UGC-MRP PROJECT

FINAL PROGRESS REPORT

‘Regulation of Alveolar Epithelial Integrity by the NRG-1/HER-2 Axis’

UGC Reference No. & Date: F. No.-43-48/2014(SR); 27/07/2015 MRP-MAJOR-BIOC-2013-37197

University Grants Commission
(Ministry of Human Resource Development, Govt. of India)
BahadurshahZafar Marg, New Delhi – 110002

SUBMISSION OF FINAL REPORT OF THE WORK DONE ON THE PROJECT - Annexure IX

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2.	Name and address of the Institution where project was undertaken	Dept of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat
3.	UGC Reference No. & Date	F. No.-43-48/2014(SR); 27/07/2015 MRP-MAJOR-BIOC-2013-37197
4.	Date of Implementation	**11/09/2015**
5.	Tenure of the Project	03 years from 2015 to 2018
6.	Total grant allocated	**13,90,000**
7.	Total grant received	**8,25,000**
8.	Final expenditure	**1185743**
9.	Title of the Project	'Regulation of Alveolar Epithelial Integrity by the NRG-1/HER-2 Axis'
10.	Objective of the Project	1) To define HER2 interactions with AJC components (β-catenin and ZO-1). 2) To identify phosphorylation sites in β-catenin critical to HER2 induced changes in alveolar barrier function. 3) Determine whether β-catenin transcriptional activation is a mechanism of HER2-induced changes in TER and permeability.

11.	Whether objectives were achieved (give details)	Enclosure I
12.	Achievement from the project	Part of the data presented as poster at International Conference Society for Reproductive Biology and Comparative Endocrinology (SRBCE), University of Hyderabad (Feb 9 – 11, 2017)
13.	Summary of the finding (in 500 words)	Enclosure II
14.	Contribution to the society (give details)	Enclosure III
15.	Whether any PhD enrolled/ produced out of the project	Mr. Ankit Khandelwal **Title** - "Role of Human Epidermal growth factor Receptor (HER) and Tight Junction Protein (TJP) interactions in regulating alveolar epithelial barrier integrity" Date of pre-registration seminar– 23-01-2017
16.	Number of publications out of the project (please attach reprints)	NIL

11. Whether objectives were achieved (give details) - **Enclosure-I**

Objective 1 was to define Her2 interactions with AJC components (β-catenin and ZO-1) is partially achieved.

Hypothesis and Rationale: β-catenin and ZO-1 are integral proteins of the AJC that maintain its stability. Our preliminary data supports our hypothesis and identifies HER2 as a new component of the AJC regulating AJC integrity and alveolar barrier function. However, the mechanisms behind the interaction are unknown. **We hypothesize that HER2 directly associates with and phosphorylates β-catenin and ZO-1, altering the AJC**. *There are no reports of HER2/β-catenin or HER2/ZO-1 interaction.* We will evaluate the HER2/ZO-1/β-catenin interaction in pulmonary epithelial cells cultured at an air-fluid interface; a human epithelial airway cell line, NuLi-1. We tested our hypothesis through independent methods – confocal microscopy, co-immunoprecipitation, and *in vitro* kinase assays:

To achieve this objective, we obtained Nuli-1 cells (Human normal immortalized alveolar primary airway epithelial cells) from our collaborator. Nuli-1 cell culture was started in the lab and initial study with Nuli-1 cells were performed. Various biochemical techniques such as Immunofluorescence microscopy, Confocal microscopy, western blot and co-immunoprecipitations were optimized with Nuli-1 cells. These instruments (Immunofluorescence microscopy, Confocal microscopy) are located in the Vikram Sarabhai Cell and Molecular Biology Institute, M. S. University of Baroda, Vadodara and Biochemistry Dept, M. S. University of Baroda, Vadodara. Our initial studies confirmed role of NRG-1 in HER2 activation (**Figure 1**). **Figure 1** showed shows Neuregulin 1 (NRG-1) induced phosphorylation of HER2 in NuLi-1 cells, which seems to peak at 30 mins after treatment.

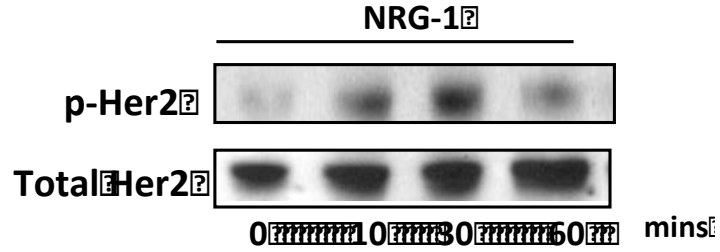


Fig 1: Neuregulin-1 (NRG-1) induced HER2 activation in NuLi-1 cells

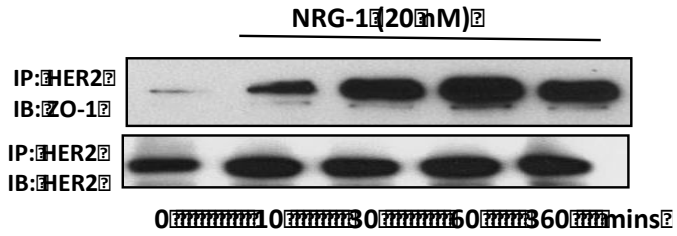
HER2 activation by neuregulin-1 (NRG-1) leads to β-catenin and ZO-1 tyrosine phosphorylation. NuLi-1 cells were grown in 6-well plates using BEBM (bronchial epithelium basal medium) supplemented with bovine pituitary extract and several growth media (Lonza) to 80-90% confluency in CO_2 incubator.

