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Engineering a Molecular Missile for Pancreatic Cancer Detection: Vector Design

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INTRODUCTION

Background, Significance, and Motivation

Pancreatic cancer is predicted to become the second leading cause of cancer-related death in the United States before 2030, despite the fact that it currently accounts for merely 3% of all cancer cases (Rahib et al., 2014) (Cancer.org, 2018). Currently, there is no one standard method for early detection of this deadly disease, and researchers are unsatisfied with the performance of current biomarkers in the detection of the most prominent form of pancreatic cancer: pancreatic ductal adenocarcinoma, or PDAC for short (Bhat et al., 2012). The most widely used PDAC biomarker is CA 19-9 --the only biomarker currently approved by the FDA (Partyka et al., 2012). By itself, CA 19-9 is unsuitable for clinical diagnosis, but it promises to be useful in combination with other diagnostic tests (Wu et al., 2013) (Herreros-Villanueva et al., 2016). Despite its shortcomings, it is currently unsurpassed by any other biomarker in terms of sensitivity and specificity (Herreros-Villanueva et al., 2016).

Biomarker detection methods are largely dependent on the use of antibodies (Chang et al, 2017). The immune system naturally produces these large proteins which form strong and specific bonds to the epitopes of their target antigen in order to destroy foreign particles (Lipman et al, 2008). (American Cancer Society, 2015). Scientists have manipulated this characteristic to create a wide range of diagnostic and therapeutic tools (Lipman et al., 2008).

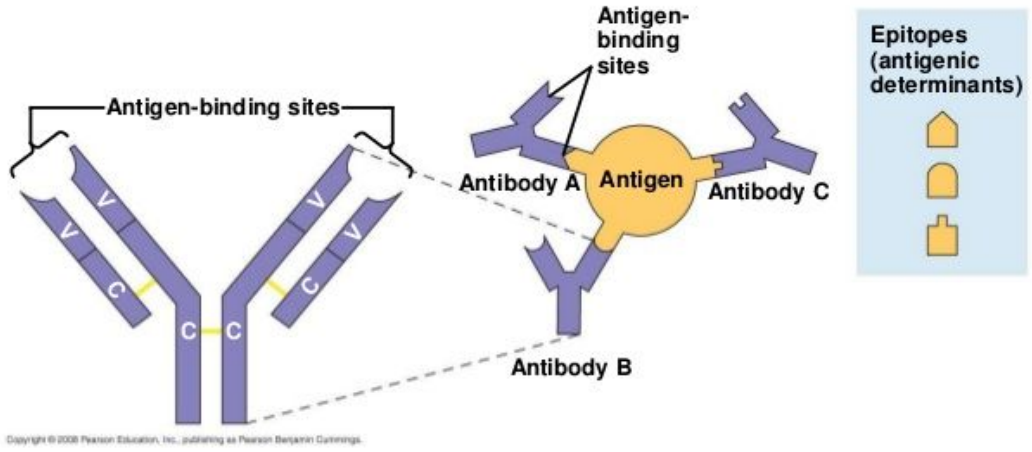

Figure 1. The interactions between an antibody and its target epitope on an antigen (Campbell, 2008).

CA 19-9 antibodies bind to a sugar epitope, rather than a protein (Partyka et al., 2012). Clinically, only a very small number of carbohydrate antigens are used as biomarkers, as

expression levels are difficult to reliably measure due to lack understanding of peptide-sugar interactions (Manimala et al., 2007).

While a CA-19-9 blood assay is currently the best biomarker-based cancer detection approach in terms of sensitivity and specificity, it is based on the use of monoclonal antibodies, or mAbs (Herreros-Villanueva et al., 2016). Monoclonal antibodies are expensive, in part due to their long production timelines, and are produced using ethically problematic methods (Lipman et al., 2005). However, synthetic antibodies appear to be a favorable alternative, as they are able to circumvent many of the shortcomings of mAbs while preserving comparable function (Wals et al., 2014).

Therefore, sugar-peptide interactions will be investigated in order to develop a "molecular missile" for the detection of sugars --that is, a macromolecule that directs itself towards strong binding to its corresponding target. By developing this synthetic antibody in E. coli and incorporating the unnatural amino acid L-DOPA, this design will to bypass the drawbacks of monoclonal antibodies and design a model for the creation of functional antibodies with the ability to target sugar biomarkers in countless other cancers and diseases.

