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Regulation of Claudin-1 Expression Via Activation of Ron Tyrosine Kinase and Transforming Growth Factor-Beta Signaling Pathways in Ovarian Carcinoma

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REGULATION OF CLAUDIN-1 EXPRESSION VIA ACTIVATION OF RON TYROSINE KINASE AND TRANSFORMING GROWTH FACTOR-BETA SIGNALING PATHWAYS IN OVARIAN CARCINOMA

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I am very blessed and grateful for the support that my wonderful family and husband have provided for me. Their continuous encouragement has helped me be where I am today and I cannot ever thank them enough.

Finally and most importantly, I would like to dedicate this work to my grandmother, Golcheen Kader, who has always been an inspiration and a source of happiness for me.

Thank you,

Saya Karim

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Abstract

Epithelial ovarian carcinoma is the most common cause of death among gynecologic malignancies in the United States. In the U.S., more than 21,000 women will be diagnosed with ovarian cancer and about 15,000 women will die of the disease this year alone. Although migration and invasion play key roles in tumor progression, the specific molecular events leading to dissemination of ovarian tumor cells have not been well delineated. Tight junctions are an integral component of epithelial junction complexes, which play a vital role in maintaining epithelial integrity and cell polarity. Disruption of tight junctions is a hallmark of epithelial cancer development and malignant progression; specifically decreased claudin-1 expression has been associated with progression to tumor malignancy. Herein, we investigate the mechanism by which claudin-1 expression is regulated via activation of the TGFβ and RON tyrosine kinase receptor signaling pathways. We propose that claudin-1 expression is regulated by the activation of the TGFβ signaling pathway (dysregulated in ovarian carcinomas), particularly SnoN/SkiL, a key TGFβ co-repressor. In ovarian carcinoma cells, we observed that treatment with TGFβ and MSP1 led to a marked reduction in claudin-1 protein expression. Further, we not only noted that siRNA targeting RON led to upregulated claudin-1 mRNA and protein expression, but similar changes in mRNA and proteins of claudin-1 were observed with SnoN and β-catenin knockdown. Strikingly, with β-catenin knockdown, SnoN levels were notably decreased suggesting that β-catenin could potentially regulate SnoN expression. Collectively, our results demonstrate that claudin-1 expression is regulated by the transcriptional regulators β-catenin and SnoN. Since identification of novel biomarkers could be beneficial in diagnosis, prognosis and prediction of patient outcomes, these observations regarding the cellular events underlying regulation of claudin-1 expression are of translational potential.

I. Introduction

A. Background

I. Ovarian Carcinoma

Epithelial ovarian cancer is the most common cause of death among gynecologic malignancies in the United States. Each year in the U.S., more than 21,000 women are diagnosed with ovarian cancer and about 15,000 women die of the disease (surveillance, epidemiology, and end results (SEER) Program of the National Cancer Institute). Early stage of the disease is associated with minimal or an absence of symptoms. Hence, most cases are diagnosed at an advanced stage when the disease has aggressively disseminated the cells from the primary tumor. Unfortunately, this results in poor prognosis for these patients.

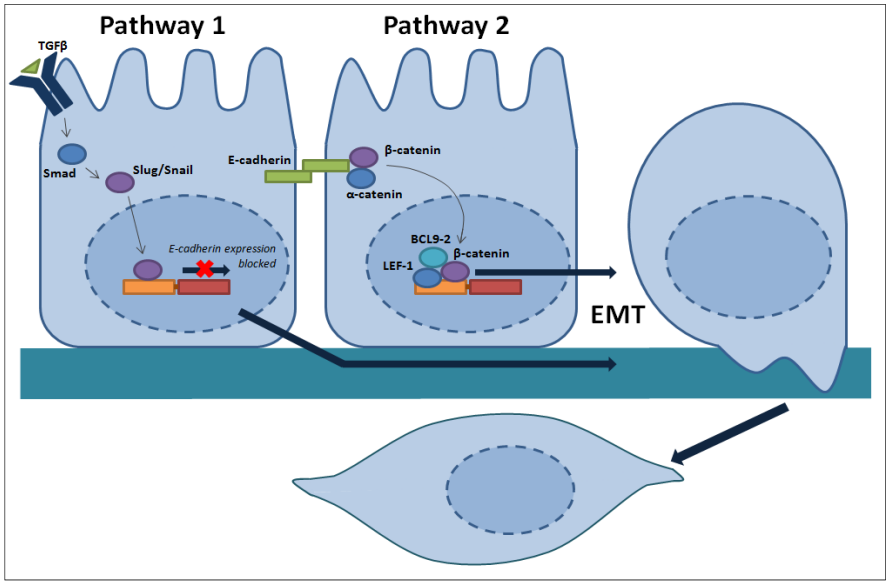

Figure 1.1-Two mechanisms through which EMT occurs in ovarian cells are shown. Cells undergoing EMT experience an altered morphology of the rigid, cuboidal epithelia to a more fibroblast-like mesenchymal phenotype.

The specific molecular events leading to metastases of ovarian tumor cells have not been well studied, but it is known that migration and invasion play a key role in the progression. These processes are carried out through epithelial–mesenchymal transition (EMT) which changes the morphology of the rigid, cuboidal epithelia to a mesenchymal form. EMT is

initiated through the dissolution of cell–cell junctions, adherens, tight and desmosomal junctions between adjacent epithelial cells[1]. This is followed by cytoskeleton reorganization, degradation of basement membranes and stroma, and enhanced growth rate[1]. Dissolution of the cell junctions may occur either as a result of the acquisition of genetic and epigenetic changes that result in mutations of their components or through repression of their expression at the transcriptional level [2].

II. Dysregulated TGFβ Signaling in Ovarian Cancer

Transforming growth factor-beta (TGFβ) is a multifunctional regulatory polypeptide that controls many aspects of cellular function such as cell proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance, and survival[3]. The actions of TGFβ are dependent on several factors such as cell type, growth conditions, and the presence of other polypeptide growth factors. TGFβ has dual functionality: (1) as a tumor suppressor (through its effects on proliferation and apoptosis) and (2) as a tumor promoter (through effects on migration, invasion, angiogenesis and the immune system)[3]. During the early stages of epithelial tumorigenesis, TGFβ inhibits tumor development and growth by inducing cell cycle arrest and apoptosis. In late stages of tumor progression, tumor cells become resistant to growth inhibition due to inactivation of the TGFβ signaling pathway or aberrant regulation of the cell cycle[3].

The effects of TGFβ are exerted through TGFβ type 1 (TGFβRI) and type 2 receptors (TGFβRII)[3]. Binding of the ligand causes the formation of heterotetrameric active receptor complexes that result in the phosphorylation of the type 1 receptor by the type 2 receptor [3]. The functional receptor complex regulates the activation of downstream Smad-dependent and Smad-independent pathways[3]. In the Smad-dependent signaling pathway, TGFβ binds TGFβRII

which activates TGFβRI[3]. The activated TGFβRI then recruits and phosphorylates receptor-regulated Smads (R-Smads), notably Smad2/3, which then form a complex with the Co-Smad, Smad4[3]. This complex then translocates to the nucleus to regulate transcription of TGFβ target genes such as cyclin-dependent kinase inhibitors (i.e. p21, involved in regulating cell survival)[3]. Thus, the overall effect of the Smad-dependent signaling is to inhibit cell growth. Interestingly, a mutation in Smad4 leads to the loss of the tumor suppressor function of TGFβ[3].

