iGEM Advisor's Meeting
4/24/12

Conference Call with Dr. Zefeng Wang_of UNC___

- Slide #2 – 2 constructs (one with PUF binding, one without). The one without the PUF binding site gives YFP because it can be translated. Dr. Wang wonders if this will this work?
- Currently there are no papers about the prokaryotic recognition sequences. Dr. Wang thinks it will not work. He recommends changing to a reporter that will work.
- Papers suggest that PUF binds to the 3' UTR in eukaryotic cells. We are unsure of if this will work in prokaryotic cells. No one knows exactly how PUF works in eukaryotes – they recruit other factors that stop translation. These other factors might not exist in prokaryotes.
- There is a paper that claims PUF has worked in prokaryotic cells, but no plasmid was included in that paper.
- Yeast 3 hybrid system works well in Dr. Wang's lab. He can provide this as a modular system to start with. This saves us the trouble of what if the recruiter doesn't work.
- Many PUF mutants can be nicely expressed in E. Coli. Some cannot be expressed.
- We can also try ASRE – artificial site specific RNA endonuclease. But we wanted to do it in E. Coli.
- Dr. Wang ran an experiment where PUF was used to repress B-galactosidase. The PUF binding occurred in the gene. The lower expression is from RNA degradation. PUF doesn't cut RNA, just bind it. Dr. Wang put the PUF together with an endonuclease that cut the RNA when PUF bound to it. But that is not PUF anymore, it's ASRE (the name of the fusion protein).
- The whole ASRE complex looks like: Flag-PUF-linker-endo
- Dr. Wang's lab has a library of these ASRE's that they are willing to share with us. But the paper is unpublished, so the data (if it works) needs to be treated confidentially. We would not release the information to the iGEM website until everything is collected and we have conveyed the results to Dr. Wang.
- Complications: We don't know what the rate-limiting step yet? The binding or the cut? Change design so that PUF binding site is between the RBS and the gene. We put the PUF binding site multiple times to try to increase the effectiveness. Could rearrange so that the reporter is under the repressor.
- Distance between binding and digestions sites is very close.
- To add multiple binding sites, where would they go? Can we add one to the 3' UTR end so that it's after the stop codon? Does it work?
- Possible death mechanism: Engineer a PUF between the start codon and the promoter. It's a highly conserved region so it will stop the manufacture of many proteins.
- We can try multiple scenarios. We want to use the existing protein and change the binding site, as opposed to using mutated PUFs.

- Dr. Wang's overriding concern is if/how the reporter will work. Is there any natural repressor in the protein? Could use Lac I and B-galactosidase. Want to arrange it so that we control gene A, gene A and YFP have inverse expression. Once this works, changing modules will be easy. We will investigate more cases like Lac I.
- Dr. Wang already sent the construct. But Dr. Wang will look into sending and MTA over to Dr. Jin.
- Dr. Wang's lab has every possible ASRE in a library. This is a good thing to look into further down the road. Then all we would do is test the readout from the different ASRE.
- Question about plasmid Erin sent: It is for eukaryotic expression but it does replicate in E. Coli. It is PUF with another fusion that changes splicing in eukaryotic cells. But now we need the new plasmid with the fusion protein ASRE.
- Erin used a step-wise amplification system to generate the library of ASRE. You can make it one-by-one, but the step-wise PCR created (in theory) all possible PUF.
- Dr. Wang is open to collaboration and will share needed plasmids with us.
- Antibiotic resistance will be Amp (maybe K? depends on which backbone).
- The current backbone has K resistance. 3 plasmids: reporter gene with YFP that is controlled by inhibitor protein. Gene 2=codes for inhibitor protein and has PUF binding site.
- 3 markers should be enough. Although we can combine to get just 2 plasmids.

