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Back in November, my dad and I were eating lunch at this quaint Vietnamese restaurant just across the street from the lab. At this point I had been working in his lab for over a year. We were discussing the experiments I had been working on; I was trying to solve an issue with a Sickle Cell correction project.

In order to fully understand the problem with the project, it may be useful to understand the project itself. DNA is made up of four nucleotides, Adenine (A), Thymine (T), Cytosine (C), and Guanine (G). Mutations are essentially a disruption of the proper sequence of these nucleotides (i.e. when certain nucleotides replace others). Sickle Cell Anemia is what is known as an A>T transversion in the hemoglobin-beta gene in your blood. This means that a single Adenine is mutated into a Thymine, causing the hemoglobin-beta protein to be misfolded in such a way that it actually becomes hydrophilic, or "afraid" of water. So, rather than dissolving into the cytoplasm of the cell as it should, it precipitates under low-oxygen conditions. This causes the red blood cells to become rigid and sickled in morphology, leading to all sorts of issues.

So, the project I was working on consisted of turning that mutated "T" back into an "A" in sickle-patient derived Induced Pluripotent Stem Cells (iPSCs). For the correction, we used a gene editing technique my dad developed in the 1990s known as Small Fragment Homologous Replacement (SFHR). We essentially took the DNA from a healthy person, amplified a small section of it surrounding the Sickle Cell mutation a trillion times, and used this piece to replace the mutated DNA in the cells from the Sickle patient.

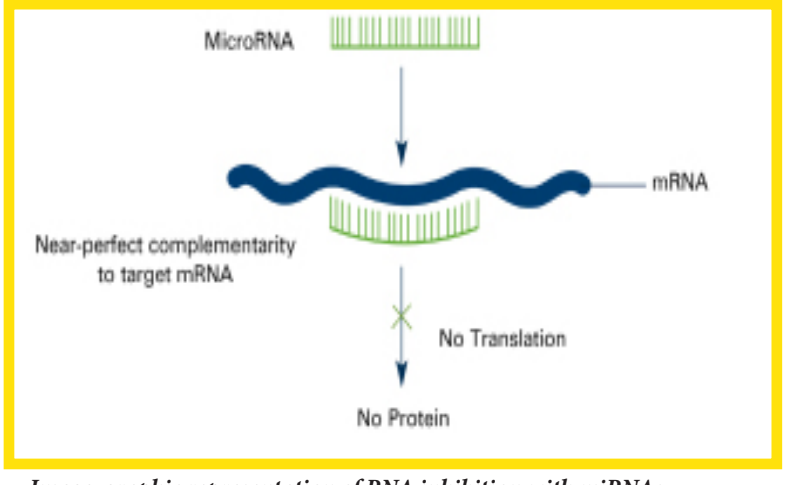

Image: graphic representation of RNA inhibition with miRNAs

Cells undergo something known as Homology-Directed Repair (HDR; formerly known as "Homologous Recombination") in order to correct breaks and mutations in their DNA. By introducing a DNA fragment with the corrected sequence, the idea is that the cells will recognize the correct sequence and swap out the mutated Sickle Cell Sequence with the corrected sequence using HDR.

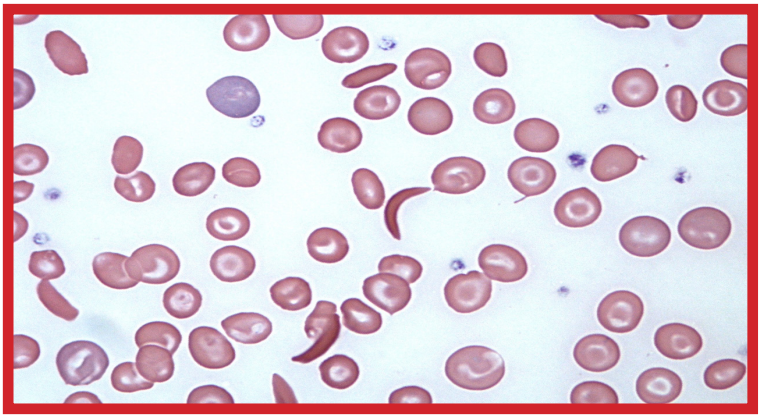

Image: sickled red blood cells amidst regular blood cells

The problem in the experiment lies around our ability to isolate the corrected cells from the uncorrected ones that are still mutated so as to grow up a pure population of only corrected cells in order for the treatment to be therapeutically viable. To help the cells facilitate this correction, an enzyme is also introduced that we designed to induce a break in the DNA near the mutation, making other repair enzymes in the cells notice and repair the break via HDR. This dramatically ups the correction frequency (by roughly 1000 times), but even with the higher frequency we still have to sort the corrected cells from the uncorrected ones. So the problem remains: how do we possibly differentiate the corrected cells from the uncorrected ones?

There is one method that exists that allows us to differentiate between corrected and uncorrected cells. This method is known as drug selection, and involves the introduction of specific sequences into the cell's DNA that make the cells resistant to deadly drugs. Because the drug-resistant sequence is added alongside the sequence correcting the mutation, the method is not what we call "footprint-free." Upon being corrected, the cells will actually incorporate the foreign DNA from the drug-resistant gene into their DNA, thus rendering the cells no

longer therapeutically viable—these cells will behave differently than the other cells in the patient's body due to the presence of this foreign DNA. Most notably, they could evoke an immune response upon being put back into the patient's body, something that we are trying to avoid. We could, theoretically, cut out the foreign sequence with an enzyme and leave just the corrected sequence in the cells, though that often leads to more mutations in the cell's DNA rendering the treatment nonviable.