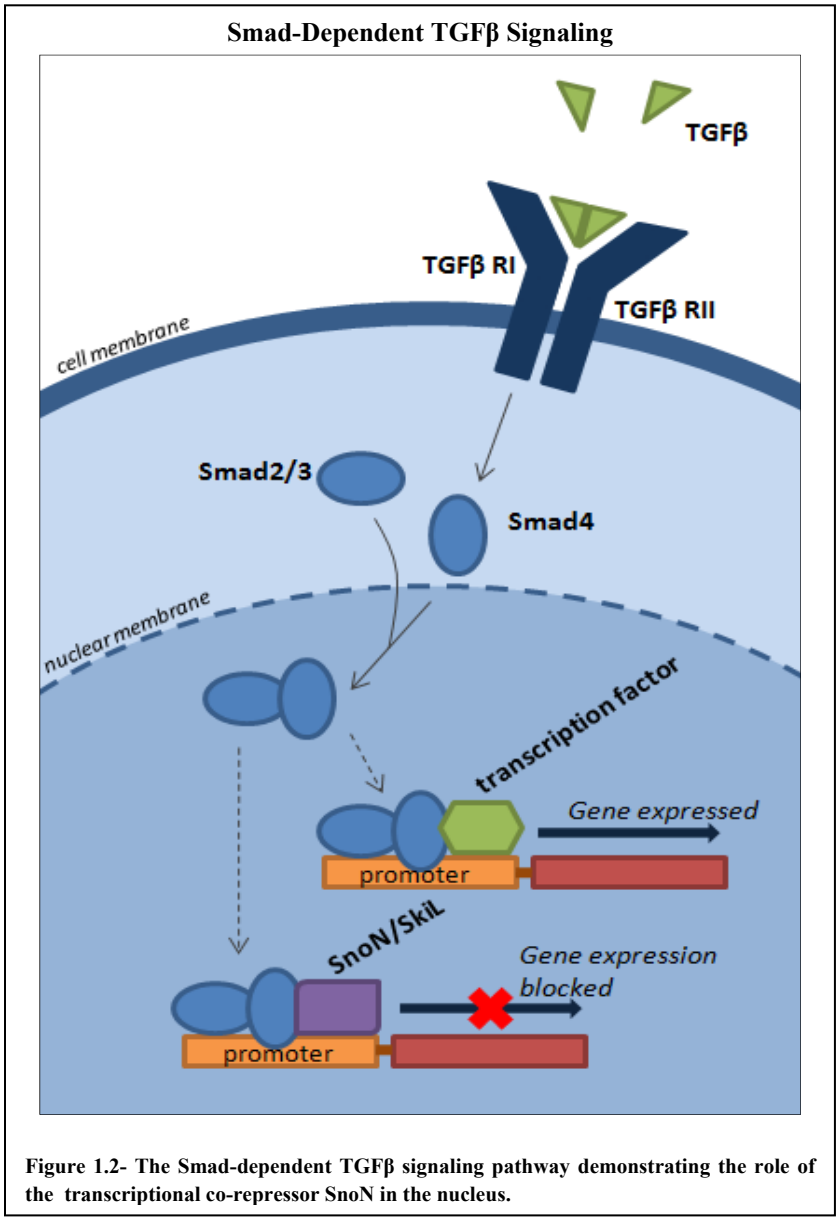

Figure 1.2- The Smad-dependent TGFβ signaling pathway demonstrating the role of the transcriptional co-repressor SnoN in the nucleus.

To date, several genes that antagonize the inhibitory effect of TGFβ in ovarian cancer have been identified (i.e. EVI1[4], SnoN/SkiL[5], AML1/RUNX1[6], PKCι[7]). Among these TGFβ antagonists, SnoN (which is a member of the SKI family of nuclear proto-oncogenes) has been well characterized[8]. In the nucleus, SnoN can elicit either pro-oncogenic or anti-oncogenic activities via the following two pathways: (1) SnoN may promote epithelial cell

proliferation by antagonizing the growth-inhibitory activity of the TGFβ/Smad pathway, or (2) very high levels of SnoN may trigger premature senescence via stabilization of p53 in a PML-dependent manner, respectively[8]. For this reason, SnoN has been associated with these pro- and anti-oncogenic activities linked to cancer progression.

III. Recepteur d'Origine Nantais (RON) Tyrosine Kinase Signaling Pathway

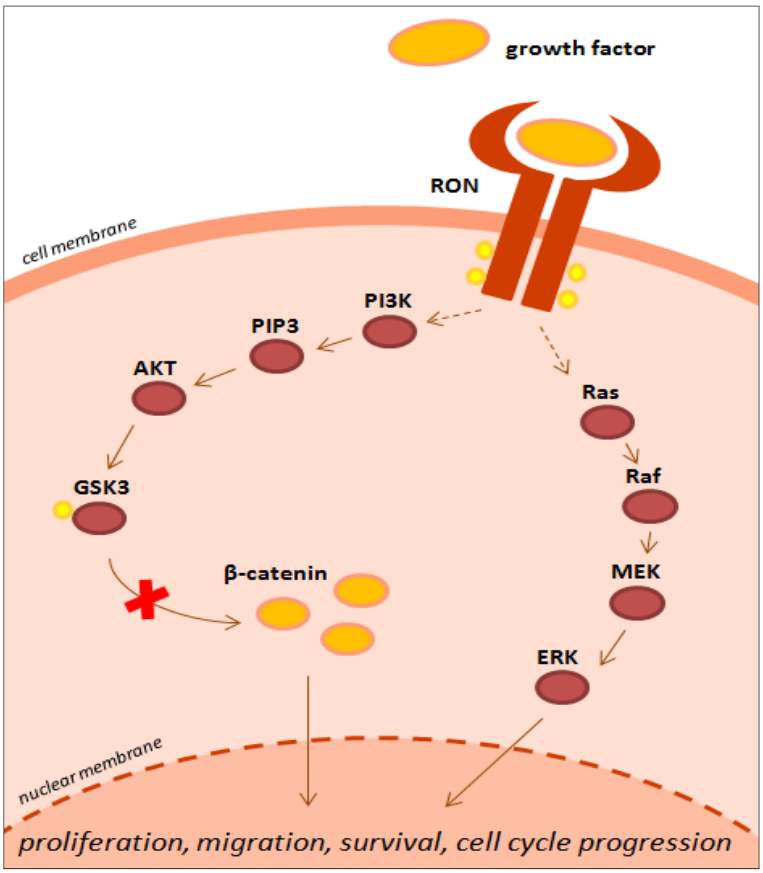

Figure 1.3- The RON tyrosine kinase signaling pathway. When activated, GSK-3 (glycogen synthase kinase-3) phosphorylates β-Catenin, targeting it for degradation in the proteasome.

Another signaling pathway that is associated with invasive cancers is that activated by Recepteur d'Origine Nantais (RON), a receptor tyrosine kinase which is a member of the MET proto-oncogene family[9]. Mature RON is a 180-kDa heterodimer composed of a 40-kDa α-chain and a 150-kDa transmembrane β-chain with intrinsic tyrosine kinase activity[9]. The ligand for RON is the macrophage-stimulating protein (MSP1)[9]. MSP1 is 725 amino acids in length and is released by

hepatocytes into the blood, where it is later proteolytically modified to a form that can bind to RON leading to activation of its tyrosine kinase activity[9]. Activation of RON by MSP1 stimulates multiple signaling pathways including PI-3 kinase, Ras, MAP kinase, DVL, GSK-3β, Smad and β-catenin leading to induction of cell adhesion, dissociation, migration and matrix invasion[10].

IV. Cross-talk between RON and TGFβ Signaling Pathways to Regulate Claudin-1, a Tight Junction Marker

Tight junctions are an integral part of epithelial junction complexes and play a vital role in creating and maintaining epithelial integrity and cell polarity[11]. Structurally, tight junctions are composed of proteins and lipids surrounding the lateral membrane of epithelial cells[12]. A group of integral membrane proteins known as claudins create the backbone of tight junctions. At present, more than 20 claudin genes have been identified[11]. Disruption of tight junctions is a hallmark of epithelial cancer development and progression towards malignancy[13]. Altered expression of claudins such as loss of claudin-1 expression has been shown to correlate with increased invasiveness and malignant progression of certain epithelial cancers[14, 15].

Various mechanisms have been implicated in regulation of claudin-1 expression. In colon cancers, Smad4 of the TGFβ pathway has been shown to repress claudin-1 transcription through modulation of β-catenin/T-cell factor/ lymphocyte enhancer factor activities[16]. Hence, there is an inverse relationship between the expression of claudin-1, a metastasis-promoting protein, and Smad4, a tumor suppressor protein, in colon cancer cell lines. With respect to the RON pathway, studies with Madin-Darby Canine Kidney (MDCK) cells have shown that activation of RON decreases E-cadherin and claudin-1 expression. Additionally, Snail and Slug, of the Snail family

of transcription factors, have been implicated as transcriptional repressors of claudin-1 in MDCK cells[17]. It has further been indicated that activation of Erk1/2 signaling cascades is the first step required for RON to exert the inhibitory effect on claudin-1 expression[17]. Ligand-dependent RON activation disrupts tight junctions and impairs their functions via diminished claudin-1 expression[17]. Hence, activation of RON has a fundamental impact on integrity and function of epithelial cell–cell junctions. Alterations of these cellular structures are vital in RON-mediated tumorigenic activities leading to malignant progression.

Previous studies have shown increased expression of RON tyrosine kinase in pancreatic tissues with Smad4 deletions in comparison to normal Smad4 expressing pancreatic cancer specimens[18]. This indicates that the Smad-dependent TGFβ signaling pathway is critically involved in transcriptional regulation of RON tyrosine kinase. Further studies have shown that that RON and TGFβ can interact collaboratively including induction of EMT[13]. Recent studies have also shown that TGFβ transcriptional mediators, EVI1 and RUNX1[4, 6], are associated with Smad and act as transcriptional co-repressors of RON tyrosine kinase [Shafiq and Nanjundan, unpublished results].