The cells after overnight starving (using media devoid of supplements) were treated with NRG-1 (2.0 nM) at different time-points (0, 10, 30, 60 minutes).

A separate set of cells were pre-incubated with HER2 pharmacological inhibitor, lapatinib (2 μM) for 30 minutes and then treated with NRG-1 at different time-points as mentioned earlier.

With this we were also able to observe the physical interaction of HER2/ZO-1 in Nuli-1 cells after NRG-1 treatment. We pulled down HER-2 protein with total HER2 antibody and immunoblotted with HER-2 and ZO-1 antibody. Figure 2 shows increased association of ZO-1 with HER-2 at 60 min by co-immunoprecipitation method.

Fig 2: Physical interaction of HER2 & ZO-1 proteins after Neuregulin-1 treatment by co-immunoprecipitation and western blot techniques:



This data shows the possible interaction of HER2 and ZO-1, a tight junction protein, and hence the role of HER2 in modulating cell-cell adhesion mechanisms.

Trans epithelial electrical conductance was monitored in Nuli-1 cells cultured in transwells These cells were grown into a monolayer and treated with NRG-1 (20 nM) and conductance was measured using electrical voltohmmeter (EVOM) instrument. The experiment was started when the instrument shows a trans-epithelial resistance (TEER) of 600 mΩ.. The cells were then treated with NRG-1 and resistance measured at different time points (2, 6, 12, 24, 48 h).

Fig 3: Neuregulin-1 induced changes in conductance in NuLi-1 cells

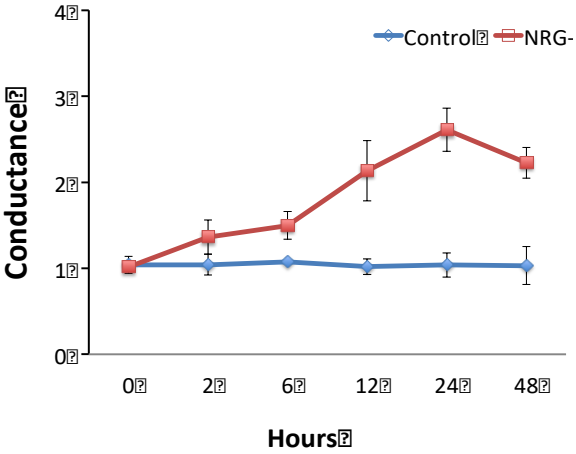


Fig 3 shows NRG-1 induced changes in conductance in NuLi-1 cells. In the figure we see increased conductance at 24 h post NRG-1 treatment. The results suggest disruption of cell-cell adhesion peaking out at 24 h as a result of NRG-1 treatment, as we observe increased conductance or otherwise less resistance (as a result of compromised cell-cell membrane integrity).

Previously the PI's group in his collaborator's lab have shown HER-2 colocalization with β-catenin in unstimulated Nuli-1 cells (**Figure 4**). They also showed HER2 activation leads to b-catenin phosphorylation and dissociation of E-cadherin from b-catenin upon NRG-1 treatment (Finigan et al 2015). Previous studies performed by PI in his collaborator's lab to generate preliminary data for this grant proposal had shown some evidence of HER2/ZO-1 and HER2/β-catenin colocalization by confocal microscopy in Nuli-1 cells (**Figure 5**).

