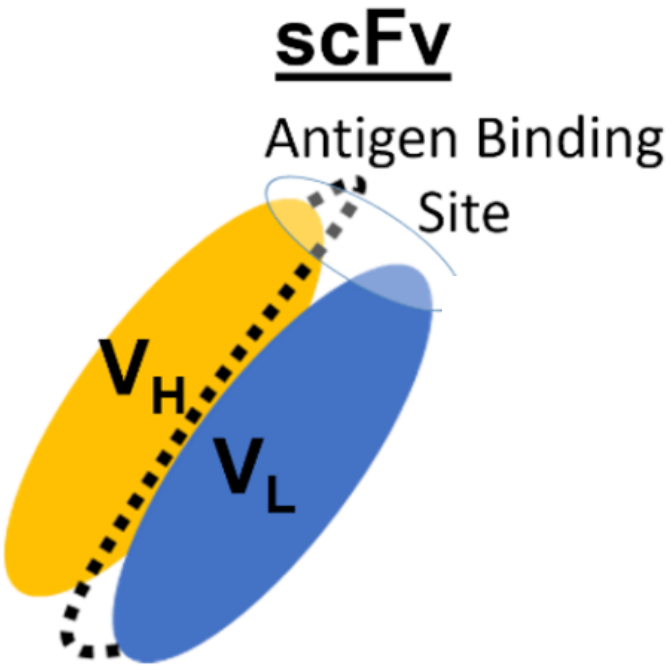

Assessing the contribution of Individual binding domains in scFv-tau interactions

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Abstract

Tauopathies are neurodegenerative disorders defined by the accumulation of misfolded tau protein in the brain, such as Alzheimer's disease, which have no official treatment. Intrabodies, or scFvs (single chain variable fragments), are antibody fragments able to be engineered and delivered as genes to target antigens. ScFvs contain both variable heavy (VH) and variable light (VL) chains, but it is unknown which one contributes more to binding. The aim of this research is to find out which, if either, domain of the scFvs dominates in tau binding, and use that to improve a weaker intrabody (scFv-2) by introducing a mutation. Pilot studies will be performed to see how the intrabodies degrade tau with both chains, and then the single-domain anti-Tau intrabodies will be cloned to create the 6 fragments of intrabodies 1, 2, and 4, broken into the VH and VL. Cells will go through Tau degradation with their separate chains, and data will be collected with imaging and western blots. To improve the function of scFv-2, the procedure will be run again with a mutation. The results will be compared in their ability to degrade tau against prior trials and controls to see if there is any decrease in fluorescent Tau, which corresponds to improvement in binding function.

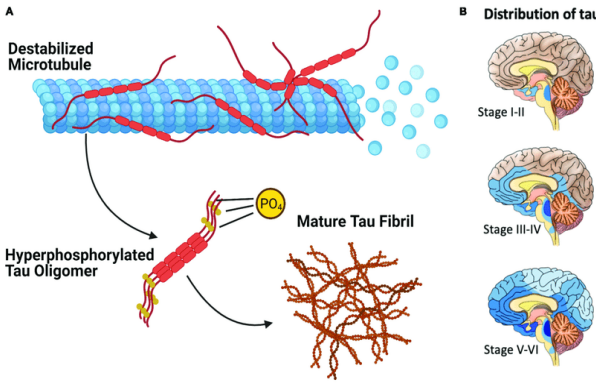
Background Information/Introduction

Antibodies are typically assembled inside the cell and secreted out to encounter antigens in the blood or other parts of the body. Intrabodies are antibody fragments that have been put back into the cell to target intracellular antigens, allowing them to have many applications (cancer proteins, virus components, toxins, other proteins not functioning correctly due to misfolding or high intracellular concentrations). The Fab variable domains, which are the parts that bind with the target antigen and are able to function independently of the whole protein, can be genetically engineered to be expressed independently which allows the small portion to have the specificity of a full-length antibody.