B. Preliminary Data

The cell line highlighted in red, BxPC-3, are Pancreatic Adenocarcinoma cells. In response to MSP1, activation of the RON tyrosine kinase has been reported. The protein expression profiling results show that expression of RON, β-Catenin, and claudin-1 are correlated positively in BxPC-3 cells. The cell line highlighted in blue, SKOV3, are ovarian cancer cells that can be activated by the MSP1 receptor. The protein expression profiling results show that RON, claudin-1 and Smad2/3 expression are highly correlated with SKOV3 cells. The cell line highlighted in green, HEY, are human ovarian carcinoma cells. The protein expression profiling results show that expression of β-catenin, claudin-1 and Smad2/3 are positively correlated in HEY cells. The cell line highlighted in yellow, H358, are squamous lung carcinoma cells that can be activated by the MSP1 receptor. The protein expression profiling

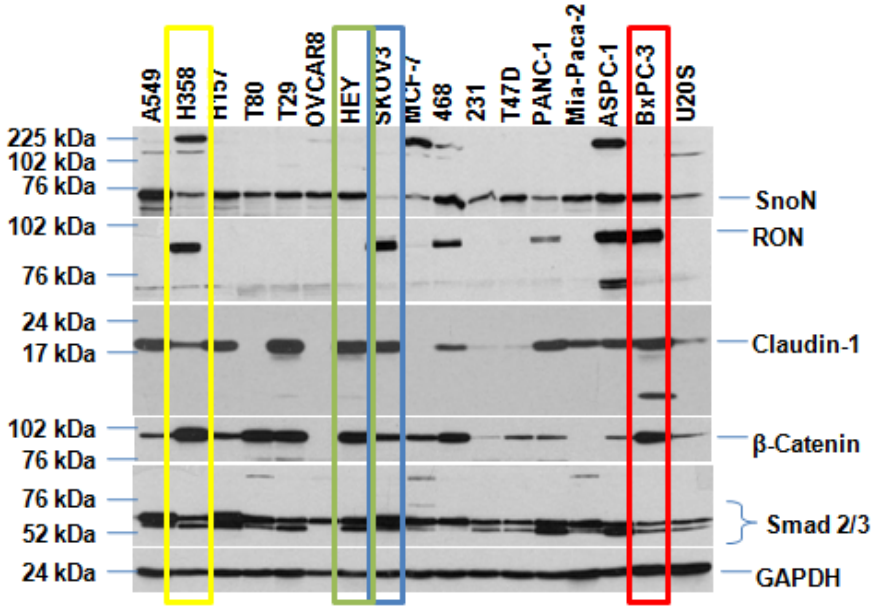

Figure 1.4- Cell line profile produced by Western analysis showing protein expression levels of various proteins. Study primarily focused on the cell lines highlighted in the figure.

results show that expression of RON, β-catenin, and Smad2/3 are positively correlated in H358 cells.

C. Objectives and Hypotheses

Tight junctions are an integral component of epithelial junction complexes and play a vital role in creating and maintaining epithelial integrity and cell polarity. Disruption of tight junctions is a hallmark of epithelial cancer development and malignant progression. The loss of claudin-1 expression has been shown to be correlated with increased invasiveness and malignant progression of certain epithelial cancers[14, 15]. Various mechanisms have been implicated in regulation of claudin-1 expression including regulators of the TGFβ and RON Tyrosine Kinase pathways. As stated in the Background, studies with colon cancers have shown that Smad4, a co-Smad involved in the TGFβ pathway, represses claudin-1 expression. Furthermore, studies in breast cancer and MDCK cells show that RON activation disrupts tight junctions and impairs their functions through diminished claudin-1 expression[17]. Hence, activation of RON has a fundamental impact on integrity and function of epithelial cell–cell junctions and alterations of these cellular structures are vital components in RON-mediated tumorigenic activities that assist malignant progression.

Undeniably, other mechanisms play a role in regulation of claudin-1 expression in cancer cell migration and invasion. Thus, we hypothesize that claudin-1 expression is regulated by the activation of the transforming growth factor beta signaling pathway, particularly through SnoN/SkiL, a key TGFβ co-repressor[5]. Scientific evidence suggests that there is cross-talk between the TGFβ pathway and receptor tyrosine kinases such as RON which is aberrantly expressed in invasive carcinomas[9, 11]. The goal of the proposed studies is to delineate the

signaling pathways that are involved in regulating the expression of this tight junction protein whose expression is dysregulated. Thus, the specific aims are as follows:

Specific Aim #1: We will test the hypothesis that altered expression of RON tyrosine kinase leads to dysregulated claudin-1 expression

Our goal is to determine whether knockdown of RON using siRNA (targeting wild type Ron Tyrosine Kinase) leads to altered claudin-1 expression via up/downregulated expression of TGFβ signaling mediators including Smad2/3, SnoN, and β-catenin.

Specific Aim #2: We will test the hypothesis that cellular treatment with TGFβ and MSP1 leads to altered expression levels of Claudin-1

Our goal is to investigate the changes in claudin-1 expression via cellular activation with TGFβ and MSP1 ligands which activate the TGFβ receptor and RON tyrosine kinase receptor, respectively.

Specific Aim #3: We will test the hypothesis that knockdown of β-catenin and SnoN upregulates claudin-1 expression

Our goal is to investigate the claudin-1 expression following cellular treatment with siRNA targeting β-catenin and SnoN in ovarian carcinoma cells.

The disruption of tight junctions is a hallmark of epithelial cancer development and malignant progression wherein decreased claudin-1 expression is involved in this progression to malignancy. Since identification of novel biomarkers could be beneficial in diagnosis, prognosis and prediction of patient outcomes, it is of great importance to elucidate the detailed cellular events underlying claudin-1 expression.

II. General Methods

1. Cell Culture

H358 Bronchial Alveolar Carcinoma and BxPC-3 Pancreatic Adenocarcinoma cell lines were obtained from ATCC while the HEY Ovarian Carcinoma and SKOV3 Ovarian Carcinoma cell lines were kindly provided by Dr. Mills (MD Anderson Cancer Center, Houston Texas). All four cells lines were maintained in RPMI 1640 containing 8% Fetal Bovine Serum and 1% penicillin-streptomycin in a 5% CO_2 humidified incubator at 37°C.

2. Cell Passage

For experiments, cells were utilized at passage numbers below 30. Cells maintained in flasks were removed from the CO_2 incubator. Media was first removed from the flasks followed by the addition of trypsin. To aid cell detachment, the flasks were gently tapped followed by the addition of complete media. The cell-media solution was then collected into aliquot tubes and centrifuged at 1,000rpm for 5 minutes which resulted in a pellet fraction that was retained while the supernatant was discarded. The pellet was resuspended in an appropriate volume of complete media and $1/5^{th}$ was then plated into new flasks.

3. Cell Seeding and treatment with TGFβ and MSP1

H358, BxPC-3, SKOV3 and HEY cells were seeded at 250,000 cells per well in 6-well plates. Twenty-four hours post seeding, cells were treated with TGFβ (50pM), MSP1 (10ng/mL), and a combination of these two ligands (50pM TGFβ and 10ng/mL MSP1), across the following time courses: a) 1 minute, 5 minutes, 1 hour, 3 hours and 6 hours (short

time course) and b) 24 hours, 48 hours, 72 hours and 96 hours (long term time course). Immediately upon completion of these treatments, samples were placed on ice to terminate cellular activities.

4. Cell Seeding and treatment for siRNA

H358, BxPC-3, SKOV3 and HEY cells were seeded at 250,000 cells per well in 6-well plates. Twenty-four hours post seeding, cells were treated either with non-targeting siRNA (control) or RON siRNA (1X). The following day, the cells were allowed to recover followed by another siRNA transfection (2X) to obtain a greater reduction in RON knockdown. The following day, cells were allowed to recover followed by cellular treatment for 24 hours with TGFβ (50pM), and MSP1 (10ng/mL). Immediately upon completion of these treatments, samples were placed on ice to terminate cellular activities.

Additional experimentations included siRNA treatment of SKOV3 and HEY cells with β-catenin siRNA and SnoN siRNA. Cells were similarly seeded at 250,000 cells per well in 6-well plates and siRNA methodology was followed as described above.

5. Protein Harvest and Quantification

a. Protein Isolation

Media was removed from each well of 6-well plates followed by the addition of 1X Phosphate Buffer Saline (PBS). The plates were gently rocked for a few seconds and the PBS was removed from each well. One-hundred μL of RPPA Lysis Buffer (containing a cocktail of protease inhibitors) was added to each well and the plates were incubated on ice for one hour. After incubation, the cells were scraped vigorously and the resulting lysate collected into Eppendorf tubes. The samples were then centrifuged at 15,000rpm for 10

minutes as 4°C. Following centrifugation, the pellet was discarded while the supernatant was retained for further analysis. Samples were kept on ice at all times to minimize protein degradation.

b. Bicinchoninic Acid (BCA) Assay for Total Sample Protein Concentration

Bovine Serum Albumin (BSA) standards (0, 25, 50, 100, 250, 500, 750, 1000, 2000μg/mL) and samples were pipetted in duplicate into a 96-well plate (2μL per well). Next, BCA Reagent A and BCA Reagent B were mixed at a ratio of 9.8ml:0.2ml. Two-hundred μL of the BCA Reagent mixture was added to each well containing a standard or sample. The plate was then placed in a 37°C incubator for 30 minutes. After incubation, the plate was read at 570nm in a BioTek Synergy 2 plate reader. The resulting data was analyzed using a Microsoft Office Excel document to generate a standard curve and determine the protein concentrations of the unknown samples. These samples were then diluted to 1-2mg/mL with lysis buffer and 6X SDS sample loading buffer to prepare gel loading sample to run of SDS-PAGE gels.

