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GENETICALLY ENCODED IRON SENSOR CELL LINE: A MODEL FOR STUDYING FERROPTOSIS AND CELLULAR IRON METABOLISM

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GENETICALLY ENCODED IRON SENSOR CELL LINE: A MODEL FOR STUDYING FERROPTOSIS AND CELLULAR IRON METABOLISM

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ABSTRACT

GENETICALLY ENCODED IRON SENSOR CELL LINE: A MODEL FOR STUDYING FERROPTOSIS AND CELLULAR IRON METABOLISM

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Cell death is a biological process that is essential in proper cellular homeostasis, development, and function. Malfunction of this system can lead to different diseases and cancers. Ferroptosis is a form of cell death that occurs as a result of the accumulation of lipid peroxides, that are dependent on intracellular levels of iron. Iron is essential to multiple metabolic processes in the cell, including the catalyzation of lipid peroxides.

Thus, intracellular iron levels must be tightly regulated. Our goal is to create a cellular model to study and detect changes in intracellular iron levels. Through the use of a genetically encoded fluorescent iron sensor, we have generated a stable cell line that provides fluorescent readout on intracellular iron levels. This model can be used to screen for genes, small molecules, and drugs that affect and regulate intracellular iron levels. These findings can then be used to discover new regulators of ferroptosis, data that could be essential to the treatment of ferroptosis-linked diseases.

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INTRODUCTION

Types of Programmed Cell Death

The regulation of the cellular population is a process that is regulated through cell division or cell death. Cell death is a biological process that is essential to many facets of life. Cell death is essential to many homeostatic processes, such as cell proliferation, embryonic development, and immune system function, among many other processes (Elmore, 2007). While at first glance cells committing "suicide" may seem counterproductive, when this programmed death does not function, it can have fatal implications to the cell and the body at large. Conditions such as "neurodegenerative diseases, autoimmune disorders, and many types of cancers" (Elmore, 2007) can arise when the cell's self-destruct mechanism is malfunctioning. Research into the field of programmable cell death has taken off due to its immense importance to the maintenance of a healthy cell population. The possibility that we can modulate, regulate, initiate, or inhibit when a cell undergoes its self-destruct protocol has implications in the treatment of numerous ailments and diseases.

There are two main distinctions of programmable cell death distinguished by the morphological features that accompany them. Apoptosis is characterized by cell shrinkage and fragmentation to apoptotic bodies (Saraste and Pulkki, 2000). Initiation of apoptosis involves the activation of caspases, which relay the recognition of an apoptotic stimuli. During apoptosis, the cell shrinks, the cytoplasm becomes denser, and the organelles become tightly packed. The cell then undergoes a process called budding, where the cell fragments separate into apoptotic bodies, which consist of the cytoplasm along with packed organelles.

These apoptotic bodies are then phagocytosed by the different cells of the immune system. Apoptosis generally occurs under two conditions. It is an important component of proper development and aging of the human body. Furthermore, the cell can use it as an immune response mechanism, where damaged or infected cells can be terminated to prevent further harm (Elmore, 2007). Apoptosis is an important step in the maintenance of homeostasis. It is an integral fail-safe in the cell's proliferation cycle, and therefore an essential step in cancer prevention. The opposite is also true, where "too much apoptosis is linked to certain pathological conditions such as acquired immune deficiency syndrome (AIDS)" (Xu et al., 2019).

The second category of programmable cell death is that of the nonapoptotic forms. One of these forms is necroptosis, an active form of cell death. Necroptosis is dependent on RIPK3 and other kinase domain-like proteins, whose activation is necessary for the cascade of necroptotic signaling pathways. Unlike apoptosis, characterized by cell shrinkage, necroptosis is characterized by the swelling of cellular organelles and increased cell size. This increase in size leads to stretching of the plasma membrane, which makes it more permeable, allowing the release of cellular contents (Park et al., 2021). Necroptosis malfunction can have many implications in many diseases such as cancer.

Ferroptosis: A Unique form of Cell Death

Ferroptosis however, is unlike other cell death types that result "from the activation of caspase-dependent apoptosis" (Dixon et al., 2012). Ferroptosis is "driven by iron-dependent phospholipid peroxidation". It is regulated and managed through metabolic processes, such as iron homeostasis, mitochondrial activity, and the metabolism of amino acids, lipids, and sugars. Certain types of cancer cells, especially in the mesenchymal state and prone to metastasis, are vulnerable to ferroptosis. Ferroptosis is especially essential in the death of cells as a result of organ injury, as well as the mechanism of death for many degenerative diseases. On that account, research into the initiation and inhibition of ferroptosis can provide insight into the treatment of "drug-resistant cancers, ischemic organ injuries, and other degenerative diseases linked to overwhelming lipid peroxidation (Jiang et al., 2021).

Mechanisms of Ferroptosis

To truly understand how and why ferroptosis has gained such traction in research we need to understand the mechanisms by which it is executed. Early work demonstrated that the deprivation of cystine can cause cell death. Cystine can be taken up by the cell in its oxidized form through the system x_c^- cystine/glutamate antiporter, or in its reduced form by the neutral amino acid transporter. Cystine is the rate-limiting substrate for the synthesis of glutathione, or GSH. "GSH is the most abundant reductant in mammalian cells, is important for iron-sulfur cluster biogenesis, and is a cofactor for multiple enzymes, [such as] glutathione peroxidases (GPX) and glutathione-S-transferases. GSH synthesis, system x_c^-, and glutathione peroxidase 4 (GPX4) can all protect cells from death triggered by

diverse oxidative stress conditions". GPX4, the hallmark protein of ferroptosis, is at the center of system x_c^-, the mechanism by which ferroptosis is executed. GPX4 is an essential enzyme catalyzing the reduction of phospholipid hyperoxides (PLOOHs), to their corresponding alcohols. For lipid peroxidation to begin, a hydrogen atom must be removed from the carbon double bond of polyunsaturated fatty acyl component on phospholipids (PUFA-PLs), which are located in the lipid bilayer. This newly formed carbon-centered radical must react with oxygen to form a peroxyl radical, which is then converted to a lipid hydroperoxide and further reduced to its corresponding alcohol. This reduction requires the catalytic selenocysteine residue of GPX4, as well as 2 electrons from glutathione (GSH). If this conversion does not occur, it leads to the accumulation of PLOOHs. PLOOHs are the executioners of ferroptosis. The buildup of PLOOHs leads to rapid and unrepairable loss of plasma membrane integrity, ultimately leading to the rupture of the cell organelles and membrane (Jiang et al., 2021).

