Learning Genotyping and Cell Culture Techniques to Perform Experiments to Study Cardiovascular Calcification

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ABSTRACT

The St. Hilaire Lab at the University of Pittsburgh studies important cardiovascular diseases such as Calcific Aortic Valve Disease (CAVD), Arterial Calcification due to Deficiency of CD73 (ACDC) and Medial Arterial Calcification (MAC). The similarity between all these conditions is the calcification of cells, which leads to the main research question of the lab: how and why does a healthy cell transition into an osteogenic cell? To determine why a healthy cell becomes calcified, we perform experiments using both in vivo and in vitro environments. However, to begin in vitro experiments that involve valuable human cells, basic techniques in cell culture such as plating and splitting are needed, and in vivo experiments require knowing the genotype of the animals being used. With these fundamental techniques, biochemical analysis such as qPCR, western blots and staining can be performed in order to help answer research questions that pertain to the lab.

Introduction

Cardiovascular disease is the leading cause of death in the United States, averaging around one in four deaths. There are various types of heart disease and in many cases, it involves calcification of either arteries, valves, or vessels. The St. Hilaire Lab focuses on the underlying cause of vascular disease and more specifically, what drives vascular and valvular calcification to develop non-surgical methods to treat these conditions. Calcific Aortic Valve Disease (CAVD) is the most common valvular heart disease and occurs when the leaflets in the valve become calcified, hardened, and thick. This phenomenon occurs in around 30% of the aging population and covers a spectrum of conditions from aortic sclerosis (hardening of valve leaflets) all the way to aortic stenosis (blood flow to aorta is blocked and results in heart failure). Currently, the treatment options for CAVD include surgical aortic valve replacement with either a mechanical or bioprosthetic valve. However, the lifespan of bioprosthetic valves is short and patients may require reoperation, indicating that surgical intervention is not the most effective option. Furthermore, the St. Hilaire Lab also studies Medial Arterial Calcification (MAC). This is a condition where calcium deposits develop along the smooth muscle layer of the arterial wall, which can eventually destroy the vessel. MAC is often associated with conditions such as diabetes mellitus, chronic kidney disease and aging. In addition to MAC and CAVD, the St. Hilaire Lab studies a rare disease called ACDC, which is Arterial Calcification due to Deficiency of CD73. Research suggests that patients with this condition have certain inactivating mutations in the gene CD73. The function of the CD73 gene is to breakdown extracellular AMP into adenosine. Without CD73 and adenosine present, patients develop vascular calcification and increased vessel tortuosity. The St. Hilaire Lab's main goal is to understand the mechanisms behind a healthy cell developing into an osteogenic cell as it occurs in these diseases. Understanding this phenomenon requires complex experiments and a strong foundation of the basic techniques used for such investigations. These techniques include genotyping and splitting/plating cells.

Methods

The St. Hilaire Lab research utilizes both in vivo and in vitro experiments. In vivo refers to working with living organisms such as mice, which are used for genotyping. In vitro refers to work that is performed outside of living organisms, such as cells. At the St. Hilaire Lab, different cells are used in cell culture, and this plays a vital role in the research being conducted.

Genotyping

Genotyping is the first technique that is learned in the lab, and it is key for experiments as it determines the differences in genetic components by allowing us to compare the DNA of the sample to a reference sequence. It is especially important to help identify correlations between genetic variations and having abnormal/normal phenotypes. At the St. Hilaire Lab, there are many different mouse lines that are used for genotyping, and these include: TERT, TERT-Tg, MGP, CD73 and ERCC1/R26R. When genotyping TERT, MGP and CD73, the goal is to determine whether mice are knockout or wild types. A knockout mouse is one whose DNA is genetically engineered to not express certain genes, whereas wild type mice express the gene in a manner that is normal and found in natural populations.

