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Parallel In-vitro and In-vivo Techniques for Optimizing Cellular Microenvironments by Implementing Biochemical, Biomechanical and Electromagnetic Stimulations

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Abstract—**Development of novel engineering techniques that can promote new clinical treatments requires implementing multidisciplinary *in-vitro* and *in-vivo* approaches. In this study, we have implemented microfluidic devices and *in-vivo*rat model to study the mechanism of neural stem cell migration and differentiation.These studies can result in the treatment of damages to the neuronal system. In this research, we have shown that by applying appropriate ranges of biochemical and biomechanical factors as well as by exposing the cells to electromagnetic fields, it is possible to improve viability, proliferation, directional migration and differentiation of neural stem cells. The results of this study can be implemented in the design of optimized platforms that can be transplanted into the damaged areas of the neuronal system.**

I. INTRODUCTION

One of the potential techniques that can result in biomedical revolution is the development of implantable platforms appropriate for the delivery of stem cells into the damaged tissues of the body. Although studied widely, there is currently limited success for transplantation of stem cells into the patients. The limited viability and directional migration of the injected cells and the lack of functional tissue formation from these cells restrict the translation of regenerative medicine and tissue engineering research into clinical therapies. To develop functional implantable platforms for clinical stem cell transplantation, combined*in-vitro* and *in-vivo* models can be employed to allow an accurate and reductionist control over the various parameters involved in determining cellular responses to environmental factors. This can result in a comprehensive understanding of the nature of complex interplay among these parameters within cellular microenvironments, one of the key factors effective in developing successful stem cell therapeutic approaches. To successfully make use of stem cells, their viability, migration, proliferation and differentiation should be strictly controlled. It is known that physical (e.g. electrical and mechanical) and chemical properties of cellular microenvironments are very important factors in cellular viability, attachment, migration and finally tissue formation. The most hopeful approach to address tissue regeneration by using stem cell transplantation is to integrate various stimuli in a single scaffold. An interdisciplinary approach is needed to provide advanced platforms for supplying the satisfactory ranges of the effectors needed for stem cell transplantation. We have implemented a combination of appropriate *in-vitro*techniques including microfluidic and microelectronic devices as well as *in-vivo* rat model to optimize the microenvironmental conditions for designing transplantable platforms for therapeutic purposes applicable forthe neuronal system injuries.

New insights into the mechanisms of nerve regeneration suggest important opportunities for the development of new therapeutics for spinal cord and brain injuries [1]. Neurons are shown to have regenerative capabilities and the lack of appropriate connection between neurons is not an intrinsic inability of the cells to form axons after being damaged, but rather the injured microenvironment is the key factor to blame for the lack of regeneration [2]. The initial step for the regeneration of damaged neuronal tissues is the directional migration of neural stem cells (NPC) towards the site of injury, so that they can start differentiation at the appropriate location to heal the damaged neuronal tissue [3]. Using time-lapse imaging and fluorescent microscopy of the neuronal cells cultured within a microfluidic device, we tested the hypothesis that a minimum gradient of neurotrophic factors (NGF and VEGF) can induce chemotaxis of these cells as well as directional navigation of their axons. These studies were performed within 3D microenvironments of collagen scaffolds with varying mechanical stiffness. An intermediate range of collagen matrix stiffness was observed to stimulate the neuronal cells to perform directional migration and axonal navigation in response to a minimum NGF gradient (20 mg/ml/mm). Continuing our studies, we assessed the viability of neuronal cells in *in-vivo* model of multiple sclerosis (MS) under the effect of electromagnetic field. We observed that specific electromagnetic field parameters can improve the repair of demyelinated plaques byincreasing proliferation and migration of neural stem cells and by preventing the cells from apoptosis in rat. Having studied the effects of these stimulators*in-vitro* and *in-vivo*, our preliminary results suggest the appropriate rangesfor biochemical, biomechanical and electromagnetic factors to induce NPCs viability, proliferation, migration and axonal navigation.

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II. METHODS

A. Design and application of microfluidic and microelectronic devices

The first step in optimizing the environmental conditions for studying stem cell growth is to develop the platforms capable of simulating stem cell niches. The lack of appropriate platforms to quantitatively analyze the simultaneous role of biochemical and biomechanical factors in a controllable and reductionist manner is the main reason for less attention towardsthese importantstudies. To overcome these limitations, we have designed a microfluidic device capable of maintaining stable concentration gradients of biochemical factors (Fig. 1)[4-6]. The cell culture chamber of this device is a static microenvironment appropriate for mammalian cell chemotaxis. While most of the available systems are appropriate for 2D cell chemotaxis studies, this device can simulate both 2D and 3D environments. This is a considerable improvement in mimicking natural tissue environments because the cells cultured on 2D microfluidic devices are subjected to relatively high shear stress of the media covering them or these devices are not capable of simulating the effect of extracellular biomechanical factors in mediating cell responses. The ability to maintain a static condition for shear-sensitive cells makes this device applicable for neural stem cell chemotaxis studies without affecting cell viability and in response to biochemical factors. Axon path-finding can also be studied within this microfluidic device.

