FGF2 Discs in Media to Produce Quality Induced Pluripotent Stem Cell Cultures

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Acknowledgements:

This endeavor would not have been possible without NeuraCell. Many thanks for providing all induced pluripotent cell lines and cultures to be used in this experiment. Additionally, I am deeply indebted to Dr. Taylor Bertucci for allowing me to contribute to her own research of methods of feeding induced pluripotent stem cells and for project guidance. Lastly, I would like to express my deepest appreciation to Susan Goderie for training me in procedures of ICC, microscope use, and lab safety. I could not have undertaken this journey without her guidance.

Abstract:

Induced pluripotent stem cells (iPSCs) have held great promise for cell therapy applications. Their ability to grow indefinitely and differentiate into almost all cell types in the human body have made them valuable tools for regenerative medicine, tissue engineering, and developmental biology. In the past decade, iPSCs have shown promise in the treatment of degenerative disorders like Alzheimer's and Parkinson's disease. It is hoped that in researching iPSCs, new ways of disease modeling can be achieved while simultaneously gaining the ability to replace degenerating tissues and impaired cells in the body. This creates a need to grow quality iPSC cultures to ensure the success of future studies using them. However, growing these cell cultures does not come without difficulty. Oftentimes iPSC colonies lose their pluripotency through spontaneous differentiation, which is when an iPSC(s) turns into a different cell within the body. This study will test a new method of feeding iPSCs through a FGF2 disc in media to increase pluripotency, decrease spontaneous differentiation, and improve iPSC cultures overall. It was found that the FGF2 disc method of feeding cells maintained similar results to that of the standard daily cultures, while maintaining a less costly and labor intensive process.

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Introduction:

Applications of induced pluripotent stem cells:

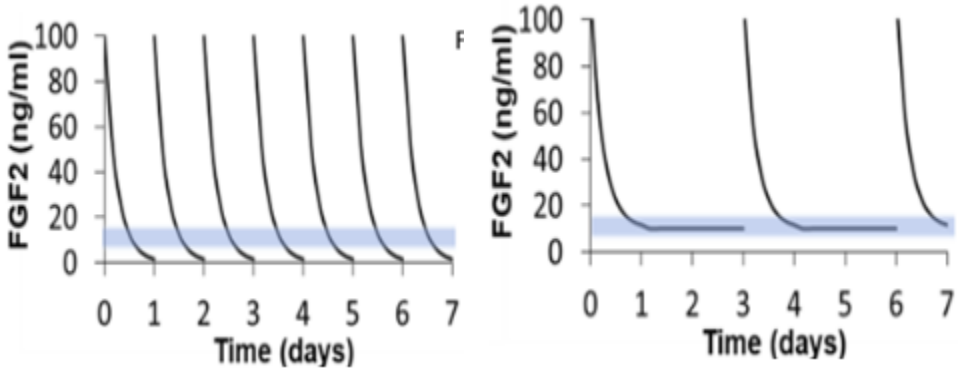
Induced pluripotent stem cells (iPSCs) hold the ability to proliferate without limit while maintaining the potential to differentiate into, theoretically, all 220 cell lineages within the human body. Additionally, these cells can be produced by reprogramming somatic cells and are not subjected to ethical issues(Takahashi, K., 2007). This makes iPSCs a valuable tool for regenerative medicine, tissue engineering, and developmental biology(Zhu, Z., 2013)(Yang, Y., 2016). Though there are many ways iPSCs can be applied, they have shown great promise in disease modeling. Creating such models from iPSCs have helped researchers further understand and potentially treat degenerative disorders such as Alziemers and Parkinson's disease(Han, C., 2018). This experiment is a part of a 5 year grant study by the Neural Stem Cell Institute. Brain organoids, produced by differentiating iPSCs, will be used to study the spread of the Tau protein, a protein found in the brain, which when missorted in the brain due to a genetic mutation is a leading cause of Alzheimer's disease(Zempel, H., 2014). To ensure the success of this subsequent study, along with other future research using disease modeling, high quality iPSC cultures must be produced.

