ABSTRACT

Title of Document: THERAPEUTIC POTENTIAL OF RNAi THROUGH ENDOCYTOTIC METHODS

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Cell Biology and Molecular Genetics

The ability to manipulate gene expression promises to be an important tool for the management of infectious diseases and genetic disorders. However, a major limitation to effective delivery of therapeutic RNA to living cells is the cellular toxicity of conventional techniques. Team PANACEA's research objective was to create new reagents based on a novel small-molecule delivery system that uses a modular recombinant protein vehicle consisting of a specific ligand coupled to a Hepatitis B Virus-derived RNA binding domain (HBV-RBD). Two such recombinant delivery proteins were developed: one composed of Interleukin-8, the other consisting of the Machupo Virus GP1 protein. The ability of these proteins to deliver RNA to cells were then tested. The non-toxic nature of this technology has the potential to overcome limitations of current methods and could provide a platform for the expansion of personalized medicine.

J Cell

THERAPEUTIC POTENTIAL OF RNAi THROUGH ENDOCYTOTIC METHODS

Team PANACEA:
Promoting A Novel Approach to Cellular (gene) Expression Alteration

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Thesis submitted in partial fulfillment of the requirements of the Gemstone Program
University of Maryland, College Park
2016

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Acknowledgments

We would like to thank the following people for their help and support throughout this project. We appreciate the time that everyone contributed towards making our project a success.

Dr. Jonathan Dinman

Dr. Denis Wafula

Dr. Vivek Advani

Dr. Frank Coale, Dr. Kristan Skendall, Vickie Hill, Gemstone Staff

Nedelina Tchangalova

Carol Marques Dos Santos Vieira

Dr. Arturas Meskauskas

Dr. Chanda Arya, Kevin Diehn

Andrea Bajcsy

Our Friends and Family

Launch UMD Donors

ACCAC (Atlantic Coast Conference Academic Consortium)

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Introduction

Numerous infectious diseases and genetic disorders negatively impact human health and are of medical concern. However, the severe side effects of many current therapeutic regimens may discourage patients from seeking treatment. One good example is cancer. Most treatments employed to combat cancer result in severe side effects and other costs that may discourage patients from seeking medical intervention: 19.3% of Americans refused treatments such as chemotherapy, radiation, and surgery despite the fact that over a third had a medical condition requiring treatment (Gavura, 2012). These statistics strongly suggest that there is a need for alternative methods of treatment that will be more widely accepted by the general public. Gene therapy represents one such form of alternative therapy. Recent surveys have indicated that over 90% of the respondents accept gene therapy as a treatment for severe illnesses (Robillard et al., 2014)

The field of genetics has made major contributions in helping researchers develop alternative treatments for genetic diseases. The ability to alter expression of specific genes in specific cells at will is a particularly useful tool that researchers are pursuing to achieve this end. However, currently available technologies, such as dendrimers, are too toxic to primary cells to be considered for effective treatment; such methods can only be used on cancerous cell lines in the laboratory (Yao & Eriksson, 2000). In addition, despite the advances in gene therapy technologies, more research is needed to develop efficient means of target certain genes in specific cell types.

Our research question revolves around the development of an efficient technique that can be used to deliver therapeutic small interfering RNA (siRNA) into primary cells.

It is based on the specific molecular interactions between cell-surface associated receptors and their ligands that enable receptor-mediated endocytosis of ligands into cells. We hypothesized that using receptor-mediated endocytosis to deliver therapeutic siRNAs into specific cells will result in measurable changes in the expression of the targeted genes. Furthermore, we hypothesized that gene expression changes will occur only in cells expressing these receptors.

To learn more about this approach to altering gene expression, we studied the cellular process of receptor-mediated endocytosis, in which cells engulf substances that are bound to receptors on the plasma membrane into the cell, as well as specific receptors that are capable of undergoing this process. We also examined the properties of siRNA, one of the components of the delivery vehicle we will be developing to achieve alteration of gene expression, as well as methods of quantifying our research, including quantitative real time polymerase chain reaction (qRT-PCR).

The project was divided into three phases. The first phase involved identifying appropriate receptor/ligand pairs and cloning desired sequences (i.e. the appropriate ligand and an RNA binding domain designed to deliver the therapeutic RNA to cells) into the parent expression plasmid. Phase two involved using *Pichia* yeast to produce recombinant proteins, optimizing synthesis of recombinant proteins, and purifying the proteins. The third phase involved testing the recombinant delivery protein in cell culture. In this phase, the negatively charged siRNA was incubated with the purified protein where it bound to the positively charged RNA binding domain through electrostatic interactions. Then, the siRNA/protein complex was introduced to the target mammalian cell cultures, and efficacy of the recombinant delivery protein was assessed.

The research presented herein explores a novel method of delivering therapeutic RNAs into cells for the treatment of a wide variety of diseases. This new technology has the potential to alter or eliminate the expression of specific genes implicated in disease. Since receptor-mediated endocytosis is a natural cellular process, it has the potential to avoid side effects that are common among other forms of treatment. Consequently, our research and proposed method of gene therapy may one day lead to advances in biomedical research and providing individualized patient care.

Literature Review

Introduction and Overview

The ability to alter gene expression in specific cells at will is a powerful tool that already has multiple applications. Many current methods used to alter gene expression, e.g. electroporation, are extremely toxic to cells. These techniques can only be used on robust cancerous cell lines and not on fragile primary cells obtained directly from healthy organisms (Niidome et al., 2002).

A technology initially developed at the National Institutes of Health (NIH) promises to give researchers the ability to alter cellular gene expression in a more targeted and less toxic manner than current commercially available techniques (Biragyn et al., 2013). The technology involves fusing RNA-binding protein domains to small protein ligands that bind to known cell surface receptors, allowing researchers to deliver therapeutic RNAs into specific cells. This literature review will discuss the mechanisms behind this novel technology, including a discussion of receptor-ligand interactions and the basic cellular processes that are targeted to alter gene expression. This review will also analyze existing gene delivery methods, assess their strengths and weaknesses, and discuss how this knowledge can be used to enhance our methodology.

Small Interfering RNA (siRNA)

Small interfering RNAs (siRNAs) are small, single-stranded RNA molecules of approximately 21-23 nucleotides in length (Maeda & Sheffield 2009). siRNAs are targeted to specific messenger RNA (mRNA) sequences through base-pairing interactions in concert with a protein complex called RNA-induced Silencing Complex (RISC). RISC then cleaves the mRNA and targets it for degradation (Maeda & Sheffield

2009). Since mRNA is the intermediary between DNA and protein, degrading a specific mRNA will decrease protein production. Using siRNAs to interfere with gene expression is known as RNA interference (RNAi) (Chiu & Rana, 2003).

Understanding the process of RNAi is important for developing the most effective methodologies for both laboratory and medical applications. In natural systems, RNAi begins with cleavage of double-stranded RNA (dsRNA) to short single-stranded siRNA (Chiu & Rana, 2003). The newly made siRNA complexes with RISC, and targets a specific mRNA for degradation.

An important avenue of siRNA research involves efforts to stabilize siRNAs as a way to increase the duration of dsRNA mediated gene silencing. To further understand the mechanism of RNAi in humans, RNAs can be chemically modified, and the resulting effects on RNAi effectiveness examined. One of the most important modifications tested the importance of the 2'-OH of the ribonucleotide that distinguishes RNA from DNA (Chiu & Rana, 2003). These studies demonstrated that the 2'-OH is not required for RNAi, indicating that structural, rather than chemical, properties of siRNA-mRNA complexes are the main factors in inducing RNAi. This series of experiments elucidated important biochemical properties of functional siRNA. In particular, it highlighted how specific chemical changes in siRNA can affect the efficiency of RNAi, and pointed to approaches designed to improve the stability and usefulness of siRNA for future RNAi applications.

We chose to utilize siRNA as a component of the delivery vehicle protein as opposed to other RNAi agents such as microRNA (miRNA) due to the specificity conferred by siRNA. siRNA are highly specific and only have one specific target, while

miRNA can have multiple targets, which can potentially lead to undesirable effects of gene expression alteration (Lam et al., 2015).

Receptor-Ligand Interactions

Receptors are proteins expressed on the surface of cells that allow the cell to interact with its external environment. These molecules bind specific ligands, i.e. essential nutrients and intercellular signaling molecules. Signaling receptors bind specific ligands and ultimately initiate pathways that lead to cellular responses involving changes in gene expression. In contrast, transport receptors bind ligands and deliver them to the interior of the cells (Shankaran, Resat, & Wiley, 2007). The internalization of the receptor-ligand complex is a process known as receptor-mediated endocytosis (RME).

Cells constantly use RME to internalize necessary extracellular molecules, such as iron or cholesterol. Proteins carrying these nutrients are ligands for these receptors. Once the receptor binds to its ligand, the receptor will move to a region of the plasma membrane containing pits coated with the protein clathrin (Goldstein et al., 1985). The clathrin coat helps to deform the plasma membrane, making it easier for the receptors and their ligands to be internalized. Once inside, ligands and their cargo can be used by the cell.

The transferrin receptor, which plays an important role in cellular uptake of iron from outside of the cell, is an example that illustrates the process of RME (Ponka 1999). Transferrin is a protein that binds to two iron ions. When a cell requires iron, it upregulates expression of the transferrin receptor. Once at the cell surface, the transferrin receptor can bind transferrin and multiple receptors will move to the clathrin-coated pit. Subsequently, the plasma membrane invaginates and the complexes are internalized

inside a membrane-bound vesicle. Finally, the vesicle fuses with an endosome. The acidic environment of the endosome causes the iron to be released from the transferrin and the receptor. The iron is then reduced from Fe^{3+} to Fe^{2+} by endosomal reductase and exits the endosome through divalent metal transporter-1 (DMT1). This iron may now be used by the cell.

Existing Gene Delivery Methods

Gene delivery is the process of introducing foreign DNA/RNA into a specific cell. Current methods of gene delivery include the use of viral vectors, liposomes, electroporation, particle-mediated gene transfer, and microseeding. While these methods can successfully deliver selected nucleic acids into cells, each method has undesirable features, which include deleterious immune responses, lack of stability and specificity of the delivery method, high rates of cell death, and insufficient diffusion into the cell. These issues necessitate the development of new, efficient and precise vectors for RNA delivery.

Two types of commonly used viral vectors are based on retroviruses and adenoviruses. Retroviral vectors are single-stranded RNA viruses which can be used to insert genetic material into cells. The technique of using retroviral vectors was first tested by transferring a human growth hormone gene to keratinocytes. Once transduced, the keratinocytes secreted the human growth hormone and were able to form an epidermal layer when grafted onto mice (Morgan, Barrandon, Green, & Mulligan, 1987). While retroviral vectors can potentially result in stable and long-term gene expression, one disadvantage of the method is that they only deliver genetic material to dividing cells.

Another disadvantage is that they may cause cell transformation as a result of random integration into the host cell chromosomes (Yao & Eriksson, 2000).

Unlike retroviral vectors, adenoviral vectors are double stranded DNA viruses that can infect both dividing and non-dividing cells. They are not incorporated into the host chromosomes. However, a major drawback to this method is that the vectors often cause strong immune responses against the transformed cells because of the presence of viral antigens (Yao & Eriksson, 2000). This immune response can be tragically severe: in September 1999, a patient given a high dose of adenoviral vector died because of the toxicity (St George, 2003). The precautionary measures required when administering adenoviral vectors to treat humans limit the scope of this approach.

Nonviral gene delivery carriers such as polycationic vectors and protein transduction domains have been developed to combat some of the safety risks associated with viral vectors. Polycationic vectors such as polythyleneimine have a strong ability to bind and deliver plasmid DNA in vitro. However, DNA transfer with polycationic vectors in vivo has not been as efficacious, and increased cytotoxicity is observed at high concentrations (An & Park, 2013). Protein transduction domains are proteins consisting of positively charged amino acids that interact with negatively charged lipids during cell membrane penetration. One type of protein transcription domain is the human transcription factor Hph-1. This protein transduction domain has demonstrated almost no cytotoxicity even at high concentrations, though its transfection efficiency was lower than that of polycationic vectors (An & Park, 2013).

