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Abstract

Human neural progenitors have a strong potential for use as cell-based biosensors for environmental toxins. Here, we report the following accomplishments in developing a neural cell-based biosensor. (1) We developed iPSC equivalents for the ESC-derived hNP1™ neural progenitor and hN2™ mixed neuronal cell lines, giving us the capability to create disease-specific neural cells from patient fibroblasts. (2) We continue to optimize our novel approach for hNP1™ differentiation into dopaminergic neurons to increase yield beyond 50%, allowing us to create a more physiologically relevant, HTS-ready *in vitro* model for Parkinson's disease research. (3) We have begun pilot studies to differentiate hNP1™ progenitors into motor neurons and astrocytes; these cell types have potential for being physiologically relevant *in vitro* models for botulinum toxin detection and neuron-glia interactions, respectively. (4) We developed HTS- and HCl-amenable assays for cell migration, cellular ATP, and neurite outgrowth. These assays have the potential as single or multiplexed assays to identify compounds with effects on neurogenesis (proliferation and differentiation) and to delineate mechanism-of-action for unknown neurotoxicants. (5) We developed an immunoblot based method for detecting botulinum toxin using the mixed neuronal hN2™ cell line, thus creating a first generation human cellular model for botulinum toxin detection – standard of comparison for existing and future models.

Scientific and Technical Objectives

The goal of this project is to develop functional neuronal networks comprised of human neurons derived from human neural progenitor (hNP1™) cells for use in a cell-based biosensor platform. hNP1™-derived neurons represent a source of renewable networks that overcome logistical issues with fielding cell-based sensors, while representing the best cell type for judging human neural toxicity. The overall objectives for this project are (1) to develop methods for accelerating neuronal differentiation and improving neural network formation and (2) to develop high throughput screening (HTS) and high content imaging (HCI) assays and microelectrode arrays as sensor elements to detect cellular responses to test compounds. These objectives have been modified from those in the original proposal.

Approach

We are currently taking a two-pronged approach to the development of a human neural cell based biosensor. The first part of the approach is to promote differentiation of neural progenitors into mature, active neurons. To achieve this objective, we are developing (1) methods for neural progenitor differentiation into functional dopaminergic and motor neurons as well as astrocytes, a glial cell type that promotes neuronal survival and formation of active neural networks, and (2) methods to generate induced pluripotent stem cell derived neural progenitors to allow the creation of disease specific neural cells for the biosensor. The second part of this approach is to evaluate the response of active hNP1™-derived neuronal networks to common environmental toxins using fluorescence based assays and microelectrode arrays (MEAs) as sensor elements. In addition to using microelectrode arrays to measure neural network activity, we are developing fluorescence based assays to monitor the effects of known and unknown toxicants on important neurodevelopmental processes, such as neurite outgrowth, proliferation, mitochondrial function, and apoptosis *in vitro*. Here we have added development of fluorescence based assays as sensor elements as a new objective due to technical difficulties with growing active neurons on MEAs.

Accomplishments

Outline

- (1) Neural progenitor isolation from induced pluripotent stem cells*
- (2) Directed differentiation of neural progenitors into neuronal and glial cell types*
- (3) Development of cell-based assays as sensor elements*

(1) Neural progenitor isolation from induced pluripotent stem cells

Our previous studies identified novel markers that could be used to identify neural progenitors within a population of induced pluripotent stem cells (iPSCs). We also developed methods to generate iPSCs using the IMR-90 human lung fibroblast cell line reprogrammed with 6 pluripotency factors (Oct4, Sox2, Nanog, Lin28, Klf4, c-Myc) in lentiviral vectors. Using these methods together, we have generated a new iPSC-derived neural progenitor line. Like the hNP1™ cells, these iPSC-derived neural progenitors were derived, propagated, and maintained as adherent monolayers using serum-, feeder-free, defined medium. They also possess a stable karyotype for multiple (>10) passages with a doubling time of ~36 hours, are robust and scalable for HTS format (96-, 384-well) assays, express proneural markers (positive for nestin, Musashi1), and can be differentiated into neurons (positive for beta-III-tubulin and MAP2) (**Figure 1**). We are now amplifying this line into working stocks for beta testing and potential commercial distribution.

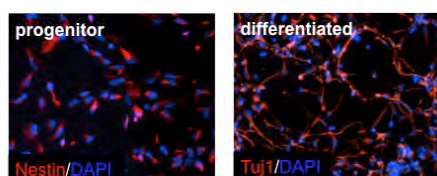


Figure 1. iPSC-derived neural progenitors (*left*) and differentiated neurons (*right*). Progenitors are stained for the proneural marker nestin and neurons are stained for the neuronal marker Tuj1. Both are also stained for nuclei (DAPI).

(2) Directed differentiation of neural progenitors into neuronal and glial cell types

We are continuing development of methods for directed differentiation of neural progenitors into dopaminergic neurons, motor neurons and astrocytic glia. The rationale for creating these particular cell types are the lack of renewable, scalable and physiologically relevant *in vitro* models for Parkinson's disease, motor neuron diseases and botulinum toxin detection, and neuron-glia interactions, respectively.

