Testing a hypothesis about a problem in human kidney tissue engineering

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1. Introduction

Currently, human kidney transplantation relies on fully-developed donor kidneys. However, the advent of induced pluripotent stem cell (iPSC) technology developed by Takahashi and Yamanaka in 2006 has led to the research aimed at creating functional kidneys that can replace *in vivo* animal testing of renal drug toxicity, and furthermore, alleviate the reliance on kidney donors for clinical transplantation. iPSCs are unique in that they can be created using human adult somatic cells, such as skin cells, and induced to maintain a pluripotent state analogous to embryonic stem cells. Similar to ES cells, iPSCs can be induced to differentiate into other somatic cell types.

The Davies lab have successfully differentiated iPSCs into renal cells in an effort to create human kidneys, but have found that the renal cells do not self-organise properly and thus fail to develop into kidneys. The proposed hypothesis is that the differentiation is imperfect; there may be irrelevant cell types 'contaminating' the cultures. These contaminating cells would likely physically obstruct properly differentiated renal cells from sorting into organised cell populations during kidney construction. This obstruction of cell migration and self-organisation presents a problem in kidney engineering; in order to produce functional human iPSC-derived miniature kidneys for drug toxicity testing, and eventually mature kidneys for transplantation, it is critical to understand the nature of the self-organisation problem.

Two approaches are taken to test whether contaminating cells can interrupt self-patterning cell systems. The first approach is to develop a computer model of the system. This model uses the idea that different cell types adhere to each other and themselves with different binding energies. As cells are allowed to migrate in the virtual "culture" as the program progresses, the system begins to pattern. The computer model allows for additional cell types, with different binding energies, to be added in order to examine the effects on pattern formation. The second approach deliberately contaminates a two-dimensional *in vitro* self-patterning system previously developed by the lab. This system uses synthetic genetic modules to fluorescently colour and visualise the locations of different cell types after the system is allowed to self-organise. Comparison of these two approaches will determine whether the computer simulation is an adequate model of the *in vitro* self-patterning culture, and furthermore, will allow future researchers to model their self-patterning systems without the labour- and time-intensive efforts of cell culture.

2. Summary of Research Activities

A self-patterning system has already been created and used by the Davies lab to allow human embryonic kidney cells expressing the tetracycline repressor protein (T-REx-293 cells) to grow and self-organise into spontaneous patterns in two-dimensional cultures (Cachat et al., 2015). This system includes tetracycline-inducible cells over-expressing Cdh1 (an adhesive surface protein) labelled with green fluorescent protein (GFP) and

Cdh3-expressing cells labelled with mCherry (a red fluorescent marker). Both cadherins Cdh1 and Cdh3 have previously been shown to be involved in cell sorting in mammalian cells (Nose et al., 1988).
This existing system was modified to include a wild type T-REx-293 cell line, which did not express either Cdh1 or Cdh3, nor did it express a fluorescent marker. This cell line represents the "contaminating," irrelevant cell line.
Varying proportions of Cdh1- and Cdh3-expressing cells and wild type cells were allowed to form monolayers in flasks over 48 hours. After 48 hours, they were imaged using a fluorescent inverted microscope and examined for pattern formation. Cultures of only Cdh1- and Cdh3-expressing cells, as well as cultures of only wild type cells, were imaged as controls.
A computer simulation, called a cell automaton, was developed to model the *in vitro* experiments described above. This simulation builds on a simulation already developed by Professor Davies (Cachat et al., 2015). Whereas his model simulated only a two cell type system (for example, a Cdh1- and Cdh3-only system), the new model includes features that allow up to 8 additional "cell types" to be added. These cell types are stored in computer memory using objects, allowing a set group of variables, or characteristics, and functions on those characteristics, to represent a particular cell type. Objects are particularly useful for initiating different instances of the same "type" of thing - for example, initialising a red cell type with an associated proportion and binding energy, and initialising a green cell type with a different proportion and binding energy. The cell automaton cycles through calculations of overall system energy, allowing cells in a grid to move if a new conformation is energetically favourable. These cells move by swapping with any of its eight surrounding neighbours. Cells along the border of the grid do not interact with the borders and can only switch with neighbouring cells.
Ideally, this automaton will allow users to modify the parameters of the system to their preference. It will also allow them to calculate Kolmogorov-Smirnov statistics at timepoints in the simulation. This statistic allows the user to compare the distribution of cells in the grid to a random probability distribution. Eventually, these calculations can be compared to Kolmogorov-Smirnov calculations for the *in vitro* model, which will determine if the computer simulation more or less accurately models the *in vitro* model.

