By

A capstone project submitted for
Graduation with University Honors

University Honors
University of California, Riverside

APPROVED

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Department of

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# Abstract

## ***Acknowledgements***

I would like to thank the Lo lab for mentoring me on this project. It was extremely helpful to be shown what research methods to use and what the next steps were in the project. These research and analytical skills will be continuously used when moving forward in this research project.

***Table of Contents***

Abstract……………………………………………………………………………………………2

Acknowledgements……………………………………………………………………………..…1

Introduction…………………………………..………………………………………………….3

Materials and Methods…………………………………………………………………………..…6

Results…………………………………………………………………………………………..……7

Discussion……………………………………………………………………………………………..16

References……………………………………………………………………………………………..17

## ***Introduction***

The intestinal epithelium is an important barrier that protects underlying tissue from antigens or bacteria that are passing through the intestine. This barrier is regulated by the apical junctional complex (AJC) and this complex is made up of tight junctions (TJ) and adheren junctions. [6]. Specifically, these tight junctions act as a semipermeable barrier to the paracellular transport of ions, solutes, water, cells and divides the apical and basolateral area of plasma membranes [13]. Tight junctions are made up of proteins such as Occludin and ZO-1, known as TJ proteins. The plasma membrane area around the AJC has been found to be the origin and destination of endocytotic vesicles.

There have been a multitude of studies that demonstrate that proteins (such as TJs) of the AJC are internalized by endocytotic processes under various conditions. These various conditions include during morphogenesis, different pathological states, and a wide range of pathogenic stimuli, including cytokines, bacteria, toxins, and oxidative stress [6]. The normal turnover of AJC proteins (regarded as "house-keeping") involves the internalization of specific junctional proteins which do not affect the AJC integrity and function. There are three types of internalization of junctional proteins in which the first type is for the internalized junctional proteins to be degraded by late endosomes (LEs), or may be delivered back to the plasma membrane via recycling endosomes (REs) [7, 5, 8]. The second type is where only TJ proteins are internalized and are recycled back to the plasma membrane by RE. The third is the internalization of both TJ and AJ proteins and are degraded or recycled. As both TJ and AJ are affected, this mechanism leads to a loss of intestinal barrier function, and of both cell-cell contacts and cell polarity [6].

In epithelial and/or endothelial cells, a variety of viruses can hijack the different components of tight junctions to complete their infection cycle. These viruses come from at least nine different families of DNA and RNA viruses and have been reported to use TJ proteins in their benefit [11]. Viruses have many types to take advantage of tight junction proteins. One type is viruses evolving so that they can use the receptors or co-receptors that are on the apical domain of epithelial cells [1]. Another type requires basolateral molecules as their attachment and entry receptors that are mostly inaccessible due to Tight Junctions covering these receptors. Viruses have evolved techniques to open the TJs to reach their receptors or these viruses use the TJ protein itself as a receptor [10, 4]. What is interesting is that the internalization of Occludin has been found to be coupled with the endocytosis of coxsackie B viruses  It is not clear if the viral particles enter the cell with Occludin molecules but there is evidence that supports the colocalization in vesicles inside cell [2]. Occludin and coxsackie B viruses enter the cell through different internalization pathways. Occludin enters the cell via macropinocytosis while the viral particles enter the cell through caveolin-mediated endocytosis [2]. Occludin is also associated with hepatitis C virus (HCV) where human cells that overexpress Occludin are not susceptible to HCV. The specific step Occludin is involved in has yet to be identified. [9] Occludin and ZO-1 have been identified to be used by rotaviruses to enter cells. When ZO-1 was silenced using RNA interference, the infectivity of the virus was significantly decreased. [12]. Despite TJs playing a role as a barrier for pathogens, viruses have evolved to use TJs as receptor to enter cells to overcome this barrier function. Since the organization of TJs is specific for each type of epithelium this could potentially cause viruses to also have some type of tissue specificity [11].

