TIGHT JUNCTION CLAUDIN-7 PROTEIN MODULATES MULTIPLE PROCESSES OF CANCER PROGRESSION IN HUMAN LUNG CANCER CELLS

BY

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Carcinogenesis usually consists of cancer initiation, promotion, and progression. Several tight junction claudin proteins have been identified as tumor suppressors or activators during cancer development. As apical proteins, tight junction claudins seal the apical sides of neighboring epithelial cells in regulating the transport of ions and fluids from the extracellular environment. However, claudin-7, which is believed to direct cell-matrix adhesion, has been found at the basal side of several human organs, including the lung. It has also been clinically reported that the low survival rate of lung cancer patients is closely associated with their low claudin-7 expression. The molecular mechanism of claudin-7 that regulates lung cancer progression is not clearly understood. In order to understand how claudin-7 is involved in lung carcinogenesis, this dissertation presents the molecular and cellular changes in human lung cancer cells upon the suppression of claudin-7 expression to study how claudin-7 modulates lung cancer cell progression, including cell proliferation, migration, and invasion, cell-matrix attachment, and cell metabolism.

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DEDICATION

To my parents, Young-Ho and Kil-Sook Kim,

and my younger brother, Do-Woong Kim

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LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl coenzyme A
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATP	Adenosine triphosphate
CD29	Integrin beta-1
CD4	Cluster of differentiation 4
CD44	Cluster of differentiation 44
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR-TK	Epidermal growth factor receptor tyrosine kinase
EMT	Epithelial mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FAK	Focal adhesion kinase
FasL	Fas ligand

FH	Fumarate hydratase (Fumarase)
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GMP	Guanosine monophosphate
GS	Glycogen synthase
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3 beta
Glut-1	Glucose transport 1 protein
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
IP_3	Inositol trisphosphate
KD	Knockdown
K-ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MDCK	Madin–Darby canine kidney
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NADPH	Nicotinamide-adenine dinucleotide phosphate
NSCLC	Non-small-cell lung cancer
N-WASP	Neuronal Wiskott–Aldrich Syndrome protein
OGD	Oxygen and glucose-deprived
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline

PC	Pyruvate carboxylase
PEP	Phosphoenolpyruvate
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PEPCK-M	Mitochondrial phosphoenolpyruvate carboxykinase
PH	Prolyl hydroxylase
PKM2	Pyruvate kinase isozymes M2
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
SCLC	Small-cell lung cancer
SDH	Succinate dehydrogenase
shRNA	Short hairpin RNA
TCA	Tricarboxylic acid
TJs	Tight junctions
VEGF	Vascular endothelial growth factor
3PG	3-phosphoglycerate

CHAPTER I. INTRODUCTION

A. Tight junction (TJ) and cell-cell communication

Cellular communication is necessary to receive, generate, and share diverse cell signals among cells for cellular events, such as cell growth, differentiation, homeostasis, and migration (reviewed in (1)). For example, cells give signals to themselves or neighboring cells (autocrine or juxtacrine) and communicate with other cells from short or long distances (paracrine or endocrine) (reviewed in (1)). In the case of a skin injury, normally penetrable cell junctions are closed adjacent to the wound sites, but emerging cell junctions upon wound closure gain permeability, suggesting that the cell junction is involved in cell communication (2). This suggests that cell junctions support cell survival. Conversely, the disruption of cellular junction could result in dysfunction of cellular function. It has been found that cancer cells lack cell-cell communication (3, 4). Thus, studying the function of cell junctions is crucial to understand the mechanism of diseases, including cancers, at the cellular level.

Cell junctions are divided into three categories depending on their role: occluding junctions, anchoring junctions, and communicating junctions (5-7). Occluding junctions are engaged in cell-cell interaction. Tight junctions (TJs) are a type of occluding junction. They mainly act as a physical barrier by sealing the apical side of neighboring epithelial cells to prevent fluid and small molecules from moving in and out through intercellular space between the neighboring cells (8). TJs also regulate the permeability of selective ions between the neighboring epithelial cells (9). TJs consist of occludins, zonula occludens (ZOs), and claudins (10). While occludin and claudin proteins both have four transmembrane domains (10), claudin proteins have 24 family members in humans (11). One of the extracellular loops of claudin proteins regulates ion permeability or

paracellular activity; however, the cytosolic carboxyl tail has been shown to interact with various signals, such as regulatory proteins, tumor suppressors, and transcription factors (11). This suggests different roles of claudin proteins beyond their usual paracellular activity.

Anchoring junctions help cells receive extracellular signals from extracellular environments, such as the extracellular matrix (ECM), through cell-matrix adhesion and respond to external environmental stimuli (12), and mechanical forces mediated by cell-cell adhesion and cell-matrix adhesion (13). Anchoring junctions build the resilient cellular structures between the cell membrane and ECM surface by connecting with either actin filaments or intermediate filaments (14, 15). For example, focal adhesion and hemidesmosome bind with transmembrane adhesion proteins, integrins, between the basal cell membrane and ECM (16). Integrins consist of 18α and 8β subunits, which give rise to 24 heterodimeric transmembrane receptors (17). The numerous heterodimeric integrins bind and interact with their various corresponding ECM components, such as collagens, laminin, vitronectin, collagen, and fibronectin (reviewed in (18)), suggesting that integrins transmit many signals depending on the context of the ECM.

Communicating junctions are also crucial to facilitate the intercellular communication between neighboring living cells (19). Gap junctions, a type of communicating junction, primarily consist of connexin subunits that create channels between neighboring cells (19). This allows adjacent cells to exchange cell signaling molecules, such as cyclic AMP, cyclic guanosine monophosphate (GMP), and inositol 1,4,5-triphosphoate (IP_3), and inorganic ions, including Ca^{2+} and Mg^{2+} (reviewed in (20)).

Although each distinct junctional complex comes together to orchestrate both physical and cellular physiological function to ensure cell survival, as discussed above, recent studies have been extensively focused on understanding the effect of the dysregulation of claudins and integrins on

the progression of diseases, including cancers (21-23).

B. Claudin expression in human cancer cells

Claudin expression has been connected to epithelial-mesenchymal transition (EMT) (24, 25). A research study focusing on claudin-1 has revealed that the *in vitro* expression of transcription factors, including Snail and Slug, has repressed claudin-1 expression in kidney cell lines and disrupted the epithelial cellular structures, resulting in the loss of other cell junction proteins, such as ZO-1 and E-Cadherin (24). The authors also conducted a follow-up study using breast cancer cell lines and human breast cancer tumor samples to demonstrate the inverse relationship between Snail and Slug levels and claudin-1 expression, suggesting that claudin regulates cancer progression.

In addition, claudin expression has been linked to cancer promotion and progression, including cell proliferation, migration, and invasion. The overexpression of claudin-6, -7, and -9 in human gastric cancer cell lines has been shown to promote cell proliferation, migration, and invasion *in vitro* (26). In addition, epidermal growth factor (EGF)-induced claudin-3 overexpression in colorectal cancer cell lines has been shown to increase cell proliferation, migration, and invasion abilities and anchorage-independent colony formation, showing the co-regulation of claudin-3 and EGFR signaling in cancer cell proliferation (27).

More importantly, claudins have also been closely related to cancer malignancy, such as cancer metastasis. The suppression of claudin-3 or claudin-4 expression *in vitro* in human carcinoma cell lines has been shown to increase cancer cell invasion *in vitro*, and the elevated metastatic ability was evidenced by micrometastatic lesions found in mouse lung tissues *in vivo* (28). However, knockdown of claudins does not always result in increasing cell invasion. Claudin-

7 knockdown (KD) in ovarian cancer cell lines has decreased cell invasion (29). These findings suggest that the correlation between claudin expression and cancer malignancy, including migration and invasion, is specific to certain cancer cell types.

Recent reports have revealed evidence that claudin proteins actively regulate cancer cell metastasis. The suppression of claudin-2 expression *in vitro* in human breast cancer cell lines has demonstrated that claudin-2 KD reduces breast cancer cell adhesion on claudin-2-expressing human hepatocytes (30). The author also demonstrated that disrupted expression in an extracellular loop of claudin-2 in breast cancer cell lines decreases the liver metastasis ability in the mouse liver *in vivo* (30), suggesting the role of claudin in regulating cancer cell adhesion and metastasis. Similarly, it has been clinically reported that the low survival rate of breast cancer patients is strongly associated with the high claudin-5 expression levels in the breast tissues of those patients (31). Furthermore, the same study has demonstrated that claudin-5 overexpression in human breast cancer cell lines increases cell proliferation, migration, and invasion *in vitro,* as evidenced by co-immunoprecipitation results showing a physical linkage between claudin-5 and neuronal Wiskott–Aldrich Syndrome protein (N-WASP), which suggests the function of claudin-5 in breast cancer cell metastasis. All these findings suggest that claudin differently regulates cancer metastasis in various cancer cell types.

C. Interconnection between claudin-7 and human lung cancer

Human lung cancer is the second leading cause of cancer patients' death in the United States (32). About 85% of human lung cancer cases are categorized as non-small-cell lung cancer (NSCLC) types, while the remaining 15% are categorized as small-cell lung cancer (SCLC) types (33). While the majority of lung cancer incidences are attributed to tobacco smoking, astonishingly,

10% of lung cancer patients in the United States have never smoked (reviewed in (34)). Numerous molecular epidemiological research studies have been conducted to identify the potential causes of lung cancer incidences from genetic mutations, including *p53*, epidermal growth factor receptor tyrosine kinase (*EGFR-TK*), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-ras*), and deoxyribonucleic acid (DNA) methylation (reviewed in (34)). However, little has been discovered regarding the cellular or molecular mechanisms of lung cancer metastasis until recently. A clinical research team has recently found that low claudin-7 expression in lung cancer patients has a close association with the low survival rate of the patients five years after physical operations (35), suggesting that the loss of claudin-7 expression may promote the malignancy of lung tumors.

D. Function of claudin-7 as basal membrane protein

Unlike other TJ proteins localized at the apical side of epithelial cells, claudin-7 has been reported to be largely localized at the basolateral membrane of epithelial cells in many human and murine organ tissues, including the uterus, kidneys, murine mammary gland and murine mammary tumors, as well as intestines (36-39). It has been demonstrated that the loss of claudin-7 in intestine tissues in claudin-7 knockout mice leads to the disintegration of the intestinal mucosa and loss of α2 integrin (40), suggesting that claudin-7 stabilizes the cell-matrix attachment through integrins. More importantly, claudin-7 appears to be engaged in complex physiological activity. It has been shown that claudin-7 expression levels are decreased in rat uterine luminal epithelial cells during early pregnancy (36). Moreover, the expressions of focal adhesion proteins, including Talin and Paxillin, were decreased on day 1 of rat pregnancy (41). These studies on pregnant rats suggest that claudin-7 regulates focal adhesion during pregnancy. The findings discussed above suggest that the basal function of claudin-7 and integrins not only establishes cell-matrix adhesion but also

participates in physiological activity.

E. Integrins in human cancers

As briefly discussed above, claudin-7 and integrins seem to contribute to the establishment of cell-matrix adhesion. Integrin heterodimers are also located at the basal side of epithelial cells, and they bind with the ECM and transduce extracellular signals (17, 18, 42). Transformed cancer cells have resulted in changes in the composition of heterodimeric integrin expressions that have been found to regulate tumor cell proliferation, tumorigenesis, and metastasis in a variety of human cancers (43), suggesting different effects on carcinogenesis depending on integrin heterodimer and cancer cell type. In addition, integrins form integrin adhesion complexes, such as focal adhesion complexes, by recruiting Talin, Vinculin, and Focal Adhesion Kinase (FAK) (reviewed in (42)), and the integrin adhesion complexes facilitate actin polymerization and assemble with actin filaments (44). This suggests that integrin adhesion supports cell migration and invasion, which could support cancer metastasis.

During cancer cell migration, integrin adhesion complexes extend their target ECM surface by extending actin filament skeletons and forming FAK through integrin clustering at the cell-matrix interface (45, 46), while integrins also sense extracellular environmental signals and regulate intracellular signaling pathways, including MAPK and protein kinase B (Akt) pathways, for cell survival (47). Evidently, cell-matrix adhesion is necessary in cancer cell invasiveness, as integrins first form focal adhesion complexes and create invadopodia precursors by building actin filament–cytoskeletal protrusions (48). Once the invasive precursors are changed to matured invadopodia (49), the matured invadopodia extend the invasive actin cytoskeletal protrusions toward the target ECM while activating proteolytic enzymes (48-50), such as MMP-2 and MMP-

9 that degrade the target ECM basement membrane during cancer metastasis (51). For the cancer dissemination process through blood circulation, cancer cells need to detach from the ECM (52) and overcome anoikis, a type of cell apoptosis induced by the disruption of cell–ECM interaction (53). Integrins appear to be one of the major players in modulating cancer metastasis.

F. Multiple stages of carcinogenesis

Claudin has recently been highlighted for regulating carcinogenesis in human cancer cells, as previous reviews of human cancer cell research demonstrate the roles of several claudin proteins in controlling metastatic potential, including cancer cell proliferation, migration, and invasion. The stages of cancer development were initially proposed and investigated by Berenblum and Schubik in 1948 (54). The process of carcinogenesis has been thought to involve three main steps, including cancer initiation, promotion, and progression (Fig. I.1.) (54). In cancer initiation, exposure to carcinogens can give rise to spontaneous cellular changes by accumulating mutations in cellular genomes and creating reactive oxygen species (ROS) that damage mitochondrial and nuclear DNA (55). Little is currently known about how changes in claudin expression lead to such DNA damages in cancer initiation.

In cancer promotion, the cells containing the initially mutated gene undergo clonal selection by propagating the population of daughter cells harboring the initial gene mutations, while they also possibly go through further gene mutations and chromosomal aberration that could establish tumor cells (54, 56). In the process, initially mutated cells are continuously transformed by accumulating additional mutations in proto-oncogenes, tumor suppressors, and DNA repair genes, all of which, in combination, lead to accelerated cell growth, suppressed cell apoptosis, and impaired DNA repair systems (56). For example, this phase of cancer progression includes

frequent mutations in genes such as *p53* (tumor suppressor) and *K-Ras* (oncogene), as previously discussed in molecular epidemiology research identifying the causative gene mutations of lung cancer patients (34). We previously discussed that changes in the expression of several claudin proteins alters cancer cell proliferation, migration, invasion, and metastasis in various human cancer cells, suggesting that claudin modulates cancer promotion and progression.

As tumor cells progress to develop more malignant subpopulations, they undergo angiogenesis and cell transformation, and develop metastatic potential (54). Rapidly growing tumor cells create tumor microenvironments where tumor cells need to receive more oxygen and nutrients for cell survival (57). Angiogenesis occurs to provide tumors with new vessels to supply enough nutrients and oxygen to keep up with the fast growth of tumor cells (57). In addition, tumor microenvironments increase the frequency of gene mutations, which also contributes to malignant transformations (58, 59). This could also promote tumor invasiveness. To initiate cancer metastasis, tumor cells need to overcome anoikis, a type of cell apoptosis induced by cell detachment from the ECM (60). A few claudin proteins have been documented to augment the resistance of tumor cells against anoikis. For example, in cell suspension, both colon cancer cell lines endogenously expressing claudin-1 and those exogenously expressing claudin-1 noticeably increased their resistance to cell death, and promoted downstream signaling for cancer cell survival, when compared to those cells with low claudin-1 expression (61), suggesting that claudin-1 expression suppresses anoikis in colon cancer cells.

The aforementioned discussion suggests that changes in claudin expression may contribute to cancer cell initiation, promotion, and progression, which also alters cell signaling to determine cell fates, including cancer cell survival and death in tumor microenvironments and during cancer metastasis.

G. Cancer metabolism

Tumor microenvironments, which usually constitute low-oxygen and low-nutrient conditions, change cancer cell metabolism, which generates cellular energy to sustain rapid tumor cell growth (62). Under hypoxic conditions, hypoxia inducible factor (HIF) is stabilized, and it facilitates aerobic glycolysis to provide tumor cells with cellular energy necessary for rapid tumor growth (63). Aerobic glycolysis, or Warburg's effect, expedites glucose uptake and its breakdown to pyruvate to produce lactate and two adenosine triphosphates (ATPs) while evading oxygen respiration by bypassing tricarboxylic acid (TCA) cycles. However, normally growing non-cancer cells convert pyruvate into acetyl coenzyme A (acetyl-CoA) that is completely oxidized by oxygen respiration to produce an additional 34 ATPs through TCA cycles in mitochondria (64). It has not been demonstrated whether claudin proteins modulate glucose metabolism in cancer cells to date.

The above discussion suggests that the tumor microenvironment plays important roles in regulating glucose metabolism for cancer cell survival during cancer cell progression.

H. Preliminary experiment

H.1. *In vivo metastatic lung tumor mouse model*

Athymic mice were purchased from Charles River Laboratory (Wilmington, NC). Adherent HCC827 human epithelial lung cancer cells were chosen, as they were derived from human lung epithelial tissues of a female NSCLC patient. A total of 2×10^6 HCC827 claudin-7 control or KD cells were suspended in 100 μl of phosphate buffer saline (PBS) and were intravenously administered via the lateral tail veins of 11 17–18-week-old mice. Changes in the body weights of mice were monitored for 4 weeks after the initial injection. Then, an additional

4×10^6 HCC827 claudin-7 control or KD cells were administered to the respective experimental mice group, and their body weights were monitored for an additional 4 weeks. Finally, 8 weeks after the initial tail vein injections, all the mice were sacrificed. Their major organs, including lungs and livers, were removed for necropsy and fixed in 10% formalin solution for preservation. This experimental design was adopted and modified from (65) in communication with Dr. Hye-Jung Han at Berkeley National Laboratory. All experimental procedures using athymic mice subjects were performed according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at East Carolina University (AUP #A-172b) (See Appendix A).

I. Preliminary experimental data that led to develop my Specific Aims

I.1. *HCC827 claudin-7 control or KD cells did not form metastatic tumors in mouse study.*

There were no symptoms of metastatic tumor formation such as shortness of breath and no significant reductions of body weight in either mouse group injected with HCC827 control or claudin-7 KD cells. Likewise, no metastatic nodules or micrometastatic lesions were found from the mice lungs and livers under a dissecting microscope upon necropsy (No publishable data available).

J. Rationale for current study

It has been clinically documented that lower claudin-7 expression has been closely correlated with a lower survival rate of lung cancer patients (35), suggesting that the loss of claudin-7 may aggravate the malignancy and invasiveness of lung cancers.

Our previous and recent studies have documented that the KD of claudin-7 in HCC827

human lung adenocarcinoma cancer cells (HCC827 claudin-7 KD cells) increases their cell proliferation but that claudin-7 overexpression in HCC827 claudin-7 KD cells reduces their cell proliferative rate to one comparable to that of HCC827 control cells (66). Cell cycle analysis has also confirmed that claudin-7 KD cells increase the dividing mitotic cell phase and reduce cell apoptosis, which was also evidenced by Western blot analysis showing a reduction in cleaved poly ADP ribose polymerase (PARP) and an increase in survivin (66). These results suggest that claudin-7 may play a role as a tumor suppressor. In contrast, the *in vivo* data obtained from mouse tumors subcutaneously injected with HCC827 caudin-7 KD cells *in vivo* showed an increase in both cell apoptotic factor (cleaved PARP) and anti-apoptotic factor (survivin), although the mouse tumor cells were much larger than the control cells (66). An *in vitro* cell culture system may allow HCC827 claudin-7 KD cells to freely access the oxygen and glucose that prevent cell apoptosis (66). However, little is known regarding how mouse tumor growth induced by HCC827 KD cells persists despite ongoing apoptotic activity in tumor microenvironments, where altered glucose metabolism could promote the survival of mouse tumors induced by HCC827 claudin-7 KD cells.

Since HCC827 claudin-7 KD lung cancer cells demonstrated greatly elevated metastatic potential based on their predominant tumor growth and survival *in vitro* and *in vivo* as well as cell detachment from the ECM *in vitro* when compared to the claudin-7 control cells (66), the claudin-7 KD cells were believed to have metastasized more predominantly than the control cells *in vivo*. However, our following *in vivo* metastatic mouse tumor study showed no significant change in the body weight of nude mice intravenously injected with either HCC827 claudin-7 control or KD cells up to a total of 8 weeks after the initial injection (Fig. I.2.), which was further confirmed by necropsy revealing no metastatic nodules or micrometastatic lesions in the tissues of sacrificed mice, including lung, liver, and lymph node tissues (unpublished data). In (67), HCC827 cell lines

could not generate bone metastatic lesions, whereas the more malignant lung cancer cell line H522 did in an *in vivo* mouse study, suggesting the possibility that using highly metastatic lung cancer cells with claudin-7 KD might metastasize more than those control cells *in vivo*.

The above discussion supports the hypothesis that HCC827 claudin-7 KD cells have increased metastatic potential, such as accelerated cell proliferation and cell cycle progression, cell detachment from the ECM, and reduced cell apoptosis (66). However, no previous study has reported the claudin-7 KD phenotype in other human lung cancer cells. In addition, we are not aware of whether the tumor microenvironment mediates claudin-7 regulation in glucose metabolism to support cell survival, which could accelerate mouse tumor growth *in vivo*. Moreover, our previous study identified that claudin-7 formed a protein complex with β1 integrin and was partially co-localized with β1 integrin at the basolateral side of HCC827 control cells and that the expression of both proteins disappeared in HCC827 claudin-7 KD cells (66). These findings suggest the co-regulation of claudin-7 and β1 integrin in cell-matrix attachment. β1 integrin is a ubiquitously present integrin that is engaged in cell-matrix adhesion and cell motility. Claudin-7 is also present at the basal side of epithelial tissues of several organs, as previously discussed (36-39). However, little is known about how claudin-7 and β1 integrin cooperate with each other and co-regulate lung cancer cell migration, invasion, and attachment at the cell-matrix interface.

Therefore, we propose that claudin-7 inhibits cell proliferation and serves as a tumor suppressor. KD of claudin-7 promotes cell survival under tumor microenvironment conditions. Claudin-7 cooperates with β1 integrin in regulating cell-matrix adhesion as well as cell migration, invasion, and attachment.

K. Specific aims

Aim 1. Determine claudin-7 KD phenotype in regulating cell proliferation and cell attachment in H358 human lung cancer cell lines.

Hypothesis: Claudin-7 KD in H358 human lung cancer cell lines increases cell proliferation and survival and reduces cell attachment, similar to the KD of HCC827 human lung cancer cells

It has been previously shown that claudin-7 KD in HCC827 human lung cancer cell lines increases cell proliferation and cell survival while reducing cell attachment (66). However, it remains unanswered whether claudin-7 KD in other human lung cancer cell lines also gives rise to the same outcomes. H358 cells are human epithelial lung cancer cells showing basal localization of claudin-7. We will treat H358 human lung cancer cells with the same lentiviral vector containing short hairpin RNA (shRNA) to suppress claudin-7 gene expression and then characterize the cellular phenotypic changes. We will focus on how the suppression of claudin-7 in the H358 cell lines modulates the cell proliferative rate, cell survival, and cell attachment and whether claudin-7 KD alters the protein expression of β1 integrin. Results from this aim will confirm whether claudin-7 KD in other human lung cancer cell lines expressing endogenous claudin-7 gives rise to cellular phenotypes similar to our previous findings of increased cell proliferation and reduced cell-matrix attachment in HCC827 human lung cancer cell lines with claudin-7 KD.

Aim 2. Characterize the role of β1 integrin in cell proliferation, migration, invasion, and attachment in HCC827 human lung cancer cells with claudin-7 KD.

Hypothesis: The overexpression of β1 integrin in HCC827 claudin-7 KD cells improves cell migration, invasion, and attachment but not cell proliferation.

Preliminary data from Aim 1 suggest that β1 integrin is co-regulated with claudin-7 by forming a

protein complex localized at the basolateral side of HCC827 human lung cancer cells. It is not clear whether β1 integrin and claudin-7 regulate cellular phenotypes, including cell proliferation, migration, invasion, and attachment synergistically or independently. We will stably transfect a β1 integrin complementary DNA (cDNA) vector into the claudin-7 KD of HCC827 human lung cancer cells to overexpress β1 integrin, and then, the cellular functions will be assayed using control and claudin-7 KD and β1 integrin-overexpressing claudin-7 KD cells. The results from this aim will clarify how β1 integrin and claudin-7 differentially regulate focal adhesion, cancer cell migration, and invasion in regulating cancer cell progression.

Aim 3. Characterize the effect of claudin-7 KD on cancer cell survival via key genes regulating glucose metabolism.

Hypothesis: Claudin-7 modulates the expression of key genes responsible for altering glucose metabolism in favor of cancer cell survival through GS kinase in tumor microenvironment conditions.

Our recent *in vivo* metastatic study in nude mice (Fig. I.2) indicates that cancer cell lines derived from human primary tumor sites, such as HCC827, do not yet have high metastatic abilities, while cancer cell lines derived from metastatic tumor sites, such as H522, usually already have metastatic abilities. Therefore, the tumor microenvironment *in vivo* must provide the tumor cells with cues or signals to move them from the non-metastatic to the metastatic state. In addition, preliminary findings from Aim 1 indicate that nude mice injected with HCC827 human lung cancer cells with claudin-7 KD exhibit expedited tumor growth in terms of both size and weight. It is not clear how KD cell tumor growth persists in the presence of apoptotic factor (cleaved PARP), although anti-apoptotic factor (survivin) is also induced. We will characterize how the claudin-7 KD contributes

to accelerated tumor cell growth at the tumor promotion stage under the tumor microenvironment. We will treat the claudin-7 KD of HCC827 human lung cancer cells under hypoxia and low glucose conditions in cell cultures to mimic the tumor microenvironment *in vivo*. This will determine whether claudin-7 KD lung cancer cells maintain the elevated levels of inhibitory phosphorylated glycogen synthase kinase 3β (GSK3β) required for cancer cell survival in regulating glucose metabolism.

