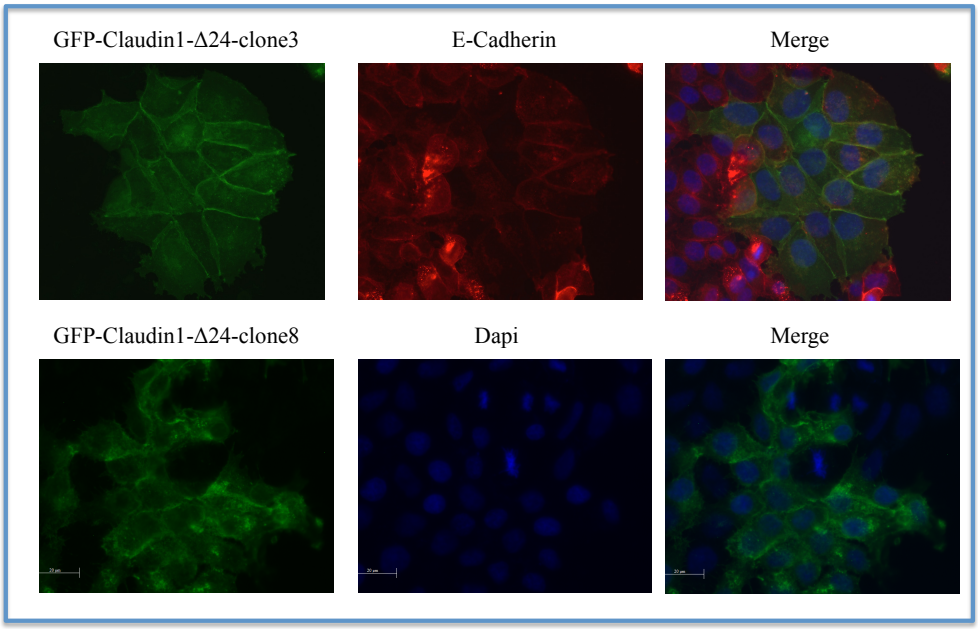

GFP-Claudin1-Δ24-clone3
E-Cadherin
Merge
GFP-Claudin1-Δ24-clone8
Dapi
Merge

Figure 13 Activation of PKC alters the protein level of claudin 1 in MCF-7 cells after 18h treatment. MCF7 cells, treated with the PKC activator TPA (200 nM for1h,4h and 18h) show an increase in claudin 1 after 18h treatment. Cells were also treated with equivalent amounts of DMSO for 1 hour, 4 hour and 18 hour as vehicle control. β-actin was used as loading control. N=3 independent experiments. Magnification=20X.