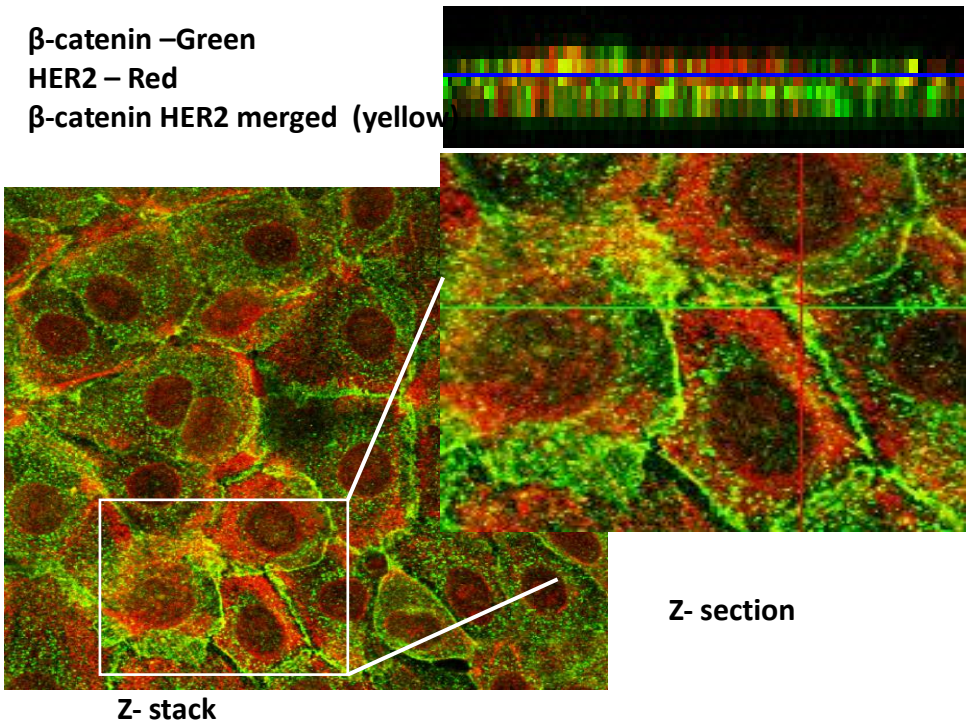


Figure 4: HER2 - β-catenin association in Nuli-1 control cells

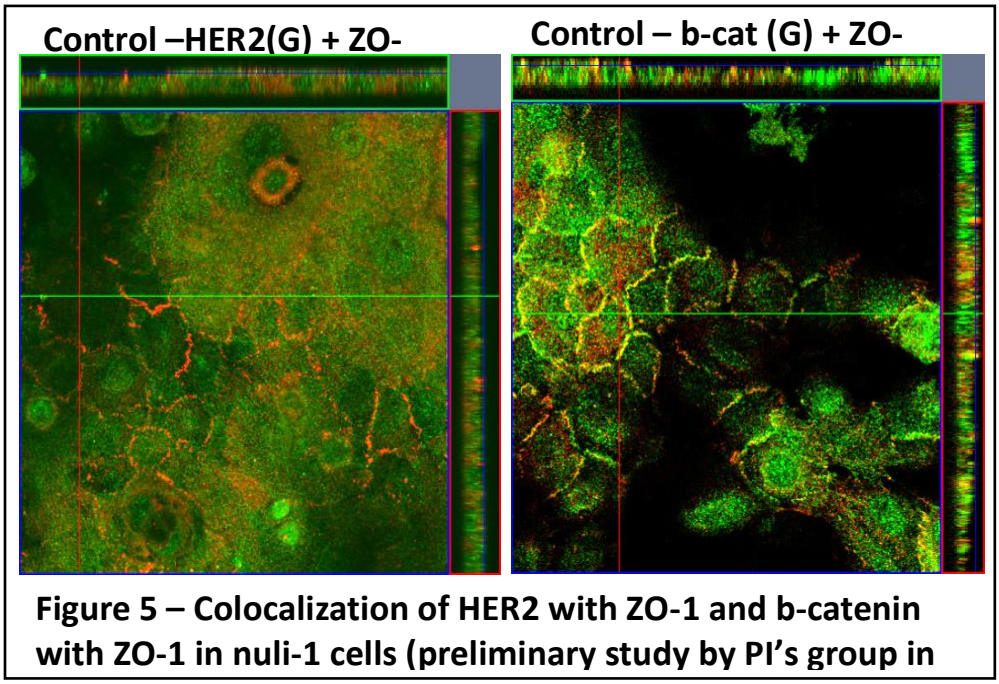


Figure 5 – Colocalization of HER2 with ZO-1 and b-catenin with ZO-1 in nuli-1 cells (preliminary study by PI's group in

In the current research project PI and junior research fellow, continue to study these interaction and role of HER2 activation and its interaction with ZO-1and β-catenin at M. S. University, Vadodara. The results from this study showed that NRG-1 treatment in Nuli-1 cells increased phosphorylation of ZO-1 (Occludin-1) protein as shown in the **figure 6.** This data shows possible mechanism of NRG-1 induced disruption of cell-cell adhesion i.e. via phosphorylating ZO-1, a tight junction protein, and hence the role of HER2 in modulating cell-cell adhesion mechanisms.

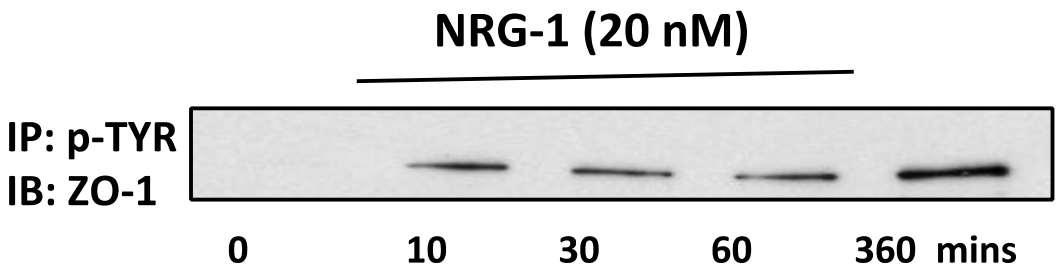


Figure 6: Neuregulin-1 induced phosphorylation of ZO-1 protein

NRG-1 treatment not only caused phosphorylation of ZO-1 but we also observed its nuclear translocation in Nuli-1 cells (**Figure 7**). This is an interesting observation, as ZO-1 is also known to act as transcription regulators and few literatures suggest that during an injury (cell), ZO-1 may translocate to nucleus inducing increased transcription of selected cytoskeletal genes.

