

SUPPRESSION OF CLAUDIN-7 ENHANCES HUMAN LUNG CANCER CELL SURVIVAL

By

Spencer M. Jackson

A Senior Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

By

Spencer M. Jackson

Greenville, NC

May 2016

Approved by:

Yan-Hua Chen, Ph.D.

Department of Anatomy and Cell Biology, Brody School of Medicine

## **Suppression of claudin-7 enhances human lung cancer cell survival**

Spencer M. Jackson and Yan-Hua Chen, PhD.

### **Abstract:**

Claudin-7 belongs to a group of tight junction membrane proteins that play vital roles in many human diseases including human lung cancer. Lung cancer is noted to be the leading cause of cancer death for both men and women in the United States, with statistics reporting mortality rates as high as 85% and five-year survival rates as low as 15%. Lung cancer is especially prominent in North Carolina. Our current study focuses on the role of claudin-7 in human lung cancer cell survival under the exposure of tumor microenvironment. Hypoxia is one of the tumor microenvironment conditions and plays an important role in cancer progression. To achieve the hypoxia condition, HCC827 human lung cancer cells with normal claudin-7 expression (control) or with claudin-7 knockdown (KD) were treated with 1% O<sub>2</sub> (hypoxic) for 3 days. The cell counting assay showed that the percentage of dead cells were significantly lower in KD cells compared to that of control cells. The immunofluorescent staining analysis also supported our finding through depicting the decreased expression of cleaved PARP in KD cells than that in the control cells ( $p < 0.05$ ). Reduced cleaved PARP expression means the cell survival is better since the cleaved PARP signal is activated in cell apoptosis. Western blot results further confirmed that the suppression of claudin-7 promoted cancer cell survival and reduced cell apoptosis. These results support our hypothesis that claudin-7 has a tumor suppression role in human lung cancer growth and suppression of claudin-7 enhances lung cancer cell survival under tumor microenvironment hypoxia

condition through inhibiting cell apoptosis. This study is supported by 2015 Undergraduate Research and Creative Activity Award from ECU Division of Research and Graduate Studies.

### **Introduction:**

Lung cancer is noted to be the leading cause of cancer death for both men and women in the United States. And, statistically, the mortality rate of this disease is as high as 85%, while according to Stella et al [1] the five-year survival rate can be as low as 15%. Lung cancer, specifically, generally arises in the cell tissue lining the bronchi, or airways. Furthermore, most lung cancers fall under the category of a carcinoma defined as a malignant tumor with origins from epithelial cells [2]. In fact in North Carolina, where this study takes place, lung cancer is especially prominent.

The claudin family, from which claudin-7 arises, is comprised of 24 members, each of which play structural and function roles on the apical membrane in the tight junctions of epithelial cells. Tight junctions are multiprotein complexes that regulate cell-cell adhesion and cell growth. They act as a barrier to modulate small molecules and ions across the epithelia sheet. Abnormal expression and mislocalization in the claudin gene sequence have been observed in cancers with epithelial origins [2]. As many claudins are expressed during embryogenesis, this could suggest that these tight junction integral proteins help in organ formation through effects on cell adhesion and proliferation [3]. In fact, previous studies have shown that claudin-7 was down-regulated in several human lung cancer cells [4].

Hypoxia treatment has been used quite often in the study of human cancer cells. Hypoxia conditions with oxygen levels of 1% (normoxia conditions 20% O<sub>2</sub>)

have significant effects on tumor cells. In normal cell culture conditions, cancer cells have sufficient nutrition and oxygen. However, research suggests fast-growing tumors have a higher cell proliferation and apoptosis rate due to the inadequate nutrition and oxygen [2]. Therefore, our hypoxia treatment simulates *in vivo* tumor cell microenvironment conditions.

We hypothesize that the suppression of claudin-7 increases human lung cancer cell survival in the hypoxia environment. To understand how the suppression of claudin-7 affects human lung cancer growth will help to elucidate the specific function of claudin-7 in lung cancer.

## **Methods:**

### **1. Cell Counting Assay (Trypan Blue Exclusion Method)**

Our cells are derived from the HCC827 cell line. It is a human lung adenocarcinoma, epithelial cell line taken on March 19, 1994 from a thirty-nine year old adult, Caucasian female. Two cohorts of cells arise from this line – our control and claudin-7 knockdown cells (KD) in which claudin-7 has been suppressed.