Liposomes are another gene transfer technology. Cationic liposomes are vesicles that have a positively charged surface, allowing them to bind to negatively charged DNA

as well as to other negatively charged molecules found on cell surfaces (Branski, Pereira, Herndon, & Jeschke, 2007). The effectiveness of this method was initially tested by creating a liposomal complex containing the beta-galactosidase gene and topically applying it to mouse skin. The liposome successfully carried the gene into the skin. The gene was expressed in the epidermis, the dermis, and hair follicles for up to seven days after the initial application (Alexander & Akhurst, 1995). Although liposomes are more stable than viral vectors and interact more readily with the plasma membrane, some constraints on their use include chemical and physical instability, short shelf-lives, and non-specific uptake by the cells (Sharma & Sharma, 1997).

Electroporation is a non-viral gene delivery technique commonly used to transfect cells. It involves electrical stimulation that increases the permeability of the plasma membranes so that large molecules such as DNA can enter the cell (Lee, Chesnoy, & Huang, 2004). One study used electroporation to deliver the TGF-β1 gene into mice. TGF-β1 encodes a growth factor that increases rates of wound-healing. Researchers found that all aspects of wound healing were enhanced in the mice. However, disadvantages to the use of electroporation include high rates of cell death, and therefore, the requirement for many cells and relatively large amounts of RNA (Gonzalez, Pfannes, Brazas, & Striker, 2007).

Gene guns, otherwise known as particle-mediated gene transfer, also deliver naked DNA to tissues or cells. In this technique, nanoscale gold particles are coated with DNA and shot through plasma membranes. This direct penetration allows the DNA to reach the cytoplasm and even the nucleus (Niidome & Huang, 2002). One experiment used gene guns to transfer human epidermal growth factor (hEGF) to wounded

keratinocytes (Andree et al., 1994). The wound fluid was later analyzed for hEGF, and the results showed that the wounds treated with the DNA-coated gold particles had a 190-fold increase in EGF concentration compared to the control wounds. They also healed 20% faster than the controls, and the DNA remained in the wound site for 30 days. This experiment indicated that gene gun technology was particularly useful for in-vivo gene transfer, such as DNA vaccinations and anti-cancer therapy (Yao & Eriksson, 2000). A constraint of this method is that it does not penetrate deep into tissues (Niidome & Huang, 2002).

An alternative delivery system that is more efficient than particle-mediated gene transfer is called microseeding. Microseeding consists of solid microneedles that are attached to a piston. Plasmid DNA is placed on the tips of the needles through tubing attached to a syringe. The size of the needles can be adjusted depending on the depth of penetration desired for the experiment, thus addressing a limitation of gene guns (Yao & Eriksson, 2000). Microseeding is also more effective than gene guns because they do not deposit foreign materials, e.g. gold beads, into the cell (Yao & Eriksson, 2000). These techniques were compared in experiments treating pig wounds with a plasmid encoding human epithelial growth factor (hEGF). The protein concentration of hEGF for each method was measured using an Enzyme Linked Immunosorbent Assay (ELISA). The assay showed that the expression of hEGF in the microseeded wounds was two to three times higher than the expression of the growth factor in the gene gun wounds (Eriksson et al., 1998). A drawback to microseeding is that the structure of the devices must be customized to the specific therapeutic RNA that is being delivered, as well as to the target

site (Reed & Lye, 2004). One device cannot be used to deliver different types of therapeutic agents, thus reducing the efficiency.

Each of these methods has successfully been shown to deliver nucleic acids into cells and produce the desired outcome: changes in gene expression. However, these techniques all have disadvantages. These problems are the driving forces behind the search for new and alternative gene delivery systems that can effectively and efficiently transfect therapeutic nucleic acids into cells without damaging the cells or tissues. Our project strives to improve the specificity of the gene delivery method, prevent damage to the cell, and promote prolonged alteration of gene expression. Our proposed technique aims to combat the limitations of these existing methods by exploiting the natural process of receptor-mediated endocytosis. Since receptor-mediated endocytosis is a natural cellular process, we predict that it will cause less harm to the cell than currently employed gene delivery methods (Wileman, Harding, & Stahl, 1985). Additionally, the current project will minimize the challenges of specifically targeting the gene of interest and preventing transfection of other healthy cells.

Receptor-Mediated Endocytosis

Receptor-mediated endocytosis is the uptake of soluble molecules into the cell (Wileman, Harding, & Stahl, 1985). For transport receptors, such as those that bind transferrin, receptor-mediated endocytosis transports the molecules inside the cell to be utilized. With signaling receptors, such as those that bind cytokines (cell signaling molecules), endocytosis internalizes the ligands that contain information and creates a new stimulus that induces a cellular response (Shankaran, Resat, & Wiley, 2007). Overall, receptor-mediated endocytosis involves the transport of specific bound ligands from the

surface of the cell to their targets in the interior of the cell. Sometimes the internalized receptors are targeted to lysosomes, where they are broken down. In other examples, the receptors are recycled back to the plasma membrane (Wileman, Harding, & Stahl, 1985).

As discussed in Alberts (2008), most receptor-mediated endocytosis depends on clathrin. Clathrin is a Y-shaped protein that associates with other proteins to form soccer ball-like latticework pits on the cytosol side of the plasma membrane. These structures are known as clathrin-coated pits. Clathrin-coated pits contain various receptors on their surface and constantly pinch off into the cell. If a ligand is bound to one of these receptors, then it is taken into the cell. This form of endocytosis is particularly useful because it allows the cell to selectively internalize molecules instead of ingesting every molecule in range of the pit.

Selected Receptor-Ligand Pairs

As a baseline investigation, we chose to assess the success of this delivery system with the receptor CXCR1 and the chemokine Interleukin 8 (IL-8). We chose a chemokine/chemokine receptor pair because Biragyn et al. (2013) demonstrated successful decreases in gene expression when delivering siRNA through the CCR4 receptor using a CCL17 delivery vehicle protein. Chemokine IL-8 is expressed throughout the body and in different types of cells, particularly by white blood cells of the immune system. IL-8 affects cells' chemical response and induces phagocytosis at sites of infection (Garofalo et al., 1996). IL-8 also contributes to angiogenesis, the creation of new blood vessels. This process is involved in diseases that deplete or damage blood vessels, and it has been linked to treatment for rheumatoid arthritis, tumor growth and wound repair (Koch, 1992).

Another receptor-ligand pair that the team examined was the Machupo virus (MACV) glycoprotein 1 (GP1), which interacts with the transferrin receptor 1 (TfR1). MACV is a pathogenic New World arenavirus that causes hemorrhagic fever and has a 10-30% fatality rate (Abraham et al., 2009). Arenaviruses are enveloped viruses that contain single-stranded and bi-segmented RNA. The glycoprotein complex associated with the surface of these viruses has three segments: the stable signal peptide (SSP), GP1, and GP2 (Abraham et al., 2009). The segment of interest for this project, GP1, is the subunit that attaches to the TfR1 receptor, through which the virus gains entry into the cell. The primary role of TfR1 is the uptake of iron into cells through endocytosis (Abraham et al., 2010). Studies on the structure of the GP1:TfR1 complex have shown that variability in the amino acid sequence in the GP1 subunit corresponds to differences in sequences of the TfR1 receptor. This suggests that that the glycoproteins are very specific and have adapted to their hosts, thus enhancing their ability to cause human disease (Abraham et al., 2010). This receptor-ligand pair is of interest in this project because of its lack of specificity: since all cells express TfR1, it is potentially capable of delivering therapeutic RNAs to all cells. In addition, there are no currently FDA-approved treatments to stop the spread of the MACV virus (Radoshitzky et al., 2011). Therefore, understanding the interactions between GP1 of MACV and TfR1 and using RNA silencing to stimulate gene repression can potentially contribute to the prevention of these viral infections and development of new treatments for those already infected.

Developing the Recombinant Proteins

The ligands and attached components were developed through the use of the yeast *Pichia pastoris* and the bacterium *Escherichia coli*. *P. pastoris* is commonly used for

industrial scale expression of recombinant proteins because it is easy to manipulate genetically. It also only secretes a limited number of proteins, which simplifies the purification of secreted recombinant proteins. (Cregg et. al., 1985). As *Pichia* is an eukaryote, it is able to do protein processing, folding, and post-translational modifications. Finally, *Pichia* generally yields higher expression levels of proteins (Higgins, 2001).

Pichia were transformed with a plasmid that codes for all of the components of the delivery vehicle. *E. coli*, commonly used in recombinant DNA research, is employed to replicate the plasmid for further use (Hill et. al., 1986).

The Project

The project proposed herein seeks to develop a better way of delivering therapeutic siRNAs into specific types of cells. To accomplish this goal, we have exploited the natural mechanism of receptor-mediated endocytosis, in which cells selectively ingest substances from their environment.

Cells interact with their environment through plasma membrane-associated receptor proteins. Some receptors are expressed on a wide variety of cells, while other receptors are specifically expressed on only one kind of cell. Each receptor has a ligand, a small specifically shaped molecule to which the receptor will selectively bind.

When a ligand binds to a receptor, it can initiate a variety of responses. The binding can trigger signaling pathways that eventually lead to some sort of cellular response, e.g. cell death, proliferation, alteration of gene expression profiles, etc. Receptor-ligand binding can also cause a cell to initiate the process of endocytosis, or the

uptake of molecules into a cell, through a process known as receptor-mediated endocytosis (Shankaran, Resat, & Wiley, 2007).

If there was a specific receptor for a specific siRNA, then it would be easy to use receptor-mediated endocytosis to deliver that siRNA into a cell and silence genes. Unfortunately, no such siRNA receptors are currently known. In order to take advantage of receptor-mediated endocytosis, the therapeutic siRNA of choice would have to be artificially coupled to a ligand with a known receptor.

This problem was addressed by Biragyn et al. (2013), who created a technique to produce this coupling. A siRNA-binding protein derived from the Hepatitis B virus was linked to CCL17, a ligand that binds to the CCR4 receptor on T-cells. The recombinant protein was then allowed to bind to siRNAs designed to target the mRNAs encoding IL-10 or FOXP3. Completed cassettes were tested *in vitro* and *in vivo* to examine whether these delivery vehicles could effectively decrease IL-10 and FOXP3 expression. It was found that these genes were temporarily inactivated for approximately 4 days. Since IL-10 and FOXP3 are involved in cancer metastasis, downregulating the two proteins was sufficient for the investigators to observe changes in cancer metastasis (Biragyn et al., 2013).

The Biragyn study was limited to CCR4-expressing T-cells, specifically a well-established cell line called CEM cells. However, many other kinds of cells are commonly used in laboratories, including HEK293T cells, HeLa cells, HUVEC cells, primary human fibroblasts, induced pluripotent stem cells, and astrocytes. Specific receptors and ligands for each of these cells have been identified in the literature. Since this delivery system is modular and can be produced with different ligands and siRNA, it can

theoretically be modified in order to deliver therapeutic RNAs to a wide variety of cells: all that is required is knowledge of a specific receptor expressed on the cell of choice, and the ligand with which it interacts.

Quantifying Gene Silencing

The use of receptor-mediated endocytosis to introduce therapeutic siRNA into cells is a useful approach, particularly when coupled with a recombinant protein designed to deliver therapeutic nucleic acids to cells expressing a specific receptor. However, it is also important to demonstrate that this procedure is not only able to introduce the siRNA into a target cell, but that it can cause changes in one specific gene expression. To ensure that the siRNA target a specific gene, siRNAs that have been verified by the manufacturer and literature to target only a specific gene will be delivered.

Two commonly used techniques to assay gene expression are quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Immunoblotting ('Western Blot').

qRT-PCR is a method of quantitatively measuring mRNA abundance. mRNA is isolated from a cell, converted to complementary DNA (cDNA) using reverse transcriptase enzyme and deoxynucleotides (dNTPs), and amplified hundreds of thousands of times using a thermal cycler. The rate of the appearance of a product is measured using either a variety of dyes or by hybridization with enzymes that produce fluorescence or luminescence. Lastly, the data are used to calculate how much of a specific mRNA was originally present in the sample (Freeman, Walker, & Vrana, 1999).

In contrast to qRT-PCR which detects RNA, Western blotting is a technique that detects the proteins produced from mRNA templates. According to Kurien and Scofield (2006), it involves extracting proteins from a sample and then separating them by size

with polyacrylamide gels. Subsequently, the size separated proteins are transferred from the gel to a membrane, effectively making a copy of the gel. The membrane can then be probed for specific proteins, typically using antibodies. To detect the bound protein-antibody complex, fluorescent, luminescent or radioactive tracers are used to produce signals that can be detected using X-ray film or charge-coupled devices (CCDs).