Literature Review and Critiques of Current Technology

Monoclonal and Polyclonal Antibodies

Determining the levels of CA-19-9 in the blood is currently the best biomarker-based PDAC detection approach in terms of sensitivity and specificity; however, it depends on the use of monoclonal antibodies (Herreros-Villanueva et al., 2016). Although many different anti-CA 19-9 mAbs have been developed, the lack of optimization of these mAbs has resulted in unsatisfactory specificity for sialyl-Lewis (Partyka et al., 2012). Monoclonal antibodies inherently have several unresolved issues stemming from their method of production: they are 1) expensive, 2) take a long time to produce, and are 3) ethically problematic (Siddiqui et al., 2010).

The hybridoma method (Figure 2) is the predominant method of mAb production (Leenaars et al., 2015). This method creates an antibody library through trial and error and revolves around injecting animals with the target antigen to stimulate natural antibody production (Leenaars et al., 2015). However, exploiting an animal's immune system by intentionally infecting them with a disease is an ethically questionable practice. Additionally, producing mAbs with the hybridoma method is an expensive pursuit, due to the use of animals and months to years required for success (Leenaars et al., 2015).

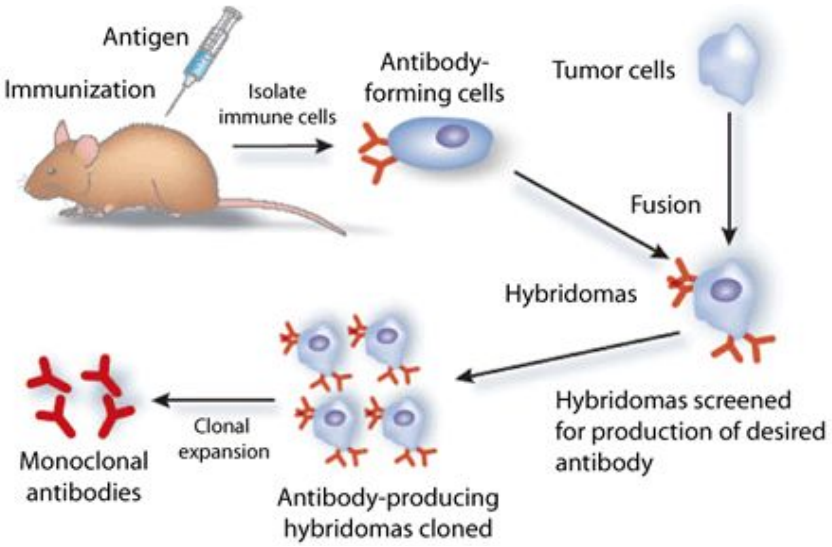

Figure 2. Traditional hybridoma method for monoclonal antibody production (Michnick, 2008).

In contrast, polyclonal antibodies (pAbs) require less time, money, and technical skill to create while maintaining the specificity possessed by monoclonal antibodies (Lipman et al., 2005). However, batch-to-batch variability is a concern in pAbs since it requires the used of multiple animals at different times (Lipman et al., 2005). Additionally, the ethical issues of mAbs are still unresolved in pAbs (Lipman et al., 2005).

Nevertheless, mAbs and pAbs are unparalleled in terms of affinity and specificity (Siddiqui et al., 2010). Therefore, this project will aim to meet the unmatched performances of mAbs and pAbs while designing a novel system to overcome their pitfalls and ethical problems via synthetic antibodies.

Synthetic Antibodies

Synthetic antibodies for diagnostics engineered to replicate the modularity and versatility of natural immunoglobulins (Deyev et al, 2009). These synthetic proteins are typically developed based on the findings of biochemical investigations focused on protein-protein interactions and can be easily manipulated and mass-produced at a low cost (Umeda et al, 2010). However, at their current stage of development, they are unsuitable for diagnostic applications due to their relatively low levels of affinity, selectivity, and efficiency (Umeda et al, 2010). Some types of synthetic antibodies bind to their target antigen via free-radical based reactions, however, this strategy tends to produce chemically heterogeneous mixtures of cross-linked peptides that convolute detection (Denison, 2004).