6. SDS-PAGE Gel (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

a. Preparation of SDS-PAGE Gels

Components for preparation of lower and upper gel were mixed in two separate 50ml aliquot tubes. The quantity used of each component (NANOpure water, gel buffer (upper or lower), 30% acrylamide, APS and TEMED) was determined by the percentage of the gel required (i.e. 8% or 12%). The lower gel mixture was first poured into a Criterion gel cassette. After lower gel polymerization, the upper gel was mixed, poured, and a comb was inserted for formation of loading wells (18 wells).

b. Running of SDS-PAGE Gels

Samples were boiled at 95°C for 5 minutes, vortexed for 2 seconds, and centrifuged for 10 seconds at 15,000 rpm. The gels were placed appropriately into the Criterion electrophoresis system. The basin of the gel cassette and the external chambers of the gel apparatus were filled with Running Buffer. 20µl of samples and molecular weight markers were loaded into the appropriate wells. The gels were electrophoresed for 2 hours at 100 volts.

c. Gel Transfer to Polyvinylidine Fluoride Membrane (PVDF) Membranes Using a Semi-Dry Transfer Method

Prior to the western blotting process, the gels were transferred to a high protein binding membrane (PVDF). For transfer, the gel was removed from the cassette and incubated briefly with transfer buffer. The membrane was activated by soaking for one minute in 100% methanol followed by rinsing 3 times with NANOpure water, and immersion in Transfer Buffer. Blotting paper was soaked in Transfer Buffer and placed on the blotting panel of the Trans-Blot apparatus. The PVDF membrane, the gel, and another piece of blotting paper were then placed on top of this blotting paper (in this order)**.** Throughout this process, care was taken to remove all bubbles from this "blot sandwich". The Trans-Blot apparatus was closed and the transfer was conducted for 2 hours with a voltage limit of 20 Volts. Depending on the number of blots transferred, a constant current of 0.11 amps (1 blot) or 0.15 amps (2 blots) was utilized.

7. Western Blotting

a. Incubation with Antibodies

After the gels were transferred to membranes, the membranes were washed in 1X Tris-Buffered Saline Tween-20 (TBST) for 10 minutes on a platform rotator. Then, the

TBST was drained and the membrane was placed on the platform rotator for 1 hour in 1X TBST with 5% milk for "blocking". The milk solution was then removed and the membrane was incubated with appropriate primary antibody on the rotator overnight at 4°C. The membrane was washed in 1X TBST for 1 hour on the rotator with the TBST being replaced every 15 minutes. Appropriate secondary antibody was then applied to the membrane and incubated with shaking at room temperature for 1 hour. The membrane was then washed in 1X TBST for 1.5 hours on the rotator with the TBST being replaced every 15 minutes.

b. Developing Western Blots

To develop western blots, the developer was turned on and warmed up at least 20 minutes in advance. Prior to use, the ECL developing reagents (HRP Luminol and Peroxide Buffer) were mixed at a 1:1 ratio. After secondary antibody incubation and subsequent 1X TBST washes, the membranes were completely covered with the ECL mixture and incubated at room temperature for 5 minutes. The blots were then placed in the developing cassette and moved to the darkroom where Kodak film was exposed to the blot and developed.

c. Western Blot Membrane Regeneration

Blots were regenerated after developing by washing in 1X TBST for 10 minutes on the platform rotator. TBST was then discarded and 20 mL of restoration buffer (Pierce) was applied to the membrane. The membrane was incubated for 1 hour at 50°C with moderate shaking, then washed in 1X TBST for 10 minutes on the platform rotator, and then blocked for 2 hours in 1X TBST containing 5% milk. The milk solution was discarded and the membrane was incubated with primary antibody on the rotator overnight at 4°C.

8. RNA Isolation

RNA isolation was performed using the QIAGEN RNeasy Mini Kit. Forty-eight hours post 2X siRNA transfection, cells were washed with 1ml of Dulbecco's Phosphate Buffered Saline (DPBS). After discarding the DPBS, 350µl of RLT buffer was added to each well. Following one minute incubation, the wells were scraped and lysates transferred into shredder columns for homogenization.

The columns were centrifuged for 2 minutes at 14,000 rpm. 300µl of 70% ethanol (RNA grade) was added to the eluate. After mixing, eluates were transferred into spin columns and centrifuged for 15 seconds at 10,000 rpm. Then, the flow-through was discarded, 700µl of RW1 buffer was added to each spin column, and centrifuged for 15 seconds at 10,000 rpm. After the flow-through was discarded again, 500µl of RPE buffer was added to each spin column, and centrifuged for 15 seconds at 10,000 rpm. Next, the flow-through was discarded, another 500µl of RPE buffer was added to each spin column, and centrifuged for 2 minutes at 10,000 rpm. The spin columns were then centrifuged for 1 minute at 14,000 rpm followed by the addition of 30µl of RNase free water. This was followed by one minute incubation and centrifugation for one minute at 10,000 rpm. The flow-through was re-added to the spin columns followed by the addition of a further 10µl of RNase free water and then centrifuged for one minute at 10,000 rpm. The spin columns were then discarded and the RNA samples were transferred into appropriately labeled Eppendorf tubes. The RNA concentration of each sample was quantified using the NANOdrop apparatus and 20ng/µl dilutions (with total volumes of 50µl) were prepared. All RNA samples were stored at -80°C until further use.

9. Real Time PCR

Real Time PCR was used to quantify the mRNA transcript levels of claudin-1 following SnoN, β-catenin, and RON knockdown using siRNA in HEY cells. β-Actin probe/primers were used to determine the β-Actin RNA levels as an endogenous control. For both the β-Actin control and genes of interest, master mixes were prepared using the components provided in the Applied Biosystems OneStep RT-PCR Kit.

The master mixes were pipetted into a 96-well plate followed by the addition of 40ng of RNA. All samples were analyzed in duplicate. PCR was run using the StepOnePlus Real-Time PCR System which was programmed as follows: (1) 48°C for 30 minutes (reverse transcriptase reaction), (2) 95°C stage for 10 minutes, and (3) 40 cycles alternating between 95°Cfor 15 seconds and 60°C for 1 minute. Results were analyzed using the following formula: $2^{-\Delta\Delta CT}$. ΔC_T= C_T value of gene of interest minus C_T value of β-Actin. $\Delta\Delta C_T$= ΔC_T minus ΔC_T of C_T of siRNA sample.

III. Results

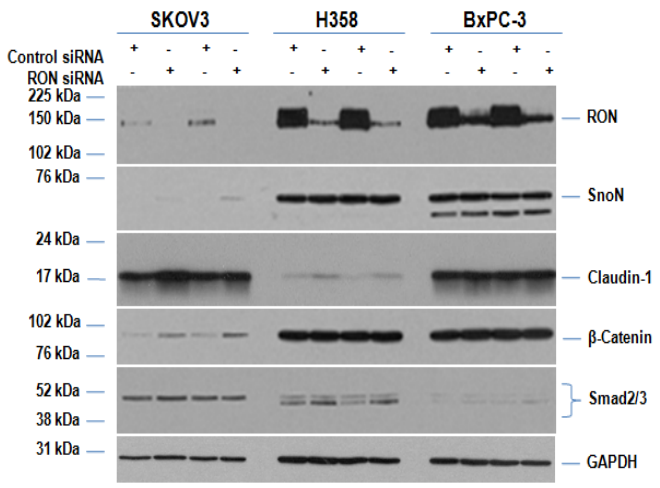

Figure 3.1- Western analysis of RON knockdown in SKOV3, H358, and BxPC-3 cells. Cells were seeded at 250,000 cells per well in 6-well plates. Twenty-four hours post seeding, cells were treated either with non-targeting siRNA (control) or RON siRNA (1X). The following day, the cells were allowed to recover followed by another siRNA transfection (2X). Protein was harvested, quantified, and run on SDS-PAGE. Following the transfer of gels to PVDF membranes, Western blotting was performed using antibodies targeting (1) RON, (2) SnoN, (3) claudin-1, (4) β-catenin, (5) Smad2/3, and (6) GAPDH used as the loading control.

Part 1: RON Knockdown Leads to Upregulated Claudin-1 Protein Levels

In **Aim #1**, we tested the hypothesis that dysregulated RON tyrosine kinase expression leads to altered claudin-1 protein levels. In high RON Tyrosine Kinase expressing BxPC-3, H358 and SKOV3 cell lines, the effects of RON knockdown using siRNA were investigated (results shown in Figure 3.1). Western blotting was performed to examine the expression of TGFβ signaling mediators (Smad2/3 and SnoN) as well as β-Catenin. RON knockdown led to greater than 80% reduction in RON protein expression in all three cell lines. Coincident with

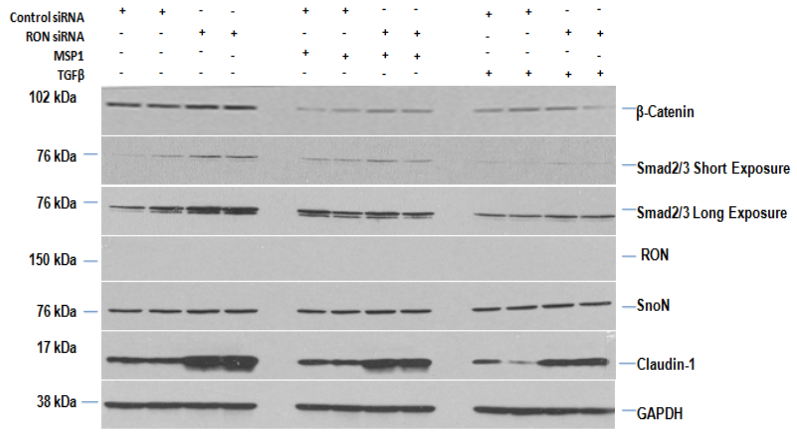

Figure 3.2- Western analysis of RON knockdown in HEY cells. Cells were seeded at 250,000 cells per well and at 24 hours post seeding, were either treated with non-targeting siRNA (control) or RON siRNA (1X). Another siRNA transfection (2X) was performed the following day to obtain a greater reduction in RON knockdown. Protein was then harvested, quantified, run on SDS-PAGE, and transferred to PVDF membranes. Western blotting was performed using antibodies targeting (1) RON, (2) SnoN, (3) claudin-1, (4) β-catenin, (5) Smad2/3, and (6) GAPDH used as a loading control.

decreased Ron levels, we observed that, (1) claudin-1 protein levels were increased in both SKOV3 and H358 cell lines, (2) β-catenin protein levels were increased in SKOV3 cells slight increase in SnoN expression in SKOV3 cells.