By using qRT-PCR and Western Blotting, the silencing effect of the delivered therapeutic RNA can be assessed at two levels: mRNA and protein.

Comparison with Other Methods of Gene Delivery

An important advantage of the receptor-mediated endocytosis based method of conducting gene therapy is its potential for use with primary cells. Electroporation in particular, has a severely adverse effect on cells: it causes many cells to die, requiring investigators to start with large amounts of cells and RNA (Gonzalez, Pfannes, Brazas, & Striker, 2007). In contrast to electroporation, all cells naturally perform receptor-mediated endocytosis. Consequently, cells do not have to undergo intensely damaging treatments and researchers can potentially alter gene expression even in fragile primary cells.

Particle-mediated gene transfer and microseeding put less stress on cells, but particle-mediated gene transfer is not reliable as it cannot fully penetrate a tissue (Niidome & Huang, 2002) and deposits foreign gold particles into a cell (Yao & Eriksson, 2000). Microseeding is more efficient, but still causes “thousands of puncture sites in a random fashion” (Eriksson et al., 1998). Receptor-mediated endocytosis, on the other hand, does not poke holes in the plasma membrane. Instead, endocytotic vesicles pinch off from the plasma membrane and maintain membrane integrity (Alberts, 2008).

Viral vectors and liposomes are relatively gentle techniques, as they do not make holes in plasma membranes, and can even be used to alter gene expression in primary cells. However, retroviral vectors run the risk of causing cancerous transformation if they randomly insert into the wrong gene, while adenoviral vectors can cause a massive immune response against the adenovirus surface antigens (Yao & Eriksson, 2000). Liposomes can and have been used *in vivo* without too many side effects. However, they only alter gene expression for about a week and are prone to transfecting any cells they are introduced to, reducing the precision and selectivity of the technique (Alexander & Akhurst, 1995).

In contrast, if the right receptor is found, the types of recombinant delivery vehicle proteins described in the current work potentially have the power to silence gene expression in specific types of cell. Although Biragyn et al. (2013) observed gene silencing for only four days, they noted that because endocytosed receptors are recycled back to the plasma membrane, this technique could be used to treat cells multiple times.

Conclusion

Gene therapy has huge implications for how injury and disease are treated. Current gene alteration methods have had some success but are damaging to cells, resulting in minimal expression or very short-term effects.

The goal of this project is to improve the efficiency of gene delivery by utilizing components and processes that are naturally employed by cells, including the cell's natural receptors and corresponding ligands. We cataloged receptors and ligands in cells of interest, produced a delivery vehicle protein, and tested our selected method of delivery for its ability to alter gene expression.

This research has potential applications both in simplifying biomedical research and in treating a wide range of diseases, including cancers and wounds. As the fear of side effects dissuades many people from taking medication, an improved delivery method for therapeutic RNA has the potential to be a milder alternative to existing medication and could one day significantly improve healthcare and disease treatment. We envision this delivery vehicle being used as a laboratory reagent to selectively deliver siRNAs for basic research more efficiently than current methods. Additionally, it has the potential to be used as a therapeutic agent in treating diseases through changing gene expression in affected cells.

Methodology

The overall methodology (Figure 1) is divided into three main phases– plasmid production (Figure 2), recombinant protein production (Figure 3), and transfection.

E. coli-based Methods

A linear vector was generated from a previously generated pPIC9 plasmid (Invitrogen) containing a sequence encoding TARC between *Xho* I and *Xma* I restriction sites. To excise the TARC sequence, the plasmid was digested with *Xho* I and *Xma* I restriction sites and the plasmid backbone was gel purified.

Sequences coding for human chemokine IL-8 (NM_000584) or Machupo virus-derived membrane protein glycoprotein 1 (GP1) were fused in frame with a single DNA/RNA-binding domain (RBD) of the core antigen of the Hepatitis B Virus (HBV) contained in the pPIC9 plasmid backbone. Both sequences were identified using the NCBI database and obtained as gBlocks gene fragments from Integrated DNA Technologies, Inc. (IDT). Sequences encoding the IL-8 and GP1 sequences fused with HPV1-RNA binding domain were cloned using the same *Xho* I and *Xma* I restriction sites as a fusion with the yeast α-factor signal sequence. These sequences were ligated into the linear vector backbone to create two plasmids named pPIC9_IL8 (Figure 4) and pPIC9_GP1 (Figure 5).

To confirm successful ligation of the IL-8 and GP1 sequences with their linear vector backbones, primers containing the *Xho*I and *Xma*I restriction sites were designed using ApE software (apesoftware.com). Both forward and reverse primers (Table 1, rows 1 and 2) were obtained from IDT. The two primers were used to amplify the sequences

coding for IL-8 or GP1 using Polymerase Chain Reaction (PCR). The PCR products for both sequences were verified by DNA sequencing (GENEWIZ).

Highly competent DH5α cells were transformed with the recombinant plasmids pPIC9_IL8 and pPIC9_GP1. The cells were spread into petri dishes containing 1% agar, Lauria broth, and 50 µg/mL carbenicillin to select for transformants. After incubation, colonies were picked and spotted onto their respective master plates. Subsequently, each respective colony was grown in liquid culture overnight, after which the candidate pPIC9_IL8 and pPIC9_GP1 plasmids were extracted. Intact plasmids were initially separated through 1% agarose gels to verify that intact plasmids were maintained in the DH5α cells. Plasmids were digested using *Xho* I and *Xma* I and run through a second 1% agarose gel to screen for recombinant plasmids. Only colonies with 297 bp fragments corresponding to IL-8, and 488 bp fragments corresponding to GP1 were selected for transformation into *Pichia*. The plasmid harboring the IL8-based construct was named pJD1965, and the plasmid harboring the GP1-based construct was named pJD1966. Bacterial strains harboring these plasmids were stored in glycerol at -80°C.

Successfully transformed E. *coli* containing pJD1965 (IL-8) and pJD1966 (GP1) were removed from -80°C storage and streaked onto for single colonies onto two LB plates containing 30 µg/mL of carbenicillin and incubated overnight at 37°C. Single colonies from each were inoculated in several LB culture tubes containing 30 µg/mL carbenicillin to ensure selection for the plasmids. The cultures were grown for twelve hours in a 37°C shaker. Plasmids were extracted using Maxiprep kit (Qiagen). The extracted plasmids were linearized using *Sal* I and compared to undigested plasmids using agarose-gel electrophoresis. The digested plasmids were then precipitated overnight

with ethanol and stored at -20°C. This process was repeated multiple times to produce a sufficient amount of the plasmids.

Pichia pastoris-based Methods

The methanol-inducible *Pichia Pastoris* expression kit from Invitrogen was used to produce both recombinant proteins. The proteins produced from pJD1965 were named IL8-ARP and the proteins produced from pJD-1966 were named GP1-ARP.

The GS115 strain *Pichia pastoris* was transformed with linearized pJD1965 and pJD1966 based on the Invitrogen protocols. Freshly grown GS115 *Pichia Pastoris* containing a defective *his4* gene were streaked onto YPAD plates and grown at 30°C. Colonies were inoculated into YPAD liquid media and serially passaged for another three days. Each day, colonies were inoculated into fresh YPAD media.

The LiCl method was used to transform the GS115. The cells were pelleted by centrifugation, supernatant was decanted, and the cell pellet was suspended in 1 mL of 100 mM LiCl. The cells were pelleted by centrifugation and LiCl was removed with pipet. The cells were resuspended in 400 uL of 100 mM LiCl and pelleted by centrifugation a third time. The LiCl was removed with pipet.

For all samples, 240 μL of 50% PEG-3350, 36 μL of 1M LiCl, and 25 μL of single-stranded fragmented salmon sperm DNA (2 mg/mL), and 50 μL of plasmid (100-200 ng/μL) were added in the order listed. As a negative control, 50 μL sterile water was used in place of the plasmid. As a positive control, yeast previously transformed with TARC were subjected to the same transformation process with 50 μL sterile water instead of a plasmid.

All samples were incubated in a 30°C water bath for one hour, then heat-shocked in a 42°C water bath. Tubes containing the cells were gently inverted every 10 minutes to prevent mixtures from separating. The cells were then streaked on solid media plates containing synthetic complete media lacking histidine (SD –His) and were grown in a 30°C incubator for three days.

The individual His^+ colonies were streaked to a –His master plate. Each transformed colony was grown in –His media for three days. The genomic DNA samples were extracted based on the Smash and Grab method and stored at -20°C (Rose, Winston & Hieter, 1990).To identify colonies in which the linearized plasmids had integrated into the HIS4 locus, polymerase chain reactions were performed with Taq polymerase master mix (Thermo Scientific) and primers for PIC9-IL8 and PIC-GP1 (Table 1, rows 5–8). PCR results were visualized using gel electrophoresis to identify colonies in which plasmid insertions into the *his4* loci on both chromosomes had occurred. All the "double insertion" colonies were amplified in liquid –His media and stored in 50% glycerol at -80°C for later use. The three PIC9-IL8 colonies were named YJD 1711-1713, and the two PIC-GP1 colonies were named YJD 1714-1715.

Protein Production

The frozen double insert colonies were streaked onto –His plates, and after three days growth, single colonies were picked and grown in 5 mL of liquid –His media for two additional days at 30°C. The *Pichia* were transferred to 2L baffled flasks containing 500 mL YPAD and grown for 18-24 hours to an O.D. 600 nm of 4-7. The cells were pelleted by centrifugation and the YPAD supernatant discarded. The pellet was re-suspended in minimal methanol media containing 400 mL water, 1 mL 500X biotin

(0.02% biotin), 50 mL 10X methanol (5% methanol), and 50 mL 10X Yeast Nitrogen Base with ammonium sulfate and without amino acids (13.4% YNB). The new mixture was incubated in a 30°C shaker for six hours. Subsequently, recombinant protein production was induced by pumping 100% methanol into the cultures at a rate of 9 microliters/minute for 36 hours at 30°C. Because the delivery vehicle plasmid has a methanol-inducible promoter, pumping methanol induced the yeast to produce the recombinant protein. Furthermore, because the delivery vehicle plasmid also has a secretion signal, the recombinant protein was secreted into the media.

Protein Purification

After undergoing 36 hour induction of recombinant protein production, the yeast cultures were centrifuged and the supernatants containing the recombinant proteins were decanted and filtered through bottle-top filters with PES membranes and 0.22 μM pore size (Fisher). 25 mL of 2.0 M $NaPO_4$ (pH 8.0) was added to every 2L of supernatant to bring the pH to approximately 7.0. The supernatant was decanted into a 2L separatory funnel connected to the separation column containing SP sepharose (Sigma Aldrich) (Figure 6).

The supernatants were allowed to pass through the column, which, due to its net negative charge, bound the recombinant proteins through non-covalent interactions with their positively charged RNA binding domains. Non-specifically bound proteins were eluted from columns using wash buffer (0.15 M NaCl, 0.05 M $NaPO_4$, pH 8.0). The recombinant proteins were eluted from columns with high salt elution buffer (1 M NaCl, 0.05 $NaPO_4$, pH 8.0). The proteins were concentrated using Amicon Ultra-15 Centrifugal

Filter Devices, stored in 30 μL aliquots, flash frozen in liquid nitrogen, and stored at -80°C. The concentration of the proteins was measured using the Bradford assay.

SDS-PAGE

Both purified recombinant proteins were separated using 12.5% SDS-PAGE gels to confirm the proteins' sizes were correct. PageRuler Prestained Protein Ladder (Thermo Scientific) was used to confirm the size of the two delivery vehicle proteins.

Testing Recombinant Protein

The ability of the recombinant proteins to deliver small RNA cargos was assayed in suspension cell culture and adherent cell culture systems.

Suspension Cell Culture

CEM cells were cultured in RPMI-1640 ++ media (10% Fetal Bovine Serum, 1X Penicillin and Streptomycin) on untreated culture flasks under sterile conditions in a tissue culture hood. Cells were incubated at 37°C and were transferred to fresh media every 2-3 days.

Suspension Cell Transfection Protocol

CEM cells were used for suspension cell transfection experiments. CEM cells were chosen because they express both the transferrin receptor and CXCR1, the IL-8 receptor. Thus, they are suitable for transfection with both GP1-ARP and IL8-ARP.

