Integrative Biology

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/ibiology

Directional migration and differentiation of neural stem cells within three-dimensional microenvironments

Amir Shamloo[1,2], Motahare Heibatollahi[1], Mohammad R. K. Mofrad[1,3,*]

[1]*Molecular Cell Biomechanics Laboratory, Departments of Bioengineering and Mechanical Engineering, University of California, Berkeley, CA 94720*
[2]*Department of Mechanical Engineering, Sharif University of Technology, Tehran*
[2]*DPhysical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720*

[*]*Corresponding author:*
Prof. Mohammad R. K. Mofrad
University of California Berkeley
208A Stanley Hall #1762
Berkeley, CA 94720
Phone: (510) 643-8165
Fax: (510) 642-5835
Email: mofrad@berkeley.edu
http://biomechanics.berkeley.edu

Keywords: Nerve regeneration; neural stem cell; Nerve growth factor; Matrix density; Microfluidics; Microenvironment; Cell-Matrix Adhesion and Mechanotransduction

Abstract

Harnessing neural stem cells to repair neuronal damage is a promising potential treatment for neuronal diseases. To enable future therapeutic efficacy, the survival, proliferation, migration and differentiation of neural stem/progenitor cells (NPCs) should be accurately studied and optimized in *in-vitro* platforms before transplanting these cells into the body for treatment purposes. Such studies can determine the appropriate quantities of the biochemical and biomechanical factors needed to control and optimize NPC behavior *in vivo*. In this study, NPCs were cultured within a microfluidic device while being encapsulated within collagen matrix. The migration and differentiation of NPCs was studied in response to varying concentrations of nerve growth factor (NGF) and within varying densities of collagen matrices. It was shown that the migration and differentiation of NPCs can be significantly improved by providing the appropriate range of NGF concentrations while encapsulating the cells within collagen matrix of optimal densities. In particular, it was observed that within collagen matrices of intermediate density (0.9 mg/ml), NPCs have a higher ability to migrate farther and in a collective manner while their differentiation into neurons is significantly higher and the cells can form protrusions and connections to their neighboring cells. Within collagen matrices with higher densities (1.8 mg/ml), the cells did not migrate significantly as compared to the ones within lower matrix densities; within the matrices with lower collagen densities (0.45 mg/ml) most of the cells migrated in an individual manner. However, no significant differentiation into neurons was observed for these two cases. It was also found that depending on the collagen matrix density, a minimum concentration of NGF caused a collective migration of NPCs, and a minimum concentration

gradient of this factor stimulated the directional navigation of the cells. The results of this study can be implemented in designing the platforms appropriate for regeneration of damaged neuronal systems.

Introduction:

Current clinical treatments for the diseased or damaged central nervous system (CNS) offer only minor symptomatic relief. New discoveries of the mechanisms of neuronal plasticity and regeneration may offer promising opportunities for the development of new therapeutics for spinal cord and brain diseases and injuries [1]. For instance, it has been shown that the adult CNS has regenerative capabilities, and the lack of appropriate regeneration is not an intrinsic deficiency, but rather the injured microenvironment is inhibitory[2]. To enable effective neuronal regeneration, specific biochemical and biophysical conditions should be provided by the cellular microenvironment[3, 4]

NPCs are multi-potential, self-renewing cells that have been known to play a critical role in regeneration of different brain damages from neurodegenerative disorders to traumatic brain injuries[5]. Transplantation of exogenous NPCs, stimulation of endogenous NPCs, and finally a combination of both approaches may be effective in promoting efficient recovery in neuronal disorders[6].

There are two major regions of the brain in which neurogenesis takes place and neural stem cells can be found: subventricular zone (SVZ) and subgranular zone (SGZ). It has been shown that hippocampal neural stem cells residing in the SGZ of the dentate gyrus can migrate short distances in response to biochemical factors[7]. Nerve growth factor (NGF) is a biochemical factor whose effect on restoring neurogenesis and improving memory has been verified[8], but it is not known how NGF can affect the activities of hippocampal neural stem cells and improve neurogenesis. NGF has also been shown to affect Dorsal Root Ganglion neurons (DRGs) by directing their axons in specific directions[9]. In this study, we investigate the effect of NGF on the migration of hippocampal neural stem cells.

The neural stem cells are reported to be able to respond to a variety of disorders by proliferation and migration into the sites of injury[10]. These cells can also differentiate into the desired neural cell types to repair the injury[11-15]. Unfortunately, the recovery process after CNS injury is interrupted by the limited ability of CNS to regenerate lost cells, replace damaged myelin sheets and reestablish functional neural connections[16]. This may be because of insufficient recruitment of NPCs due to disturbed signaling pathways, migration difficulties or the lack of differentiation of NPCs into the desired cell type at the sites of neuronal damages[16,17]. For example, when the neuronal axons are damaged in the spinal cord injuries, NPCs differentiation into neurons is desirable while the cell differentiation into other neural cell types may be problematic. The enhancement of NPC proliferation, migration and differentiation may improve the repair of the neuronal injured regions.

Employing exogenous neural stem cells to repair brain damages is one of the inspiring areas of biomedical investigations[6]. Unfortunately multiple pathological insults are involved in most brain disorders, which provide a hostile environment for cell implantation and consequently the survival, migration and differentiation of these cells. Thus, the therapeutic benefits of NPC transplantation are limited by a high degree of donor cell death[18]. To successfully make use of neural stem cells, their migration, proliferation, and final differentiation should be strictly controlled. Various types of soluble biochemical factors and also biomechanical cues are known to influence NPC function[3, 4], but there is still no effective therapeutic method for implementing NPCs for clinical applications. To improve neural stem cell transplantation, different scaffolds have been designed to reduce inhibitory factors and increase stimulatory cues[19-23]. It seems that these scaffolds, if designed appropriately, can potentially improve the functional transplantation of NPCs by

promoting neuronal differentiation, enhancing the expansion of neuronal processes, promoting the connection between the cells and reduction of inflammation and scarring[24]. To improve NPC transplantation, scaffolds with appropriate biochemical and biomechanical factors may be utilized to improve the outcome[25-27]. In this study, these factors are characterized in a quantified manner for optimal migration and differentiation of NPCs.

Collective cell migration has recently been shown to be very critical during the formation of different tissues such as endothelial sprouts. It refers to the migration of the cells while they are in contact with each other physically and functionally. This ensures cell-cell junction integrity and results in their cohort migration. Since NPCs are found to have a great potential in the field of nerve regeneration, guiding them towards the site of injury in a collective manner can be of great importance. During this process, NPC connections may improve the formation of an integrated neural network at the desired location. As an example, neural crest cells are known to migrate collectively in response to a chemoattractant[28]. Another mechanism called co-attraction maintains cell-cell interactions in which the complement factor C3a and its receptor C3aR are found to be responsible for cell contacts[29]. A chemoattractant stabilizes the protrusions in its direction and a steady polarity will be achieved. Therefore, the cells migrate in group towards the source of the chemoattractant[30]. Cell adhesion molecules are also critical during the process of neural migration since very high or very low level of cell adhesion results in migration difficulties[31]. The importance of contact dependent cell polarity in directional collective migration of neural crest cells has been also investigated[32]. It was shown that in response to stromal cell-derived factor 1 (sdf1), cell polarity was enhanced due to cell-cell interactions.

As a result, cell protrusions were stabilized causing collective chemotaxis. In another study, vascular endothelial growth factor (VEGF) was used as the chemoattractant and its role in the migration of neural crest cells was examined both *in vitro* and *in vivo*[33]. The results of this study indicated that VEGF increased proliferation and induced migration *in vitro*. This result was also seen *in vivo* where neural crest cells diverted from random migratory pathways and experienced polarized directional migration towards the VEGF-soaked beads. This chemotactic response was suggested to be due to neurophilin-1-VEGF interactions.

In the present study, we aim to quantitatively investigate the nature of cell-matrix and cell-ligand relationships for the migration and differentiation of neural stem cells and suggest the optimal conditions for improving NPC ability to migrate directionally and differentiate into neurons. Neural stem cells should initially migrate to the locations of injury and disease, or spread within these regions, and subsequently differentiate into the appropriate neural cell types[5]. Several studies have determined the extracellular factors needed for effective differentiation of neural stem cells[34-40], but few studies have considered directional migration of NPCs[41-43]. Development and utilization of appropriate platforms to quantitatively analyze the simultaneous role of biochemical and biomechanical factors in a controllable and reductionist manner would enhance our knowledge of NPC migration. We have devised a microfluidic device for studying NPC migration and differentiation in response to a biochemical factor (NGF) and within 3D matrices of varying collagen densities. This microfluidic device can provide a 3D microenvironment with controllable concentrations of biochemical factors and allow 3D directional navigation of NPCs in response to the gradients of these factors. The appropriate range of NGF

concentrations and collagen matrix density that can stimulate NPC migration and differentiation are investigated using our microfluidic device.