Problems with the standard method of culture:

The proper maintenance of iPSC cultures minimizes spontaneous differentiation and preserves pluripotency throughout each stem cell. Enhancing methods to maintain pluripotency within iPSC cultures is essential in increasing the number of iPSCs produced and the potential to subsequently differentiate them into a lineage of choice for future applications. Fibroblast growth factor 2 (FGF2) is a fundamental medium component of many types of stem cells, including iPSCs(Amit, M., 2000). Standard culture methods to maintain undifferentiated iPSCs require a daily culture medium change, even on weekends and holidays, which can be problematic for laboratories in the industry. This accepted culture method of replacing media daily is not only costly and labor intensive, but it does not fully eliminate spontaneous differentiation of iPSCs in culture(Amit, M., 2002). It is observed that the soluble FGF2 used in daily medium is highly liable(Levenstein, M., 2006) and, as a result, levels of this protein fluctuate dramatically between

each medium replacement, which can be stressful for the iPSCs. Differentiation within iPSC cultures can be attributed to these fluctuations in the levels of soluble FGF2 within media.

Disc method of culture:

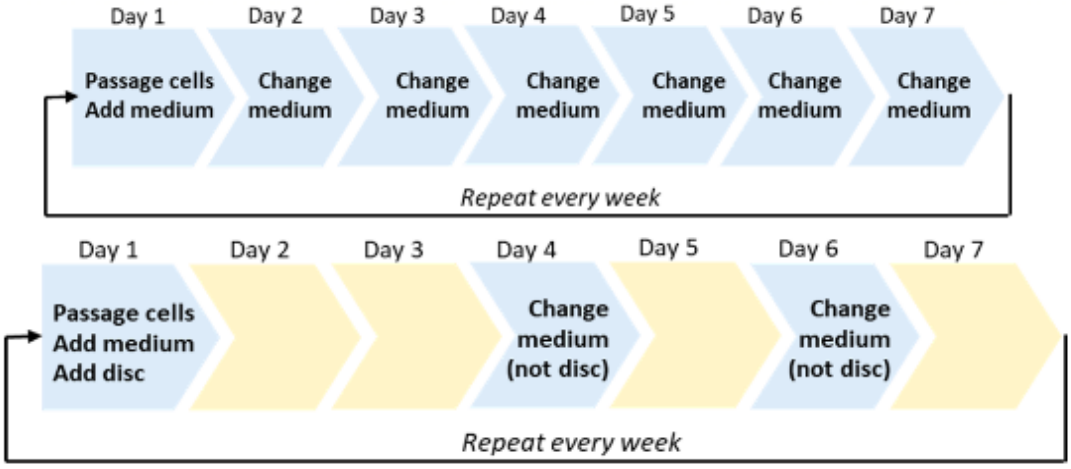


This figure represents the levels of FGF2 in media. The graph on the left shows the levels of FGF2 in media for iPSCs cultured with the mTESR daily method, and the graph on the right shows the levels of FGF2 in media for iPSCs cultured with the disc method. Soluble FGF2 has a half life of about 4 hours, so iPSCs culture with mTESR daily are subjected to low FGF2 levels daily.It is found that more sustained levels of FGF2 improves the maintenance of iPSC cultures(Lotz S., 2013). In this experiment we hypothesized that FGF2 discs in media of iPSC cultures will result in increased pluripotency, decreased spontaneous differentiation, and improved iPSC cultures overall. Additionally, this new method culture only requires one disc replacement per week and medium changes twice per week, making maintenance of iPSC cultures less costly and labor intensive.

Methods:

Culture

There are many different cell lines of iPSCs depending on the genome of the fibroblast donor. In this case, I utilized 6 different cell lines to test our hypothesis, GIH7-A01, GIH7-B12, GIH7-F02, GIH7-C1, GIH7-C3, and F11350.1. All GIH7 lines came from the same donor, but were put through CRISPR/Cas9 editing as a way to correct or induce genetic mutations that are known to cause Alziemer's, resulting in different cell lines. The "wildtype" line F11350.1 was taken from a donor without a mutation and did not go through the CRISPR/Cas9 process. We used two different culture methods per cell line, FGF2 discs in media as our test sample and the standard culture method, mTSER daily, as our control. mTSER daily requires a medium change every day of the week, while the FGF2 disc method only requires a disc change at the beginning of the week and medium changes twice a week(see fg. 1). Levels of FGF2 in media for both conditions were monitored. The iPSCs were thawed and grown for 3-4 weeks, and fixed three days post passage. Complications with culture for line GIH7-C3 under mTESR daily conditions resulted in only the disc cultured condition being seated, therefore there is no data on the daily condition for that cell line.