The day before transfection, cells were spun down and resuspended in fresh media to a concentration of $5x10^5 – 7x10^5$ cells/mL. The day of transfection, cells were spun down and resuspended to a concentration of $1x10^6$ cells/mL. 250 μL cells were added to each well of a 48-well untreated plate.

The transfection reagent mix was prepared the day of transfection (250 μL reagent per well). To make the transfection mix, 2 μL 20 μM siRNA were added to 246 μL RPMI-1640 (No FBS, no antibiotics. Then, 2 μL of HiPerfect (Qiagen) or 2 ng of recombinant protein were added to the siRNA and RPMI mixture. AllStars Hs Cell Death Control siRNA (Qiagen) was used as the experimental siRNA. AllStars Negative Control siRNA (Qiagen) was used as the negative control siRNA.

Transfection reagents were incubated for 15-20 min at room temperature in the tissue culture hood. 250 μL of transfection reagent were subsequently added to each well. The cells were incubated at 37°C and samples were taken for analysis at specific time points (12, 24, 48 hours).

Adherent Cell Culture

HEK 293T cells were cultured in DMEM++ media (10% Fetal Bovine Serum, 1X Penicillin and Streptomycin) on treated culture flasks under sterile conditions in a tissue culture hood. Cells were incubated at 37°C and split once they reached 80% confluency.

Adherent Cell Transfection Protocol

$1x10^5$ cells were seeded into each well of a 48-well treated plate and allowed to grow overnight until they reached 70-90% confluency. Before transfection, the old media was replaced with fresh DMEM++.

The transfection reagent mix was prepared the day of transfection (250 μL reagent per well). To make the transfection mix, 2 μL 20 μM siRNA were added to 246 μL DMEM (No FBS, no antibiotics. Then, 2 μL of HiPerfect (Qiagen) or 2 ng of recombinant protein were added to the siRNA and DMEM mixture. AllStars Hs Cell

Death Control siRNA (Qiagen) was used as the experimental siRNA. AllStars Negative Control siRNA (Qiagen) was used as the negative control siRNA.

Transfection reagents were incubated for 15-20 min at room temperature in the tissue culture hood and added by drops to each well. The cells were incubated at 37°C for the appropriate time points (12, 24, 48 hours). Before harvesting, cells were examined under a light microscope for signs of stress. Cells were trypsinized and harvested from the plate.

Quantifying Transfection

The fraction of live cells was assayed at 12, 24, and 48 hour time points. Cells were stained with trypan blue and counted using the BioRad TC20 automated cell counter.

Results

Cloning of IL-8 and Machupo Virus GP1 Sequences Into pIC9

The IL-8 and GP1 gene sequences were successfully cloned into the *Xho* I and *Xma* I restriction sites of pPIC9. The pPIC9_IL8 plasmid map (Figure 4) shows the location of the newly inserted IL-8 gene sequence in the pPIC9 backbone. Similarly, the pPIC9_GP1 plasmid map (Figure 5) shows the location of the newly inserted GP1 gene sequence in the pPIC9 backbone.

Sequential digestion of the pPIC9_IL8 and pPIC9_GP1 plasmids with the *Xho*I and *Xma*I restriction enzymes shows two distinct bands on the 1% agarose gel (Figure 7). The IL-8 insert migrated through the gel as an approximately 300 bp fragment and the GP1 fragment migrated as about a 500 bp fragment. The actual base pair lengths of the IL-8 and GP1 inserts are 297 bp and 488 bp, respectively.

Plasmid Linearization and *Pichia* Transformation

The plasmids contained within each *E.coli* strain conferred resistance to carbenicillin. Through this, we ensured that selective pressure drove retention of plasmids. After the plasmids were extracted, purified, and linearized with *Sal* I (Thermo Scientific), agarose gel electrophoresis confirmed that the plasmid was linearized (Figure 8). The supercoiled DNA of unlinearized plasmids is visible in lanes 6 and 7, while the linearized plasmids in lanes 2 through 5 show singular bands at of the expected sizes.

The plasmids were then integrated in to yeast genome through homologous recombination into the *his4* locus. The transformed colonies were selected for on –His plates (Figure 9). The negative control, mock-transfected cells, did not grow on –His

medium. PCR analysis further confirmed that the sequence of the protein vehicle was integrated successfully. After extraction and purification of genomic DNA from the transformed cells, PCR was used to amplify sequences near the IL-8/GP1 protein sequence (Table 1, rows 3 and 4). The PCR results suggest that the amplified sequence contained the correct sequence inserted into the *his4* loci of the transformed cells (Figure 10 and Figure 11). The negative control, i.e. untransformed GS115 cells, showed no PCR product (lane 14) because this genome lacks sequences complementary to the primer sites. The positive control, i.e. pure plasmids, shows these PCR products (lane 13). Because *Pichia* is diploid, it is possible for plasmid integration to occur at one, the other or both *his4* loci. The double insertions colonies can be identified by the presence of only the larger PCR products, i.e. lanes 4, 6, and 11, while the presence of two bands indicates integration into only one of the *his4* loci.

Verification of Recombinant Protein

Protein concentrations were measured using the Bradford Assay. Concentrations ranged from 700 μg/mL to 2700 μg/mL, but most batches of proteins produced were at a concentration of 1000 μg/mL.

SDS-PAGE was performed on the proteins to check their size. The IL-8 delivery vehicle protein is visible in a band that roughly matches the 11 kDa size of the IL-8 protein (Figure 12, lanes 4 through 6). The GP1 delivery vehicle protein is visible in a band approximately 11 kDa (Figure 12, lanes 2 and 3), which does not match the size of the GP1 protein (22 kDa). There is a faint 22 kDa band on the first sample of GP1 (Figure 12, lane 2), which may indicate the protein was degraded or the protein was modified by *Pichia*. Even in its truncated form, there may have been a sufficient portion

of the GP1 recombinant protein to induce receptor-mediated endocytosis, which was why it was tested in cell culture.

Transfection Experiment Results

Preliminary CEM cell experiments produced highly variable results (Figure 13). The cells transfected with delivery vehicle protein and cell-death inducing siRNA did not die at a significantly higher rate. However, the HiPerfect positive control was also unable to successfully deliver cell-death inducing siRNA.

Preliminary HEK293T cell experiments produced promising results (Figure 14). At 24 hours post-transfection, the cells transfected with the GP1 delivery vehicle protein and cell death siRNA had lower viability than cells transfected with the GP1 delivery vehicle protein and scrambled siRNA negative control. At 48 hours, there were no significant differences between the experimental group and negative control.

Discussion

The sequences for the IL-8 and GP1 HPV1-RNA binding protein delivery vehicles were successfully inserted and retained in the plasmids, which were then transfected into yeast. The histidine selectable marker in the DNA of the plasmids ensured that only colonies containing the plasmids were able to grow. The methanol inducible promoter included in the sequences of the plasmids initiated the production of the proteins in the presence of methanol and the secretion signal facilitated their release from the cells. Achieving successful transformations of the correct sequences to produce the proteins signified a marked improvement in technique optimization.

Limitations

There are some important limitations to our methodology that remain to be addressed. We only had a year and a half to complete the research portion of our project. With this limited amount of time, we were not able to test a large variety of receptor-ligand and cell type combinations as would be preferred. However, the two ligands we studied met our goal of initiating the development of a catalogue of working receptor-ligand-cell combinations that will be later expanded upon by other researchers.

Another limitation of our methodology is that the uptake of RNA into the cell depends on a protein ligand interacting with a cell surface receptor. There are many types of ligands including protein, hormone, and sugar ligands, but because of the way we created our cassette, only proteins could be ligated. This somewhat limits this method of RNA delivery and lessens its versatility as a method for gene therapy. However, since there are hundreds of known protein ligand-receptor combinations, there are still a wide variety of potential cell types to which this method can be applied.

The greatest limitation to our project is that it was done *in vitro,* which does not accurately simulate the conditions of the human body. The whole point of the utility of this method of RNA delivery is that the only cells that should be affected by the treatment are those that express a surface receptor that binds to the ligand of the delivery vehicle. This allows for targeted delivery of siRNA to only certain cell types, something current gene delivery methods lack. We tested the efficiency of our RNA delivery vehicle in a medium containing cells ranging from a single cell type to three or four cell types. This did not completely simulate the environment of the human body and the results we obtained from these test may be different than what would happen *in vivo*. However, this is an issue that can easily be addressed by future research once it is proven that this RNA delivery method works effectively for singular cell types.

Challenges

We had to overcome several hurdles throughout the course of this project. We had trouble creating the plasmids for the recombinant proteins as the starting pPIC9 plasmid was very large. Its size made it difficult to achieve successful transformations in *E. coli*. We had the same problem when transfecting Pichia with these clones. It took several months to create *Pichia* strains that successfully integrated our linearized plasmids. On several occasions, improper handling of reagents led to successful clones dying before they could be properly stored, setting the project back months at a time.

Our second major challenge lay in protein production, as our GP1 protein was about 10 kDa too short. In this project, we verified insertion of the GP1 plasmid into *Pichia* using PCR-based techniques. Future experiments to sequence the insert at the

HIS4 locus are needed. Protein folding studies to determine proper folding of the ligand part of the delivery vehicle protein could also be helpful.

Additional RNA binding assays to determine the binding kinetics of the recombinant protein could be also useful in troubleshooting, as would studies to visualize the release of siRNA once the delivery vehicle protein-siRNA complex is internalized into the cell.

Our last major challenge lay in optimizing the design of our initial transfection experiments. The first experiments involved transfecting CEM cells with AGO1 siRNA to reduce expression of the AGO1 gene. Because AGO1 is part of the RNA interference pathway, knocking down AGO1 led to unanticipated downstream effects and inconclusive data about the success of the transfection.

To overcome these challenges, we shifted the focus of the project slightly. Instead of delivering AGO1 siRNA, we delivered apoptosis-inducing siRNA. The benefits of delivering apoptotic siRNA were two-fold. First, there are no unknown effects to apoptosing cells, as cells can only remain alive or die. The second benefit was a significantly expedited analysis of our transfection experiments. To analyze the AGO1 siRNA experiments, we had to extract mRNA and quantify levels of AGO1 mRNA through qRT-PCR, a very time-consuming and labor-intensive process. In contrast, for the cell death siRNA transfections, we were able to simply measure the percentage of live cells. This relative ease of analysis allowed us to take measurements and get a more detailed look at the effect of our protein over time.

We had additional trouble with the CEM cells, which are notoriously difficult to transfect using methods other than electroporation (Fyrberg & Lotfi, 2010). Even when

using a commercial reagent as a positive control, only a fraction of the initial CEM transfections were successful. Also, the Trypan Blue assay used to measure CEM cell viability may have under-measured the percentage of dead cells. Due to these limitations, we moved to testing the recombinant delivery vehicle protein in HEK293T cell culture, which produced promising results at 24 hours post-transfection.

As our cell culture experiments were in their preliminary stages, further optimization of siRNA concentrations, protein concentrations are needed. Additional experiments to determine why the HiPerfect positive control was not working, or experiments using a different positive control reagent, are also needed. The time points measured also need to be optimized. The HEK293T cells showed significant decreases in cell viability 24 hours post-transfection, but not 48 hours post-transfection. One explanation for these results could be that the cells recovered from the first delivery of cell-death inducing siRNA by the 48 hour time point. Therefore, the optimal window to measure transfection efficacy needs to be determined. Finally, experiments with multiple deliveries of siRNA could also be highly informative.

Significance of Research

This research project enhanced the specificity of techniques altering gene expression so that only particular cells of interest would be targeted. Existing treatments, i.e. chemotherapy, target cells that share similar characteristics, which result in healthy cells also being killed by the treatment. By studying the IL8 receptor and ligand, we aimed to demonstrate the specificity of the delivery vehicle protein and turn off gene expression only in cells containing the IL8 receptor. The specificity of this receptor-ligand pair was compared to that of the GP1 protein, which targets the transferrin

receptor found on all cells. By identifying receptors specific to particular cells, therapeutic RNAs can be utilized to target solely those cells, thereby preventing harm to other healthy cells. In cancer treatment, this technology can be used to target a receptor only found on the tumor cells, which will allow other fast-growing cells such as hair cells targeted in current treatments to survive.