<u>Discussion</u>

- It is clear that binding is not enough. We need the PUF binding and endonuclease activity. Dr. Wang's lab will send us their contruct.
- The concern right now is the location of the binding site. Can we choose one or can we engineer one?
- There are many types of repressors to choose. We need to research which would be the best choice. For next week we will remodel the construct and find different repressors. Also look for a biobrick part.
- It's a big help that they already have the whole PUF library.
- For the RBS we will use the Biobrick. This is because we want to submit a standard part, so we should standardize everything. Because we have to ligate everything, it's a problematic idea to put the binding site between the RBS and the gene.
- Other idea: Remove the loop area from the end of the mRNA. The 3' UTR is always involved in stability, so if our PUF binds on the loop it would be more susceptible to the nuclease.

<u>Cara's phat project</u>

- Buy resveratrol and a cytochrome P450 (several have been found, a human one and a mutant Bacillus one. However the Bacillus one may not be available for purchase online.) Sigma Aldrich is our potential vendor.

- Resveratrol ranges in price and available styles (approx $100 for 100 mg, approx $300 for 500 mg)
- The goal is to create mutant piceatannols. So we mutate the cytochrome P450 to see if we can get mutant piceatannols.
- It's hard to work with cytochrome P450 in the prokaryotic system because it's made to work in eukaryotes. That's why we can the bacillus cytochrome.
- Three available ways to make mutant enzymes: site specific mutagenesis by a company, arichrome PCR, chemical mutagenesis
- Overall idea for a lab plan: We put the mutated cytochrome in the e. coli and feed it resveratrol. We get a chip that binds to the insulin receptor and see what has binded to our chip to see what is happening.
- Is this too ambitious? Or is it totally possible? There are conflicting opinions.
- Biggest problem is the low solubility. So all of the assays and screenings are not easy.
- Our ideal mutant would be more soluble, because then it would avoid degradation is the bloodstream. This random mutagenesis is a bit of a game of luck. How many mutations do we need to go through before we find something good? How many residues are there?
- If we have directed group addition or the making of derivatives, it might be better and faster and potentially yield better results for our mutated piceatannols.
- Feedback inhibition occurs when resveratrol and piceatannol inhibit the cytochrome that creates the mutants.
- The painful part is where we need to evaluate each clone by an individual assay. Is there a way to create a florescence detection method? This is much easier. That way if the chemical is modified we see a color. Then we can do a ton of wells and only examine the wells that have changed color.
- What is our desired endpoint? We don't know what our final product A is. We're just looking for the best product, so how can we know for sure? Extensive testing is needed. There are 2 unknowns (the enzyme and the compound.)
- This had morphed into a pharmaceutical project, which is time consuming.
- Still a good side project if we can obtain and test various derivatives.
- Can we get constructs from people who have published? Then we can make sure that it will actually work as expected in E. coli.
- 2 important things: We made a novel chemical and we made it in a commercially viable amount (g/L). We need to engineer the cell to produce it in proper amounts. So look at making P450 more stable so it can produce more.
- Purdue paper: They did their research in cell cultures, but mentioned degradation in the body. So does it degrade in the cell culture?

<u>Announcements</u>

-New advisors: Kori Dunn (3rd year graduate student from Prof. Rao's lab), Amet (also a 3rd year grad student from Prof. Rao's lab), Prof. Joe Bradley for Entrepreneurship.

Agenda for next weekly meeting

- We are wrapping up for summer next week! It is important that we make the most of our meeting time, so come on time and come prepared!
- Sunday Meeting agenda:
    1) Angela finishes up the biobrick talk
    2) Vote on projects. Do we want 2 equal projects? A main project and a side project? Which one is which?
    3) Vote on bootcamp. Do we want a bootcamp? Will we split into pairs? How long will it last and what lab procedures will we cover?
    4) Create a solid wetlab plan. Make one for any and all projects that we have. Start simply yet thoroughly and let the gold medal guildelines determine our progress. Make sure everyone is clear on this plan and understands all of the constitutive components.
    5) Vote on vacation time. When will we reconvene for summer? Before coming back to work, what should everyone have done? (Reading papers, awareness of lab procedures, just take a brain break).
    6) If time permits, general announcements, including an update on reading day's social outing.