Unnatural Amino Acids

However, a more recent development in the field bioengineering that has allowed the potential of synthetic antibodies to skyrocket is the development of unnatural amino acids capable of being incorporated into recombinant proteins synthesized in Escherichia coli, or E. coli (Lital et al., 2003). These unnatural amino acids replicate the structure of post-translationally modified amino acids, and with continual development, this technology has the potential to render obsolete one of the biggest drawbacks to using E. coli in synthetic biology --the inability of E. coli to perform post-translational modifications.

Before an unnatural amino acid can be incorporated into a peptide, an unnatural tRNA and tRNA synthetase must be introduced into the cell manufacturing the peptide through a vector, such as a plasmid (Wals et al., 2014). The unnatural tRNA is typically designed so that the amber stop codon (TAG/UAG) signals for the incorporation of this unnatural tRNA when mRNA is translated in the cell (Wals et al., 2014). Of the three stop codons found in nature, the amber stop codon used the least frequently in E. coli --only 7% of the time (Wals et al., 2014) Since it is rarely used in essential proteins, using this stop codon minimizes the possibility of killing the cell or severely disrupting function as an unintended side effect of unnatural amino acid incorporation (Wals et. al, 2014).

Specifically, the work done by Umeda et al in the site-specific incorporation a redox-active amino acid 3,4-dihydroxy-L-phenylalanine (l-DOPA) is of particular usefulness in the development of synthetic antibodies (Umeda et al., 2010). This synthetic antibody rectifies the issue of weak binding affinities of synthetic antibodies by supplementing the peptide interactions with the target antigen with an irreversible covalent bond. See Figure 3 below for the mechanism of its function.

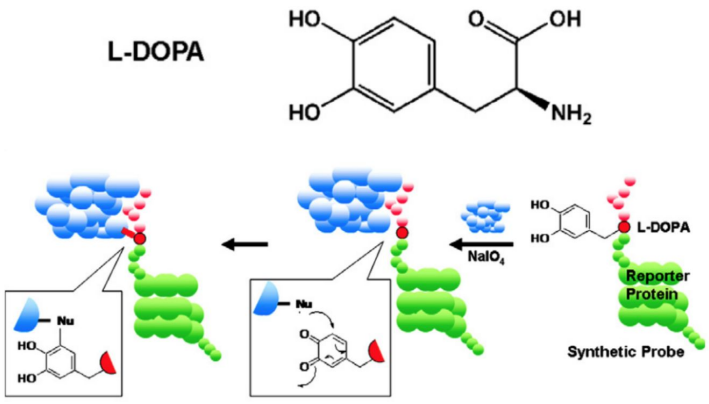

Figure 3. Mechanism of l-DOPA covalent bond formation. The peptide (red molecule) binds to the antigen (blue molecule). Figure adapted from Umeda et al (2010).

While several papers have demonstrated the utility of l-DOPA incorporation for strengthening both protein-protein (Umeda et al., 2009) and peptide-protein bonds (Umeda et al., 2010), researchers have yet to use l-DOPA to strengthen the bond between peptide-carbohydrate interactions. However, upon analyzing the structure of carbohydrates the mechanism of l-DOPA in strengthening protein-peptide interactions, its success in this application appears to be likely. The nucleophilic hydroxyl groups that are characteristically plentiful in carbohydrate structures will provide l-DOPA many potential locations for covalent bond formation.

The pAC-DHPheRS-6TRN plasmid, which codes for l-DOPA tRNA and tRNA synthetase, has already been used in previous senior design projects, and it will be used in this project as well (Therriault et al. 2016) (Nguyen et al., 2017). With this plasmid, the amber stop codon will signal for the incorporation of l-DOPA (Therriault et al., 2016).

This ability to mimic post-translational modifications with unnatural amino acids is significant, as using E. coli for recombinant protein production has many advantages. E. coli's doubling time is only 20 minutes, which is must faster than other cell type options such as yeast, insect, or mammalian cells, which all have a doubling time on the range of hours or even days (Rosano et al., 2014) (Murakami et al., 2009) (Thermo Fisher Scientific, 2017) (Sitton et al., 2008). E. coli is also favorable due to a simple lab setup, cheap growth media, ease of transformation, and those using this cell type also avoid the ethical issues that arise when using animals to produce recombinant proteins (Rosano, 2014). While recombinant proteins produced by E. coli are more susceptible to genetic mutations, minimizing the length of the peptide used in the design will allow for DNA sequencing to be used to check for these potential spontaneous mutations (Wals, 2014).