Materials and Methods:

NPC preparation and culture:

NPCs were isolated from the hippocampi of six-week-old female rats (Fisher 344, Charles River) and were cultured on plates coated with poly ornithine/laminin. The media used for NPC culture was DMEM/F12 (Life Technologies, Carlsbad, CA) containing N2 supplement (Life Technologies, Carlsbad, CA) and 20 ng/ml FGF-2 (PeproTech, Rocky Hill, NJ). Accutase (Phoenix Flow Systems, San Diego, CA) was used for subculturing NPCs upon reaching 80% confluency. Passages of 2-7 were used for studying NPC migration. Since a high density of NPCs was required to be injected into the microfluidic device, the cells in the form of neurospheres were cultured in the last passage before their injection into the device. NPC neurospheres with the sizes of ~ 50 microns were formed when the cells were cultured on the plates without any coating after two days as discussed in the paper by Babu et al[44]. These neurospheres were then mixed with collagen matrix and injected into the microfluidic device.

Matrix rheology:

Rheological tests were performed on collagen matrix samples using an oscillatory rheometer (Anton-Paar, Graz, Austria) in parallel-plate geometry (8 mm diameter disk). The appropriate amplitude of deformation was determined to be 5% by performing multiple strain sweeps under varying frequencies (1-100Hz). Storage and loss moduli were relatively constant when increasing the amplitude up to 5% for the mentioned range of frequency. Storage and loss moduli (G' and G'', respectively) were measured at 37°C at

frequencies ranging from 0.1-100 rad/s. All samples exhibited plateau moduli between 0.2-1 rad/s.

Scanning electron microscopy (SEM):

Samples were fixed with 4% paraformaldehyde overnight and prepared for SEM analysis using critical point drying. Fixed matrices were saturated with ethanol of increasing purity up to 100% using a series of intermediate equilibration steps. The samples were placed in a critical point dryer (Auto Samdri 815, Rockville, MD) in which the ethanol solution was replaced with liquid carbon dioxide. The temperature and pressure of the system were adjusted to bring the carbon dioxide to its critical point. Next, the system temperature was raised above the critical point to completely vaporize the carbon dioxide and dry the sample. The samples were sputter coated with gold and palladium (Tousimis) and imaged by SEM (Hitachi S-5000).

NPC preparation and culture within the microfluidic device:

Stock collagen (Collagen type I, BD Biosciences, San Jose, CA) was mixed with neurobasal media containing NPC neurospheres and transformed into the gel phase by the addition of sodium hydroxide (0.5 N stock solution, 5% volumetric mixture with stock collagen) at 37 °C. Different 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. Inlet and outlets of the cell culture reservoir were pin-plugged, and the device was covered with media to avoid gel evaporation. Collagen gel with the same density as the one

injected into the cell culture reservoir was made by mixing collagen stock solution with neurobasal media without NPCs, yielding a gel with the same characteristics in the cell culture reservoir. This gel was injected into the cell culture chamber of the microfluidic device, and the inlets and outlets of this environment were pin-plugged.

Neurobasal media containing varying concentrations of NGF (PeproTech, Rocky Hill, NJ) was continuously supplied to the source and sink channels of the microfluidic device using a syringe pump (World Precision Instruments, Saratosa, FL). The injection flow rate was 20 nL/ min. The entire system (microfluidic device, syringe pump, and syringes) was placed in the incubator during the 2 days of cell culture. Syringes were filled with fresh media every day. NPC migration was observed by time-lapse phase contrast microscopy over two days in the intervals of 12 hrs. In each time step, images from all the regions within the microfluidic device were taken to study the cell migration progress over time.

Immunocytochemistry:

NPCs within collagen gels were fixed by injecting 4% paraformaldehyde into the source and sink channels and incubating at 4ºC for ~1 hr. The samples were washed at least four times by injecting PBS into the source and sink channels. Cell nuclei were stained using DAPI. The cells were blocked with 10% normal goat serum in PBST (0.3% Triton X-100 in PBS solution) for ~3 hrs. MAP2 protein as a marker for differentiated neurons was labeled overnight at 4ºC using Alexa Fluor555-conjugated MAP2 (Invitrogen, Carlsbad, CA).

Statistical analysis:

For each condition reported, at least n=3-5 independent experiments were performed. Two-tailed, non-paired, Student T-tests were used to determine the statistical significance of differences between pairs of conditions. To quantify the cell density and average migration distances within each collagen gel density, fluorescent images of the migrating cells showing their nuclei were compared to phase contrast images of these cells. The number of the cells per unit area and the distance of the cell nuclei from the beginning of the cell culture chamber were calculated for each microfluidic device and averaged for the microfluidic chambers. To quantify the directional migration of NPCs, a statistical parameter, directional migration index (DMI) was calculated by taking the average of distances of the cells nuclei from the sink channel where NGF gradient has the lowest quantity and normalizing this parameter by dividing it to the width of the channel, so this parameter remains between zero and one. The higher this parameter (closer to one), the farther the cells from the sink channel. That is, higher values for DMI indicate better directional migration of NPCs in response to NGF gradient.

All experiments were performed in compliance with the relevant laws and institutional guidelines of the University of California.

Results:

Microfluidic device design for studying NPC migration:

A microfluidic device was designed to provide an appropriate microenvironment for NPC migration and differentiation. This device is composed of a cell culture reservoir and a cell culture chamber that are connected to each other by a series of small microchannels called

cell microcapillaries (Fig.1A). This microfluidic device includes a source and sink channels that are connected to the cell culture chamber by other narrower microcapillaries. The diffusion of soluble factors through these narrower microcapillaries from the source channel into the cell culture chamber and finally into the sink channel allows the formation of concentration gradients of biochemical factors within cell culture chamber of the microfluidic device (see Supplemental Figs. S1, S2) similar to prior reports[45-49]. By injecting NGF to both source and sink channels, it is also possible to form a uniform NGF concentration within the cell culture chamber of the microfluidic device.

The time it took for NGF to diffuse from the source and sink channels into the cell culture chamber and form a uniform concentration was analyzed using fluorescent microscopy and was measured to be less than two hours . The time for NGF to diffuse from the source channel into the cell culture chamber and then into the sink channel and form a stable concentration gradient within the cell culture chamber was also measured (Supplemental Fig. S2). Supplemental Fig. S1 shows the fluorescence of FITC-dextran with relatively the same molecular weight as NGF (~10 KDa) after 3 hrs of NGF injection into the source channel. As can be seen in this figure, fluorescence intensity within the cell culture reservoir is negligible that shows NGF concentration is very close to zero after 3hrs. After this time NGF gradient does not vary within the cell culture chamber for all the collagen matrix densities and a NGF gradient would exist between the cell culture reservoir and the cell culture chamber (Supplemental Fig. S2). This means that the migration of the cells within cell microcapillaries can be considered to be chemotaxis, but their migration is motogenic within the cell culture chamber in a uniform NGF gradient. Due to critical role of directional migration in directing the cells to the site of injury and because of the

importance of motogenic migration in distributing the cells at the target site, we investigated both directional and motogenic migration of NPCs within the microfluidic device. Although the time required for the stabilization of NGF gradient within the cell culture chamber is dependent on the collagen matrix density (70 min for gel density = 0.45mg/ml, 90 min for gel density = 0.9 mg/ml and 120 min for gel density =1.8 mg/m), the difference in the stabilization times for the highest and lowest gel densities remains below one hour, which is much less than the time scale required for the cell migration (24-48 hrs).

NPCs were initially mixed with collagen matrix and injected into the cell culture reservoir in the form of neurospheres in a 3D gel. In the next step, collagen matrix without any NPCs but with the same density was injected into the cell culture chamber of the device. Nerve growth factors were injected into the source channel of the microfluidic device and diffused into the collagen matrix filling the cell culture chamber over time. The cells in the cell culture reservoir were seen to migrate into the cell microcapillaries in response to NGF concentration.