Inducers of Ferroptosis

There are two main compounds that can inactivate/inhibit this pathway, erastin and RSL3. Through their inhibitory effect, they go on to propagate and induce ferroptotic death. They both inhibit GPX4, either directly or indirectly. RSL3 directly inhibits GPX4 and is not dependent on system x_c^-. During RSL3 inhibition, GSH concentration was unaffected, but it was found that RSL3 directly binds to GPX4, inactivating its peroxidase activity (Yang et al., 2014). Erastin indirectly inhibits GPX4 through the inhibition of cystine transport. When the cell is deprived of cystine, which is an essential antioxidant and building block of glutathione (GSH), it is unable to synthesize GSH to contribute electrons to the

reduction of PLOOHs.

The Role of Iron in the Ferroptotic Pathway

Iron and iron metabolism plays a crucial role in lipid peroxide formation. Lipoxygenases (LOXs) and cytochrome P450 oxidoreductase (POR) are metabolic enzymes that aid in lipid peroxidation. LOXs and POR require iron for catalysis. In addition to its role in the catabolism of enzymes essential to lipid peroxidation, iron can also further propagate PLOOH production.

Excess amounts of free iron are toxic to the cell. Excess iron in the cell can act as free radicals, which are able to remove the hydrogen atom from the carbon double bond of PUFA-PLs. Hence, excess free iron plays a direct role in the formation of lipid peroxides. It is therefore justified that elevated levels of iron in the cell propagate and enhance the formation of PLOOHs, which lead to ferroptotic death when not reduced into their corresponding alcohols.

Given the many ferroptotic implications that iron is involved in, it is not unfair to assume that intracellular iron levels have some role in the sensitization of the cell to ferroptosis. Research indicated that ferroptosis is dependent upon the transferrin receptor, which is responsible for the import of iron into the cell. Once iron has been imported, the cell's labile iron pool is stored using ferritin, a protein responsible for the storage of intracellular iron. When the cell is starved of cystine, it leads to the "autophagic degradation of ferritin". Without ferritin, the cell is unable to store the free labile iron, which leads to excess labile iron in the cytosol. Due to this increase in labile iron, as aforementioned, there is more iron present to contribute to the catabolism of enzymes essential to lipid peroxidation, as well as more iron to react with PUFA-PLs to produce PLOOHs (Jiang et al., 2021).

Iron and Ferroptosis Linked Diseases

Understanding ferroptosis is especially important due to its relevance in the pathologies and treatments of numerous cancers and diseases. Research has shown that elevated levels of intracellular iron are associated with resistance to chemotherapy. This is a reason why this research, and the creation of this model to study iron levels in cells, is important. Firstly, this model can be used to find iron chelators that will decrease levels of cellular iron so that chemotherapy is effective. Secondly, research done with this model can lead to the discovery of new ferroptosis inducing drugs that work by changing iron levels in the cell. Thus, we can induce ferroptotic death on the cancer cells so that chemotherapy is not needed (Bajbouj et al., 2019). Certain autoimmune diseases are also directly linked to ferroptosis. One such disease is Systemic lupus erythematosus (SLE), which is an autoimmune disease that is the result of the overproduction of autoantibodies that attack cellular components in the nucleus, cytoplasm, and membrane. The deposition of these immune complexes into tissues results in organ damage. In patients with SLE, conditions such as “neutropenia, proteinuria, and the production of anti-ds DNA antibodies”, can result when iron metabolism is dysregulated and ferroptosis takes place. Ferroptosis also plays a role in Rheumatoid Arthritis. However, in this case, it is the inhibition of ferroptosis that is at fault. Oxidative stress increases the production of ROS, which induces the proliferation of synovial cells, leading to joint destruction in patients with Rheumatoid Arthritis. When synovial fibroblasts are prevented from undergoing ferroptosis, through increased ROS production, the joints of RA patients remain inflamed.

Ferroptosis also has dangerous implications in diseases of the intestinal epithelium. Inflammatory Bowel Diseases such as Crohn's disease (CD) and Ulcerative Colitis (UC) are the result of chronic inflammation, causing cell death. "Over-supplementation of iron increases its deposition in the intestine, resulting in excessive production of ROS" (Lai et al., 2022). Therefore, identifying new methods of influencing intracellular iron levels is of the utmost importance, as it will allow for the regulation of ferroptotic-linked diseases.

METHODS AND RESULTS

Benefits of a Genetically Encoded Iron Sensor

Research by (Chen et al., 2019) demonstrated another method of measuring the labile iron in a cell. They first washed cells in PBS and then incubated with calcein-acetoxymethyl ester. The cells were washed again with PBS. Some cells were then incubated with deferiprone and the rest were left untreated. These cells were then analyzed with a flow cytometer, where they measured the calcein fluorescence. To determine the amount of labile iron in the cell, they measured "the difference in the mean cellular fluorescence with and without deferiprone incubation". While this method can be used to measure labile iron levels, for our purposes this method is too time consuming. Since we will aim to screen numerous small molecules, drugs, and genes, we need a system that allows for high throughput screening. Thus, if we can create a stable cell line with a genetically encoded iron sensor that will provide a fluorescent readout, we can then use flow cytometry and fluorescent microscopy to screen a multitude of drugs, small molecules, and genes. Furthermore, inserting a sensor into the genome of the cell will allow us to monitor intracellular iron levels without having to terminate our cells, allowing us monitor iron levels over a longer period of time.