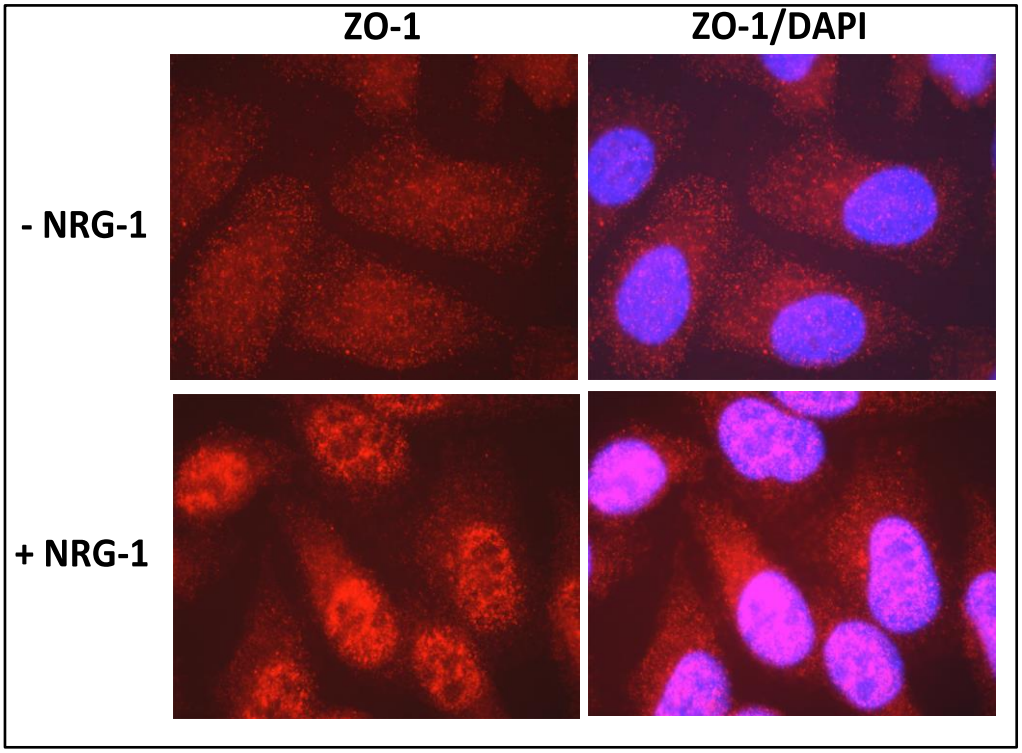
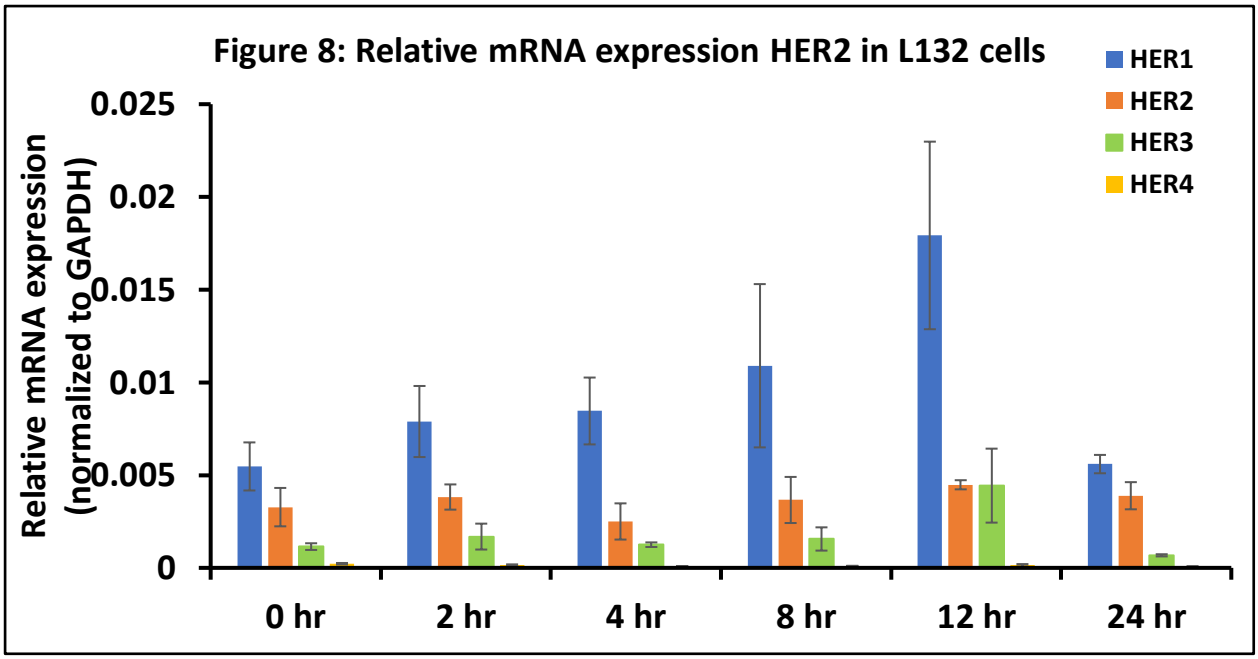