There are three main aspects to genotyping: DNA extraction, polymerase chain reaction (PCR) and gel electrophoresis. As the first step, the DNA must be obtained from the mice samples. To do this, a small portion of the mice's tails are snipped and a certain amount of DNA lysis reagent as well as proteinase K is added to the tail to create a mixture. The purpose of DNA lysis reagent is to act as a buffer solution and break open cells to obtain DNA. The addition of proteinase K inactivates nucleases or other substances that might degrade the DNA by digesting protein and removing contamination. After adding the mixture of DNA lysis reagent and proteinase K to each tail, the tubes are then put into a heat block, allowing for the denature of proteins and the actual extraction of DNA. The next step in the genotyping process is PCR. PCR is comprised of three steps: denaturation, annealing and extension. The denaturation of the template is completed in the first step by DNA extraction where the double stranded DNA is separated into single strands. Annealing is the process where primers, which are small molecules of DNA, bind to regions of the complementary single DNA strands. The final step is extension, where the DNA polymerase extends the primer from the 3' end all the way to the end of the amplicon. With these three steps, the target region of the DNA that is needed for observation is amplified. After PCR, gel electrophoresis occurs to check whether PCR was successful and to determine the size of each DNA sample. This is done by comparing the DNA samples to the DNA ladder, which indicates known base pair lengths of DNA. In addition to genotyping, another aspect of the St. Hilaire Lab's research is in vivo mouse dissections. Mice are quite similar to humans in aspects such as anatomy, physiology and genetics. Since the mouse genome is closely related to a human's genome, this makes genetic research helpful for the study of different conditions, and in this case CAVD and MAC. During these dissections, the main objective is to remove the aorta of the mouse, which is the main artery that carries blood away from the heart and transfers it to the rest of the body. This is done so that the cells from the aorta can be extracted and studied through performing experiments in cell culture. After the aorta is extracted, the tissues are sent to pathology to be put in paraffin blocks. Next, there will be the opportunity to learn how to stain slides from these blocks.

Cell Culture

In addition to working in vivo, the St. Hilaire Lab also works in vitro by experimenting with various kinds of cells. Cell culture is an important technique in molecular biology as it allows researchers to study the biology, chemistry, and physiology of wild type cells as well as diseased cells. Further, we had the opportunity to learn methods in working with cells such as splitting, plating and collecting cells. However, the significance in working with cells is using sterile

technique. It is essential to spray everything with ethanol before bringing those items under the chemical hood, as this will prevent bacterial and fungal contamination of the cells.

To split cells, it is essential to look at the confluency, which is the percentage of the surface of a plate or dish that contains cells. If the cells are around 90% confluent, they are ready to be split. If cells are not split in time, it can result in them growing on top of each other or they will stop growing all together. When cells grow on top of each other, the cells at the bottom have little access to nutrients, causing them to die while the ones on top may detach and float. To split cells, the media (contains nutrients for the cells) and trypsin (breaks down proteins that enable cells to stick to the vessel) is placed in the water bath to keep them warm at room temperature. After the media and trypsin are warmed to room temperature, the process starts by aspirating the old media and washing the cells with Phosphate Buffered Saline (PBS) twice. PBS is used opposed to water as it prevents cells from either rupturing or shrinking due to osmosis. Next, the trypsin is added, and the plate is left to incubate for a few minutes. Once the plate is done incubating, it should be visible under the microscope that the cells have been lifted off the plate suggesting that they are ready to be split into separate wells or plates. Before they are split into separate wells/plates, media is added to neutralize the trypsin reaction. Furthermore, plating cells is important for future experiments.

To plate cells, the number of cells is counted using a machine and then split evenly into a six well plate to use for future experiments. If there are any remaining cells, they are mixed with a freezing solution and re-stored in the liquid nitrogen tank for the next use. Splitting and plating cells are significant for following experiments such as qPCR, western blots, and staining. qPCR is utilized for determining the actual amount of PCR product that is present in each cycle. The process uses a fluorescent report in the PCR reaction, allowing to measure DNA generation. The main difference between PCR and qPCR is that PCR is a qualitative technique that shows the absence or presence of DNA, whereas qPCR is a quantitative technique that determines the amount of DNA amplified after each cycle. Moreover, western blotting is a method that detects particular protein molecules among many proteins. The protein molecules are separated by size through gel electrophoresis. Western blotting is a method of gel electrophoresis; however it deals with proteins as opposed to DNA. Lastly, cell staining is a technique that is used to have a clearer visual of cells and their components under a microscope. Cell components are stained differently to allow for comparison depending on what the researcher is studying. For example, at the St. Hilaire Lab, cells have been stained using healthy cell markers, osteogenic cell markers and intermediate markers (indicate the process of a healthy cell undergoing transition into an osteogenic cell) to see the process of a cell becoming osteogenic more clearly. These learning techniques, such as genotyping and the basis of cell culture, can be utilized for projects that will involve qPCR, western blots and staining in order to study how a healthy cell transforms into a calcifying cell.

