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Enhancement of neurite outgrowth in neuron cancer stem cells by growth on 3-D collagen scaffolds

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ABSTRACT

Collagen is one component of the extracellular matrix that has been widely used for constructive remodeling to facilitate cell growth and differentiation. The 3-D distribution and growth of cells within the porous scaffold suggest a clinical significance for nerve tissue engineering. In the current study, we investigated proliferation and differentiation of neuron cancer stem cells (NCSCs) on a 3-D porous collagen scaffold that mimics the natural extracellular matrix. We first generated green fluorescence protein (GFP) expressing NCSCs using a lentiviral system to instantly monitor the transitions of morphological changes during growth on the 3-D scaffold. We found that proliferation of GFP-NCSCs increased, and a single cell mass rapidly grew with unrestricted expansion between days 3 and 9 in culture. Moreover, immunostaining with neuronal nuclei (NeuN) revealed that NCSCs grown on the 3-D collagen scaffold significantly enhanced neurite outgrowth. Our findings confirmed that the 80 μm porous collagen scaffold could enhance attachment, viability and differentiation of the cancer neural stem cells. This result could provide a new application for nerve tissue engineering and nerve regeneration.

1. Introduction

Collagen is one component of the extracellular matrix that has been widely used for constructive remodeling to facilitate cell growth and differentiation. The widespread use of collagen across many clinical applications is due to its bio-inductive, mechanical, degradative and material properties, as well as the host tissue response to naturally occurring ECM [1–6].

Collagen-based biomaterials have been formed into fibers, films, sheets, sponges and dispersions of fibrils. Collagen sponges serve as cellular scaffolds in tissue regeneration. These sponges support physiologic processes in the course of healing and provide a template for the development of 3-D, functionally intact cell constructs and vascular in-growth, which is indispensable for nutrient supply [7]. Biomaterial polymers immobilized with collagen can be used for neuronal stem cell culture [8] and have been reported to improve neurite growth of PC12 cells [9–11]. The 3-D distribution and growth of cells within the porous scaffold are of clinical significance for nerve tissue engineering [8–13]. Here, we established an 80 μm porous collagen scaffold to validate the application of nerve regeneration. Human neuroblastoma SH-SY5Y cells, which are neuron cancer stem cells (NCSCs), were used to study the effects of the collagen scaffold on cell growth and neurite extension. The SH-SY5Y cells have been shown to undergo neuronal differentiation when they are exposed to nerve growth factor or retinoic acid, which makes these cells a useful model system to study neuronal differentiation [14–16]. However, cell growth and neuronal differentiation on a 3-D scaffold have not been fully studied.

In the current study, we designed a green fluorescence expression system using the simian immunodeficiency virus (SIV) lentiviral vector to generate stably expressed GFP-human neuron cancer stem cells (GFP-NCSCs). This system will allow us to verify growth and morphology changes in cells during growth on the 3-D collagen scaffold. The effect of the collagen scaffold on proliferation, adhesion, and differentiation of neurite outgrowth in GFP-NCSCs will be evaluated by Alamar Blue dye reduction assay, inverted phase-contrast microscopy, laser scanning confocal microscopy, scanning electron microscopy, and NeuN immunostaining.

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2. Materials and methods

2.1. Synthesis of the collagen scaffold

The base collagen solutions were prepared as previously described [6]. Briefly, 0.55 g of Type I bovine collagen was added to 200 ml of 0.5 M acetic acid solution (2.75 mg/ml). A porous collagen scaffold that mimics the extracellular matrix was generated from a lyophilized collagen solution. The optimized pore size of 80 μM for cell seeding was obtained from a 7% collagen solution.

2.2. Generation and secretion of GFP expressing SH-SY5Y cells

Neuroblastoma SH-SY5Y cells, which are neuron cancer stem cells, were purchased from ATCC and cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (Gibco BRL), 2 mM glutamine (Invitrogen, USA), 100 mg/ml streptomycin (Invitrogen, USA) and 100 U/ml penicillin (Invitrogen, USA) at 37 °C in a 5% CO_2 atmosphere. For generation of GFP expressing SH-SY5Y cells (GFP-NCSCs), 1×10^5 cells were seeded in 6-well plates in media containing Lentivirus-carrying GFP (National RNAi Core Facility, Taiwan) and 8 μg/ml polybrene (Sigma–Aldrich, St. Louis, MO). The culture media were removed at 24 h post-infection, and GFP-NCSCs were identified as those growing in the presence of 5 μg/ml puromycin (Sigma–Aldrich, St. Louis, MO) for an additional 48 h.

