INFLUENCING DIFFERENTIATION AND GROWTH OF NEURAL PROGENITOR CELLS WITH GENE SILENCING AND LAMININ

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INTRODUCTION

Damage to the central nervous system (CNS) caused by injury or illness can be debilitating. One novel potential treatment for CNS injury is the use of cell replacement therapy utilizing neurons derived from induced pluripotent stem cells (iPSCs). In order to utilize iPSCs as an effective treatment, researchers must be able to control the differentiation of iPSCs into neuronal lineages. Stem cell fate commitment is driven by a variety of factors both physical and chemical. The ECM molecule laminin is known to be of particular importance to the proliferation of neural stem cells [1]. In this study we seek the optimal concentration of laminin necessary for cell seeding, as determined by cell number.

In addition to laminin, culturing cells on 3D electrospun scaffolds may provide the appropriate topography cues for differentiation. Another challenge in the direction of stem cell fate commitment is the presence of repressive pathways that guard against lineage commitment. Previous study has demonstrated the efficiency of the small interfering ribonucleic acid (siRNA) RE-1 silencing transcription factor (REST) for the enhancement of neuronal fate commitment [2]. One known method of modulating neuron development is the use of retinoic acid (RA) and purmorphamine(PMN) [3]. Therefore we chose to examine the effects of REST siRNA treatment on existing protocols for NPC to motor-neuron progenitor cells (MNPs) differentiation.

METHODS

Laminin Concentration Study

The first portion of the study was concerned with determining the amount of laminin coating needed for seeding iPSC derived NPCs. To induce differentiation into a neural lineage, iPSCs were cultured in a specific NPC medium that contained the following components:

- N2: 100x (0.5 ml)
- B27: 50x (1.0 ml)
- Glutamax: 100x (0.5 ml)
- P&S: 1%/100x (0.5 ml)
- BSA: 50000x (0.001 ml)
- hLIF: 1000x (0.05 ml)
- SB431542: 1000x (0.05 ml)
- y-27632: 1000x (0.05 ml)
- CHIR99021: 1000x (0.05 ml)
- DMEM/F12: 23.65 ml
- Neurobasal: 23.65 ml

Cells were regularly passaged in a 1:3 ratio at full confluence. Excess cells were stored in cryostor CS10 according to manufacturer protocol. Thawing of cells for further experiment was done according to manufacturer protocol.

Aligned nanofibrous PCL scaffolds were electospun onto aluminum foil then cut to fit 24 well plates. Scaffolds were pre-wet in 70% EtOH and washed in DI water before coating with 0.5 mg/ml of poly-dopamine for 4 hours at room temperature followed by poly-DL-ornithine coating for one hour at 37°C. Coated scaffolds were washed 3 times and lyophilized overnight.

Next scaffolds were UV sterilized prior to coating with laminin for 2 hours at room temperature. Six different weights of laminin were tested: 10 µg, 5 µg, 2.5 µg, 1.25 µg, 0.625 µg, and 0 µg. Each laminin coated scaffold was seeded with 50,000 iPSC-NPC, and cultured in motor neuron progenitor medium (N2B27 + retinoic acid (RA) + purmorphamine + rock-inhibitor). After 3 days cells were counted and stained with a Live/Dead Assay kit.

MNP Study

Scaffolds and coverslips were prepared for culture of iPSC-NPCs. Cells were seeded onto either aligned electrospun scaffold or 2D coverslip. Four differentiation protocols were used for 2D culture and two were used for scaffolds. For the 2D, Group 1 received culture medium with PMN+RA, Group 2 received PMN only, Group 3 received RA for 4 days then PMN+RA for 10 days, and Group 4 received normal medium for 4 days then PMN+RA for 10 days. Scaffold Group 1 received medium with PMN+RA and Group 2 received RA for 4 days then PMN+RA for 10 days.