Carcinogenesis

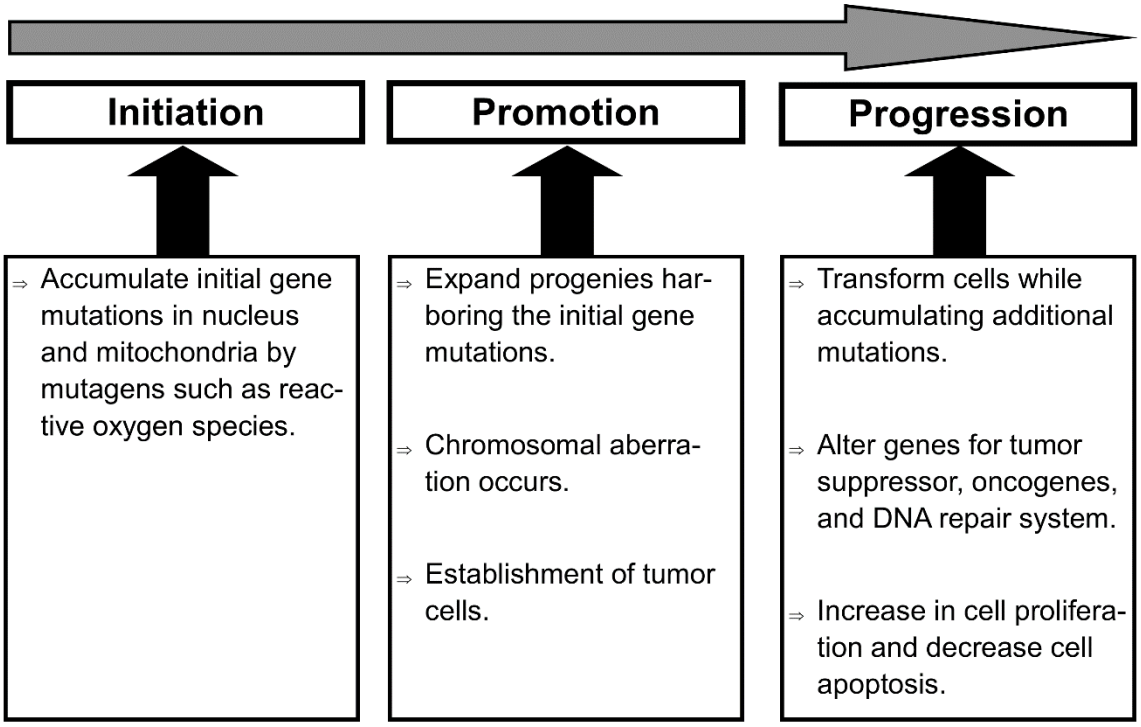

Figure I.1 Carcinogenesis

Carcinogenesis usually consists of cancer initiation, promotion, and progression. Cancer initiation occurs with the accumulation of mutations in nuclear and mitochondrial DNA by mutagens, such as ROS. In cancer promotion, the cells with the initial gene mutation are selectively propagated while chromosome aberration occurs, which could establish tumor cells. As the cancer cells progress, they are further transformed while adding more mutations on genes and surveilling genome integrity, cell proliferation, and DNA repair mechanisms. Alteration on these genes contributes to accelerating cell proliferation and decreasing cell apoptosis.

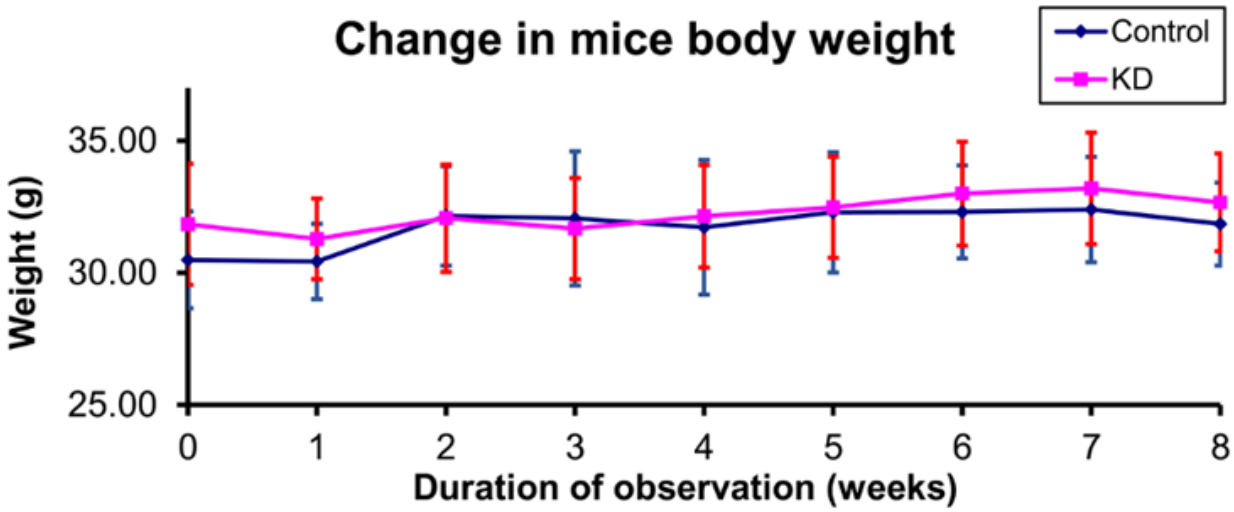
Change in mice body weight
Control
KD
Weight (g)
35.00
30.00
25.00
0
1
2
3
4
5
6
7
8
Duration of observation (weeks)

Figure I.2 *In vivo* metastatic study using athymic nude mice

In total, 2×10^6 HCC827 control or claudin-7 KD cells were intravenously administered to 11 17–18-week-old athymic mice through their lateral tail veins. Up to the first 4 weeks after the initial injections, there was no substantial reduction in body weight as a metastatic behavioral symptom from either mice group administered with claudin-7 control or KD cells. Thus, second injection of 4×10^6 HCC827 claudin-7 control or KD cells were administered to the experimental mice group. Still, their body weights still revealed no remarkable changes during the second 4 weeks. After the total of 8 weeks, all the mice were sacrificed. Their major organs, including lungs and livers, were removed for necropsy and fixed in 10% formalin solution for preservation. No metastatic nodules or micrometastatic lesions were found from the mice lungs or livers under a dissecting microscope upon necropsy (Unpublished data). All experimental procedures using athymic mice subjects were performed according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at East Carolina University (AUP #A-172b) (See Appendix A).

CHAPTER II. A NON-TIGHT JUNCTION FUNCTION OF CLAUDIN-7 – INTERACTION WITH INTEGRIN SIGNALING IN SUPPRESSING LUNG CANCER CELL PROLIFERATION AND DETACHMENT

This chapter is modified and reprinted from a journal article previously published on June 17, 2015, in *Molecular Cancer*, 14, 120. (2015) ((66), DOI:/10.1186/s12943-015-0387-0) under Creative Commons Attribution License 4.0 adapted by license agreement of BioMed Central and with permission of all other co-authors on the fair and appropriate use of the contents of this manuscript. As the first co-author of this manuscript, I revised and edited the manuscript, including the introduction, materials and methods, results, and discussion sections. I also conducted experiments to generate the revised figures and the "supplementary data" of this manuscript (See Appendix B).

A. Summary

Claudins are a family of TJ membrane proteins involved in a broad spectrum of human diseases, including cancer. Claudin-7 is a unique TJ membrane protein, in that it has a strong basolateral membrane distribution in epithelial cells and tissues. Therefore, this study aims to investigate the functional significance of this non-TJ localization of claudin-7 in human lung cancer cells. Previously, we found that HCC827 knockdown (KD) cells showed an increase in cell cycle and cell proliferation and a reduction in cell apoptosis, cell attachment, and β1 integrin expression. We also confirmed that claudin-7 formed a protein complex with β1 integrin and co-localized with β1 integrin at the basolateral side of HCC827 control cells. However, their presence disappeared in HCC827 KD cells. In addition, our *in vivo* mouse tumor study confirmed larger

tumor growth in mice injected with HCC827 KD cells when compared to those injected with HCC827 control cells. In order to confirm whether the claudin-7 KD phenotype found in the HCC827 cell line is also found in other human lung cancer cell lines, we generated the claudin-7 KD phenotype in NCI-H358 human lung cancer cell lines using the same lentiviral shRNA empty vector (H358 control cells) or shRNA vector targeting the claudin-7 gene (H358 KD cells). We reproduced the claudin-7 KD phenotype in NCI H358 cell lines showing an increase in cell proliferation and a reduction in cell apoptosis and cell attachment. Claudin-7 and β1 integrin were also partially co-localized of and formed a protein complex in H358 control cells. These similar findings further support the hypothesis that claudin-7 KD increases the cell proliferative rate and decreases cell attachment by interacting with β1 integrin.

B. Introduction

Lung cancer is the leading cause of cancer death in the United States, and the five-year survival rate is 15% (33). The majority of lung cancer evolves from lung epithelial lining the air passages. TJs are the most apical component of the junctional complex, and they provide the critical support of cell-cell adhesion in epithelial cells (68). The disruption of cell adhesion promotes cancer progression, invasion, and metastasis (11, 69). TJs serve as a barrier regulating the ions and small molecules through the paracellular pathway (70). Both the downregulation and upregulation of TJ proteins can alter the organized arrangement of TJ strands, allowing more nutrients and growth factors to cancerous cells, thus acquiring the cells with invasive behavior (71).

Claudins are tetraspan proteins with molecular weights of 20–27 kDa (72), and they are the major structural and functional components of TJs. Abnormal expression and mislocalization of claudins are frequently observed in epithelial-derived cancers (73-76). Moldvay et al. have

analyzed the expression profile of different claudins in lung cancers and found that claudin-7 is downregulated in several types of lung cancers, including squamous cell carcinoma, at the messenger RNA (mRNA) level (77). Our previous study demonstrates that claudin-7 is strongly expressed in benign bronchial epithelial cells with a predominant cell-cell junction staining pattern, while it is either altered with discontinued weak expression or completely absent in lung cancers (78). However, the exact roles of claudin-7 in lung tumorigenesis are largely unknown.

Although claudins are well-known apical TJ proteins, recent antibody-based studies have indicated that several claudins, including claudin-7, are not only localized at the apical TJs but also have a strong basolateral membrane distribution in the epithelial cells of various tissues (37, 40, 79). These observations suggest that claudins could be involved in cell-matrix interactions. The principal proteins at the basolateral membrane responsible for anchoring cells to ECM proteins are integrins (80). Integrins are heterodimers with α and β subunits that play essential roles in cell attachment, survival, migration, and invasion (81, 82).

In this study, we revalidated our previous finding from HCC827 claudin-7 KD cells (66), in that claudin-7 also co-localized and formed a protein complex with β1 integrin in H358 human lung cancer cells. The suppression of claudin-7 not only promoted cell proliferation but also disrupted the localization and downregulated the expression of β1 integrin at the protein level, resulting in severe defective cell attachment. Thus, our current findings further support the non-TJ function of claudin-7 in regulating cell attachment through β1 integrin in human lung cancer cells.

C. Materials and methods

C.1. *Antibodies and reagents*

The rabbit polyclonal anti-claudin-7 antibody (Cat. 18875) was obtained from Immuno-

Biological Laboratories (Japan). The rabbit polyclonal anti-phospho-FAK (Cat. 3283S), anti-phospho-extracellular signal-regulated kinase (ERK)1/2 (Cat. 9101S), anti-ERK 1/2 (Cat. 4695), and anti-cleaved PARP (Cat. 5626) were purchased from Cell Signaling Technology (Beverly, MA). The mouse monoclonal and the goat polyclonal anti-β1 integrin antibodies were purchased from BD Biosciences (Cat. 610467, Bedford, MA) and Santa Cruz Biotechnology (Cat. sc-6622, Santa Cruz, CA), respectively.

C.2.*Cancer cell culture and lentivirus shRNA KD of claudin-7*

Lung carcinoma cell line NCI-H358 (H358) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 culture medium containing 10% FBS, 100 units/ml of penicillin, and 100 μg/ml streptomycin in a humidified air (5% CO_2) atmosphere at 37^oC. H358 cells were epithelial human lung cancer cells and expressed claudin-7 and β1 integrin, similar to HCC827 human lung cancer cells. Three lentivirus claudin-7 shRNA vectors were found as following nucleotide sequences (sequences #1: 5'-TTCCAAGGAGTATGTGTGA-3'; #2: 5'-GGCTATGGGAGTGTCTAGA-3'; #3: 5'-TCCCTACCAACATTAAGTA-3'). The #2 lentivirus claudin-7 shRNA vector was used to target claudin-7 in HCC827 cells (66). Likewise, the same #2 lentivirus claudin-7 shRNA vector was also introduced to suppress claudin-7 expression of H358 cells and generate H358 claudin-7 KD cells. All the lentivirus vectors contained a GFP expression sequence and puromycin resistance gene (66). Thus, H358 cells transfected with an off-target shRNA were designated as H358 claudin-7 control cells and showed GFP expression as positive controls for successful lentivirus transfection. After 48 h incubation, successfully stably transfected cells were selected in 1 μg/ml puromycin. The stable transfects displayed green fluorescence, indicating successful lentivirus infection as positive controls.

Similar to HCC827 KD cells, 80% of claudin-7 expression was also decreased in H358 KD cells when compared to the control cells.

C.3.*Cell number count and cell cycle analysis by flow cytometry*

A total of 5×10^3 H358 control or claudin-7 KD cells were seeded into each well of 24-well culture plates and then trypsinized on days 2, 4, and 6 after plating. A total of 1×10^5 6-micron AlignFlow Plus beads (Molecular Probes, Eugene, OR) were added to each sample, and the relative ratio of beads versus cells was obtained by the flow cytometer. The total cell number for each sample was then calculated.

HCC827 control or claudin-7 KD cells untreated or treated with 5 μg/ml aphidicolin for 24 h were harvested, washed with PBS, and fixed in 70% ethanol. Prior to analysis, cells were washed with PBS and then resuspended in the propidium iodide staining solution. Samples were analyzed on the flow cytometer.

C.4.*Immunofluorescence*

H358 control or claudin-7 KD cells grown on poly-D-lysine-coated glass coverslips (BD Biosciences) were fixed in 100% methanol for 8 min at -20ºC and washed with PBS for 5 min before being blocked in 5% bovine serum albumin for 60 min at room temperature. After blocking, cells were incubated with primary antibody (dilution ratio 1:100). All antibodies were diluted in PBS containing 2.5% bovine serum albumin (BSA). After washing, cells were incubated with corresponding secondary anti-rabbit FITC-conjugated (Green) or anti-mouse Cy3-conjugated (Red) antibody (dilution ratio 1:400) for 45 min at room temperature. Staining cells only with either secondary antibody displayed no fluorescence signals as negative controls. Coverslips were

mounted with ProLong Antifade Kit (Molecular Probes). Samples were photographed using a Zeiss Axiovert S100 or Zeiss LSM 510 laser confocal scanning microscope (Carl Zeiss Inc., Thornwood, NY) and analyzed by MetaMorph software (Molecular Devices, Sunnyvale, CA).

C.5.*Co-immunoprecipitation*

H358 control cells were washed three times with ice-cold PBS and then lysed in RIPA buffer. After centrifugation, the supernatants were incubated with either anti-claudin-7 or anti-β1 integrin antibody at 4ºC overnight. Protein A or protein G beads were then added to the mixture, followed by incubation at 4ºC for 3 h. The beads were washed twice with RIPA buffer, once with high salt buffer (0.5 M NaCl), and once with Tris buffer (10mM Tris, pH 7.4). Bound proteins were eluted from the beads in SDS sample buffer and analyzed by Western blot. For the positive control, protein lysates were immunoprecipitated overnight with either primary anti-claudin-7 rabbit antibody or anti-β1 integrin mouse antibody. Then, each lysate was subject to Western blot analysis and was successfully detected using the same primary anti-claudin-7 rabbit antibody or anti-β1 integrin mouse antibody at 22 kDa or 130 kDa in size, as shown in Fig. II.6.B.

C.6.*Process of mouse tumors harvested from in vivo tumor xenograft model*

An *in vivo* mouse tumor growth study was previously conducted. In brief, five-week-old male athymic nude mice were obtained from Charles River Laboratory and used for human tumor xenografts. A total of 2×10^6 HCC827 control or claudin-7 KD cells were suspended in the culture medium and injected subcutaneously into the left and right flanks of each nude mouse. All the mice were sacrificed 8 weeks after injection. The tumors were removed, weighed, and frozen at -80°C. The animal experiments were performed according to the animal use protocol (AUP)

approved by East Carolina University. The frozen mouse tumors were ground using a pestle under liquid nitrogen and then lysed in RIPA buffer to prepare for Western blot analysis.

C.7.*SDS-PAGE and Western blot*

Whole cells were lysed in RIPA buffer (1% Triton-100, 0.5% deoxycholate, 0.2% sodium dodecyl sulfate, 150 mM sodium chloride, 2 mM ethylene diamine tetraacetic acid, 10 mM sodium pyrophosphate, 20 mM sodium fluoride) supplemented with a complete protease inhibitor cocktail tablet (Cat. 11836153001, Roche Diagnostics, Indianapolis, IN). After cell debris from the protein lysate was removed by centrifugation at 4°C, the protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Cat. 23225, Thermo Scientific, Grand Island, NY). Proteins (20 μg per lane) were separated by SDS-PAGE gel, transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare) by electrophoresis, and blocked and immuno-blotted with appropriate primary antibodies (dilution ratio 1:1000, see section C.1.) followed by peroxidase-conjugated secondary antibodies (dilution ratio 1:2500). Protein bands were visualized using an ECL detection reagent (GE Healthcare, Buckinghamshire, UK) and photographed using an X-ray film developer.

C.8.*Statistical analysis*

Statistical analysis was performed using Origin50 (OriginLab, MA) or SigmaPlot (SPSS Science, Chicago, IL) software. For each *in vitro* experiment, at least three independent experiments were performed. All data were recorded and expressed as means ± SE. The differences between two groups were analyzed using the unpaired Student's *t*-test. All statistical tests were two-sided, and a value of $P < 0.05$ was considered significant (*).

D. Results

D.1. *Increased cell proliferation in claudin-7 KD cells*

Our previous results confirmed the successful KD of claudin-7 using a #2 shRNA vector against claudin-7 gene expression in HCC827 cells (66). Using the same viral vector, we have also knocked down claudin-7 expression in NCI-H358 (H358) human lung cancer cells (Fig. II.1).

As it has previously been found that claudin-7 KD in HCC827 cells increases the cell proliferative rate (66), we performed a cell counting experiment to investigate the cell growth rates of both H358 control and claudin-7 KD cells. After claudin-7 was knocked down in H358 cells, the cell proliferation rate increased. Starting from day 4, the number of claudin-7 KD cells was more than that of control cells, and the number of claudin-7 KD cells was almost twice that of control cells on day 6 (Fig. II.2.A). Thus, we confirmed that claudin-7 expression was associated with decreased cell proliferation.

Then, we examined several proteins involved in cell proliferation, survival, and apoptosis by Western blot. Similar to the previous Western blot results from HCC827 claudin-7 KD cells (66), H358 claudin-7 KD cells showed increased levels of phospho-ERK1/2, phospho-Bcl-2, and survivin but decreased levels of cleaved PARP when compared to H358 control cells (Fig. II.2.B).

To confirm our previous finding that cell apoptosis was reduced as indicated by decreased levels of cleaved PARP, we further investigated the reduced cell apoptosis of HCC827 claudin-7 KD cells (66) by performing TUNEL (terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling) staining in both HCC827 control and claudin-7 KD cells. Although the total population of apoptotic cells in both HCC827 control and claudin-7 KD cells was low, the claudin-7 KD cells showed a lower percentage of apoptotic cells than the control cells, as shown in Fig. II.3.

D.2.*Impaired cell attachment and decreased β1 integrin expression in H358 claudin-7 KD cells*

Our previous study demonstrated that claudin-7 KD in HCC827 human lung cancer cells showed defects in cell adhesion and attachment when compared to control cells. Thus, in this current study, we examined whether claudin-7 KD also weakened cell adhesion and attachment of H358 lung cancer cells. We found that H358 claudin-7 KD cells formed spheroidal colonies when plated on uncoated glass coverslips, whereas control cells adhered well after 2- and 5-days plating on the glass surface (Fig. II.4.A). Cell attachment assay results also revealed that only 15% of H358 KD cells were attached to the culture plate when compared to the control cells four h after plating on the culture plate (Fig. II.4.B). These results suggest that claudin-7 KD also results in losing cell adhesion and attachment, similar to the previous HCC827 claudin-7 KD study.

The observed defect in cell attachment upon claudin-7 KD also indicates a non-TJ function of claudin-7 in the cell-matrix adhesion of human lung cancer cells. Among the various transmembrane protein domains, integrins are the principal receptors responsible for binding the cells to the ECM (81, 82). In our previous study, we found that β1 integrin was greatly decreased at both mRNA and protein levels, and the phosphorylation level of FAK was also substantially decreased at the protein level in HCC827 claudin-7 KD cells. Similarly, our previous claudin-7 knockout mouse lung tissues also showed a reduction in signals of β1 integrin and phospho-FAK. All these results suggest that claudin-7 modulates β1 integrin and phospho-FAK.

In order to rule out co-localization of claudin-7 with β1 integrin in HCC827 control cells due to bleed-through of immunofluorescent signals, single immunofluorescent staining of β1 integrin or claudin-7 on HCC827 cells was performed (Figs. II.5.A and II.5.B). The images suggest that both claudin-7 and β1 integrin appear to be localized at the cell-cell contact area in the

HCC827 control lung cancer cells. Next, we determined whether the respective fluorescence signals of claudin-7 and β1 integrin were found by performing separate immunofluorescence staining with their corresponding anti-secondary antibody-only in HCC827 control cell culture. The weak auto-fluorescence images ruled out the possibility of nonspecific binding of either anti-secondary antibody to HCC827 control lung cancer cells (Figs. II.5.A and II.5.B).

Thus, we were able to perform double immunofluorescence staining using primary antibodies against claudin-7 and β1 integrin, and we confirmed their partial co-localization in H358 control cells, which disappeared in the H358 claudin-7 KD cells (Fig. II.6.A). Co-immunoprecipitation experiments further demonstrated that claudin-7 interacted with β1 integrin and formed a protein complex in H358 control cells (Fig. II.6.B). These results were consistent with similar results previously shown in HCC827 control and KD cells (66), supporting the hypothesis that claudin-7 regulates β1 integrin expression. Western blot results also revealed that H358 claudin-7 KD cells decreased the levels of β1 integrin and phospho-FAK when compared to control cells (Fig. II.6.C), suggesting that claudin-7 KD in human lung cancer cells may result in the reduced focal adhesion.

All these results demonstrated that claudin-7 KD in H358 human lung cancer cells reduces cell adhesion and cell attachment, which was also evidenced by the reduced expression of focal adhesion proteins, including β1 integrin and phospho-FAK (Fig. II.6.C). This is also consistent with those previously shown in HCC827 claudin-7 KD cells (66), supporting the hypothesis that claudin-7 regulates cell attachment through interaction with β1 integrin in human lung cancer cells.

D.3.*Claudin-7 suppressed tumor growth in vivo*

We previously investigated whether claudin-7 inhibited tumor cell growth *in vivo* by

administering subcutaneous injections of HCC827 control or claudin-7 KD cells into the flanks of nude mice (66). The harvested mouse tumor tissues induced by the claudin-7 KD cells were significantly larger and heavier than those induced by the control cells, indicating that claudin-7 inhibited the tumor growth *in vivo*.

This current study further investigated the reason why HCC827 claudin-7 KD cells accelerated mouse tumor growth *in vivo* using Western blot analysis on previously harvested tumor tissues (66). The claudin-7 KD cell-induced mouse tumors significantly increased the protein expression levels of phospho-ERK1/2, survivin, and cleaved PARP when compared to those of control cell-induced tumors (Fig. II.7). This result demonstrated that mouse tumors with claudin-7 KD cells substantially increased cell apoptosis and survival. This was in contrast to our previous Western blot analysis in an *in vitro* cell culture study showing a significant decrease in apoptosis (cleaved PARP) but an increase in anti-apoptosis (survivin) in HCC827 claudin-7 KD cells when compared to HCC827 claudin-7 control cells. This suggests that claudin-7 KD-induced mouse tumors support cell proliferation by inducing anti-apoptotic expression (survivin), which suppresses the cancer apoptosis initiated by apoptotic expression (cleaved PARP).

E. Discussion

In this study, we demonstrated that the KD of claudin-7 significantly reduced β1 integrin expression and its partial co-localization in NCI-H358 lung cancer cells (Figs. II.6 A and II.6 C). This result is comparable to our previous results from claudin-7 KD in both HCC827 lung cancer cells and breast cancer cell line T-47D displaying the severe cell-matrix adhesion defect (66). Confocal immunofluorescence microscopy and co-immunoprecipitation also revealed that claudin-7 interacted with β1 integrin and formed a protein complex in the HCC827 claudin-7

control cells (66). These results suggest that the cell-matrix adhesion defect can be rescued by transfecting claudin-7 or β1 integrin into the claudin-7 KD cells in HCC827 and NCI-H358 lung cancer cells.

Our previous findings on HCC827 claudin-7 KD cells also suggest that the KD of claudin-7 in NCI-H358 cells promoted cell proliferation and cell cycle progression, which was also indicated by cell counting assays and Western blot analysis on H358 claudin-7 KD cells showing increased cell proliferation and reduced levels of apoptosis (cleaved PARP), as shown in Fig. 2. In addition, we further conducted a Western blot analysis using the harvested mouse tumors from our previous *in vivo* mouse tumor growth experiments based on HCC827 control and claudin-7 KD cells (66). Interestingly, while HCC827 claudin-7 KD cells previously demonstrated enlarged and heavier tumor growth in nude mice *in vivo* (66), apoptotic expression (cleaved PARP) was coincidentally highly expressed with an elevated anti-apoptotic factor (survivin). These results suggest that survivin suppresses downstream cascades of apoptotic signaling. It is also known that fast-growing tumors have a higher cell proliferation rate as well as a higher cell apoptosis rate due to the lack of nutrition and hypoxia within the tumor tissue (57, 83, 84). In the cell culture system, cancer cells have sufficient nutrition and oxygen. This could explain why a higher expression level of cleaved PARP was observed in claudin-7 KD cell-induced tumors (Fig. II.7), while claudin-7 KD cells in culture previously exhibited a decreased level of cleaved PARP. Our current results suggest that claudin-7 could be a tumor suppressor in lung cancer, inhibiting tumor cell growth both *in vitro* and *in vivo*.

