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FINAL REPORT:

MICROFABRICATION OF A NEURAL STEM CELL DIFFERENTIATION SYSTEM

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The following narrative represents a description of progress made during the funding period of our NJCSCR grant, the goal of which was to develop high throughput methods to optimize protocols that can differentiate embryonic stem cells into neurons and oligodendrocytes. Our long-range objective is to ultimately use these protocols to produce reasonably large quantities of these cells for use in cell-based therapies for spinal cord injury. As indicated below, we have made excellent progress on a number of fronts, including advancing collaborations with Drs. David Shreiber, Martin Grumet, and Mehmet Toner.

Specific Aim 1: To develop a microfluidic bioreactor for high- throughput characterization of embryonic stem cell differentiation into neural cells.

Prior to 2004, many protocols had been developed to differentiate stem cells along neuronal lineages and/or promote neuron extension. The studies originally proposed in this aim were intended to design a develop a microfabricated microfluidic device capable of generating concentration gradients as an ideal system for systematically analyzing the combinatorial effects of immobilized cell matrix proteins and soluble growth factors on these cells. We have successfully generated such a microfluidic device which can be used to as a stem cell differentiation environment.

The design of the the device can be summarized as follows. The microfluidic device consists of microwells with four to six microchannels extending out radially from each microwell. Perpendicular to each microchannel is a microfluidic gradient generator, which is composed of a parallel network of interconnected microchannels, with fluid inlet and outlet ports located in opposite corners. The gradient generators are located in a plane above the microchannel extending from the microwell.

Finite Element Analysis: A growth factor gradient generator was designed based on results from Finite Element Analysis (FEA). In the analysis, the diffusivity of the growth factor was assumed to be 1×10^{-8} cm^2/sec. When the height and width of all the channels is 100 micron, and the velocity of the flow in all the channels is 100 micron/sec, a growth factor gradient is generated. These results show that growth factor levels can be altered over a 2 order of magnitude range within a distance of less than 1 mm.

Microfabricated Bioreactor: Based on our simulation results described above, we designed and constructed a set of devices to generate appropriate growth factor gradients. The devices were made using photolithography and a polydimethylsiloxane (PDMS) molding techniques. Using colored dyes, we have demonstrated gradient generation along the main channel using a cross-flow technique. A red colored dye was added to the medium flow through the main channels, while blue dye was added to the cross-flow (gradient generator) channels. We have

observed that blue dye appears more intensely as one goes downstream in the main channel, and that by adjusting cross-flow, exquisite control of the gradient is possible.

Biocompatibility Testing and EB Formation on Device Substrate: In order to more efficiently seed EBs into the microdevices, we developed a patterning technique for in situ EB formation. Briefly, surfaces similar to that used for the microdevices but devoid of microfluidic channels were seeded with embryonic stem (ES) cells through a removable stencil. The size of holes in the stencil governs EB size. After removal of the stencil, EBs started to spread, as is observed when suspension culture generated EBs are placed on a biocompatible surface that supports cell attachment.

The methodology described above leads to EBs that eventually spread onto the neighboring surfaces. By a slight modification of the technique which involves coating the spaces in-between EBs with bovine serum albumin, spreading can be significantly retarded, thus allowing further maturation before spreading occurs or harvesting of EBs for other applications.

Assessment of Neuronal EB Differentiation in Microdevices: A microbioreactor device was injected with a collagen solution to coat the culture substrate before EB seeding. A hanging-drop EB was seeded in each well of the microbioreactor. After 11 days in culture, cells migrated out of the EB, and undergo differentiation, as shown by expression of the mature neuron marker, neurofilament on Day 18 of differentiation.