The two cell cultures (control and KD) were then taken and placed in a two-by-three cell well. The top row of three contained the control cells, and the bottom row contained the KD cells. These cells were then treated under hypoxia conditions (at 1% O<sub>2</sub>) for three days. On the third full day, the cells were taken out of the hypoxia machine to begin the process of a cell counting assay. This was performed as follows: floating cells were removed through aspiration. Then, cell cultures were trypsinized and placed in the incubator for five minutes. The cells were then removed from the incubator and the attached cells were detached by carefully

pipetting the existing trypsin onto the back on the cell culture dish. The cells were then well mixed with a pipette and ten microliters of each cell type was removed and placed into a separate test tube (one for Control and one for KD). Trypan Blue stain was then added to each of the test tubes, and the cells and stain were well mixed approximately thirty times with a pipette. Ten microliters of the mixture was placed on each side of a Countess slide and then the counting assay was performed, which gave the total count, live count, and dead count. This procedure was then repeated twice over the next two weeks for a total of three experimental sets.

At the end of the third set, a statistical analysis was performed comparing percent live and percent dead. Regular bar graphs were created to compare the Control and KD cells. Microsoft Excel and the Data Analysis ToolPak were used to calculate statistical differences.

## **2. Immunofluorescence (IF) Staining**

HCC827 control or claudin-7 KD cells grown on poly-D-lysine coated glass coverslips (BD Biosciences, Bedford, MA) were fixed in 100% methanol for 8 min at -20°C and washed with PBS for 5 min before blocked in 5% bovine serum albumin for 60 min at room temperature. After blocking, cells were incubated with primary antibodies. All antibodies were diluted in PBS containing 2.5% BSA. After washing, cells were incubated with corresponding secondary antibody for 45 min at room temperature. Coverslips were mounted with Pro-Long Antifade Kit (Molecular Probes Inc., OR). Samples were photographed using Zeiss Axiovert S100 (Carl Zeiss Inc., Thornwood, NY) and analyzed by MetaMorph software (Molecular Devices, Sunnyvale, CA).

A cell count was then performed of the IF stained Cleaved PARP cells. Cells were then counted by hand (a count of total and dead for control and KD) and then a cell assay was performed using Analysis ToolPak on Microsoft Excel.

### **3. Western Blot**

Protein samples were separated through electrophoresis and blotting according to normal techniques. The protein was transferred to Hybond ECL. The non-specific binding sites were blocked by immersing the membrane in 5% non-fat dried and 0.1% Tween 20 in PBS overnight in a refrigerator at 2°C. The membrane was briefly rinsed using two changes of wash buffer. The primary antibody was diluted in PBS, and the dilution factor was determined empirically for each antibody. The membrane was incubated in diluted primary antibody for one hour at room temperature on an orbital shaker. The membrane was then rinsed with six changes of wash buffer at room temperature. The secondary antibody was diluted, and the dilution factor was determined empirically for each corresponding antibody. The membrane was incubated in the diluted secondary antibody for one hour at room temperature on an orbital shaker. The membrane was washed five times at room temperature. An equal volume of detection solution was mixed. The excess wash buffer was drained from the washed membranes. The mixed detection reagent was pipetted on to the membrane and allowed to incubate for one minute at room temperature. Holding the membrane gently with forceps and touching the edge against a tissue drained off the excess detection reagent. The blots were then placed protein side down on to a fresh piece of SaranWrap. The blots were then placed the wrapped blots protein side up in an X-ray film cassette. The cassette was then

exposed for times ranging between 15 seconds to 10 minutes and then allowed to develop.

**Results:**

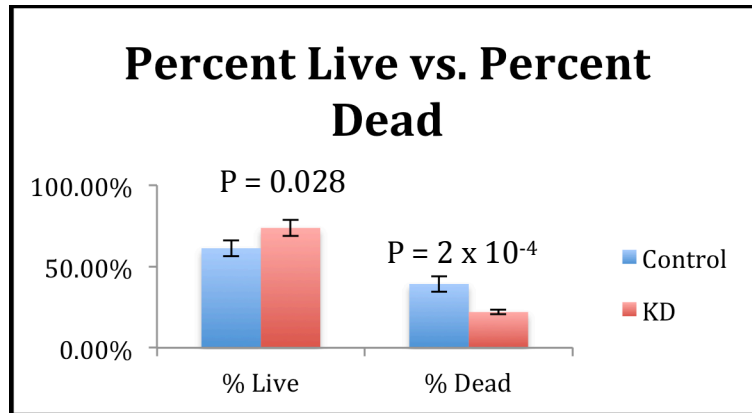


Figure 1. Cell counting assay comparing percent live and percent dead. P-value for Percent live was 0.028. P-value for Percent dead was 0.0002.