Immunocytochemistry

IPSCs have many transcription factors and surface markers that can prove its pluripotency. In this case, we used immunocytochemistry(ICC) to stain for the transcription factors OCT3/4, SOX2 and NANOG, which should be present in the nuclei of all

undifferentiated iPSCs. Cells were blocked with a .01% Triton X solution in PBS, 3% bovine serum albumin, and 10% normal donkey serum. Stains of OCT3/4 were at a dilution of 1:100 for the primary antibody and a dilution 1:1000 for the secondary antibody. Stains of SOX2 had a 1:100 dilution of the primary antibody and a dilution of 1:300 for the secondary antibody. Stains of NANOG were at a dilution of 1:100 for the primary antibody, and at a dilution of 1:1000 for the secondary antibody. In this case, we used the colors Alexa 488 (green) for OCT/4, Alexa 647 (far red) for SOX2, and Alexa 546 (red) for NANOG. The cells went through three layers of staining on the same day, and left overnight to image the next day.

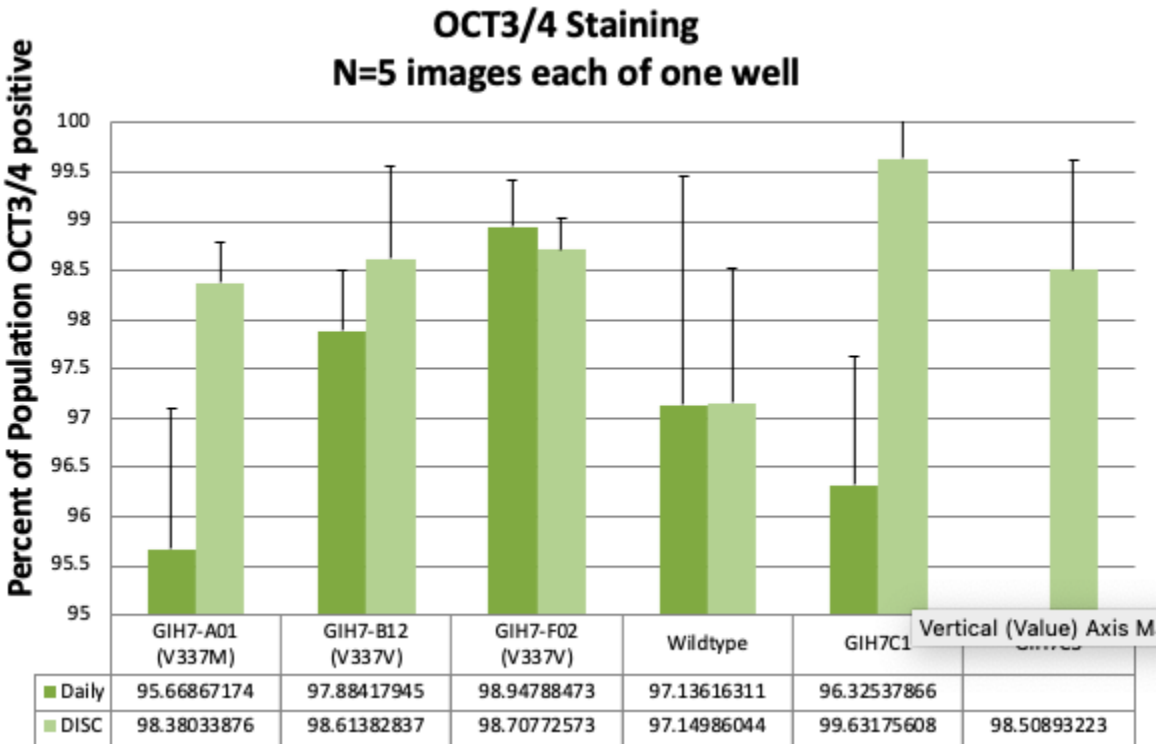
Imaging

We used the Axio Observer and Axiovision software to take representative images of each cell line cultured with the daily and disc method for data collection (See fg.) We took 5 images per one well at a magnification of 320x. Each