Results

After gel electrophoresis, the mice samples can be genotyped. ERCC1 is a gene that is genotyped as either wildtype or flox. Flox refers to the sandwiching of a DNA sequence by two loxP sites. This utilizes the Cre-loxP system, where Cre is a protein that can catalyze the recombination of DNA between specific sites. These sites are the loxP sequences, which contain specific binding sites for Cre. Cre will then get rid of the loxP sites so that the DNA sequence in between those sites can be translated.

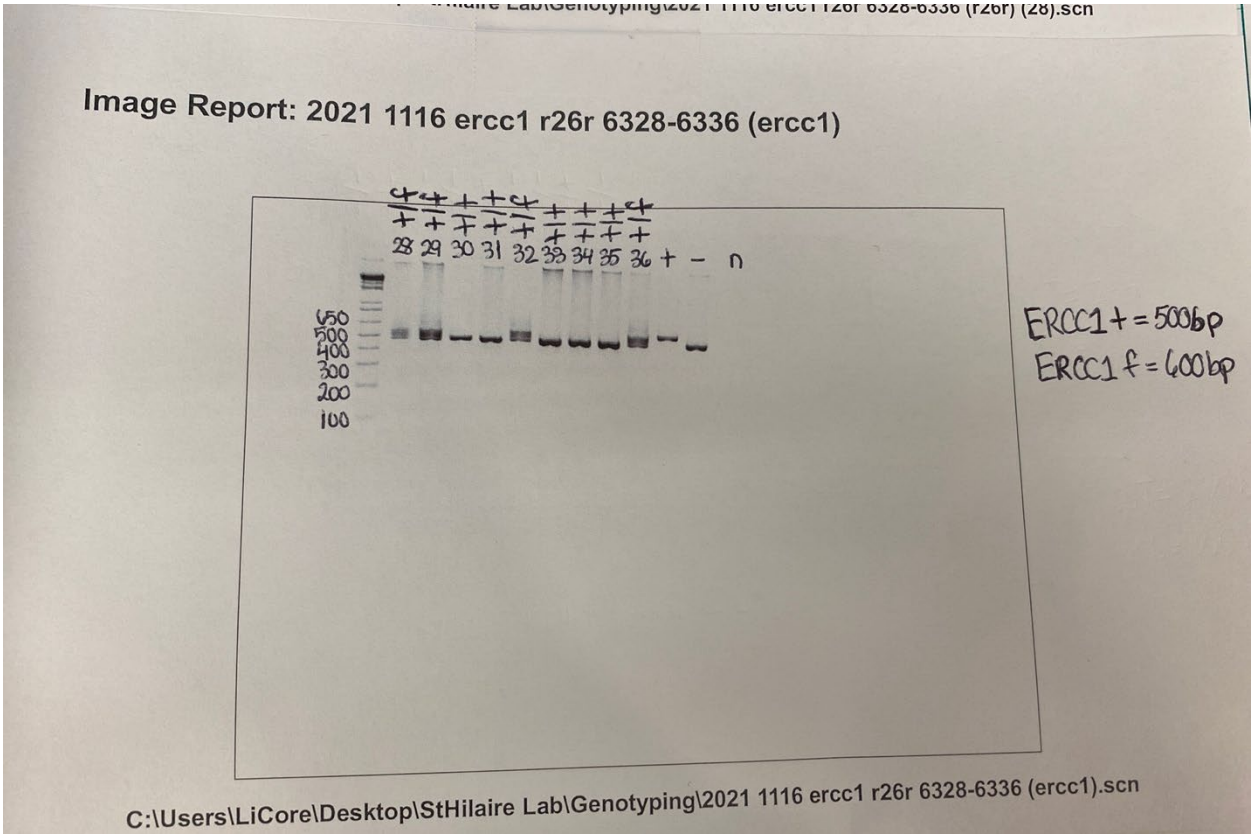

Figure 1: ERCC1 genotyping results. Both + labels indicate that the mouse is wildtype, one + and one f label indicates that it has one loxP site and having two f labels indicates that there are two loxP sites. The positive control represents the allele as flox and the negative control represent the allele as wild type. The no template control at the end is water and it important for detecting contamination or the lack of amplification.