Many diseases are caused from inside cells, cancer being a major one, and using highly specific molecules inside cells allows for a new variety of drugs and druggable targets.

Tau is a microtubule-binding protein found in neurons and is responsible for functions such as the stabilization of internal microtubules involved in maintaining cell shape and tracks for axonal transport. They also play key roles in establishing links between the microtubules and cytoskeleton. The gene from chromosome 17 in the brain splices tau into 6 different isoforms by mRNA splicing mechanisms that have varying ratios and are expressed in neurodegeneration. The accumulation of tau continues throughout the course of the entire disease, spreading through oligomer 'seeds' that travel across a synapse. There is a connection between the growth of the neocortical areas of the brain (largest part responsible for attention, through, perception, and memory) and the growing severity of dementia as tau proteins are markers of the neurodegenerative process of disease stage and severity. In Alzheimer's, tau breaks away from the microtubules and attaches to other tau proteins, creating tangles inside of neurons that block delivery and microtubules of the transport system, harming the communication between neurons. This accumulation and misfolding leads to neurodegeneration and tauopathies, disorders characterized by abnormal amounts of tau.

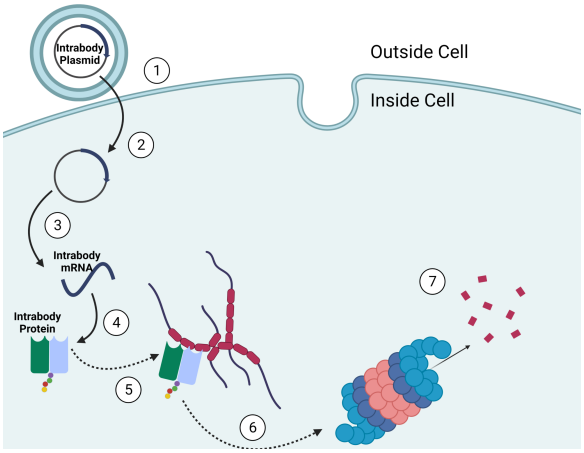
Fig. 1, Mah



Since intrabodies are intracellular, modifying the proteins inside the cell is useful in targeting intracellular antigens as usually the origins of neurodegenerative diseases are misfolded proteins. Intrabodies can be used in altering the misfolding, as well as manipulating turnover, preventing nuclear accumulation, and interactions with abnormal proteins. Intrabodies can also be engineered and delivered as cells. When in the cell, the intrabody is directed to the

proteasome, which breaks down proteins tagged with ubiquitin, which degrades the intrabody and tau, clearing up the clumps (Fig. 2).

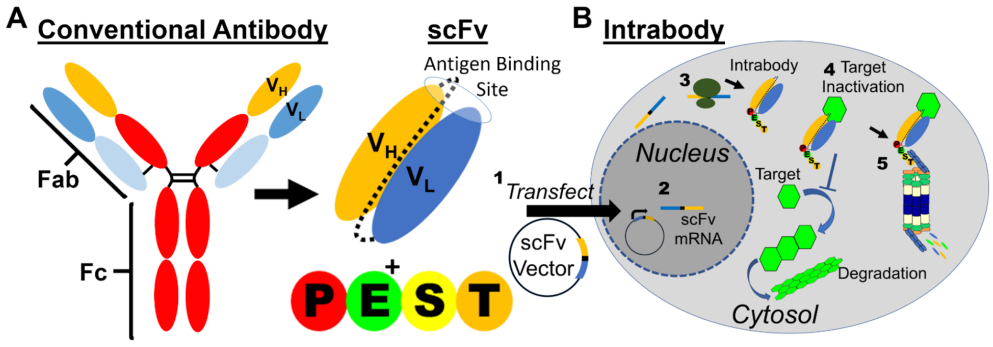
Fig. 2, Unknown



1. We transfect cells with antibody-encoding DNA.
2. The DNA enters the cell.
3. DNA is transcribed into mRNA (in the nucleus)
4. mRNA is translated into protein (our intrabody has been made).
5. Intrabody binds intracellular target (Tau).
6. Intrabody is directed to the proteasome (by a sequence of amino acids on the end of the protein, the colorful tail in the diagram).
7. The proteasome degrades intrabody+cargo (Tau), clearing up these toxic clumps or "aggregates."