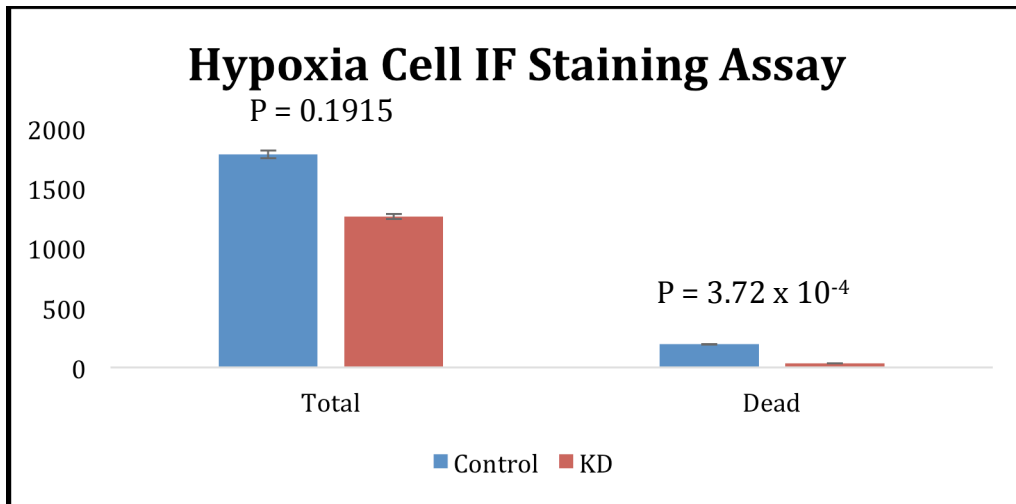
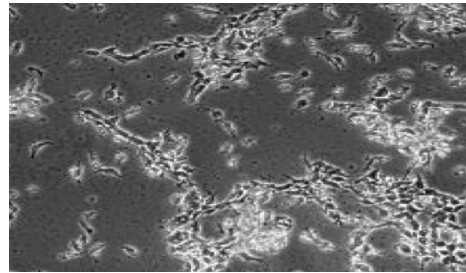
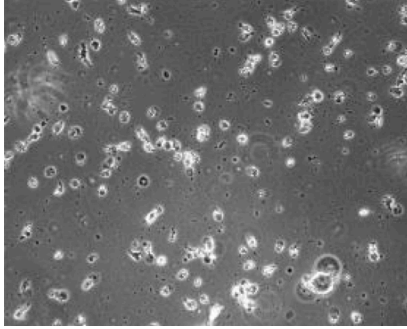


Figure 2: Comparison of Total and Dead. P-value for Total was 0.1915. P-value for Dead was 0.000372.

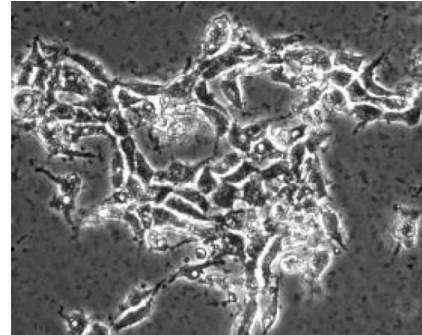
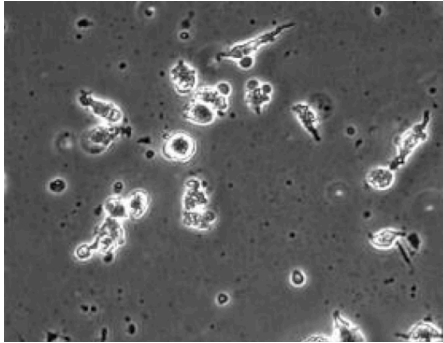
**Control**