A minimum NGF concentration is required to direct NPCs into the cell culture chamber:

To stimulate the directional collective migration of NPCs with NGF, a collagen density of ~1 mg/ml was provided within the device. The specific design of the cell culture reservoir with a sharp bending (Fig. 1A) caused the injected cells to be trapped in the region near the cell microcapillaries. The inlet and outlet of the cell culture reservoir were pin-plugged before injecting collagen matrix without neurospheres into the cell culture chamber. Since

the outlets of the cell culture reservoir were closed during this step, the collagen matrix and NPCs within this environment remained in a static condition during injection. Finally, neurobasal media with a specific NGF concentration (40 ng/ml) was injected into the source and sink channels of the device to create a uniform NGF concentration within the cell culture chamber of the microfluidic device. Although NGF concentration is uniform within the cell culture chamber, there exists a gradient between the cell culture chamber and cell culture reservoir along cell microcapillaries. This NGF gradient stimulated the cells within the cell culture reservoir to migrate in a collective manner towards the cell culture chamber. The width (20 μm) and height (20 μm) of the cell microcapillaries allow the migration of NPCs while the cells are in contact with each other. As can be seen in Fig. 1B, the cells entered the cell microcapillaries after ~6 hrs, and entered the cell culture chamber of the microfluidic device after ~12 hrs (Fig. 1C).

The first parameter studied was the minimum NGF concentration required to direct the cells into the cell culture chamber. Migration studies were conducted with four different uniform NGF concentrations in the cell culture chamber (10, 15, 20 and 40 ng/ml). The minimum NGF concentration that stimulated NPC migration towards the cell culture chamber was found to be ~ 20 ng/ml. Below this NGF concentration level (e.g. 15 ng/ml), although some of the cells initially migrated into the cell culture chamber, they did not continue their migration after 24 hrs (Supplemental Fig. S3). This shows that a minimum concentration of a neurotrophic factor (NGF) may be required to stimulate NPCs to migrate within 3D collagen matrix.

NPC migration within collagen matrices of varying densities:

To study the effect of collagen matrix density on NPC migration and differentiation, NPC neurospheres were encapsulated within 3D matrices of varying densities (0.45, 0.9, 1.8, and 2.5 mg/ml). The mechanical stiffness of each collagen matrix was measured via rheometry. As expected, it was observed that the matrix stiffness increases with increasing matrix density. Specifically, the mechanical stiffness of the matrices with 0.45, 0.9, 1.8 and 2.5 mg/ml were found to be 20, 60, 170 and 400 Pa, respectively. SEM of critical point dried samples confirmed that the denser matrices resulted in scaffolds with less porosity, and greater fibril entanglements (Fig. 2). The cells were encapsulated within each type of matrix independently, and analyzed for migration behavior within the microfluidic device. Within matrices of higher density (2.5 mg/ml), the cells had a minor tendency to migrate but did not travel significantly during 48 hrs of imaging (Supplemental Fig. S4). Within the matrices with larger mesh sizes and lower density (concentration= 1.8 mg/ml), however, the ability of the cells to enter into the cell culture chamber and migrate within this environment was increased (Fig. 3 A-D). Although these cells were able to migrate into the cell microcapillaries and enter into the cell culture chamber after 24 hrs, they did not continue migration within the cell culture chamber significantly (Fig. 3C, D). In this condition, the migrated cells were very packed together within the cell culture chamber, and no significant protrusions were observed around the cells after 48 hrs.

The migration ability of the cells was increased within collagen matrix of 0.9 mg/ml density (Fig. 4A-D). The cells migrated into cell microcapillaries and continued their migration within the cell culture chamber while being adjacent to each other. The cells

continuously migrated into the cell culture chamber over 48 hrs (Fig. 4A-D). This continuous cell migration trend was not similar to NPC migration within the matrix of higher density (1.8 mg/ml) in which the cells stopped movement after 24 hrs (Fig. 3). As can be seen in Fig. 4, NPCs not only migrated in a collective manner within the cell culture chamber during 48 hrs, but also the cells extended protrusions while being in contact to the other cells (Fig. 4E).

Within lower matrix densities (0.45 mg/ml), most of the cells could migrate into the cell culture chamber (Fig. 5A-D), but most of these cells migrated with minimal intercellular contacts during the 48 hrs of movement. As can be seen in this figure, the cells within less dense collagen matrix did not show a collective migration, as did the ones migrating within intermediate matrix density (0.9 mg/ml). Relative individual movements were observed for most of these cells that implies their inability to have efficient interactions with their neighboring cells during migration.

By measuring the number of cells per unit area and the average cell migration distance, NPC distribution within collagen matrices of varying densities was quantified (Supplemental Fig. S5). Analysis of the number of the cells per unit area confirmed that the cell density increases significantly by increasing the collagen gel density. This is mainly because the cells have less migratory ability in higher collagen densities and as a result they remain closer to each other within a smaller region. As can be seen in Fig. 6A, at a collagen density of 1.8 mg/ml, the density of the cells is significantly higher compared to the cell densities within collagen matrices of 0.45 and 0.9 mg/ml densities ($p < 0.01$). Within intermediate collagen densities (0.9 mg/ml), a significantly higher number of the cells per

unit area was observed compared to this parameter within matrices with a lower collagen density (0.45 mg/ml, $p < 0.05$). By quantifying the average distances that the cells can migrate within the cell culture chamber, it was found the cells within collagen matrices with a density of 0.9 mg/ml can migrate ~ 300 microns within the uniform NGF concentration which is significantly higher compared to the cell migration distance within a matrices with a collagen density of 1.8 mg/ml and lower compared to the ones within matrices with a collagen density of 0.45 mg/ml (Fig. 6B, $p < 0.05$). If the length of cell microcapillaries (~150 microns) is added to this migration distance, the total cell migration can be obtained.

NGF concentration gradient effect on NPC directional navigation in the cell culture chamber:

In the next step, we investigated the response of NPC to the NGF gradients. NGF gradients were generated within the cell culture chamber of the microfluidic device by injecting NGF into the source channel of the device and allowing this factor to diffuse into the cell culture chamber and finally into the sink channel. By testing varying NGF concentration gradients within the collagen density of 0.9 mg/ml (that induced the formation of cellular connections in uniform NGF concentration), it was found that a minimum NGF concentration gradient of ~15 ng/ml/mm between the source channel and sink channel (with lower and higher absolute NGF concentrations of 14 and 29 ng/ml respectively) is required for the directional navigation of NPCs and the formation of aligned protrusions in the cell culture chamber of the microfluidic device (Fig. 7). Aligned protrusions were formed in the top region of the cell culture chamber where the absolute NGF concentration was greater than

~20 ng/ml (Fig. 7). This verifies the initial results that a minimum NGF absolute concentration of ~20 ng/ml is also necessary for directional navigation of NPCs. Lower NGF concentration gradients did not cause the formation of aligned NPC protrusions even though the absolute NGF concentration was maintained above 20 ng/ml. This was examined by keeping the maximum NGF concentration to ~29 ng/ml, but decreasing NGF concentration gradient to 10 ng/ml/mm (Supplemental Fig. S6).

To investigate the effect of varying NGF gradients on the directional migration of NPCs within collagen matrices with higher and lower densities, a statistical parameter, directional migration index (DMI) was calculated whose higher values shows better migration of NPCs towards NGF gradient as explained in the Materials and Methods section. As shown in Supplemental Figure S7, the cell nuclei were further away from the sink channel and closer to the source channel when the cells migrated towards NGF gradient. Table 1 shows DMI as a function of NGF gradients and collagen matrix density. Although NPC migration exhibited a rather individual behavior within the lower matrix densities (0.45 mg/ml), the quantification of the results showed that within this matrix density, lower NGF gradients (10 ng/ml/mm) could induce the directional migration of the cells (DMI = 0.73). As mentioned previously, at this NGF concentration NPCs did not show a directional migration within higher matrix densities. At the collagen matrix density of of 1.8 mg/ml, NGF gradient of ~50 ng/ml/mm induced the directional migration of NPCs (DMI =0.76) while lower NGF concentrations did not induce a significant directional migration (DMI ~0.5). These results show that the lower collagen matrices require shallower minimum NGF gradients to permit NPC migration, although the pattern of NPC

migration (individual vs. collective) was not observed to depend on the NGF gradient steepness. It can also be found that NPC directional migration can be rescued by increasing NGF concentration gradient within collagen matrices with higher densities.