Iron Regulatory System

The basis for the operation of our genetically encoded iron sensor is found in the regulatory system of intracellular iron. One of the main components of this regulatory system is the interaction between iron regulatory proteins (IRPs) and iron regulatory elements (IREs). This interaction helps to regulate intracellular iron on a genetic level. The genes encoding for the iron storage protein, ferritin, are

regulated through an IRE in the 5' UTR, where the interaction of this IRE with IRPs control the ferritin expression in response to changes in intracellular iron levels. This same interaction also takes place with IREs in the 3' UTR of mRNA for the transferrin receptor, which controls the import and export of iron. IRP1 and IRP2 are two cytoplasmic proteins that aid in the regulation of intracellular iron metabolism due to their high binding specificity to IREs. IRP1 in the cytoplasm is the counterpart of mitochondrial aconitase, which converts citrate to isocitrate through a catalytic cluster. In cells with high iron levels the cluster is assembled and IRP1 displays aconitase activity. In cells with low levels of iron, the cluster is not formed and IRP1 functions as an RNA binding protein. When the cell is deficient in labile iron reserves, IRPs bind to IREs and stabilize transferrin mRNA. Under the same iron deficient conditions, IRPs also bind to IREs upstream of the ferritin gene to decrease ferritin expression. Due to an increase in transferrin expression, and a decrease in ferritin expression, more iron is imported into the cell and less of that iron becomes ferritin bound for storage. The opposite effect occurs when iron is in excess. High levels of intracellular iron decrease IRP-IRE binding, destabilizing transferrin mRNA and promoting ferritin expression. This leads to lower amounts of iron being imported into the cell, and more labile iron being bound to ferritin for storage (Cairo and Recalcati, 2007). It is this iron dependent IRP-IRE interaction that is used to measure labile iron levels.

Iron Sensor Operation

Our sensor which we received from our collaborator at Memorial Sloan Kettering Cancer Center, Dr. Xuejun Jiang, has two different fluorescent signals (Figure 1). In the 5' end of the sensor, an IRE is encoded. Downstream of this IRE

is the mCherry gene. By placing the mCherry gene downstream of an IRE, the expression of mCherry is dependent on the IRP-IRE interaction, which is further dependent on the intracellular levels of iron. The red fluorescent signal from mCherry expression allows us to measure the level of iron in the cell based on the expression level and intensity of the mCherry signal. Towards the 3' end of the sensor, GFP is encoded. Upstream of this GFP gene, is an internal ribosome entry site (IRES). The IRES-GFP combination is essential for two functions. Firstly, due to the IRES, GFP expression is not dependent on intracellular iron levels. Therefore, it should be expressed regardless of cellular iron content. This GFP signal provides us with confirmation of the uptake and expression of the sensor in our cells. Secondly, the GFP signal serves as a base for us to measure our iron dependent signal, mCherry, against. The ratio between GFP and mCherry expression will tell us whether or not labile iron levels have increased or decreased. An increase in mCherry expression and intensity indicates an increase in intracellular iron levels, while a decrease in mCherry expression and intensity indicates a decrease in intracellular iron levels.

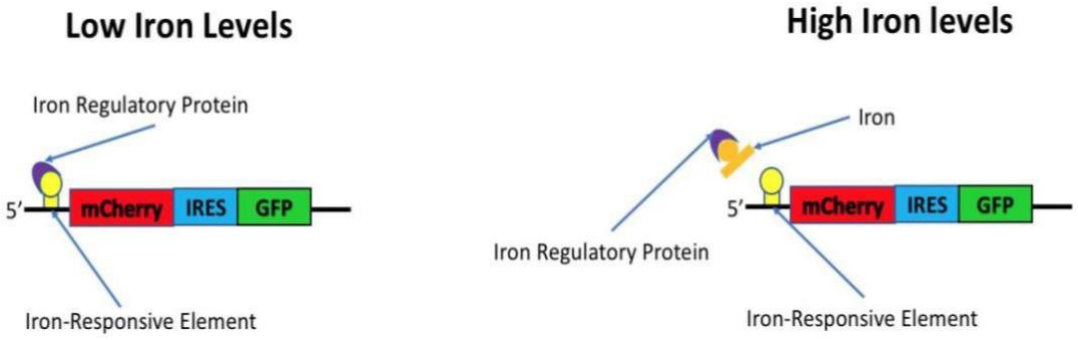


Figure 1: Blueprint of Genetically Encoded Iron Sensor. Blueprint of the sensor showing the location of all fluorescent marker genes, the internal ribosome entry site, and the iron regulatory element. The left side shows the binding of the iron regulatory protein and the iron responsive element, halting the expression of mCherry when iron levels are low. The right side shows the binding of the iron regulatory protein with iron, leaving the iron responsive element unbound, allowing for the expression of mCherry when iron levels are high.

Confirmation of the Operation of the Iron Sensor

After receiving the sensor, we needed to check to ensure that the sensor is operating correctly and giving us the readout we expected. We transfected our sensor into 293T cells. The following day we observed GFP signal, which indicates a successful insertion of our sensor into the cells (Figure 2a). We then treated different cell populations with different conditions. We left one set untreated as a control (Figure 2b), we treated one with DFO, an iron chelator (Figure 2c), and supplemented the last set with additional iron (Figure 2d). We observed that in relation to the control, cells supplemented with iron had greater and more intense mCherry signal. We did not see a convincing decrease in mCherry expression and intensity in the DFO treated cells, relative to the control. We hypothesized that the iron content in DMEM, which was used as the cell media during testing phases, had a base level of iron that could not be reduced further. However, due to other gradient iron supplementation tests performed in the lab, we were able to see the expected result of decreasing the iron content of the cell. This data validated our sensor and gave us the confidence to move forward to the creation of a stable model expressing our operational sensor.