The gene R26R works similarly to ERCC1. The mutant gene contains loxP sites, which surround a specific part of the DNA sequence. However, what is different is that the stop sequence after the loxP site contains the enhanced yellow fluorescent protein gene that is expressed with the implementation of the Cre-loxP system. With the addition of Cre removing the loxP sites, the stop sequence is then deleted, and the yellow fluorescent protein is expressed in the mutant gene.

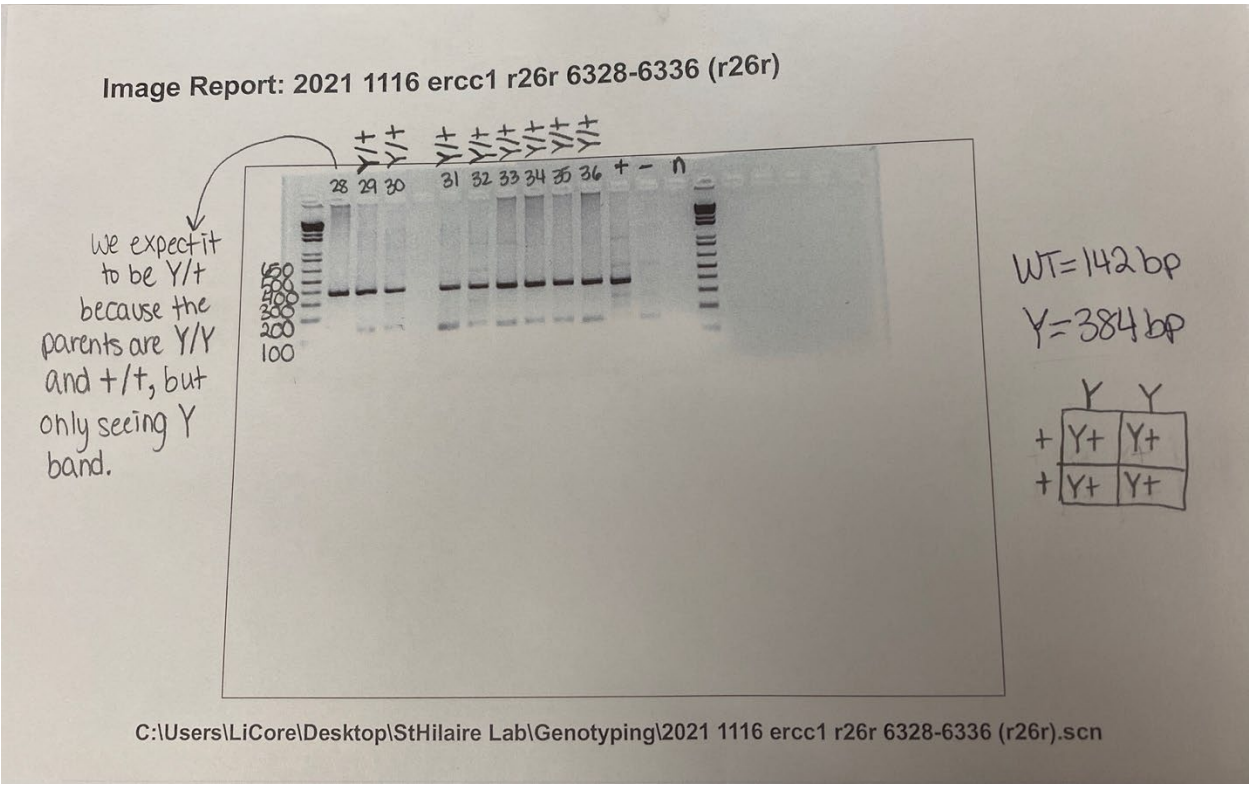

Figure 2: R26R genotyping results for the same mice samples as ERCC1. Both + labels indicate that the mouse is wildtype, one + and one Y label indicates that it has one loxP site and having two Y labels indicates that there are two loxP sites. The positive control represents the allele as Y and the negative control represent the allele as wild type. The no template control at the end is water.

Tert-Tg is a line that is transgenic, meaning that the mouse has been genetically engineered with an extra piece of DNA added to its genome. When genotyping this line, there is only one genotype that can occur. It is expected to see a band at both 200 base pairs and 600 base pairs. The first band at 200 base pairs is an internal control that just checks if PCR ran and was successful. The band at 600 base pairs indicates that the mouse is transgenic.