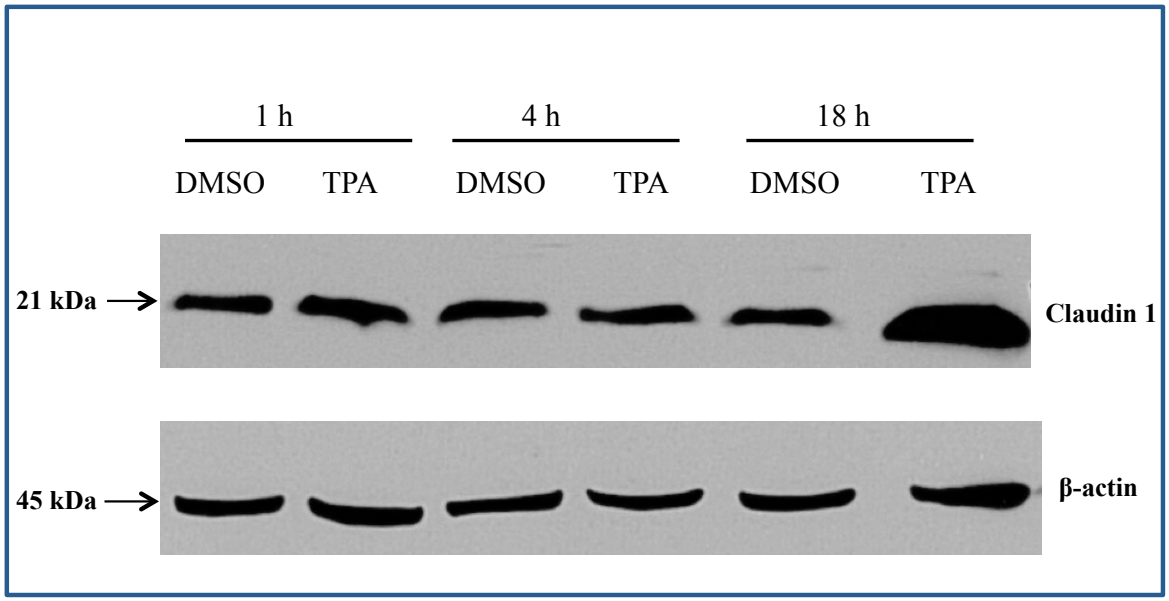


Figure 14. Subcellular localization of full-length claudin 1 and the mutants in stably transfected cells. (A) Overexpression of full length claudin 1 and mutants S192D and T195D in MCF10A$_1$ cells showed both membrane and cytoplasmic localization. (B) Overexpression of full length claudin 1 and mutants S192D and T195D in T47D human breast cancer cells localized in membrane and cytoplasm. Nucleus were stained with DAPI (blue). N=3 independent experiments. Magnification=20X.