Integrins are cell surface receptors that lack intrinsic tyrosine kinase activity. FAK is constitutively associated with β1 integrin of the integrin receptors. The binding of β1 integrin to ECM proteins leads to the activation of FAK through its auto-phosphorylation at the tyrosine 397

site. It has been reported that claudin-2 facilitates cell-matrix adhesion by increasing the cell surface expression of α2β1 and α5β1-integrin complexes in breast cancer cells, promoting the formation of breast cancer liver metastases (85). Similar to HCC827 KD cells, we also found that the KD of claudin-7 reduced β1 integrin at protein levels and led to the disappearance of the protein complex formation of claudin-7 with β1 integrin in H358 KD cells. It remains largely unknown how claudins affect integrin expression. We were unable to detect claudin-7 in the nuclei of HCC827 or H358 cells by immunofluorescence staining (66); however, we cannot rule out the possibility that claudin-7 translocated into the nuclei at a level that was beyond the immunofluorescence detection sensitivity. Western blot analyses on nuclear extracts are needed to determine whether claudin-7 is present in nuclei and whether it can potentially affect β1 integrin transcriptional expression. On the other hand, claudin-7 could indirectly regulate β1 integrin transcription by regulating the transcription factors of β1 integrin, such as Pax6 (86), Hox D3 (87), hypoxia-inducible factor (HIF) (88), and c-Myc (89). One approach to investigate the potential transcription factors involved is to apply cDNA microarrays in the future.

The suppression of claudin-7 was thought to not only disrupt cell-matrix adhesion but also promote higher cell proliferation. Thus, we previously investigated which of these two consequences was the primary effect of claudin-7 KD in HCC827 lung cancer cells (66). Frequent cell division can promote cell detachment. During mitosis, cells undergo large morphologic changes: They become detached, they form retraction fibers while the cell margin moves inward, and then they are finally rounded up and prepared for division (90). This indicates that cell-matrix detachment could be a consequence of rapid cell division. However, inhibiting the rate of cell cycle progression of HCC827 claudin-7 KD cells—either using drug treatment, such as aphidicolin or genistein, or culturing in type IV collagen-coated plates—could not recover the β1 integrin

expression of the HCC827 KD cells (66). Type IV is the structural backbone of the basement membrane of several solid organs, including the lung, and it has the ability to interact with cell surface adhesion molecules, such as integrins (91). These results mentioned above suggest that cell attachment depends on both cell proliferation and β1 integrin levels; however, cell proliferation does not depend on cell attachment. Both the enhanced cell proliferation rate and the decreased β1 integrin level are the primary results of claudin-7 suppression in HCC827 and H358 lung cancer cells.

To conclude, in this study, we have discovered a novel function of claudin-7 as a basolateral protein in regulating cell proliferation and cell-matrix adhesion in lung cancer cells. This study extends our current understanding of the roles of claudins in carcinogenesis. Both claudins and integrins play essential regulatory roles in tumor proliferation, invasion, and metastasis. Understanding the molecular mechanisms of lung cancer cell-matrix adhesion could lead to the identification of novel therapeutic strategies to target tumor cells. This novel basolateral function of claudin-7 engaging β1 integrin in human lung cancer cells could provide a previously unidentified therapeutic target in the future.

Our study suggests a tumor suppression role of claudin-7 in lung cancer growth and identifies a new function of claudin-7 in maintaining epithelial cell attachment through interaction with β1 integrin.

F. Competing interests

The authors declare that they have no competing interests.

G. Authors' contributions

ZL: Acquisition of data, analysis and interpretation of data, writing of the manuscript, DHK: Acquisition of data, analysis and interpretation of data, revising of the manuscript, JF: Acquisition of data, analysis and interpretation of data, QL: analysis and interpretation of data, writing of the manuscript, KV: analysis and interpretation of data, writing of the manuscript, LD: Acquisition of data, RR: Acquisition of data, YHC: Securing of funding, conception and design, study supervision, writing and revising of the manuscript. All authors read and approved the final manuscript.

H. Acknowledgements

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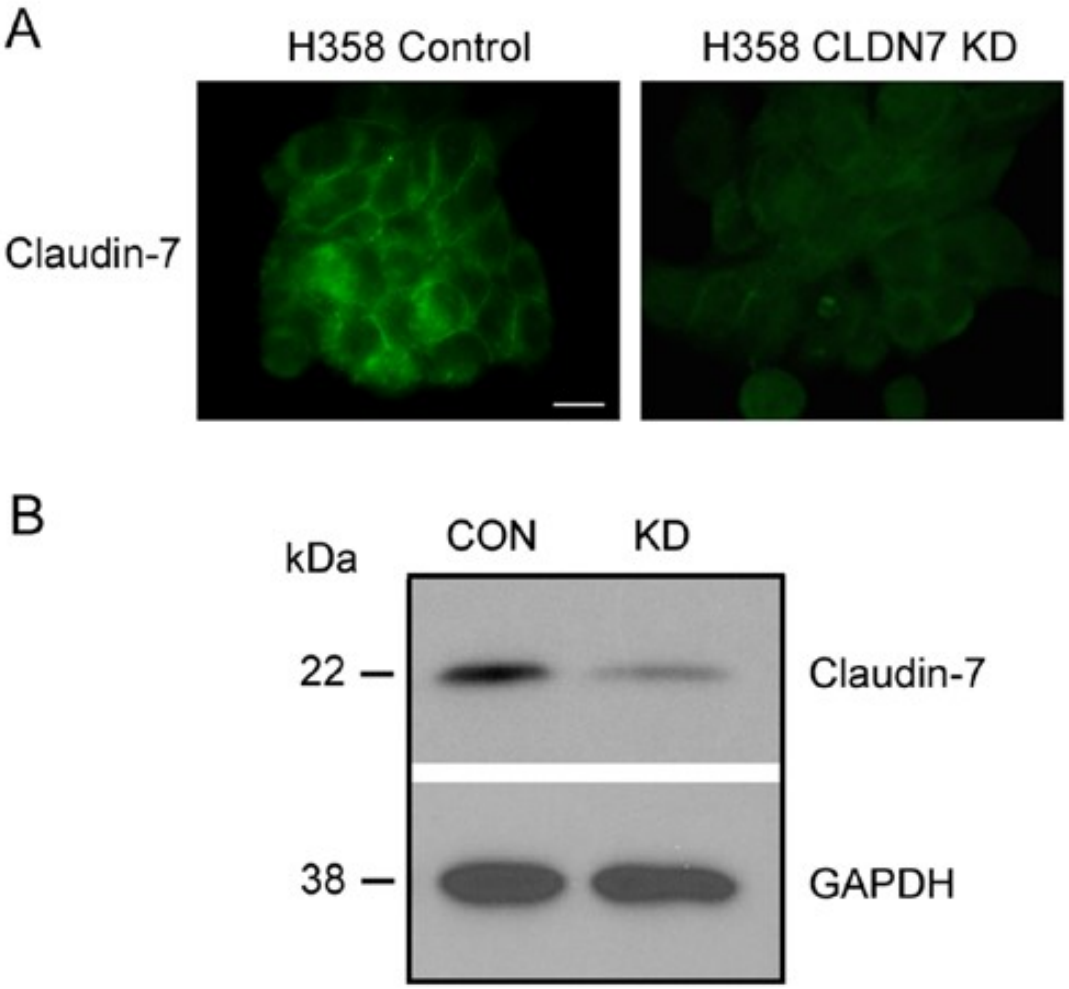
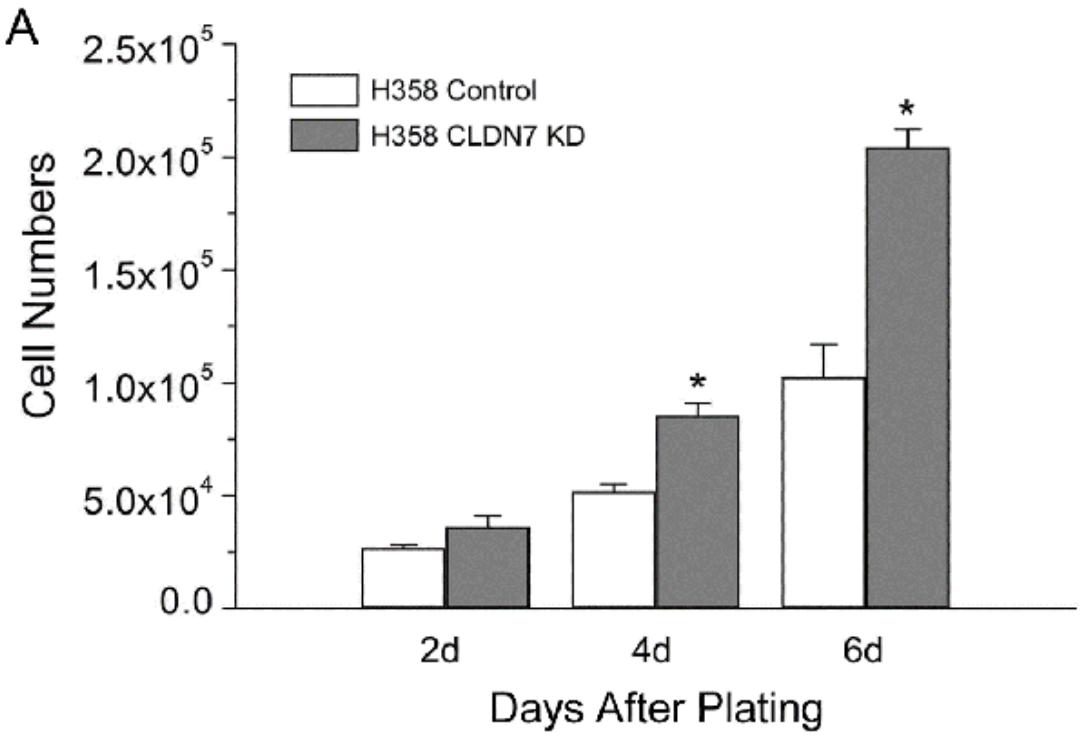

A
H358 Control
H358 CLDN7 KD
Claudin-7
B
kDa
CON
KD
22
Claudin-7
38
GAPDH

Figure II.1 KD of claudin-7 using #2 shRNA lentivirus construct against claudin-7 in H358 lung cancer cells.

A. Immunofluorescence images of H358 control and claudin-7 KD cells using anti-claudin-7 antibody. The cells were fixed with 100% methanol. Scale Bar: 20 μm. **B.** Western blot shows that the expression level of claudin-7 is greatly reduced in the KD cells compared to that of the control cells.


A
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0.0
Cell Numbers
H358 Control
H358 CLDN7 KD
*
*
2d
4d
6d
Days After Plating

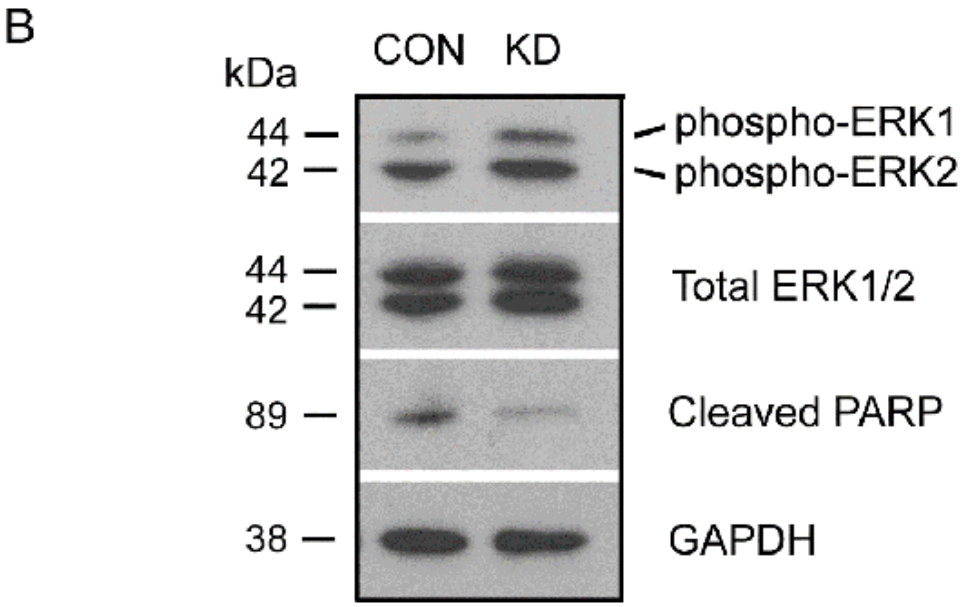

B
kDa
CON
KD
44
42
phospho-ERK1
phospho-ERK2
44
42
Total ERK1/2
89
Cleaved PARP
38
GAPDH

Figure II.2 Increased cell proliferation in H358 claudin-7 KD cells.

A. In total, 5×10^3 H358 control and claudin-7 KD cells were seeded into 24-well plates. The cell numbers were counted 2, 4, and 6 days after the cells were plated. Claudin-7 KD cells displayed a significantly higher proliferation rate compared to the control cells on days 4 and 6. *$P<0.05$. **B.** Representative Western blots show an increased level of phospho-ERK1/2 and a decreased level of cleaved PARP in claudin-7 KD cells when compared to those of the control cells, while the total ERK1/2 was unchanged.

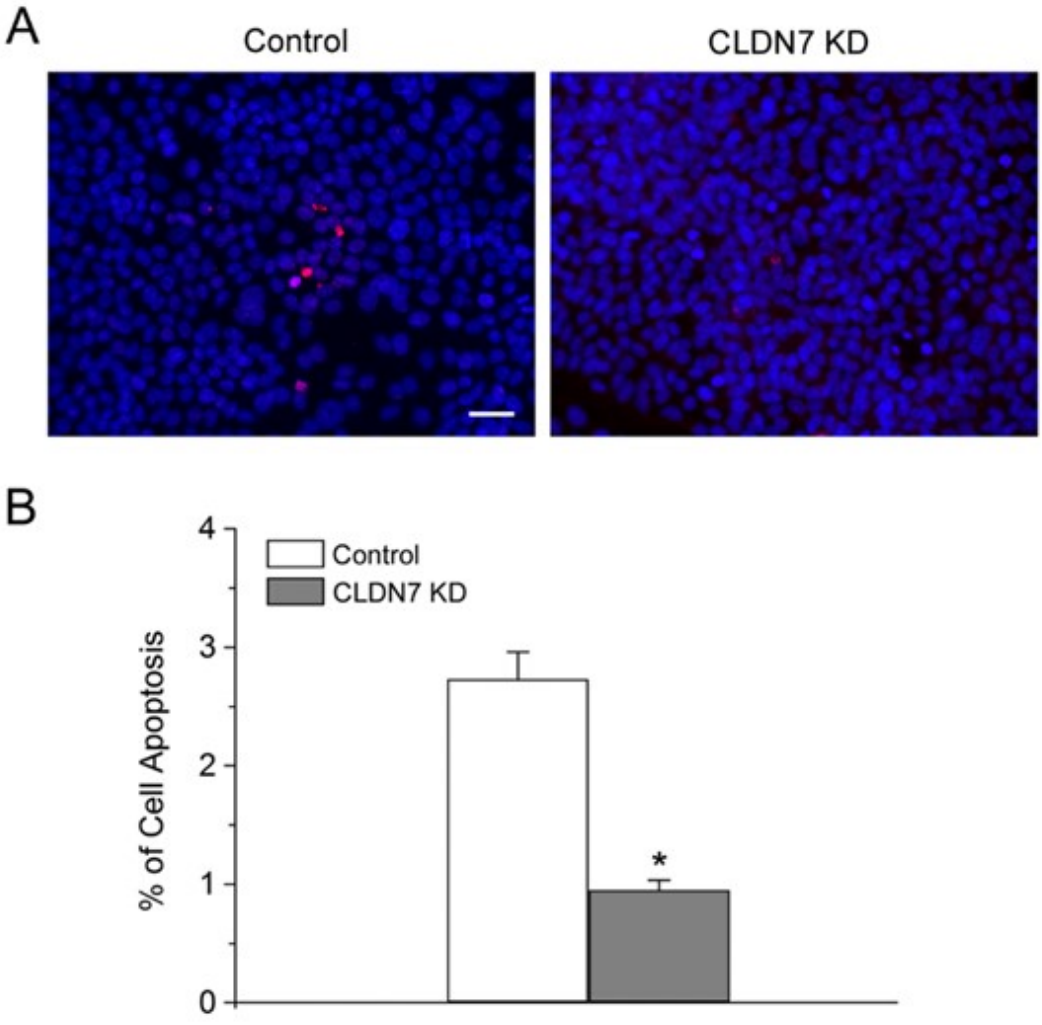
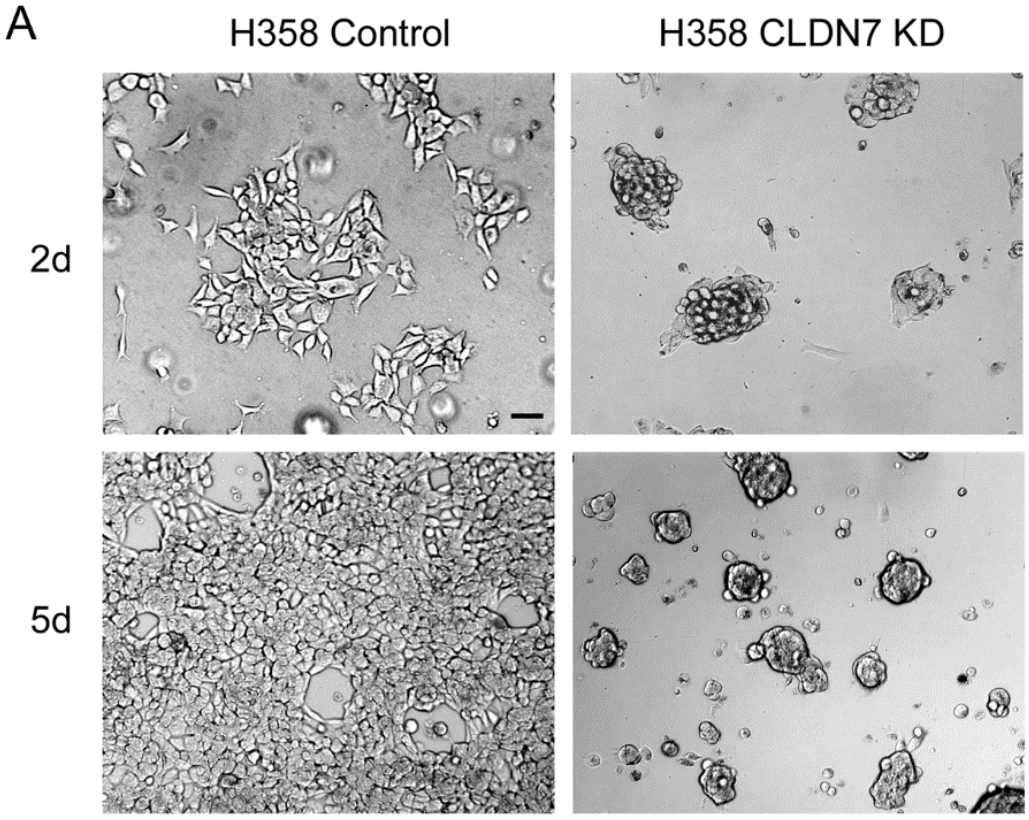

A
Control
CLDN7 KD
B
Control
CLDN7 KD
% of Cell Apoptosis
0
1
2
3
4
*

Figure II.3 Reduced cell apoptosis in HCC827 claudin-7 KD cells.

A. HCC827 control and claudin-7 KD cells were fixed in 100% methanol and incubated with 10% BSA in PBS for 30 min at 37ºC before applying the TUNEL reaction mixture (Roche Diagnostics, Cat. 12156792910) to the cells for 1 h at 37ºC. The red signal indicates the apoptotic cells. The blue signal is the nuclear staining. Scale bar: 50 μm. **B.** The percentage of cell apoptosis was significantly lower in HCC827 claudin-7 KD cells compared to that of the control cells. Data were analyzed from five different samples. *P<0.05.


A
H358 Control
H358 CLDN7 KD
2d
5d

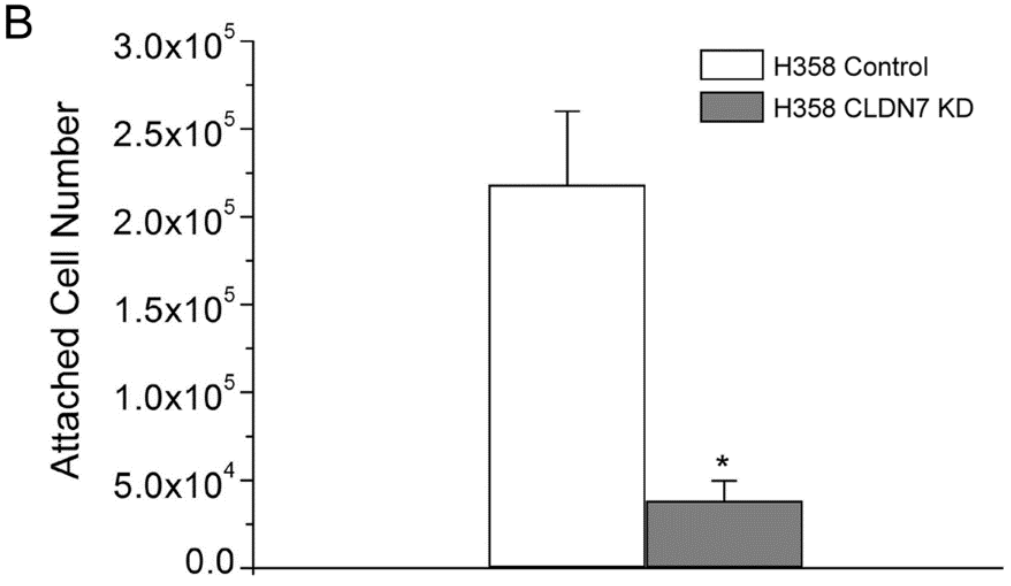

B
Attached Cell Number
3.0x10^5
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0.0
H358 Control
H358 CLDN7 KD
*

Figure II.4 Cell attachment defect in H358 claudin-7 KD cells.

A. When cultured on uncoated glass coverslips, H358 claudin-7 KD cells formed spheroids on both 2-day (2d) and 5-day (5d) cultures, while the control cells were able to spread out and form a monolayer. Scale Bar: 30 μm. **B.** In total, 2×105 H358 control and KD cells were plated to each well of 24-well plates. After 4h, the unattached cells were washed off, and the attached cells were trypsinized and counted. Claudin-7 KD cells showed significantly reduced cell attachment compared to that of the control cells. *$P<0.05$.

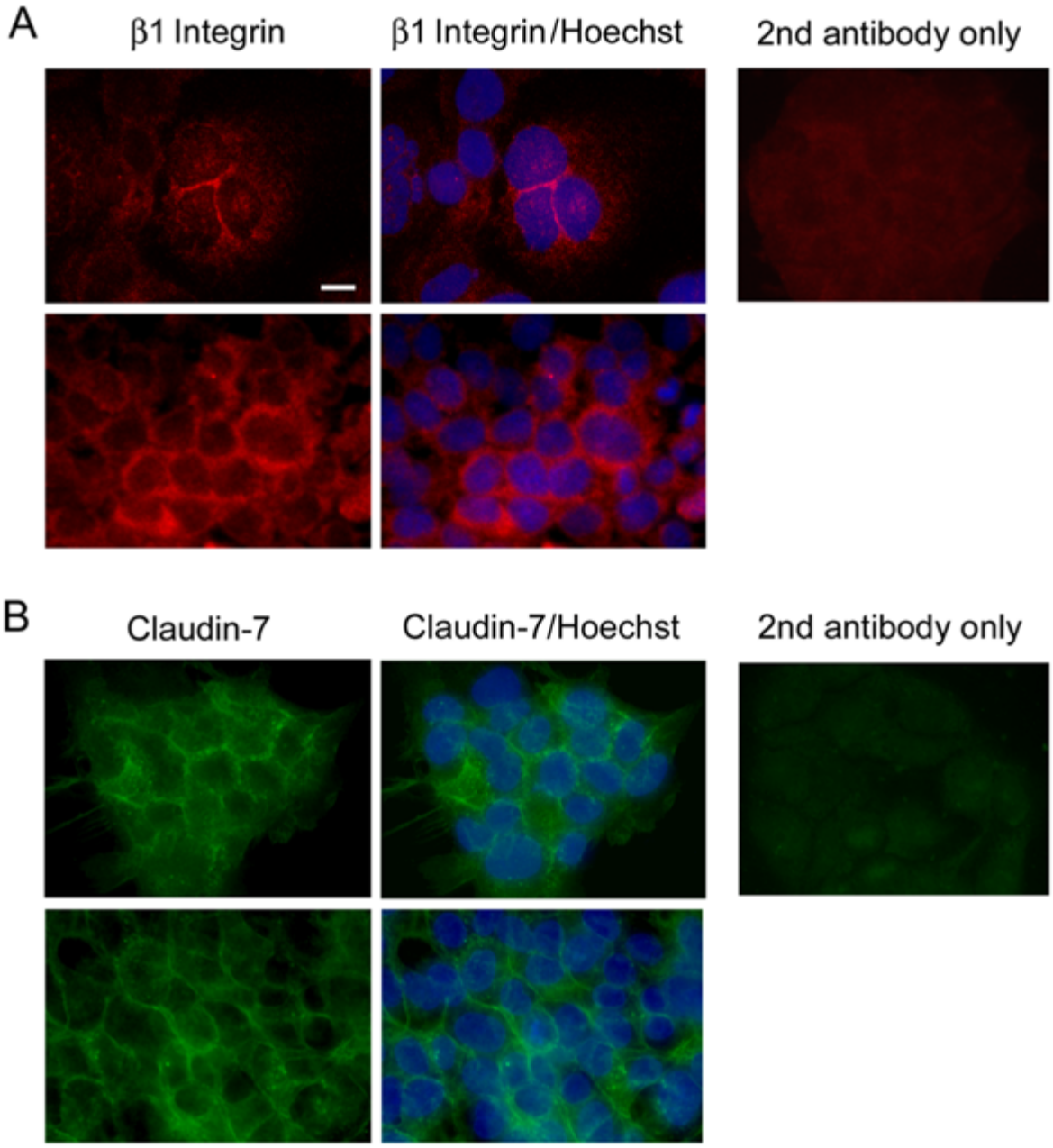

A
β1 Integrin
β1 Integrin/Hoechst
2nd antibody only
B
Claudin-7
Claudin-7/Hoechst
2nd antibody only

Figure II.5 Single immunofluorescent staining of β1 integrin and claudin-7 on HCC827 cells.