To further validate these changes in an independent cell line, we selected HEY cells, a highly metastatic ovarian cancer cell line (see Figure 3.2). Similar to SKOV3 and H358 cells (Figure 3.1), we observed that claudin-1 protein levels were increased with RON knockdown in HEY cells. Furthermore, similar to SKOV3 cells (Figure 3.1), β-catenin protein levels were increased with RON knockdown in HEY cells. However, SnoN expression levels appear to be unchanged in HEY cells in contrast to SKOV3 cells.

Part 2: Reduction of Claudin-1 Protein Expression Upon Cellular Treatment with TGFβ and MSP1

The goal of Aim #2 was to investigate changes in claudin-1 protein expression following cellular activation with TGFβ and MSP1 ligands which activate the TGFβ receptor and RON

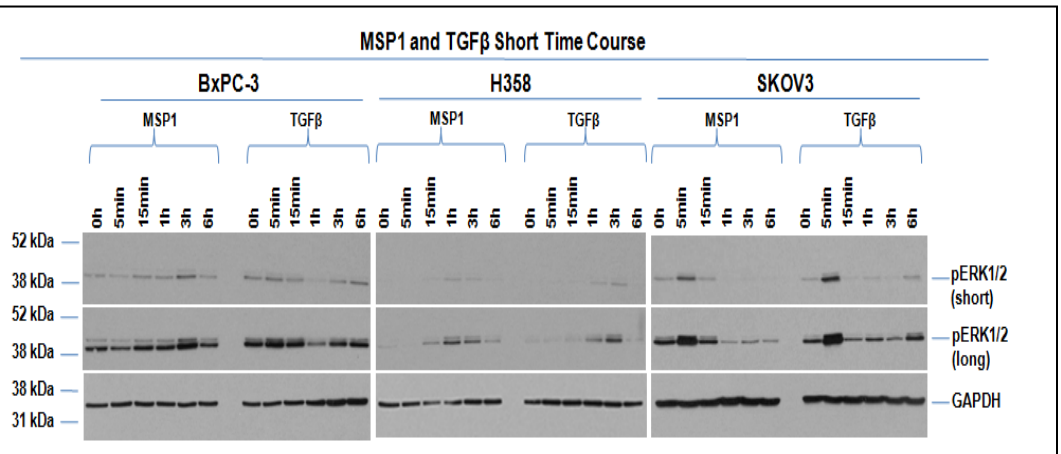

Figure 3.3-Effects of MSP1 and TGFβ on ERK activation (pERK1/2) in H358, BxPC-3, and SKOV3 cells. Cells were seeded at 250,000 cells per well and twenty-four hours post seeding, cells were treated with TGFβ (50pM) and MSP1 (10ng/mL) across the following time course: 1 minute, 5 minutes, 1 hour, 3 hours and 6 hours (short time course). Western blotting was performed using antibodies targeting (1) pERK1/2 and (6) GAPDH used as a loading control.

tyrosine kinase receptor, respectively. The effects of MSP1 and TGFβ activation on claudin-1

expression were investigated across a series of short (up to 6 hours) and long (up to 96 hours) time course treatments. In the short-term time course, Western blotting was performed to examine the activation of pERK1/2 (also known as MAPK), as shown in Figure 3.3. We observed a marked activation of MAP kinase at 3 hours post treatment in BxPC-3 cells, 1 hour post treatment in H358 cells and 5 minutes post treatment in SKOV3 cells. This was detected using an antibody against MAP kinase which specifically detects its phosphorylation status.

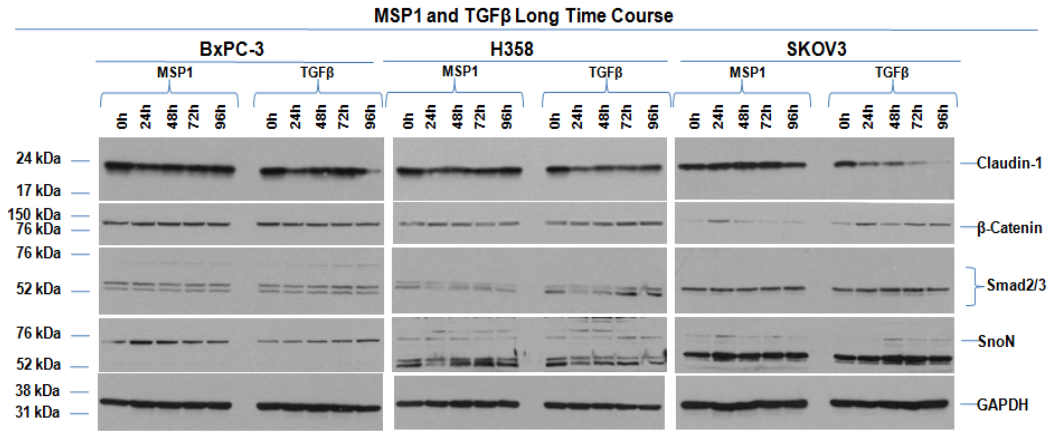

Figure 3.4- Effects of long term cellular treatments with MSP1 and TGFβ treatment H358, BxPC-3, and SKOV3 cells. Cells were seeded at 250,000 cells per well in 6-well plates. Twenty-four hours post seeding, cells were treated with TGFβ (50pM), MSP1 (10ng/mL), across the following time course: 24 hours, 48 hours, 72 hours, and 96 hours. Protein was harvested, quantified, separated by SDS-PAGE, and transferred to PVDF membranes. Western blotting was then performed using antibodies targeting (1) claudin-1, (2) β-catenin, (3) Smad2/3, (4) SnoN, and (4) GAPDH used as the loading control.

In the long-term time course, expression levels of claudin-1, β-catenin, Smad2/3, and SnoN were investigated up to 96 hours post MSP1 and TGFβ addition. As shown in Figure 3.4, we observed decreased claudin-1 expression 24 hour post treatment with MSP1 and TGFβ in both BxPC-3 and H358 cells. Furthermore, we observed decreased claudin-1 expression 24 to 96 hours post TGFβ treatment in SKOV3 cells. β-catenin protein levels were increased 24 hours post treatment with both MSP1 and TGFβ in SKOV3 cells. SnoN expression levels were increased 24 hours post treatment with combinatorial treatment of both MSP1 and TGFβ in SKOV3 cells. In contrast, we did not detect any marked changes in Smad2/3 expression.

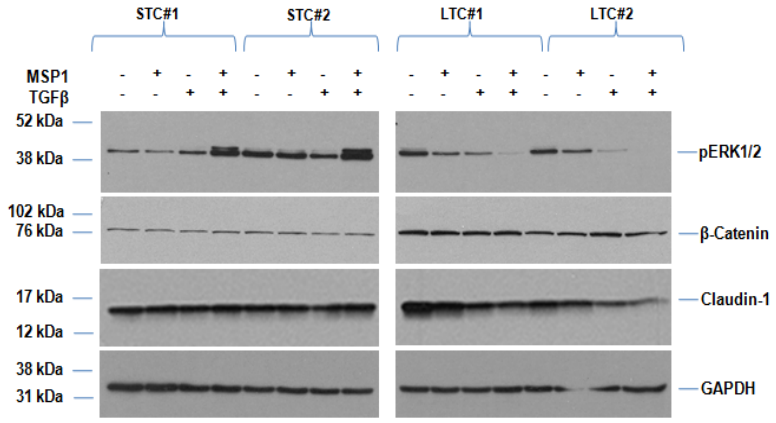

Figure 3.5- Combinatorial short and long-time course cellular treatment with MSP1 and TGFβ in SKOV3 cells. Cells were seeded at 250,000 cells per well and twenty-four hours post seeding, cells were treated with TGFβ (50pM) and MSP-1 (10ng/mL), or a combination of these two ligands (50pM TGFβ with 10ng/mL MSP1) either at 5 minutes (short time course, STC) or at 24 hours (long time course, LTC). Cell lysates were harvested, protein quantified, separated by SDS-PAGE, and transferred to PVDF membrane. Western blotting was using antibodies detecting (1) pERK1/2, (2) β-catenin, (3) claudin-1, and (4) GAPDH as the loading control.