Peyer's Patches are organized lymphatic tissues that are part of the mucosal immune system and located within the Small Intestine. M cells are found within the epithelial border in Peyer's

Patches. The function of M cells is to sample luminal microbes and envelop them via endocytosis. M cells then hand these particles off to the mucosal immune system via transcytosis. Studies have provided evidence that TJs could potentially be recruited to endosomes as part of the capture of luminal microparticles [3]. One possibility of how TJs participate in M cell surveillance is that TJs are incorporated into the endosome membrane and can alter the geometry of the endosome which can allow M cells to survey particularly large bacterial particles [3]. Since viruses rely on TJs such as Occludin and ZO-1 as cellular receptors to invade epithelial cells, perhaps TJs that are recruited into M cell endosomes could be taken advantage of by viruses to gain better access into the body [3]. If TJs are incorporated in M cell function, it leaves many questions.

## ***Materials and Methods***

**Animals**

Four- to 6-month-old both male and female Occludin eGFP, PGRPdsRed/Occludin eGFP, and ZO-1 RFP mice were used. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under assurance number A3873–1.

**Live mounting**

Small intestine was extracted from the mouse and briefly flushed with 10X PBS then flushed again with 4% paraformaldehyde in PBS. Peyer's Patches (PP) were collected from the intestines and were fixed in 4% paraformaldehyde in PBS for 60 min. After fixation, the Peyer's Patches were sliced open and laid onto slides for imaging.

**Microscopy**

Peyer's Patches on slides were viewed by confocal microscopy on a Zeiss confocal microscope. Images were collected with a Photometrics camera using Micro-Manager image software. Intensity measurement values were obtained from analysis of multiple fields in duplicate images on the confocal microscope. Image analysis was performed by measuring the area and manually counted using ZEN software.

**Statistical Analysis**

Prism software was used to conduct a column/box and whiskers plot. To test the significance, a parametric and unpaired with Welch's correction t-test was performed and is indicated on the figures (*)

## ***Results***

While examining the PP in the small intestine of Occludin in PGRP Occludin-GFP transgenic mice, the phenomenon of tight junction proteins fluorescence in the center of epithelial cells was observed. The pattern of fluorescence was seen to be in compact areas assumed to be vesicles. This is interesting because the literature mentions that tight junctions are likely to be at the border of epithelial cells.  To capture this phenomenon, Z-stacks of this Occludin phenomenon were captured through confocal microscopy and a snapshot from the Z-stack is in Figure 1A/B. In Figure 1, the tight junction protein, Occludin is fluorescing green due to GFP in the PP tissue of an Occludin-GFP/PGRP mouse. Occludin is clearly not restrained to the border of the cells, rather it is sprinkled across the cells. The next question was to see if these vesicles could be observed in other parts of the small intestine such as Villi.