**KD**

**100X**



**320X**

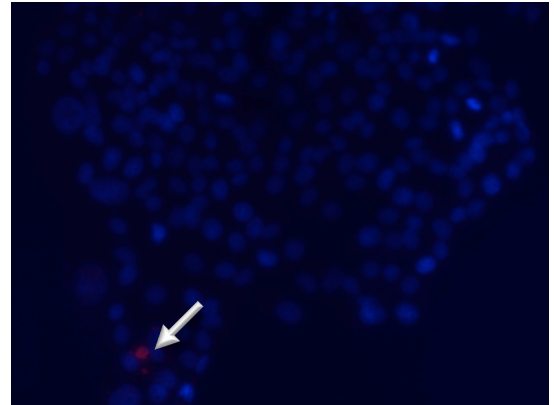
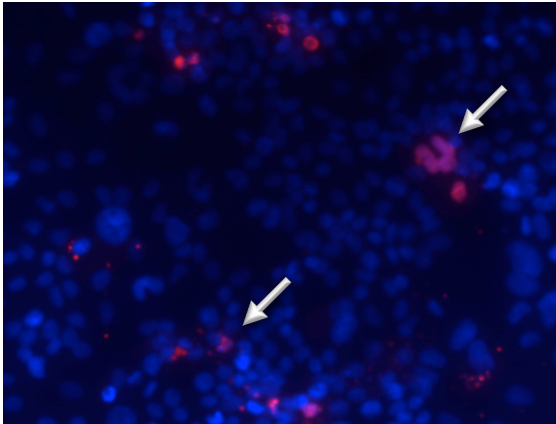


Phase images (1% hypoxia for 72 hours).

**Control**

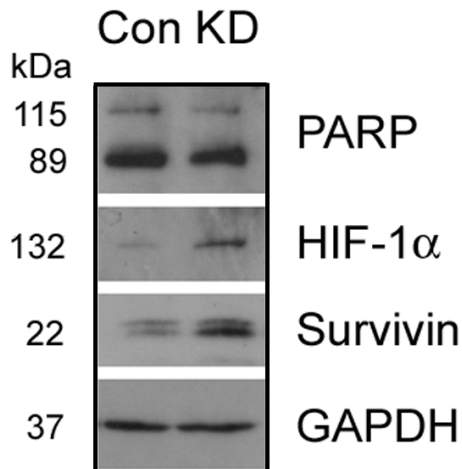
**KD**

**Cleaved PARP/DAPI**



Reduced Cell Apoptosis in KD Under Hypoxic Conditions. Images depict IF Staining after one day. Blue Coloring represents DAPI stain of the nucleus. Red stain is Cleaved PARP signal (dead cells or those cells preparing to undergo cell apoptosis.). White arrows indicate sample areas of cell apoptosis.





Western Blot: Protein expression of Cleaved PARP (89 kDa), Hypoxia Induced Factor-1 Alpha (132 kDa), and Survivin (22 kDa) comparison between Control and KD cells under hypoxic conditions.

The cell counting assay totaled three experiments over several weeks. Our data was then compiled to create a statistical analysis. Figure 1 provides evidence of a significant difference between both the Percent Live and the Percent Dead. These values are quite important as they support our hypothesis. There are reduced levels of dead cells in the KD than in the control, while there is a reduced rate of live cells in the control than in the KD.

The Phase Images taken after our Cell Counting Assay present a phenotypic difference between the Control and the KD. There is a larger grouping of the KD cells in comparison to the controls. This is evident in the 320x magnification. It is also important to note that there are far more cells per unit area in KD. This can be observed in the 100x magnification.

Our data for Figure 2 was compiled over a one-day period after the IF staining. Cleaved Poly ADP Ribose Polymerase (Cleaved PARP) plays an important role in the Cell Apoptosis Mechanism, as it is a marker for cell death. The cleavage of PARP facilitates cellular disassembly leading to DNA fragmentation and eventually cell death. As these images of our IF staining were taken after one day, it is

important to note the already increased expression of Cleaved PARP in the Control cells in comparison to the KD cells.

Our western blotting data was also important. As mentioned above, Cleaved PARP plays an important role in the Cell Apoptosis Mechanism. Increased expression of Cleaved PARP indicates increased cell death. At 89 kDa, there is an increased expression of Cleaved PARP in the control. At 132 kDa HIF-1 $\alpha$  is the Hypoxia Induced Factor. This antigen is used to provide evidence of hypoxia, as it is unregulated in hypoxic environments. This is seen for both Control and KD. Survivin is expressed in many human cancers and inhibits proteolytic activity. The up-regulation of survivin indicates greater survival. At 22 kDa, there is a greater expression of survivin in KD than in the control. Lastly, GAPDH (or Glyceraldehyde 3-phosphate dehydrogenase) is a loading control. It is not being tested in our experiments.

### **Discussion:**

In our study, we found that the down-regulation of claudin-7 led to an increase in tumor cell survivability for the HCC827 human lung adenocarcinoma cell line. To investigate tumor survivability we used a combination of techniques and methods including the Trypan Blue Exclusion Method, IF Staining, and western blot analysis. We demonstrated that not only do tumor cells respond to the suppression of claudin-7, but also that the introduction to the hypoxia environment leads to decreased survivability as it mimics *in vivo* tumor cell microenvironment.