Fig 7: Neuregulin-1 induced changes in ZO-1 translocation

Unfortunately, we lost the Nuli-1 cell culture after using these cells for over 1.5 years due to cell culture contamination and were not able to revive frozen cells after that. Hence, we obtained another lung epithelial cell line L132 from NCCS Pune, Maharashtra, India and decided to use this cell line for our future studies. These cells were evaluated for various Her family of receptors expression. We observed similar HER receptor family expression in L132 cells also (**Figure 8**). Relative expression of HER1 was higher than HER2 and HER3 and HER4 expression was lowest among all.



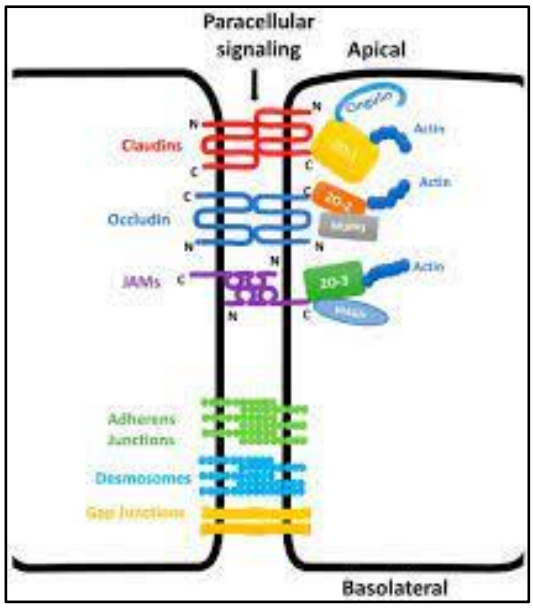


Figure 10 – Apical junction complex proteins (Image from web source)

Multiple proteins interact with each other to form apical complex. We observed ZO-1 protein expression in L132 cells by immunofluorescence and just like in Nuli-1 cells, NRG-1 treatment for 30 min also led to decrease in ZO-1 protein staining indicating disruption of APC on the cell membranes in L132 cells (**Figure 11**).

We further wanted to evaluate the expression of other apical junction proteins in L132 cells. Hence expression of different claudins, JAM-A/JAM-B/JAM-C, MARVAL proteins along with ZO-1(TJP-1)/ ZO-2 (TJP-2) and ZO-3 (TJP-3) mRNA expression was determined by real time PCR method.

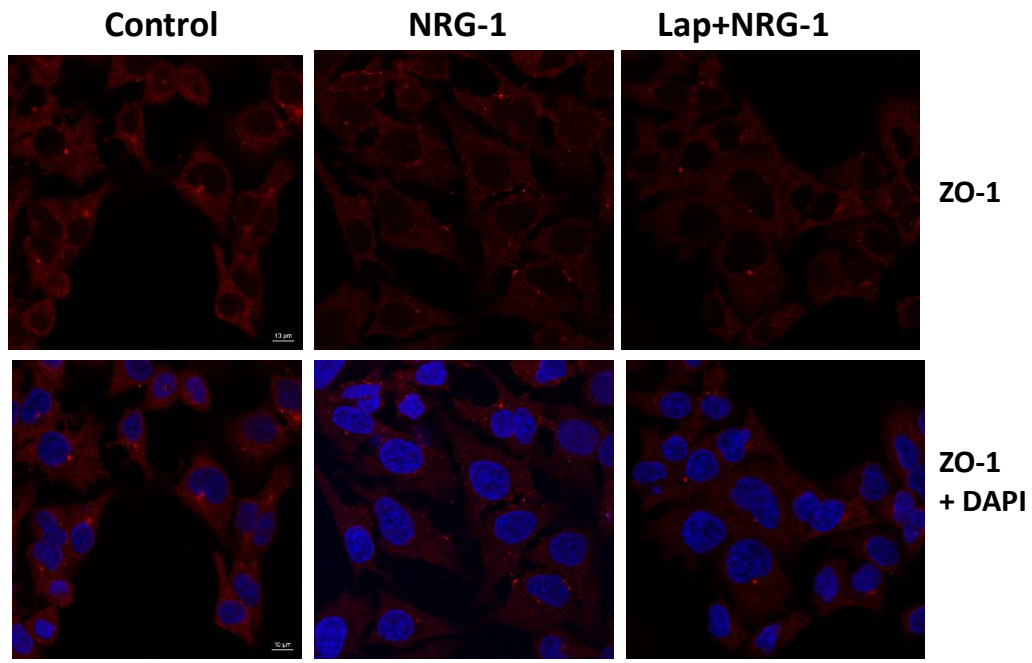
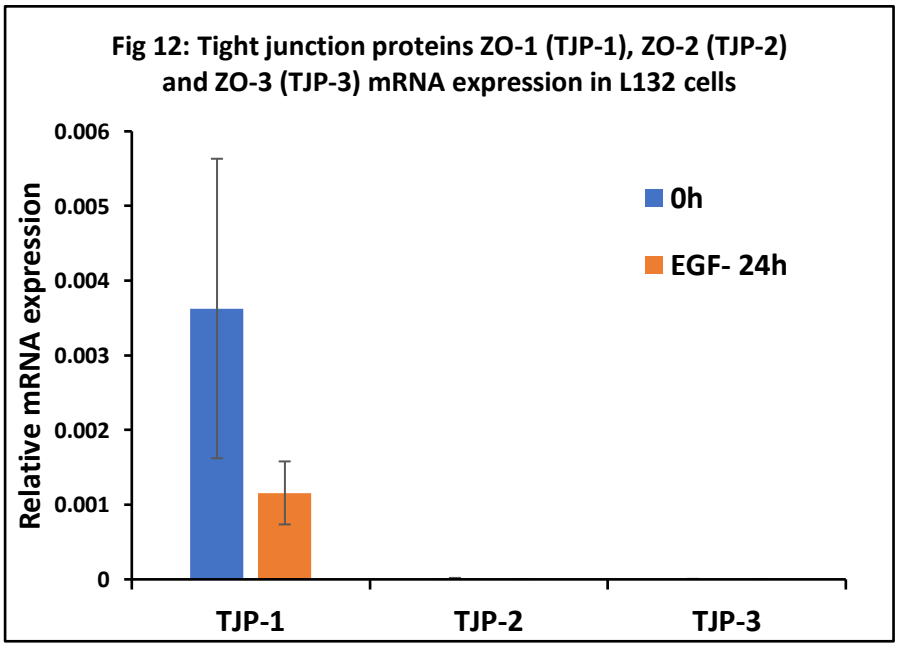