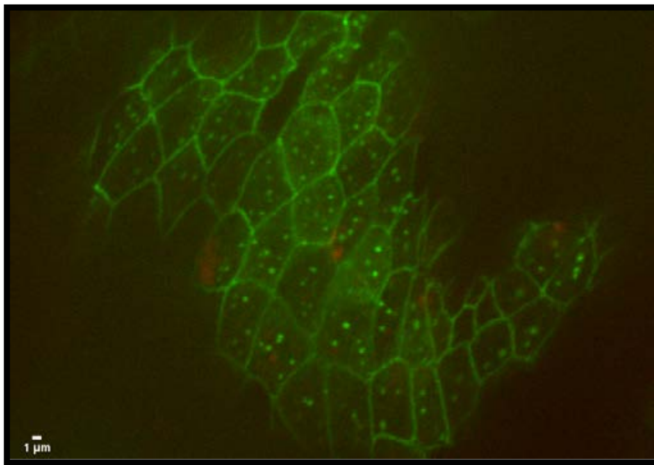


**Figure 1A**

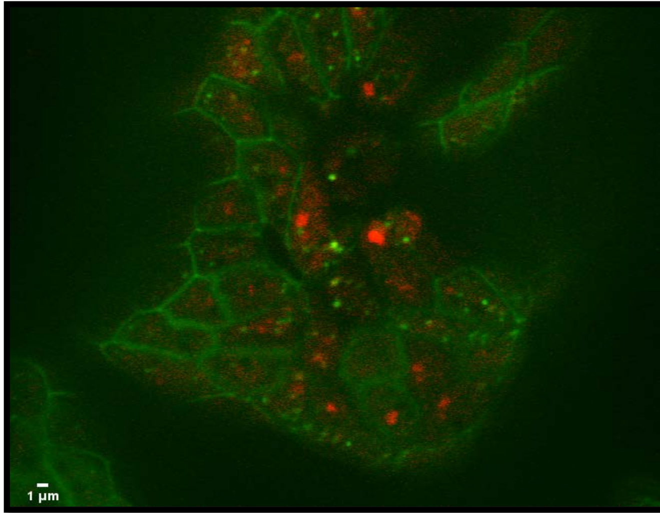


**Figure 1B**

*Figure 1A-B. The confocal microscope was used to acquire these images. They are snapshots of Zstacks taken of the Peyer's Patches found within the small intestine from an Occludin-GFP/PGRP-dsRED mouse. The green fluorescence represents the Occludin tight junction protein. The red fluorescence is from the RFP attached to the PGRP. The red fluorescence in the image represents M cells which are commonly found in Peyer's Patches. In the images, you can see multiple vesicles within each epithelial cell that have enveloped Occludin. In addition, the Occludin is also at the tight junctions which has been commonly seen in literature.*

After looking at PP, Villi from Occludin-GFP mice were also seen to have Occludin-GFP packaged into vesicles like PP tissue [Figure 2A/B]. What was interesting when examining the Occludin that is within these vesicles was that the Occludin varied in size and number in PP and villi. From comparing the two types of tissue for Occludin, it seemed that the Occludin vesicles were larger in size for PP tissue but were more numerous per cell in Villi.

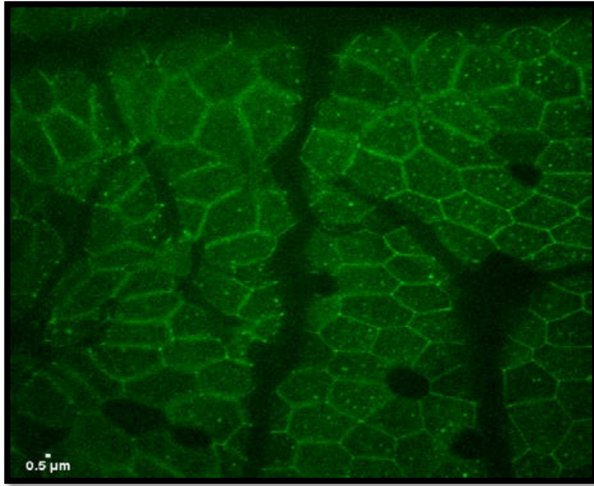


**Figure 2A**

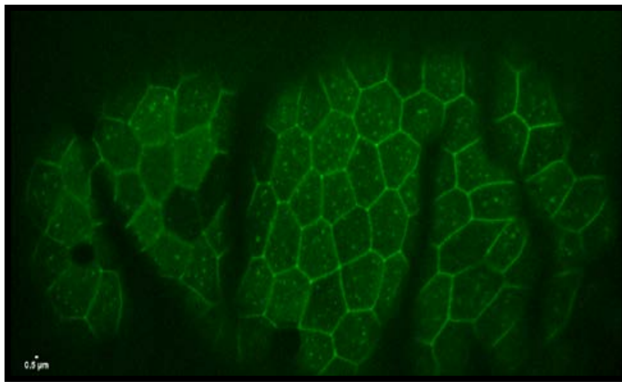

**Figure 2B**

*Figure 2A-B. The confocal microscope was used to acquire these images. These are snapshots of Zstacks taken of the villi found within the small intestine from an Occludin-GFP mouse. Like Figure 1, the green fluorescence represents the Occludin tight junction protein. In the images, Occludin is found within the vesicles that are sprinkled across within each epithelial cell and at each tight junction like the Peyer's Patch images.*

To see if this observation was true, the software Zen was used to measure the cross-sectional area (uc^2) of a subset of vesicles in PP and Villi. The area was taken by manually drawing a circle on the vesicle in a Z-stack image that displayed the vesicle to be at its largest and brightest. Therefore, each area measurement of a specific vesicle is at different Z-stacks since vesicles vary in brightness and size in between Z-stack images. The data from the vesicle size for PP and Villi was inputted into Prism and a box-and-whisker plot was created and is Figure 3. From Figure 3, PP vesicles tend to be statistically larger than Villi. Going back to Figures 1 and 2, besides vesicle size, the number of vesicles per cell seemed higher in Villi compared to PP within the small intestine.