Specific Aim 2: To regulate embryonic stem cell differentiation into mature neurons and oligodendrocytes using immobilized and soluble factors

Microarray analysis of embryoid body cultures: In addition to the microbioreactor work described above, we have also used traditional hanging drop cultures to test for the presence of neuronal lineage cells using microarray analysis. Independent culture conditions were assessed 17 days post differentiation induction. EB culture induces mixed population lineages. However, various neural specific genes under spontaneous (i.e. growth factor-/collagen-) and induced conditions (i.e. growth factor+/collagen+) indicate the presence of neural cells within the mixed population including NCAM and Neurofilament.

Neuronal differentiation and enrichment via EB culture: We have adapted the hanging drop technique to generate EBs from the ES cell line, ES-D3 (ATCC, Manassas, VA), which we have previously used to induce hepatocyte differentiation. In the presence of retinoic acid, we successfully induced neurofilament expressing neuronal cells. In addition, retinoic acid induced the differentiation of cells expressing a variety of neural markers on Day 8 (Figure 1). Among these cells, over 60% expressed the progenitor marker A2B5. While EB differentiation is efficient enough to commit ES cells into neuronal precursor cells, it still yields a heterogeneous cell population. In an effort to isolate committed subpopulation as a source of transplantable cells following nerve injuries, we have utilized dual-color fluorescence-activated cell sorting (FACS) with lineage selection surface markers, including PSA-NCAM neuronal-restricted progenitor marker and A2B5 glial-restricted progenitor marker. Retinoic acid treated EB cultures were disassociated on Day 8, immunostained with anti-A2B5 antibody and anti-PSA-NCAM, and sorted by FACS (Figure 2). We were able to distinguish different cell populations, including A2B5+/PSA-NCAM- cells as oligodendrocyte progenitors and PSA-NCAM+ cells as neuronal progenitor cells The cells were maintained for over a month.

Alginate encapsulation: In order to develop a method to differentiate large numbers of ES cells, an alginate culture system was developed. The encapsulation system implements an electrostatic droplet approach, and resulted in a bead diameter of 500 μm at an applied voltage of 6.4 kV. We were able to maintaine a solid bead core, thus maintaining the property of a single cell suspension. Cells were recovered by depolymerizing alginate capsules in sodium citric solution. Thus far we have demonstrated the spontaneous differentiation of both hepatocyte and neuronal lineage cells in this system. In the absence of retinoic acid only 5% cells express NCAM during the first 14 days post encapsulation. As hepatocyte differentiation increases, NCAM expression disappears. However, in the presence of Retinoic Acid, the differentiation progression is altered. Neuronal lineage cells persist throughout the 20 day encapsulation period, including A2B5 (progenitor marker), NF 150kD (neuronal marker) and O1 (oligodendrocyte marker). The percent positive cells range from 35-55%. The alginate encapsulation system provides an alternative differentiation environment to test a range of soluble and electrical environments to control neuronal lineage differentiation.

Neuroectodermal Specification of Embryonic Stem Cells: A primary goal of our work is to develop the know-how for controlling and enhancing specific differentiation along particular pathways. A recent study performed by Ying et al. (*Nat. Biotech.* 21: 183, 2003) described the generation of a knock-in embryonic stem cell line that reports the expression of the Sox1 transcription factor, the earliest known transcription factor expressed by the developing neuroectoderm and, therefore, a marker of germ layer specification. In this study, it was observed

that a serum free medium consisting of specific supplements could generate high yields of Sox1-GFP$^+$ cells (~77%) in a monolayer culture, with the remaining Sox1-GFP$^-$ cells expressed an undifferentiated phenotype. This suggests that these culture conditions lead to either one of two cell fates. This presents the first model system to clearly study lineage commitment of embryonic stem cells without confounding lineage commitment into other germ layers. In addition, the authors comment that "not all cells behave identically...these observations might indicate that there is a stochastic component, a community effect, or both in lineage commitment."

In order to study this community effect clearly, we devised cell pair experiments using microfabrication techniques. Microwells were fabricated using polyethylene glycol (PEG) photolithography. The gelatin-coated wells were seeded with mouse ESCs expressing green fluorescent protein (GFP) under the regulation of the Sox1 gene promoter, the earliest known transcription factor expressed by the developing neuroectoderm and, therefore, a marker of germ layer specification.