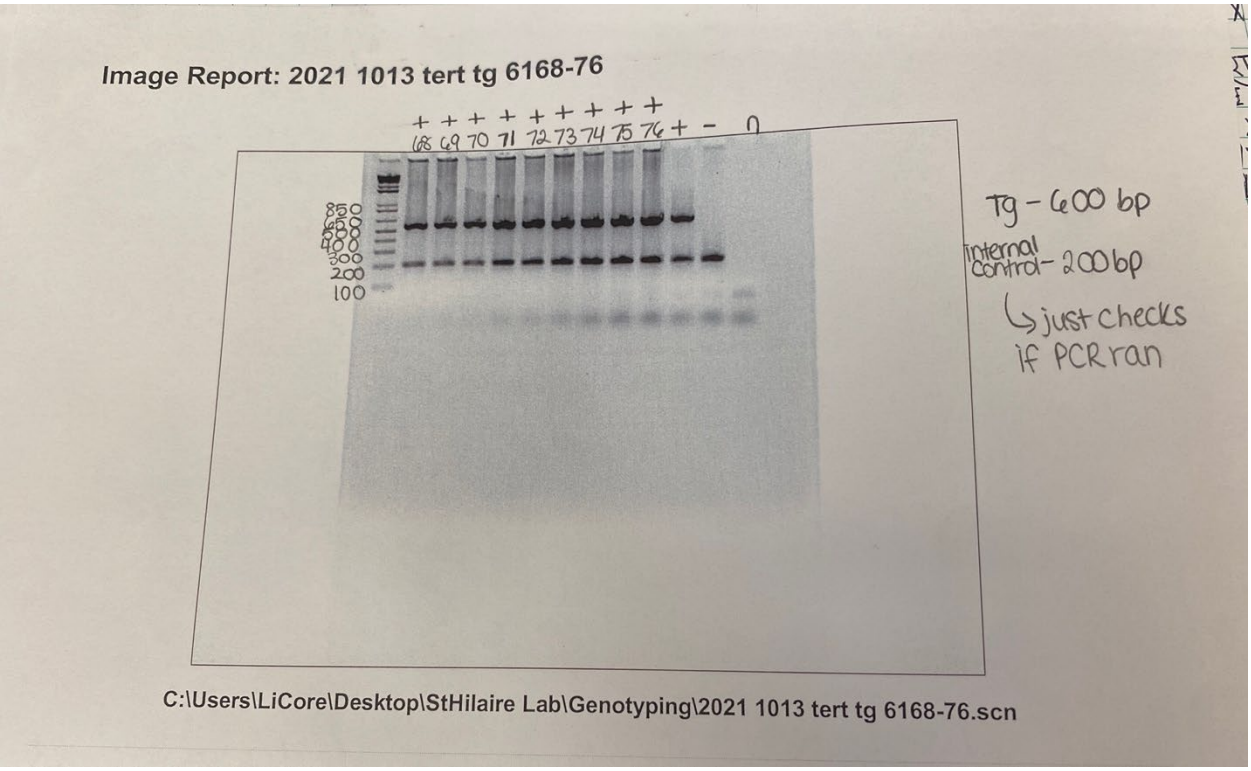

Figure 3: TERT-Tg genotyping results. Unlike TERT, MGP, CD73 and ERCC1/R26R, this gene has only one possibility in genotyping and it is a simple +, confirming that the mouse is transgenic. The positive control represents the allele as transgenic and it includes the internal control. The negative control represents the allele as the internal control. The no template control at the end is water.

TERT, CD73 and MGP are mouse lines that are all genotyped the same way. For example, +/+ indicates that the mouse is a wildtype, +/- indicates that the mouse is heterozygous (one normal allele and one mutated allele) and -/- indicates that the mouse is a knockout.