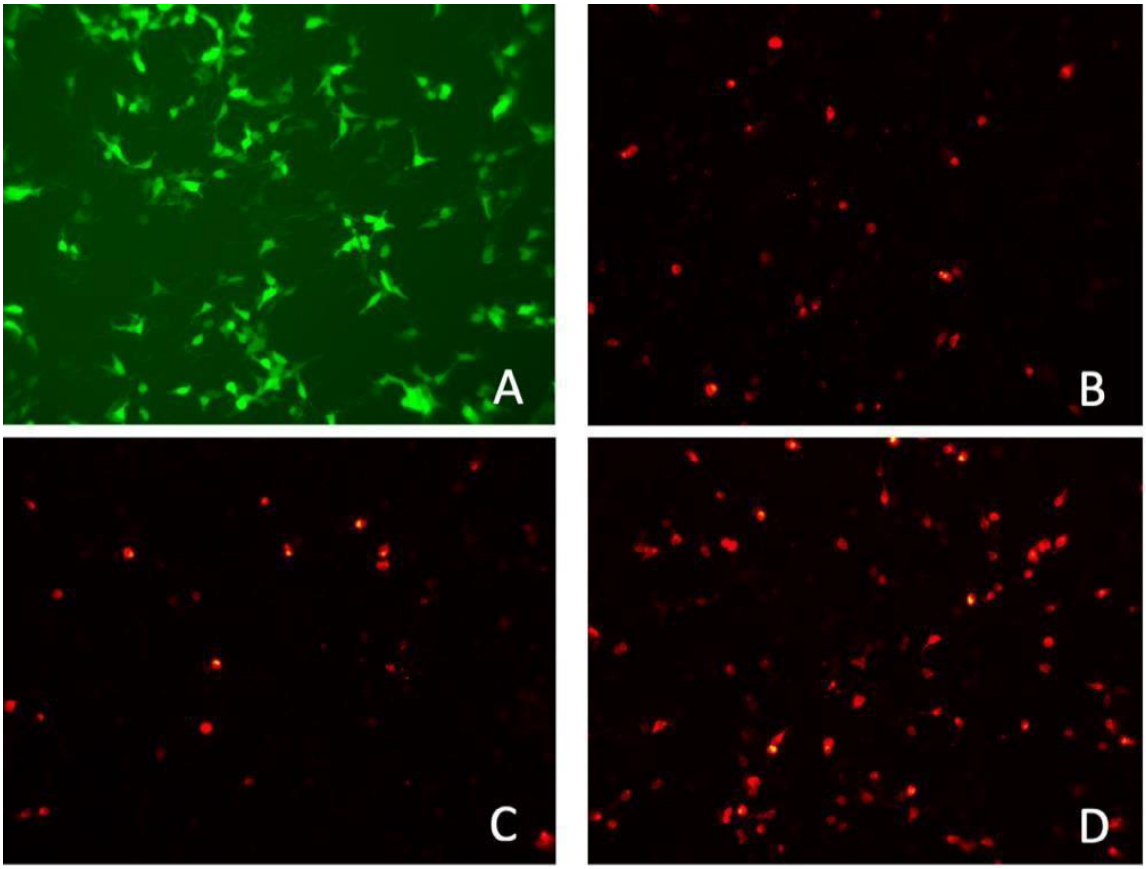


Figure 2: Confirmation of the Operation of the Iron Sensor. A) GFP signal indicates that transfection was successful, and cells are expressing our sensor. B) Control cells left untreated. C) Cells treated with DFO. D) Cells supplemented with additional iron.

Retroviral Production Method

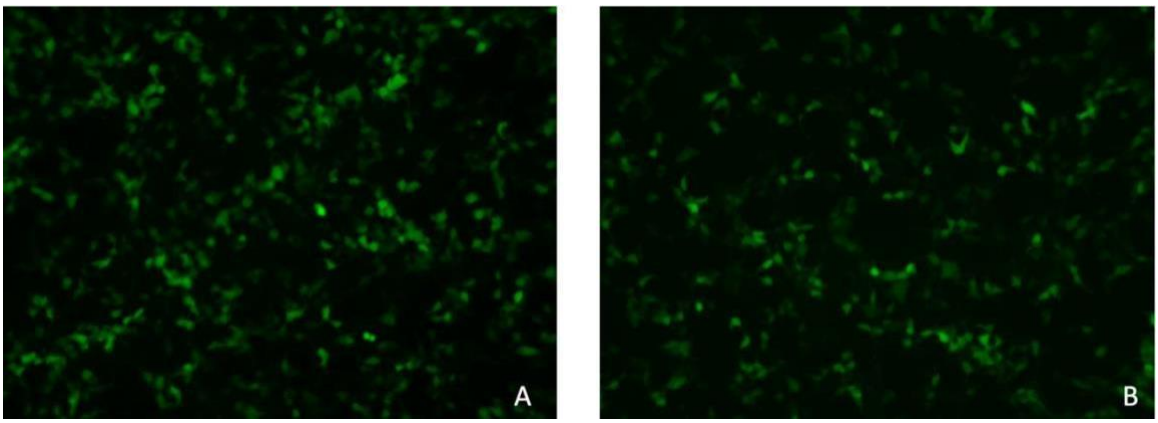
In order to create our stable cell line, we needed a method of transfection that will permanently insert our sensor into the cell's genome. We determined that retrovirus transfection would be best suited for this purpose. We decided to use PLAT-GP cell line as our production cell line. PLAT-GP (Platinum-GP) is a retroviral packaging cell line based on the 293T cell line. These cells are engineered to express essential retroviral structure proteins, gag and pol. We first seeded these PLAT-GP cells so that the dish would be almost confluent at the time of transfection. Then, we transfected PLAT-GP cells with a plasmid containing our sensor construct, along with VsVg, an envelope plasmid. The next day, cells were

checked for GFP expression to confirm positive cellular uptake, and the cell media was replaced. The following day we collected retroviral particles by collecting the media, and then replaced the media once again. The next day, we collected the media, harvesting the last set of viral particles. We then centrifuged the viral solution to collect any cells or cell fragments that remained in the viral solution. The solution was then filtered to further purify. The solution was then ultra-centrifuged to collect the viral particles. The supernatant was decanted, and the viral particles were resuspended in media. This concentrated virus was either used immediately or stored at -20^{o} C. The concentrated virus was then used to infect 293T cells, through a spin infection. The spin infection was used to aid in the uptake of the viral particles by the cells.