Project Objectives

The overall goal of this project is to create a proof-of-concept model for designing a synthetic antibody capable of binding to sugars by developing a synthetic antibody for CA 19-9 detection in PDAC. In targeting a sugar, this project aims to provide insight into a relatively under-researched domain. In using synthetic monoclonal peptides, the benefits of monoclonal antibodies will be maintained while circumventing the cost-effective and ethical drawbacks of such manufacturing.

The main components of the proposed synthetic antibody are outlined in Figure 4 below.

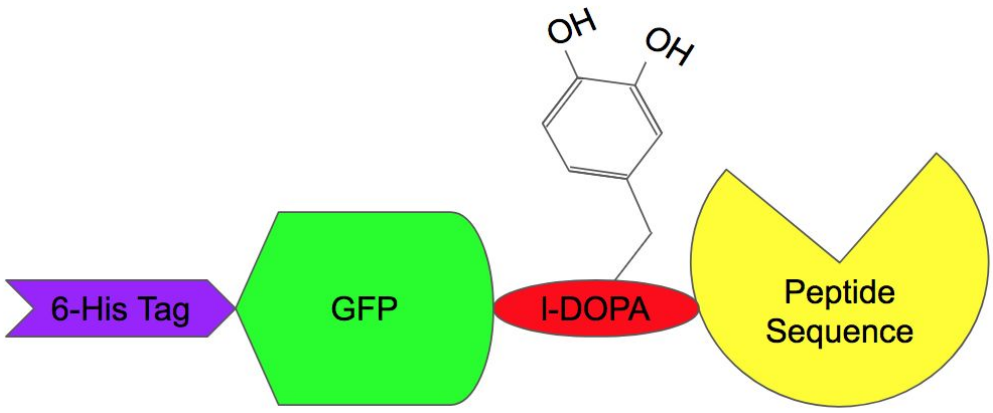

Figure 4. Diagram of synthetic antibody design. Not to scale.

The peptide sequence will be responsible for binding to the epitope of the biomarker, the l-DOPA will strengthen the ability of the peptide to bind to the biomarker, the GFP segment will serve as a fluorescent reporter, and the 6-His tag facilitates the protein purification process. This is based on the modular design presented in Umeda et al (Umeda et al., 2010), which has been used in previous senior design projects as well (Therriault et al., 2016) (Nguyen et al., 2017).

Project Milestones and Expected Results

The first milestone for this project is identifying a target biomarker. CA 19-9 was chosen as the target biomarker based on its high specificity and sensitivity to PDAC, particularly in comparison to other proposed biomarkers. Since CA 19-9 is a carbohydrate, a parallel objective of the project is to elucidate peptide-sugar interactions.

The second milestone is identifying a peptide sequence that will facilitate binding to the target antigen through a bioinformatics study. This involves pinpointing monomeric proteins that bind to CA 19-9's epitope sialyl-Lewis a, and selecting a protein to base the peptide sequence off of with the aim of minimizing the length of the peptide sequence required for binding.

The third milestone is designing the plasmid for vector insertion into E. coli. This plasmid includes the DNA sequence corresponding to the amino acid sequence of the synthetic antibody as diagrammed in Figure 4. The E. coli will translate the DNA sequence contained in this plasmid to manufacture this synthetic antibody.

Backup plan

If L-DOPA does not strengthen antibody affinity as expected, the target biomarker will be

switched from a carbohydrate to a protein, as l-DOPA been already proven to enhance peptide-protein bonds (Umeda et al., 2010).

Significance

The development of a proof-of-concept model for a synthetic antibody capable of binding to sugars will not only benefit the scientific community at large, but also the field of medicine. This accomplishment will help expand the knowledge of peptide-sugar interactions of the scientific community, a domain that is markedly under-researched. This specific project can help the CA 19-9 blood test become cheaper and more widely-available to PDAC patients, thereby increasing future possibilities for early detection in more patients, and consequently improving overall PDAC survival outlooks (Berquist et al., 2016).

However, the potential benefits for the medical field do not stop here. By modularizing the design of synthetic antibodies capable of binding strongly with sugars, this project can greatly expand the diagnostic and therapeutic applications of antibodies in an ethical manner. With this development, a whole new category of antigens can now be targeted. The GFP reporter molecule included in this design can be theoretically swapped for a therapeutic payload, equipping the antibody to be used in targeted drug deliveries and other therapies.