Figure 3.5 shows the western analysis of short and long-term time courses of combinatorial treatments of both MSP1 and TGFβ. Since the main focus of this study is ovarian carcinomas, we selected SKOV3 cells to examine the effects of combinatorial treatments of these two ligands at 24 hours post-treatment. MAP kinase activation (via detection of phosphorylated ERK1/2) is observed in short time course TGFβ treatment but not in combination treatment with both MSP1 and TGFβ. In the long time course, activation of MAP kinase decreased with both MSP1 and TGFβ but more dramatically upon combination of both of these two ligands. Consistent with our previous observations, claudin-1 expression levels decreased with only MSP1 or TGFβ alone; however, the reduction in claudin-1 was more marked when cells

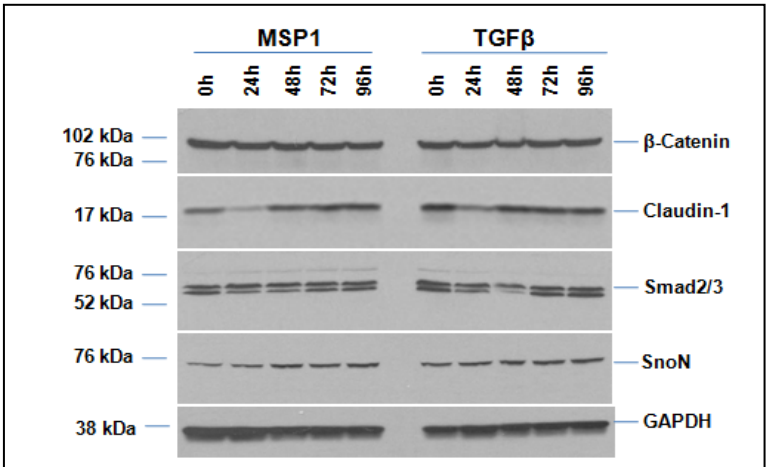

Figure 3.6- Long time course treatment of MSP1 and TGFβ in HEY cells. Cells were seeded at 250,000 cells per well and twenty-four hours post seeding, cells were treated with TGFβ (50pM), and MSP-1 (10ng/mL), across the following time course: 24 hours, 48 hours, 72 hours and 96 hours. Protein was then harvested, quantified, separated by SDS-PAGE, and transferred to PVDF membranes. Western blotting was performed using antibodies detecting (1) β-catenin, (2) claudin-1, (3) Smad2/3, (4) SnoN, and (5) GAPDH as a loading control.

were treated with both TGFβ and MSP1. In contrast to these dramatic changes in claudin-1 protein levels, β-catenin expression levels were unchanged.

To further validate these observations and determine whether these results can be generalizable to other carcinoma cell lines, we repeated these studies using HEY cells. Figure 3.6 displays the Western analysis of the long time course while Figure 3.7 displays the Western analysis of the combination treatments of MSP1 and TGFβ for two selected time points (i.e. 24 and 48 hours). In the long term time course in HEY cells (Figure 3.6), claudin-1 expression levels decreased 24 hours post treatment with MSP1 and TGFβ. Smad2/3 expression levels decreased 48 hours post treatment with TGFβ while no changes were seen in SnoN or β-catenin expression levels. In the combination study (shown in Figure 3.7), we observed a marked reduction in claudin-1 expression following 24 hours post treatment with MSP1, TGFβ, and the combination of these two ligands. In contrast, the protein expression levels of Smad2/3 were increased 24 hours post treatment with MSP1, TGFβ, and the combination of these two ligands. SnoN expression levels were slightly increased post 24 and 48 hours treatment of MSP1, TGFβ, with a more marked increase with the combinatorial treatment at 48 hours post treatment. Similarly, β-catenin protein expression levels were increased at 24 and 48 hour post treatment with TGFβ.

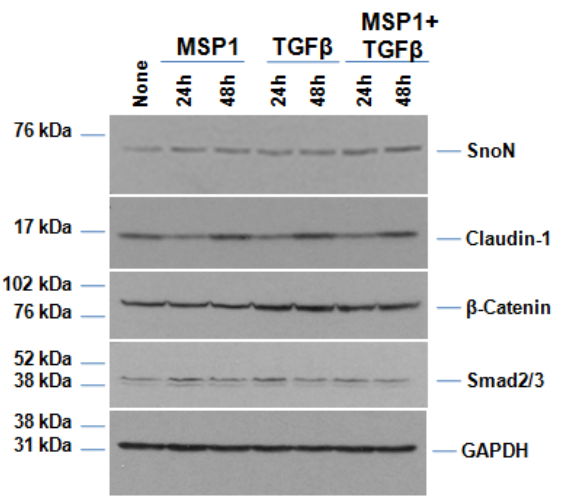

Figure 3.7- Long time course treatment of MSP1, TGFβ and the combination of the two ligands in HEY cells. Cells were seeded at 250,000 cells per well and twenty-four hours post seeding, cells were treated with TGFβ (50pM) and MSP-1 (10ng/mL), and a combination of these two ligands (50pM TGFβ and 10ng/mL MSP-1) at 24 and 48 hours. This was followed by protein harvesting, quantification, separation by SDS-PAGE, and transferred to PVDF membranes. Western blotting was performed using antibodies detecting (1) SnoN, (2) claudin-1, (3) β-catenin,(4) Smad2/3, and (5) GAPDH as a loading control.

Part 3: SnoN and β-Catenin Knockdown Leads to Upregulated Claudin-1 Protein Levels

Aim #3 of this study was to test the hypothesis that knockdown of β-catenin and SnoN upregulates claudin-1 expression. Initially, SKOV3 cells were used to investigate the claudin-1 expression following cellular treatment with siRNA targeting β-catenin.

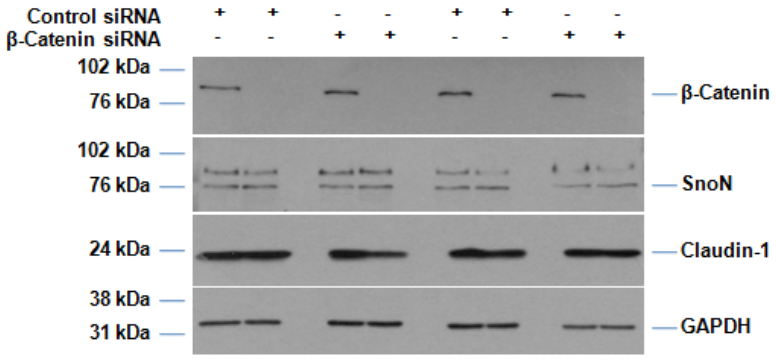

Figure 3.8- Effects of β-catenin knockdown in SKOV3 cells. Cells were seeded at 250,000 cells per well in 6-well plates. Twenty-four hours post seeding, cells were treated either with non-targeting siRNA (control) or β-catenin siRNA (1X). The following day, the cells were allowed to recover followed by another siRNA transfection (2X). Protein was harvested, quantified with BCA, and run on SDS-PAGE. Following the transfer of gels to PVDF membranes, Western blotting was performed using antibodies detecting (1) β-catenin, (2) SnoN, (3) claudin-1, and (4) GAPDH as a loading control.

However, SKOV3 cells (as shown in Figure 3.8) were extremely slow growing and thus, the protein amounts were suboptimal for further analyses. Hence, HEY cells were used since they yielded an abundance of protein for Western analyses.

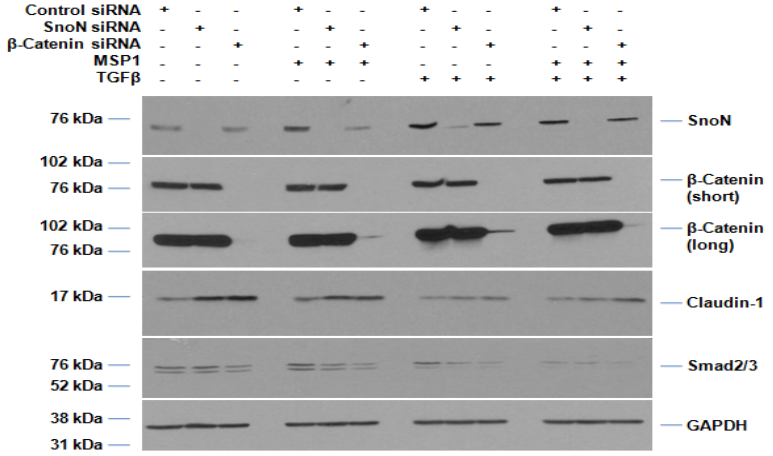

Figure 3.9- Effects of β-catenin knockdown in HEY cells. Cells were seeded at 250,000 cells per well in 6-well plates. Twenty-four hours post seeding, cells were treated either with non-targeting siRNA (control) or SnoN siRNA (1X), or β-catenin siRNA (1X). The following day, the cells were allowed to recover followed by another siRNA transfection (2X). The following day, cells were allowed to recover followed by cellular treatment for 24 hours with TGFβ (50pM), MSP1 (10ng/mL), and a combination of the two ligands (50pM TGFβ and 10ng/mL MSP1). Protein was harvested, quantified, and run on SDS-PAGE. Following the transfer of gels to PVDF membranes, Western blotting was performed using antibodies detecting (1) SnoN, (2) β-catenin, (3) claudin-1, (4) Smad2/3, and (5) GAPDH as a loading control.