Matrix density effect on NPC differentiation:

NPC differentiation has been previously studied in 2D matrices with varying stiffness levels and it has been shown that a specific matrix stiffness can induce the differentiation of NPCs[3]. Here, the differentiation of NPCs into neurons was investigated within varying collagen matrix densities in 3D. This was done by labeling and counting the cells that expressed MAP2 protein as a marker for neurons after 48 hrs of NPC injection into the device. As can be seen in Fig. 8A, the number of the cells that expressed MAP2 within the collagen matrix of 1.8 mg/ml was not very significant. NPC differentiation into neurons was significantly increased for the ones within collagen matrices of 0.9 mg/ml. Most of the cells within this matrix density showed MAP2 expression demonstrating the high tendency of them to differentiate into neurons after their migration to the cell culture chamber (Fig. 8B). A few of the cells encapsulated within collagen matrix of lower density (0.45 mg/ml) showed MAP2 expression as a signal for differentiation into neurons. Fig. 9 shows the quantified percentage of NPCs that differentiated into neurons within each collagen matrix density. As can be seen in Fig. 9, approximately 78% of the cells within intermediate matrix density (0.9 mg/ml) showed MAP2 expression, which was significantly higher compared to those in lower and higher matrix densities ($p < 0.01$). This could be due to the effect of matrix stiffness on the differentiation of NPCs, which is in agreement with other reported studies that showed the matrix stiffness effect on the differentiation of stem cells.

Conclusion:

Microenvironmental factors within NPC niches are the key regulators of NPC growth, migration, and differentiation. The response of NPCs to various biochemical cues has been widely investigated; for example, chemoattractant stromal cell-derived factor-1 (SDF-1) is shown to be an important factor for homing SVZ NPC to the vascular niches through SDF-1/Cxcr4 signaling[50, 51]. Microfluidic devices have also been used to implement stable gradients of chemokines in order to study the 2D migration of pretreated NPCs[43]. In the present study, a new microfluidic device was devised for investigating the 3D NPC microenvironment to determine the appropriate conditions for NPC migration and differentiation in a complex interplay of biochemical and biomechanical factors.

Two types of mobility for NPCs have been reported: radial migration and tangential migration. In radial migration, NPCs move alongside radial glial fibers, whereas in tangential migration NPCs follow pathways orthogonal to the radial glial processes. Directional migration of NPCs is a result of tangential migration in response to chemotactic gradients[52]. The initial step for the regeneration of damaged neurons is directional migration of NPCs towards the site of injury so that the cells can initiate differentiation at the appropriate location for potential cell replacement therapy. The ability of NPCs to migrate directionally is an important parameter in determining their potential to reach to the site of injury in the neuronal system[53]. In this study, the net migration of NPCs was investigated in response to NGF within collagen matrices of varying densities. Using time-lapse imaging, we tested the hypothesis that a minimum gradient of a neurotrophic factor (both across the microcapillaries and across the cell culture chamber) and an optimal collagen matrix density can induce the chemotaxis of NPC within a 3D microenvironment.

Although the minimum necessary absolute concentration of some chemoattractants has been evaluated in other studies[54], no comprehensive study has examined the effect of a varying range of biochemical and biophysical factors on NPC migration in a quantified manner. By investigating NPC responses to varying biochemical and biomechanical factors in our controllable microenvironment, we were able to determine the appropriate conditions that may result in the functional migration and differentiation of these cells. The results of this study showed that a specific collagen matrix density (0.9 mg/ml) and a minimum NGF absolute concentration and concentration gradient (20 ng/ml and 15 mg/ml/mm respectively) is required for optimal migration of neural stem cells, their differentiation into neurons and finally the formation of aligned protrusions (Fig. 4, 7-9).

As can be observed in Figure 4, the cells within collagen matrix of 0.9 mg/ml started to migrate towards the microfluidic cell culture chamber after 12 hrs and proceeded into this environment for the next 24 hrs. Then, they started to extend their axon-like protrusions after ~36 hrs. Time-lapse imaging of the cells showed a uniform NPC collective migration into the cell culture chamber of the microfluidic device within this intermediate collagen matrix density. In collective migration, a group of cells divide into leader and follower cells. The driving force of migration comes from the cells inside the group and the leader cells only dictate the direction of motion[55]. This collective migration as well as the effect of matrix stiffness may also be hypothesized to be the reasons for an efficient differentiation of the cells into neurons that were encapsulated within the intermediate matrix density (Fig. 8, 9). Our results show that the cells with greater ability of collective migration can differentiate more efficiently into neurons, confirming a correlation between NPC optimized migration and differentiation. We will investigate the hypothesis of the

effect of NPC collective migration on their differentiation into neurons in our future studies with further details.

While testing matrices with higher densities than 0.9 mg/ml (1.8 mg/ml), only a low percentage of the cells could continue their migration into the cell culture chamber of the microfluidic device. Although these cells initially started to have collective migration within this matrix density, they stopped to migrate and no sign of forming neuronal connections was observed. The cells were completely packed together during migration (Fig. 3), allowing them a very slow movement within the cell culture chamber of the device. Finally, the cells did not show a significant differentiation into neurons within this matrix density (Fig. 8). At lower matrix densities, the cells could migrate into the cell culture chamber of the device in response to NGF concentration but their migration did not occur in a collective manner and individual moving cells were observed in this microenvironment (Fig. 5). The cells migrated rather quickly, causing them to show an individual rather than collective migration. NPCs with minimum contact to the other cells did not also show a significant tendency to differentiate into neurons (Fig. 8). It was also observed that NPC encapsulated within collagen matrices with higher densities requires steeper NGF concentration gradients to exhibit directional migration. This shows that a complex biochemical and biomechanical interaction governs the directional navigation of these cells in a 3D microenvironment, which is in agreement with recent studies on cell-matrix adhesion and mechanotransduction[56-60].

The results of this study confirm the significance of biochemical and biomechanical factors in NPC collective migration. Finding the optimal matrix density and appropriate

range of biochemical factors that can support neural cell collective migration is therefore essential in designing efficient biomaterials that are conducive to healing of the neuronal damages in the spinal cord and brain injuries. In this study, it was found that the matrix density along with appropriate NGF concentrations can mediate NPC migration and differentiation. The results of this study can be translated into better designs of optimized platforms with potentials for transplantation into the damaged areas of the neuronal system and introduce a new method for 3D studies of stem cells *in-vitro*.

Acknowledgements:

Financial supports through a National Science Foundation CAREER award CBET-0955291 (M.R.K.M.) and a Siebel Stem Cell Institute post-doctoral fellowship (A.S.) are gratefully acknowledged. The authors would also like to thank the UC Berkeley Stem Cell Center and QB3 Shared Stem Cell Facilities for their assistance throughout this project.