Furthermore, as mentioned earlier existing gene therapy techniques can cause significant changes to the cell, i.e. damage to the membrane or cell death. The developed protein described in this paper prevents these harmful side effects by utilizing the process of receptor-mediated endocytosis in order to be internalized into the targeted cell. Since this is a natural cellular process, it is less likely cause damage to the cell. Studies have shown that receptor-mediated endosomal uptake of therapeutic RNAs is the most efficient uptake pathway since it eliminates the risk of cell membrane disruptions by direct cell membrane penetration methods (Haussecker 2014). Thus, not only does the developed delivery vehicle protein contribute to the specificity of gene expression alteration, it also promises more efficient delivery of therapeutic RNA.

Future Directions

The next step in advancing this technology is to expand the catalogue of receptor-ligand pairs that have been tested. As data is gathered on receptors of additional cell types, the technology can be applied to an expanding number of disease states in the body. For example, Chemokine Receptor type 4 (CXCR4) is involved in many disease states including 23 types of cancer and several immunodeficiency disorders. In the example of cancer, when cancer cells express CXCR4, the receptor is still able to interact with CXCL12. This leads to cancerous cell retention in tissues full of CXCR12 and

allows for the growth and spread of tumors. By using receptor-ligand interaction, we can introduce RNA that inhibits CXCL12 receptors, which will in turn stop the growth and spread of tumor cells.

This method can also be applied where other forms of gene therapy have not been effective. For example, mutations in skin grafts produced as a result of retroviral vectors could be avoided by treating existing cells to minimize damage to the skin (Yao and Eriksson, 2000). Another common method, electroporation, used to deliver skin-healing growth factors (Lee, Chesnoy, & Huang, 2004), resulted in high rates of cell death that could be avoided by this method, which is much gentler because it utilizes natural cell processes (Gonzalez, Pfannes, Brazas, & Striker, 2007). As this technology is expanded, it must also be optimized.

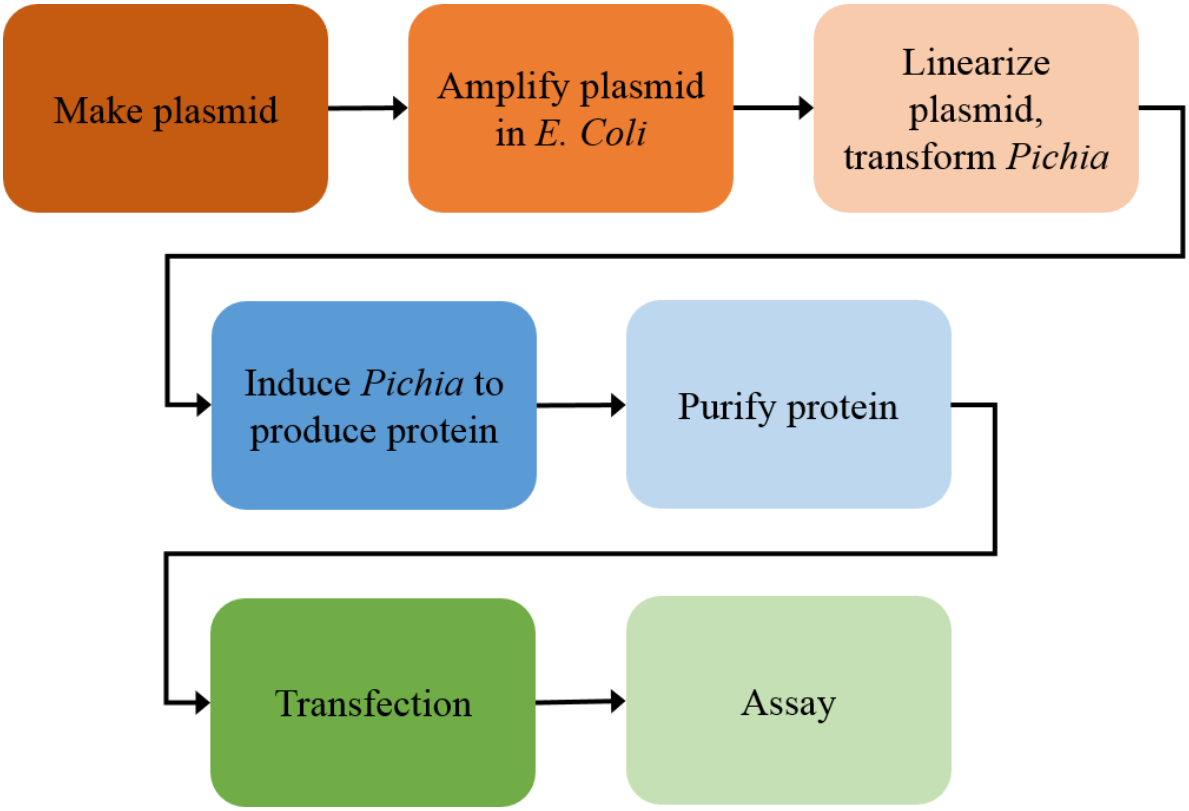
Another possible optimization comes from the ligand. There are many smaller peptides and molecules that can bind to receptors on cell surfaces as well as the full length peptides of our ligands. For example, OX26, a transferrin receptor monoclonal antibody, could be used instead of GP1 for binding to transferring receptor. The OX26 has been used extensively in similar delivery into cells with transferrin receptor (Ulbrich et al., 2009). The ligand has smaller size than natural ligand, as well as having higher binding affinity.

Following many iterations and significant testing over the course of the next few years, the project will be developed enough to consider its clinical applications. One rising clinical application of gene therapy is ex vivo treatment, removing cells from the body to treat them with gene therapy and then replacing the healthy cells into the body, particularly in the case of treatments for immune diseases (Hacein-Bey-Abima et al.,

2002). When ex vivo methods are not yielding positive results, another growing use in treatment is intraoperative gene therapy (Mann et al., 1999). Progression toward clinical applications will also include validating the specificity of the treatment in vivo. One approach to increasing the reach of the project is to produce the protein commercially as an intermediate step. This way it can be marketed for in vitro laboratory uses in order to receive feedback that can contribute to the optimization of the technology. The mechanism for marketing the product exists in the framework of Birich Tech, the start-up company that currently holds the patent for the origin paper on the method.

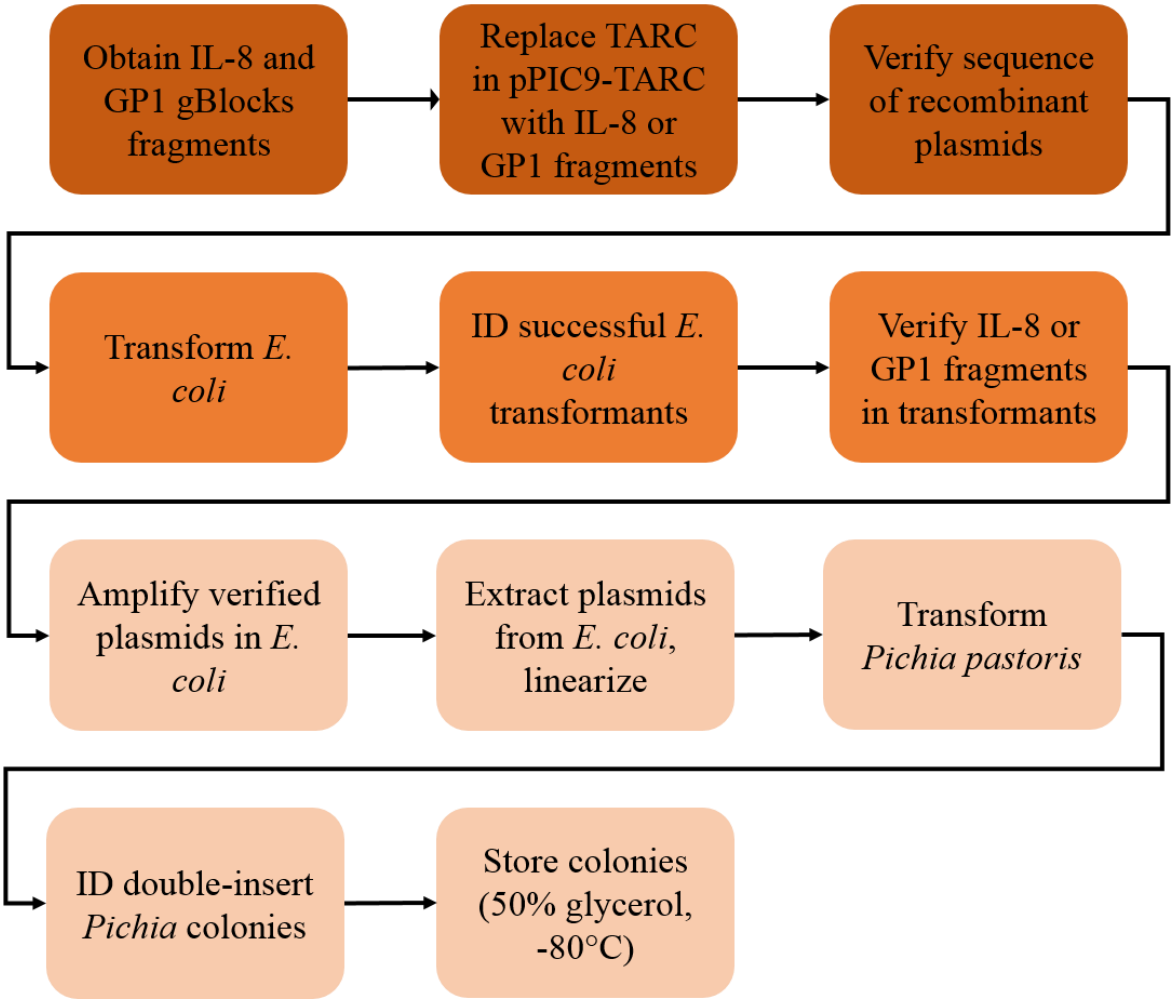
Appendix A: Figures and Tables

Figure 1



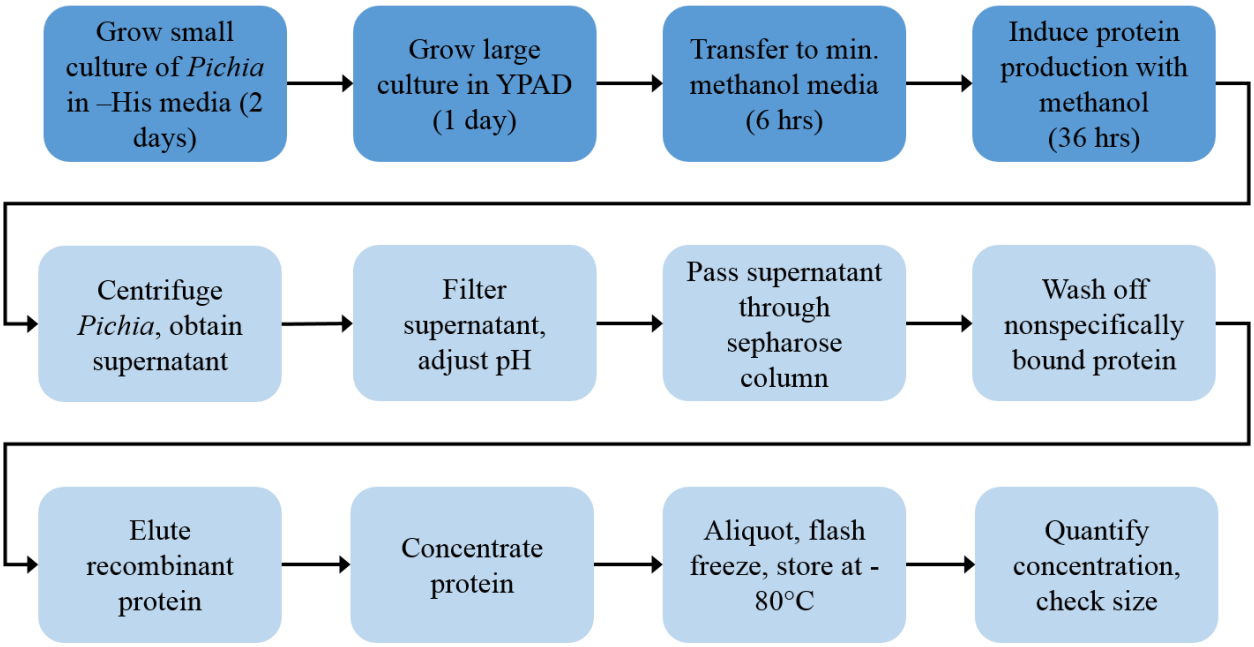
Project overview.

