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Mutagenesis of Claudins to Probe in vivo Interactions and Assemblies

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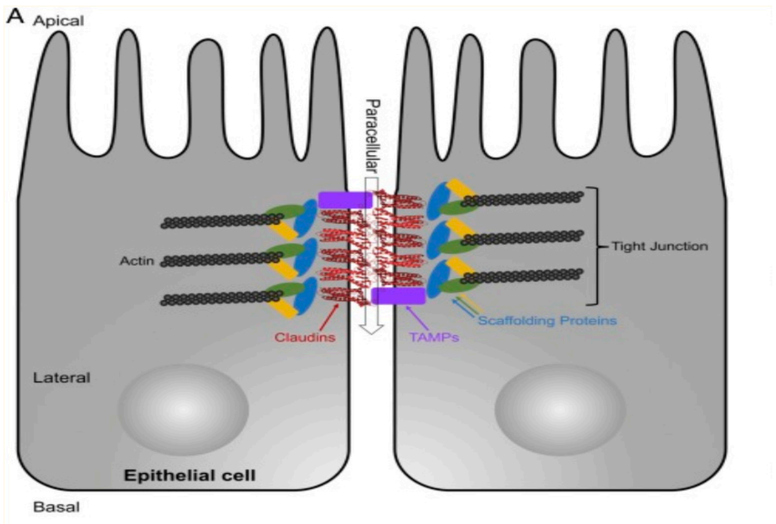
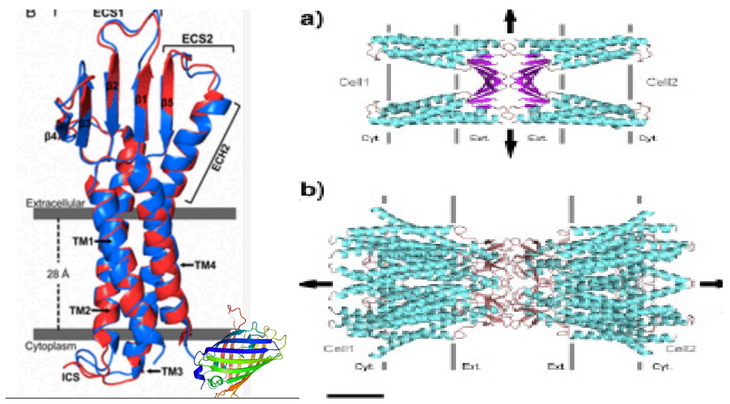
BACKGROUND AND SIGNIFICANCE

Paracellular transport of solutes and the control of the flow of molecules through the intracellular space in vertebrate epithelia is directed by tight junctions (TJs). As integral components of TJs, Claudins form paracellular barriers and pores that determine tight junction permeability. Disruption to the assembly and folding of claudins directly correlates to diseases. Yet, still much is unknown about the assembly and disassembly of Claudins into TJs is unknown.The investigation within the Vecchio Lab will be in an attempt to explain the molecular bases for destruction and reconstruction of tight junctions within epithelial cells, occurring via both natural and disease-causing mechanisms. This interdisciplinary research is important in the advancement of our understanding of human biology and health, where knowledge gained can be applied to solving problems in these areas. Different disruptions to epithelial tight junctions, in many tissues, are hallmarks of many severe human diseases. Examples include Alzheimer's, multiple sclerosis, Parkinson's, renal wasting, inflammatory bowel disease, as well as cancer. The research we will conduct is vital to clarify the various roles that the specific proteins play in human disease and for defining the molecular bases that underlie epithelial tissue specialization and claudin interaction.



ABOVE: Schematic model of two epithelial TJs formed utilizing TJ-associated MARVEL proteins (TAMPs; purple), claudins (red), and actin (black). Scaffodling proteins are various colors.



ABOVE RIGHT: Within the formation and function of a tight junciton, there are two types of interactions, cis-interactions and trans-interactions. Cis-interactions occur between two proteins in the same membrane, where trans-interactions occur between two proteins in opposing plasma membranes. Tight junctions are able to seal the plasma membrane, forming vital between differing tissue compartments, consisting of TAMPs and claudins.
(Suzuki 2013)
ABOVE LEFT: A structure of Claudin-9 tagged with GFP is presented. When inserted into the vector, the GFP gene can be used as a visual tag for the expression of other genes

UCARE OBJECTIVE:

The overall objective was to take previously made Claudin containing vectors (pEGFP-N3) containing a Green Fluorescent Protein (GFP) sequence and utilize site specific mutations to change the sequences to express Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP). Proteins tested in this experiment were Human Claudins 1, 9, 19, Tricellulin, and Occludin. Once the specified claudins and TAMPs are tagged with CFP and YFP, FRET microscopy techniques can be utilized to determine generic interactions within the formation of TJs, forming a baseline for future research. This will expand our ability to understand these important interactions.