PEPTIDE SEQUENCE DESIGN

Introduction

By performing a bioinformatics study, I determined the peptide sequence for the portion of the synthetic antibody that will be responsible for creating a bond with the CA 19-9 epitope sialyl Lewis. This bond will be strengthened with l-DOPA. The DNA sequence that codes for this amino acid sequence will be inserted into an experimental plasmid in order to engineer E. coli to synthesize the molecular missile, which targets CA 19-9. The other components of this molecular missile, namely l-DOPA, the GFP reporting molecule, and the His tag are all well-defined structures with predetermined peptide sequences.

Design Constraints

The peptide sequence chosen will have to be proven to selectively bind to sialyl Lewis a. Specific binding would be optimal, but an unrealistic goal considering the limited amount of research on this topic. Future work could use peptide library creation and directed evolution to make the proposed synthetic antibody specific. The peptide sequence also must be able to be expressed in E. coli, the chosen cell type for recombinant protein production. Additionally, the peptide sequence must be a functional monomeric protein, as multimeric proteins are too complicated to express in E. coli given the laboratory resources available to SCU students.

There are also different limitations regarding the length of the chosen peptide sequence. Realistic technical capabilities include either (1) going to a company to synthesize the vector, (2) piecing together two short oligos using a Klenow reaction, or (3) using high-fidelity PCR to amplify a fragment of DNA from a plasmid. The first option grants the most flexibility in both length and customizability, but it is an expensive option. For the second option, the corresponding DNA sequence is limited to one's imagination, so long as it is very short, ideally less than roughly 20 amino acids long. If the peptide sequence is longer, the last option is the most favorable, however, the corresponding DNA sequence is limited to those included in commercially available plasmids.

Methodology Constraints

This bioinformatics search is limited to what information is available for free through online search engines and the SCU library database.

Back-Up Plan

If the bioinformatics search for a suitable peptide sequence is unsuccessful, the target antigen will have to be switched to a biomarker whose binding patterns are more thoroughly researched than CA 19-9.

Results

After a thorough search on several SCU library databases, including *SciFinder*, I found a 1998 paper by Torgersen et al that described a short (44 amino acids) peptide sequence that was proven to bind to sialyl-Lewis x, a close relative of the target antigen sialyl-Lewis a. While the authors of the paper did not test the ability of the peptide sequence to bind to sialyl-Lewis a themselves, the peptide sequence they expressed in E. coli did contain the binding regions required for binding to sialyl-Lewis a, as described in a 2008 paper by Veluraja, et al. As illustrated in Figure 5 below, these "hotspots" include Tyr94, Arg97, Glu98, Lys99, Asp100, and Lys111 (Veluraja, et al., 2008).

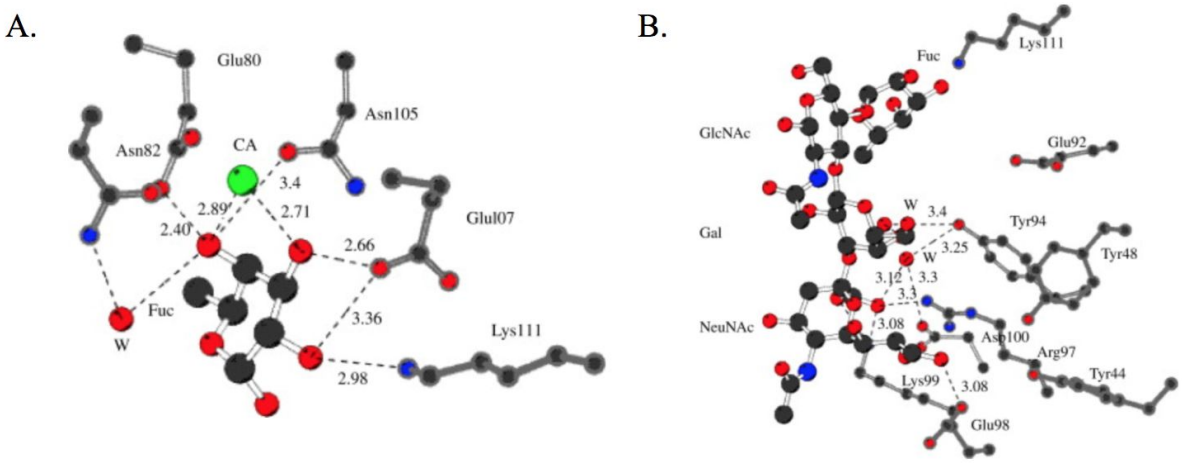

Figure 5. E-selectin binding to sialyl-Lewis a. Both essential and non-essential interactions are shown. Figure adapted from Veluraja, et al.