image was taken at a set exposure of 2000ms for OCT3/4 in Alexa 488, 3500ms for SOX2 in Alexa 647 and 5000ms for NANOG in Alexa 546. We also used DAPI, a nuclei marker present in all cells regardless of pluripotency, as a control. At this point in the experiment, it was clear that there were complications in the ICC process for the NANOG transcription factor for all cell lines, so further imaging for that transcription factor was halted.

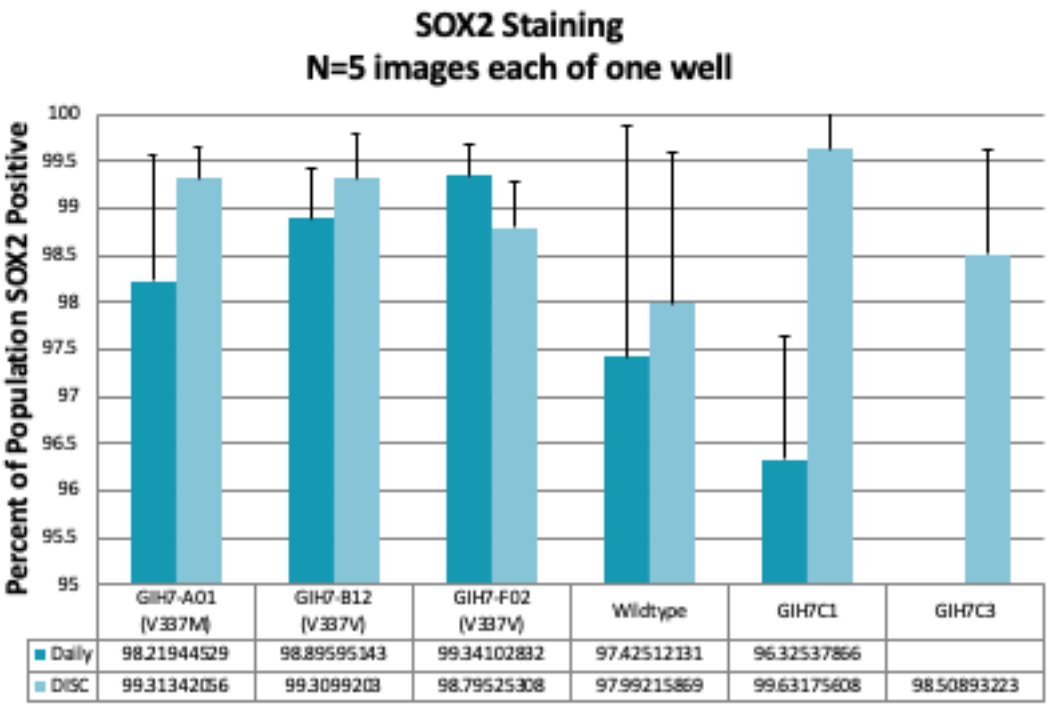
Data Analysis

The method of data analysis we utilized was using the manually taken representative images of all iPSC lines and conditions and manually counting each cell positive for OCT3/4 and SOX2, then marking cells that were only positive in only one pluripotency marker, comparing all cells to our DAPI control and ultimately determining what percentage of the iPSC colonies maintained pluripotency. All data was collected into Microsoft Excel to calculate percent and standard deviation. Other factors, such as observed cell death and mitosis, were also analyzed with manual counts. Let it be known that mitosis and death counts were observed and therefore subjected to human error. Data analysis on the transcription factor NANOG was not conducted due to complications with ICC.