The cell pair consisted of a Sox1-GFP+ cell and an undifferentiated cell (with the ability to express GFP upon Sox1 transcription) with or without cell contact. Cell pair criteria were verified by cell tracking methods. The GFP expression of the undifferentiated cell was monitored after two days of coculture using fluorescence microscopy. The independent variable in the experiment was the duration of differentiation culture (0-17 days) experienced by the Sox1-GFP+ cell prior to coculture with the undifferentiated cell. The dependent variable (termed yield) was the number of cell pairs, in which Sox1-GFP expression was induced in the undifferentiated cell, divided by the total number of cells pairs monitored.

A coculture of two undifferentiated cells served as a negative control and showed a baseline yield of 42 +/- 8%, which can be attributed to the differentiation medium and

substratum. A plot of yield versus differentiation culture time followed a sigmoidal trajectory for contact-dependent experiments, increasing to a maximal yield of 72 +/- 7% on Day 7 and to a minimal yield of 31 +/- 12% on Day 14. Contact-independent experiments followed a saturating exponential trajectory with a threshold yield of 56 +/- 2% on Day 7. The contact-dependent data could be fit to a cooperativity model of receptor-ligand binding kinetics with associated parameters. Hill analysis of the data revealed a Hill coefficient of 2.4 suggesting positive cooperativity between the transformed variables (yield vs. time^{-1}). Qualitatively, these results show consistent trends suggesting that a soluble factor, rather than cell contact, plays a major role in neuroectodermal specification. Statistical analysis using a paired student's t-test showed significance between the two test populations on Day 7 and Day 17. These time points may be indicative of lineage committed states where cell-cell interaction may have a cooperative (Day 7) and inhibitory (Day 17) effect on a primitive cell.

Analysis of cell morphology in differentiating ES cells prior to coculture show distinct changes in cell junctional complexes, which may correlate with the cooperative/inhibitory effect of cell contact stated previously.

Specific Aim 3: To introduce electrically charged microenvironments into the embryonic stem cell/neural bioreactor differentiation system: The studies proposed in this aim were designed to engineer intracellular regulation of stem cell differentiation. We have targeted this goal in two ways:

1) We have studied the effect of electrical stimulation on the differentiation of a model neuronal cell line, PC12. These studies indicate that we can increase neuronal extension in the presence of nerve growth factor and electrical fields. We have also applied electrical field stimulation to embryoid bodies and have observed an effect on cell distribution on the periphery of the differentiating embryoid body. These studies indicate that we can change the spatio-temporal interaction in the developing EB, which is important in regulating lineage commitment.

2) We have begun to assess the metabolic changes that accompany differentiating neuronal lineage cells. In order to quantitatively measure these changes we have developed a protocol for enriching and secondarily culturing these cells. We have demonstrated for example, that we can isolate A2B5 expressing cells (early oligodendrocyte precursors) and can maintain them in secondary culture fro more than 30 days. We have also quantified the lactose production and glucose consumption rates during the differentiation of ES cells into neural lineages.

We have found in our hepatocyte differentiation systems that these parameters can be used as good indicators of differentiation and/or maturation. During the early stages of differentiation, increases in anaerobic respiration are indicated by increased lactose production and glucose consumption. As functional maturity is reached, lactose production declines relative to glucose consumption and mitochondrial development progresses. These approaches were also applied to our neuronal differentiation system. Cells were enriched from the differentiating neuronal population following supplementation with retinoic acid, using the FACS, as described above. Cells were secondarily cultured and their supernatants assessed for metabolic changes using a bioanalyzer. These studies demonstrated similar increases in lactate production in A2B5 cells relative to undifferentiated cells. While these studies are still ongoing, this approach will provide a valuable option for intracellular regulation of neuronal differentiation in the presence and absence of electrical field stimulation.