3. Meaning and significance of results

3.1 Novel findings

Although the object-oriented Java software and interface is not yet complete and functional, the program generates a random grid of up to ten different cells. The cells are then able swap places in computer memory; however, I have not yet programmed the visual representation of the sorting (figure 1).
Because the wild type cell line does not produce a Cdh1 nor Cdh3 and a fluorescent marker such as GFP or mCherry, its growth rate may be slightly faster than the other two cell lines. Therefore, although the three cell types are seeded at equal amounts (33.33% Cdh1, 33.33% Cdh3, an 33.33% WT) at t=0, the WT cells are more abundant than either Cdh1 or Cdh3 cells at t=48 hours (figure 2, a). In fact, at 48 hours, the proportions of each cell type when seeded equally at t=0 are approximately 25% Cdh1, 25% Cdh3, and 50% WT. This was calculated by analysing pixel colour using ImageJ-Fiji image analysis software on photographs of plates at 48 hours.

The correct seeding proportions in order to achieve approximately equal proportions at 48 hours were determined to be 37.5% Cdh1, 37.5% Cdh3 and 25% WT (figure 2, b).
In the absence of quantitative analysis in the form of Kolmogorov-Smirnov statistics, it is not possible to definitively say whether the addition of wild type T-REx-293 cells hindered the self-organisation of Cdh1- and Cdh3-expressing cells over time. However, basic analysis of the images allowed some determinations to be made about the behaviour of cells when irrelevant, wild type cells are introduced. For example in a mixture seeded with 45% Cdh1, 45% Cdh3, and 10% wild type, red Cdh3-expressing cells appear to form large clusters with rounded boundaries, compared to green Cdh1-expressing cells, which form clusters with rougher edges interrupted with wild type (black) cells (figure 3, b). By contrast, when wild type cells are not present in a mixture of 50% Cdh1- and 50% Cdh3-expressing cells, green Cdh1-expressing cells form the largest clusters, mostly surrounding islands of smaller red clusters (figure 3, a).
Because of the apparent effects of wild type cells on cluster formation, the next step was to determine whether wild type cells tended to preferably border either Cdh1- or Cdh3-expressing cells. However, there did not appear to be a difference in the amount of red or green cells bordering wild type populations, only a difference in the shape of those border regions.

3.2 Previous findings

Previous findings in this research group have confirmed that iPSCs that have been successfully differentiated, but that approximately 40% of these differentiated cells were non-renal. Furthermore, these differentiated iPSCs do not form expected embryonic kidney structures well (Davies, unpublished). Whether the reason is that the improperly differentiated cells physically obstruct renal cells from self-organising was tested by these experiments.
Preliminary data indicate that irrelevant cell types (contaminating cells), such as improperly differentiated cells in iPSC-derived renal cell cultures or wild type T-REx-293 cells in these experiments, have some effect on cell patterning and sorting, although the quantitative nature of the effect remains unclear. This will be the subject of future research and modelling.
Further research will investigate whether cultures contaminated with an irrelevant cell type have a random distribution of Cdh1- and Cdh3-expressing cells, compared to cultures without the irrelevant cell type. These results may further confirm that physical obstruction contributes to self-organisation failure in the Cdh1 and Cdh3 culture experiments, which can lead to further experimentation that confirms that improperly differentiated cells in iPSC-derived renal cell cultures contribute to failure of kidney structure formation.
However, there is an alternative hypothesis that has yet to be tested. While these improperly differentiated cells may physically obstruct renal cells from self-organising to form kidney structures, they may also secrete signals into the surrounding region that prevents proper organisation and structure formation. This hypothesis would require alternative *in vitro* experiments involving specially conditioned media for cell culture. However, the nature of the alternative hypothesis lends itself well to computational modelling. While the computational model built for this project takes into account adhesion energies between cells, another computational model could secrete virtual “particles” that affect cells of certain types in a given radius, thus modelling the secretion hypothesis.