2.3. Cell proliferation of cultured NCSCs on 3-D collagen scaffolds

5 mm thickness of collagen scaffolds were generated in 24 well Petri-dish and each scaffold was seeded with 5×10^3 GFP-NCSCs. Cell proliferation was monitored using the Alamar Blue dye reduction assay at 37 °C for 8 days. Briefly, the cell-scaffold complexes were incubated with assay reagent in 2 ml of complete media containing 5% Alamar Blue (BioSource International, CA, USA) for 3 h. The aliquots were transferred into the wells of a 96-well plate

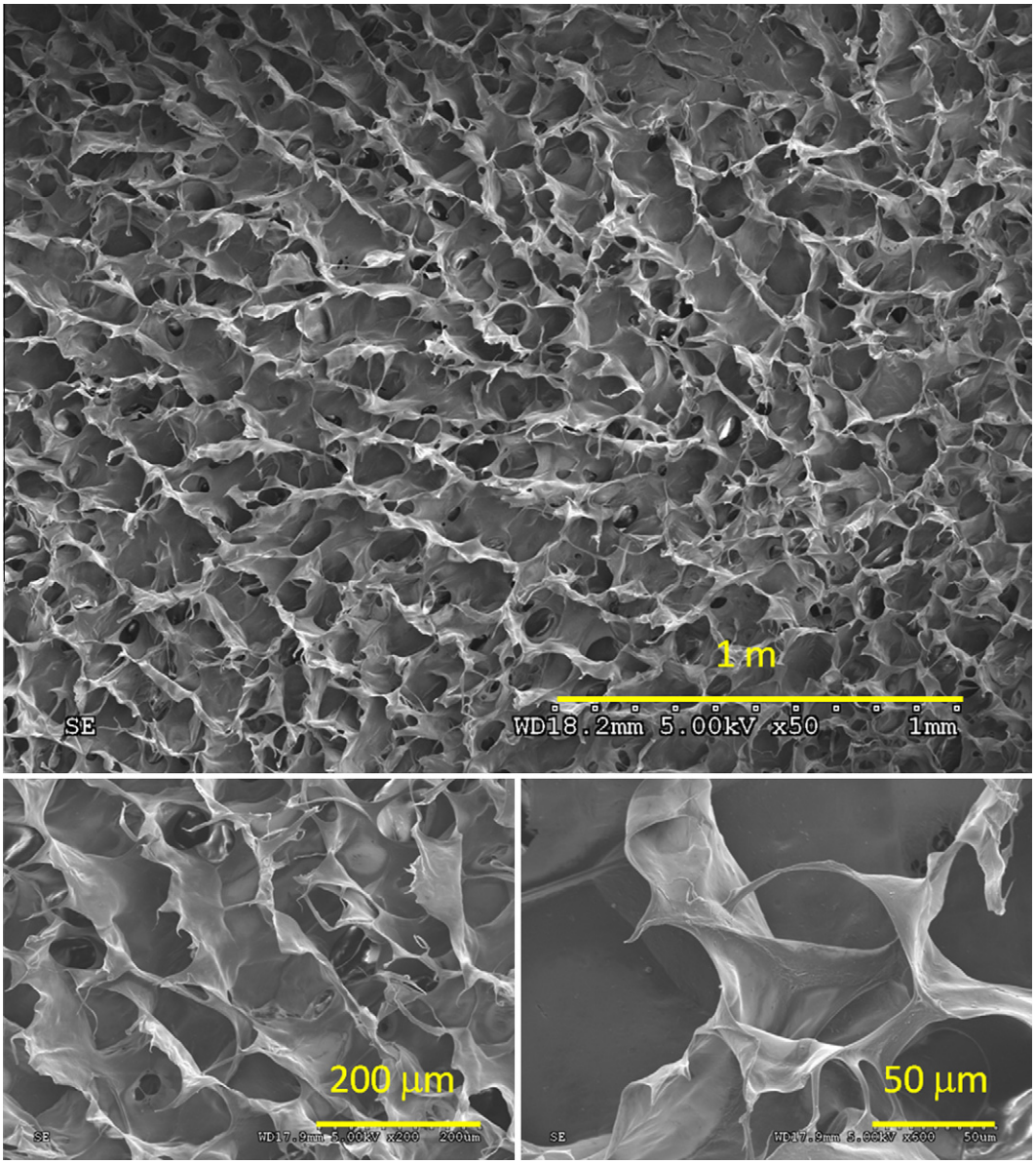

Fig. 1. The 3D architecture and pore size of porous collagen scaffolds were confirmed by scanning electron microscopy (SEM).

