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Engineered High Affinity IGF1R Imaging Agents from the Novel WNM Protein Scaffold

With the support of the Undergraduate Research Opportunities Program (UROP), I set out to construct a novel imaging agent using the WNM scaffold. The WNM protein, based off the T7 phage protein Gp2, was identified earlier for its high β-sheet character, small size (wild type: 64 amino acids), and loop regions in the secondary structure capable of binding, making it an ideal candidate for a molecular imaging probe [1]. Using this basic scaffold, I selectively evolved this protein to exhibit high-affinity binding to the protein receptor IGF1R, a marker commonly overexpressed in a number of carcinomas [2]. The final product produced in the time allotted by the UROP was not sufficient to be tested in a biodistribution model, but only requires minimal refinement to reach a final binder.

The means to screen the selective binding potential of a protein is a process known as yeast surface display. This system utilizes yeast with the ability to express the protein of interest bound to the cell wall via a mechanism between the anchoring proteins, Aga1p/Aga2p [3]. After the initial random mutation of the wild type WNM scaffold, the resulting library was fed through two rounds of magnetic bead sorting, which selected for yeast displaying proteins with no affinity to bare magnetic beads and magnetic beads conjugated to naturally occurring protein, transferrin [4]. The final selection for each sort was for yeast showing specific binding to IGF1R. The approximate diversity following two rounds of selection was estimated at approximately 20,000 cells according to a series of dilution plates of the 0.2 (after 2nd sort) populations.

To ensure mutations achieved thus far have not yielded truncated proteins, analysis was conducted using a C-terminal c-myc epitope. I took my 0.2 population to Fluorescence Activated Cell Sorting (FACS) with a fluorescent marker which binds to this expressed tag and collected only yeast that showed fluorescent signal. The resulting population was comprised of 30,000 yeast cells, which no doubt included multiples of selected clones from the 0.2 population. A portion of these cells were then lysed in order to recover their DNA. Using a combination of amplification and error-prone PCR (to induce mutations) [5], I created two samples of plasmids, one comprised of mutations solely in the loop regions and one of mutations to the full gene of interest. Only the loop regions should be an active area in the binding event between WNM and IGF1R; however, a low error-rate mutagenesis of the entire gene ensures local maximums are escaped, due to possible structural inhibition.

The final stage in the first cycle of yeast surface display is the infusing of the mutated 0.2 DNA into the next generation of yeast cells in a process known as electroporation transformation. Yeast surface display has a number of advantages over bacterial methods of expression, but these benefits are weighted largely against the difficulty in plasmid uptake for eukaryotic cells. Within the scope of the Hackel lab, electroporation transformations were at a point of near hiatus, with the average total of successfully transformed cells lower than 0.5-1 million; this is far below optimal levels. Protocol failures at this stage could be derived from any number of variables, and troubleshooting is an arduous process. Eventually, after successive failures, I accepted a 1.0 population of approximately 1 million cells and proceeded with the second cycle of yeast surface display.

After two magnetic bead sorts, the media in which I cultured my yeast started to show pollution by some outside bacterial population. Another constant threat when using yeast surface display instead of other display technologies is the frequency of contamination. After each step, the yeast requires a rich media that inherently possesses the ability to cultivate bacteria as well as facilitate the yeasts' growth. At this point, I have reconstructed the 1.0 library with a new electroporation transformation. A recent transformation success within the research group, with diversity of 61 million, prompted me to reattempt my transformation. Initial estimates suggest my new diversity is around

10 million between both the loop mutation and full gene mutation library, which is more than sufficient to continue sorting.

My initial goal was to have a functioning, purified protein by the conclusion of my UROP project. The pathway to this goal was optimized and assumed no major disruption of the protocols involved. Electroporation transformation proved to be a very challenging step in my process and took much trial and error to reach the bare minimum of passable transformants. Furthermore, the contamination issues slowed me further, forcing me to rebuild my 1.0 library.

Yeast surface display is designed to produce highly specific binders through multiple cycles. Within our lab, dependent both on the protein scaffold and target, an acceptably evolved protein (generally, sub-nanomolar affinity) has emerged after anywhere between one and six cycles of yeast surface display. WNM has only been recently identified so evolutionary speed has not yet been documented. My project was projected for two full cycles of yeast surface display- further affinity assays are required to determine my approach to an adequate binding affinity.

My project fell short of the final goal but is continuing nevertheless. I will continue working with yeast surface display to further mature my scaffold to achieve a workable imaging agent. This is the final of four scaffolds with the ability to bind to IGF1R within the Hackel lab (scaffolds: antibody, affibody, Fibronectin, and WNM). The next phase in this line of research is a biodistribution study, looking into how protein structure affects the distribution of molecular imaging agents throughout the body. This will give us insights into which characteristics of a protein allow for the best molecular imaging agent *in vivo*.

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References

[1] Cámara, Beatriz, et al. "T7 phage protein Gp2 inhibits the Escherichia coli RNA polymerase by antagonizing stable DNA strand separation near the transcription start site." *Proceedings of the National Academy of Sciences*107.5 (2010): 2247-2252.

[2] Bähr, Corinna, and Bernd Groner. "The insulin like growth factor-1 receptor (IGF-1R) as a drug target: novel approaches to cancer therapy." *Growth hormone & IGF research: official journal of the Growth Hormone Research Society and the International IGF Research Society* 14.4 (2004): 287.

[3] Hackel, Benjamin J., and Dane Wittrup. "Yeast Surface Display in Protein Engineering and Analysis." *Protein Engineering Handbook, Volume 1 & Volume 2* (2009): 621-648.

[4] Ackerman, M., Levary, D., Tobon, G., Hackel, B., Orcutt, K. D. and Wittrup, K. D. (2009), Highly avid magnetic bead capture: An efficient selection method for de novo protein engineering utilizing yeast surface display. Biotechnol Progress, 25: 774–783. doi: 10.1002/btpr.174

[5] Cirino, Patrick C., Kimberly M. Mayer, and Daisuke Umeno. "Generating mutant libraries using error-prone PCR." *Directed Evolution Library Creation*. Humana Press, 2003. 3-9.