3.3 Biomedical significance

As previously noted, the practical uses of iPSCs in human kidney tissue engineering are widespread. Further research in the field can lead to applications ranging from drug toxicity testing to clinical transplantation. However, it will be critical to determine what causes the failure of sorting in differentiated iPSCs. Our experiments contribute to the first step in this process; they identify that a potential factor contributing to self-organisation failure is the presence of irrelevant cell types, and develop two models to test whether these irrelevant cell types cause self-organisation failure in known, controlled systems.

While the preliminary data from our experiments only represent a first step in discovering what prevents cell sorting in iPSC-derived renal cell cultures, further research confirming the effects of irrelevant cell types on iPSC-derived renal cell cultures may lead to changes in the protocol that is currently used to build iPSC-derived mini-kidneys.

On a larger scale, the computational model represents a generalised adhesive cell sorting automaton. While its design was based on the *in vitro* experiments and the hypotheses regarding irrelevant cell contamination as it relates to kidney formation, the simulation can ultimately be used to model any self-sorting cell system; it is not specific to either renal cells or cadherin-expressing cells.

Furthermore, both myself and the rest of the members of the Davies lab strongly believe that such simulations, as well as research results and methods, should be made freely available so that other students and researchers can use, build upon, and comment on work that has already been done. To that effect, I have made the code for the self-sorting cellular automaton - still a work in progress - available at https://github.com/cerog232/contaminated-cell-automaton. There, it can be modified, commented on, and freely used by anyone. In particular, we value scientific collaboration and cooperation, and would encourage people to add features, interfaces, or parameters that might be useful to their research interests and the research interests of others.

4. Replacement of animal experiments

The Davies lab has an interest in the replacement of animals in research. The long-term goal of the research carried out for this project involves directly replacing animal models used for *in vivo* drug toxicity testing. Nephrotoxicity - or toxicity to renal cells - has a long history of *in vivo* experimentation. Countless studies have tested potential drugs for nephrotoxicity in rat (Mengs and Stotzem 1993; Baliga et al., 1998), mouse (Portilla et al., 2006), zebrafish (Ding and Chen 2012), and many other animal models. The long-term product of the research carried out for this project results in eliminating the need for animal models of drug toxicity; with human kidney tissue derived from iPSCs, not only are animals spared drug testing, but the data from nephrotoxicity screens on human kidney tissue will be much more accurate and applicable to human disease than screens on animal tissue.

The project also directly replaces animals by using a human embryonic kidney cell line to model a cell sorting system, rather than using an animal cell line or animal renal cells.

4.1 Animal experiments

Originally, we attempted to use EX-CELL 293 animal serum-free medium to culture T-REx-293 cells. However, cell growth and adhesion in this medium was limited compared to Dulbecco's Modified Eagle's Medium (DMEM), the standard medium used in the lab for cell culture (figure 4). Even with the addition of calcium to aid the formation of adhesions, cell growth and monolayer formation failed after multiple passages and we had to seek special permission to switch to DMEM.

Beyond this discrepancy, the results obtained using T-REx-293 human embryonic kidney cells in DMEM medium for cell organisation experiments aligned with previous results in similar studies.
It must be noted that the reason this study was carried out is because of a discrepancy in animal and human experiments. While “mini-kidneys” have previously been developed by the lab using stem cells derived from mouse foetal tissue, the protocol fails when it is applied to human induced pluripotent stem cells. The hypothesis tested - that irrelevant cell types physically obstruct sorting cells - concerns a direct effect of the transition from animal models to non-invasive, replenishable human iPSC lines.