Protocol Optimization

After observing the efficacy of this method, we needed to optimize the protocol to produce a virus that infects with the greatest efficiency in a majority of the cell population. This need for greater transfection efficiency is due to our use of the cell sorter to isolate our stable cell line. The cell sorter was used to isolate cells positively expressing GFP, and thus our sensor construct, from those that are not. Therefore, we meticulously tested each variable of the retrovirus production. We tried to double the amount of plasmid DNA during initial transfection to try to improve the efficiency of the virus. We initially used 1.8ug of plasmid DNA for transfection, as suggested by the SignaGen protocol, which we tested against using 3.5ug of plasmid DNA (Figure 3). After changing this one variable and maintaining all others, we found that using the suggested amount of plasmid DNA instead of doubling the amount, gave us the most efficient virus. We hypothesized that this was

because too much DNA can be toxic.



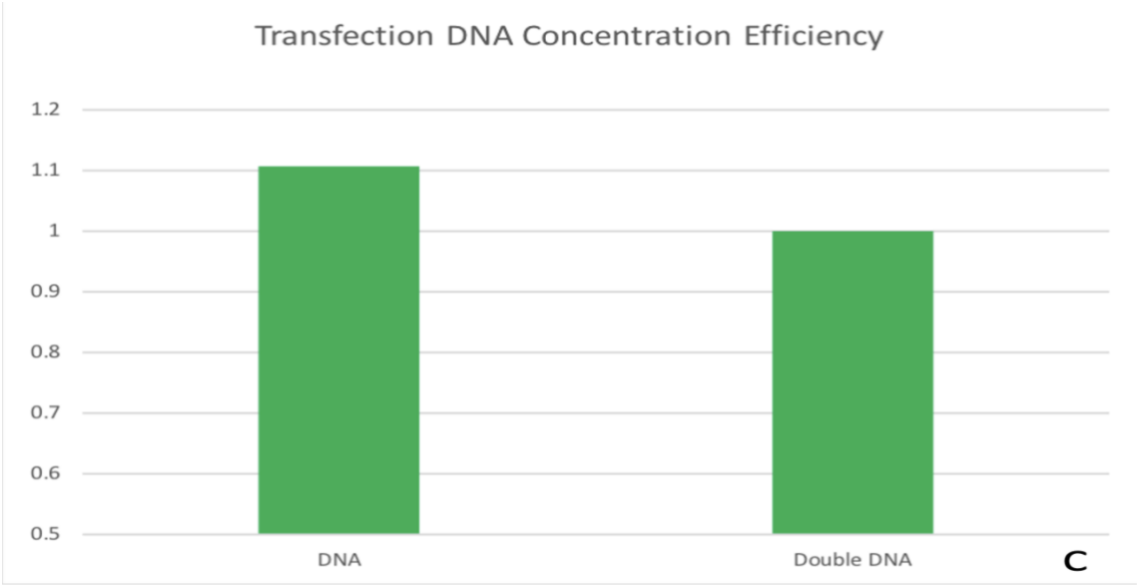


Figure 3: Plasmid DNA Transfection Concentration Test. Images taken after transfection of plasmid during retrovirus production. A) GFP expression using 1.8ug of DNA. B) GFP expression using 3.5ug of DNA. C) Relative comparison of GFP expression between normal and double transfection DNA amount.

Transfection reagents can sometimes be toxic to cells as well. Therefore, we decided to test our current transfection reagent, PolyJet, against FuGENE 6. After producing the virus and infecting into 293T cells, we did not observe any noticeable difference in the efficiency of the virus between the two reagents (Figures 4a-4c). When observing the virus titer, the amount of concentrated virus needed to express the genes of interest, PolyJet narrowly fared better (Figures 4d-4e). Since the evidence was not conclusive, we decided to continue using PolyJet.