Figure 2



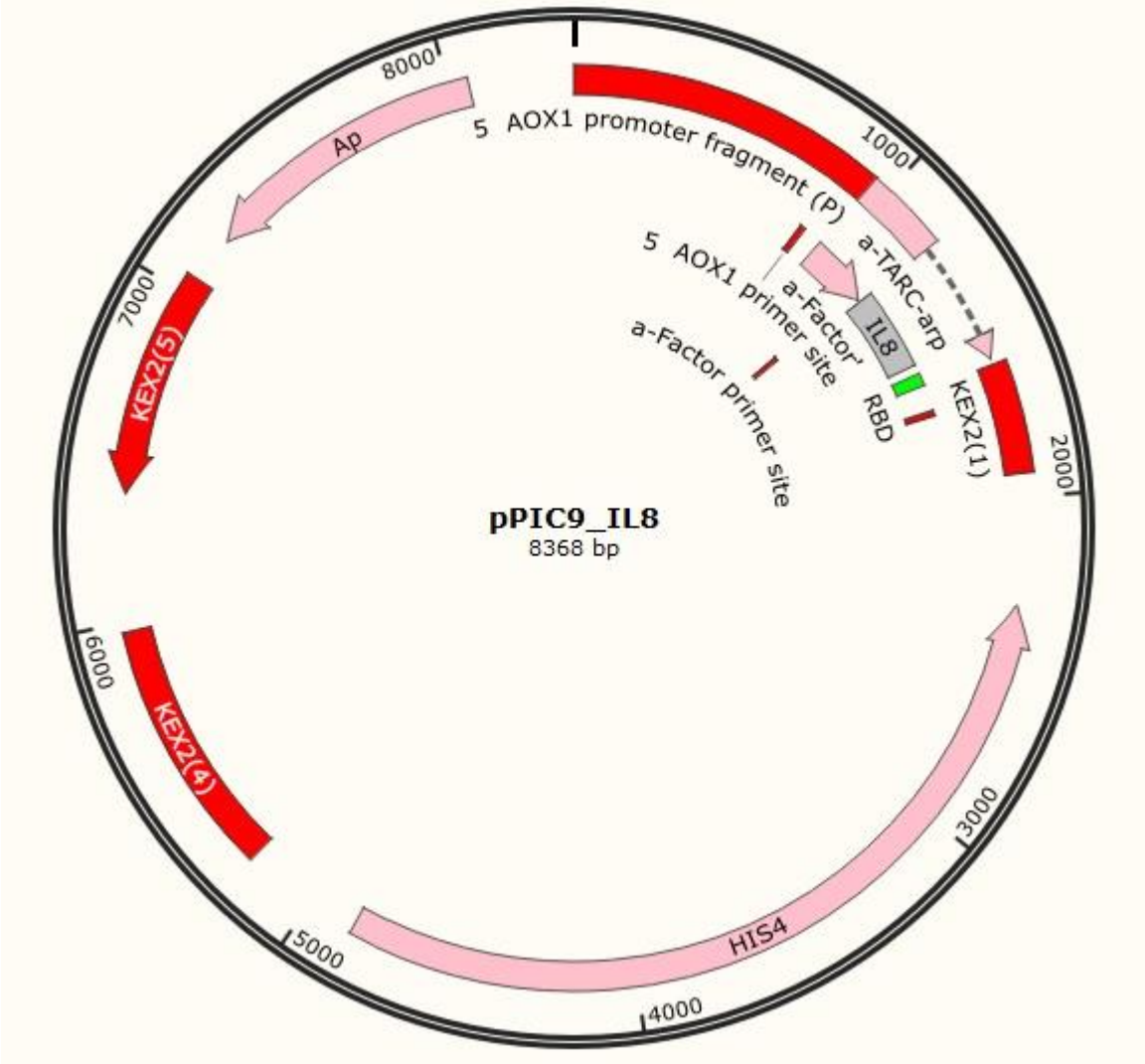
Overview of *E. coli* and *P. Pastoris* based methods.

Figure 3



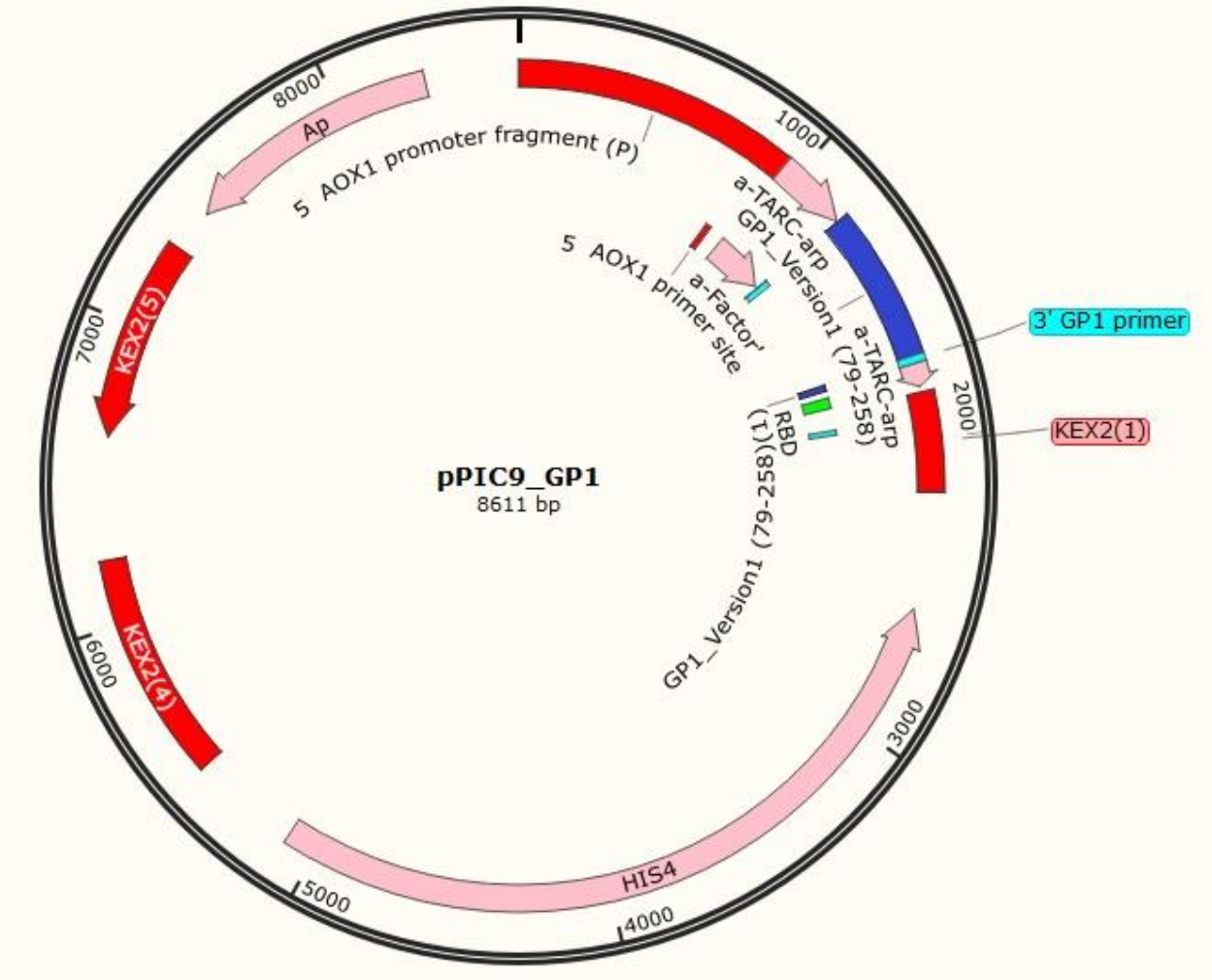
Overview of protein production and protein purification.

Figure 4



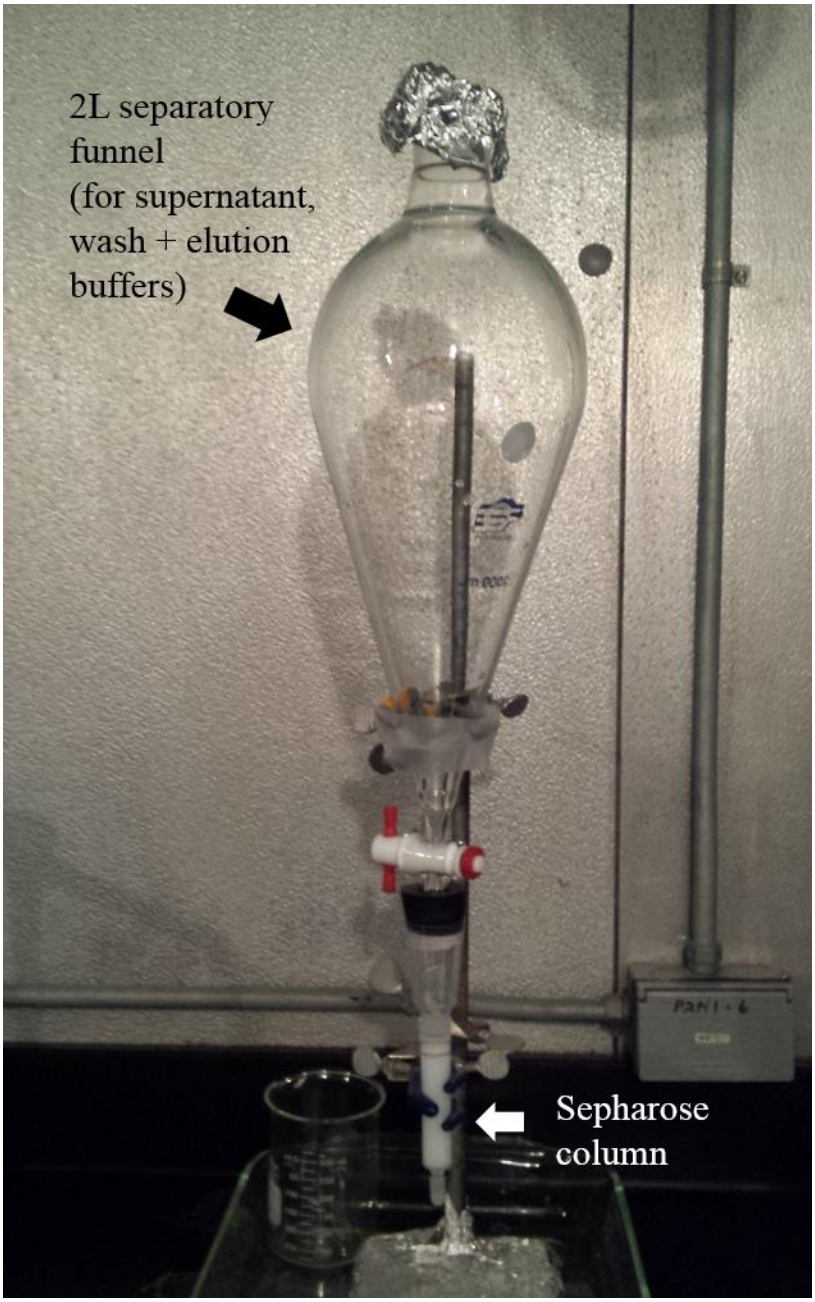
Plasmid map of newly cloned PIC9_IL8 plasmid containing Interleukin 8 sequence. Snapgene software used to create plasmid image.

Figure 5



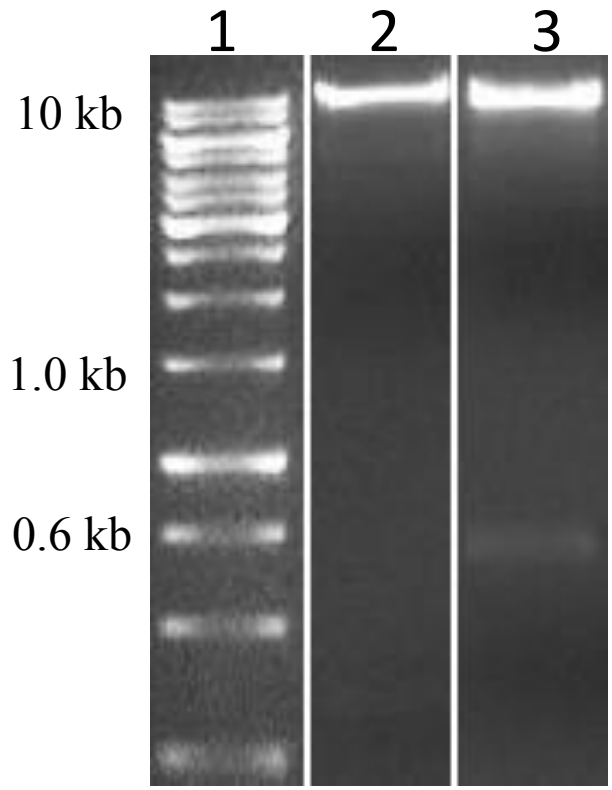
Plasmid map of newly cloned PIC9_GP1 plasmid containing Glycoprotein 1 sequence. Snapgene software used to create plasmid image.