A. HCC827 control cells were grown on poly-D-lysine-coated glass coverslips and then fixed in 100% methanol for 8 min at -20°C. After blocking, cells were incubated with mouse anti-β1 integrin antibody for 1 h at room temperature. Coverslips were mounted with a ProLong Antifade Kit, and samples were photographed using a Zeiss Axiovert S100. Both low-density (top) and high-density (bottom) cells were shown. Scale Bar: 15 μm. **B.** HCC827 control cells were treated in the same condition as in **A.**, except that the primary antibody was the rabbit anti-claudin-7 antibody. The secondary antibodies were anti-mouse Cy3-conjugated antibody (Red color) and anti-rabbit FITC-conjugated antibody (Green color).

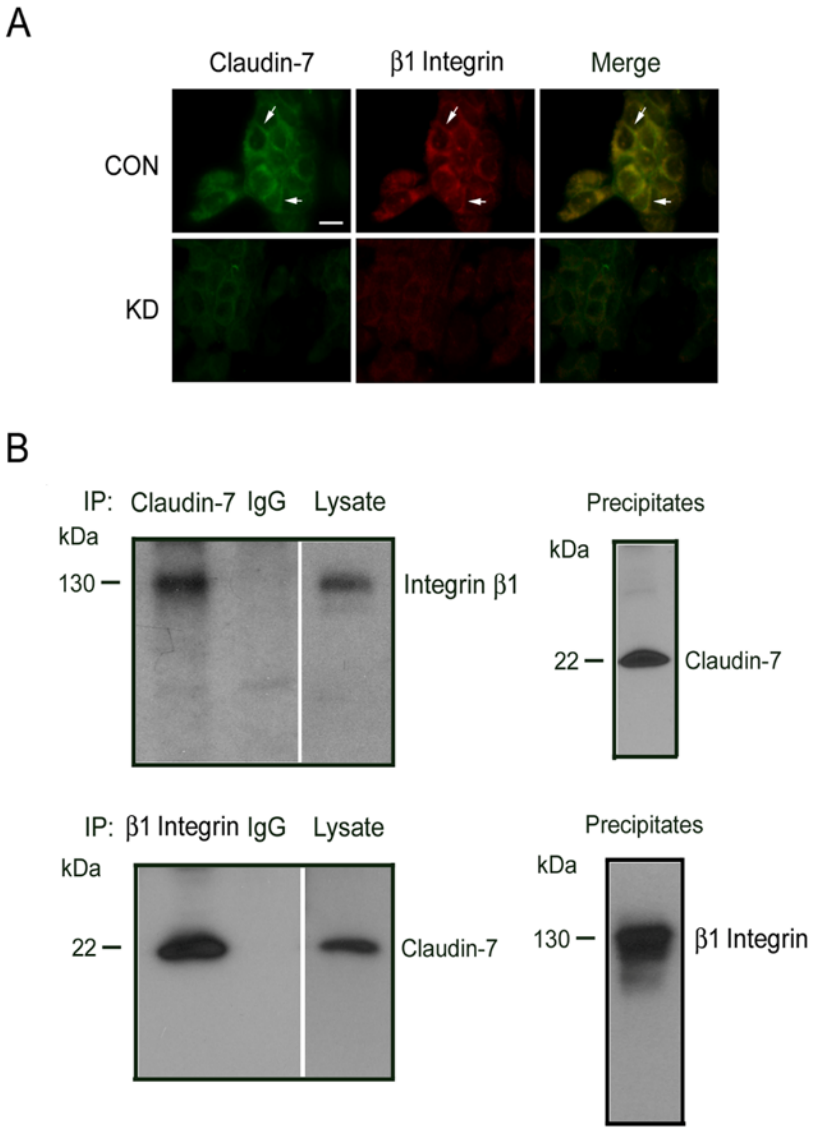

A
Claudin-7
β1 Integrin
Merge
CON
KD
B
IP: Claudin-7 IgG Lysate
kDa
130
Integrin β1
Precipitates
kDa
22
Claudin-7
IP: β1 Integrin IgG Lysate
kDa
22
Claudin-7
Precipitates
kDa
130
β1 Integrin
C
kDa
CON
KD
22
Claudin-7
130
β1 Integrin
125
p-FAK Tyr397
38
GAPDH

Figure II.6 Reduced β1 integrin expression level in H358 claudin-7 KD cells.

A. Double immunofluorescence staining of claudin-7 and β1 integrin in H358 control cells. Arrows in control cells indicate the partial co-localization of claudin-7 with β1 integrin. Cells were incubated with mouse anti-β1 integrin antibody and rabbit anti-claudin-7 antibody together (dilution 1:100) at room temperature for 1 h. Then, secondary antibodies against anti-mouse Cy3-conjugated antibody (Red color) and anti-rabbit FITC-conjugated antibody (Green color) were used (dilution 1:400). Scale bar: 20 μm. **B.** Claudin-7 co-immunoprecipitated with β1 integrin. Control cells were lysed in RIPA buffer without SDS and immunoprecipitated with either anti-β1 integrin or anti-claudin-7 antibody. The membrane was probed with either claudin-7 or β1 integrin. **C.** Western blots show that β1 integrin and phospho-FAK levels were decreased in H358 claudin-7 KD cells compared to the control (CON) cells.

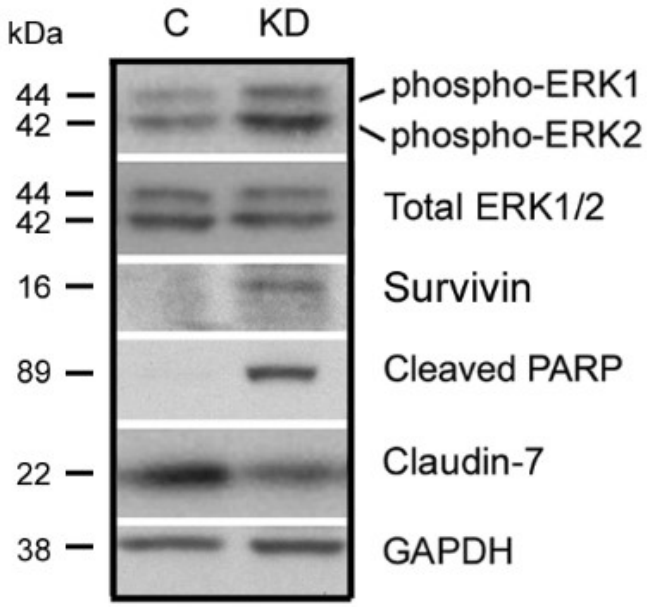

kDa
C
KD
44
42
phospho-ERK1
phospho-ERK2
44
42
Total ERK1/2
16
Survivin
89
Cleaved PARP
22
Claudin-7
38
GAPDH

Figure II.7 Apoptotic effect of claudin-7 on tumor growth in nude mice *in vivo*.

Western blot results showed increased expression levels of phospho-ERK1/2, total ERK1/2, survivin, and cleaved PARP in claudin-7 KD cell-induced tumors when compared to those of control cell-induced tumors. For SDS-PAGE and Western blot analysis, 20 μg/μl of protein was loaded per well. Claudin-7 was probed to validate the effect of claudin-7 KD on mouse tumor growth *in vivo*. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control protein.

CHAPTER III. CLAUDIN-7 MODULATES CELL-MATRIX ADHESION THAT CONTROLS CELL MIGRATION, INVASION, AND ATTACHMENT OF HUMAN LUNG CANCER CELLS

A. Summary

Claudins, a family of TJ proteins, play important roles in the epithelial barrier, selective ion transport, and cancer metastasis. The role of claudin-7 in cancer is unclear. However, a recent clinical study has shown that claudin-7 is associated with the survival of lung cancer patients after surgery. Our previous studies have shown the protein complex of β1 integrin and claudin-7 in HCC827 lung cancer cells. Moreover, *in vitro* claudin-7 KD reduced β1 integrin expression and increased cell proliferation, whereas claudin-7 re-overexpression in the KD cells decreased cell proliferation**.** Whether claudin-7 and β1 integrin synergistically or independently regulate cell proliferation and metastasis remains unclear. In this study, we found that β1 integrin overexpression in claudin-7 KD lung cancer cells did not reduce cell proliferation. However, β1 integrin overexpressing cells migrated more effectively (*$p < 0.05$) in wound healing and cell invasion assays, and they were more adhesive (*$p < 0.01$) in cell attachment assays when compared to claudin-7 KD cells. This indicates that claudin-7 controls cell proliferation directly, while it regulates cell motility and attachment partially through β1 integrin. Additionally, claudin-7 overexpression in claudin-7 KD cells resulted in a greater ability to attach to cell culture plate surfaces (*$p < 0.05$) and higher expression of focal adhesion proteins when compared to claudin-7 non- KD control cells, further supporting the role of claudin-7 in cell motility and adhesion. These data suggest that claudin-7 regulates cell motility through β1 integrin, providing additional

insight into the roles of claudins in carcinogenesis and metastasis.

B. Introduction

Human lung cancer is the second leading cause of mortality in the United States (32). The lung expresses various TJ proteins depending on their compartments, including claudin-1, -2, -3, -5, -7, -8, and -18 (92). TJ proteins are one of the cellular junctional proteins located at the apical side of epithelial cells, and they regulate paracellular permeability between neighboring epithelial cells (93). One of the TJ proteins is the claudin family that consists of four transmembrane spanning proteins (93). Although the function of claudins as epithelial barriers for the maintenance of cell polarity and selective ion transport has been well established (94), their role in diseases, including cancer, is unclear. However, a possible link between TJs and metastasis has been recently demonstrated using human colon cancer cell lines *in vitro* (25, 95). The varying levels of claudin expression may be correlated to cancer progression (96). Additionally, claudin-5 has been shown to form a protein complex with ROCK and N-WASP and promote actin cytoskeletal movement in breast cancer cells (31), suggesting that TJ proteins are crucial for cancer cell motility.

A recent clinical research study has shown that claudin-7 expression is associated with the survival of lung cancer patients after surgery (35), suggesting the role of claudin-7 in cancer progression. Results from our previous study demonstrated that claudin-7 KD (claudin-7 KD) in HCC827 human lung cancer cell lines increased cell proliferation and reduced β1 integrin expression and cell adhesion (66). Interestingly, claudin-7 was able to form a protein complex with β1 integrin and was partially co-localized at the basolateral membrane of HCC827 control cells (66). This suggests a possibility that claudin-7 and β1 integrin co-regulate cellular events, including cell proliferation and adhesion; however, this has not been fully explored. Several studies

have shown the basal localization of claudin-7 in the epithelial cells of several organs, including the mammary gland, kidney, and uterus, suggesting the roles of claudin-7 in cell-matrix adhesion (36-38) and vesicle trafficking (38). In this study, we examined whether β1 integrin and claudin-7 independently or synergistically functioned on cell proliferation, adhesion, migration, invasion, and attachment. Our results demonstrate that β1 integrin overexpression partially recovers the defective cell adhesion, migration, invasion, and attachment, but not cell proliferation, of claudin-7 KD cells.

C. Materials and methods

C.1. *Antibodies*

Rabbit polyclonal anti-phospho-Y397-FAK (Cat. 3283S), anti-FAK (Cat. 3285), anti-phospho-Y118-Paxillin (Cat. 2541), and anti-GAPDH (Cat. 2118, clone 14C10) were purchased from Cell Signaling Technology. Goat polyclonal anti-β1 integrin antibody was obtained from Santa Cruz Biotechnology (Cat. sc-6622). Mouse monoclonal anti-Paxillin antibody (Cat. 610051) was obtained from BD Transduction Laboratories (San Jose, CA). The secondary anti-mouse (Cat. W4021) and anti-rabbit (Cat. W4011) antibodies tagged with HRP were purchased from Promega (Madison, WI). Rabbit polyclonal anti-claudin-7 antibody (Cat. 18875) was obtained from Immuno-Biological Laboratories, and mouse monoclonal anti-Myc (Cat. 46-0603) antibody was obtained from Invitrogen (Carlsbad, CA).

C.2. *Cell lines and reagents*

The HCC827 human NSCLC cell line was obtained from ATCC and cultured in RPMI-1640 medium (Cat. 11875-093, Gibco, Long Island, NY) supplemented with heat-inactivated 10%

fetal bovine serum (Cat. SH3007001, HyClone, Logan, UT, USA), 1% 10,000 U/ml penicillin, and 10,000 μg/mL streptomycin (Cat. 15140-122, Gibco, Carlsbad, CA) in a 37°C, 5% CO_2 humidified incubator. HCC 827 control or claudin-7 KD cell lines were previously established [1].

C.3.*Overexpression of β1 integrin or claudin-7 in claudin-7 KD cells*

In order to establish the stable transfection of β1 integrin in HCC827 KD cells (KD+b1 cells), the β1 integrin cDNA vector (Transomics, Huntsville, AL) was digested at EcoRI and NotI restriction enzyme sites. The size of the *β1 integrin* cDNA insert was confirmed from DNA electrophoresis. The insert was gel-purified using a Gel Extraction kit (Cat. 28704, Qiagen, Valencia, CA) and then sub-cloned to a pcDNA3.1 vector at EcoRI and NotI restriction sites. After the pcDNA3.1-*β1 integrin* cDNA vector was transfected to HCC827 KD cells using Amaxa Nucleofector™ Kit V reagent (Cat. VCA-1003, Lonza, South Plainfield, NJ) by electroporation, the stably transfected cells were selected at 600 μg/ml Geneticin (G418) for 4 weeks. The stable transfectants were maintained in the culture medium containing 300 μg/ml G418. For the transient transfection, the pcDNA3.1-*claudin-7*-myc (mouse *claudin-7* cDNA) vector was transfected to HCC827 KD and KD+b1 cell lines, and the transfectants were incubated and recovered overnight under an antibiotic-free medium. The transfectants were given fresh media the next day and used for the experiment within 72 h.

C.4.*SDS-PAGE and Western blot*

Whole cells were lysed in RIPA buffer (1% Triton-100, 0.5% deoxycholate, 0.2% sodium dodecyl sulfate, 150 mM sodium chloride, 2 mM ethylene diamine tetra-acetic acid, 10 mM sodium pyrophosphate, 20 mM sodium fluoride) supplemented with a complete protease inhibitor

cocktail tablet (Cat. 11836153001, Roche Diagnostics). After cell debris from the protein lysate was removed by centrifugation at 4°C, protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Cat. 23225, Thermo Scientific). Proteins (20 μg per lane) were separated by SDS-PAGE gel, transferred to the nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare, Piscataway, NJ) by electrophoresis, and blocked and immuno-blotted with appropriate primary antibodies (dilution ratio 1:1000) followed by peroxidase-conjugated secondary antibodies (dilution ratio 1:2500). Protein bands were visualized using ECL detection reagent (GE Healthcare) and photographed using an X-ray film developer.

C.5.*Cell proliferation counting assay*

A total of 2×10^4 HCC827 control, claudin-7 KD, and KD+b1 cells were seeded in 6-well plates, and the total cell numbers were counted after 2, 4, and 6 days by the trypan blue exclusion method using a Countess™ automated cell counter (Cat. C10227, Invitrogen).

C.6.*Cell attachment assay*

A total of 2×10^5 or 1×10^5 HCC827 control, claudin-7 KD, and KD+b1 cells were seeded in a 12-well plate or a 24-well plate, respectively, to assess the effect of β1 integrin or claudin-7 overexpression on KD cell attachment, respectively. After 4 h incubation at 37°C in a 5% CO_2 humidified chamber, the culture medium was removed, and each well was washed briefly with PBS buffer to remove unattached cells. Then, the remaining attached cells were trypsinized and mixed with 6-micron AlignFlow Plus beads (Molecular Probes) at a concentration of 1×10^5 beads/ml. Total cell numbers were calculated from the estimated relative ratio of beads to cells using a FACScan flow cytometer.

C.7.*Wound healing migration assay*

The control, claudin-7 KD, and KD+b1 cells were plated in a 6-well plate until confluent. The cells were then cultured in serum-free media for 22 h. After creating a scratch using a pipet tip, serum-containing media was given, and the gap distance was photographed using an inverted Zeiss light microscope (Carl Zeiss Inc.) and analyzed by MetaMorph software (Molecular Devices, Sunnyvale, CA) at time points of 0, 3, 6, 12, and 24 h. The closed gap distance per time point was normalized to its respective initial gap distance at time point 0 per cell line.

C.8.*In vitro cell invasion assay*

A total of 2×10^5 HCC827 control, KD, and KD+b1 cells were suspended and seeded in 500 µl of serum-free RPMI1640 on the membranes of the inner chamber in 6-well BD Matrigel plates (Cat. 354481). The outer chamber was also filled with serum-free media. After 24 h serum starvation, serum-containing culture media was added in the outer chamber. After 53 h incubation at 37°C in a 5% CO_2 humidified incubator, the membranes from the inner chamber were scrubbed using a medium-wetted cotton swab. Counter cell staining was performed using modified protocols from the Hema-3 Stain Kit (Fisher Scientific, Pittsburgh, PA). In brief, the membranes were first fixed with fixative (Hema-3 Fixative) for 4 min, then stained in red color solution I (Hema-3 Solution I) for 10 min (cytosol staining), and then stained in blue color solution II (Hema-3 Solution II) for 10 min (nucleus staining). After two brief washes with de-ionized water, the membranes were quickly dried in air and mounted with glycerol on glass slides. Five areas per membrane sample were randomly selected under inverted light microscopy at 200 × magnification using a Zeiss Axiovert S100 microscope (Carl Zeiss Inc.) and photographed using Axiovision 4.6

imaging software (Carl Zeiss Inc.).

C.9.*Statistical analysis*

All experiments were performed at least three times and data were presented as means ± standard error of means. Student's t-test was performed to compare samples from two cell lines, and a one-way analysis of variance (ANOVA) was performed in samples from three cell lines followed by a post-hoc comparison using IBM SPSS software. We considered differences statistically significant when $*p < 0.05$.

D. Results

D.1.*β1 integrin overexpression did not alter the cell proliferative rate of claudin-7 KD cells*

Our previous study showed that HCC827 claudin-7 KD lung cancer cells increased the cell proliferative rate, decreased the expression of a variety of ECM components (including collagen IV and cell adhesion proteins, such as β1 integrin), and displayed reduced cell adhesion compared to HCC827 cells with no claudin-7 KD (claudin-7 control cells) (66). Although the reduced cell adhesion and ECM components appeared to accelerate the proliferative rate of the KD cells, supplementing the KD cells with collagen IV improved cell attachment but did not change the cell hyper-proliferative phenotype (66). This suggests that cell adhesion proteins such as β1 integrin may be more important in regulating cell adhesion and migration, as protein complexes of claudin-7 and β1 integrin were partially co-localized at the basolateral membrane (66). β1 integrin can form heterodimers with various α integrin partners, and it regulates cell proliferation and cell-matrix attachment and binds to the actin cytoskeleton for cell motility (97). This suggests that the re-expression of β1 integrin may adjust the cell proliferative rate of claudin-7 KD cells in a manner

comparable to that of control cells. Thus, we transfected *β1 integrin* cDNA into the claudin-7 KD cells (KD+b1) to see whether β1 integrin overexpression decreased the KD cell hyper-proliferative rate. Cell numbers were expressed as means ± standard error of means. The cell numbers of the KD and KD+b1 cells were not different on day 4 ($8.1\times10^4 \pm 1.7\times10^3$ vs. $7.4\times10^4 \pm 1.0\times10^4$) or day 6 ($1.5\times10^5 \pm 1.2\times10^4$ vs. $1.5\times10^5 \pm 1.8\times10^4$). However, the numbers of KD and KD+b1 cells were far more than those of control cells on day 6 ($6.8\times10^4 \pm 4.3\times10^3$) (*$p < 0.05$) (Fig. III.1). This result showed that β1 integrin expression did not rescue the claudin-7 KD cell hyper-proliferative phenotype, suggesting that β1 integrin may not be directly involved in regulating the cell proliferation of claudin-7 KD cells.

D.2.*β1 integrin overexpression partially recovered the cell adhesion of claudin-7 KD cells*

Although claudin-7 KD cells increased the cell proliferative rate, they exhibited a reduction in cell adhesion and β1 integrin expression (66). In addition, β1 integrin and claudin-7 formed a protein complex, and they were co-localized at the basolateral membrane of HCC827 control cells (66). This suggests the possibility of claudin-7 regulation of cell adhesion through β1 integrin. Therefore, we overexpressed β1 integrin in the claudin-7 KD cells to examine whether claudin-7-regulated cell adhesion occurs through β1 integrin. Similar to control cells, KD+b1 cells also formed a monolayer on glass coverslips, although a spheroidal clump remained, indicating incomplete cell spreading, whereas claudin-7 KD cells displayed spheroidal colonies only, as previously reported (Fig. III.2.A) (66). Likewise, KD+b1 cell layers around the scratch site were less stripped off than claudin-7 KD cell layers, similar to control cell layers when using pipet tips (Fig. III.2.B).

These results demonstrate that β1 integrin overexpression partially improved the reduced

ability of cell attachment to the dish in the claudin-7 KD cells.

D.3.*β1 integrin overexpression enhanced cell migration and invasion ability of claudin-7 KD cells*

We showed that β1 integrin overexpression partially recovered the cell adhesion of claudin-7 KD cells, but the effects of claudin-7 via β1 integrin on cell motility have not been studied before. Thus, we next examined whether β1 integrin overexpression recovered the reduced cell migration and invasion of claudin-7 KD cells using wound healing and cell Matrigel invasion assays, respectively. Wound healing assays showed that KD+b1 cells migrated faster than claudin-7 KD cells ($*p < 0.05$) but slower than control cells ($*p < 0.05$) at all time points. At 24 h, control cells filled up the initial gap by 82.3% ± 6.8%, while KD cells and KD+b1 cells filled up the initial gap by 50.2% ± 1.9% and 59.3% ± 3.0%, respectively (Figs. III.3.A and III.3.B). This indicates that β1 integrin partially compensates for the defect in KD cell migration caused by claudin-7 KD. Additionally, KD+b1 cells invaded more efficiently than claudin-7 KD cells ($*p < 0.05$) but less efficiently than control cells ($*p < 0.01$) (Figs. III.4.A and III.4.B). The average numbers of invading control cells were 29.2 ± 2.9, while those of KD and KD+b1 cells were 6.6 ± 0.5 and 17.5 ± 2.5, respectively. This indicates that β1 integrin overexpression also partly recovers the defective cell invasiveness of KD cells.

D.4.*β1 integrin overexpression partially restored defective cell attachment of claudin-7 KD cells*

β1 integrin overexpression significantly improved the cell adhesion of claudin-7 KD cells, indicating that claudin-7 may result in an increase in cell-matrix attachment via β1 integrin at cell-matrix interactions. Thus, we first investigated whether the overexpression of β1 integrin sufficiently improved the cell-matrix attachment of claudin-7 KD cells compared to control cells.

The number of KD+b1 cells that remained attached was significantly higher than that of claudin-7 KD cells (*$p < 0.01$) but significantly lower than that of control cells (*$p < 0.001$) in the cell attachment assay (Fig. III.5.A). This indicates that β1 integrin overexpression partially restores the defect in the cell attachment of claudin-7 KD cells.

In order to fully restore the defective KD cell attachment, we transiently transfected *claudin-7* cDNA-containing Myc-tag into the KD cells to generate KD+cldn7 cells. The cell attachment results showed that the number of KD+cldn7 cells that remained attached was significantly higher than that of control cells (*$p < 0.05$) and claudin-7 KD cells (*$p < 0.01$) (Fig. III.5.B), indicating that overexpression of claudin-7 strengthened the cell-matrix attachment of the KD cells far better. Western blot analysis confirmed the overexpression of β1 integrin or claudin-7 in KD cells (Fig. III.5.C). Although β1 integrin expression moderately increased the level of phospho-FAK in KD cells compared to that of the control cells, claudin-7 overexpression greatly elevated the expression levels of phospho-FAK, phospho-Paxillin, as well as β1 integrin in KD+cldn7 cells when compared to control cells (Fig. III.5.C). This result indicates that both β1 integrin and claudin-7 synergistically support cell attachment, although claudin-7 appears to have a greater effect than β1 integrin.

Taken together, these results suggest that β1 integrin overexpression in claudin-7 KD cells partially recovered the control cell adhesion, migration, and invasion but not cell proliferation.