Specifically we modifiedthe previous version of our microfluidic device to make it appropriate for culturing neural stem cells in a 3D microenvironment by adding a microwell for culturing NPCs in the form of encapsulated neurospheres (Fig. 1). In the first step, we studied NPCs migration and differentiation in response to biochemical and biomechanical factors. Also, a microelectronic system has been added to the microfluidic system that allows the application of electrical signals to the cells. As can be seen in Fig. 1, this microelectronic system is composed of microelectrodes and electrical pads that are located beneath the microfluidic channels. Within this combined microfluidic-microelectronic device, neuronal cell migration, differentiation and axonal path-finding can also be studied in response to electrical field stimulations.

B. Electromagnetic field application in in-vivo rat model

Application of electromagnetic fields (EMFs) has been widely studied in recent years for therapeutic purposes.Also, EMF stimulation for improving the diseases of central nervous system (CNS) has been widely studied. In this study, EMF was implemented in the rat model of multiple sclerosis to stimulate neural stem cells. One of the most devastating disabilities that can disable young adults isMultiple Sclerosis (MS). During this disease, Myelin loss happens as the most common pathological feature [7]. There is not a reliable treatment for MS yet, possibly because of not completely known mechanism of this disease [8]. Here, we showed that EMFs stimulation can significantly improve remyelination of neuronal cells in rat model of MS. This study was performed by myelin staining and comparing the results in MS rat model exposed and not exposed toEMF.

III. RESULTS

To result in an effective neuronal regeneration, specific biochemical and biophysical conditions should be provided by the cellular microenvironment. Neural stem cells should initially migrate to locations of injury and disease before they can differentiate into the appropriate neural cell type and form neuronal networks [3]. There have been studies devoted to the discovery of extracellular factors affecting differentiation of neural stem cells [9, 10], but there are not enough studies for targeted neural stem cell migration and axon path-finding in a 3D environment.

Here, the microfluidic device was implemented forneuronal cell migration, differentiation and path-finding studies in a 3D microenvironment (Fig. 2). As can be seen in this figure, neural stem cells were cultured as neurospheres and encapsulated within collagen matrix. Stock collagen was mixed with neurobasal media containing NPC neurospheres and transformed into the gel phase by adding sodium hydroxide (0.5 N stock solution and5% volumetric mixture with stock collagen). The matrix and the encapsulated cells were injected into the microwell of the microfluidic device (also see Fig. 1) and the cells were allowed to move towards the cell culture chamber of the device by migrating through the microchannels that connect the microwell and the cell culture chamber. A concentration gradient of Nerve Growth Factor (NGF) was generated in the cell culture chamber of the device by injecting this factor into the source channel [4].

It was observed that the NPCs can migrate appropriately towards the cell culture chamber when they are encapsulated within an intermediate range of collagen matrix (0.9 mg/ml). Preliminary results also showed the differentiation and axon path-finding of NPCs in response to a minimum NGF concentration gradient of 20 mg/ml/mmafter cell migration into the cell culture chamber. Time-lapse imaging microscopywas implemented to record the migration and axon path-finding of NPCs as a function of time (Fig. 2). In order to investigate the effect of matrix density of collagen matrix on NPCs migration and differentiation, varying densities of collagen gel were made by altering the collagen concentration. Immediately after mixing, ~20 μL of the cell-hydrogel solution was injected into the cell culture reservoir of the microfluidic device and gelation occurred in 5-10 minutes. As can be seen in Fig. 3, collagen matrices with varying densities show different fiber distributions. By increasing collagen matrix density, the entanglement of these fibers increases and the mechanical stiffness of the matrix becomes higher (measured by performing rheology test of collagen matrices). It was observed that within collagen matrices of intermediate density (0.9 mg/ml), NPCsmigration and differentiation was significantly higher compared to higher and lower matrix densities. As can be observed in Fig. 2, within this range of collagen matrix density, NPCs can differentiate into neuronal cells with clear axons after migrating into the cell culture chamber of the microfluidic device. Axonal navigation of the differentiated cells can also be observed in response to NGF concentration gradient. Within the lower collagen matrix densities, NPC migration was more random and less directional while within higher matrix densities, very few cells could migrate into the cell culture chamber of the microfluidic device.