Figure 6



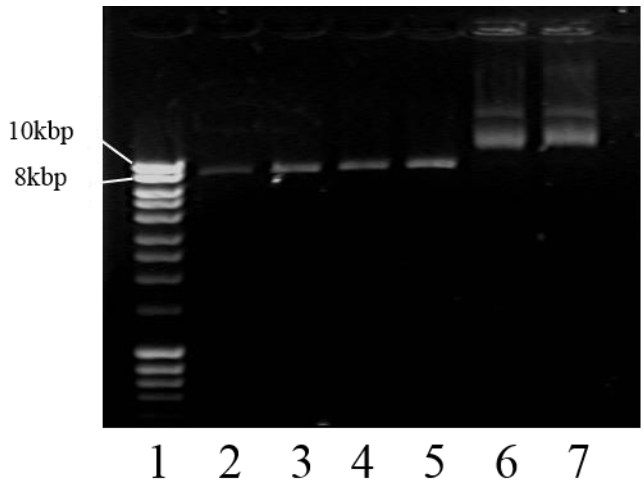
Sepharose column and separatory funnel setup for protein purification.

Figure 7



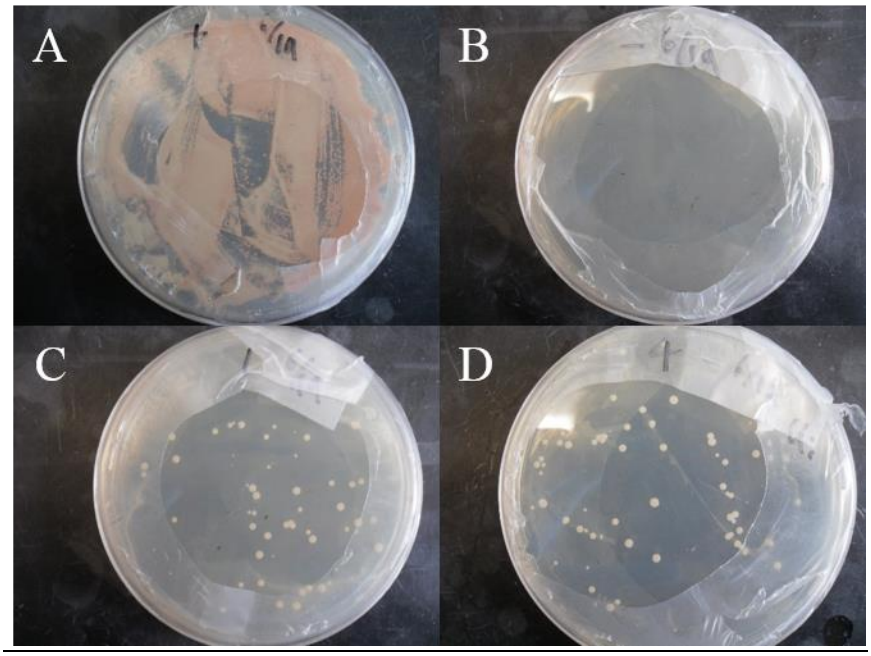
Lane 1: 1 kb ladder; **Lane 2:** pPIC9_IL8 double digested; **Lane 3:** pPIC9_GP1 double digested. Both lanes 2 and 3 were double digested with *Xho* I and *Xma* I. The expected sizes of the pPIC9_IL8 and pPIC9_GP1 double digested are 297 bp and 488 bp respectively.

Figure 8



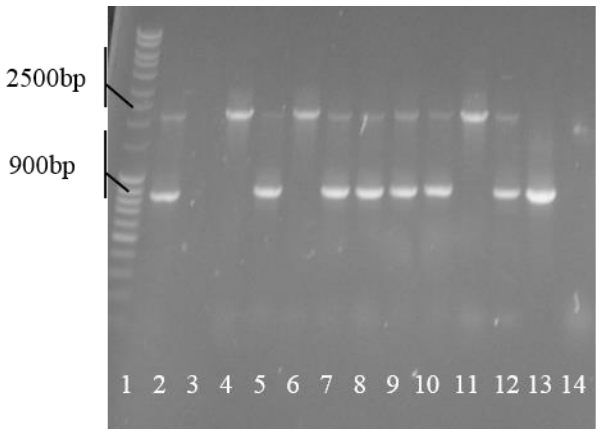
Linearized plasmids on a 1% electrophoresis gel. Lane 1 shows the MassRuler DNA Ladder Mix from Thermo Scientific. Lane 2 and 3 contain linearized PIC9-IL8, which matches the actual size of the digested PIC9-IL8 (8368 bp). Lane 4 and 5 contain linearized PIC9-GP1, which matches the actual size of PIC9-GP1 (8611 bp). The Sal1 digest enzyme was used to linearize all plasmids. Lane 6 contains unlinearized PIC9-IL8, and lane 7 contains unlinearized PIC9-GP1.

Figure 9



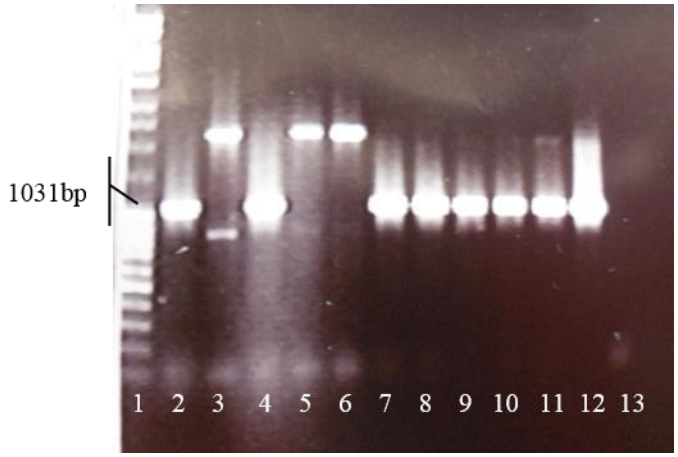
Transformation of GS115 *Pichia*, plated on –His plates. **(A)** Previously successfully transformed *Pichia* containing TARC. The cells already were able to grow in environment without histidine, and were used as positive control. The red coloring indicates overgrowth. **(B)** Mock-transformed *Pichia.* These Pichia were used as a negative control. There was no growth, as no plasmids were added to cells during transformation. **(C)** and **(D)** show colonies of *Pichia* transformed with PIC9-IL8 and PIC9-GP1, respectively

Figure 10



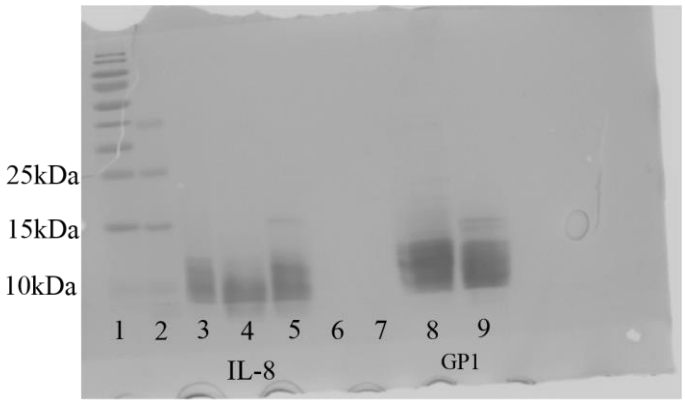
PCR of GS115 *Pichia* transformed with pPIC9-IL8. Lane 1 shows the MassRuler DNA Ladder Mix from Thermo Scientific. Lanes 2 through 12 are transformed colonies. Lanes 4, 6, and 11 show colonies with the pPIC9-IL8 plasmid inserted on both chromosomes (double insertion). Lane 13 shows the original plasmid, used here as the positive control. Lane 14 contains the untransformed GS115 genome as the negative control.

Figure 11



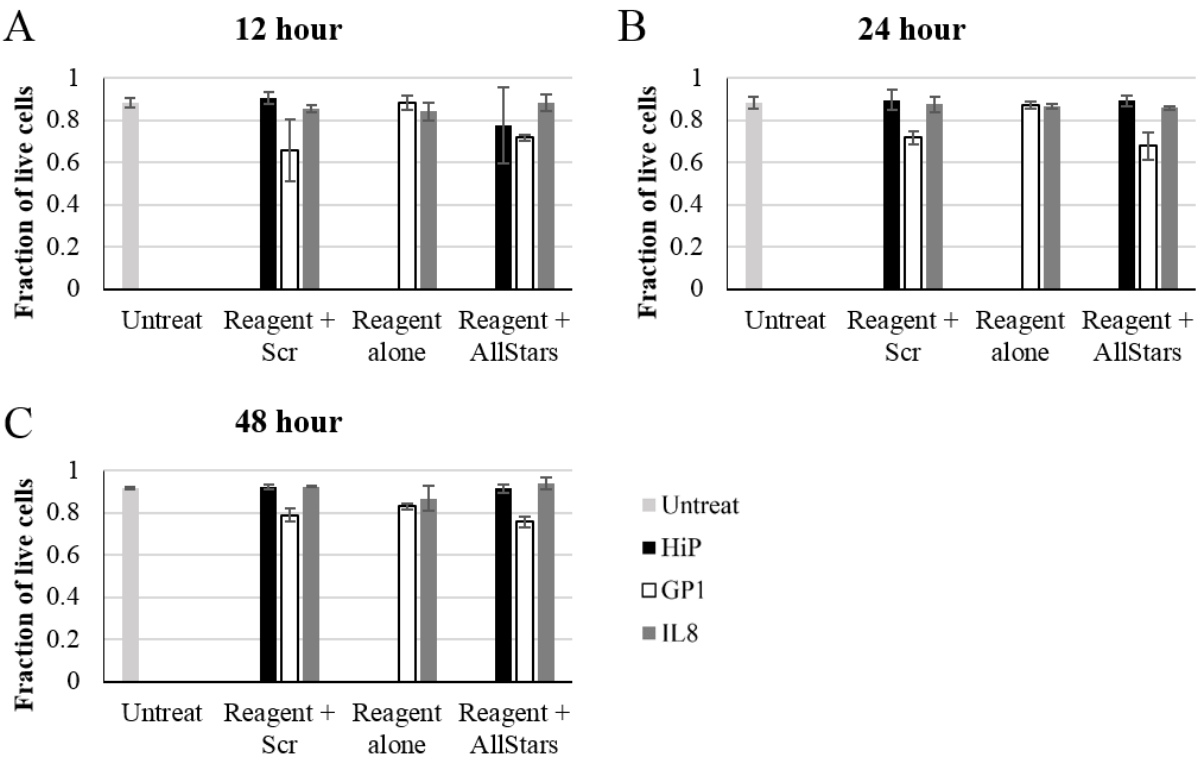
PCR ofGS115 *Pichia* transformed with pPIC9-GP1. Lane 1 shows the MassRuler DNA Ladder Mix from Thermo Scientific. Lanes 2 – 11 are transformed colonies. Lane 5 and 6 show colonies with the GP1 plasmid inserted on both chromosomes. Lane 12 shows the original pPIC9-GP1 plasmid, used here as the positive control. Lane 13 contains the untransformed GS115 genome as the negative control.

Figure 12



SDS PAGE was performed on all batches of delivery vehicle protein produced to confirm expected size. Lane 1 and 2, ladder. Lane 3 through 5, IL8-ARP. Lane 8 and 9, GP1-ARP. The IL-8 ARP bands approximately match the 11kDa expected size of IL-8 (11kDa). The GP1-ARP bands do not match the 22kDa expected size of GP1.