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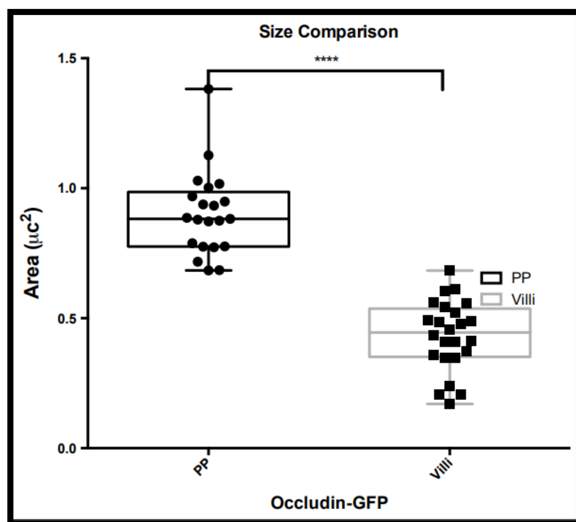


**Figure 3**

*Figure 3. Box and whisker plots were created to compare the sizes of the vesicles that encapsulate the tight junction proteins between Villi and PP. This graph is comparing the vesicle size difference in Occludin tight junction proteins. PP tissue has a higher size average than villi tissue. The asterisk represents the statistical significance of this difference tested by a t-test. In this graph, there is a strong, significant difference indicated by having four asterisks.*

Zen software was used again to examine if the numbers of vesicles per cell varied between PP and Villi tissues for Occludin. The procedure involved randomly picking a few dozens of cells in the collected images and individually counting the number of vesicles within that chosen cell and recording it into a spread sheet. This data was uploaded into prism and another box-and-whiskers plot was created and is Figure 4. As it can be seen, PP has a significantly smaller number of vesicles per cell in comparison to Villi. Therefore, both observations originally seen in the Z-stacks collected from PP and Villi within PGRP Occludin-GFP and Occludin-GFP typed transgenic mice.

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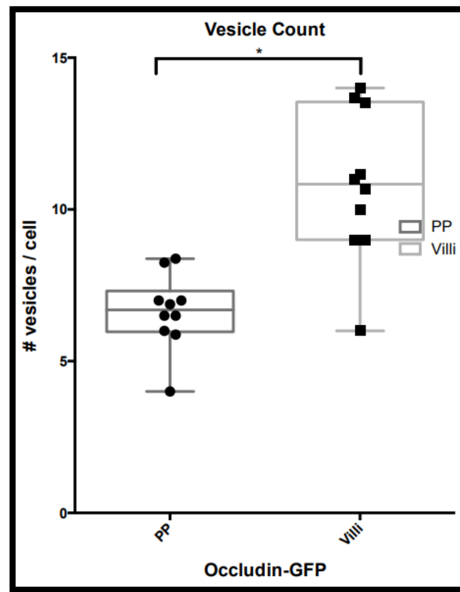


**Figure 4**

*Figure 4. Box and whisker plot was created to compare the number of vesicles in each epithelial cell on average between PP and Villi tissue. Figure 6B is comparing Occludin tight junction protein found within vesicles between PP and Villi. There are more Occludin vesicles within each cell in comparison to PP. A t-test was conducted to test the statistical significance of these results and is indicated by the asterisk. From the t-test, it was concluded that the differences between the number of vesicles per cell between Villi and PP is significant in Occludin.*

Since this phenomenon has been seen in Occludin tight junction protein, it was then interesting to see if it applied to other tight junction proteins. Thus, ZO1-RFP transgenic mice were used to investigate the characteristics of the ZO1 protein. When PP and Villi tissue were examined with the confocal microscope, Z-stacks visually showed the same phenomenon with the Occludin protein and is Figure 5A/B (PP) and Figure 6 (Villi). It was apparent that PP vesicles had larger size whereas Villi had more vesicles per cell.