So, the question remained: what method can we come up with that remains footprint-free but also allows us to differentiate between corrected and uncorrected cells? First, we needed to figure out a way to selectively kill any uncorrected cell. Well, kill genes kill cells… so what if we could put a kill gene into all of the uncorrected cells? Hmm that would work, but how could we possibly differentiate between the uncorrected and corrected cells and express the kill gene in only the uncorrected cells? And then it hit us: the answer lied within the power of noncoding RNA.

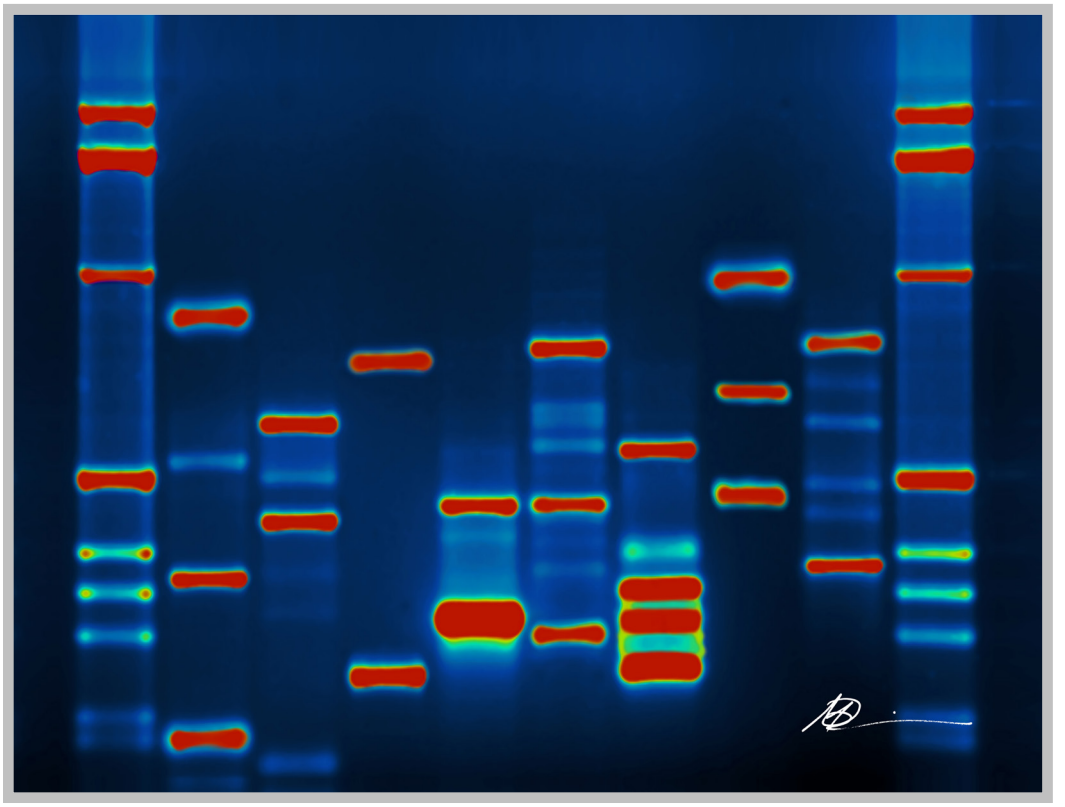

Image: a southern blot of electrophoresis -seperated human DNA

Our new method was simple: introduce what's known as an episomal plasmid (something that allows us to express any gene of our liking in a cell without leaving any foreign DNA permanently in the cell) containing a kill gene on it that could be inhibited by the presence of a micro RNA (miRNA--NOT to be confused with mRNA which denotes the messenger RNA that codes for a protein).

miRNAs are brilliant little things. They essentially work on the mRNA of a protein, binding to the mRNA and preventing it from being read and turned into a protein. So, the idea is that the plasmid codes for the messenger RNA (mRNA) for a kill gene, but if the corresponding miRNA is present, the translation of the kill gene protein will be prevented (miRNAs have shown to reduce protein production by up to 98%).

Our idea was that we would change the sequence of the correction fragment and tack a manually-designed, artificial miRNA sequence onto the end of it. This miRNA would have a complementary sequence to a section of the messenger RNA for the kill gene. The plan was to put the artificial miRNA sequence in the area of the fragment that corresponds with an intron (the portion of the gene that doesn't code for a protein), so that the foreign sequence would not actually have any bearing on the production of proteins. Also, the sequence of the miRNA would be unique to this fragment/mRNA pair, so it would not affect any other DNA sequence in the cell. Thus, any cell producing this specific miRNA would not die in the presence of the kill gene, as the miRNA would prevent expression of the gene. That said, however, any cell not producing the miRNA would die in the presence of the kill gene. So, after adding the correction fragment, we would treat with the kill gene a week later to kill off any uncorrected cells.

Now, this method was all fine and good as it was applied--it would allow us to speed up the process of isolating a corrected clone. But we soon realized it actually had many broader implications. If, for example, we could actually activate the kill gene in the presence of certain noncoding RNAs, we could potentially create very novel therapies. Any disease that is caused by the mutation of a noncoding RNA that leads to the upregulation of responsible genes (e.g. cancer) could be treated in this way.

So, naturally, we got very excited, ran back up to the lab (yes, we were still eating lunch), and formalized the idea into a project proposal that we have now submitted for funding. It's funny how science works—how one conversation can lead to a project proposal; how one lunch can lead to the next five years of your professional life being planned out. Spontaneity is a beautiful thing.