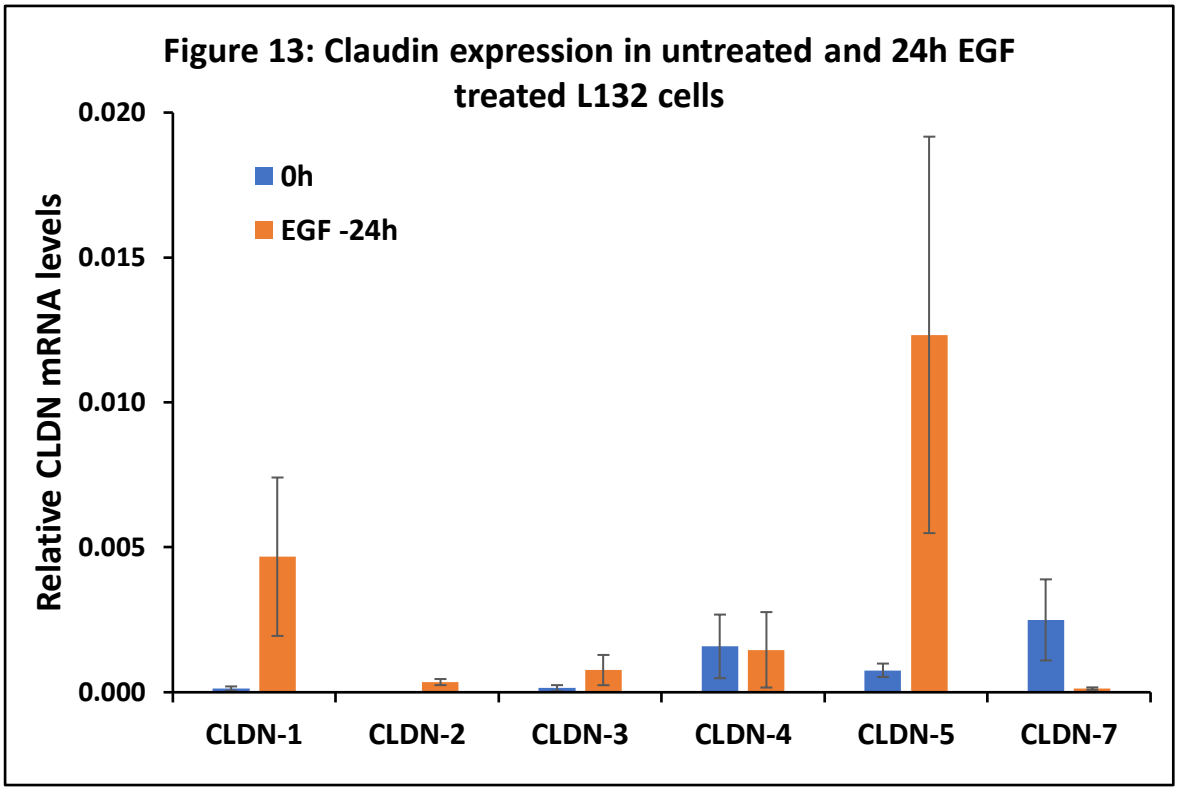
Figure 11 - L-132 cells were p-HER2 inhibitor Lapatinib treated for 24 hours and then stimulated with NRG-1 for 30 minutes. ZO-1 expression was evaluated in the cells by immunofluorescence.