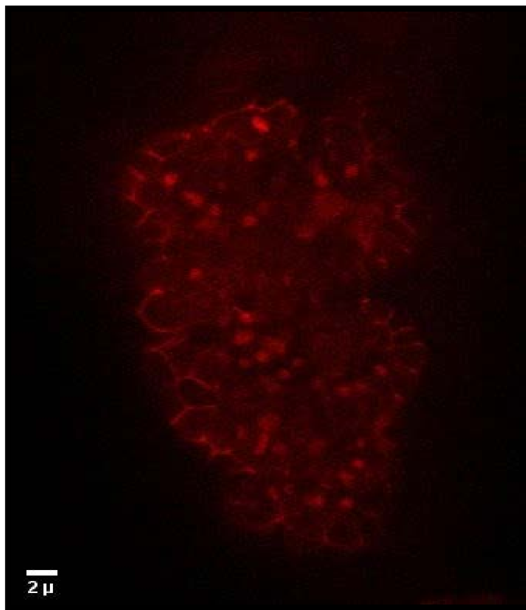


**Figure 5A**

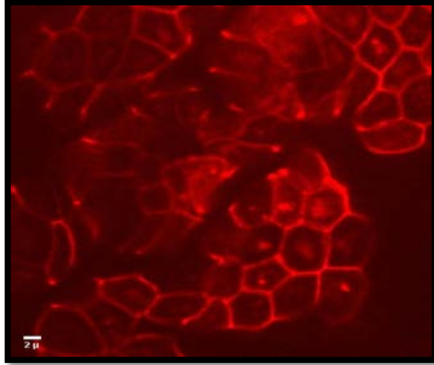

**Figure 5B**

*Figure 5A-B. The Confocal microscope was used to take images of PP tissue. These are snapshots of Zstacks taken of the PP found within the small intestine from a ZO1-RFP mouse. The red fluorescence represents the ZO1 tight junction protein. In the images, ZO1 is found within the vesicles and at the tight junction of the epithelial cells. The vesicles within the PP tissue are exceptionally large in comparison to villi.*

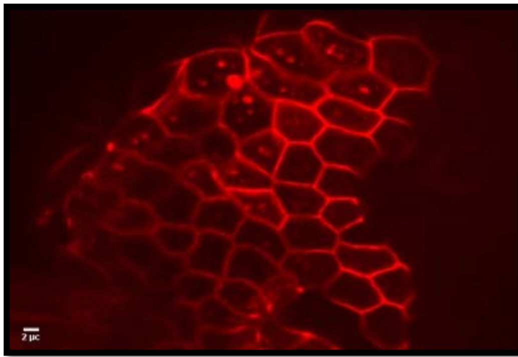

**Figure 6**

*Figure 6. Confocal microscope was used to capture images of collected villi tissue. These are snapshots of Zstacks taken of the villi found within the small intestine from a ZO1-RFP mouse. Like figure 4, the red fluorescence represents the ZO1 tight junction protein. In the images, ZO1 is found within the vesicles and at the tight junction of the epithelial cells. The vesicles within the villi tissue are considerably smaller and more in number compared to vesicles found within PP tissue.*

As with the studies on Occludin, Zen microscopy was used to measure the area per each vesicle with the same technique and was used to make a box-and-whiskers graph with Prism and is Figure 7. Like Occludin, ZO1 vesicles are larger in PP in comparison to Villi. Next, the number per cell was counted with the same technique used for Occludin and the data was used to make a box-and-whiskers graph and is Figure 8. Clearly, the number of vesicles per epithelial cell is larger within Villi in contrast to PP. Thus, the same phenomenon seen with the Occludin is also apparent with ZO1.