References

1. H. G. Kuhn, T. D. Palmer and E. Fuchs, *European archives of psychiatry and clinical neuroscience*, 2001, 251, 152-158.
2. P. J. Horner and F. H. Gage, *Nature*, 2000, 407, 963-970.
3. L. Little, K. E. Healy and D. Schaffer, *Chemical reviews*, 2008, 108, 1787-1796.
4. L. M. Yu, N. D. Leipzig and M. S. Shoichet, *Materials today*, 2008, 11, 36-43.
5. R. Jagasia, H. Song, F. H. Gage and D. C. Lie, *Trends in molecular medicine*, 2006, 12, 400-405.
6. P. Riess, C. Zhang, K. E. Saatman, H. L. Laurer, L. G. Longhi, R. Raghupathi, P. M. Lenzlinger, J. Lifshitz, J. Boockvar and E. Neugebauer, *Neurosurgery*, 2002, 51, 1043-1054.
7. T. Namba, G. l. Ming, H. Song, C. Waga, A. Enomoto, K. Kaibuchi, S. Kohsaka and S. Uchino, *Journal of neurochemistry*, 2011, 118, 34-44.
8. W. Zhu, S. Cheng, G. Xu, M. Ma, Z. Zhou, D. Liu and X. Liu, *Drug delivery*, 2011, 18, 338-343.
9. R. Gundersen and J. Barrett, *Science*, 1979, 206, 1079-1080.
10. T. Yamashita, M. Ninomiya, P. H. Acosta, J. M. García-Verdugo, T. Sunabori, M. Sakaguchi, K. Adachi, T. Kojima, Y. Hirota and T. Kawase, *The Journal of neuroscience*, 2006, 26, 6627-6636.
11. B. Nait-Oumesmar, N. Picard-Riéra, C. Kerninon and A. Baron-Van Evercooren, *Journal of the neurological sciences*, 2008, 265, 26-31.
12. A. Rice, A. Khaldi, H. Harvey, N. Salman, F. White, H. Fillmore and M. Bullock, *Experimental neurology*, 2003, 183, 406-417.
13. M. A. Sherafat, M. Heibatollahi, S. Mongabadi, F. Moradi, M. Javan and A. Ahmadiani, *Journal of Molecular Neuroscience*, 2012, 48, 144-153.
14. O. Thau-Zuchman, E. Shohami, A. G. Alexandrovich and R. R. Leker, *Journal of Cerebral Blood Flow & Metabolism*, 2010, 30, 1008-1016.
15. P. Thored, A. Arvidsson, E. Cacci, H. Ahlenius, T. Kallur, V. Darsalia, C. T. Ekdahl, Z. Kokaia and O. Lindvall, *Stem cells*, 2006, 24, 739-747.
16. M. LaPlaca, C. Simon, G. Prado and D. Cullen, *Progress in brain research*, 2007, 161, 13-26.
17. A. E. Davis, *Critical care nursing quarterly*, 2000, 23, 1-13.
18. D. A. Shear, C. C. Tate, M. C. Tate, D. R. Archer, M. C. LaPlaca, D. G. Stein and G. L. Dunbar, *Restorative neurology and neuroscience*, 2011, 29, 215-225.
19. M. Cooke, K. Vulic and M. Shoichet, *Soft Matter*, 2010, 6, 4988-4998.
20. C. T. W. P. Foo, J. S. Lee, W. Mulyasasmita, A. Parisi-Amon and S. C. Heilshorn, *Proceedings of the National Academy of Sciences*, 2009, 106, 22067-22072.
21. K. J. Lampe, A. L. Antaris and S. C. Heilshorn, *Acta biomaterialia*, 2013, 9, 5590-5599.
22. K. S. Straley, C. W. P. Foo and S. C. Heilshorn, *Journal of neurotrauma*, 2010, 27, 1-19.
23. A. Khademhosseini, *Biomaterials,* 2007**,** *28*, 5087-5092.

24. K. I. Park, Y. D. Teng and E. Y. Snyder, *Nature biotechnology*, 2002, 20, 1111-1117.
25. B. Ananthanarayanan, L. Little, D. V. Schaffer, K. E. Healy and M. Tirrell, *Biomaterials*, 2010, 31, 8706-8715.
26. R. A. Brown and J. B. Phillips, *International review of cytology*, 2007, 262, 75-150.
27. D. K. Cullen, M. C. Lessing and M. C. LaPlaca, *Annals of biomedical engineering*, 2007, 35, 835-846.
28. C. Carmona-Fontaine, H. K. Matthews, S. Kuriyama, M. Moreno, G. A. Dunn, M. Parsons, C. D. Stern and R. Mayor, *Nature*, 2008, 456, 957-961.
29. C. Carmona-Fontaine, E. Theveneau, A. Tzekou, M. Tada, M. Woods, K. M. Page, M. Parsons, J. D. Lambris and R. Mayor, *Developmental cell*, 2011, 21, 1026-1037.
30. E. Theveneau and R. Mayor, *Small GTPases*, 2010, 1, 113-117.
31. S. J. McKeown, A. S. Wallace and R. B. Anderson, *Developmental biology*, 2013, 373, 244-257.
32. E. Theveneau, L. Marchant, S. Kuriyama, M. Gull, B. Moepps, M. Parsons and R. Mayor, *Developmental cell*, 2010, 19, 39-53.
33. R. McLennan, J. M. Teddy, J. C. Kasemeier-Kulesa, M. H. Romine and P. M. Kulesa, *Developmental biology*, 2010, 339, 114-125.
34. L. A. Flanagan, L. M. Rebaza, S. Derzic, P. H. Schwartz and E. S. Monuki, *Journal of neuroscience research*, 2006, 83, 845-856.
35. J. Gerardo-Nava, T. Führmann, K. Klinkhammer, N. Seiler, J. Mey, D. Klee, M. Möller, P. D. Dalton and G. A. Brook, *Nanomedicine*, 2009, 4, 11-30.
36. F. Guilak, D. M. Cohen, B. T. Estes, J. M. Gimble, W. Liedtke and C. S. Chen, *Cell stem cell*, 2009, 5, 17-26.
37. N. D. Leipzig and M. S. Shoichet, *Biomaterials*, 2009, 30, 6867-6878.
38. K. Saha, A. J. Keung, E. F. Irwin, Y. Li, L. Little, D. V. Schaffer and K. E. Healy, *Biophysical journal*, 2008, 95, 4426-4438.
39. S. Han, K. Yang, Y Shin, J. S. Lee, R. D. Kamm, S. Chung, S. W. Cho, *Lab Chip,* 2012**,** 12, 2305-2308.
40. C. R. Kothapalli, R. D. Kamm, *Biomaterials,* 2013**,** 34*,* 5995-6007.
41. B. G. Chung, L. A. Flanagan, S. W. Rhee, P. H. Schwartz, A. P. Lee, E. S. Monuki and N. L. Jeon, *Lab on a Chip*, 2005, 5, 401-406.
42. P. Rajan and E. Snyder, *Methods in enzymology*, 2006, 419, 23-52.
43. H. Xu and S. C. Heilshorn, *Small*, 2013, 9, 585-595.
44. H. Babu, J.-H. Claasen, S. Kannan, A. E. Rünker, T. Palmer and G. Kempermann, *Frontiers in neuroscience*, 2011, 5.
45. F. Kuhnert, M. R. Mancuso, A. Shamloo, H.-T. Wang, V. Choksi, M. Florek, H. Su, M. Fruttiger, W. L. Young and S. C. Heilshorn, *Science*, 2010, 330, 985-989.
46. A. Shamloo and S. C. Heilshorn, *Lab on a Chip*, 2010, 10, 3061-3068.
47. A. Shamloo, N. Ma, M.-m. Poo, L. L. Sohn and S. C. Heilshorn, *Lab on a chip*, 2008, 8, 1292-1299.
48. A. Shamloo, M. Manchandia, M. Ferreira, M. Mani, C. Nguyen, T. Jahn, K. Weinberg and S. Heilshorn, *Integrative Biology*, 2013, 5, 1076-1085.
49. A. Shamloo, H. Xu, S. C. Heilshorn, *Tissue Engineering Part A,* 2012**,** 18*,* 320-330.

50. E. Kokovay, Y. Wang, G. Kusek, R. Wurster, P. Lederman, N. Lowry, Q. Shen and S. Temple, *Cell stem cell*, 2012, 11, 220-230.
51. B. Z. Barkho, A. E. Munoz, X. Li, L. Li, L. A. Cunningham and X. Zhao, *Stem Cells*, 2008, 26, 3139-3149.
52. O. Marín, M. Valiente, X. Ge and L.-H. Tsai, *Cold Spring Harbor perspectives in biology*, 2010, 2, a001834.
53. R. Gordon, N. Mehrabi, C. Maucksch and B. Connor, *Experimental neurology*, 2012, 233, 587-594.
54. R. J. Gordon, A. L. McGregor and B. Connor, *Molecular and Cellular Neuroscience*, 2009, 41, 219-232.
55. X. Trepat, M. R. Wasserman, T. E. Angelini, E. Millet, D. A. Weitz, J. P. Butler and J. J. Fredberg, *Nature physics*, 2009, 5, 426-430.
56. M.R.K. Mofrad, R. D. Kamm Eds. *Cellular Mechanotransduction: Diverse Perspectives from Molecules to Tissues*, 2014, Cambridge University Press.
57. Z. Jahed, H. Shams, M. Mehrbod, M. R. K. Mofrad. *International Review of Cell and Molecular Biology*, 2014;310:171-220.
58. K. S. Kolahi, M. R. K. Mofrad. *Wiley Interdiscip Rev Syst Biol Med.* 2010 Nov-Dec;2(6):625-39.
59. Y. Jamali, T. Jamali, M. R. K. Mofrad. *Journal of Computational Physics.* 2013; 244:264–278.
60. M. Mehrbod, S. Trisno, M. R. K. Mofrad. Biophysical Journal, 2013 Sep 17;105(6):1304-15.