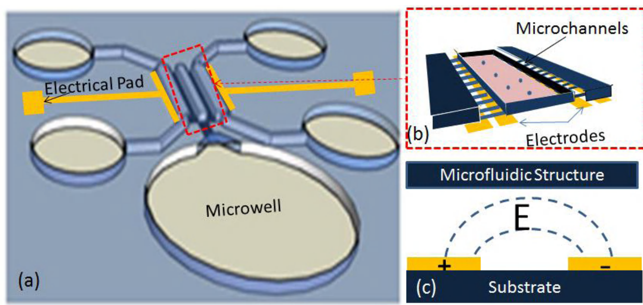

Fig. 1.(a) Microfluidic device with ability to generate concentration gradients ofbiochemical factors and culturing NPCs in a 3D microenvironment. (b) 3D schematic of the microfluidic device with microelectronic system at the bottom. (c) The microelectronic system for applying electrical field to NPCs.

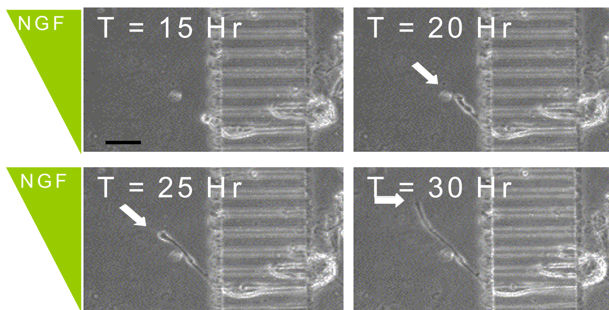

Fig.2.Time-lapse imaging of NPCs within the microfluidic device. The image sequences show NPCs migration and the axonal growth cone path-finding in response to nerve growth factor (NGF) and within collagen matrix.

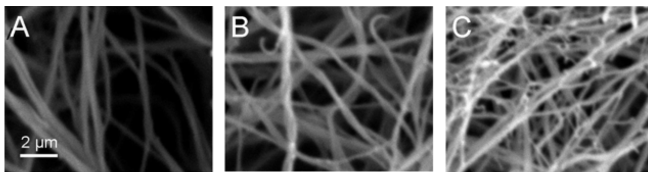

Fig. 3. Collagen fibril formation within collagen gels of varying densities. Scanning electron microscopy demonstrated that the density of collagen fibrils shows a significant increase by increasing collagen density. The mesh size of the gels with concentration of 1.5mg/ml (C) shows a significant increase compared to mesh size of the gels with concentration of 0.9 mg/ml (B) and 0.5 mg/ml (A).

To include the electrical signals in conjunction with other biochemical and biomechanical signals, we have combinedthe microfluidic device with a microelectronic system for studying neuronal axon navigation in a 3D microenvironment. Electrical stimulator fabricated within the cell culture chamber of the microfluidic device can provide a controllable amount of electrical current and has been implemented for generating tunable electrical fields in this microenvironment. Theamplitude, duration of electrical signals, current polarity and the best time for signal initiation are varied in this tunable device to study the role of localized electrical voltages on NPCs migration and differentiation. The results of these studies can be used for optimizing the parameters of electrical stimulations when combined with biochemical and biomechanical factors for developing clinically implantable microdevices.

Having this microfluidic-microelectronic system, we arealso assessing the efficiency of neuronal network formation within the cell culture chamber of the microfluidic device. This efficiency will be measured by applying electrical voltages to the neuronal cell bodies at specific parts of the neuronal circuit while measuring the electrical outputs from other parts of this circuit. This design allows us to apply electrical voltage to specific partitions of the device where the neural stem cells bodies are initially located and to measure the output signal where the cell's axons are extended. After NPCs differentiation, their axons are allowed to extend through tiny microfluidic features (Fig. 2). The appropriate connections among the cells within different device partitions will determine the functionality of the neuronal network for each specific biochemical and biophysical condition. This efficiency will be quantified by comparing the input and output voltages at the two different positions of the neuronal network.

To assess and complete the results of *in-vitro* studies, we developed an *in-vivo* technique in parallel to test the effect of biochemical, biomechanical and electromagnetic stimulators simultaneously. In the first step, we examined how electromagnetic field may improve the viability, proliferation and migration of neural stem cells.To study the mechanism of remyelination in MS rat model, we investigated whether the proliferative neural stem cells that reside in the subventricular zone can be effective in the remyelination process and whether EMF can improve this effect. It was observed that the number of BrdU+ cells had a significant increase in the rat models exposed to EMF on days 7 and 14 post lesion (Fig. 4). This shows that the proliferation of neural stem cells in MS rat model can be improved when the samples are exposed to EMF. Also, it was observed that EMF stimulation significantly increases the number of SVZ-nestin positive cells, indicating higher number of neural stem cells in SVZ in the presence of EMF. This increase was in parallel with a decrease in the level of apoptosis in demyelinated lesions. So, our *in-vivo* studies confirmed the hypothesis that EMF stimulation can enhance remyelination of demyelinated MS plaques and increase the number of the proliferating cells in SVZ while it can prevent the apoptosis of demyelinated plaquesin MS rat model. We will use the results of the *in-vitro* experiments to combine the biochemical and biomechanical stimulators with the EMF stimulation and fabricate transplantable platforms to be tested *in-vivo*. Finally, the optimized conditions obtained from these studies can be provided in the platforms implemented for clinical purposes.