E. Discussion

In this study, we noticed that β1 integrin overexpression in claudin-7 KD cells did not lead to claudin-7 expression, but claudin-7 overexpression in KD cells sufficiently revived β1 integrin expression. Our previous study also demonstrated that β1 integrin and claudin-7 formed a protein

complex co-localized at the basolateral membrane of HCC827 control cells (66). These results suggest that claudin-7 and β1 integrin may cooperate with each other, although claudin-7 seems to have a greater ability to control cellular phenotypes. For example, β1 integrin overexpression did not change the hyper-proliferative rate of claudin-7 KD cells. However, claudin-7 overexpression in KD cells reduced the cell proliferation rate (66). This indicates that claudin-7 may enable β1 integrin at the basolateral membrane to properly receive signaling from the ECM to control lung cancer cell proliferation. This does not align with the current dogma of β1 integrin signaling that regulates cell proliferation and survival (98). For example, deletion of β1 integrin reduces the cell proliferation of the mammary gland at the cellular level (99, 100). At the organ level, β1 integrin-knockout mice die before birth (99) probably due to the absence of β1 integrin survival signaling. In contrast, our claudin-7 KD cells suppressed β1 integrin expression but accelerated cell proliferation (Fig. III.1). Other β integrins could compensate for the lack of β1 integrin signaling to promote cell proliferation. In a breast cancer cell study, β3 integrin signaling was activated to increase cell proliferation and invasion when β1 integrin was suppressed (101). Future investigation is thus warranted to clarify the compensatory effect of other β integrins on claudin-7 KD cells.

Second, claudin-7 also synergistically collaborates with β1 integrin to control cell adhesion, migration, invasion, and attachment. We demonstrated that KD+b1 cells have improved cell-matrix adhesion when compared to claudin-7 KD cells (Fig. III.2.B), indicating that β1 integrin overexpression supports the role of claudin-7 in cell-matrix adhesion through the basolateral membrane of KD cells. However, KD+b1 cells still showed a reduction in the rate of cell migration when compared to control cells. Cell migration requires proper cell-matrix adhesion; focal adhesion, which primarily consists of integrin clustering through the cell-matrix interface,

maintains an extended actin cytoskeletal protrusion in the direction of cell migration, and it is further stabilized by nascent adhesion along with lamellipodia at the leading cell edge (45, 46). The consequentially skewed cell shape accumulates contractile force, which eventually drives cell migration when rear cell adhesion is released (45, 46). When claudin-7 is low in quantity, focal adhesion through the basolateral membrane may not be properly established, inhibiting the subsequent process of cell migration. This suggests that claudin-7 cooperatively regulates cell migration via β1 integrin.

Similar to the cell migration process, cell-matrix adhesion is also essential in cell invasiveness: Integrins form focal adhesion complexes, which results in the creation of an actin cytoskeletal protrusion as an invadopodia precursor (48). In addition, β1 integrin recruits integrin-linked kinase (ILK) that transforms the invadopodia precursor into adhesion ring-containing matured invadopodia (49), which allows the extension of the actin cytoskeletal protrusions and activates ECM degradation activity (48-50). The low amount of claudin-7 may reduce the number of focal adhesion complexes and prevent further processes of cell invasiveness. Although β1 integrin overexpression in KD+b1 cells may increase the amount of integrin clustering, the low quantity of claudin-7 in the KD+b1 cells may not effectively anchor the β1 integrins through the basolateral membrane and thus make it unable to fully recover the intensity of focal adhesion and degradation enzyme activity as it does in the control cells.

Likewise, cell-matrix adhesion is crucial in cell-matrix attachment. Claudin-7 overexpression in claudin-7 KD cells (KD+cldn7) recovers cell attachment ability at a far higher level than that of control cells. The increased cell attachment in claudin-7 overexpression is due to the excessive level of focal adhesion proteins confirmed by Western blot results, suggesting that the claudin-7 overexpression at the basolateral membrane of KD+cldn7 cells may bind stronger to

the target ECM surface than that of control cells. It is likely that claudin-7 overexpression also inhibits cell migration and invasion by creating excessive cell-matrix adhesion, which could also result in the reduced metastatic potential of lung cancer cells (78). However, we were unable to test the metastatic possibility *in vivo* due to the intrinsically poor metastatic potential of HCC827 cell lines (67). Future experiments on whether claudin-7 overexpression inhibits cancer metastasis using metastatic lung cancer cell lines *in vivo* will distinguish the different roles of β1 integrin and claudin-7 in coordinating cancer cell motilities.

Interestingly, claudin-7 proteins may serve as anchoring domains to recruit other cell adhesion proteins at cell membranes, as previously shown for β1 integrin (66) as well as other cell adhesion proteins, including cluster of differentiation 44 (CD44) and epithelial cell adhesion molecule (EpCAM) (102, 103), all of which could contribute to the overall cell-matrix adhesion of lung cancer cells. This may explain, in part, why β1 integrin overexpression in claudin-7 KD cells did not fully recover the cell-matrix adhesion, migration, invasion, and attachment.

The ability of claudin-7 interaction with β1 integrin to establish cell-matrix adhesion appears to be important to understand the molecular mechanism of Human Immunodeficiency Virus (HIV) infection. It has been reported that HIV infected a cluster of differentiation 4 (CD4)(-)) T cell subpopulation when the claudin-7 gene was introduced to the HIV particles *in vitro* (104). However, it remained unclear whether surface ligands in the CD4(-) T cells could interact with claudin-7 of the viral particle. Recently, it has also been reported that peripheral blood lymphocytes from normal patients show the high-level β1 integrin (CD29) surface marker in 66% of the CD4(-) T cell subpopulation (105). This suggests that claudin-7 proteins in the HIV-1 viral coat may interact with the CD29 present in the host CD4(-) T cells, which could establish mutual membrane adhesion that allows mediation of the membrane fusion process for entry of the HIV particles to

CD4(-) T cells.

In this report, we conclude that claudin-7 functions as a cell adhesion protein that modulates cell-matrix adhesion and regulates cellular processes, including lung cancer cell migration, invasion, and attachment.

F. Acknowledgement

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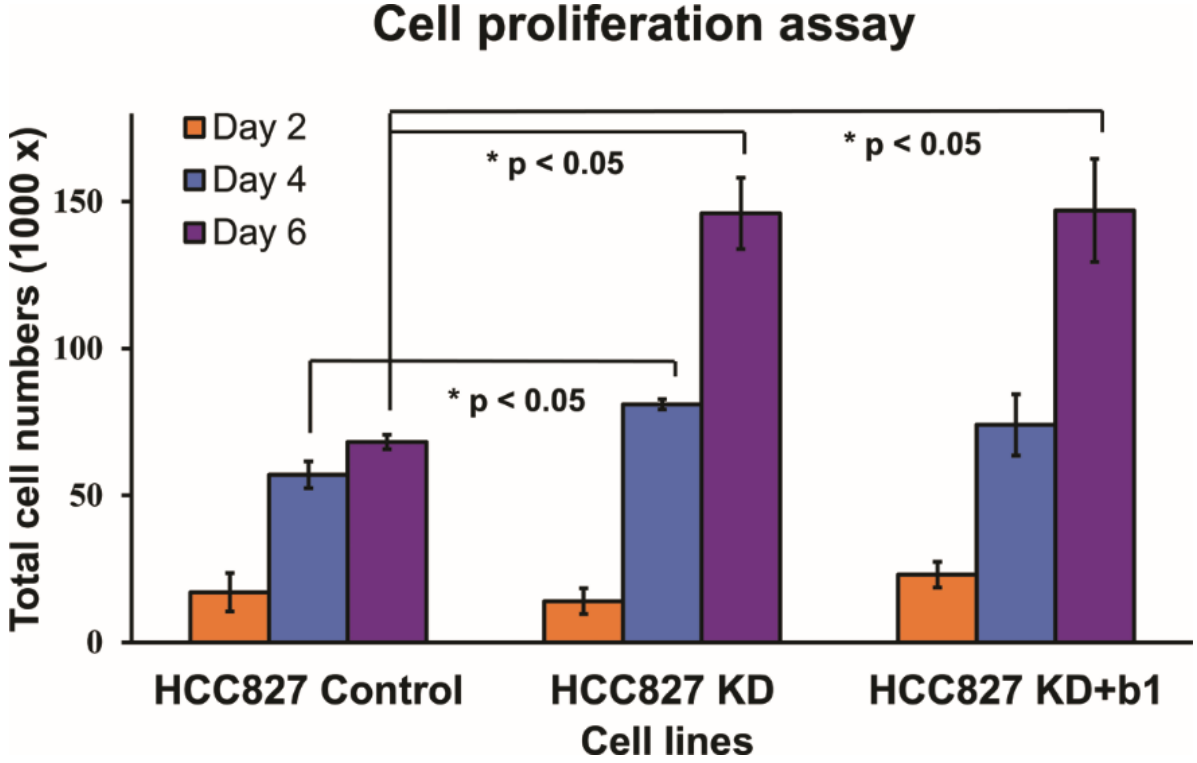
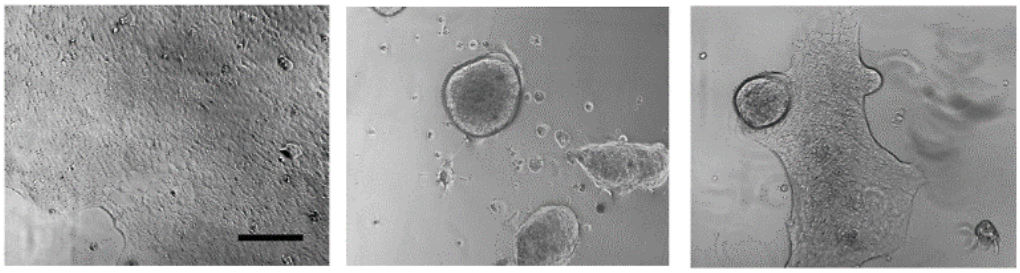

Cell proliferation assay
Day 2
Day 4
Day 6
* p < 0.05
* p < 0.05
* p < 0.05
Total cell numbers (1000 x)
150
100
50
0
HCC827 Control
HCC827 KD
HCC827 KD+b1
Cell lines

Figure III.1 Exogenous β1 integrin expression did not reduce hyper-proliferation of claudin-7 KD cells.

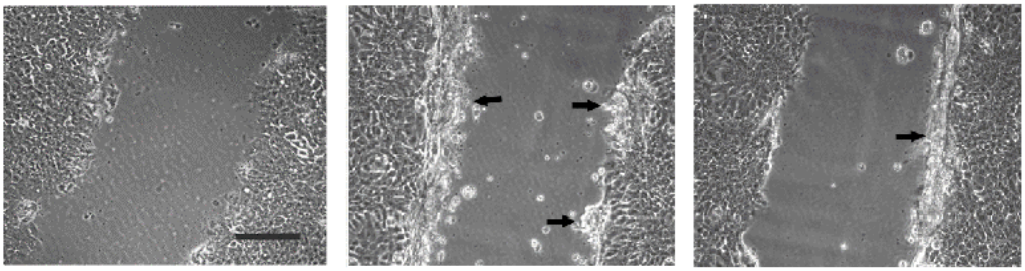
A total of 2×10^4 control, KD, and KD+b1 cells were seeded in 12-well plates, and the cell numbers were counted for each sample 2, 4, and 6 days after the initial cell culture date. KD+b1 cells did not show any significant change in cell proliferation rate when compared to claudin-7 KD cells during the entire cell growth period. Both KD and KD+b1 cells showed significantly higher proliferating cell numbers when compared to the control cells on day 6 ($*p < 0.05$). At least three independent experiments were performed for statistical analysis.

A



HCC827 Control | HCC827 KD | HCC827 KD+b1

B



HCC827 Control | HCC827 KD | HCC827 KD+b1

Figure III.2 Exogenous β1 integrin expression improved cell-matrix adhesion of claudin-7 KD cells.

A. Cells were cultured on glass coverslips. The control cells developed a complete monolayer, while all claudin-7 KD cells showed spheroid colonies. KD+b1 cells formed a partial monolayer with a spheroid cell clump, indicating incomplete cell spreading. The phase images were photographed at 100 × magnification. Scale bar: 10 μm. **B.** A scratch was created using a pipet tip on each confluent control, KD, and KD+b1 cell monolayer. Both control and KD+b1 cell layers were attached to the plate fairly well, whereas claudin-7 KD cells were stripped off along the scratch (shown as black arrows).

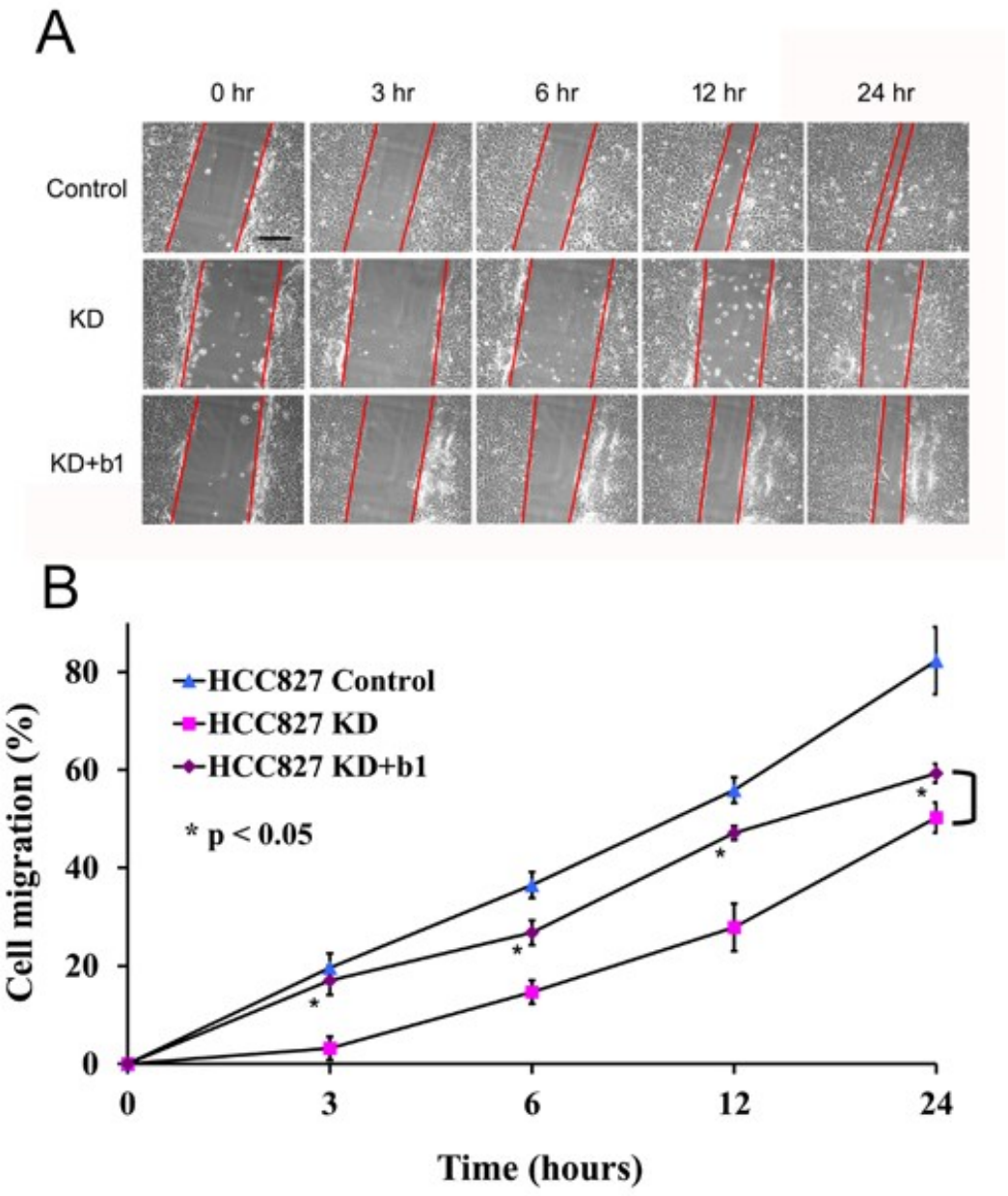
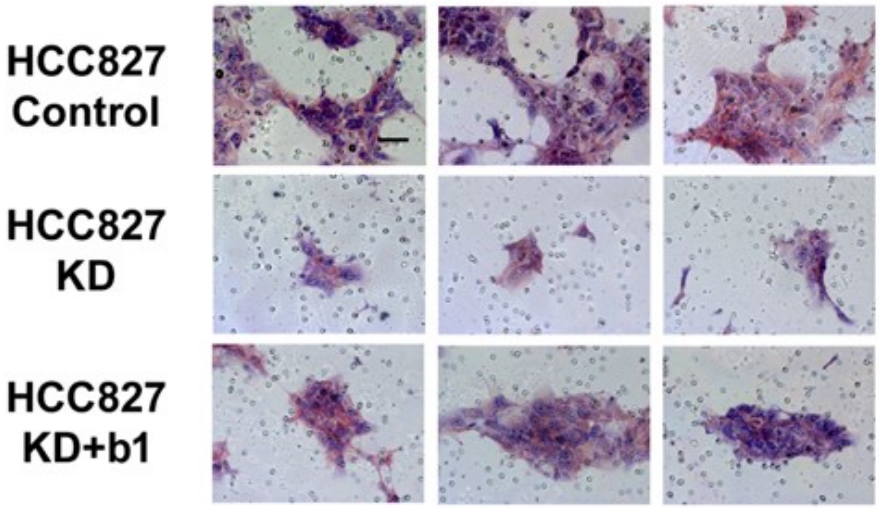

A
0 hr
3 hr
6 hr
12 hr
24 hr
Control
KD
KD+b1
B
HCC827 Control
HCC827 KD
HCC827 KD+b1
* p < 0.05
Cell migration (%)
Time (hours)
0
20
40
60
80
0
3
6
12
24

Figure III.3 Exogenous β1 integrin expression partially recovered cell migration ability of claudin-7 KD cells.

A. A panel of representative light phase images of control, KD, and KD+b1 cells in wound healing assay plates was photographed at 200 × magnification at a specified time point. Scale bar: 10 μm. **B.** Relative cell migration rate on each cell line in wound healing assay. The relative cell migration distance was estimated as the ratio of the closed gap distance at a given time point over the initially created gap distance at the 0 time point. KD+b1 cells migrated more effectively than claudin-7 KD cells (*$p < 0.05$). At least three independent experiments were repeated for statistical analysis.

A


HCC827
Control
HCC827
KD
HCC827
KD+b1

B

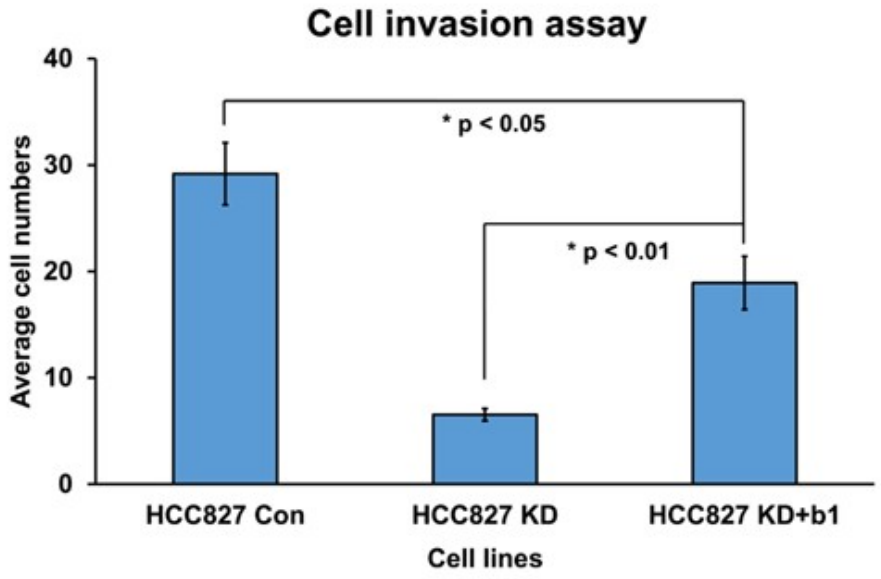

Cell invasion assay
Average cell numbers
40
30
20
10
0
* p < 0.05
* p < 0.01
HCC827 Con
HCC827 KD
HCC827 KD+b1
Cell lines

Figure III.4 Exogenous β1 integrin expression partially recovered the cell invasion ability of claudin-7 KD cells.

A. A panel of representative light phase images of control, KD, and KD+b1 cells was photographed at 200 × magnification. Scale bar: 20 μm. **B.** Cell invasion assay. The numbers of nuclei stained by blue dye (Hema-3 Solution II) were counted and averaged from five randomly chosen areas per cell line from three independent experiments. Red-dye (Hema-3 Solution I) staining reflected cytosols. At least three independent experiments were performed for statistical analysis.

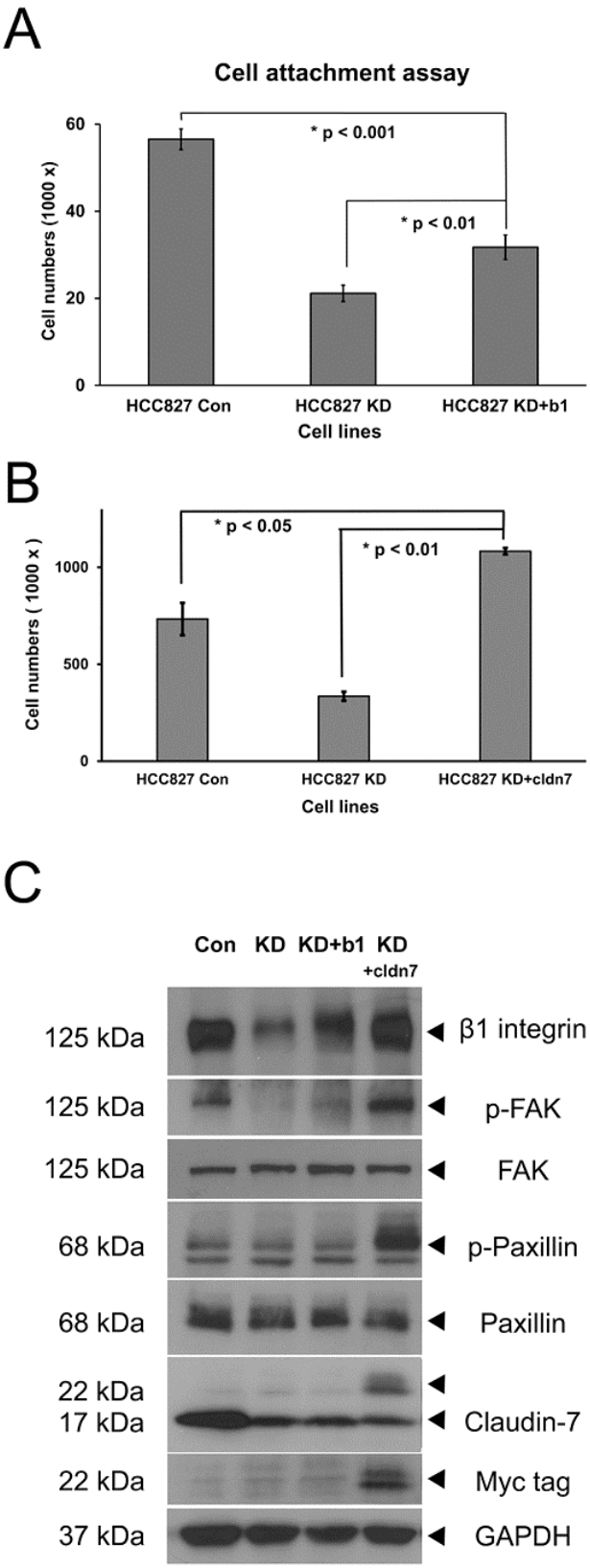

A
Cell attachment assay
Cell numbers (1000 x)
60
40
20
0
* p < 0.001
* p < 0.01
HCC827 Con
HCC827 KD
HCC827 KD+b1
Cell lines
B
Cell numbers (1000 x)
1000
500
0
* p < 0.05
* p < 0.01
HCC827 Con
HCC827 KD
HCC827 KD+cldn7
Cell lines
C
Con
KD
KD+b1
KD +cldn7
125 kDa
β1 integrin
125 kDa
p-FAK
125 kDa
FAK
68 kDa
p-Paxillin
68 kDa
Paxillin
22 kDa
17 kDa
Claudin-7
22 kDa
Myc tag
37 kDa
GAPDH

Figure III.5 Both β1 integrin and claudin-7 improved the cell attachment capability of claudin-7 KD cells to different degrees.

A. Cell attachment assay testing the effect of β1 integrin overexpression in KD cells. A total of 2 × 10^5 cells per cell line were seeded in a 12-well plate and incubated for 4 h at 37°C in a 5% CO_2 humidified incubator. After unattached cells were washed briefly with PBS buffer, the remaining attached cells were trypsinized and counted for evaluation. KD+b1 cells showed significantly more attached cells than claudin-7 KD cells (*$p < 0.01$) but significantly fewer than control cells (*$p < 0.05$). At least three experiments were repeated for statistical analysis. B. Cell attachment assay testing the effect of claudin-7 overexpression in claudin-7 KD cells. A total of 1 × 10^5 control, KD, and KD+cldn7 cells were seeded in a 24-well plate and incubated for 4 h at 37°C in a 5% CO_2 incubator. Unattached cells were washed briefly using PBS buffer, and remaining attached cells were trypsinized and counted for evaluation. KD+cldn7 cells increased the attached cell numbers significantly more than both KD cells (*$p < 0.01$) and control cells (*$p < 0.05$). At least three repetitive experiments were performed for statistical analysis. C. Representative Western blot result on protein expression levels of focal adhesion proteins in control, claudin-7 KD, KD+b1, and KD+cldn7 cells. KD+b1 cells increased the expression levels of β1 integrin and phospho-FAK but not phospho-Paxillin when compared to KD cells. KD+cldn7 cells increased the protein expression levels of β1 integrin, phospho-FAK, and phospho-Paxillin. KD+cldn7 cells showed both an exogenous claudin-7 band at 22 kDa with Myc-tag and endogenous claudin-7 expression at 17 kDa. GAPDH served as a loading control.