Additional studies included siRNA treatment of HEY cells with β-catenin siRNA and SnoN siRNA, as noted in Figure 3.9. With β-catenin siRNA, the combination treatment of MSP1 and

TGFβ led to increased claudin-1 expression compared to β-catenin siRNA cells treated with only MSP1 or TGFβ. SnoN expression decreased in cells treated with β-catenin siRNA. Smad2/3 expression is decreased with MSP1, with a further marked decrease upon TGFβ treatment as well as with the combination of MSP1 and TGFβ. An even more dramatic reduction in protein levels was observed with Smad3 in contrast to Smad2.

Part 4: RON, SnoN, and β-Catenin Knockdown Leads to Upregulated Claudin-1 mRNA Levels

Table 3.1- RNA concentrations and purity of samples.

Sample ID	ng/µl	λ260	λ280	260/ 280
Control siRNA-1	855.03	21.376	10.372	2.06
Control siRNA-2	966.05	24.151	11.73	2.06
RON siRNA-1	1061.38	26.535	12.854	2.06
RON siRNA-2	1063.95	26.599	12.885	2.06
SnoN siRNA-1	842.19	21.055	10.18	2.07
SnoN siRNA-2	661.59	16.54	8.024	2.06
β-Catenin siRNA-1	1187.45	29.686	14.429	2.06
β-Catenin siRNA-2	715.44	17.886	8.71	2.05

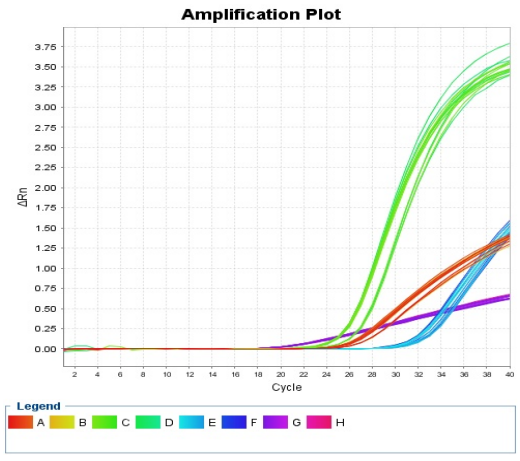

Figure 3.10 – PCR Amplification Plot of RNA levels of SnoN, claudin-1, and RON in siRNA treated HEY cells.

To validate the results we obtained in the above sections where we examined protein levels, we performed RNA analysis to determine whether these changes occurred at the transcriptional level. HEY cells were transfected with control, SnoN, β-catenin, and RON siRNA. RNA was then isolated and quantified (A260/A280=2.0). The RNA concentrations for these samples are presented in Table 3.1. The RNA concentrations for all samples were standardized to 20ng/µl and

qPCR (quantitative PCR) was then performed using probes specific to claudin-1, SnoN, and RON mRNA transcripts. β-Actin probes/primers were used as an endogenous control.

Table 3.3 – Raw Data and Calculation of CT values from qPCR.

Probe	Sample	C_T	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$
Claudin-1	Control siRNA-1	32.72	9.48	0	1
Claudin-1	Control siRNA-2	32.87	9.58	0.096	0.935
Claudin-1	SnoN siRNA-1	31.99	9.22	-0.266	1.203
Claudin-1	SnoN siRNA-2	31.98	9.34	-0.139	1.101
Claudin-1	β-Catenin siRNA-1	31.78	8.91	-0.575	1.490
Claudin-1	β-Catenin siRNA-2	31.52	8.58	-0.904	1.871
Claudin-1	RON siRNA-1	31.71	8.71	-0.772	1.708
Claudin-1	RON siRNA-2	31.79	8.79	-0.693	1.617
SnoN	Control siRNA-1	28.29	5.057	0	1
SnoN	Control siRNA-2	28.25	4.952	-0.104	1.075
SnoN	SnoN siRNA-1	29.42	6.648	1.591	0.332
SnoN	SnoN siRNA-2	29.37	6.731	1.674	0.313
SnoN	β-Catenin siRNA-1	28.16	5.282	0.226	0.855
SnoN	β-Catenin siRNA-2	28.19	5.248	0.192	0.876
SnoN	RON siRNA-1	28.20	5.195	0.138	0.909
SnoN	RON siRNA-2	28.31	5.309	0.252	0.840
RON	Control siRNA-1	33.93	10.70	0	1
RON	Control siRNA-2	34.20	10.909	0.212	0.863
RON	SnoN siRNA-1	34.02	11.242	0.544	0.686
RON	SnoN siRNA-2	33.95	11.309	0.612	0.654
RON	β-Catenin siRNA-1	34.69	11.816	1.119	0.461
RON	β-Catenin siRNA-2	34.91	11.964	1.266	0.416
RON	RON siRNA-1	34.82	11.818	1.121	0.459
RON	RON siRNA-2	34.61	11.603	0.906	0.534

Table 3.2- Averaged β-actin C_T Values.

Probe	Sample	C_T
β-Actin	Control siRNA-1	23.23
β-Actin	Control siRNA-2	23.29
β-Actin	SnoN siRNA-1	22.78
β-Actin	SnoN siRNA-2	22.63
β-Actin	β-Catenin siRNA-1	22.88
β-Actin	β-Catenin siRNA-2	22.95
β-Actin	RON siRNA-1	23.00
β-Actin	RON siRNA-2	23.00

Table 3.4 - Average and SD of Data.

Probe	Sample	Mean	Standard Deviation
Claudin-1	Control siRNA	0.967	0.046
Claudin-1	SnoN siRNA	1.152	0.072
Claudin-1	β-Catenin siRNA	1.681	0.270
Claudin-1	RON siRNA	1.662	0.064
SnoN	Control siRNA	1.038	0.053
SnoN	SnoN siRNA	0.323	0.013
SnoN	β-Catenin siRNA	0.865	0.014
SnoN	RON siRNA	0.874	0.049
RON	Control siRNA	0.932	0.097
RON	SnoN siRNA	0.670	0.022
RON	β-Catenin siRNA	0.438	0.032
RON	RON siRNA	0.497	0.052

The PCR study yielded an Amplification Plot (Figure 3.10) from which C_T (Threshold Cycle) values were obtained. Table 3.2 shows β-Actin C_T values. We performed comparative qPCR analysis where we subtracted the C_T value of β-actin from the C_T value of claudin-1. This was similarly performed for the other genes of interest whose transcript levels were quantified. This resulted in a ΔC_T value. $\Delta\Delta C_T$ was calculated by normalizing samples to a reference sample. For example, Control-1 would be subtracted from SnoN-1 to obtain the $\Delta\Delta C_T$ for SnoN. To determine the RNA-fold change, the following equation was used: $2^{-\Delta\Delta CT}$. Sample

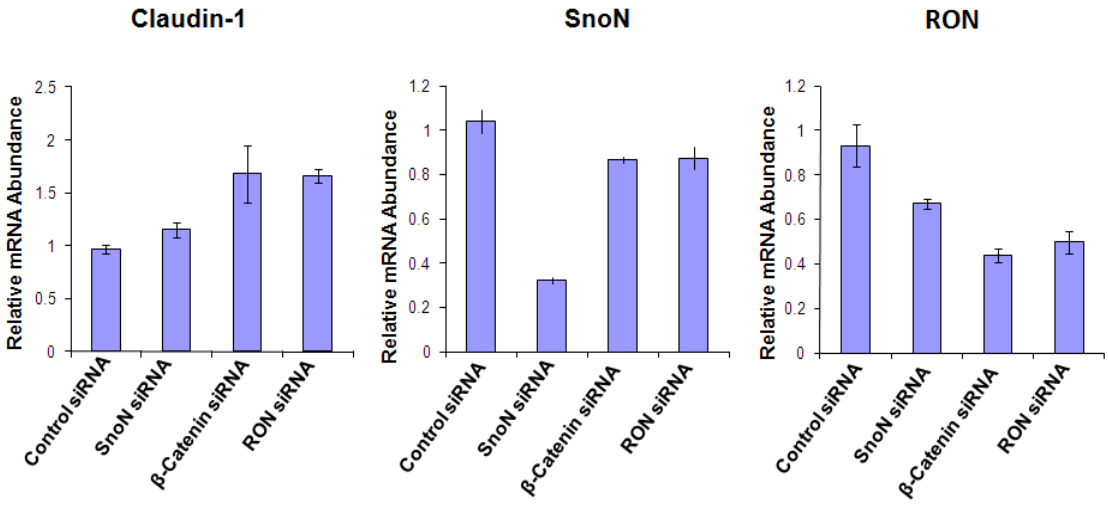

Figure 3.11 – Relative mRNA transcript levels of (A) claudin-1, (B) SnoN, and (C) RON resulting from SnoN, β-catenin, and RON siRNA transfection in HEY cells.

calculations are shown in Table 3.3. Finally, the resulting values for each sample were averaged and standard deviations were derived (Tables 3.4).