An intrabody is made up of the variable heavy (VH) and variable light (VL) chains, but is unknown which one contributes more to binding. As shown in Figure 3, the scFv (single chain variable fragment, both the VH and VL, smallest fragment size that retains complete antigen binding) combines with the PEST degron, which is a signal for protein degradation. The variable region refers to the parts of the antibody that are the most variable, allowing antibodies to recognize a variety of antigens and get better at recognizing antigens after repeated exposure (like vaccines, called 'affinity maturation'). When in the cell, after binding with tau, the proteasome receives the pest degron signal on the antibody showing that it's ready to be degraded, and degrades the intrabody and tau, clearing up the clumps and thus lowering tau levels.

Fig. 3, Butler



Problem/Aims

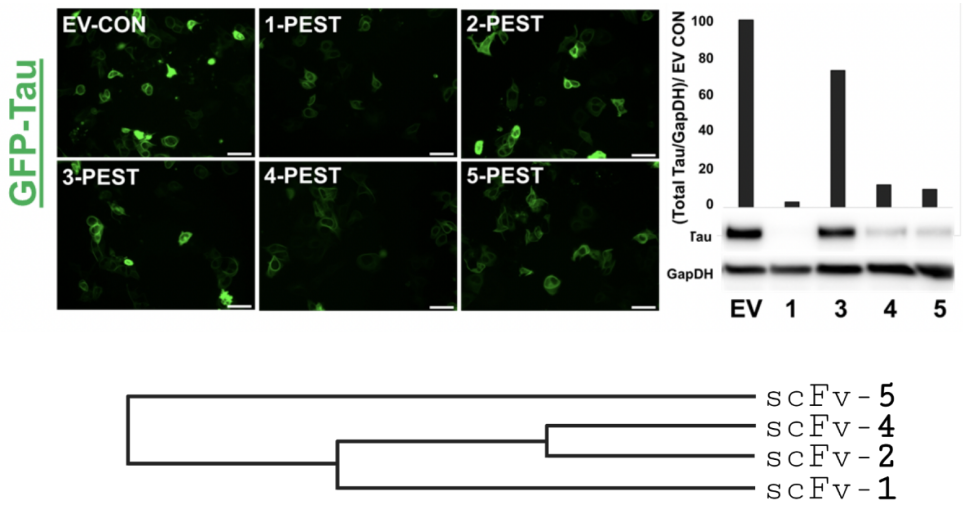


Fig. 4

Why does scFv-2 not function given its sequence homology to the other intrabodies? In Figure 3, GFP-Tau (or green fluorescent protein tagged tau which allows it to be observable) is present in the cells and the 5 different intrabodies and the empty vector (negative control with no changes made to it). The empty vector has the largest concentration of tau shown by the brightness of the green and the concentration, similar to scFv-2, while intrabodies 1, 4 and 5 are all comparatively low. The homology tree above (Fig. 4) shows that intrabodies 1, 2 and 4 are all similar, but still produce very different results.