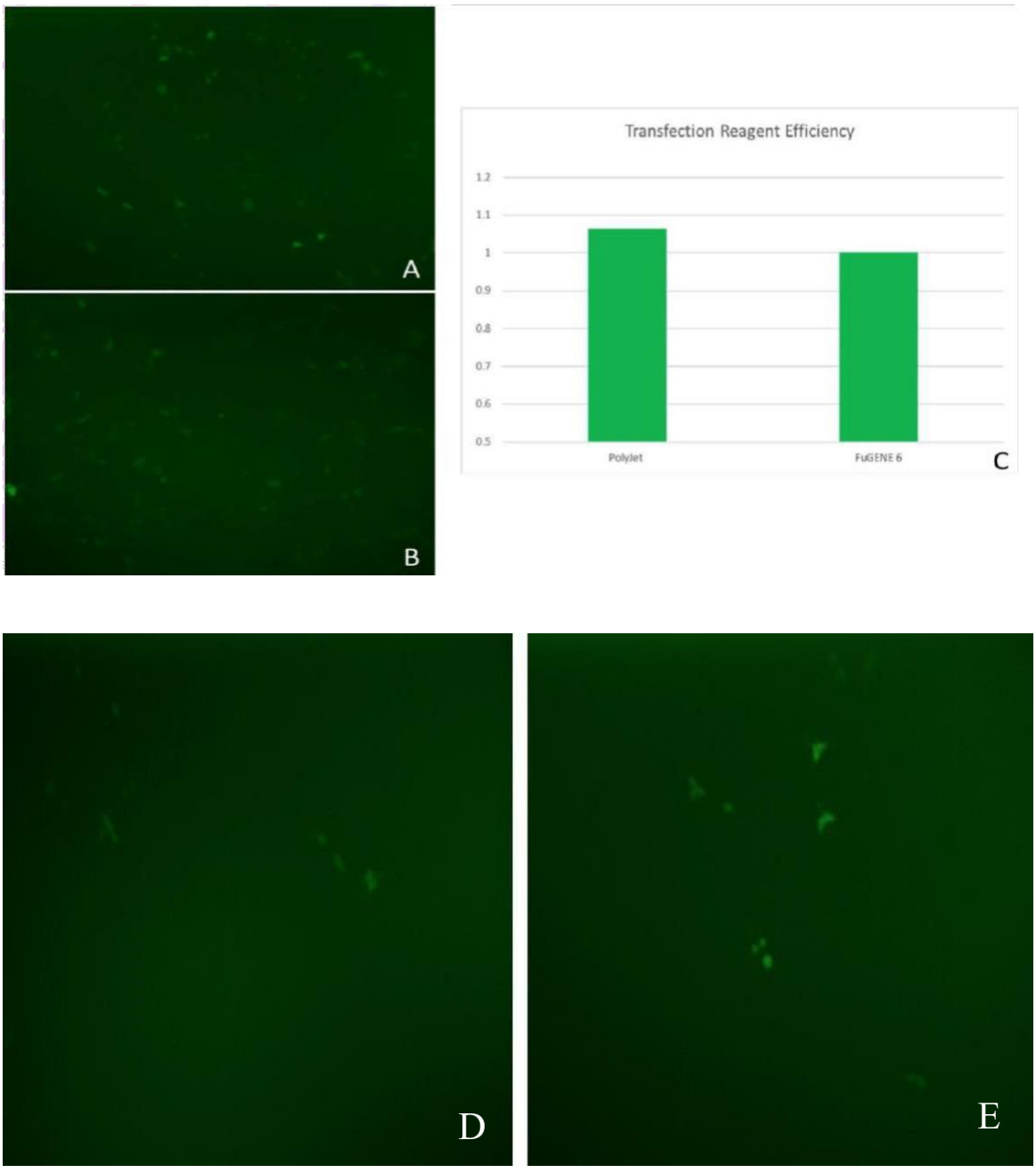


Figure 4: Transfection Reagent Test. Images taken after infection of each respective virus into target 293T cells. A) GFP expression from PolyJet produced virus. B) GFP expression of FuGENE 6 produced virus. C) Relative comparison of PolyJet and FuGENE 6 produced virus. D) Sixth well of 96-well plate where expression of PolyJet produced virus was observed. E) Fifth well of 96-well plate showing FuGENE 6 produced virus GFP signal.

The next variable we wanted to test was the production cell line. We wanted to test using 293T cells along with a packaging plasmid containing gag-pol genes against the PLAT- GP cell line engineered to express gag-pol (Figure 5). We wondered if the manual transfection of the packaging plasmids would increase the virus potency. After infection into 293T cells we determined that the virus produced in PLAT-GP cells were more efficient.