These data were then graphed (Figure 3.11). The knockdown of SnoN, β-catenin, and RON led to an increase in the abundance of claudin-1 mRNA transcripts (Figure 3.11a). However, this increase is observed most markedly with β-catenin and RON siRNA than SnoN siRNA. Additionally, β-catenin and RON knockdown resulted in a decrease in the abundance of SnoN mRNA transcripts (Figure 3.11b). Interestingly, SnoN and β-catenin knockdown resulted

in a marked decrease in RON transcript levels (Figure 3.11c). Furthermore, the reduction is greater with β-catenin siRNA than with SnoN siRNA.

IV. Discussion

Tight junction proteins such as claudin-1 are an integral component of epithelial junction complexes and play a vital role in maintaining epithelial integrity and cell polarity. Disruption of tight junctions is a hallmark of epithelial cancer development and malignant progression. The loss of claudin-1 expression has been shown to be correlated with increased invasiveness and malignant progression of certain epithelial cancers[14, 15]. Herein, we investigated whether claudin-1 expression is regulated by activation of mediators in the TGFβ signaling pathway, particularly SnoN/SkiL, a key TGFβ co-repressor, in ovarian cancer cells. This hypothesis was investigated through three specific aims: (1) to determine whether altered expression of RON tyrosine kinase leads to dysregulated claudin-1 expression; (2) to determine whether cellular treatment with TGFβ and MSP1 leads to altered expression levels of claudin-1; and (3) to determine whether knockdown of β-catenin and SnoN upregulates claudin-1 expression.

In Figure 3.1 and 3.2, we observed that claudin-1 protein levels were increased with RON knockdown in HEY and SKOV3 cells, respectively. Similarly, as reported in breast cancer and MDCK cells, RON activation disrupts tight junctions and impairs their functions through reduced claudin-1 expression[17]. Interestingly, we also noted that β-catenin and SnoN levels were slightly increased in both SKOV3 and HEY cells. We propose that these changes may be independent of the effect of RON knockdown on claudin-1 levels since increased SnoN levels are expected to counter the effects of reduced RON expression. However, we did not observe these effects in two independent cell lines, H358 and BxPC-3 cells, suggesting that these effects are likely unique to ovarian carcinoma cells.

In Figure 3.3, we investigated the immediate changes in the activation status of certain cascades following RON and TGFβRII/I receptor activation. We primarily focused on the MAP kinase signaling cascade since it is known to be a major player in numerous signaling pathways and modulates β-catenin activity[19-22]. Cells treated with MSP1 and TGFβ led to a marked phosphorylation of pERK1/2. We also noted that TGFβ is more potent in altering claudin-1 levels in contrast to MSP1. In addition, long-term treatments (Figure 3.4 and 3.5) of these cells with these ligands led to marked reduction of claudin-1 levels. Thus, we propose that these changes in expression of these tight junction proteins may be due to the activation of the MAP kinase signaling cascade. We have observed an increase in SnoN levels and further, the inverse relationship between claudin-1 expression levels and SnoN supports the data presented in Figure 3.9. Undeniably, disruption of tight junctions is indeed a hallmark of cancer development as noted in various cancer subtypes including colon cancer and as our work has potentially shown, in ovarian cancer development.

We observed that siRNA targeting RON tyrosine kinase led to a marked elevation in claudin-1 protein. As shown in Figure 3.11, the changes that we observed in protein expression also occurred at the RNA level. This indicates that claudin-1 is regulated at the transcriptional level and since SnoN is a transcriptional co-repressor, this could be a potential pathway by which claudin-1 levels are modulated. The mechanism by which this could potentially occur is shown in Figure 4.1. Various mechanisms have been implicated in regulation of claudin-1 expression including regulators of the TGFβ and RON Tyrosine Kinase pathways. Studies with colon cancers have shown that Smad4, a co-Smad, involved in the TGFβ pathway represses claudin-1 expression[18]. Activation of kinase linked receptors (i.e. RON and TGFβRII/I) normally occur following ligand binding (i.e MSP1 or TGFβ) followed by activation of intricate intracellular

signaling cascades (including MAP kinase activation, Figure 3.3) which leads to the progressive movement of the signal into the nuclear compartment. Within the nucleus, the activity of transcriptional co-repressors and co-activators modulate transcriptional induction. Both β-catenin and SnoN are mediators in such intracellular signaling pathways.

One mechanism of β-catenin activation involves GSK3 (glycogen synthase kinase 3) which phosphorylates β-catenin and targets the protein for proteasome mediated degradation[19-22]. Thus, dephosphorylated β-catenin will promote its cytoplasmic and thus, nuclear accumulation where it could potentially modulate the transcription of target genes such as claudin-1[19-22]. On the other hand, SnoN is regulated by phosphorylation via TAK1 which promotes its ubiquitination via E3 ubiquitin ligase and eventually determines the quantity of SnoN protein that is present[23]. Interestingly, we also noted that β-catenin and SnoN altered claudin-1 protein levels. These changes were observed at the RNA levels implicating transcriptional regulation of claudin-1.

With knockdown of SnoN and β-catenin, changes were observed in claudin-1 expression independent of the presence of ligand or receptor activation. With β-catenin knockdown, SnoN levels were reproducibly decreased suggesting that β-catenin may regulate SnoN expression either 1) directly via protein-protein interaction (via C-terminal binding protein which has been reported to interact in a complex with both SnoN and β-catenin) or 2) potentially indirectly via modulation of SnoN transcription as a β-catenin target gene. Strikingly, we noted that the morphology of the β-catenin knockdown HEY cells were more epithelial-like and polarized (results not shown). This observation is consistent with the role of claudin-1 in maintaining cell polarity in cell-cell interactions. This was not observed in RON or SnoN knockdown in cells; this indicates that there are some other critical components which aid or promote cell polarity such as

zonula occludens (ZO-1), gap junctions, and/or desmosomes. It is presently unknown whether the signaling mechanism by which β-catenin alters claudin-1 levels is through alteration in activities of SnoN transcription co-repressor activities.

RON and TGFβ Transcriptional regulation of Claudin-1 Expression

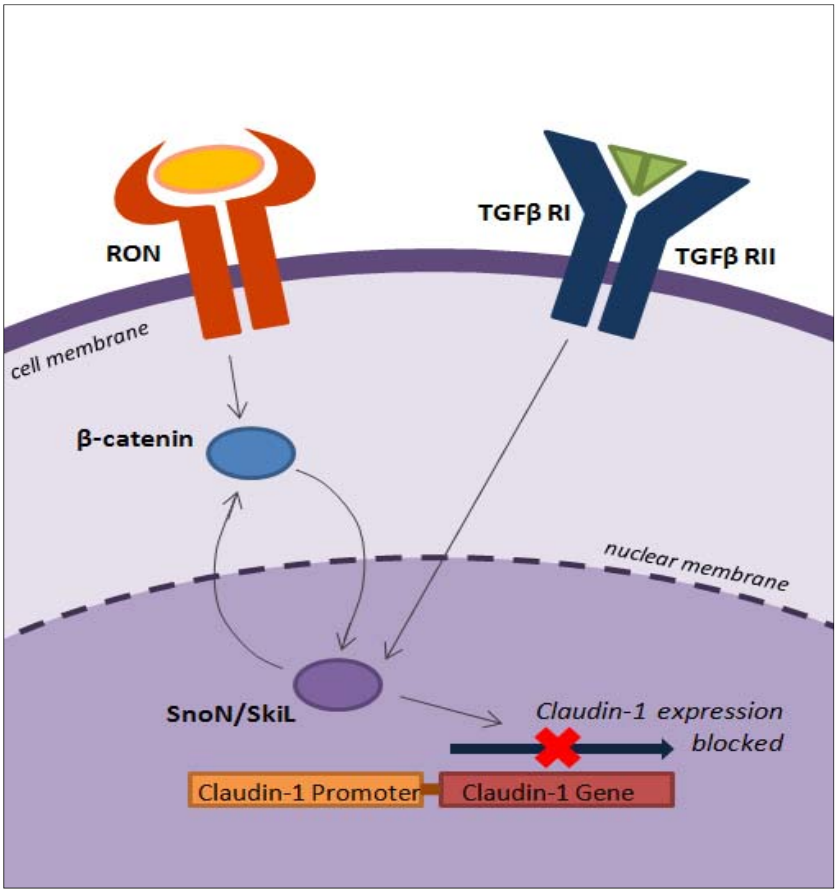

Figure 4.1-Proposed mechanism of claudin-1 regulation via SnoN demonstrating potential cross-talk between the TGFβ signaling pathway and RON tyrosine kinase.

Future studies will investigate the effects of a MAP kinase inhibitor (PD98059) as well as other pathways including the PI3K/AKT, p38, and JNK pathways to further identify the signaling pathways leading to modulation of claudin-1 transcript levels. Thus, further elucidation of the detailed signaling events underlying regulation of claudin-1 expression will certainly be invaluable in future studies to identify novel biomarkers in ovarian cancer detection and treatment.

V. References

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