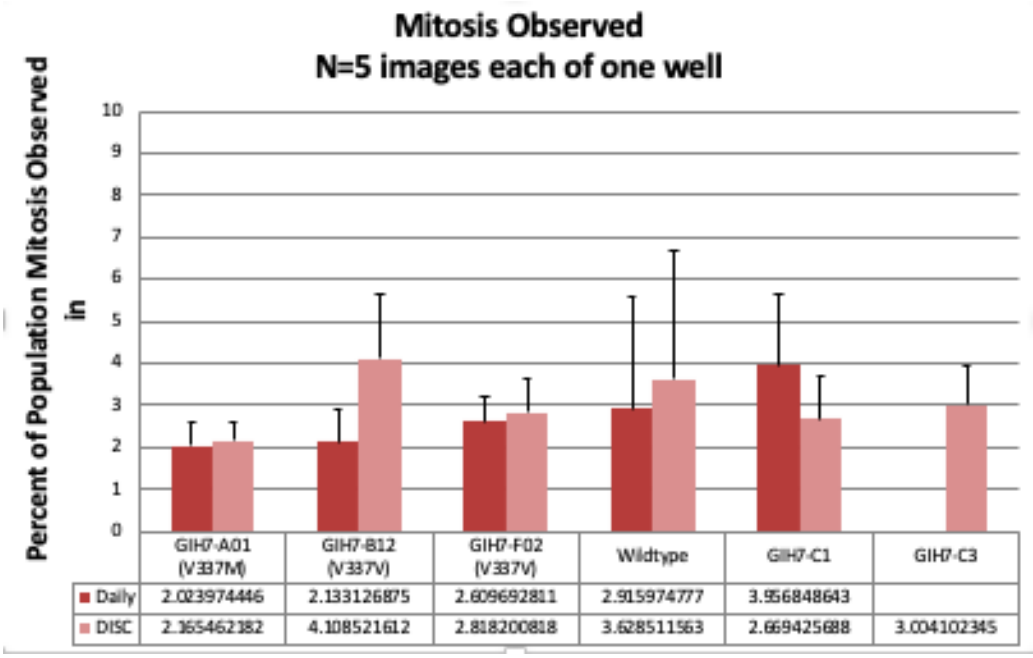
Results:



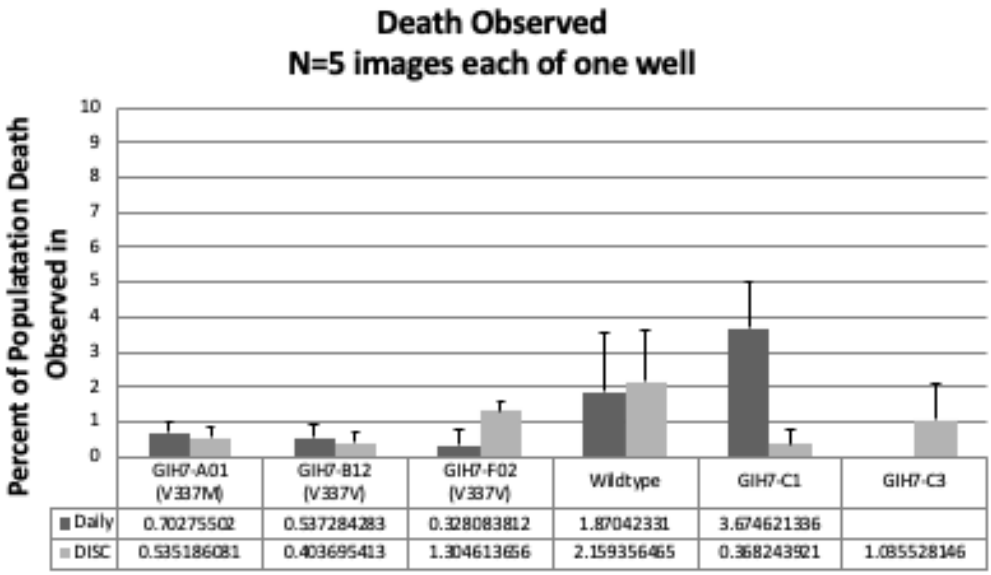
This graph (fg.3) shows the percent per population that is positive for the transcription factor OCT3/4 for each cell line. The dark green bar represents the iPSCs that were cultured with mTSER daily and the light green bar represents the iPSCs cultured with the FGF2 disc in media. Error bars represent standard deviation. Daily standard deviations range from 0.4778-1.4339 and disc standard deviations range from 0.3258-1.3738.