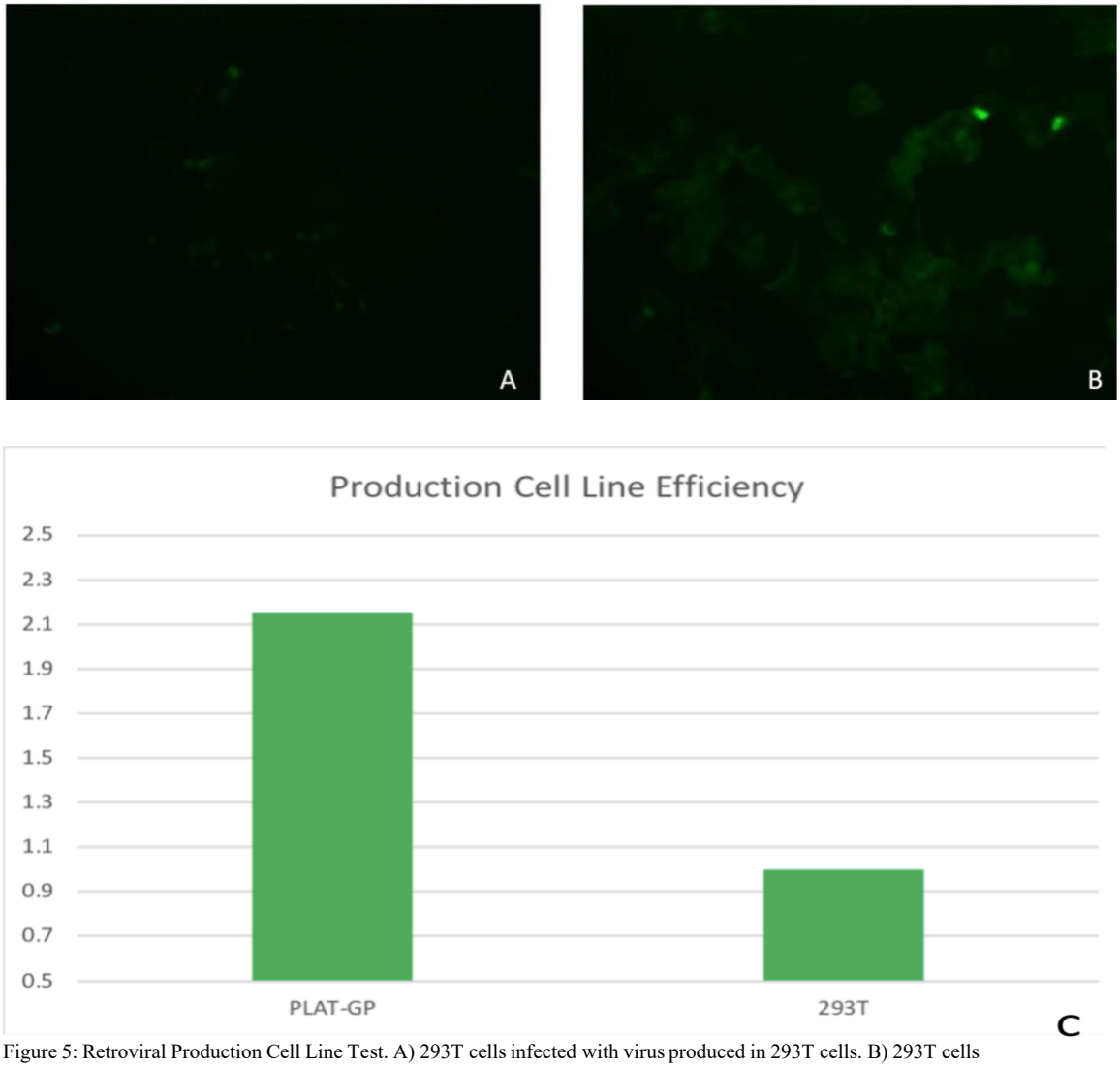


Figure 5: Retroviral Production Cell Line Test. A) 293T cells infected with virus produced in 293T cells. B) 293T cells infected with virus produced in PLAT-GP cells. C) Relative comparison of PLAT-GP and 293T produced virus.

The last variable we tested was in the spin infection step. During spin infection, the target cells are treated with a polycation, which is used to neutralize the charge repulsion between the viral particles and the cell membrane, allowing for easier uptake. So far, we have been using polybrene. We decided to test another polycation, DEAE-dextran (Figure 6). After observing the results, we determined that polybrene was the better polycation.

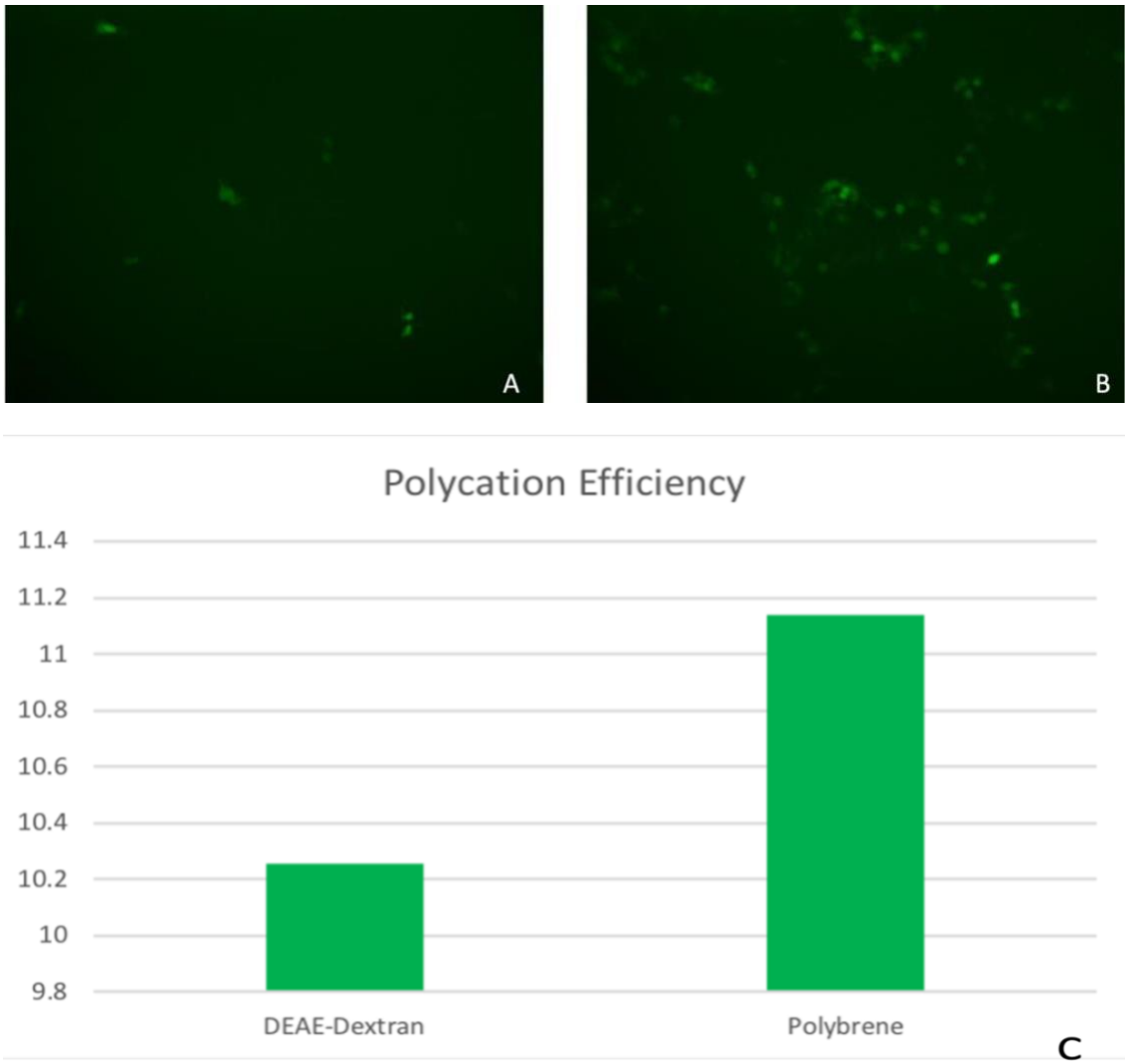


Figure 6: Polycation Infection Test. A) 293T cells treated with DEAE-dextran during spin infection. B) 293T cells treated with polybrene during spin infection. C) Comparison of GFP intensity between DEAE-dextran and Polybrene treated cells.

Ultimately, this meticulous optimization gave us the most efficient retrovirus production protocol that we used to generate our stable 293T cell line that can express our operational sensor. We used PLAT-GP as the production cell line, the SignaGen protocol suggested amount of DNA, PolyJet as the transfection reagent, and finally used a spin infection supplemented with polybrene as the method of infection.