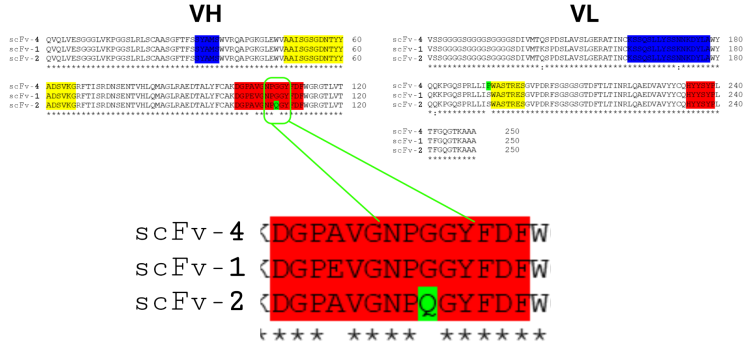


Fig. 5

Given the sequence alignments for the heavy chain on the left, and light chain on the right (Fig. 5), the differences between the two are highlighted in green, with the specific amino acid that differentiates N (scFv-1) and K (scFv-3) is zoomed in. The goal here is to generate a mutation that will change the Q to a G. The other where N and K differ is not being changed (yet) as the hypothesis is that the glutamine (Q) to glycine (G) mutation in the scFv-3 will restore its function, indicating that this is a critical residue for activity. Future experiments could address the proline (P) in the VL region depending on results.

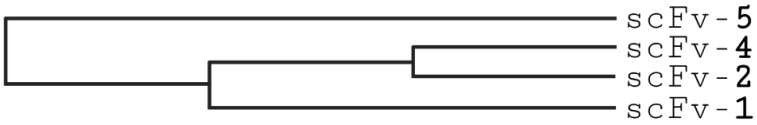


Fig. 6

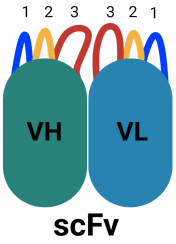


Fig. 7

F (scFv-5) is not included in this alignment because it has a much different sequence (Fig. 6).

There are two different ways of showing an scFv with the 6 Complementarity Determining Regions colored in Blue (CDR1), Yellow (CDR2), and Red (CDR3) (Fig. 7). Each domain contains 3 CDRs, which are the 'hypervariable' loops where antigen (Tau) binding happens and the question is to figure out if either domain's CDRs contribute more to this binding interaction. Often CDR3 is the most influential of the 3 CDRs in terms of binding to its target. In scFvs 1 and 4, there is a G (glycine) in place of the Q, so this is the ideal place to target when looking to improve function. We could also mutate N or V in the same spot to see if there's a loss of function.

The major goals are to find out **which, if either, domain of the scFvs dominates in Tau binding interaction,** as knowing which chain dominates in binding function leads to the possibility of simplifying project methods, as knowing if one does more work limits the potential for error and more complications. The second goal is to see **if the scFv-2 'K' intrabody can be improved by introducing a mutation in its CDR3 antigen binding site**, as that is where the differing amino acid is located.

Procedure

Pilot studies were performed using the full scFvs to judge how they perform without any changes to use as our positive control. DNA was designed to be transfected (inserting DNA into the cells) into the cells for the intrabodies. The cells were transfected and grown. Once grown, the cells were lysed open and analyzed on how they binded with Tau using Western blotting. Western blotting is used to separate and identify proteins based on molecular weight (and type) though gel electrophoresis.

Then, cells are to be co-transfected (multiple transfections at a time) with the fragments and fluorescent Tau to see which, if any, fragments decrease the levels of fluorescent Tau in the cells. Each component will be compared against the full scFv, the positive control to see if there were any changes of tau in the cells, representing improvement in binding function, and allowing us to tell which, if any, chain works better.