Table 1. Directional migration index (DMI) for NPC within varying collagen matrix densities and exposed to different NGF concentration gradients.

Case	Bottom concentration (ng/ml)	Top concentration (ng/ml)	Gradient (ng/ml/mm)	DMI for collagen density = 0.45	DMI for collagen density = 0.9	DMI for collagen density = 1.8
1	19	29	10	0.73	0.47	0.53
2	14	29	15	0.77	0.80	0.48
3	28	58	30	0.70	0.75	0.57
4	47	97	50	0.66	0.73	0.76

Figure Captions

Fig. 1. (A) A new microfluidic platform devised for studying 3D migration and differentiation of NPCs. This device is composed of a cell culture chamber as well as source and sink channels for generating concentration gradients of biochemical factors. There is also a cell culture reservoir for the culture of NPCs that is connected to the cell culture chamber using cell microcapillaries. NPCs were initially mixed with collagen matrix and injected into the cell culture reservoir in the form of neurospheres in a 3D gel. Collagen matrix with the same density was prepared and injected into the cell culture chamber of the microfluidic device. **(B)** The cells started to migrate into the cell microcapillaries in response to NGF after ~6hrs. **(C)** NPCs entered the cell culture chamber after ~ 12 hrs.

Fig. 2. Three different collagen gel matrices were prepared by changing collagen densities **(A)**: 0.45 mg/ml, **(B)**: 0.9 mg/ml and **(C)**: 1.8 mg/ml). SEM images showed that the entanglement of collagen fibers significantly increased when collagen density was higher.

Fig. 3. A-D: NPC migration within collagen matrix of 1.8 mg/ml density. NPCs migrated into the cell culture chamber but their migration was very slow in this environment. Migrated cells were highly packed together and did not show protrusions similar to neural axons.

Fig. 4. A-D: NPC migration within collagen matrix of 0.9 mg/ml density. NPCs entered into the cell culture chamber after ~12 hrs and continued their collective migration during 48 hrs. E. NPCs formed protrusions similar to neural axons after ~48 hrs.

Fig. 5. A-D: NPC migration within collagen matrix of 0.45 mg/ml density. NPCs migrated into the cell culture chamber after ~12 hrs. Most of the migrating cells were moving within the cell culture chamber in a separate manner and did not form connections to their neighboring cells.

Fig. 6. Variation of cell density **(A)** and average cell migration distance **(B)** within varying collagen matrix densities. (**A)** Cell density was significantly higher within collagen density of 1.8 mg/ml compared to the ones within lower matrix densities (** $p < 0.01$). Cell density was also significantly higher within collagen density of 0.9 mg/ml compared to the cell density within lower matrix density (* $p < 0.05$). **(B)** Average cell migration distance within collagen matrix of 0.9 mg/ml density was significantly higher compared to this parameter within higher collagen density (* $p < 0.05$) and lower compared to this parameter within lower matrix density (* $p < 0.05$).

Fig. 7. NGF gradient (15 mg/ml/mm) caused directional navigation of NPCs and the formation of aligned axon-like structures in top part of the cell culture chamber where NGF absolute concentration was above ~20 ng/ml.

Fig. 8. The expression of MAP2 protein was detected by fluorescent labeling (red). Cell nuclei were stained with dapi (green). **A.** Within collagen matrix of 1.8 mg/ml density, very few cells showed MAP2 expression. **B.** Within collagen matrix of 0.9 mg/ml density, a high percentage of the cells

expressed MAP2 protein. **C.** Within collagen matrix of 0.45 mg/ml density, few cells showed MAP2 expression.

Fig. 9. The percentage of NPCs showing MAP2 expression for the cells encapsulated within collagen matrices of 0.45, 0.9 and 1.8 mg/ml densities. The cells within collagen matrix of 0.9 mg/ml density showed significantly higher MAP2 expression demonstrating higher tendency of NPCs to differentiate into neurons compared to higher and lower matrix densities (**p <0.01).

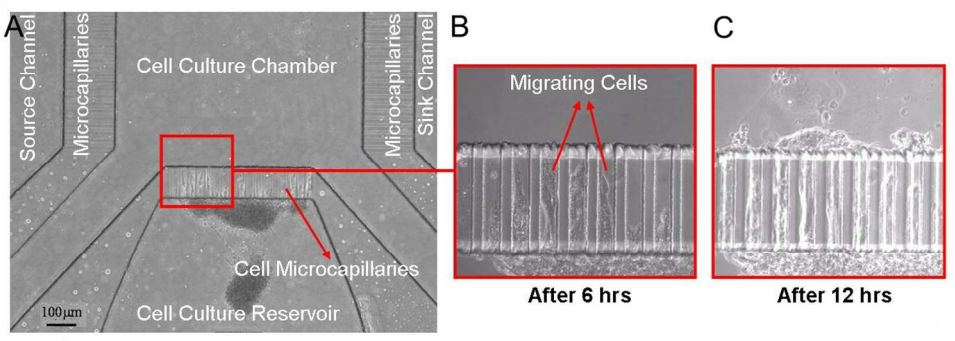

Fig. 1. A. A microfluidic device was implemented for studying 3D migration and differentiation of NPC. This device is composed of a cell culture chamber as well as a source and a sink channel for generating concentration gradients of biochemical factors similar to what explained in Ref. 46. There is also a cell culture reservoir for culturing of NPC that is connected to the cell culture chamber using cell microcapillaries. NPC was initially mixed with collagen matrix and injected into the cell culture reservoir in the form of neurospheres in a 3D gel. Collagen matrix with the same density was prepared and injected into the cell culture chamber of the microfluidic device. B. The cells started to migrate into the cell microcapillaries in response to NGF after ~6hrs. C. NPC entered the cell culture chamber after ~ 12 hrs.
372x133mm (96 x 96 DPI)

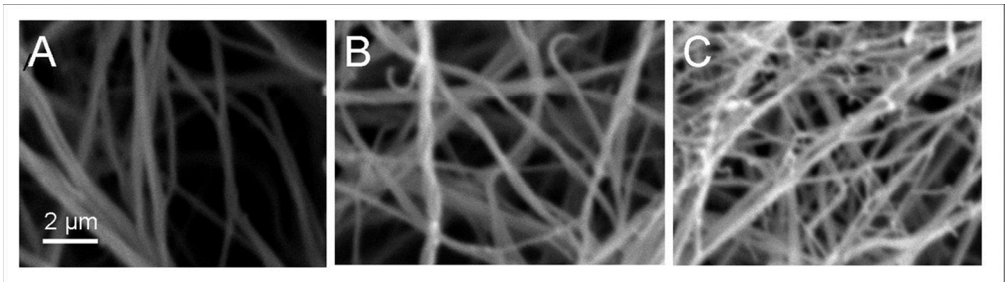

Fig. 2. Three different collagen gel matrices were prepared by changing collagen densities (A: 0.45 mg/ml, B: 0.9 mg/ml and C: 1.8 mg/ml). SEM images showed that the entanglement of collagen fibers significantly increased when collagen density was higher.
222x61mm (150 x 150 DPI)