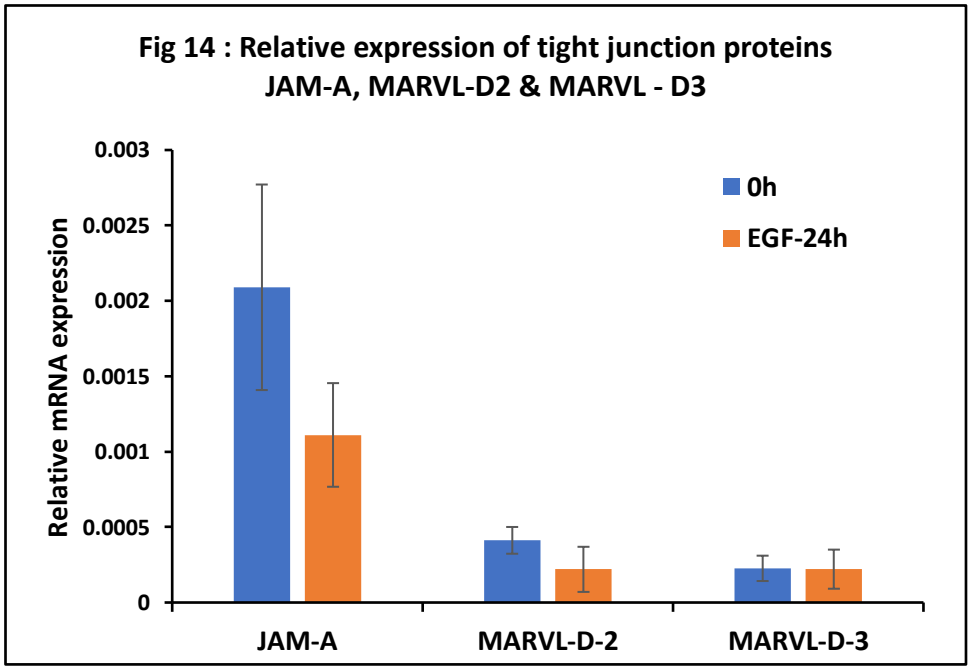
We observed ZO-1 (TJP-1) expression in L132 cells and relative expression on ZO-2 and ZO-3 was negligible compared to ZO-1. These cells mainly expressed ZO -1 as member of apical junction complex proteins (**Figure 12**).



Different claudin expression was also monitored and it was found that Claudin-7 expression was highest then followed by Claudin 4 and 5. Claudin 1 and 3 were also expressed in these cells and claudin 2 had lowest expression among all claudins studied in L132 cells (**Figure 13**).

Similarly, we found other AJ complex proteins like MARVL-A expression levels were high compared to MARVL-B and MARVL-C. Other AJ complex proteins which were expressed good amount in L132 cells are MARVL-D2 and MARVL-D3 (**Figure 14**).





As we lost the Nuli-1 cells after 1.5 year when project started and then switched to L132 cells and other human lung epithelial cells.

Due to lack of time (project ended in March 2019), HER2 interaction with ZO-1 and β-catenin by Co-immunoprecipitation and kinase assay could not be performed in either L132 or Nuli-1 cells.

Objective 2: **To identify phosphorylation sites in β-catenin critical to HER2 induced changes in alveolar barrier function (Could not be achieved).**

Hypothesis and Rationale: We hypothesize that HER2 mediated phosphorylation of β-catenin Y654 is required for loss of β-catenin/E-cadherin association, reduction in trans-epithelial electrical resistance (TEER) and increased permeability across an alveolar epithelial monolayer.

To test this hypothesis, we will use GFP tagged, mutant β-catenin cDNA clone with a tyrosine to phenylalanine substitution at Y654 (Y654F Mutant). We predict that HER2 phosphorylates β-catenin on Y654, decreases β-catenin/E-cadherin association, thereby decreasing cell adherence and TEER while increasing permeability. However, in the β-catenin^{F654} mutant cell lines HER2 will be activated but unable to phosphorylate β-catenin^{F654}, β-catenin^{F654}/E-cadherin association will be preserved and cell-cell adherence, TEER and permeability will not be affected. These results would confirm that NRG-1 and IL-1β induced changes in TEER and permeability are regulated through phosphorylation of β-catenin's Y654.These results will validate Y654 as a critical β-catenin phosphorylation site that is required for HER2 induced changes in alveolar barrier function.

We had performed TEER assay in Nuli-1 cells expressing wild-type endogenous β-catenin (**Figure 3)** and obtained GFP tagged β-catenin lentiviral construct to knockdown endogenous b-catenin and another construct to over express β-catenin which had tyrosine to phenylalanine substitution at Y654 (Y654F Mutant). This project ended in March 2019, hence could not perform transfection and generation stable cell line generation experiments to study this second objective.