CHAPTER IV. CLAUDIN-7 REGULATES GLUCOSE METABOLISM FOR CELL SURVIVAL OF HUMAN LUNG CANCER CELLS

A. Summary

Claudins are a family of TJ membrane proteins involved in human lung cancers. It has been documented that lung cancer patients with high claudin-7 expression survive longer than those with low claudin-7 expression after physical surgery. Our previous study has shown that suppressing claudin-7 expression in a human lung adenocarcinoma HCC827 cells significantly increased cell proliferation in culture and tumor growth in mice. To investigate how the tumor microenvironment facilitates claudin-7 regulation of cell survival, we treated the control and claudin-7 KD cells under hypoxia (1% O_2) for 3 days in light microscopic and cell counting studies and for 1 day in an immunofluorescence staining study to count the cells with cleaved PARP indicating cell death. The results showed that claudin-7 KD reduced cell death more effectively in cell counting ($p < 0.01$) and immunofluorescence staining assays ($p < 0.001$). The KD cells displayed fairly normal-looking morphologies, whereas the control cells displayed shrunken cell morphologies. To examine how claudin-7 modulates glucose metabolism for cell survival, the cells were first pre-treated with a normal (2 g/L) glucose-containing medium in atmospheric oxygen conditions (normoxia) for 2 days. The cells were sub-cultured with a normal or low (0.2 g/L) glucose-containing medium in either hypoxia or normoxia up to an additional 24 h for the short-term hypoxia study, or the cells were subject to low glucose and hypoxic conditions up to an additional 72 h for the chronic hypoxia study. In the Western blot analysis, the short-term hypoxia study revealed substantial inhibition (or phosphorylation) of a negative regulator of glycogen synthesis, GSK3β, and a dramatic decrease in a cellular energy sensor, phospho-AMP kinase, in claudin-7 KD cells, indicating enhanced glycogen synthesis when compared to control cells. In the

chronic hypoxia study, the KD cells greatly reduced glucose-6-phosphate dehydrogenase (G6PD) and increased phospho-pyruvate kinase M2 isoform, suggesting a prompt decrease in DNA synthesis and glycolysis. We conclude that claudin-7 regulates the cell survival and glucose metabolism of human lung cancer cells.

B. Introduction

Lung cancer is one of the leading causes of mortality in the United States (32). Of all cases, 85% were diagnosed as NSCLC, and the five-year survival rate is 15% (33). Lung tissue expresses various TJ proteins depending on their compartments, including claudin-1, -2, -3, -5, -7, -8, and -18 (92). TJ proteins are major cellular junctional transmembrane proteins mainly located at the apical side of the epithelial cells, and they regulate fluid exchange, called paracellular permeability activity, between neighboring cells (106) and the uptake of selective ions (93, 106). Claudins are one of the TJ proteins; they consist of four transmembrane spanning proteins (93), and they interact with other TJ proteins, including Zonula Occludens, Occludins, and actin cytoskeletons, to establish apical cell polarity (106). It has been well established that claudins function as physical and physiological epithelial barriers for maintenance, cell polarity, and water and selective ion transport (93, 106), but their roles in disease, including cancer, remain unclear.

Several studies have shown an interconnection between TJ proteins and cancer cell growth. Dhawan et al demonstrated that overexpressing claudin-2 in colon cancer cells resulted in increased tumor cell numbers in culture *in vitro* and tumor size in mice *in vivo* (107). The authors also found that providing EGF in the colon cancer cell culture increased claudin-2 expression levels *in vitro*. Claudin-7-overexpressing cervical tumor cell lines have also been found to exhibit elevated cell proliferation (108). Conversely, therapeutic antibody treatment targeting claudin-4

protein in human bladder cancer cell lines has been shown to decrease cell proliferation and increase cell apoptosis *in vitro* (109). Although these reports suggest that claudins support cancer cell growth, whether claudin expression always contributes to cancer cell growth and the way in which claudin regulates cancer cell growth and apoptosis remain unclear. Recently, it has been clinically reported that the survival rate of lung cancer patients is strongly inversely associated with their level of claudin-7 expression within five years of physical surgery (35), suggesting that the loss of claudin-7 expression appears to expedite lung cancer progression. These findings discussed above suggest that correlation between cancer progression and claudin expression depends on cancer type.

Similarly, our previous study has demonstrated that claudin-7 KD in HCC827 human lung cancer cells increases cell proliferation in cell culture *in vitro* and mouse tumor growth in nude mice *in vivo* (66). In the *in vivo* mouse study, mouse tumor tissues derived from claudin-7 KD cells substantially increased both cell apoptosis (cleaved PARP) and the inhibitor of cell apoptosis (survivin) when compared to those derived from control cells (66). This suggests that claudin-7 KD cells induce an anti-apoptotic factor to prevent tumor cell death, although the KD cells do not fully inhibit the apoptosis caused by expedited mouse tumor growth that could also establish a tumor microenvironment, such as low oxygen (hypoxia) and low nutrient (including glucose) conditions.

A shift in cancer metabolism to generate cellular energy occurs in order to sustain the massive tumor cell growth (62). In the hypoxic environment, stabilized HIF changes glucose metabolism to aerobic glycolysis, which permits fast cancer growth by providing cellular energy in an inefficient manner but at a faster rate when compared to normally growing non-cancer cells regardless of oxygen (63). This altered metabolic pathway expedites glucose uptake and its

breakdown to pyruvate, which leads to the production of lactate and 2 ATPs at a faster rate in cancer cells, whereas normally growing non-cancer cells direct pyruvate to the TCA cycle for the oxidative phosphorylation of pyruvate in mitochondria, which produces an additional 34 ATPs (64).

Changes in the cellular energy level are monitored by an energy sensor kinase called adenosine monophosphate kinase (AMPK) (62, 63). It is activated by phosphorylation at the threonine 172 site (phospho-AMPK) when adenosine monophosphate (AMP) is high or cellular energy is low (110). The activated AMPK promotes ATP generation through anabolic metabolism, such as fatty acid oxidization, and inhibits fatty acid synthesis (111, 112). AMPK also induces autophagy that breaks down and recycles cellular components to generate ATP (113). Interestingly, it has been reported that AMPK expression in lung cancer patients is correlated with their survival rate (114), suggesting that AMPK promotes anti-tumor activity.

In order to avoid impaired tumorigenesis, cancer cells are capable of storing glycogens by HIF-1α or HIF-2α induction under hypoxia conditions for cancer cell survival in the event of glucose withdrawal conditions (115). In addition, the breakdown of glycogen by glycogen phosphorylase under limited glucose conditions has been prominently reported to protect cancer cells from undergoing either cancer apoptosis or premature senescence induced by ROS (116, 117). This suggests that glycogen stored within cancer cells is used to provide glucose for glycolysis in generating cellular energy and for the pentose pathway in synthesizing antioxidants, such as nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH), which neutralize ROS. However, the way in which claudin is linked to cancer cell metabolism for cell survival has not been extensively investigated.

In the current study, we investigate whether claudin-7 in lung cancer cells alters cell death

and the way in which claudin-7 modifies glucose metabolism in the tumor microenvironment *in vitro*. We demonstrate that claudin-7 KD lung cancer cells have the potential to synthesize glycogen as an energy source upon hypoxia. Our report shows that claudin-7 KD cells further reduce cancer cell apoptosis by reprogramming glucose metabolism in favor of lung cancer cell survival when compared to control cells.

C. Materials and methods

C.1. *Antibodies*

Rabbit polyclonal anti-Histone H3 (Cat. No. 9715), anti-phospho-Thr172-AMPKα (Cat. 2531), anti-AMPKα (Cat. 2795), anti-phospho-Ser9-GSK3β (Cat. 9336), anti-phospho-Y105-pyruvate kinase isozymes M2 (PKM2) (Cat. 3827), anti-PKM2 (Cat. No. 3198), anti-G6PD (Cat. 8866), and mouse monoclonal anti-α-tubulin (Cat. 3873) were purchased from Cell Signaling Technology. Rabbit polyclonal anti-claudin-7 antibody (Cat. 18875) was obtained from Immuno-Biological Laboratories. Rabbit polyclonal anti-HIF-1α (Cat. SC-10790) and anti-Glut1 antibodies (Cat. PA1-16152) were purchased from Santa Cruz Biotechnology and Thermo Scientific, respectively. Mouse monoclonal anti-GSK3β (Cat. 610201) antibodies from BD Transduction Laboratories were a kind gift from Dr. Qun Lu at East Carolina University. Secondary anti-mouse (Cat. W4021) and anti-rabbit (Cat. W4011) antibodies conjugated with HRP were obtained from Promega.

C.2. *Cell lines and reagents*

HCC827 human NSCLC cell lines were obtained from ATCC and cultured in RPMI-1640

medium (Cat. 11875-093, Gibco, Long Island, NY) supplemented with heat-inactivated 10% fetal bovine serum (Cat. SH3007001, HyClone) and 1% 10,000 U/ml penicillin and 10,000 μg/mL streptomycin (Cat. 15140-122, Gibco, Carlsbad, CA) at 37°C in a 5% CO_2 humidified incubator in atmospheric oxygen conditions. HCC827 control and claudin-7 KD cell lines were established as previously described [1]. For hypoxia treatment, cells were treated at 1% O_2 and harvested inside the Invivo$_2$ 400 hypoxia workstation (Baker Ruskinn, Sanford, ME).

C.3.*Treatment of cell culture to mimic tumor microenvironment* in vitro

In order to mimic the tumor microenvironment *in vitro*, HCC827 control and claudin-7 KD cells were pre-cultured in a poly-D-lysine-coated 8-well plate (Cat. 354632, BD Biocoat, Rockford, IL) in normal (2 g/L) glucose-containing medium and at 37°C in a 5% CO_2 humidified incubator in atmospheric oxygen conditions (normoxia) for 2 days. They were then sub-cultured in either normal or low (0.2 g/L) glucose-containing medium at 37°C in a 5% CO_2 humidified incubator in 1% oxygen conditions (hypoxia) up to an additional 1 day for the short-term hypoxia study or 3 days for the chronic hypoxia study.

The chronic hypoxia study was conducted in hypoxia and low glucose culture conditions in order to mimic the tumor microenvironment *in vitro*, without replenishing with fresh cell culture media. Six sites per cell line were randomly selected at 200 × magnification using a Zeiss Axiovert S100 inverted light microscope (Carl Zeiss Inc.) and photographed using Axiovision 4.6 imaging software (Carl Zeiss Inc.).

C.4.*SDS-PAGE, Western blot, and cell fractionation*

Whole cells were lysed in RIPA buffer (1% Triton-100, 0.5% deoxycholate, 0.2% sodium

dodecyl sulfate, 150 mM sodium chloride, 2 mM ethylene diamine tetraacetic acid, 10 mM sodium pyrophosphate, 20 mM sodium fluoride) supplemented with a complete protease inhibitor cocktail tablet (Cat. 11836153001, Roche Diagnostics) and phosphatase inhibitors, including 1 mM sodium orthovanadate and 1 mM hydrogen peroxide. Supernatants from the protein lysate were separated from cell debris by centrifugation at 4°C, and protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Cat. 23225, Thermo Scientific). Next, 20 μg of protein were loaded and separated for each well of SDS-PAGE gel, transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare) by electrophoresis, and then blocked and immuno-blotted with appropriate primary antibodies followed by peroxidase-conjugated secondary antibodies. Protein bands were detected using ECL detection reagent (GE Healthcare) and photographed using an X-ray film developer. Cell fractionation was performed to separate cytosolic and nuclear extracts to probe for HIF-1α in the Western blot according to the instructions of NE-PER Nuclear and Cytoplasmic Extraction Reagents (Cat. 78833, Thermo Scientific). Histone H3 and α-tubulin served as loading control proteins in nuclear extracts and cytosolic extracts, respectively.

C.5.*Cell counting assays*

HCC827 control and claudin-7 KD cells cultured for 2 days in normal glucose and normoxic conditions were sub-cultured under hypoxia (1% O_2) in an 8-well plate up to 24 h or 72 h. Dead and total cell numbers were counted for the 72-h hypoxia study using the trypan blue exclusion method in a Countess™ automated cell counter (Cat. C10227, Invitrogen). The proportions of dead cells per cell line were obtained in at least triplicate.

C.6. *Immunofluorescence staining*

The cells sub-cultured for 1 day under hypoxia were fixed in -20°C ice-cold methanol for 5 min and briefly washed with phosphate buffered saline (PBS). They were blocked with 3% BSA, incubated with poly rabbit anti-cleaved PARP antibody solution (1:200) and anti-rabbit Cyt3-tagged antibody solution (1:400) for 1 h each in series at room temperature, and then treated with Hoechst (1:5000) for nucleus staining for 1 min. The labeled cells were mounted with Antifade solution (Invitrogen). Six random spots per cell line were selected at 200 x magnification using a Zeiss Axiovert S100 laser scanning microscope (Carl Zeiss Inc.) and photographed using Axiovision 4.6 imaging software (Carl Zeiss Inc.). Proportions of cell populations with PARP cleavage were estimated by the ratio of cleaved PARP numbers over total nucleus numbers per spot.

C.7. *Statistical analysis*

All experiments were performed at least three times, and data were presented as means ± standard error of means. Student's *t*-test was performed to compare samples from two cell lines using IBM's SPSS program. We considered differences statistically significant when $p < 0.05$.

D. Results

D.1. *Claudin-7 KD cells showed reduction in cell death under hypoxia conditions*

Our previous study has shown that claudin-7 KD in HCC827 lung cancer cells decreased pro-apoptosis protein (cleaved PARP) *in vitro*, whereas our *in vivo* mouse study results revealed an increase in both pro-apoptotic protein and anti-apoptotic protein (survivin) from mouse tumor tissues derived from claudin-7 KD cells (66). This suggests that mouse tumors derived from

claudin-7 KD cells are more resistant to cell death despite the presence of apoptotic expression (cleaved PARP). To examine whether claudin-7 altered hypoxia-induced cancer cell death, we subjected both claudin-7 control and KD cells to 1% oxygen (hypoxia) conditions without replenishing with new cell culture media for 3 days.

Light microscopy revealed that the claudin-7 KD cells maintained normal-looking cell layers. However, given the same cell culture conditions, claudin-7 control cells showed shrunken shapes, suggesting significantly more apoptotic cells (Fig. IV.1.A). The cell counting assays based on trypan blue exclusion further confirmed that the proportion of the dead cell population was significantly increased in claudin-7 control cells (55%) when compared to KD cells (20%) (* $p < 0.01$) (Fig. IV.1.B), indicating that the KD cells were more resistant to cell death than the control cells.

In order to examine whether the resistance of claudin-7 KD cells against cell death was attributed to reduced apoptotic events, we evaluated the proportion of the cell population that underwent cell apoptotic events based on the apoptotic marker protein cleaved PARP after short-term hypoxia treatment for 1 day. The immunofluorescence staining images demonstrated that the proportion of the claudin-7 KD cell population expressing cleaved PARP was also significantly decreased when compared to that of the control cell population (*$p < 0.001$) (Figs. IV.2.A and IV.2.B). All the results suggest that claudin-7 KD cells are able to inhibit cell apoptosis in hypoxia (1% O_2) and that claudin-7 KD cells are resistant to hypoxia-induced cell death.

D.2.*Claudin-7 KD cells maintain phosphorylation of GSK3β in hypoxia and low glucose culture conditions*

As previously mentioned, we found that HCC827 claudin-7 KD cells were largely

protected from apoptotic events when compared to control cells under chronic hypoxia for up to 3 days. This result suggests that claudin-7 KD cells overcome apoptosis in the tumor microenvironment by switching their cancer metabolism in support of cancer cell survival.

To investigate whether different tumor microenvironments changed the regulation of the claudin-7 phenotype in glucose metabolism and cellular energy *in vitro*, both HCC827 claudin-7 KD and control cells were initially cultured in normoxia (atmospheric 21% O_2) and normal glucose-containing culture conditions for 2 days as time point 0. The pre-cultured cell lines were sub-cultured in four different culture conditions, including either normoxia or hypoxia (1% O_2) and normal (2g/L) or low (0.2g/L) glucose-containing media conditions, for up to an additional 24 h. The short-term hypoxia experiment was conducted to first examine whether claudin-7 regulates HIF, which controls the expression of several key enzymes, by regulating glycolysis for up to 24 h.

In the Western blot analysis, the HIF-1α level declined rapidly in claudin-7 KD cells regardless of oxygen and glucose levels by 24 h, whereas claudin-7 control cells maintained relatively high levels of HIF-1α in hypoxia and normal glucose culture conditions (Figs. IV.3.A and IV.3.B). On the other hand, glucose transport 1 protein (Glut-1) on both claudin-7 control and KD cells were consistently expressed in both normal and low glucose cultures in normoxia and hypoxia conditions regardless of claudin-7 phenotypes (Figs. IV.3.A and IV.3.B), indicating that claudin-7 does not extensively modulate Glut-1 protein levels to regulate glucose uptake in hypoxia treatment for 24 h, although levels of HIF-1α changed depending on oxygen and glucose levels.

Next, we examined whether the claudin-7 phenotype modulates GSK3β, a negative regulator for glycogen synthase (GS). In hypoxia and normal glucose culture conditions, the level

of inhibitory phosphorylation of GSK3β at the serine 9 site (phospho-GSK3β) in claudin-7 KD cells was substantially higher than that of control cells for up to 24 h (Left panel, Fig. IV.3.A). A similar observation was also made in hypoxia and low glucose culture conditions for 24 h (Right panel, Fig. IV.3.A). Interestingly, HIF-1α, which is known to support glycogen synthesis in hypoxic conditions (118), almost disappeared in the claudin-7 KD cells in hypoxia conditions by 24 h regardless of glucose concentration (Fig. IV.3.A). This result suggests that claudin-7 KD cells chiefly inhibit the activity of GSK3β so as to keep GS in active status, while HIF-1α is not available to sustain glycogen synthesis in hypoxia and low glucose conditions

In normoxia and normal glucose culture conditions, inhibitory phospho-GSK3β levels were not noticeably different between claudin-7 control and KD cells at all time points (Left panel, Fig. IV.3.B). Similarly, no significant difference was found in levels of phospho-GSK3β between claudin-7 control and KD cells in normoxia and low glucose culture conditions. However, phospho-GSK3β levels of both claudin-7 control and the KD cells were slightly and equally increased at the 8-h time point when compared to those levels of both cells at the 0-and 24-h time points (Fig. IV.3.B). This result suggests that both claudin-7 control and KD cells activate GS 8 h after new low glucose culture media is provided, but both cells become adapted and decrease GS levels back to normal levels by 24 h in normoxia and normal glucose conditions. As expected, HIF-1α expression was not remarkably detectable at either the 8- or 24-h time points in both claudin-7 control and KD cells due to the presence of atmospheric oxygen, suggesting that phospho-GSK3β is not greatly regulated by HIF-1α or claudin-7 in normoxia conditions.

D.3.*Claudin-7 KD cells downregulate AMPK activation under hypoxia and low glucose culture conditions*

The results of our current study using four cell culture conditions has shown that GSK3β levels of claudin-7 control and KD cells change differently in hypoxia conditions, suggesting that claudin-7 modulates glucose metabolism in hypoxia conditions. Thus, we further examined whether claudin-7 altered AMP kinase (AMPK), which senses cellular energy levels (62, 63, 110).

In hypoxia and normal glucose culture conditions (Left panel, Fig. IV.3.A), a Western blot analysis first revealed that claudin-7 control cells slightly increased levels of both active phospho-AMPK and total AMPK by 8 h and maintained their levels for up to 24 h. Claudin-7 control cells also showed substantially lower levels of both active and total AMPK than claudin-7 KD cells at the 8- and 24-h time points, respectively. On the other hand, claudin-7 KD cells significantly increased levels of both active phospho-AMPK and total AMPK by 8 h. While the total AMPK level later remained the same, the active phospho-AMPK level quickly declined by 24 h under hypoxia and normal glucose culture conditions.

On the other hand, in hypoxia and low glucose culture conditions (Right panel, Fig. IV.3.A), the total AMPK form of claudin-7 control cells remained at constantly lower levels than that of claudin-7 KD cells for up to 24 h. Similarly, claudin-7 control cells kept the highly elevated level of the active phospho-AMPK form for up to 24 h, but the level of the active AMPK form rapidly increased by 8 h and quickly dropped by 24 h in claudin-7 KD cells. A similar fluctuating pattern of changes in total AMPK levels was also found in the KD cells by 8 and 24 h.

All the experimental results from the hypoxia conditions mentioned above recapitulate the hypothesis that the claudin-7 KD phenotype decreases AMPK activation differently depending on glucose level. When normal glucose is provided, claudin-7 KD cells decrease the level of active

phospho-AMPK while maintaining the total AMPK level. However, when glucose levels are low, claudin-7 KD cells decrease levels of both active phospho-AMPK and total AMPK. This indicates that a decrease in the level of total AMPK is attributed to the decreased level of active AMPK. AMPK is a cellular energy sensor kinase that shifts glucose metabolism in a way that generates cellular energy, increases catabolic processes, and decreases anabolic processes when the AMP level is high (119). In this sense, the results of our hypoxia treatment experiments suggest that claudin-7 KD cells decrease active AMPK levels to promote anabolic processes in hypoxia treatment for up to 24 h.

Next, we proceeded to execute our cell culture experiments in normoxia and normal glucose culture conditions (Left panel, Fig. IV.3.B). Both claudin-7 control and KD cells slightly and comparably increased the levels of both active and total AMPK forms by 8 h and decreased the levels of both active and total AMPK forms by 24 h. This indicates that claudin-7 control and KD cells equally increase both active and total AMPK levels in order to facilitate the uptake of freshly provided glucose for 8 h, and both claudin-7 control and KD cells later decrease their levels of both active and total AMPK by 24 h in normoxia (21% O_2) and normal (2 g/L) glucose culture conditions.

A similar shifting pattern of changes in total AMPK levels was also seen when both claudin-7 control and KD cells were cultured in normoxia and low glucose culture conditions for up to 24 h (Right panel, Fig. IV.3.B). However, the elevated active phospho-AMPK levels of both claudin-7 control and KD cells at the 8-h time point were kept at comparably high levels for up to 24 h in normoxia and low glucose culture conditions. This suggests that low glucose conditions keep activating AMPK in both claudin-7 control and KD cells for up to 24 h.

Thus, the results from the normoxia and low glucose condition experiment demonstrated

that both claudin-7 control and KD cells dramatically and equally decreased the levels of active phospho-AMPK by 24 h in normoxia and normal glucose culture conditions. However, both control and KD cells comparably increased active AMPK levels by 8 h and maintained the levels for up to 24 h in normoxia and low glucose culture conditions. These observations clearly indicate that glucose level controls active phospho-AMPK levels in claudin-7 control and KD human lung cancer cells in normoxia conditions. Similarly, several previous *in vitro* and *in vivo* studies have documented that high glucose treatment inhibits active phospho-AMPK levels (120). Therefore, the results from our current normoxia treatment experiment suggest that normoxia conditions do not differentially regulate levels of active phospho-AMPK between claudin-7 control and KD cells at all time points.

In summary, we found that HCC827 claudin-7 KD cells greatly decreased phospho-AMPK levels to a further lower level when compared to that of claudin-7 control cells after 24 h hypoxia and low glucose culture treatment *in vitro* (Right panel, Fig. IV.3.A).

D.4.*Claudin-7 KD cells showed some reduction in glycolytic and pentose phosphate pathway in chronic hypoxia conditions*

All the data illustrated above point out that claudin-7 KD cells decreased cancer cell apoptosis and inactivated AMPK activity, and inhibited (or phosphorylated) GSK3β, which possibly suppressed the activity of GS in hypoxia and low glucose conditions *in vitro*.

Interestingly, it has been previously documented that the β subunit of AMPK binds to glycogen, and it has been suggested that AMPK may interact with enzymes regulating glycogen synthesis, such as GS, GSK3β, and glycogen phosphorylase (121). AMPK has also been reported to block the activity of GS (120). These findings suggest that both AMPK and GSK3β possibly

inhibit GS. Interestingly, our current experimental results have shown that claudin-7 KD cells not only intensely decreased active phospho-AMPK but also greatly inhibited (phosphorylated) GSK3β in hypoxia and low glucose conditions for up to 24 h (Right panel, Fig. IV.3.A). This suggests that the suppression of both GSK3β and AMPK in claudin-7 KD cells results in potentially activating GS in hypoxia and low glucose conditions for up to 24 h. In addition, we have demonstrated that claudin-7 KD cells showed a significant reduction in cell apoptosis (cleaved PARP) when compared to control cells after 24 h of hypoxia treatment. These experimental results suggest that claudin-7 KD cells protect against cell apoptosis more efficiently than claudin-7 control cells by potentially increasing the activity of GS through modulating GSK3β and AMPK.

Based on the possible changes in glucose metabolism, we next examined whether claudin-7 KD cells also manipulated other key genes regulating the glucose metabolic pathways, such as the glycolysis and pentose phosphate pathways, for cancer cell survival in chronic hypoxia and low glucose culture conditions for up to 3 days.

Western blot results (Fig. IV.4.A) revealed that phosphorylation of pyruvate kinase isoform M2 at the tyrosine 105 site (phospho-PKM2) was slightly increased in claudin-7 KD cells after 3 days of hypoxia and low glucose culture treatment, indicating that KD cells lead to an earlier reduction in the conversion of phosphoenolpyruvate (PEP) to pyruvate in aerobic glycolysis. In addition, claudin-7 KD cells further decreased the expression level of G6PD earlier than the claudin-7 control cells from 2 days of hypoxia and low glucose culture treatment. This result also suggests that claudin-7 KD cells decrease the pentose phosphate pathway for nucleotide synthesis earlier than the control cells in chronic hypoxia and low glucose conditions.

Taken together, we conclude that claudin-7 KD cells more efficiently support cancer cell

survival by inhibiting cell apoptosis in the tumor microenvironment *in vitro*. They do so by coordinately regulating the overall glucose metabolic pathways by potentially activating GS, decreasing the conversion of PEP to pyruvate, and inhibiting nucleotide synthesis.