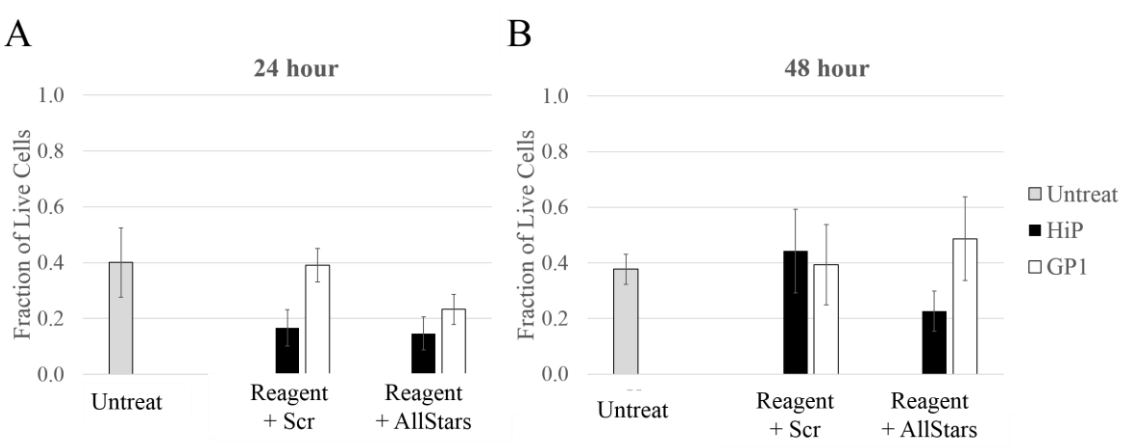
Figure 13



CEM cells were transfected with HiPerfect, a commercially available transfection reagent, as a positive control. CEM cells were also transfected with delivery vehicle proteins GP1-ARP and IL8-ARP. A scrambled RNA sequence (Scr) with no biological activity was used as a negative control. AllStars Cell Death siRNA (AllStars), a commercially available cell-death reagent, was used as the experimental siRNA. Cell Viability was determined by counting the fraction of live cells in Trypan blue. **(A)** depicts CEM cell viability 12 hours post-transfection, **(B)** depicts viability 24 hours post-transfection, and **(C)** depicts viability 48 hours post-transfection.

All experimental groups were performed in triplicate.

Figure 14



HEK293T cells were transfected with HiPerfect, a commercially available transfection reagent, as a positive control. HEK293T cells were also transfected with delivery vehicle proteins GP1-ARP. A scrambled RNA sequence (Scr) with no biological activity was used as a negative control. AllStars Cell Death siRNA (AllStars), a commercially available cell-death reagent, was used as the experimental siRNA. Cell Viability was determined by counting the fraction of live cells in Trypan blue. **(A)** depicts HEK293T cell viability 24 hours post-transfection, **(B)** depicts viability 48 hours post-transfection.

All experimental groups were performed in triplicate.

Table 1

Oligo Name	**Target Plasmid**	**Sequence 5' to 3'**
5'*Xho*I to *Xma*I Primer	pPIC9_IL8 and pPIC9_GP1	actattgccagcattgctg
3'*Xho*I to *Xma*I Primer	pPIC9_IL8 and pPIC9_GP1	ttctacgcgattgagacctt
5' AOX1 Primer	PIC-IL8 and PIC-GP1	gactggttccaattgacaagc
3' AOX1 Primer	PIC-IL8 and PIC-GP1	ggatgtcagaatgccatttgc
5' IL-8 Primer	PIC-IL8	atgacttccaagctggcc
3' IL-8 Primer	PIC-IL8	tgaagagggctgagaattca
5' GP1 Primer	PIC-GP1	agacttttagctaaccattcaaatga
3' GP1 Primer	PIC-GP1	gaagtaagacacatctcaactttgaa

The table of PCR primer sequences.

Appendix B: Glossary

Adenovirus (n) – Any of a group of DNA-containing viruses that cause conjunctivitis and upper respiratory tract infections in humans (thefreedictionary.com)

Adherent cell line (n) – cells that continue to grow in vitro until they have covered the available surface area (sigmaaldrich.com)

Allele (n) – a member of a pair of genes that is located on an important portion of the chromosome (freedictionary.com)

Anion Affinity Column (n) – a chromatographic technique in which biological molecules bind reversibly to ligands of a negative charge on the column (freedictionary.com)

Antigen (n) – any substance that, when injected into the body, will cause the body to produce antigens. Common antigens include toxins, bacteria, and implanted organ cells (freedictionary.com)

Antisense strand (n) – A strand of nucleotide sequence that is complementary to the messenger RNA (mRNA) (freedictionary.com)

Assay (v) – To examine by testing or experimenting (freedictionary.com)

Bradford Assay (n) – a spectroscopic technique that measures the concentration of all proteins in a solution (freedictionary.com)

Cassette (n) – A DNA sequence encoding multiple genes of interest between one or more sets of restriction sites (freedictionary.com)

CEM cells (n) – a human T cell lymphoblast-like cell line (abcam.com)

Consumption controlled (adj) – response control depends on the ability to internalize surface-bound ligand (freedictionary.com)

Cytokines (n) – regulatory proteins that are released by the immune system to act as messengers between cells during a immunological response (freedictionary.com)

Endocytosis (n) – Process in which the cell brings substances past the membrane and into itself through the folding of the plasma membrane (freedictionary.com)

***Escherichia coli* (n)** – a rod-shaped bacterium commonly used in recombinant DNA research (freedictionary.com)

Enzyme-Linked Immunosorbent Assay (ELISA) (n) – an assay that uses an enzyme bound to an antibody or antigen to detect a specific protein (freedictionary.com)

Glycoprotein 1 (n) – a protein found in the Machupo virus that binds to the transferrin receptor (uniprot.org)

Growth factors (n) – any protein within the cell that supports the growth, organization, and maintenance of the cell; large amount of growth factor could lead to cancer (freedictionary.com)

HEK293T cells (n) – cells derived from the human embryonic kidney cell line 293 and commonly used in transfections (freedictionary.com)

Homologous recombination (n) – A type of genetic recombination is which sequences of nucleotides are exchanged between two similar segments of DNA (everythingbio.com)

Interleukin 8 (n) – a cytokine produced by different cell types that activates neutrophils and is involved in inflammation (free dictionary.com)

Interleukin 8 receptor/CXCR1 (n) – a receptor found on neutrophils that selectively binds interleukin 8 (freedictionary.com)

Keratinocytes (n) – the predominant cell in the epidermis, the outermost layer of the skin, constituting over 90% of the epidermis cells (freedictionary.com)

Ligands (n) – Proteins that bind to the receptor sites of the cell (freedictionary.com)

Lipoprotein (n) – a structure on the cell membrane that regulates water flow into and out of the cell (freedictionary.com)

Lysosomes (n) – an organelle of the cell that breaks down unneeded substances, like worn organelles and food particles, and harmful substance, like bacteria and viruses (freedictionary.com)

Messenger RNA/mRNA (n) – RNA that is transcribed from DNA in the nucleus and moves to the cytoplasm, where it is translated to protein (freedictionary.com)

Mid-Logarithmic Phase (n) – The phase in the growth of a cell culture characterized by cells doubling with each successive round of replication (masfield.osu.edu). This phase is the time when cells most efficiently take up plasmid DNA (goldiesroom.org)

Nucleotide (n) – Any of various compounds consisting of a nucleoside combined with a phosphate group and forming the basic constituent of DNA and RNA (thefreedictionary.com)

Plasmid (n) – A circular, double-stranded unit of DNA that replicates within a cell independently of the chromosomal DNA. Plasmids are most often found in bacteria and

are used in recombinant DNA research to transfer genes between cells (freedictionary.com)

***Pichia pastoris* (n) –** a species of yeast used for protein production using recombinant DNA (pichia.com)

Polymerase Chain Reaction/PCR (n) – A technique for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase (freedictionary.com)

Quantitative Real-Time Polymerase Chain Reaction/qRT-PCR (n) – A method for quantifying the amount of a specific RNA sequence (freedictionary.com)

Recombinant DNA (n) – DNA molecules that are extracted from different sources and chemically joined together. For example, DNA comprising an animal gene may be recombined with DNA from a bacterium (free dictionary.com)

Recombinant Protein (n) – a protein derived from genetically engineered DNA (freedictionary.com)

Restriction Enzymes (n) – Enzymes that cut nucleic acids at specific sites on the strand and produce discrete fragments (freedictionary.com)

Ribonucleotide (n) – The basic building block of RNA (freedictionary.com)

RNA binding domain (n) – a region of RNA binding proteins that binds to double or single-stranded RNA in cells (nlm.nih.gov)

Small RNA (n) – RNA such as small interfering RNA or microRNA that dynamically represses gene expression (Journal of Cell Science)

Small interfering RNA (siRNA) (n) – A type of small RNA that requires complete complimentary sequence to silence the matching mRNA strand (Journal of Cell Science)

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis/SDS-PAGE (n) – The separation of proteins by migration through a solution under the influence of an applied electric field. Proteins are treated with SDS reagent to linearize them and give them a negative charge (freedictionary.com)

Supernatant (n) – The liquid lying above a solid residue after crystallization, precipitation, centrifugation, or other process (google.com)

Transcribe (v) – To find corresponding messenger RNA (mRNA) nucleotide sequence that matches a particular sequence of DNA; reverse transcribe is the transcription of mRNA to DNA (freedictionary.com)

Transferrin (n) – iron-binding glycoproteins that control the level of iron in the blood (freedictionary.com)

Transferrin receptor 1/TfR1 (n) – A receptor that mediates the uptake of iron from transferrin into the cell via endocytosis (Journal of Biochemistry and Cell Biology)

Vector (n) – A bacteriophage, plasmid, or other agent that transfers genetic material from one cell to another (thefreedictionary.com)

Appendix C: Protocols

Gibson Assembly Protocol* (Gibson, 2013**)**

1. Prepare 5X ISO buffer. Six mL of this buffer can be prepared by combining the following:

3 ml of 1 M Tris-HCl pH 7.5

150 µl of 2 M MgCl2

60 µl of 100 mMdGTP

60 µl of 100 mMdATP

60 µl of 100 mMdTTP

60 µl of 100 mMdCTP

300 µl of 1 M DTT

1.5 g PEG-8000

300 µl of 100 mM NAD

Add water to 6 ml

Aliquot 100 µl and store at -20 °C

2. Prepare an assembly master mix. Combining the following:

320 µl 5X ISO buffer

0.64 µl of 10 U/ µl T5 exo

20 µl of 2 U/µl Phusion pol

160 µl of 40 U/µl Taqlig

Add water to 1.2 ml

Aliquot 15 µl and store at -20 °C. This assembly mixture can be stored at -20 °C for at least one year. The enzymes remain active following at least 10 freeze-thaw cycles. This mixture is ideal for the assembly of DNA molecules with 20-150 bp overlaps.

3. Thaw a 15 µl assembly mixture aliquot and keep on ice until ready to be used.

4. Add 5 µl of DNA to be assembled to the master mixture. The DNA should be in equimolar amounts. Use 10-100 ng of each ~6 kb DNA fragment. For larger DNA segments, increasingly proportionate amounts of DNA should be added (e.g. 250 ng of each 150 kb DNA segment).

5. Incubate at 50 °C for 15 to 60 min (60 min is optimal).

6. If cloning is desired, electroporate 1 µl of the assembly reaction into 30 µl electrocompetent E. coli.

Mammalian Cell Freezing Protocol (Freezing Cells, 2013)

Prepare freezing medium and store at 2° to 8°C until use. Note that the appropriate freezing medium depends on the cell line.

For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Resuspend the cells in complete medium required for that cell type.

Determine the total number of cells and percent viability using a hemacytometer, cell counter and Trypan Blue exclusion, or the Countess® Automated Cell Counter. According to the desired viable cell density, calculate the required volume of freezing medium.

Centrifuge the cell suspension at approximately 100–200 × g for 5 to 10 minutes. Aseptically decant supernatant without disturbing the cell pellet.
Note: Centrifugation speed and duration varies depending on the cell type.

Resuspend the cell pellet in cold freezing medium at the recommended viable cell density for the specific cell type.

Dispense aliquots of the cell suspension into cryogenic storage vials. As you aliquot them, frequently and gently mix the cells to maintain a homogeneous cell suspension.

Freeze the cells in a controlled rate freezing apparatus, decreasing the temperature approximately 1°C per minute. Alternatively, place the cryovials containing the cells in an isopropanol chamber and store them at –80°C overnight.

Transfer frozen cells to liquid nitrogen, and store them in the gas phase above the liquid nitrogen.

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