5. Adherence to original plan of investigation

As noted above, cell growth failure in the original animal serum-free medium combined with time constraints required us to switch to regular medium containing serum. This change was made primarily because cells grown in the animal serum-free medium failed to adhere to one another. With the addition of calcium, which mediates cadherin adhesion, cells formed three-dimensional blobs (figure 4, b) (Nose et al., 1988). Imaging the cell cultures for patterning required the cells to adhere to one another in a two-dimensional monolayer, something that could not be achieved without animal serum medium.
Time constraints also cut the project short before all the goals had been completed. For one, Kolmogorov-Smirnov statistics were not calculated for the *in vitro* model. This process requires specialised materials and techniques for acquiring images of the cell culture, as well as manually counting cells in the image in order to carry out the test.
While I was able to build on the original program Professor Davies wrote to model cell sorting, the new simulation will take more time to complete. My goal was to increase the interactivity of the simulation, allowing users to graphically change parameters, add cell types, take screen captures, and calculate proportions and statistics. The transition from Professor Davies’s program to an object-oriented program in Java took much more time than expected, and the new simulation and interface is not yet complete. Moving forward, I hope to refine the sorting algorithm, design a user interface, integrate Kolmogorov-Smirnov statistic calculations at intervals for user export, and adapt the model for three-dimensional systems.

6. Final summary

This research was an important first step in identifying the issues that lead to cell organisation failure in iPSC-derived renal cell cultures. Understanding that the failure could, in part, be due to physical obstruction by irrelevant cell types will allow us to ameliorate the renal cell differentiation process to minimise the amount of irrelevant cell types. While this research did not lead to many quantifiable results, visualising an *in vitro* model allows us to form hypotheses that can be statistically tested, and paves the way for further experimentation.
The cell automaton that I began developing for this project can be compared to further *in vitro* experimentation, and eventually used as an accurate model for the system. This will be useful, especially given the time-consuming nature of cell culture. Together, the two models that were built for this project will further research leading to functional kidneys for toxicology studies and clinical transplantation.

7. Personal experience

This was the first project that I worked on that had the specific goal to replace animals in research. In the past, I had been involved in studies using iPSCs for various reasons, and many of these studies, by nature, did not involve animal products, *in vivo* animal models, or animal cell lines. However, they also did not actively pursue research that would eliminate animal products in future research. Jamie Davies's lab's interest in eliminating animal products from research was a common theme throughout my time there, and I was intrigued and impressed that they have taken such bold strides in the effort to eliminate the use of animals in nephrotoxicity testing. In general, I intend to continue working with labs that make use of induced pluripotent stem cells, as the technology both eliminates animal models for human disease and paves the way for more personalised medicine with huge clinical applications.

This was also my first bioinformatics research project that allowed me to conduct wet lab research and simulation research. I had never dealt with modelling systems before. It was a huge learning curve, and ultimately took more time than I expected, but I truly enjoyed working on the program for the cell automaton, and hope to continue exploring the field of computational modelling.

8. Future prospects

Going forward, this research will be continued and built upon by other members of the Davies lab, as I was an exchange student and have now returned to my home university. In particular, statistical testing will be carried out on the *in vitro* cultures of Cdh1, Cdh3, and wild type cells. This will allow the *in vitro* model to be compared to the computational model.

As new students with computational modelling and programming experience join the lab, the cell automaton program will continue to be developed. I will remain in communication with the lab and any further lab members who take up work on the program. In the interim, I will continue to work on the program and add features and interfaces from Montreal, Canada, where I am currently finishing my BSc in computer science and biology at McGill University. I hope to make use of the computational modelling skills I learned during my time in the Davies lab in future projects I undertake at McGill and beyond.

9. Publications

As of now, there are no publications arising from this work. This may change in due course and we will, of course, notify the DHT.

I would like to express my deepest gratitude to Jamie Davies for hosting me in his lab this summer, and to lab members Elise Cachat, Joanna Sharman, Melanie Lawrence, and Chris Mills for all their help.

I would also like to thank the Dr. Hadwen Trust for their generosity and support of student research.

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10. Photographs

Figure 1 - the output of the program, showing a 100x100 grid of randomly distributed computer “cells.”