E. Discussion

Since the discovery of aerobic glycolysis or Warburg's effect, which is still commonly accepted as facilitated glycolysis to produce lactate for cancer cell growth and metabolism (62), several broad attempts to identify other types of altered cancer metabolism have been reported. In the meantime, the overexpression of TJs, including claudins, in several different cancer cell lines has been shown to promote cancer progression, which decreases cancer cell apoptosis and increases cancer cell growth and metastasis *in vitro* or *in vivo* (29, 107, 122).

Interestingly, several experiments using claudin overexpression do not always seem to support cancer cell proliferation. Cunniffe et al reported that claudin-1 and -7 overexpression in ovarian cancer cells did not increase cell proliferation (108). In contrast, our previous studies have demonstrated that claudin-7 overexpression decreases lung cancer cell proliferation *in vitro* (66). These results suggest that the interconnection between claudin expression and cancer cell growth is specific to certain cancer cell types. However, it is unclear whether TJs modulate cancer metabolism. Thus, this current study has focused on how the TJ protein claudin-7 regulates glucose metabolism in human lung cancer cell lines in order to promote cancer cell survival in the tumor microenvironment *in vitro*.

Our previous *in vivo* study has shown that claudin-7 KD in lung cancer cell lines induces PARP cleavage, indicating apoptosis, but it also promotes survivin expression, reflecting anti-

apoptosis (66). Although the apoptotic event was thought to suppress mouse tumor growth, the mouse tumor inoculated with claudin-7 KD cells still grew into a large mouse tumor when compared to that inoculated with claudin-7 control cells (66), suggesting that anti-apoptotic expression (survivin) may inhibit apoptotic cascades by binding terminal effector cell death caspase-3 or -7 cleavage (123). Conversely, our previous study showed that cleaved PARP was almost abolished while survivin was still highly expressed in claudin-7 KD cells in cell culture *in vitro*. The disparity in the induced level of cleaved PARP between *in vivo* and *in vitro* studies could be explained by the fact that cell culture conditions offer the free exchange of oxygen and nutrients to cells, which inhibits cell apoptosis (66). Overall, it is likely that tumor microenvironments, such as oxygen and glucose-deprived (OGD) conditions, promote better survival of claudin-7 KD cells by suppressing the apoptotic process, which could also allow for the acceleration of larger mouse tumor growth, as shown in our previous *in vivo* mouse study (66). Therefore, this current study has further investigated the way in which claudin-7 KD cells decrease cancer cell apoptosis in OGD conditions that mimic tumor microenvironments *in vivo*.

The next main question of this current study is whether claudin-7 KD lung cancer cells modulate glucose metabolism to evade cell apoptosis *in vitro*. We observed that claudin-7 control cells significantly increase not only apoptosis (Figs. IV.2.A and IV.2.B) but also the potential for autophagy, shown as a shrunken cell shape (Fig. IV.1.A). The claudin-7 control cells seem to break down their cellular components often in generating cellular energy for cell survival, possibly due to their low cellular energy levels. In contrast, claudin-7 KD cells maintain normal-looking epithelial cells in the same hypoxia condition for 3 days, suggesting a possible shift in glucose metabolism in the claudin-7 KD cells in securing their cellular energy level required for cell survival.

The fate of cell survival is determined in the context of the cellular environment. For example, hypoxia conditions stabilize HIF, which increases glucose uptake and promotes aerobic glycolysis for lactate production, while HIF activates pyruvate dehydrogenase kinase 1 (PDK1) to inhibit pyruvate dehydrogenase (PDH) that converts pyruvate to acetyl-CoA for oxidative phosphorylation in the mitochondria (124). On the other hand, low glucose conditions increase active phospho-AMPK, which then promotes autophagy by recycling cellular components to produce the cellular energy required for cell survival (125, 126). However, severe hypoxia and low glucose conditions in combination causes high cellular stress that activates AMPK and induces autophagy, leading to cell death (127).

Interestingly, the combinatory effect of both low oxygen and low glucose conditions also appears to induce ROS production. It has been reported that lens epithelial cells create ROS via protein kinase RNA-like endoplasmic reticulum kinase (PERK) signals in OGD conditions (128).

Similarly, OGD conditions are not beneficial for the survival of certain cancer cells. For example, it has been shown that in hypoxia conditions, C2C12 myoblast cancer cells in low glucose conditions substantially reduce lactate production when compared to those cells in high glucose conditions (129). This suggests that aerobic glycolysis in hypoxia conditions is compromised due to limited glucose sources. In low glucose culture conditions, the β-oxidation of fatty acids, which produces acetyl-CoA, could be an alternate pathway to produce ATP. Consuming the acetyl-CoA to produce ATP requires TCA cycles through mitochondrial oxygen respiration. Thus, hypoxia and low glucose culture conditions eventually hinder the pathway of β-oxidation of fatty acids.

Our data suggest that under OGD conditions, claudin-7 KD cells rapidly decrease active phospho-AMPK and increase inhibitory phospho-GSK3β, which possibly supports the activity of

GS, but control cells increase active phospho-AMPK and decrease inhibitory phospho-GSK3β, which possibly suppresses the activity of GS. Thus, it appears that claudin-7 KD cells could synthesize more glycogen than control cells in hypoxia conditions, although we have not directly measured the content of glycogen within both claudin-7 control and KD cells. Therefore, we need to conduct a follow-up investigation to evaluate the amount of stored glycogen in both claudin-7 control and KD human lung cancer cells.

Although some glucose is necessary to maintain HIF-1α in hypoxia conditions (130), claudin-7 KD cells significantly reduced HIF-1α expression unlike the control cells after 24 h of hypoxia and normal glucose culture treatment (Left panel, Fig. IV.3.A). It is possible that in the HCC827 claudin-7 KD cells, aerobic glycolysis in hypoxia conditions may not be in high demand to maintain sufficient cellular energy levels for cell survival by increasing inhibitory phospho-GSK3β. Similarly, we found that the active phospho-AMPK level was dramatically reduced in claudin-7 KD cells when compared to control cells at the 24-h time point in OGD conditions (Right panel, Fig. IV.3.A). These results suggest that claudin-7 KD cells inactivate (or dephosphorylate) AMPK to evade the possible cell apoptosis resulting from the high cellular stress caused by OGD conditions. The increase in phospho-GSK3β and decrease in phospho-AMPK may constitute a metabolic phenotype of claudin-7 KD cells. Interestingly, it has been well established in a clinical report that reduced AMPK levels are strongly associated with lower survival rates of lung cancer patients (114). Another clinical group has also documented that low claudin-7 expression results in reduced survival rates of lung cancer patients (35). Considering that claudin-7 KD cells exhibit substantially decreased activity of AMPK by reducing either active phospho-AMPK or total AMPK levels in claudin-7 KD cells depending on glucose levels, it seems that claudin-7 and AMPK are correlated to each other. However, whether claudin-7 synergistically co-regulates

AMPK or vice versa in lung cancer progression remains unclear.

Next, we also investigated how claudin-7 KD cells modulated glucose metabolism in order to avoid cell apoptosis-induced cell death under chronic hypoxia conditions for up to 3 days, as claudin-7 KD cells maintained normal-looking cell layers whereas control cells shrunk (Fig. IV.1.A). It has been well established that cancer cells regulate PKM2 to reroute glucose flux from the glycolytic pathway to the pentose phosphate pathway in order to build up nucleotides and protect cancer cells against ROS by generating the antioxidants NADPH and GSH for cancer cell survival (131). As expected, our experimental results revealed that phospho-PKM2 levels were slightly increased in claudin-7 KD cells when compared to control cells after 3 days of OGD conditions (Fig. IV.4.A). However, G6PD was substantially reduced in the KD cells from day 2 (Fig. IV.4.A), suggesting that claudin-7 KD cells slow down glucose flux toward the pentose pathway earlier than control cells under OGD conditions. Consequently, claudin-7 KD cells are likely to reduce levels of antioxidants, including NADPH and GSH, due to the possibly decreased pentose phosphate pathway. This observation has raised the question of how claudin-7 KD cells can protect against cell death by increased ROS in OGD conditions without obtaining sufficient levels of antioxidants through the pentose phosphate pathway.

It is possible that claudin-7 KD cells immediately decrease nucleotide synthesis in order to avoid severe DNA damage by ROS in OGD conditions that could result in apoptosis. In addition, claudin-7 KD cells could also produce more GSH and NADPH through an altered glucose metabolic pathway to protect themselves against DNA damage. For example, it has been established that a glucose metabolite, 3-phosphoglycerate (3PG), can be used to synthesize serine and glycine, both of which are conjugated with glutamate to synthesize GSH (132).

In addition, claudin-7 KD cells may utilize TCA cycles to generate cellular energy by

modulating HIF. We found that claudin-7 KD cells rapidly reduced HIF-1α levels by 24 h in hypoxia treatment when compared to claudin-7 control cells (Left panel, Fig. IV.3.A). HIF-1α activates PDK, which inhibits PDH (133). PDH is responsible for converting pyruvate into acetyl-CoA for its entry to the TCA cycle (133). In this sense, claudin-7 KD cells seem to gain access to TCA cycles in order to produce more ATP due to their abolished HIF-1α expression 24 h after hypoxia treatment. It has been documented that several advanced-staged cancer cells access the TCA cycle by activating PDH in addition to aerobic glycolysis (134).

For NSCLC cells, pyruvate carboxylase (PC) has also been documented to convert from pyruvate to oxaloacetate in order to participate in the TCA cycle, which also generates GSH to protect lung cancer cells against ROS and support their cell proliferation (135). In addition, cancer cells under nutrient-deprived conditions have been demonstrated to induce endoplasmic reticulum (ER) stress, which activates mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) that converts from oxaloacetate in mitochondria to PEP for gluconeogenesis (136). This metabolic shift also provides TCA cycles with the majority of oxaloacetate and pyruvate that originates from lactate (136). These findings suggest that lung cancer cells can also use lactate to fuel the TCA cycle in generating cellular energy ATP and antioxidant GSH in nutrient-deprived conditions. Moreover, the accumulation of fumarate and succinate in the TCA cycle due to mutations in fumarate hydratase (FH) and succinate dehydrogenase (SDH) in cancer cells has been reported to suppress the degrading activity of prolyl hydroxylase (PH) for HIF-1α (137). We are not aware of whether claudin-7 directly regulates the genes encoding for FH and SDH enzymes. However, claudin-7 KD cells almost abolished HIF-1α expression, unlike control cells, after 24 h of hypoxia (Fig. IV.3.A). Thus, the KD cells are likely to preserve the degradative activity of PH for HIF-1α even in hypoxia conditions for up to 24 h, possibly by activating the genes of FH and SDH in

running the TCA cycle, which could decrease the amount of available fumarate and succinate. Likewise, claudin-7 KD cells could also modulate the TCA cycle in such a way that more antioxidant GSH is generated and the ROS generation caused by ER stress in OGD conditions through TCA cycles is minimized, as previously discussed (128).

We conclude that claudin-7 regulates the genes responsible for glucose metabolism for the survival of lung cancer cells under tumor microenvironments.

F. Acknowledgement

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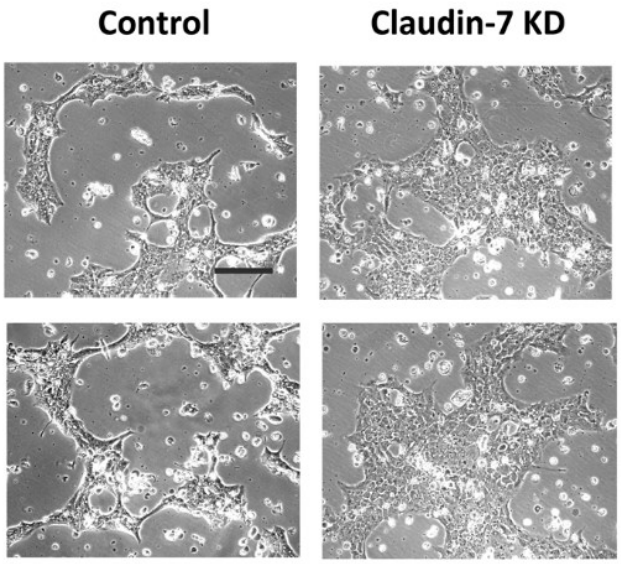
A


Control
Claudin-7 KD

B

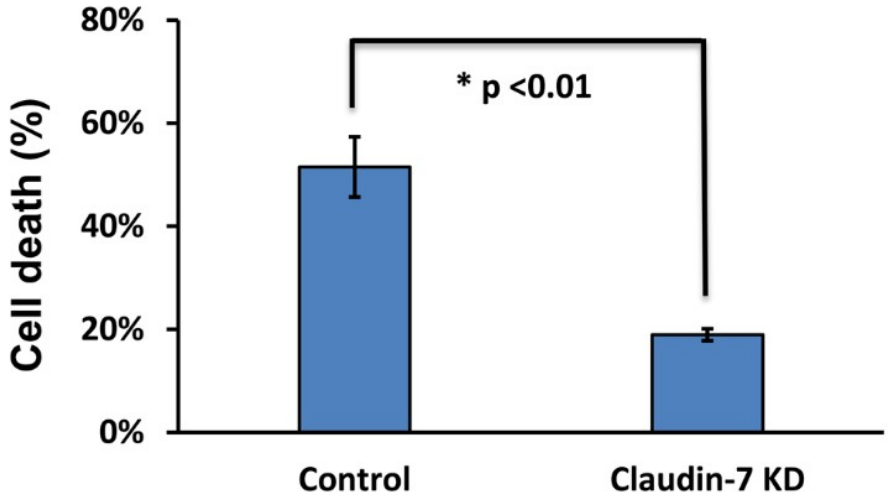

80%
60%
40%
20%
0%
Cell death (%)
* p <0.01
Control
Claudin-7 KD

Figure IV.1 Claudin-7 KD inhibited hypoxia-induced cell death.

Both claudin-7 control and KD cells were cultured in poly D-lysine-coated 8-well plates under normoxia and normal (2 g/L) glucose-containing media conditions for 2 days. Then, both cells were sub-cultured in hypoxia for 3 days. **A.** Three days after hypoxia treatment, six random spots of cell shapes per cell line were photographed at 100 × magnification using a Zeiss Axiovert S100 inverted light microscope (Carl Zeiss Inc.) and photographed using Axiovision 4.6 imaging software (Carl Zeiss Inc.). Scale bar: 10 μm. **B.** Claudin-7 KD cells showed a significant reduction in the proportion of the cell death population when compared to control cells (*$p < 0.01$). At least three experiments were performed for statistical analysis.

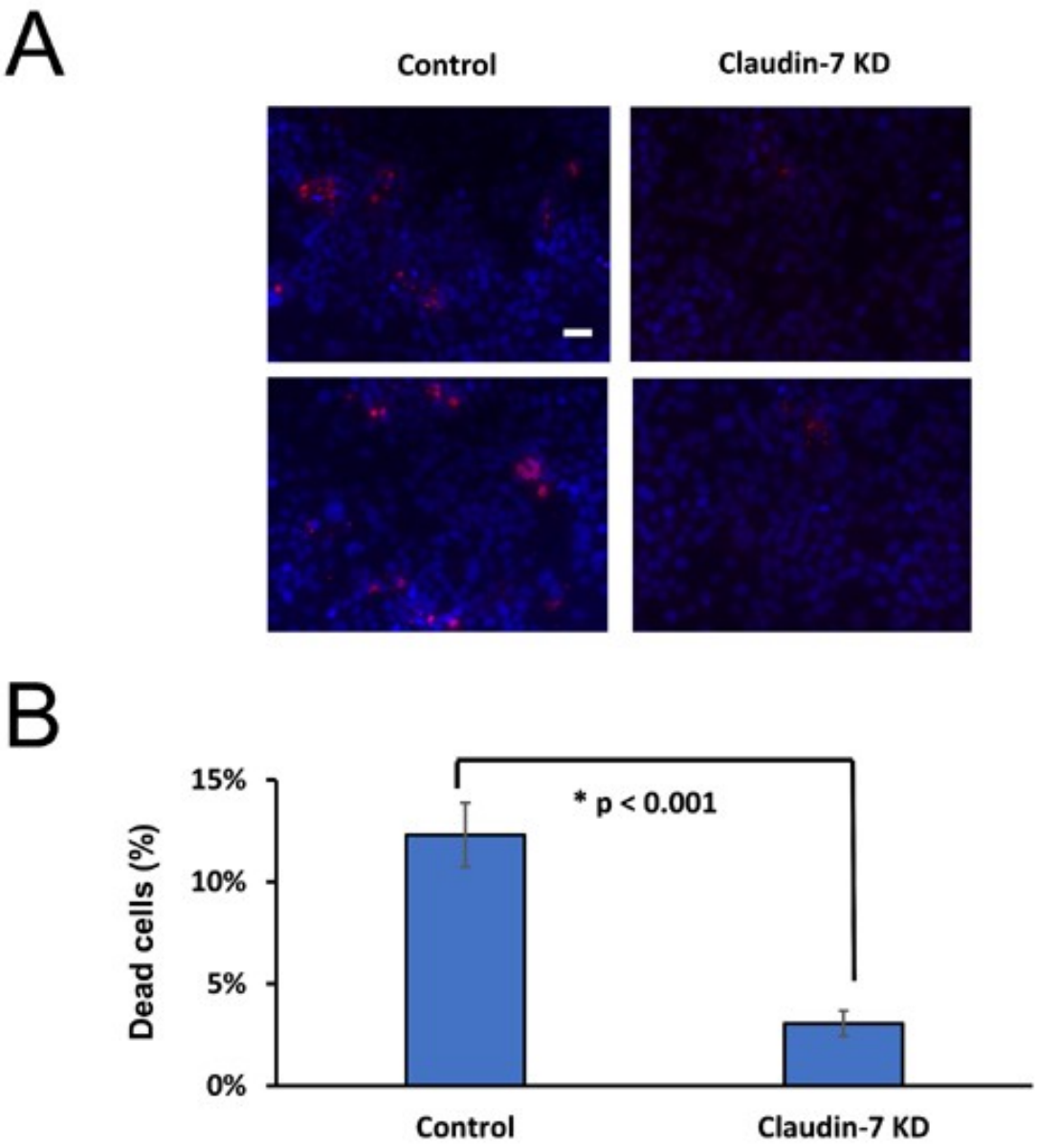

A
Control
Claudin-7 KD
B
Dead cells (%)
15%
10%
5%
0%
* p < 0.001
Control
Claudin-7 KD

Figure IV.2 Claudin-7 KD cells reduced cell apoptosis.

Both claudin-7 control and KD cells were cultured in poly-D-lysine-coated 8-well plates under normoxia and normal (2 g/L) glucose-containing media culture for 2 days. Then, both cells were sub-cultured in hypoxia for 1 day and fluorescently labeled for PARP cleavage indicating cell apoptosis. **A.** Representative immunofluorescent images. Cleaved PARP and nuclei were respectively double-labeled as red and blue spots. The proportion of the apoptotic cell death population was estimated by the ratio of numbers of cleaved PARP over numbers of nuclei. Six random spots per cell line were selected at 200 x magnification using a Zeiss Axiovert S100 laser scanning microscope (Carl Zeiss Inc.) and photographed using Axiovision 4.6 imaging software (Carl Zeiss Inc.). Scale bar: 50 μm. **B.** Claudin-7 KD cells showed a dramatically decreased proportion of the apoptotic cell population when compared to control cells (*$p < 0.001$) At least three experiments were performed for statistical analysis.

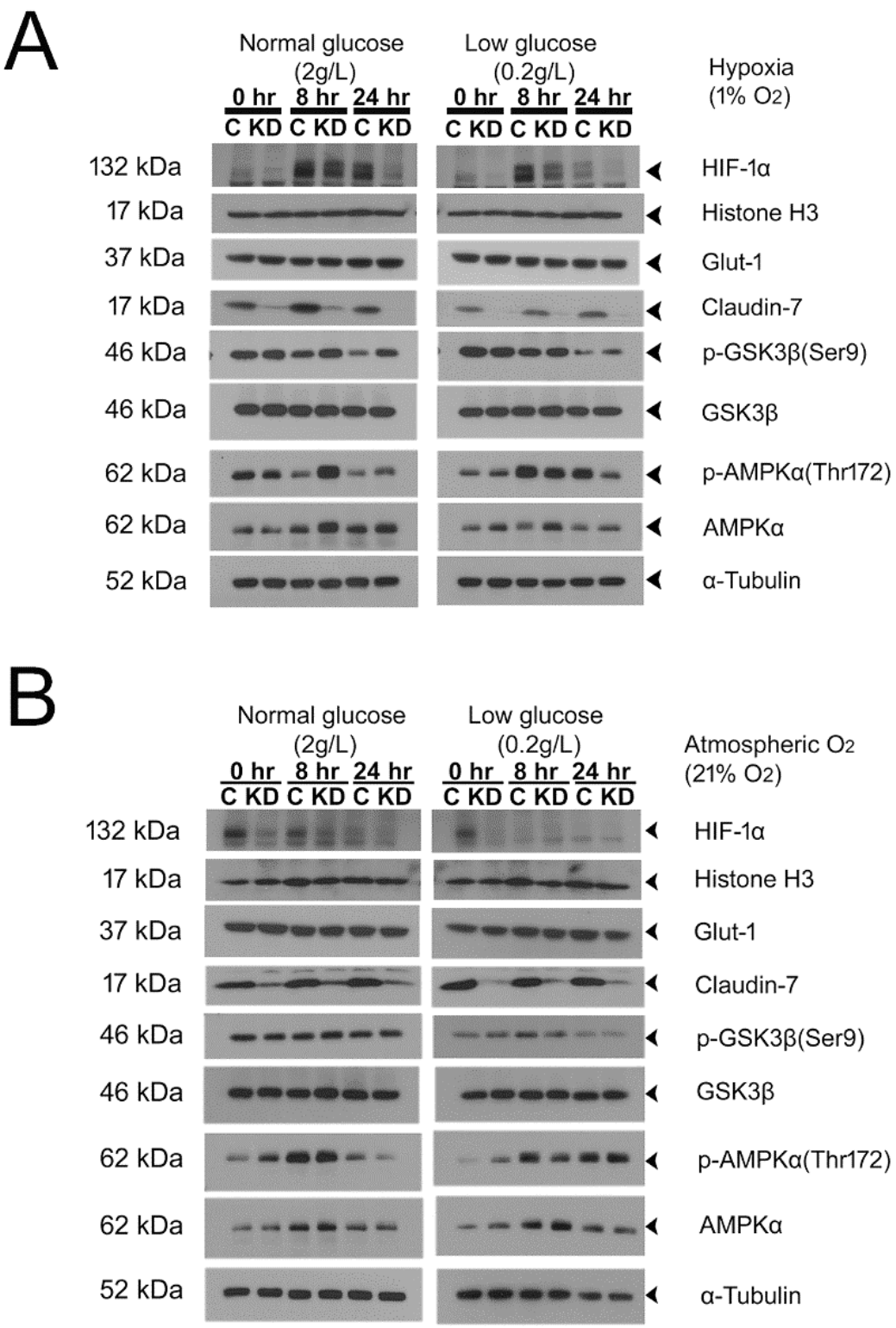
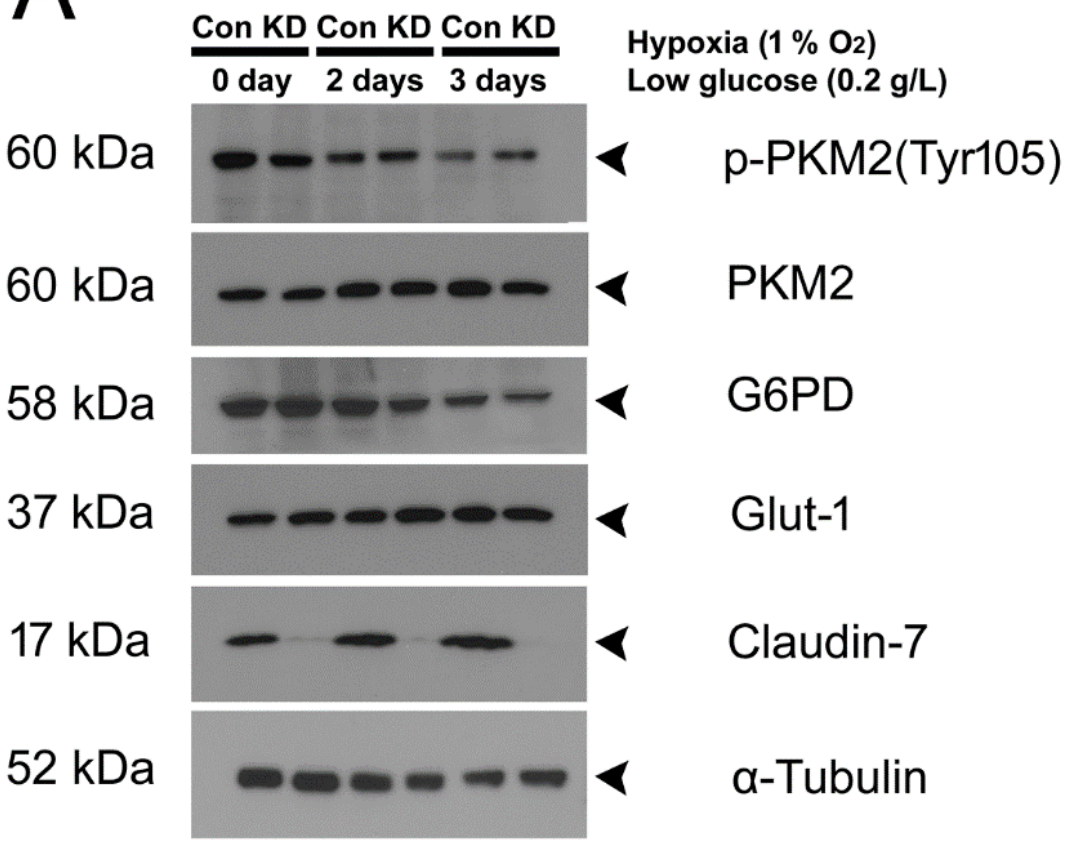

A
Normal glucose (2g/L)
Low glucose (0.2g/L)
Hypoxia (1% O2)
0 hr 8 hr 24 hr
C KD C KD C KD
132 kDa
HIF-1α
17 kDa
Histone H3
37 kDa
Glut-1
17 kDa
Claudin-7
46 kDa
p-GSK3β(Ser9)
46 kDa
GSK3β
62 kDa
p-AMPKα(Thr172)
62 kDa
AMPKα
52 kDa
α-Tubulin
B
Normal glucose (2g/L)
Low glucose (0.2g/L)
Atmospheric O2 (21% O2)
0 hr 8 hr 24 hr
C KD C KD C KD
132 kDa
HIF-1α
17 kDa
Histone H3
37 kDa
Glut-1
17 kDa
Claudin-7
46 kDa
p-GSK3β(Ser9)
46 kDa
GSK3β
62 kDa
p-AMPKα(Thr172)
62 kDa
AMPKα
52 kDa
α-Tubulin

Figure IV.3 Claudin-7 KD cells modulated genes of glucose metabolism in hypoxia and low glucose culture conditions for cancer cell survival.

