Small Compounds and Microfluidic Culture for Inducing Maturation of hiPSC-derived Hepatocyte-Like Cells

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Hepatocytes play a critical role in drug metabolism in the human body, and thus culturing these cells *in vitro* would allow for humane safety testing of new drugs. Instead of using human primary hepatocytes, causing human induced pluripotent stem cells (hiPSCs) to differentiate into hepatocyte-like cells (HLCs) is one method of obtaining cells for culture. Additionally, it is a promising treatment for liver disease. However, current methods have produced cells resembling fetal hepatocytes more closely than adult ones, and do not express enzymes critical for drug metabolism. We hypothesize that using 3D cell culture as well as incorporating tensile stress and small-molecule compounds into the culture environment might better mimic *in vivo* conditions and thus produce more mature HLCs from hiPSCs. To study 3D cell culture and chemical compounds, we introduce six compounds to a microfluidic culture environment. These compounds are thought to play a role in signaling pathways related to hepatocyte differentiation and maturation. Of them, five have been used in other types of culture, while one is entirely new to differentiation research. Hepatocyte-committed hiPSCs at 16 days of growth are cultured in microfluidic wells with different combinations of the compounds added to a hepatocyte-growth basal medium. At day 22, cells are harvested and gene expression will be evaluated using immunocytochemistry and quantitative reverse transcription polymerase chain reaction (RT-PCR) to determine which compounds produce HLCs most similar to adult hepatocytes. We expect that the final results will help in further refinement of HLC maturation procedures.

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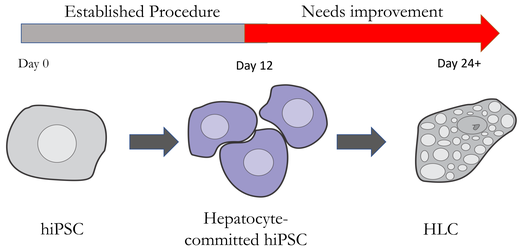
Introduction

- Liver cells (hepatocytes) play a critical role in human drug metabolism
- In vitro hepatocyte culture would allow for humane drug safety testing
- Causing human induced pluripotent stem cells (hiPSCs) to differentiate into hepatocyte-like cells (HLCs) allows for in vitro culture and is also a promising treatment for liver disease
- However, current differentiation procedures produce cells more like fetal hepatocytes than adult ones



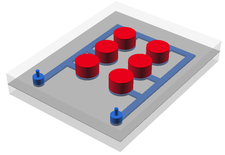
By more closely mimicking in vivo conditions, our lab is trying to produce HLCs that are more mature.

Examine Three Aspects of Cell Culture:

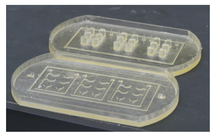
- **3D culture**: cells are traditionally cultured on a flat surface, but in the body are part of 3D environments.
- **Small-molecule compounds**: signaling pathways triggered by chemical cues cause cells to change state.
- **Tensile stress**: physical cues may also play a role in differentiation.

Microfluidics

Polydimethylsiloxane (PDMS) polymer can be poured into a mold to make culture environments of desired shape.
Clear polymer allows for live image analysis, has stiffness in physiological range (unlike glass / plastic), and is permeable to O_2.

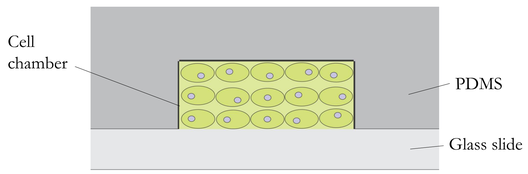


CAD model of mold



3D-printed, reusable mold

Cross-section of microfluidic well



Traditional Cell Culture
- Only supports 2D culture
- Large volumes and cell counts
- Limited adaptability to different experimental designs

Microfluidics
- Can support more realistic 3D culture
- Small well mimics scale of microstructures in the human body
- Flexibility in shape and design

Compounds in Microfluidic 3D Culture

Identified six small-molecule compounds to examine:

- Valproic acid (VPA)[1]
- 8-bromo cyclic adenosine monophosphate (8-Br-cAMP)[2]
- Dexamethasone (Dex)[3]
- Functional Proliferation Hit 1 (FPH1)[4]
- Functional Hit 1 (FH1)[4]

} Successful in other culture types

- Beberine[5]

} New to hepatocyte differentiation

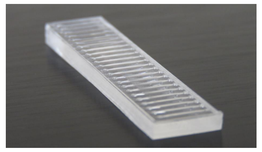
Experimental Design

Day 0-16: established differentiation procedure → Day 16-21: culture in microfluidics with compounds → Stain for DAPI and A1AT → Measure A1AT fluorescence intensity

x 6 (positive control)

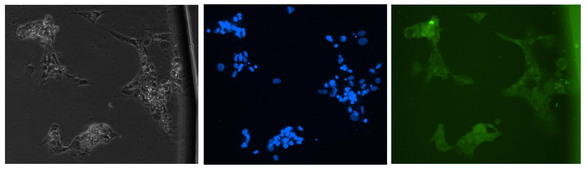
x 0 (negative control)

x 5 (6 conditions, 1 compound missing from each)

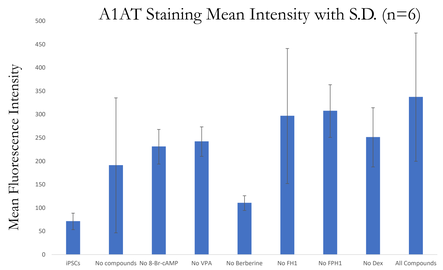


Microfluidics chip has many small wells[6]. The high precision of soft lithography allows them to be placed close together.