METHODS

Making Primers:
Using primer design programs, both forward and reverse primers were selected to have an ideal nucleotide length, GC% content, as well as the proper primer melting temperature (Tm), in order to generate a large amount of mutated DNA during PCR amplification.
Mutations Needed:
- GFP to CFP: Y66W, N146I, M153T, V163A
- GFP to YFP: L64F, T65G, V68L, S72A, T203Y

5'-ctgcacgccccaggtcagggtggt-3'	Tm=74.5℃
5'-accaccctgacctggggcgtgcag-3'	24 nt: A=3.0 T=4.0 C=8.0 G=9.0 CG=70.83%

ABOVE: The forward (top nucleotide sequence) and the reverse (bottom nucleotide sequence) primers for the Y66W mutation are shown. The Tm, or the primer melting temperature, designated the temperature where one-half of the DNA will be dissociated into single stranded DNA, indicating the Primer stability. Tm is dependent on the primer's GC content, as CG content rises, the Tm is increased, as more hydrogen bonds are present between the DNA.

PCR Amplification:
In two separate PCR microtubes, either the forward primer or the reverse primer were mixed with water, PCR master mix, pEGFP-N3 template strand, and DMSO. By raising and lowering the temperature on a cycle, denaturation of the pEGFP-N3 template occurs, allowing the primer to anneal, and causing extension of these primers, inserting the primer into the plasmid vector. Dpn-1 was used to digest the parental DNA vectors.
Transformation:
Competent E. coli cells (DH5 α) were used to maximize transformation efficiency. After being incubated on ice for 30 minutes, the mixture was heat shocked in a water bath at 42°C. After being heat shocked the mixture was incubated on ice for 5 minutes. 700µL of a nutrient rich bacterial growth liquid was added, and incubated at 37°C. After the growth period, 100µL was spread a plate of LB agar supplemented with 100 µg/ml Tetracycline (pEGFP-N3 contains tetracycline resistant sequence).
Plasmid Mini-Prep:
Isolated colonies were chosen for Mini Preps. Detergents were used in Resuspension, Lysis, Neutralization, Washing, and Elution of the purified DNA. Concentration of DNA was tested using NanoDrop, with most samples containing around 300 ng/µL of DNA.