A Western blot analysis was performed in four different cell culture environments, including low glucose and low oxygen conditions mimicking the tumor microenvironment *in vitro*. Cell fractionation was performed to probe for HIF-1α, and Histone H3 served as a loading control protein in nuclear lysate. α-Tubulin served as a loading control protein in whole lysate, and it was not detected in nuclear lysate. Glut-1 expression levels were shown to be equal over all four cell culture conditions. **A.** Representative Western blot images on protein expression levels of phospho-Ser9-GSK3β and phospho-Thr172-AMPK in hypoxia (1%O_2) and either normal (2 g/L) or low (0.2 g/L) glucose culture conditions. HIF-1α was rapidly decreased in claudin-7 KD cells when compared to control cells at 24 h, particularly in hypoxia and low glucose culture conditions. Claudin-7 KD cells maintained higher levels of phospho-GSK3β than control cells up to 24 h after hypoxia treatment. The KD cells also dramatically decreased phospho-AMPK levels when compared to control cells at the 24-h time point in hypoxia and low glucose culture conditions. **B.** Representative Western blot images on protein expression levels of phospho-GSK3β and phospho-AMPK in normoxia and either normal (2 g/L) or low (0.2 g/L) culture conditions. Neither phospho-AMPK nor phospho-GSK3β differed dramatically from claudin-7 control and KD cells in either normal or low glucose culture and normoxia conditions up to an additional 24 h.

A


Con KD Con KD Con KD
0 day 2 days 3 days
Hypoxia (1 % O2)
Low glucose (0.2 g/L)
60 kDa
p-PKM2(Tyr105)
60 kDa
PKM2
58 kDa
G6PD
37 kDa
Glut-1
17 kDa
Claudin-7
52 kDa
α-Tubulin

B

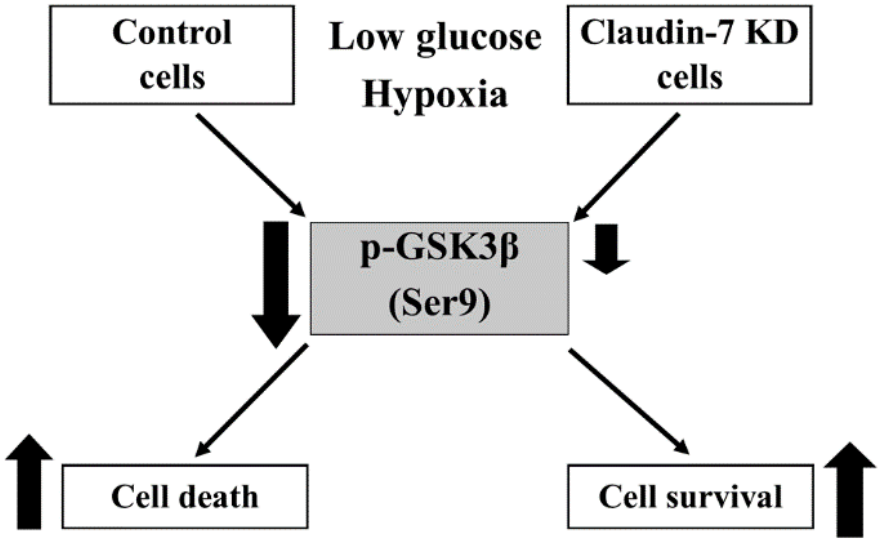

Control cells
Low glucose
Hypoxia
Claudin-7 KD cells
p-GSK3β (Ser9)
Cell death
Cell survival

Figure IV.4 Claudin-7 KD cells suppress genes of glycolysis and nucleotide synthesis in chronic hypoxia and low glucose culture.

A Western blot analysis was performed in both claudin-7 control and KD cells cultured in hypoxia (1%O_2) and low (0.2 g/L) glucose conditions for up to an additional 3 days. α-Tubulin served as a loading control protein. Glut-1 expression levels were shown to be equal for up to 3 days. **A.** Representative Western blot images on protein expression levels of G6PD and phospho-PKM2 in hypoxia and low glucose culture conditions for up to an additional 3 days. G6PD levels were further decreased in claudin-7 KD cells when compared to control cells from day 2. In contrast, phospho-PKM2 was maintained at a slightly higher level in KD cells when compared to control cells on day 3. **B.** Schematic diagram of relationship between lung cancer cell survival and glycogen synthesis kinase 3β. In hypoxia and low glucose culture conditions, claudin-7 KD cells maintain higher levels of the inhibitory phosphorylated form of GSK3β than control cells in support of cancer cell survival.

CHAPTER V. CONCLUSIONS AND DISCUSSION

Cells generate and receive various cell signals from one another to regulate their cellular functions of proliferation, differentiation, homeostasis, and migration (1). Understanding cellular communication is essential to dissect the processes of diseases, including cancers. Recently, it has been brought to our attention that the TJ protein claudin modulates carcinogenesis in several human cancers (26-31). However, the roles of TJ proteins in human lung cancer cells have not been extensively investigated.

Human lung cancer is the second leading cause of cancer patients' death in the United States (32). About 85% of human lung cancers are categorized as NSCLC types, while the remaining 15% are categorized as small-cell lung cancer (SCLC) types (33). Numerous molecular epidemiological research studies have successfully identified potential causes of lung cancer incidences from alteration in genes responsible for tumor suppression as well as cell proliferation and survival, such as *p53* and *K-ras* in addition to epigenetic modification such as DNA methylation (34), whereas the underlying cellular mechanisms of lung carcinogenesis remain unknown. Recently, a clinical research team found that low claudin-7 expression in lung cancer patients was closely associated with their low survival rate after physical surgery (35). Like other TJ proteins, claudin proteins are usually located at the apical side of human epithelial cells (8). However, claudin-7 has been found at the basal side of several human organs and tissues, including uterine, kidney, mammary gland, and intestines (36-39). Claudin-7 has also been found to be involved in the physiological activity of pregnant female rats by altering the claudin-7 protein expression level at the basal side of the rat uterus (36, 41). Therefore, claudin-7 in epithelial tissues seems to be actively involved in the cell-matrix adhesion mentioned above. In this regard, we have

strived to dissect the unknown function of claudin-7 in lung carcinogenesis, including cancer initiation, promotion, and progression, in this dissertation.

Chapter II was published as my co-first-authored manuscript documenting that claudin-7 regulates lung cancer cell proliferation, cell survival, and cell-matrix attachment through β1 integrin using human lung cancer cells (66). These findings offer numerous clues on the roles of the TJ claudin-7 protein in potentially regulating lung carcinogenesis. First, the hyper-cell proliferation found in the claudin-7 KD of human lung cancer cells appears to fit the characteristics of cancer promotion (56), whereas cell detachment from the ECM and reduction in β1 integrin are well suited to cancer progression and metastasis (60). Based on these results of how claudin-7 regulates cell-matrix adhesion in Chapter II, we examined how claudin-7 and β1 integrin co-regulate cell-matrix adhesion and the cell migration, invasion, and attachment of human lung cancer cells in Chapter III, which elucidates how claudin-7 contributes to lung cancer progression and metastasis. Next, as claudin-7 KD in human lung cancer cells promoted cell survival, as described in Chapter II, we also extended our investigation of whether claudin-7 regulates cell metabolism for cancer cell survival under tumor microenvironments in Chapter IV. This also provides evidence of how claudin-7 augments cancer metastatic potential, including resistance to cancer cell apoptosis, in lung cancer promotion and progression.

The first distinguishable feature of claudin-7 KD in human lung cancer cells is concomitant dysregulation of β1 integrin expression (66). β1 integrin is a ubiquitous major anchoring junction (66) that helps cells receive extracellular signals from extracellular environments such as the ECM through cell-matrix adhesion and respond to those external environmental stimuli (138). β1 integrin also regulates cell migration and invasion through cell-matrix adhesion (45, 46, 48).

In the following current study shown in Chapter III, we overexpressed β1 integrin to

enhance the cell-matrix adhesion of claudin-7 KD cells and evaluated whether the overexpression of β1 integrin strengthens cell migration and invasion and the attachment of KD cells. Our results demonstrated that the overexpression of β1 integrin highly improved cell migration and invasion and the attachment of claudin-7 KD cells. However, β1 integrin overexpression in claudin-7 KD cells could not fully recover focal adhesion proteins, unlike the re-expression of claudin-7 in claudin-7 KD cells. This suggests that claudin-7 has a far greater influence than β1 integrin on controlling cell-matrix adhesion, cell migration and invasion, and the attachment of human lung cancer cells through the cell-matrix interface.

For cancer metastasis, cancer cells need to detach from the ECM before entering the blood circulation (52). Thus, claudin-7 KD in human lung cancer cells is thought to augment the metastatic potential, although it suppresses β1 integrin, focal adhesion, and cell-matrix attachment. Claudin-7 KD human lung cancer cells also show additional evidence that support an increased metastatic potential. In normal cells, the loss of β1 integrins has been documented as a negative consequence, such as reduced cell proliferation in the mammary glands (99, 100) and death of β1 integrin-knockout mice before birth (99). These findings indicate that β1 integrin is necessary for the cell proliferation and survival of normal epithelial cells. In contrast, claudin-7 KD human lung cancer cells have been shown to retain hyper-proliferation and reduced apoptosis despite significant reductions in β1 integrin (66). This suggests that the increased metastatic potential of claudin-7 KD cells inhibits anoikis, a type of cell apoptosis induced by cell detachment from the ECM (53).

The ability of cancer cells to survive without cell-matrix-adhesion seems important for cancer metastasis through blood circulation. Our previous *in vivo* mouse tumor growth study first illustrated that a mouse tumor subcutaneously injected with claudin-7 KD human lung cancer cells

showed expedited tumor growth in terms of both size and weight when compared to those injected with claudin-7 control cells (66). This suggests that a significant reduction in β1 integrin in claudin-7 KD human lung cancer cells leads to anchorage-independent growth regardless of the context of the extracellular environment *in vivo*. In addition, the enlarged mouse tumor formation induced by claudin-7 KD lung cancer cells could be due to angiogenesis. We previously found that claudin-7 KD in both HCC827 lung cancer cells and T84 breast cancer cells similarly increased the gene expression of metalloproteinases (MMPs) such as MMP3 *in vitro* (40). MMP3 has been well known to activate other MMPs, including collagenase, gelatinase, and matrilysin, all of which can promote ECM remodeling (139). MMP3 gene expression levels have also been strongly associated with the angiogenesis factor vascular endothelial growth factor (VEGF) at the plasmid level (140). These findings suggest that HCC827 claudin-7 KD human lung cancer cells are able to promote cancer metastasis and facilitate angiogenesis.

In addition, our current experiment in culturing HCC827 claudin-7 KD human lung cancer cells in hypoxia and low glucose culture conditions has illustrated that claudin-7 KD cells were more resistant to cell apoptosis and increased inhibitory phospho-GSK3β. As we previously discussed in Chapter IV, one plausible explanation is that claudin-7 KD cells survive better than the control cells in such a way that glycogen synthesis may be indirectly promoted through potentially active GS, which is stimulated by preserving elevated levels of inhibitory phospho-GSK3β under tumor microenvironments. More importantly, GSK3β plays a crucial role in upholding cancer metastasis. It has recently been reported that human gastrointestinal (GI) cancer cells increased inhibitory phospho-GSK3β when Fas ligand (FasL) induced an epithelial mesenchymal transition (EMT), which subsequently upregulated the transcriptional activity of β-catenin and Snail to increase MMP9 (141). The authors also clinically demonstrated that the high

expression of inhibitory phospho-GSK3β in GI patients was closely associated with their low survival rate (141), indicating that phospho-GSK3β is connected to the malignancy of human GI cancer. Furthermore, Snail has been said to inhibit cell cycles during embryogenesis, and high Snail-expressing Madin–Darby canine kidney (MDCK) cell lines have been shown to develop resistance to cell death under serum-deprived culture conditions (142). Interestingly, high Snail-expressing MDCK cells have also increased cell death in low glucose culture media and reduced several genes of enzymes responsible for TCA cycles, whereas high Snail-expressing cells have survived better than control vector-expressing cells in hypoxia and sufficient glucose culture conditions (143). However, the authors did not report on whether low glucose media also induced the cell death of the high Snail-expressing cells in hypoxia conditions.

All these findings indicate that cancer cells under EMT increase phospho-GSK3β levels, induce high Snail expression, and become resistant to cell death in hypoxia when glucose is provided at an appropriate level, whereas those cells are highly dependent on glucose levels for cell survival in normoxia. These features, similar to those of cancer cells undergoing EMT, also appear in our claudin-7 KD human lung cancer cells from the following observations. Our current study has first revealed that the claudin-7 KD cells were more resistant to cell death in hypoxia and normal glucose culture conditions for 3 days. Next, claudin-7 KD cells showed highly elevated levels of phospho-GSK3β expression when compared to claudin-7 control cells in hypoxia and normal or low glucose conditions for up to 24 h. Lastly, we also found that claudin-7 KD cells started to reduce the expression level of G6PD earlier than the control cells from 2 days after hypoxia and low glucose treatment. This last observation seen in OGD conditions particularly appears to reflect the possibility of the EMT of the claudin-7 KD cells inhibiting nucleotide synthesis for cell division. These three distinctive features of claudin-7 KD cells seem to resemble

the characteristics of cancer cells undergoing EMT discussed above. Future investigation will clarify possible EMT induction in claudin-7 KD lung cancer cells in OGD conditions.

In the meantime, we unexpectedly observed the rapid reduction of HIF-1α expression in the claudin-7 KD cells when compared to the control cells in hypoxia and normal glucose culture conditions up to 24 h. HIF-1α activates PDK, which inhibits PDH (133), and PDH is responsible for converting pyruvate into acetyl-CoA for its entry to the TCA cycle (133). Thus, it is possible that rapidly disappearing HIF-1α allows the claudin-7 KD to gain access to the TCA cycle earlier than the claudin-7 control cells 24 h after hypoxia and normal glucose culture treatment. In this sense, however, oxidative phosphorylation through the TCA cycle is also likely to generate more ROS, which could result in more significant cell death in the claudin-7 KD lung cancer cells. This could not explain why claudin-7 KD lung cancer cells significantly reduced cell death in hypoxia and normal glucose conditions for 3 days in our current study. One possible explanation is that claudin-7 KD cells might modify the TCA cycle in such a way that cellular energy and antioxidants including NADPH and GSH are generated while oxygen respiration is minimized, as described in Chapter IV.

Alternatively, it has recently been documented that lymphoma cells with high Myc expression drive glutaminolysis to consume glutamate in generating cellular energy and GSH in the TCA cycle in a glucose-independent manner, and the inhibition of glutaminolysis has increased ROS (144). In addition, in the same study, lymphoma cells in hypoxia and glucose-withdrawal conditions did not proliferate but continuously consumed glutamate to maintain cancer cell viability. Interestingly, c-Myc is highly expressed in HCC827 human lung cancer cells (145). In a similar way, claudin-7 KD is also likely to drive HCC827 cells to glutaminolysis in hypoxia and low glucose conditions in order to maintain cell viability, although further investigation is

necessary to clarify this possible metabolic pathway using isotope-labeled glutamate in low glucose and hypoxia conditions.

As we discussed above, claudin-7 is likely to perform multiple functions in regulating lung cancer cell progression based on our previous and current studies. Firstly, claudin-7 rather than β1 integrin mainly modulates cell proliferation, migration, invasion, and attachment. Secondly, claudin-7 also alters glucose metabolism to support cancer cell survival in tumor microenvironments *in vivo* and *in vitro*. Despite the promising characteristics of elevated lung cancer metastatic potential on the claudin-7 KD of human lung cancer cells, our *in vivo* metastatic mouse tumor study did not show any metastatic nodules in lymph nodes or micrometastatic lesions in mouse tissues, including the lung and liver (Fig 1.2.). It has been reported that HCC827 parental lung cancer cells are of low metastatic potential (67). Thus, the low metastatic potential of HCC827 lung cancer cells may be attributed to the failure to generate metastatic nodules in athymic nude mice. Conversely, claudin-7 KD may promote stages of cancer promotion and progression but not metastasis. Future *in vivo* experiments are needed to characterize the roles of claudin-7 KD using high metastatic human lung cancer cells in cancer progression.

The concomitant reduction of β1 integrin upon claudin-7 KD in human lung cancer cells is thought to indicate increased lung cancer metastatic potential, including hyper-proliferation, cell detachment from the ECM, and β1 integrin reduction. In addition, a clinical research team has found a strong association between low claudin-7 expression of lung cancer patients and their low survival rate (35). However, it is not clearly understood how low claudin-7-expressing highly metastatic lung cancer cells can metastasize to secondary sites through blood circulation, although the ubiquitously present β1 integrin may not be highly present to support cell-matrix adhesion-based cell migration. It has recently been documented that melanoma cells rearrange the actin

cytoskeleton to use friction as a mode of amoeboid cell migration when the cells pass through physically confined areas and non-adhesive environments *in vitro* (146). Likewise, human lung cancer cells could also metastasize by decreasing claudin-7 to abolish focal-adhesion-based migration in order to adapt fast amoeboid migration while they are circulating through blood vessels for lung cancer metastasis.

In conclusion, we first report that a TJ protein, claudin-7, participates in multiple functions and modulates human lung cancer metastatic potential and carcinogenesis. Claudin-7 controls cell-matrix adhesion that also regulates cell migration and invasion and the attachment of human lung cancer cells at the ECM surface for cancer metastasis. For cancer promotion and progression, claudin-7 adjusts cell proliferation to expedite lung tumor growth by altering cell metabolism for cell survival under tumor microenvironments.

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APPENDIX A: ANIMAL USE PROTOCOLS

East Carolina University

Animal Care and Use Committee
212 Ec Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

December 16, 2011

Yan-Hua Chen, Ph.D.
Department of Anatomy
Brody 7N-100
ECU Brody School of Medicine

Dear Dr. Chen:

Your Animal Use Protocol entitled, "Claudin-7 Gene Targeting and Paracellular Chloride Permeation" (AUP #A172b) was reviewed by this institution's Animal Care and Use Committee on 12/16/11. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

November 6, 2013

Yan-Hua Chen, Ph.D.
Department of Anatomy
Brody 7N-100
ECU Brody School of Medicine

Dear Dr. Chen:

The Amendment to your Animal Use Protocol entitled, "Claudin-7 Gene Targeting and Paracellular Chloride Permeation", (AUP #A172b) was reviewed by this institution's Animal Care and Use Committee on 11/6/13. The following action was taken by the Committee:

"Approved as amended"

**Please contact Dale Aycock prior to any hazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

February 11, 2014

Yan-Hua Chen, Ph.D.
Department of Anatomy
Brody 7N-100
ECU Brody School of Medicine

Dear Dr. Chen:

The Amendment to your Animal Use Protocol entitled, "Claudin-7 Gene Targeting and Paracellular Chloride Permeation", (AUP #A172b) was reviewed by this institution's Animal Care and Use Committee on 2/11/14. The following action was taken by the Committee:

"Approved as amended"

**Please contact Dale Aycock prior to any hazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

March 11, 2014

Yan-Hua Chen, Ph.D.
Department of Anatomy
Brody 7N-100
ECU Brody School of Medicine

Dear Dr. Chen:

The Amendment to your Animal Use Protocol entitled, "Claudin-7 Gene Targeting and Paracellular Chloride Permeation", (AUP #A172b) was reviewed by this institution's Animal Care and Use Committee on 3/11/14. The following action was taken by the Committee:

"Approved as amended"

**Please contact Dale Aycock prior to any hazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

June 10, 2014

Yan-Hua Chen, Ph.D.
Department of Anatomy
Brody 7N-100
ECU Brody School of Medicine

Dear Dr. Chen:

The Amendment to your Animal Use Protocol entitled, "Claudin-7 Gene Targeting and Paracellular Chloride Permeation", (AUP #A172b) was reviewed by this institution's Animal Care and Use Committee on 6/10/14. The following action was taken by the Committee:

"Approved as amended"

**Please contact Dale Aycock prior to any hazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 16, 2014

Yan-Hua Chen, Ph.D.
Department of Anatomy
Brody 7N-100
ECU Brody School of Medicine

Dear Dr. Chen:

Your Animal Use Protocol entitled, "Claudin-7 Gene Targeting and Paracellular Chloride Permeation" (AUP #A172c) was reviewed by this institution's Animal Care and Use Committee on 12/16/14. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

APPENDIX B: PERMISSION LETTERS FROM CO-AUTHORS TO PUBLISH

RE: Need response from you!

卢哲 <zhelu84@126.com> Wed, Jan 6, 2016 at 6:38 AM
To: "Chen, Yan-Hua" <CHENY@ecu.edu>
Cc: "Lu, Qun" <LUQ@ecu.edu>, "Verbanac, Kathryn" <VERBANACK@ecu.edu>, 丁磊 <dinglei1005@126.com>, "Kim, Do Hyung" <KIMDO11@students.ecu.edu>

Dear Do Hyung,

You have my permission to use appropriate portions of the co-authored paper "A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment" published in Journal of Molecular Cancer.

Sincerely,
Zhe Lu

[Quoted text hidden]

/RE: 1824572509144988 A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment

fjmelite <fjmelite@163.com> Tue, Dec 22, 2015 at 3:28 AM
To: "Kim, Do Hyung" <KIMDO11@students.ecu.edu>

Hi Do Hyung,

You have my permission to use appropriate portions of the co-authored paper referenced in your email.

Merry Christmas and Happy New Year !

Sincerely,

Junming

发自我的小米手机
[Quoted text hidden]

Thesis material copyrights

1 message

Lu, Qun <LUQ@ecu.edu> Tue, Jan 5, 2016 at 1:30 PM
To: "Kim, Do Hyung" <KIMDO11@students.ecu.edu>
Cc: "Chen, Yan-Hua" <CHENY@ecu.edu>, "Lu, Qun" <LUQ@ecu.edu>

Dear Do Hyung,

You have my permission to use appropriate portions of the co-authored paper "A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment" published in Journal of Molecular Cancer.

Sincerely,

****

Qun Lu, PhD, MS, BS.
Professor of Anatomy and Cell Biology
Director
The Harriet and John Wooten Laboratory
for Alzheimer's and Neurodegenerative Diseases Research
The Brody School of Medicine at East Carolina University
252-744-2844 (Office)
luq@ecu.edu
http://www.ecu.edu/cs-dhs/anatomy/faculty/lu.cfm
http://www.ecu.edu/cs-dhs/wootenlab/contact.cfm

RE: Need response from you!

Verbanac, Kathryn <VERBANACK@ecu.edu> Tue, Jan 5, 2016 at 2:37 PM
To: "Kim, Do Hyung" <KIMDO11@students.ecu.edu>
Cc: "Chen, Yan-Hua" <CHENY@ecu.edu>

Dear Do Hyung,

You have my permission to use appropriate portions of the co-authored paper "A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment" published in Journal of Molecular Cancer.

Sincerely,

Kathryn Verbanac, PhD
Assistant Vice Chancellor, Interim
Director, Office of Postdoctoral Affairs
Research and Graduate Studies
Professor of Surgery
Brody School of Medicine
East Carolina University
Greenville, NC 27834
Ph (252) 744-3689; (252) 737-4812

re paper

1 message

丁磊 <dinglei1005@126.com> Wed, Jan 6, 2016 at 8:46 AM
To: KIMDO11@students.ecu.edu

Dear Do Hyung,

>

>You have my permission to use appropriate portions of the co-authored paper "A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment" published in Journal of Molecular Cancer.

>

>Sincerely,

Lei Ding

/RE: 1824572509144988 A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment

Renegar, Randall H <RENEGARRA@ecu.edu> Mon, Dec 21, 2015 at 1:39 PM
To: "Kim, Do Hyung" <KIMDO11@students.ecu.edu>, "Chen, Yan-Hua" <CHENY@ecu.edu>
Cc: Zhe Lu <zhelu84@126.com>, Junming Fan <fjmelite@163.com>, "Lu, Qun" <LUQ@ecu.edu>, "Verbanac, Kathryn" <VERBANACK@ecu.edu>, Lei Ding <dinglei1005@126.com>

Do Hyung,

Congratulations on reaching this point in your training. You have my permission to use appropriate portions of the co-authored paper referenced in your email.

Best wishes,

Randy Renegar
[Quoted text hidden]

/RE: 1824572509144988 A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment

Chen, Yan-Hua <CHENY@ecu.edu> Wed, Dec 23, 2015 at 2:58 PM
To: "Kim, Do Hyung" <KIMDO11@students.ecu.edu>

Hi Do Hyung:
You have my permission to use appropriate portions of the co-authored paper "A non-tight junction function of claudin-7 - Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment"
published in journal of Molecular Cancer.

Best wishes,

Yan-Hua Chen

[Quoted text hidden]