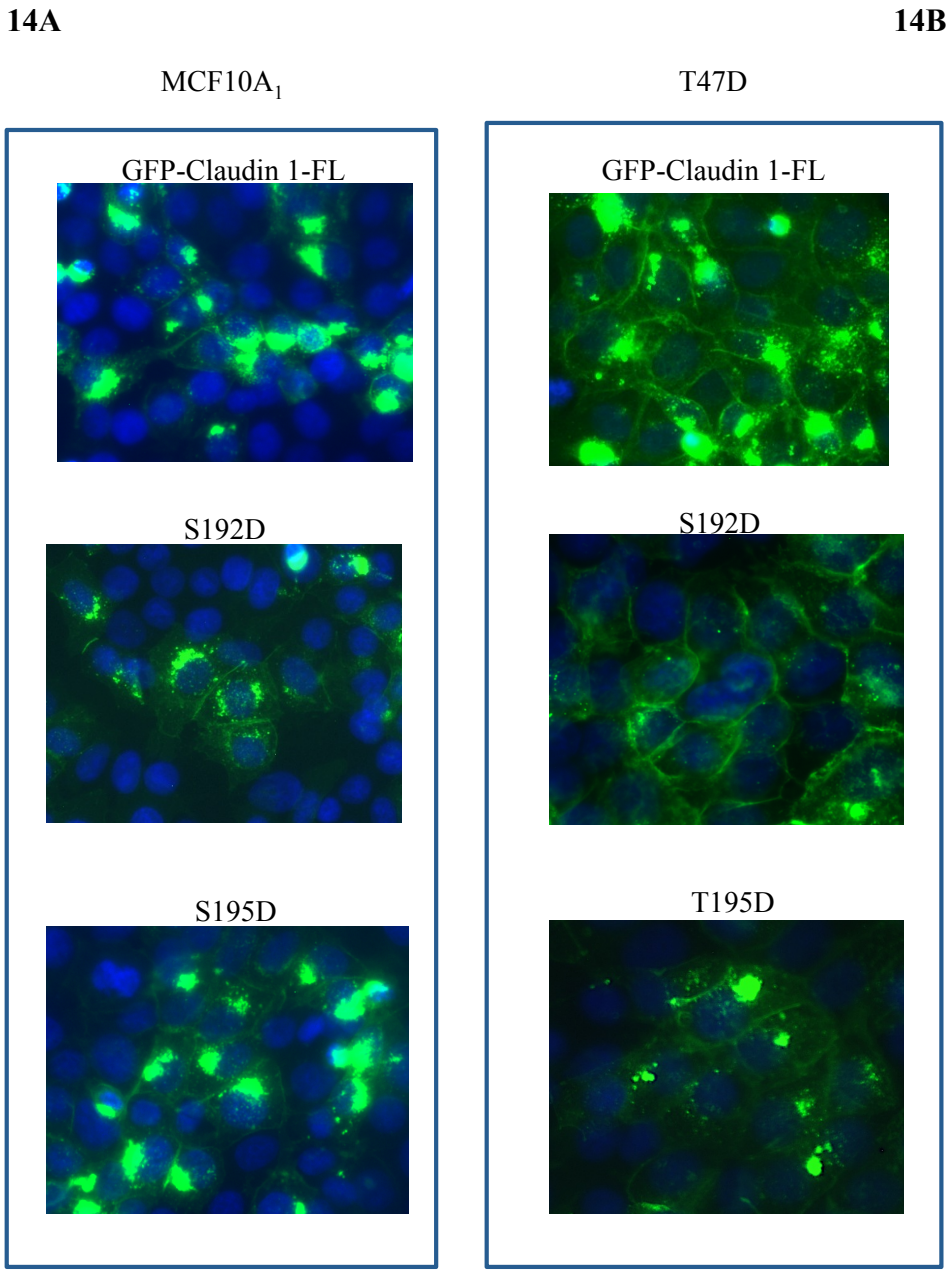
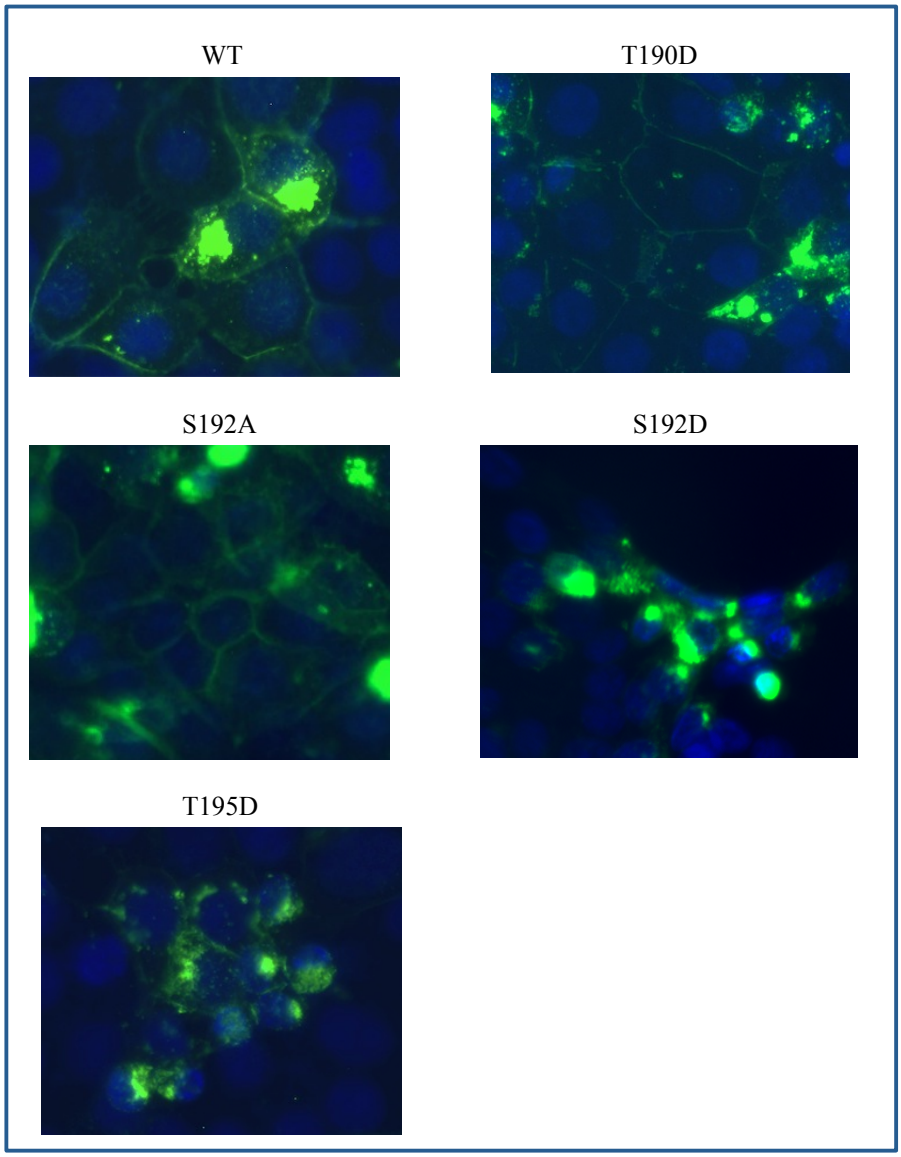