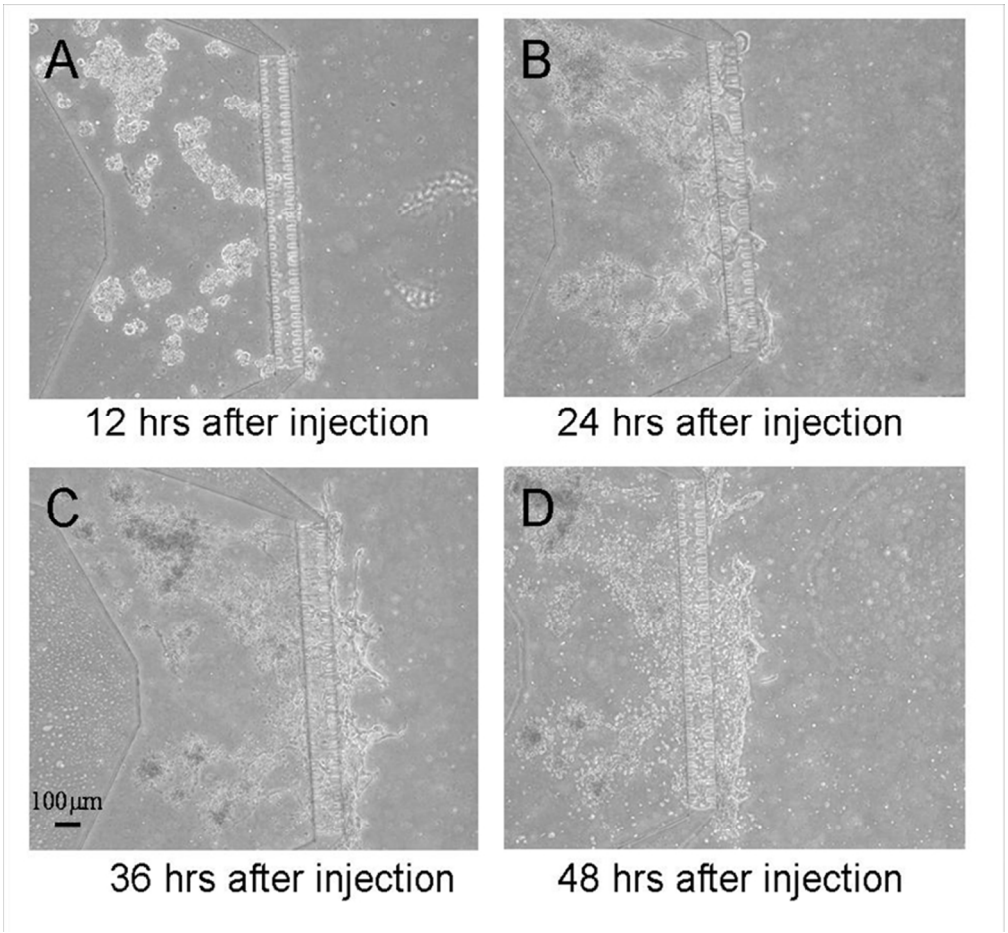

Fig. 3.A-D: NPC migration within collagen matrix of 1.8 mg/ml density. NPC migrated into the cell culture chamber but their migration was very slow in this environment. Migrated cells were very packed together and did not show protrusions similar to neural axons.
146x135mm (150 x 150 DPI)

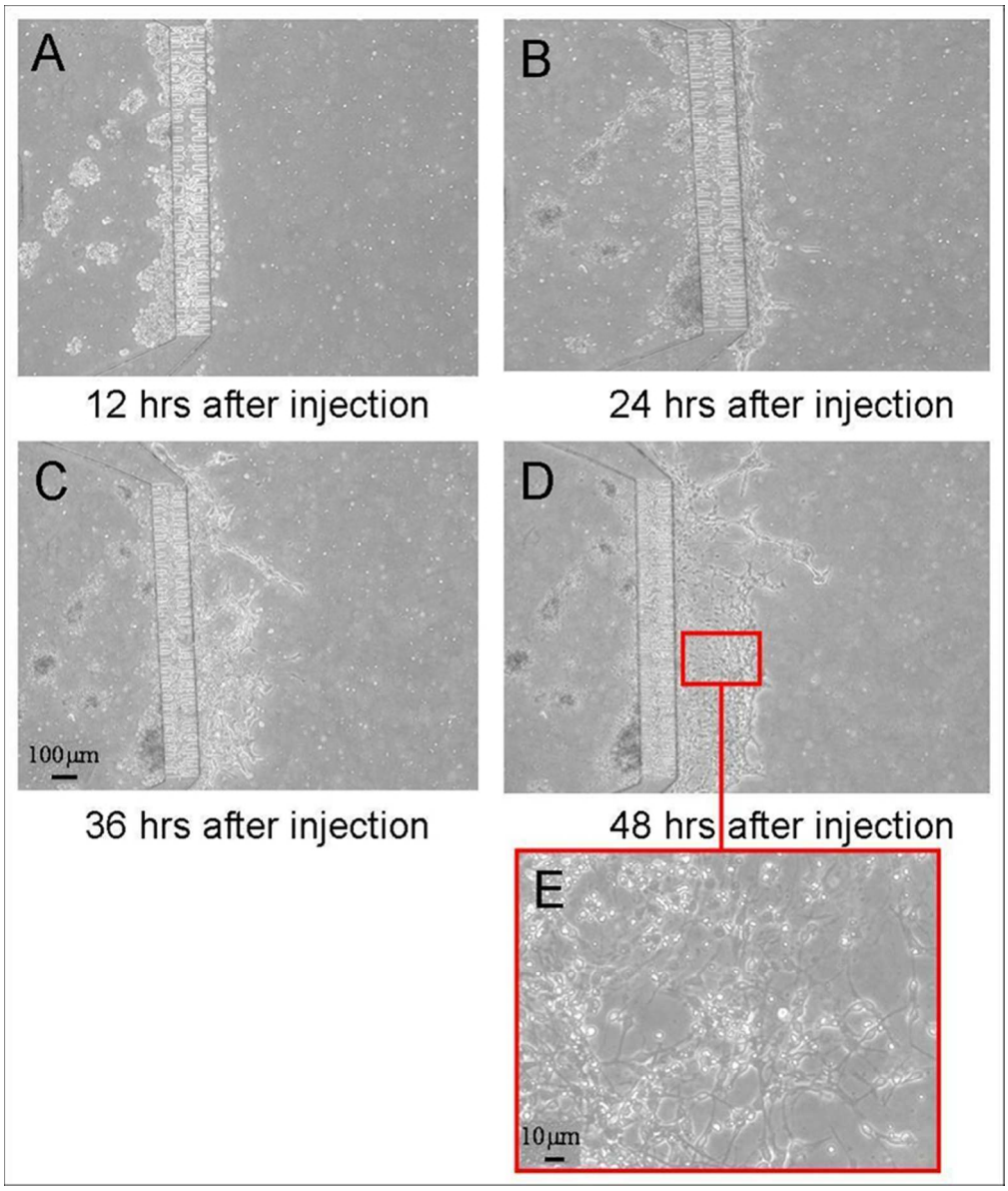

Fig. 4.A-D: NPC migration within collagen matrix of 0.9 mg/ml density. NPC entered into the cell culture chamber after ~12 hrs and the cells continued their collective migration during 48 hrs. E. NPC formed protrusions similar to neural axons after ~48 hrs.
134x158mm (150 x 150 DPI)

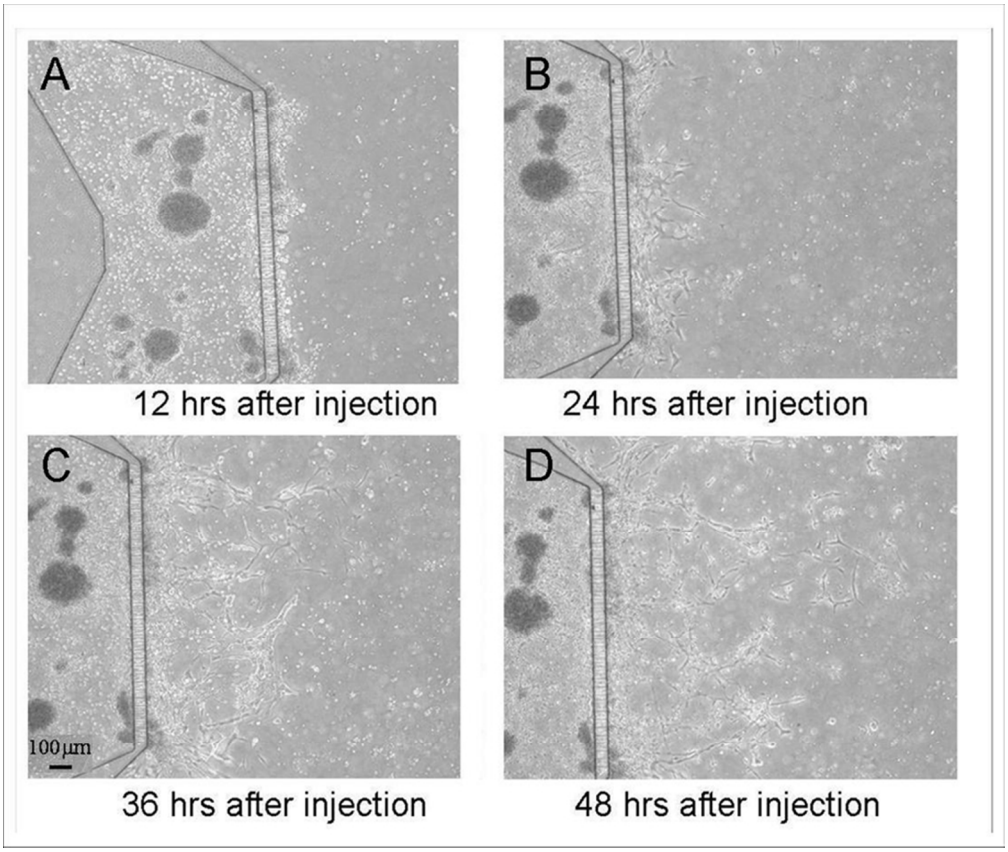

Fig. 5.A-D: NPC migration within collagen matrix of 0.45 mg/ml density. NPC migrated into the cell culture chamber after ~12 hrs. Most of the migrating cells were moving within the cell culture chamber in a separate manner and did not form connections to their neighboring cells.
241x203mm (96 x 96 DPI)