Dopaminergic neurons: A high-yield method (~50%) to differentiate neural progenitors into dopaminergic neurons was developed (Young, et al., 2010). We are currently adapting this approach for commercialization. Accordingly, we are establishing new screening assays in addition to the standard markers tested for dopaminergic differentiation and continue to optimize the differentiation protocol to increase yield beyond 70% for HTS applications. Also, we are in the midst of beta testing a dopaminergic progenitor cell line developed for potential commercial distribution.

Motor neurons: We are currently working establishing a robust differentiation protocol for motor neurons using published approaches as a starting point. Experiments are ongoing.

Astrocytes: Substantial progress has been made with establishing a robust astrocyte differentiation procedure; recent work continues to optimize conditions necessary for establishing an accelerated high-yield differentiation protocol.

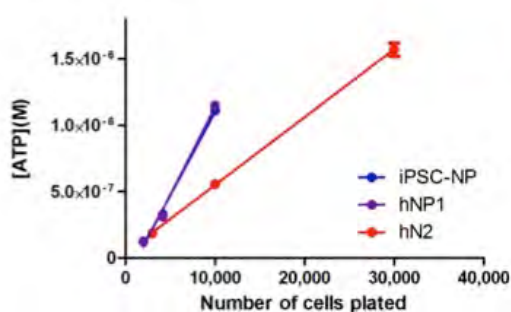
(3) Development of cell-based assays as sensor elements

We are developing fluorescence based assays to examine mitochondrial health, reactive oxygen species generation and cell migration in our neural progenitor and differentiated neural cells. These assays would serve as sensor elements in cell-based biosensors. We continue to make rapid, substantial progress in developing fluorescence based assays as sensor elements in cell-based biosensors.

Cellular ATP assay: We are in the midst of developing a cellular ATP assay for both hNP1™ and hN2™ cells. The assay shows excellent signal-to-noise and a marked difference between the proliferative hNP1™ and post-mitotic hN2™ cells, suggesting a strong potential as both a proliferation assay and an indicator of cellular metabolism. We are currently exploring the ability of this assay to measure both parameters, thus increasing the assay's utility.

Alamar Blue assay: We have optimized conditions for using both hNP1™ and hN2™ cells with the Alamar Blue assay, which measures mitochondrial reductase activity in both cell types. While the assay shows excellent signal-to-noise, it shows only a small difference between the proliferative hNP1 and post-mitotic hN2 cells, suggesting a limited potential as a proliferation assay. However, the Alamar Blue assay may still be a good indicator of cellular metabolic activity – further testing is needed. We have also assembled a small set of known neurotoxins to complete proof-of-concept studies for this assay.

Cellular ATP assay



Alamar Blue assay

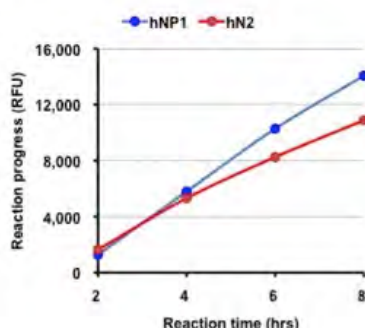


Figure 2. Application of hNP1™ neural progenitors and hN2™ differentiated neurons to cellular ATP (*left*) and Alamar Blue (*right*) assays. Cells were plated, incubated for 48 hours, and then tested in each assay.

ROS assay: To measure reactive oxygen species (ROS) generation in hNP1™ and hN2™ cells under conditions that induce oxidative stress, we are developing an assay that uses hydrocyanines specifically detect mono- and di-oxygen radicals. Preliminary studies at Georgia Tech showed low background and robust signal-to-noise in vitro and in vivo. However, instability of dye during transport has presented as a major hurdle for assay development, and the project is currently on hold. Additional optimization of the dye chemistry is needed to overcome this problem.

Neurite outgrowth assay: We previously established a neurite outgrowth assay for detecting neurotoxicants using the already differentiated, mixed neuronal hN2™ cell line. Now, we are expanding the utility of this assay using the high content imaging platforms. We are currently testing the assay's ability to measure not only toxicant effects on neurite outgrowth in the differentiated hN2™ neuronal cells and but also growth factor effects on neurite outgrowth on the differentiating hNP1™ neural progenitors. The expanded capability would allow detection of both positive and negative modulators of neuronal differentiation.

Cell migration assay: We have developed an HTS-amenable cell migration assay using hNP1™ neural progenitors, since migration of neural progenitors during CNS development can be affected by environmental toxins.

Our results show that cytochalasin D, an actin polymerization inhibitor, can block hNP1™ migration while certain growth factors can have potent chemokinetic effects. These results demonstrate that the assay can identify inhibitors and stimulators of migration. Moreover, under proliferative conditions, >70% of hNP1™ cells in detection zone are positive for mitotic markers, indicating the assay's potential for measuring proliferation using high content imaging. We are currently exploring the expansion of the assay's utility accordingly. We have assembled a small set of known neurotoxins to complete proof-of-concept studies for this assay. We are in the process of marketing our neural progenitors with the migration assay as a screening tool for drug discovery as well as toxicology investigators.