and cell viability was measured at 490 nm using a Polarstar microplate reader (FLUOstar OPTIMA; BMG Labtech, Offenburg, Germany) at 37 °C.

2.4. Analysis of cell adhesion, proliferation, and morphological extension of GFP-NCSCs cultured on 3-D collagen scaffolds

To analyze the growth of GFP-NCSC colonies on 3-D collagen scaffolds, 1×10^3 GFP-NCSCs were seeded, and cell images were taken every 24 h using an inverted phase-contrast microscope with an Olympus digital camera. To evaluate cell morphological extension, GFP-NCSCs were fixed with 3% paraformaldehyde and then incubated with Hoechst 33258 nucleus staining solution for 30 min. The images were taken using a laser scanning confocal microscope (LSCM, Fluoview FV300, Olympus). To study scaffold architecture, cell adhesion and extended neurite morphology, the scaffolds, which were in the presence or absence of NCSCs, were fixed with 3% paraformaldehyde, embedded in paraffin sections coated with gold metal, and then analyzed by scanning electron microscopy (SEM, JSM-5800LV, JEOL, Tokyo Japan). Image analysis software was used to measure ranges of neurite outgrowth diameters, as well as pore sizes of collagen scaffolds on the SEM micrographs (ImageJ; National Institutes of Health, Bethesda, MD).

2.5. Measurement of neurite outgrowth by NeuN immunostaining

After 1, 3 and 5 days in culture, the neurite outgrowth of NCSCs on 3-D collagen scaffolds (i.e., the 3-D collagen culture group) and