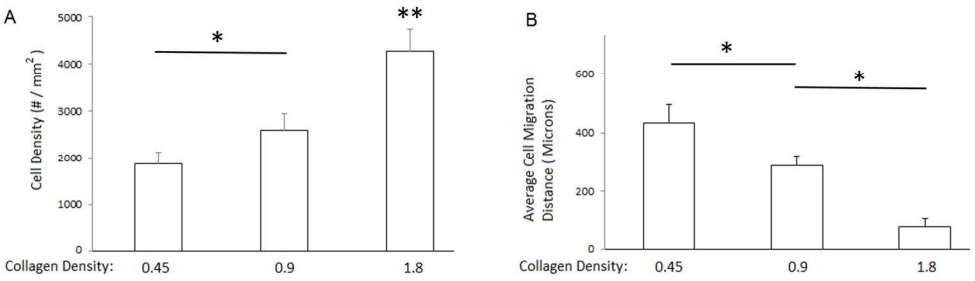

Fig. 6. Variation of cell density (A) and average cell migration distance (B) within varying collagen matrix densities. A. Cell density was significantly higher within collagen density of 1.8 mg/ml compared to the ones within lower matrix densities (** $p < 0.01$). Cell density was also significantly higher within collagen density of 0.9 mg/ml compared to the cell density within lower matrix density (* $p < 0.05$). B. Average cell migration distance within collagen matrix of 0.9 mg/ml density was significantly higher compared to this parameter within higher collagen density (* $p < 0.05$) and lower compared to this parameter within lower matrix density (* $p < 0.05$).
456x153mm (96 x 96 DPI)

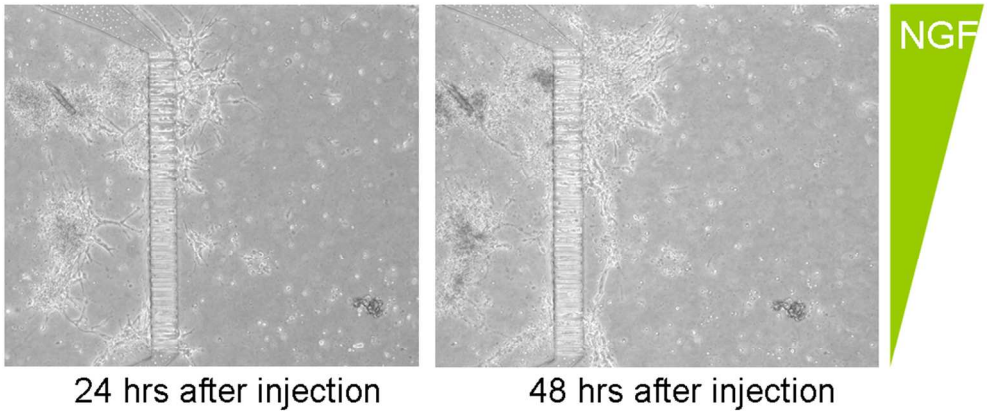

Fig. 7. NGF gradient (15 mg/ml/mm) caused directional navigation of NPC and the formation of aligned axon-like structures in top part of the cell culture chamber where NGF absolute concentration was above ~20 ng/ml.
186x82mm (150 x 150 DPI)

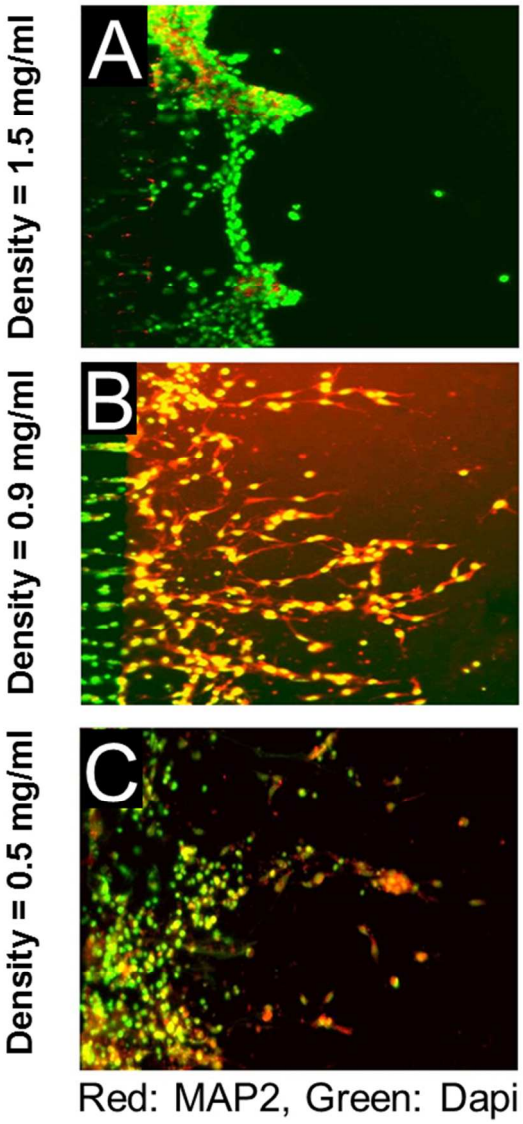

Fig. 8.The expression of MAP2 protein was detected by fluorescent labeling (red). Cell nuclei were stained with dapi (green). A. Within collagen matrix of 1.8 mg/ml density, very few cells showed MAP2 expression. B. Within collagen matrix of 0.9 mg/ml density, a high percentage of the cells expressed MAP2 protein. C. Within collagen matrix of 0.45 mg/ml density, few cells showed MAP2 expression.
120x264mm (96 x 96 DPI)

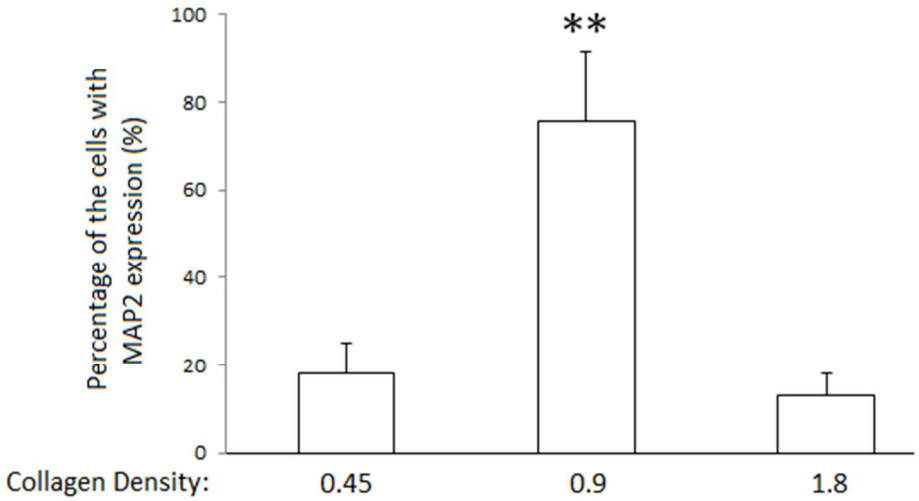

Fig. 9. The percentage of NPC showing MAP2 expression for the cells encapsulated within collagen matrices of 0.45, 0.9 and 1.8 mg/ml densities. The cells within collagen matrix of 0.9 mg/ml density showed significantly higher MAP2 expression demonstrating higher tendency of NPC to differentiate into neurons compared to higher and lower matrix densities (**p <0.01).
156x91mm (96 x 96 DPI)