Results and analysis

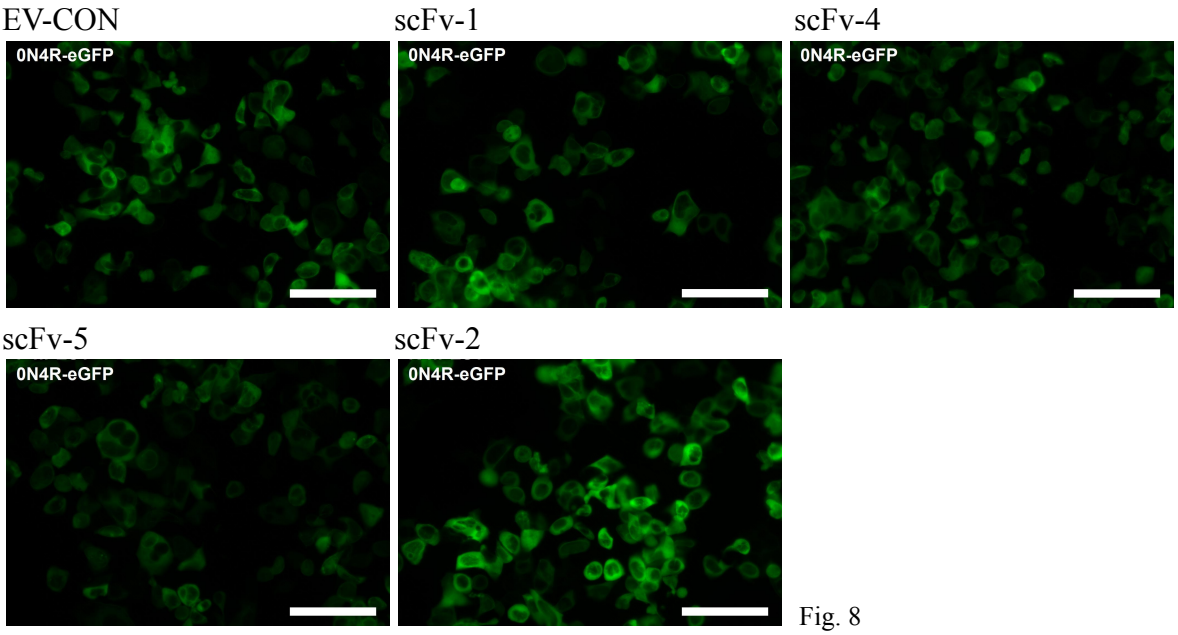


Fig. 8

As pictured above (Fig. 8), scFv-2 does not appear to reduce tau levels, which can be seen as the tau that is present (shown in green) has been reduced by the other intrabodies when compared to the empty vector (negative control), but not by scFv-2.

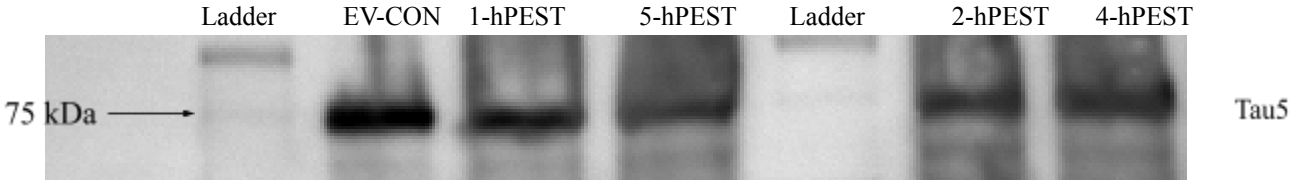


Fig. 9

Further Investigation

For the next steps and experimental design moving forward, the goal is to assess scFv-2-Q107G for its ability to degrade tau. For the experimental design, the unknown will be the scFv-2-Q107G, positive control the scFv-4, negative control the empty vector, and the target being GFP-Tau. The single-domain (VH only) anti-Tau intrabodies will be cloned, and assessed for their degradation of Tau, with the overall goal to compare GFP-Tau with the empty vector control, and the new scFv-2 variant with the mutation to see if there are any improvements in its binding function.