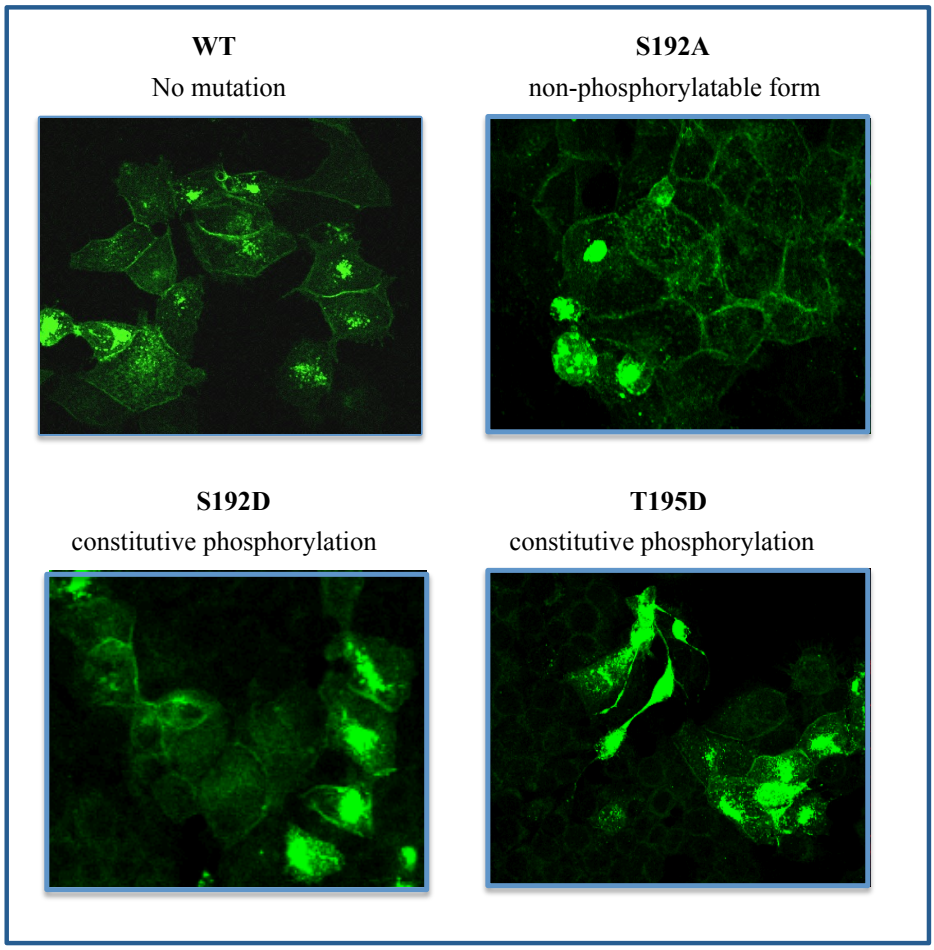
Figure 15. Phosphorylation of claudin 1 protein contributes to its cytoplasmic localization in MCF-7 cells. Site-directed mutagenesis rendering the potential PKA/C target site (S192) on claudin 1 protein non-phosphorylatable (S192A) resulted in membrane localization of the protein, while mutation mimicking constitutive phosphorylation (S192D & T195D) caused more cytoplasmic localization and less membrane localization using fluorescence microscopy (A) and confocal microscopy (B). Mutation mimicking constitutive phosphorylation on site T190 showed membrane localization of the protein similar with the observation of GFP-Claudin 1-FL and the mutant S192A (A). N=3 independent experiments. Magnification=20X.

15A

MCF7



15B


WT
No mutation
S192A
non-phosphorylatable form
S192D
constitutive phosphorylation
T195D
constitutive phosphorylation

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