Tumor migration, commonly referred to as metastasis, has been linked to cell-cell adhesion reduction. As noted in Lu Z et al [5], the disruption of cell-cell

junctions, such as tight junctions in epithelial cells in the case of claudin-7, may cause the release of cancer cells from the original cancer sites and induce the invasive properties on a tumor.

Many studies have reported the down-regulation of claudin-7 in human cancers. Yu Usami DDS et al [6] reported reduced expression of claudin-7 correlates with the invasion and metastasis of squamous cell carcinoma in the esophagus. Furthermore, Moustafa et al [7] found that claudin-7 had been suppressed in head and neck squamous cell carcinomas when compared to control cells. In another example, Kominsky et al [8] described the loss of claudin-7 as it was interconnected with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast. With this evidence, Lu Z et al [5] hypothesized that claudin-7 may be acting as a tumor suppressor gene in human lung cancers. Our data can be added to further support this argument. In our claudin-7 knockdown cells, we proposed the increased survivability in comparison to the normal cells. Not only does our data support this (refer to Figures 1 and 2), but it correlates to the data presented by Lu Z et al [5]. There is, therefore, a likely possibility that claudin-7 plays a role as a tumor inhibitor in human cancers, including human lung cancer. In fact, in a recent publication, Lu Z et al [2] suggests a tumor suppression role of claudin-7 in lung cancer cell growth. They found the suppression of claudin-7 not only disturbed cell-matrix adhesions, but also lead to increased cell apoptosis. It is well documented that claudin-7 is involved in the cell apoptosis mechanism through Cleaved PARP, as the cleavage of PARP leads to cellular disassembly, DNA fragmentation, and eventually cell apoptosis. We noted in this study there was

increased signaling of Cleaved PARP in the control cells in comparison to KD. After one day in the hypoxia environment, there was increased signaling of Cleaved PARP in the control cells in comparison to KD. Western blotting data also supports this hypothesis, as there was increased expression of Cleaved PARP in the control and increased expression of survivin in KD.

To conclude, in this study, we have indicated that claudin-7 suppression leads to increased tumor cell survival. This study further extends our understanding of the roles of claudin-7 as it applies to human lung cancers. Claudins play structural and functional roles in tumor cell proliferation and apoptosis. This function of claudin-7 could provide evidence of a curative treatment in the future of human lung cancer research.

### **Conclusion:**

In conclusion our current study demonstrates that the suppression of claudin-7 in the hypoxia microenvironment increases human lung cancer cell survival through reducing cell apoptosis. This conclusion is supported by cell morphology, the cell counting assay, IF antibody staining, and the western blotting data.

### **References:**

1. Stella GM, Luisetti M, Inghilleri S, Cemmi F, Scabini R, Zorzetto M, et al. Targeting EGFR in non-small cell lung cancer: lessons, experiences, strategies. *Respir Med.* 2012, 106:173-83.
2. Lu Z, Kim DH, Fan J, Lu Q, Verbanac K, Ding L, Renegar R, and Chen YH. A non-tight junction function of claudin-7 interaction with integrin signaling in

- suppressing lung cancer cell proliferation and detachment. *Mol. Cancer* 2015, 14:120.
3. Khairallah H, Andalousi JE, et al. Claudin-7, -16, and -19 during mouse kidney development. *Tissue Barriers* 2014, 2(4).
  4. Moldvay J, Jackel M, Paska C, Soltesz I, Schaff Z, and Kiss A. Distict claudin expression profile in histologic subtypes of lung cancer. *Lung Cancer*. 2007;57:159-67.
  5. Lu Z, Ding L, Hong H, Hoggard J, Lu Q, and Chen YH. Claudin-7 inhibits human lung cancer cell migration and invasion through ERK/MAPK signaling pathway. *Exp Cell Res*. 2011;13:1935-46.
  6. Usami Y, Chiba H, Nakayama F, Ueda J, Matsuda Y, Sawada N, Komori T, Ito A, Yokozaki H. Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. *Hum Pathol*. 2006;37:569-77.
  7. Al Moustafa AE, Alaoui-Jamali MA, Batist G, Hernandez-Perez M, Serruya C, Alpert L, Black MJ, Sladek R, Foulkes WD. Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelia and squamous carcinoma cells. *Oncogene*. 2002;21:2634-40.