Results and Analysis



Example cell images: brightfield (left), DAPI nuclear staining (center), DAPI staining (right)



- A1AT is a protein commonly used to stain only liver cells
  Undifferentiated iPSCs had the lowest mean fluorescence, while the 6-compound treatment had the highest.
- The treatment without berberine had significantly less intensity than other treatments, suggesting that berberine was a critical factor in improving expression of A1AT.

Genetic Analysis of Promising Compounds

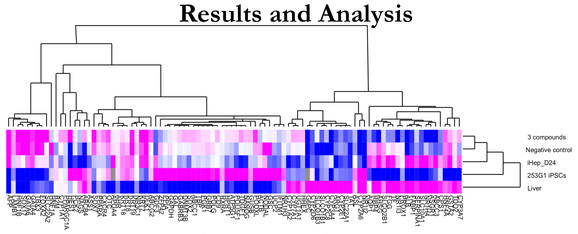
Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) allows gene expression to be evaluated quantitatively. 90 compounds relevant to hepatocyte development were chosen to measure.

RT-PCR Process

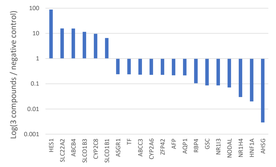
Collect RNA → Reverse transcription: cDNA → Amplify cDNA with cycles of PCR → Fluorescent cDNA detector reaches threshold after certain number of cycles

Cell counts in microfluidic wells are too small for this procedure, so one compound combination (berberine, FH1, and valproic acid) was chosen for culture on a larger, traditional plastic plate.

Results and Analysis



Gene expression clustering

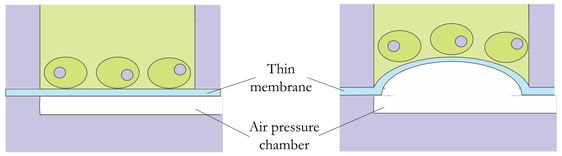


Genes with greatest change in expression

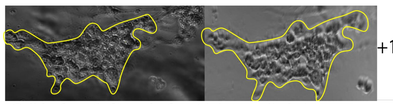
- Gene expression suggests that the 3-compound treatment did not improve overall similarity of hiPSCs to mature hepatocytes.
- However, expression of 7 out of 9 CYP family genes (involved in drug metabolism) was upregulated by the treatment.

Tensile Stress

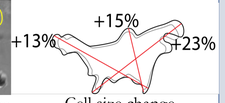
Cells are cultured on a thin membrane that is stretched via air pressure applied from below. The spacing of the culture wells along the air tube creates a gradient of tension. This experiment is currently in progress.



Without pressure — Air pressure applied



Cell before (left) and after (right) applied air pressure



Cell size change

Conclusions

- A1AT staining of cells grown in microfluidics with small-molecule compounds suggests that the use of small-molecule compounds may have improved resemblance of HLCs to mature hepatocytes.
- RT-PCR results suggest that use of VPA, dex, and FH1 did not improve resemblance of HLCs to mature hepatocytes compared to existing methods.
- VPA, dex, and FH1 did improve expression of many CYP-family genes
- The no-berberine condition resulted in significantly lower A1AT expression compared to other conditions, suggesting berberine might improve HLC maturation.
- The microfluidic chip designed to apply tensile stress to cells successfully applies tension in 2D.

Discussion

- Cell counts in the microfluidics decreased over the course of the experiment and only a few cells remained in many of the wells at day 21. This limits the applications of cells produced using this method.
- Conditions with highest A1AT fluorescence also had very low cell counts, suggesting that these cells were more susceptible to death and/or weakened attachment.

Future Directions

- Extend quantitative RT-PCR experiment to other combinations of compounds.
- Optimize compound concentration.
- Design a culture chip that allows for both microfluidic 3D culture and tensile stretching.
- To create a more realistic *in vitro* model of *in vivo* differentiation conditions, unite the 3D culture, chemical compound, and tensile stress approaches into one model.
- Find a way to scale up cell production so that once successful hepatocyte-like cells are obtained, they can be used in applications.

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Acknowledgments

This research project was conducted as part of the 2017 Nakatani RIES Fellowship for U.S. Students with funding from the Nakatani Foundation. For more information see http://nakatani-ries.rice.edu/. Thank you to Kamei-sensei and Nicolas for their incredible mentorship. Thank you as well Professor Kono for his excellent advising and to Sarah Phillips, Ogawa-san, Endo-san, and Professor Kono for making this program possible!