G-blocks, commercially made double-stranded DNA fragments of varying lengths, for the 7 different intrabodies (1, 2, and 4 heavy and light chains, as well as the mutated antibody) were designed to be transfected with GFP-Tau into empty vectors. To get the genes inside of the plasmid pcDNA3.1, the process of cloning (Fig. 10) was performed to insert the DNA into an expression vector. The DNA will be prepared, cut using Restriction Enzyme Digestion to make sure there are overhangs at the ends of the double-stranded DNA to be compatible with the ones in the plasmid so they can be ligated later on. The DNA was then isolated with Agarose gel electrophoresis and purified, and extracted with a DNA kit. Then with DNA ligation,the vector and the DNA will be combined with a ligase to join them back together. Then, the DNA will be transformed into competent E.coli, and the cells will be grown under 'selective' pressure to increase DNA production. The bacteria will be 'heat shocked' to take in the surrounding plasmid, grow and spread with ampicillin to kill anything not taken up by the plasmid, and any unwanted bacteria. Then a midiprep will be performed to take the DNA out of the E.Coli by lysis, making glycerol stocks and isolating the plasmid of interest, and sent to confirm sequences, to ensure the plasmids prepared are the original plasmids cut, not the wanted product with the plasmid, as well as checking if the DNA is at a high enough concentration to use for future experiments.

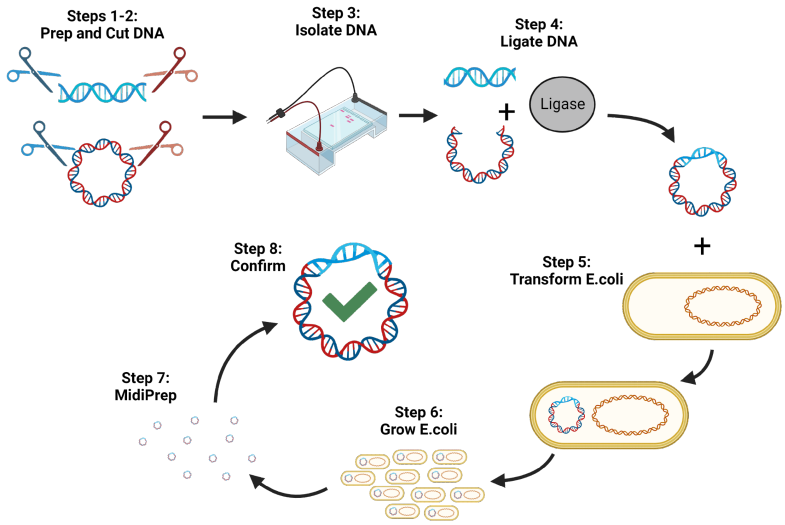


Fig. 10, Unknown

Of the three outcomes, (both regions are critical, VH functions independently or VL functions independently), our hypothesis is that the VH functions independently as an intrabody better than the VL chain. Based on that, we also hypothesize the binding function of the scFv-2 intrabody will improve with the addition of the Q107G mutation in the VH sequence, as CDR3 is the most influential of the 3 CDRs in terms of binding to its target, and the only difference between the two intrabodies (Q→G) is in CDR3.

Conclusion

"V" and "F" are antibody fragments that bind to Tau and can target it for degradation inside of cells. They consist of two domains, the VL and VH, standing for variable light and variable heavy respectively. These domains are found on every antibody and are highly mutation-prone, enabling the body to make a ton of new antibodies whenever a new foreign disease arrives.

Sometimes only one of the chains is necessary for binding, but they are not always stable alone, except for a few unique exceptions. Single domains are preferable as they are much smaller, and more applicable in certain cases. In this experiment, antibodies V and F were broken into their single chain domains, V-VL, V-VH, F-VL, and F-VH, to be tested compared to the larger antibodies, or scFvs, with both chains connected. The ultimate question to be answered is if the single chain domains are sufficient in binding and degrading tau on their own. This answers many therapeutic, biotechnical, or basic biological questions about the nature of antibodies and their interactions with tau, and also has the possibility to help with future modifications or improvements on antibodies.

Being able to improve an antibody by introducing mutations is an important function as antibodies are central to the body's response to a viral infection.

Acknowledgements

I would like to thank the Neural Stem Cell Institute for helping me with the research presented and providing resources, as well as taking high school interns and allowing us to get this valuable experience.

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