RESULTS

Using a custom sequencing primer (pEGFPN3ModR) our mutated DNA sequenced utilizing radioactive labeling. Utilizing the Clustal Omega Sequence alignment, we compared the known Amino Acid (AA) sequence of pEGFP-N3 to our mutated sequences. Asterisks indicate similar amino acids, conserved over the two sequences, whereas spaces or colons indicate differences in sequence.

```
pEGFP-N3    MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT   60
CLDN9-3     MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT   60
            ************************************************************

pEGFP-N3    LVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL   120
CLDN9-3     LVTTFGYGLQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL   120
            ****: **:***********************************************:****

pEGFP-N3    YGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIEL 126
OCLN-1      WGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIEL 240
            :************************************************************

pEGFP-N3    KGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQN 186
OCLN-1      KGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQQN 300
            ******************** ******* *********.**********************

pEGFP-N3    KLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF   101
CLDN1-1     KLTLKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF   180
            ************************:***********************************

pEGFP-N3    KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNG   161
CLDN1-1     KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNG   240
            ************************************************* *******

pEGFP-N3    IKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVL   221
CLDN1-1     IKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVL   300
            **.************************************************************
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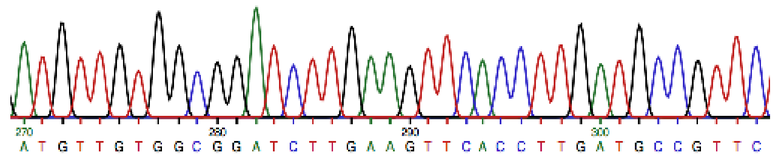
ABOVE: The figure shows the AA sequence analysis of Claudin 9 (Top strand) compared to the original unmutated pEGFP-N3 vector (bottom strand). The mutations L64F, T65G, and V68L are exhibited, starting the conversion of GFP to YFP.

ABOVE: With respect to changing the parent vector amino acid sequence from expressing GFP to expressing CFP, all necessary mutations are present in this Occludin sequence. The Y66W mutation is exhibited on the first row whereas the remaining N146I, M153T, and V163A mutations are on the second row of sequencing.

ABOVE: Claudin 1 is shown to have the Y66W mutation denoted by the semicolon in the first row of nucleotides. The M153T and V163A mutation are shown in the second and third rows, denoted by a space. The last necessary mutation to the sequence to a CFP-expressing sequence is the N146I mutation.

RESULTS (continued)



ABOVE: After sequencing, a four-color chromatogram showing the results of the sequencing run is produced. Each color represents a nucleotide, with amplitude indicating signal strength. Successful Chromatograms show evenly-spaced peaks, with one color at each peak.

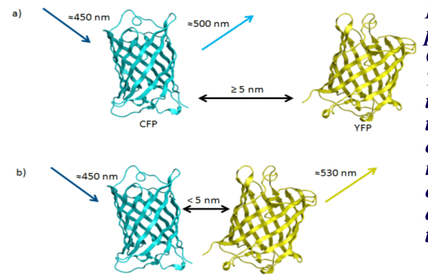
Table of Completed Mutations

Initial Protein	Target Protein:CFP	Y66W	N146I	M153T	V163A	
Claudin-1-GFP	CFP	X		X	X	
Claudin-9-GFP	CFP	X	X	X	X	
Claudin-19-GFP	CFP		X	X		
Occludin-GFP	CFP	X	X	X	X	
Tricellulin-GFP	CFP	X	X	X		
	Target Protein:YFP	**L64F**	**T65G**	**V68L**	**S72A**	**T293Y**
Claudin-1-GFP	YFP	X	X	X		
Claudin-9-GFP	YFP	X	X	X		
Claudin-19-GFP	YFP					
Occludin-GFP	YFP	X	X	X		
Tricellulin-GFP	YFP					

The above table denotes with an X, the proposed mutations that have been completed and confirmed utilizing the previously mentioned sequence analysis technique. When research resumes, the final mutations will be placed in the vector utilizing the same techniques. The two highlighted rows show highlighted rows show constructs ready for FRET.

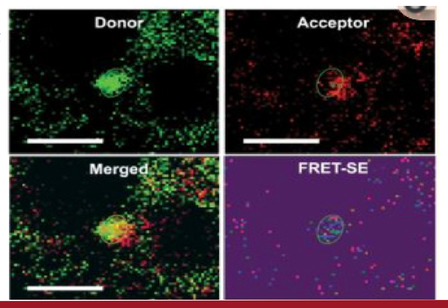
FUTURE RESEARCH

After the Claudins contain successful mutations of the GFP vector to both YFP and CFP, Förster resonance energy transfer (FRET) assays can be utilized. FRET can be used for proximity analysis in the plasma membrane even outside of cell–cell contacts. Energy transfer from a fluorescent donor to an acceptor that can take place only when the two fluorophores (YFP and CFP) are situated at distances <~10 nm. If this interaction occurs, further experiments can be done, with emphasis on mutations within these areas, expanding our knowledge of the oligomerization, folding, and assembly of these claudins.



LEFT: Illustration of the FRET phenomenon using the traditional CFP/YFP donor/acceptor pairing. Top reaction shows that they are too far away to interact, so exiting the donor only produces emission of the donor molecule. The bottom reaction shows when the excitation of the donor is propagated to the acceptor molecule, and emission of the acceptor molecule is shown.

RIGHT: As mentioned before the FRET phenomenon occurs when a donor fluorophore is close enough to transfer some of its energy to an acceptor fluorophore.This image shows an example of FRET being used to analyze the distance between specific DNA regions. FRET-SE (sensitized emission) is able to reveal energy transfer. (Gandhi 2012)



Literature

Gandhi, Manoj et al. "Frequency of close positioning of chromosomal loci detected by FRET correlates with their participation in carcinogenic rearrangements in human cells." Genes, chromosomes & cancer vol. 51,11 (2012): 1037-44. doi:10.1002/gcc.21988

Suzuki, Hiroshi & Tani, Kazutoshi & Tamura, Atsushi & Tsukita, Sachiko & Fujiyoshi, Yoshinori. (2014). Model for the Architecture of Claudin-Based Paracellular Ion Channels through Tight Junctions. Journal of Molecular Biology. 427. 10.1016/j.jmb.2014.10.020.

Vecchio, Alex J, and Robert M Stroud. "Claudin-9 structures reveal mechanism for toxin-induced gut barrier breakdown." Proceedings of the National Academy of Sciences of the United States of America vol. 116,36 (2019): 17817-17824. doi:10.1073/pnas.190892911