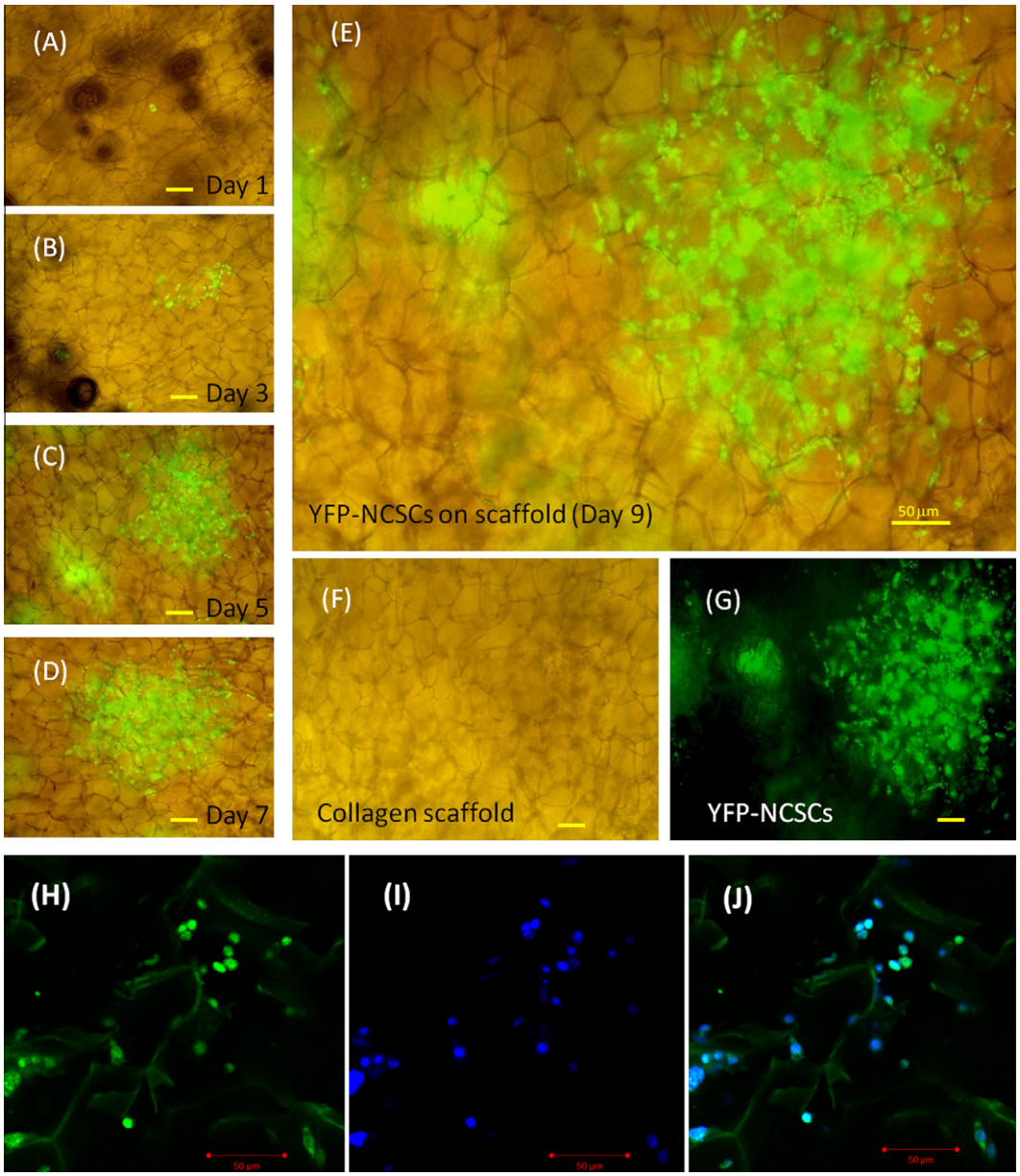

Fig. 2. Elapsed time monitoring of the growth of GFP-NCSCs on 3D collagen scaffold by inverted Olympus phase contrast microscope observation (A–G). After GFP-NCSCs were seeding on 3D collagen scaffold, the images of cell adherence morphology and colonies growth were taken every 24 h using phase-contrast plus green fluorescence emission (A–E). The photographs of phase-contrast (F) and dark-field (G) was taken for incubation on day 9. Effect of collagen scaffold in nesting 3D space on neurite outgrowth of GFP-NCSCs. The surface of porous (H), counter stain DAPI (I) and merge (J). Scale bar, 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on 2-D collagen coating slides (i.e., the 2-D collagen culture group) was measured by immunostaining with a neuronal marker, NeuN. The immunostaining procedure was performed as previously described [17]. Briefly, the 3-D collagen scaffold with NCSCs was embedded with paraffin, and 5-μm sections were mounted on microscope slides. The sections were then deparafinized. The deparaffinized sections and 2-D collagen culture slides were immersed in mouse anti-NeuN (diluted at 1:200, Sigma) for 1 h and then incubated with secondary antibody and Novolink polymer (Novocastra, Leica). The peroxidase reaction was performed by using 3,3′-diaminobenzidine tetrahydrochloride (DAB), and counterstaining was performed with Harris hematoxylin. Neurite outgrowth of the differentiated NCSCs seeded on scaffolds was observed in five independent high-magnification (200×) fields.

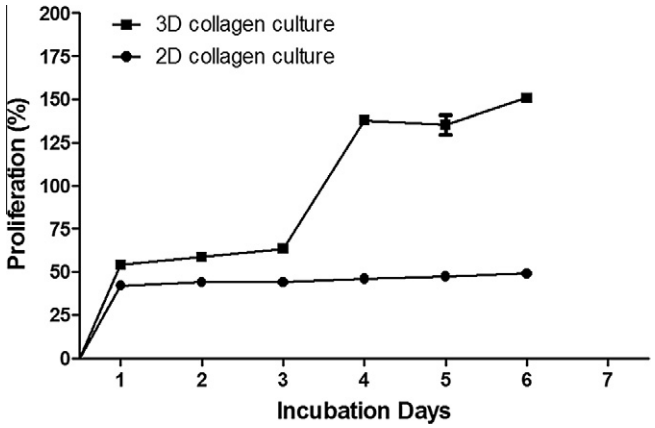

Fig. 3. Effect of the 3D collagen scaffold- and 2D collagen-culture of GFP-NCSCs on proliferation. The cell proliferation of GFP-NCSCs on 3D collagen scaffold- and 2D collagen culture were examined using Alamar Blue assay. Each point represents the mean ± SEM from three experiments.