<u>Objective 3</u>: Determine whether β-catenin transcriptional activation is a mechanism of HER2 induced changes in TEER and permeability (could not be achieved).

<u>Hypothesis and Rationale</u>: We hypothesize that β-catenin induced new gene transcription induces changes in TEER and permeability. The rapidity of TEER loss after NRG-1 and IL-1β exposure suggests that initial events are not dependent on new gene expression. However, new gene expression may account for late TEER effects when HER2 activation has resolved.

Our first analysis was going to determine whether β-catenin transcriptional activity is induced by HER2 using Lef/Tcf reporter activity (TOPFlash). In the TOPFlash reporter system, activated β-catenin binds to the Lef/Tcf promoter motif resulting in luciferase expression. We anticipated that β-catenin activation will be HER2 dependent as HER2 activation leads to increase in β-catenin transcriptional activity compared to controls.

We were going to obtain these reporter constructs from our collaborator but due to lack of time, we could not obtained these constructs and perform these experiments before March 2019 (project end date).

<table>
<tr><th></th><th>Year 1</th><th>Year 2</th><th>Year 3</th><th></th></tr>
<tr><td>Objective 1</td><td colspan="4" rowspan="4">|------Partially achieved----------|
|----Could not be achieved -----|
|----- Could not be achieved ----------|</td></tr>
<tr><td>Objective 2</td></tr>
<tr><td>Objective 3</td></tr>
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Executive Summary of the finding (in 500 words) **Enclosure-II**

Acute Respiratory Distress Syndrome (ARDS) contributes more than 6,000,000 hospital days/year to health care costs and leads to over 133,000 deaths/year. Incidence of ARDS increases in patients who are in ICU and are mechanically ventilated. ARDS in India has a high mortality rate ranging between 30% to 60%. Disruption of the alveolar epithelium's barrier and the resulting pulmonary oedema play an important role in inflammatory lung diseases such ARDS. Currently there is gap in the knowledge to understand molecular mechanism of alveolar barrier function and lack of effective therapy to protect the alveolar barrier function and prevent or reduce pulmonary oedema formation in ARDS patients.

Previous studies by PI's group had shown the role of HER-2/ NRG-1/ ADAM17 axis in increasing alveolar epithelial permeability in ARDS. Their data further support HER2 as a novel component of the AJC (Apical junction complex), and suggest that HER2 activation also alters the AJC through tyrosine phosphorylation of AJ proteins – specifically β-catenin. However, how HER2 interacts with other AJ proteins like ZO-1 and alters AJ function is unknown.

To understand the molecular mechanisms behind the HER2 induced alveolar leak in ARDS, we studied the **role of HER2 phosphorylation and its interaction with ZO-1 and β-catenin as integral TJ (tight junction) and AJ (adherens junction) proteins respectively**. Our results showed that NRG-1, which increases during ARDS in lungs, induced HER2 phosphorylation in lung epithelial cells. We also observed NRG-1 treatment increased HER2 association with ZO-1 (AJ protein) and ZO-1 phosphorylation. We were not able to confirm HER2 association β-catenin in this study due to lack of time but we observed a novel phenomenon with relation to ZO-1 that NRG-1 treatment led to nuclear translocation of ZO-1. The biological significance of ZO-1 nuclear translation in relation with initial disruption of epithelial barrier integrity and later restoration of epithelial barrier integrity needs to further investigation in future.

Contribution to the society (give details) **Enclosure-III**

Disruption of the alveolar epithelium's barrier and the resulting pulmonary edema play an important role in inflammatory lung diseases. This is most evident clinical problems that is observed in Acute Respiratory Distress Syndrome (ARDS. ARDS contributes more than 6,000,000 hospital days/year to health care costs and leads to over 133,000 deaths/year. ARDS in India has a high mortality rate ranging between 30% to 60%. Currently there is no effective therapy is available to prevent or reduce pulmonary barrier function in ARDS patients.

we have a limited understanding of the molecular mechanisms that regulate the alveolar barrier. Because of this knowledge gap we currently have no therapy to protect the alveolar barrier function and prevent or reduce pulmonary edema formation. For functional recovery, restoration of the alveolar barrier is dependent on a number of responses by both alveolar pneumocytes – regeneration of cell-cell adherence, restoration of the Apical Junctional Complex (AJC) and reformation of the alveolar epithelial barrier.

This research study was performed to understand the molecular interaction between HER2 and AJC proteins like b-catenin and ZO-1. Specially how HER2 activation would alter its interaction with AJC proteins - b-catenin and ZO-1 and how this interaction will impact the epithelial barrier permeability. These data further support HER2 as a novel component of the AJC, and suggest that HER2 activation also alters the AJC through tyrosine phosphorylation of AJ proteins – specifically ZO-1.

By understanding the detail molecular mechanisms behind impairment of epithelial barrier function and identifying key molecular interactions/ mechanisms will provide novel targets to design an effective therapy to prevent or reduce pulmonary edema in ARDS and acute lung injury (ALI) patients.