Discussion

Replicating the mutated MBP (mannose-binding protein) peptide fragment as described in a 1998 paper authored by Torgersen et al (Figure 6) initially seems to be a desirable option. This mutated MBP peptide fragment contains domains from another protein, E-selectin, that are important to binding to the CA 19-9 epitope. E-selectin is a protein that interacts with sialyl-Lewis a, among other ligands, to trigger an immune response (Mann et al., 2011). Given that this peptide fragment contains the "hotspots" needed for binding to sialyl-Lewis a, one can reasonably hypothesize that the given fragment will be able to also bind to sialyl-Lewis a in addition to sialyl-Lewis x. Fine-tuning of the fragment through iterative experimentation would need to be done before specific binding to sialyl-Lewis a could be accomplished, but the selectivity of this relatively short amino acid sequence is suitable for this project's prototypic goals.

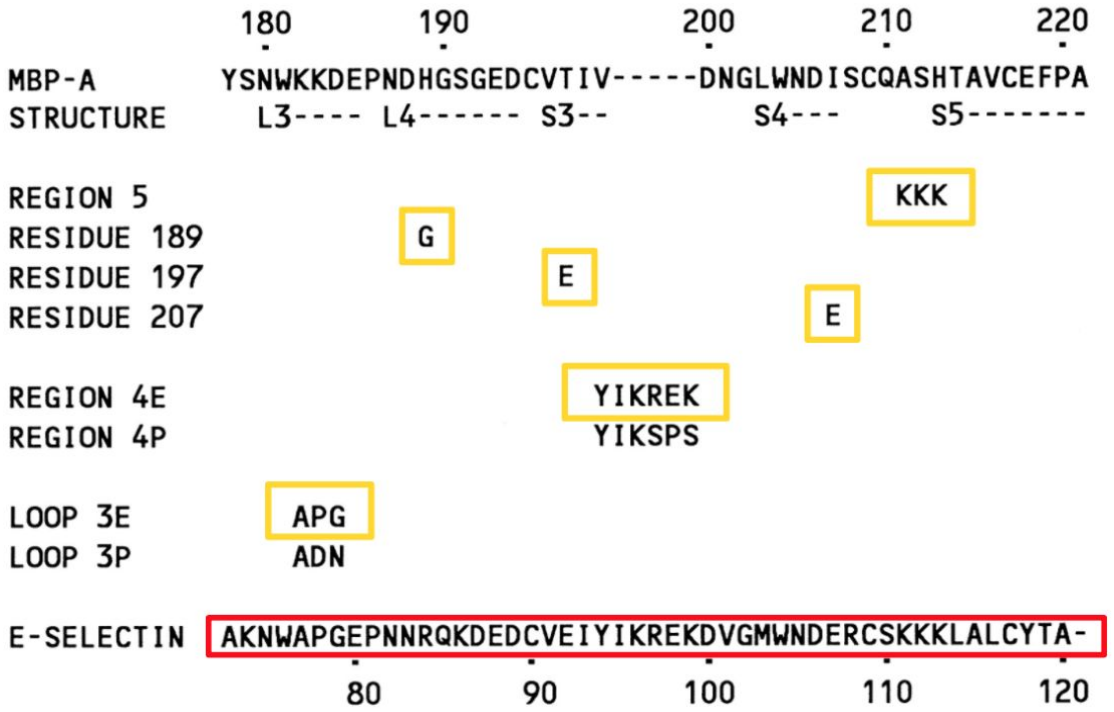

Figure 6. Aligned amino acid sequences from relevant fragments of MBP and E-selectin. Yellow boxes indicate the locations where MBP-A was mutated to contain the corresponding E-selectin sequence, which is boxed in red. Adapted from Torgersen et al.