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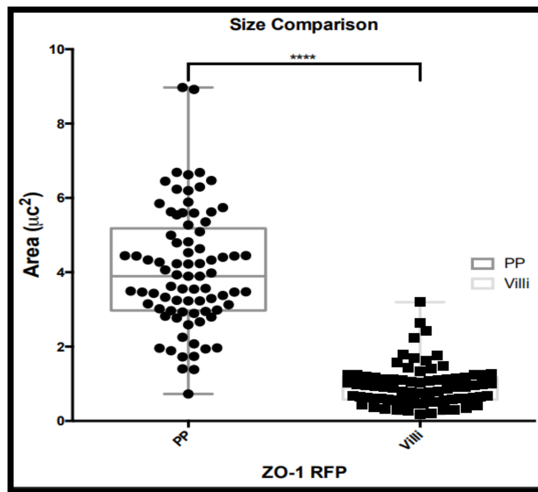


**Figure 7**

*Figure 7. Box and whisker plot were created to compare the sizes of the vesicles that encapsulate the tight junction proteins between Villi and PP. Figure 5 is comparing the size difference of ZO1 tight junction protein. From the data, PP tissue tends to have a larger vesicle size than villi tissue. In this graph, there is a strong, significant difference indicated by having four asterisks.*

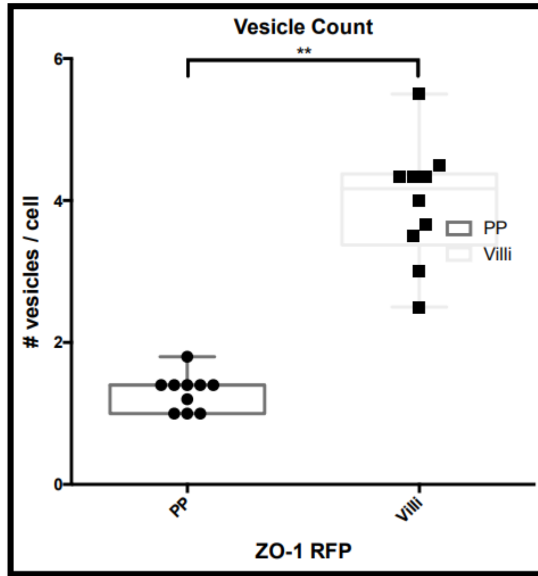


**Figure 8**

*Figure 7. Box and whisker plots were created to compare the number of vesicles in each epithelial cell on average between PP and Villi tissue. Figure 6A is comparing ZO1 tight junction protein found in vesicles between PP and Villi. It is apparent that Villi tends to have more vesicles per cell when compared to PP tissue. For ZO1, there are more vesicles within each cell in comparison to PP. A t-test was conducted to test the statistical significance of these results and is indicated by the asterisk. From the t-test, it was concluded that the differences between the number of vesicles per cell between Villi and PP is significant.*

## Discussion

When first examining Occludin-GFP and ZO1-RFP mice under the confocal microscope, it was intriguing to see that these tight junction proteins are encapsulated within vesicles. Previous literature suggests that this is due to endocytic recycling. This recycling mechanism helps maintain the important barriers between the apical and basolateral area of plasma membranes of the small intestine. What was more unusual was to visually see differences of vesicles in size and number between Villi and PP tissues. To quantify these differences, Z-stack images taken by the confocal microscope were analyzed with the software, prism (Figure 1-4). From the graphs (Figure 5-6), you can see statistically, how PP tissues are quite larger in size and smaller in number when compared to Villi tissue in Occludin-GFP and ZO1-RFP mice. It is hypothesized that the reason PP tissue has a smaller number of vesicles per epithelial cell is due to having sizable vesicles. It could be that, the larger the vesicle indicates more efficiency in the recycling mechanism and therefore less requirement of vesicles.

The hallmark of PP tissue is that it is the place in the Small intestine where M cells are located. Studies have shown that M cells use TJ proteins in their function of particle capture and transcytosis. To which, viruses take advantage of this by hijacking the TJs receptors to invade the epithelial cells. When it comes to the process of M cell use of TJs, it would be compelling to answer the question of whether TJs help accompany the particles endocytosed all the way to the basolateral side of the M cell. Or instead, do these TJs are quickly recycled back to Tight Junctions during early endocytosis and this is what is being seen in these captured Z-stacks? Furthermore, exploring how Viruses impact these recycling mechanisms utilized by M cells and the entire PP is important. It would be interesting to conduct further research on how the vesicle size and number between Villi and PP fluctuates based on the presence of a virus.

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