HT1080 Iron Sensor Operation Test

Despite having a stable, functioning 293T cell line, we decided to attempt to stably insert our sensor into another cell line, HT1080, which is more susceptible to ferroptosis, due to a harbored Ras mutation. Due to their greater susceptibility to ferroptosis, we attempted to generate a stable HT1080 sensor expressing cell line. As we did with 293T, we first needed to establish the transient operation of the sensor in the cell line. We transfected our sensor into HT1080 cells and observed good GFP signal. However, mCherry signal was not as expected (Figure 7). Most of the observed mCherry signal came from rounded, detached, dead cells. The attached cells that we saw GFP expression in, had little to no mCherry expression. We tried to increase the intensity of the mCherry signal through iron supplementation. In spite of the additional iron, we did not see an increase in mCherry signal (Figure 8). Therefore, we do not think that HT1080 cells are able to express our sensor as well as 293T cells, and thus, we decided that our stable 293T sensor expressing cell line is the best model for studying iron metabolism and ferroptosis in cells.

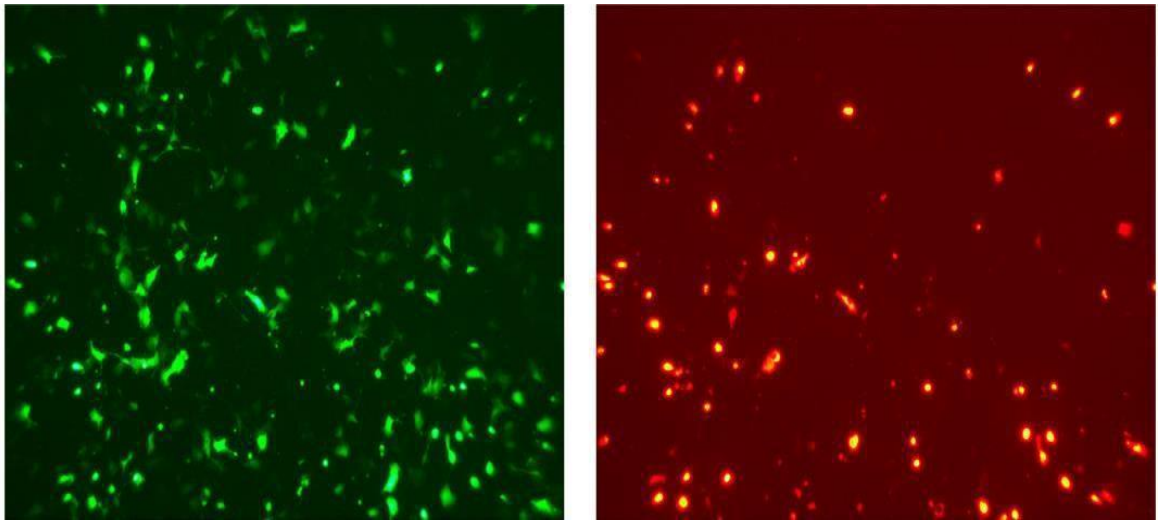


Figure 7: HT1080 Iron Sensor Operation Test. HT1080 cells expressing GFP, but most live cells (cells attached to the dish) are not expressing mCherry.

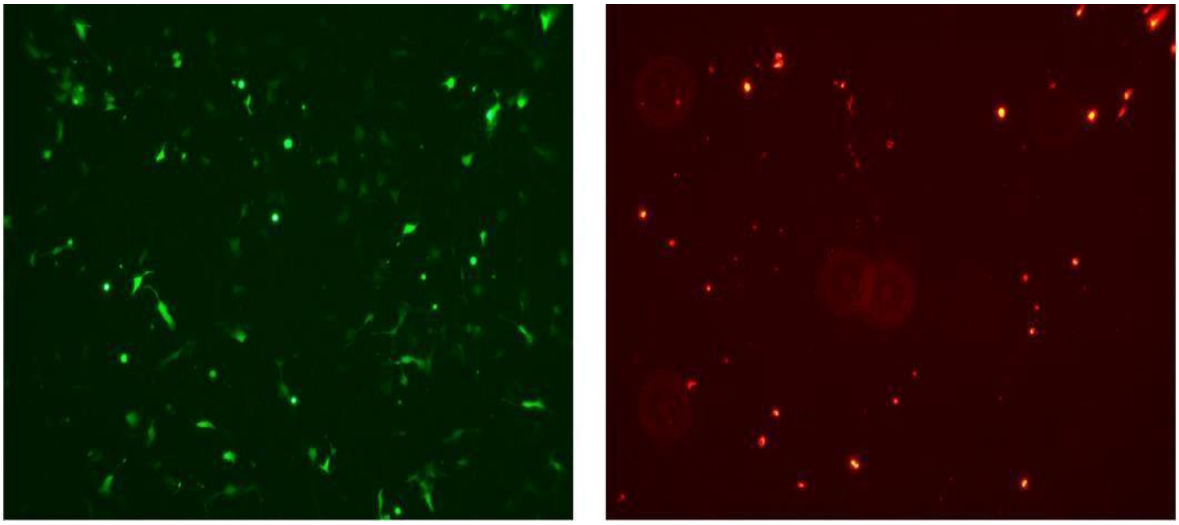


Figure 8: Attempted Rescue of HT1080 mCherry Signal. HT1080 cells expressing lower GFP signal, and the addition of iron did not improve the mCherry signal.

DISCUSSION

Since we have now obtained a stable model, future experimentation is limitless. The model will be used to screen for small molecules and drugs that regulate intracellular iron levels. While the main purpose for creating the 293T iron sensor cell line was to screen small molecules and drugs, we also aim to be able to perform genetic screens to search for genetic regulators of iron metabolism and ferroptosis. We plan to do this by using CRISPR Cas9 gene editing system. We are currently working on generating another 293T cell model that expresses both our sensor and the Cas9 protein. Using this model, along with our current one, we will be able to perform all types of screening. Using the high throughput fluorescent readout provided by our genetically encoded sensor, we can screen thousands of samples and quickly identify regulators of iron metabolism and ferroptosis. The data gathered from theses screens can provide insight into treatment of iron deficient and iron abundant diseases, along with treatments of ferroptosis linked diseases.

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