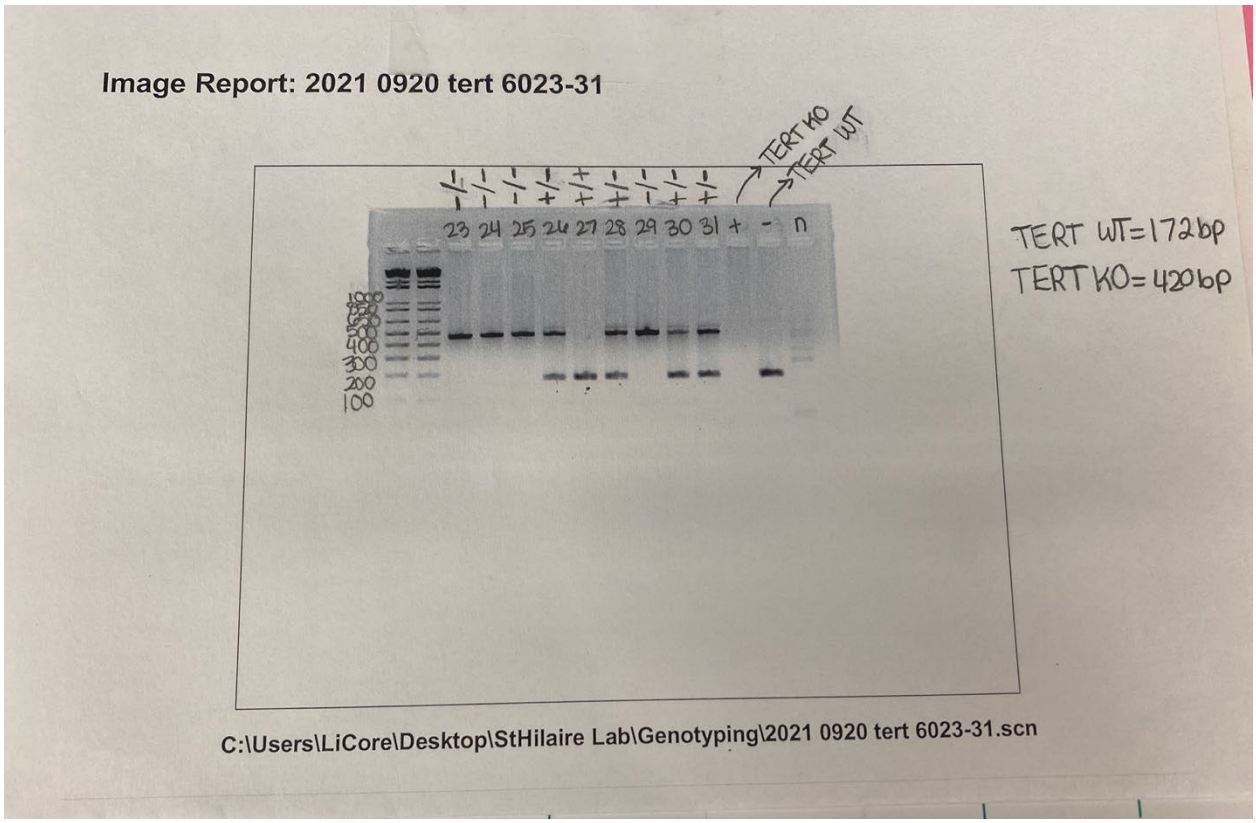

Figure 4: TERT genotyping results. The positive control represents the allele as knockout and the negative control represent the allele as wild type. The no template control at the end is water.

In vitro experiment results and techniques involve taking pictures from the microscope to track how cells are doing. It is important to take pictures before splitting the cells to ensure they are confluent enough. It is just as crucial to keep checking in on the cells even after a day or so of splitting them, to make sure that the cells are growing properly and have enough nutrients to do so.