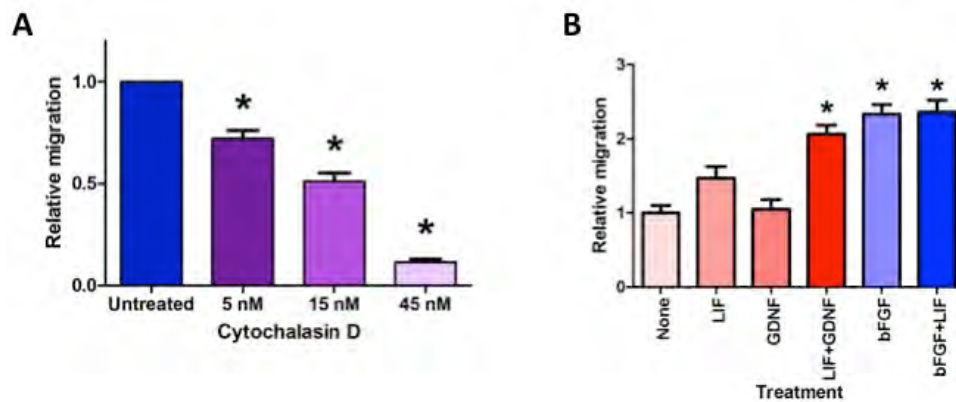


Figure 3. Modulation of hNP1™ neural progenitors by cytochalasin D (A) and recombinant growth factors (B).

Botulinum toxin assay: There has been substantial progress in the development of an hN2™ cell based sensor for botulinum toxin (BoNT) detection. hN2™ cells are derived from hNP1™ progenitors using a proprietary differentiation method. We demonstrated that hN2™ cells express SNAP-25, the target for BoNT proteolytic activity, and that treatment of hN2™ with BoNT-A caused SNAP-25 cleavage in a dose-dependent fashion. This cleavage was blocked by co-treatment with *T. vulgaris* lectin, an inhibitor of BoNT action, indicating the specificity of the BoNT effect. The manuscript of these findings has been submitted for publication and is under review.

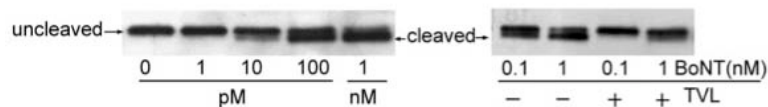


Figure 4. Immunoblot of SNAP-25 from hN2™ differentiated neurons treated with indicated concentrations of BoNT and *T. vulgaris* lectin. Uncleaved and cleaved SNAP-25 are indicated by the arrow and the arrowhead, respectively.

In summary, we have established several HTS- and HCL- amenable assays for cell migration, cellular metabolism, and neurite outgrowth using hNP1™ neural progenitors and hN2™ differentiated neurons. These assays have the potential as single or multiplexed assays to identify compounds with effects on neurogenesis (proliferation and differentiation) and to delineate the mechanism-of-action for neuroactive compounds of interest. We have also developed an immunoblot based method for detecting botulinum toxin using the mixed neuronal hN2™ cell line. As a result, we have created a first generation human cellular model for botulinum toxin detection that will serve as a standard of comparison for existing and future models.

CONCLUSIONS

Our work here strongly suggests that human neural progenitors have a strong potential for use as cell-based biosensors for environmental toxins. Our ongoing efforts to develop methods to differentiate progenitors into multiple neural subtypes will increase the versatility of the resulting biosensor by incorporating cell type specificity. Moreover, our development of HTS- and HCL-amenable assays as 'sensor elements' will diversify the utility of a human neural cell-based biosensor by examining multiple cellular processes and allowing for

mechanistic interrogation of test compounds. The combination of diverse cell types with diverse sensor elements forms a powerful and versatile platform for the detection and mechanistic analysis of novel neurotoxicants.

SIGNIFICANCE

We believe the work described here will have significant impact beyond the scope of this project. We have developed iPSC equivalents for the ESC-derived hNP1™ neural progenitor and hN2™ mixed neuronal cell lines, giving us the capability to create disease-specific neural cells from patient fibroblasts. We have developed a novel approach to direct hNP1™ differentiation into dopaminergic neurons using GDNF with ~50% yield; this will allow the creation of a more physiologically relevant in vitro model for Parkinson's disease research. We have begun pilot studies to direct differentiation of hNP1™ progenitors into motor neurons and astrocytes and have potential for being physiologically relevant in vitro models for botulinum toxin detection and neuron-glia interactions. We have also developed HTS- and HCI- amenable assays for cell migration, cellular ATP, and neurite outgrowth; these assays have the potential as single or multiplexed assays to identify compounds with effects on neurogenesis (proliferation and differentiation) and to delineate compound mechanism-of-action. Finally, we have developed an immunoblot based method for detecting botulinum toxin using the mixed neuronal hN2™ cell line and in so doing created a first generation human cellular model for botulinum toxin detection – standard of comparison for existing and future models

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- Abstracts at conferences for International Society for Stem Cell Research, Society of Toxicology, Society for Neuroscience (20 total)

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One of the Most Influential Georgians (Georgia Trends Magazine, 2010)