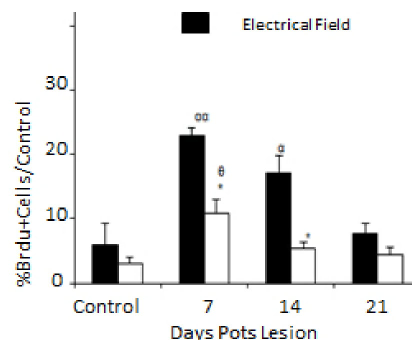

Fig. 4.Proliferation of the NPCs was increased in MS rat model when exposed to EMF after 7 and 14 days post lesion.

IV. DISCUSSION AND CONCLUSION

The combination of *in-vitro* and *in-vivo* techniques may be a key factor to facilitate testing multiple ideas in an efficient manner. While *in-vivo* techniques require time consuming experiments and costly materials, *in-vitro* techniques can be beneficial to test numerous varying experimental conditions with much lower expenses. The optimized conditions obtained from *in-vitro* experiments should be confirmed by *in-vivo* techniques. So, developing parallel algorithms to combine these two techniques together for answering a specific biological question can result in the fabrication of innovative transplantable devices for biomedical purposes.

In this study, we are recruiting both these techniques to find the appropriate conditions for the viability, directional migration, differentiation and axon navigation of neural stem cells (NPCs). We have developed a microfluidic-microelectronic platform that is capable of simulating 3D microenvironment of NPCs. Using this combined device, we can determine the optimized biochemical, biomechanical and electrical factors appropriate for the formation of neuronal networks *in-vitro*.In parallel, we also have developed an *in-vivo* technique for testing the hypotheses obtained from *in-vitro* experiments in MS rat model. In the first step, we found that the matrix density can be a critical factor for the migration of NPCs in response to the gradients of biochemical factors. Our *in-vivo* experiments also confirmed the positive effect of electromagnetic field on the viability andproliferation of NPCs. Having developed these two platforms, we can now test varying biophysical and biochemical conditions in parallel to optimize NPCs niches for an appropriate neuronal network formation.

In conclusion, the role of biochemical and biophysical factors on neural stem cell microenvironment were described and demonstratedusing *in-vitro* and *in-vivo* models. However the main goal of this approach is to study the role of combinatorial factors that can result in the development of optimized transplantable platforms applicable for healing the damages tothe neuronal system during spinal cord and traumatic brain injuries or diseases such as MS.These studies are underway by implementing parallel *in-vitro* and *in-vivo* techniques.

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REFERENCES

[1] P. Rajan, E. Snyder " Neural stem cells and their manipulation"*Adult Stem Cells,*vol 419, 2006, pp. 23-52.

[2] P. J. Horner, F. H. Gage " Regenerating the damaged central nervous system"*Nature,*vol 407, 2000, pp. 963-970.

[3] R. Jagasia, et al "New regulators in adult neurogenesis and their potential role for repair" *Trends in Mol Med*, vol. 12, 2006, pp. 400–405.

[4] A. Shamloo, et al " Endothelial cell polarization and chemotaxis in a microfluidic device"*Lab Chip,*vol 8, 2008, pp. 1292-1299.

[5] F. Kuhnert, et al "Essential regulation of CNS angiogenesis by the orphan G protein-coupled receptor GPR124" *Science*, vol330, 2010, pp. 985-989.

[6] A. Shamloo,S. C. Heilshorn, "Matrix density mediates polarization and lumen formation of endothelial sprouts in VEGF gradients" *Lab on a Chip,*vol 10, 2010, pp. 3061-3068.

[7] J. H. Noseworthy, et al. "Multiple sclerosis."*New Engl. J. Med.*,vol 343, 2000, pp. 938-952.

[8] A. Thompson, et al. "Pharmacological management of symptoms in multiple sclerosis: current approaches and future directions*." Lancet Neurol.,*vol 9, 2010, pp. 1182-1199.

[9] K. Saha, et al,"Substrate Modulus Directs Neural Stem Cell Behavior" *Biophys J*, vol. 95, 2008, pp. 4426–4438.

[10] L. A. Flanagan, et al"Regulation of human neural precursor cells by laminin and integrins". *J of Neuroscie Res*, vol. 83, 2006, pp. 845–856.