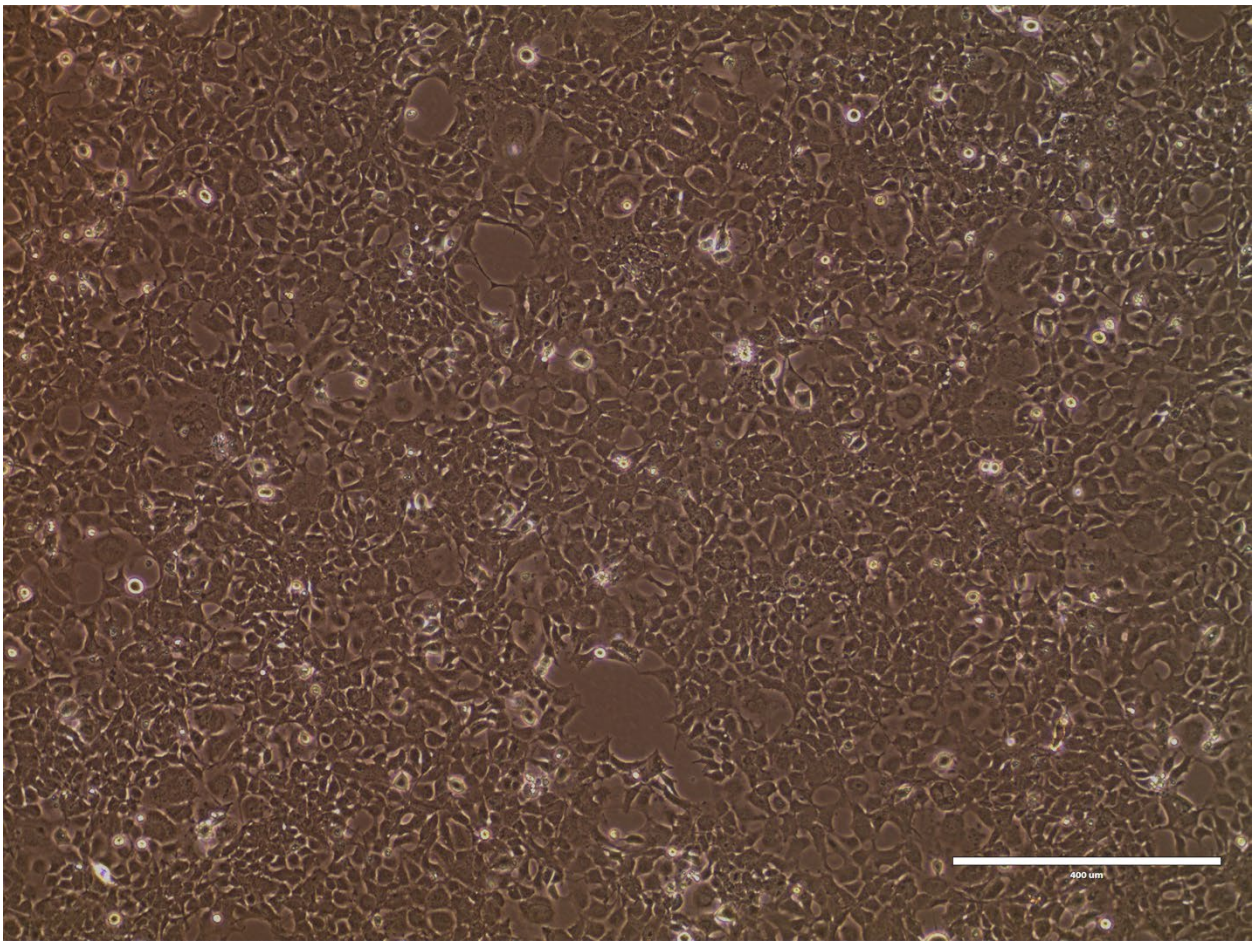

Figure 5: Cells are around 90% confluent and ready to be split.

The picture above shows HEK 293 cells, which are human embryonic kidney cells. These cells look around 90% confluent and are ready to be split so that they do not grow on top of each other, which can cause many problems. The picture below shows the cells one day after splitting them. The cells are much less confluent compared to the picture above, the day that the cells were ready to be split.