This fragment is a favorable choice as the aforementioned paper demonstrated that it possesses binding capabilities suitable for selectively targeting sialyl Lewis a and can be successfully expressed in E. coli. However, the 44 amino acid length of the fragment meant that the required DNA fragment length (132 bp) would be much too long to be created using DNA oligo synthesis. Although high-fidelity DNA polymerase could be used to amplify the DNA sequence that encodes this protein fragment from a plasmid for insertion into the experimental plasmid, there is not commercially available plasmid that contains the mutated MBP peptide as described in Torgersen et al.

However, it is possible to procure a plasmid that contains the entire sequence for E-selectin, including the binding domain the mutated MBP protein was designed to mimic. This sequence is 43 amino acids long and is boxed in red in Figure 6 above. Although this option is not as preferable, as the expression of E-selectin in E. coli has proven to be rather difficult, studies have shown that it is possible to express fragments of E-selectin in E. coli (Kuwano, 2013). A plasmid that contains the entire E-selectin sequence capable of E. coli expression, such as pGL3-ELAM-luc can be used to obtain the corresponding DNA sequence to be inserted into the experimental vector.

The final vector design is depicted Figure 6 below:

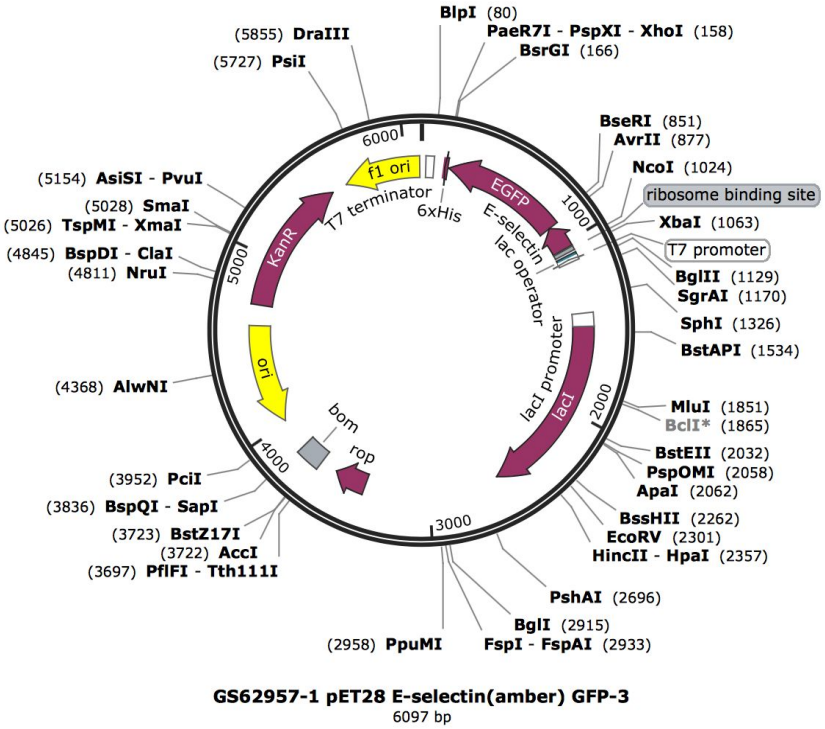

Figure 6. Plasmid map of the experimental vector. Image generated by Epoch Life Science, Inc.

In this design, the DNA sequence that codes for E-selectin fragment will be directly followed by the amber stop codon (TAG), which will be immediately followed by the DNA sequence for EGFP, which is followed by the DNA sequence for the 6-His tag.

Conclusion

The design peptide sequence responsible for binding to CA 19-9 will consist of the 43 amino acid-long sequence as boxed in red in Figure 6. This proposed design meets the requirements set forth by the "Design Constraints" section of this chapter. The chosen sequence selectively binds to the epitope of CA 19-9 in a monomeric form. Research is supportive of the feasibility of expression in E. coli. This design can be manufactured in a couple of ways. The vector design can be sent to a company for production, or the DNA sequence that encodes this selected peptide fragment can also be obtained by performing high-fidelity PCR on pGL3-ELAM-luc, using the appropriate forward and reverse primers. Using the appropriate restriction enzymes, the DNA sequence can then be inserted into the vector pET28b-P1+TAG+GFP+His, which contains code for rest of the design.

Acknowledgments:

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