This graph (fg. 4) shows the percent per population that is positive for the transcription factor SOX2. The dark blue bar represents the iPSCs that were cultured with mTESR daily and the light blue bar represents the iPSCs with the FGF2 disc in media. Error bars represent standard deviation. Daily standard deviations range from 0.3249-2.4300 and disc standard deviations range from 0.3248-1.5926.



This graph (fg. 5) shows the percent of population mitosis is observed in. There are clear ways to tell when a cell is dividing within fluorescent images. Oftentimes the fluorochrome used will bind in a non-specific area around the cell because the genome of the iPSC becomes exposed and the shape of the cell changes during division. This non-specific binding and shape change is how cells in mitosis are determined. The dark red bar represents the iPSCs that were cultured with mTESR daily and the light red bar represents the iPSCs with the FGF2 disc in media. Error bars represent standard deviation. Daily standard deviations range from 0.5556-2.6515 and disc standard deviations range from 0.4173-3.0244.



This graph (fg. 6) represents the percent of population cell death is observed in. When iPSCs die, they leave behind cytoplasm and cell debris. The excess cytoplasm and debris is how cell death was determined within the iPSC colonies. The black bar represents the iPSCs that were cultured with mTESR daily and the gray bar represents the iPSCs with the FGF2 disc in media. Error bars represent standard deviation. Daily standard deviations range from 0.3156-1.6891 and disc standard deviations range from 0.2412-1.4756.

Discussion and Conclusion:

It can be concluded that both daily and disc culture methods for GIH7 lines and wildtype line F11350.1 yielded high levels of pluripotency above 95% positive for OCT3/4 and SOX2. It is clear that the disc method of culturing iPSCs produces results that match the standard and accepted culture method of mTESR daily, while maintaining a more inexpensive and less labor intensive process. It could not be determined in this experiment whether the FGF2 disc method improved pluripotency within the iPSC cultures due to statistically insignificant results from a small sample size. Nonetheless, the disc culture method seems to be an effective method for culturing iPSCs.

An iPSC colony's growth and death rate can say a lot about the health and quality of the colonie itself. In this case, a good colonie should have high levels of cell division and a low number of dead cells. It can not be concluded if the disc culture method yields healthier cell colonies due to insignificant results and the amount of death, but our results give insight to the health of these colonies at the time they were fixed.

Future direction:

There are many future directions to be taken from this study. All cell lines used in this experiment are known to be “cooperative” in culture and usually express high levels of pluripotency regardless of the culture method. With this in mind, it would be beneficial to conduct the same experiment on more “uncooperative” cell lines that are known to spontaneously differentiate with the mTESR daily culture method to possibly see more significant differences in pluripotency between the two methods.

Another future experiment could include other pluripotency markers. There are many other transcription factors and surface markers that can be used to mark pluripotency, such as SSEA4 and TRA-1-60. When iPSCs begin to differentiate they may still express some pluripotency markers until later stages in differentiation. The transcription factor SOX17 can be used to mark differentiated iPSCs, which is an even clearer way to recognize differentiated iPSCs even if they are positive for some pluripotency markers. Overall, collecting data across

more pluripotency or differentiation markers would be beneficial when conducting this experiment to determine the effects of the FGF2 disc in culture.

It became apparent in this experiment that there could be errors with the ICC process, as seen with the results of the transcription factor NANOG. Even after re-staining the iPSCs for NANOG to collect data for this experiment the results were very weak, which would normally signify all the iPSCs were negative for NANOG. After many smaller experiments were conducted to see why NANOG was not showing up well in the images, we were able to determine there were no issues with the primary or secondary antibodies used and that it must be an issue with the iPSCs themselves. However, considering the high levels of expression OCT3/4 and SOX2 in the all iPSC cell lines, it was clear the colonies maintained their pluripotency and therefore should also be positive for NANOG. After consulting scholarly articles on the topic, the negative NANOG results could be attributed to how the iPSCs were originally reprogrammed from fibroblasts(Schwarz, B., 2014). Further research on this topic could be beneficial in determining whether NANOG is a good pluripotency marker to use when testing the pluripotency of iPSC colonies.

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