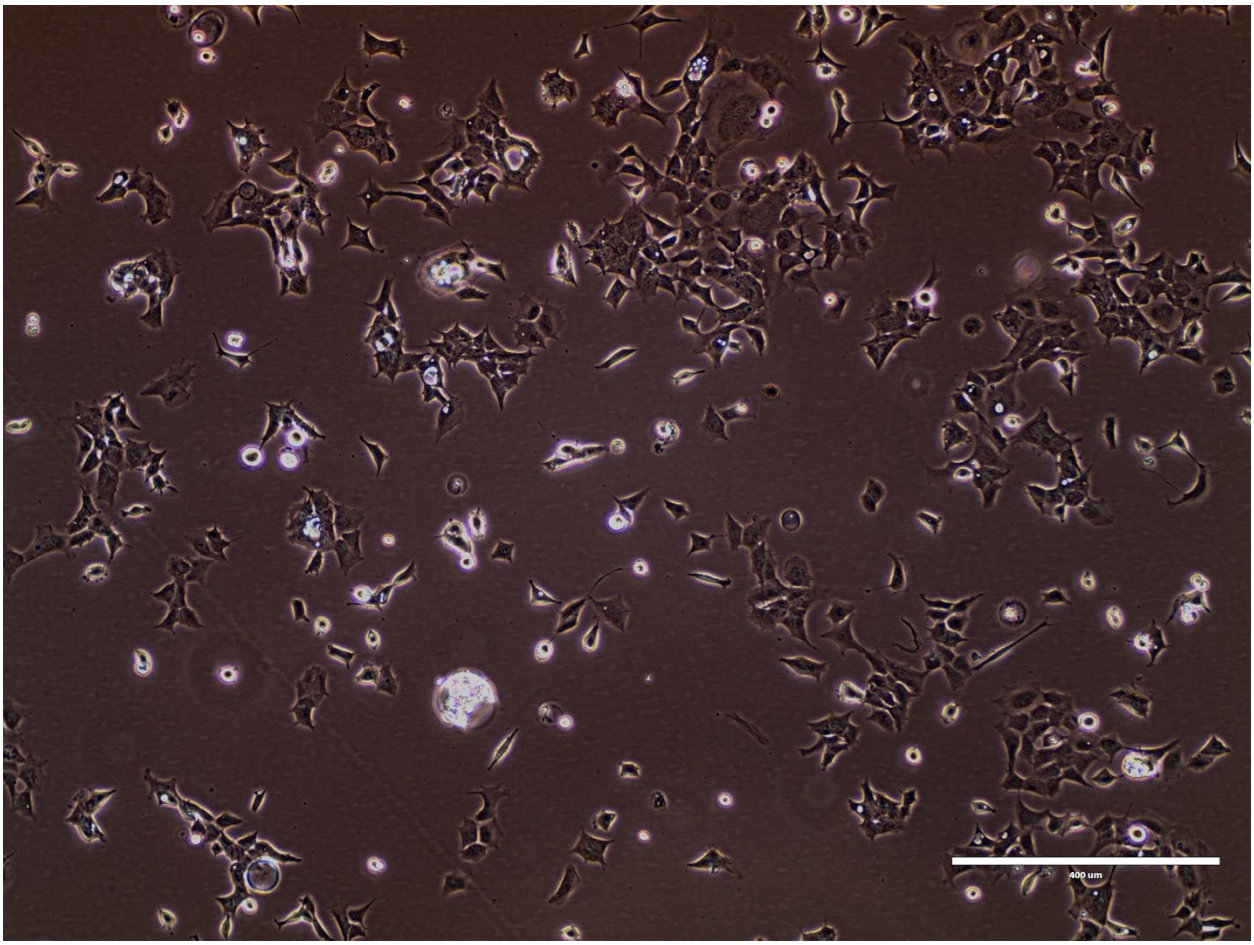

Figure 6: One day after the cells have been split; the cells are less confluent and have more space for nutrients to grow.

Conclusion

From the results, it can be concluded that both genotyping and cell culture techniques such as splitting, plating, and collecting cells are very important for future complex experiments. Genotyping helps researchers understand the hereditary behind an organism's genome and how it can be compared with the combination of genes that are inherited from the organism's parents. Furthermore, the process of genotyping allows researchers to see if there is a break or lesion in the DNA and how that will have an impact on the organism, such as having a disease. On the other hand, cell culture helps researchers understand the biology, physiology, and chemistry behind cells. Sterile practice is vital in cell culture to ensure that bacterial/fungal contamination does not occur, which will greatly impact results. Practices such as spraying objects and media with ethanol before bringing them under the chemical hood will guarantee that the cells are in a sterile environment. Basic techniques in cell culture can help with experiments in qPCR, staining and western blots, which can be used to study how a healthy cell transitions to a calcified cell.

References

Losurdo, Fabrizio, et al. "Medial Artery Calcification: The Silent Killer of the Leg." *American College of Cardiology*.

Yutzey, Katherine E., et al. "Calcific Aortic Valve Disease." *American Heart Association Journals*, vol. 34, no. 11, 4 Sept. 2014.

"St. Hilaire Laboratory Cardiovascular Calcification and Remodeling." *Sthilairelab.pitt.edu*, www.sthilairelab.pitt.edu/?page_id=71. Accessed 12 Dec. 2021.