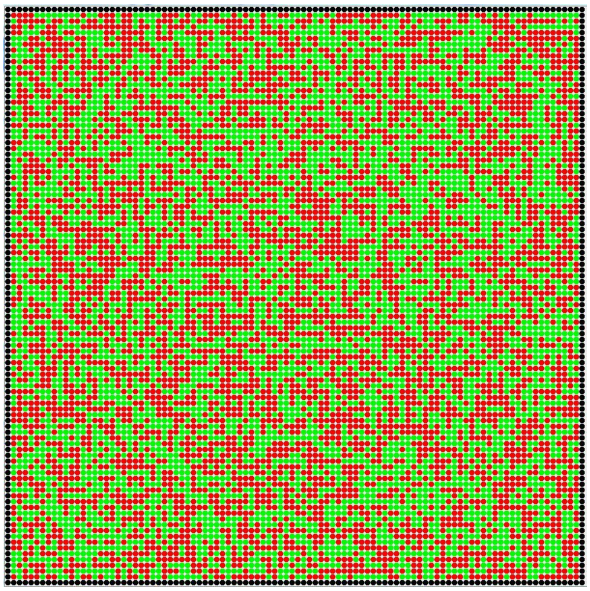


Figure 2, a

An equal mixture of Cdh1, Cdh3, and WT cells seeded at t=0. At 48 hrs, it was 25% Cdh1, 25% Cdh3, and 50% WT.

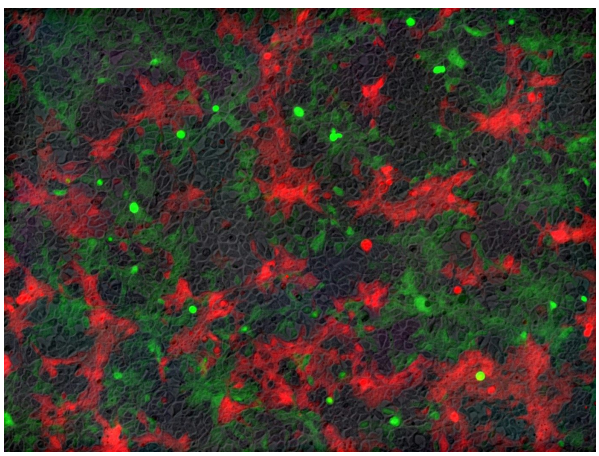


Figure 2, b

A mixture of 37.5% Cdh1, 37.5% Cdh3, and 25% WT seeded at t=0. At 48 hrs, it was an equal mixture of all three.

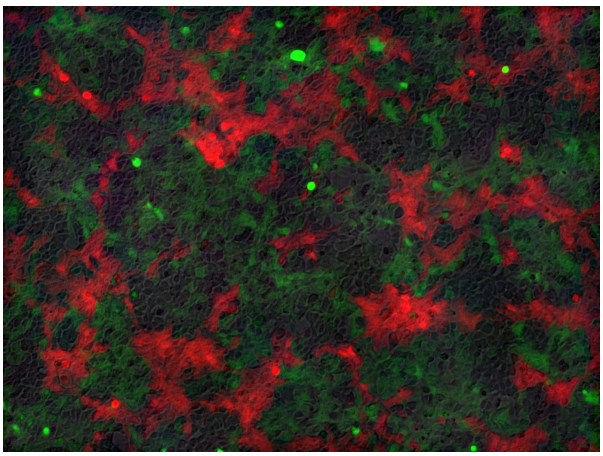


Figure 3, a
An equal mixture of Cdh1 and Cdh3.

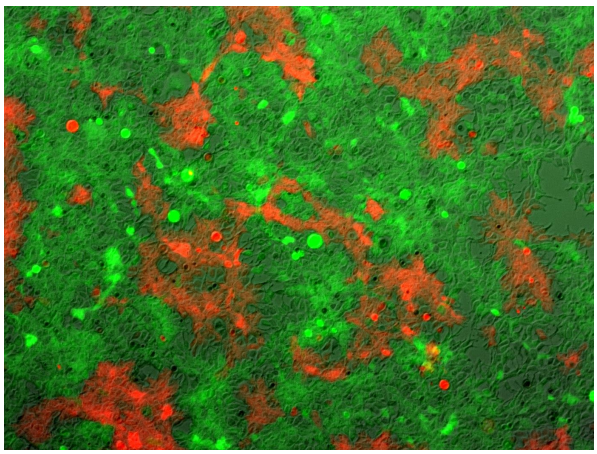


Figure 3, b
A mixture of 45% Cdh1, 45% Cdh3, and 10% WT.