Groups were subdivided and treated with each of: REST siRNA, scrambled siRNA (scr-siRNA), or no additional differentiation cues (no treatment, n.t.). After 14 days cells are fixed and immunostained for neuronal lineage markers: TUJ1, MAP2, Nestin, and Olig2.

RESULTS

As seen in Figure 1, cell number was higher among the 10 µg, 5 µg, 2.5 µg, and 1.25 µg groups.

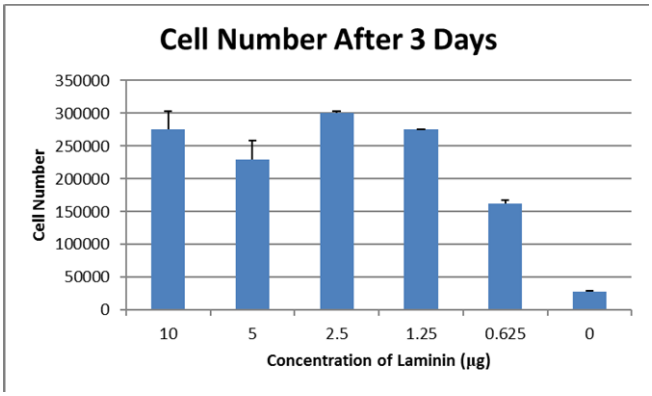

Figure 1: Plot of cell number vs laminin concentration. Error bars represent SEM.

Figure 2 below displays the results of staining for Nestin and Olig2 on the 2D culture groups. All of the 2D culture groups stained positive for Nestin and negative for Olig2. Stains for TUJ1 and MAP2 were also negative for all of the 2D culture groups. Scaffold Group 2-Rest and Group 2-Plain stained positive for Nestin. All scaffold Group 2 subgroups stained positive for Olig2 and negative for TUJ1. Group 1-Plain and Group 1-Rest stained positive for MAP2.

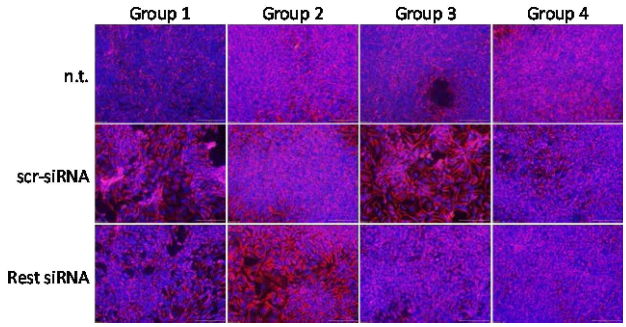

Figure 2: Immunofluorescent staining for neuronal markers Nestin and Olig2. Nestin is shown in red, DAPI in blue, Olig2 in green.

DISCUSSION

Too low mass of laminin is detrimental to cells as seen by lower cell numbers in the 0.625 µg and 0 µg groups. In the 2D culture experiments on the differentiation of NPC to MNP cells, positive staining for Nestin, an NPC marker, in all groups indicates a lack of differentiation. Negative stains for MNP cell markers TUJ1, Olig2, and MAP2 highlight that there are no MNP cells present in the culture. In MNP scaffold experiments, presence of MAP2 and Olig2 suggests that some of the NPCs have differentiated into MNP cells. Increased MNP marker signal in REST siRNA+scaffold subgroups relative to control+scaffold subgroups indicated that REST siRNA enhances iPSC-NPC differentiation. Together, these results suggest that a combination of topographical cues and silencing of REST is most effective in inducing differentiation of NPCs.

Since many cells were negative for MNP markers, we also consider the possibility that cells not expressing the chosen markers have already passed through the MNP stage and fully differentiated into neurons. In future studies we will examine this possibility by testing cells for neuronal markers such as Islet1.

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ACKNOWLEDGEMENTS

This work was funded by the Swanson School of Engineering, the University of Pittsburgh Office of the Provost, the University of Pittsburgh Study Abroad Scholarship, and the Nanyang Technological University Summer Research Internship Program.