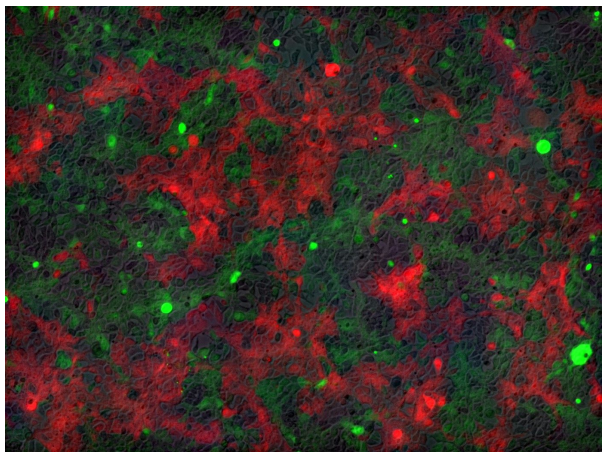
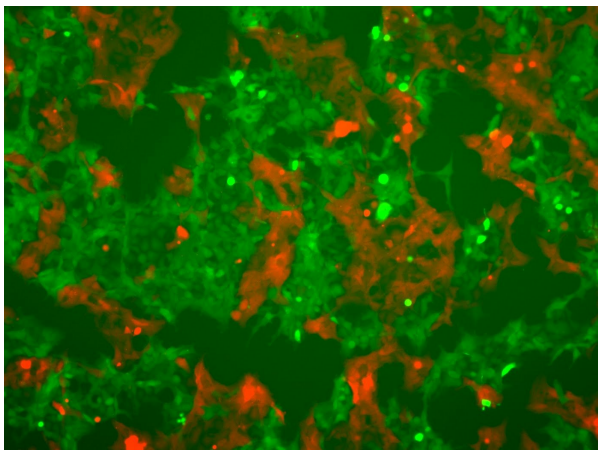
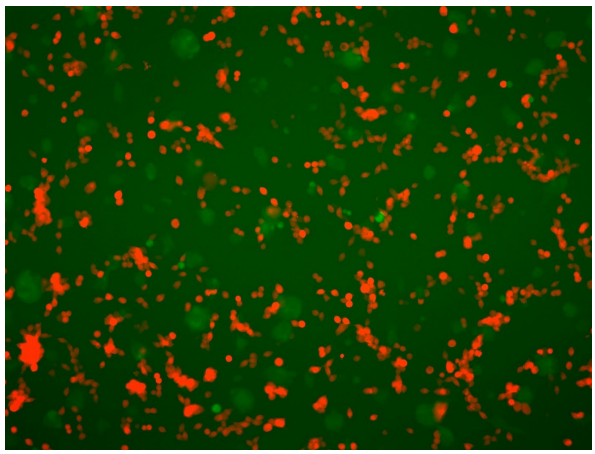


Figure 4 a, b, and c
4, a: Cdh1 and Cdh3 cells growing in normal, DMEM medium.
4, b: Cdh1 and Cdh3 cells growing in animal serum free medium with 0.1 mM calcium.
4, c: Cdh1 And Cdh3 cells growing in animal serum free medium without calcium.

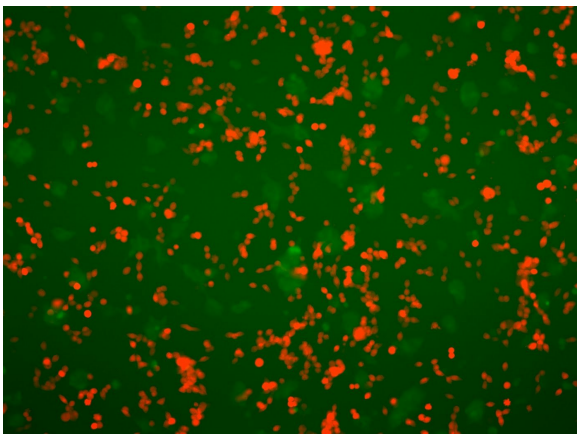


a



b

c



Photos of student