2.6. Statistics

All data presented are expressed as mean-standard deviation (SD). ANOVA single factor analysis was conducted, and the level of statistical significance is defined as $p < 0.05$. Each parameter was conducted with three samples ($n = 3$). Cell viability assay results by Alamar Blue dye reduction consist of three samples with triplicate readings.

3. Results

3.1. Growth of GFP-NCSCs on a collagen scaffold

A porous collagen scaffold that mimics the extracellular matrix was generated from a lyophilized 7% collagen solution. The 3-D architecture and the uniform 80 μm pore size of the porous collagen scaffold were confirmed by SEM (Fig. 1). To monitor the behavior and cellular morphological changes during cell growth on the 3-D collagen scaffold, neuron cancer stem cells were engineered with GFP using a Lentivirus encoding GFP. After 3 days of infection, over 90% of NCSCs had a strong prevalence of GFP expressed in the cytoplasm as observed by fluorescence microscopy. These cells were designated as GFP-NCSCs.

To analyze the growth of GFP-NCSC colonies on the 3-D collagen scaffolds, 1×10^3 GFP-NCSCs were seeded, and cell adherence was observed and photographed every 24 h for 9 days using an inverted Olympus phase contrast microscope attached to an Olympus digital camera. After 3 days of incubation, scatter colonies were grown from independent cells, and various cell populations were observed in a time-dependent manner (Fig. 2A–G). After 8 days of incubation, a rapidly growing number of colonies were displayed

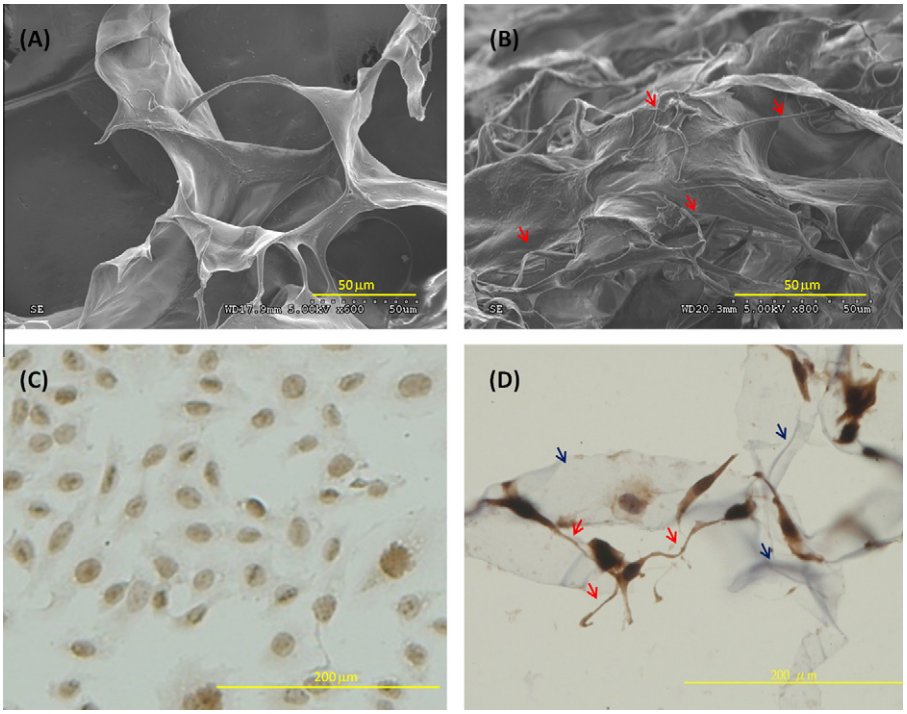

Fig. 4. The SEM analysis for the neurite outgrowth of GFP-NCSCs on a 3D collagen scaffold (A) as compared with cell-free 3D collagen scaffold (B). Scale bar, 50 μm. Characterization of neurite outgrowth of NCSCs grows on 2D collagen (C) or nested on 3D collagen scaffold (D). The neurite outgrowth of NCSCs was characterized by NeuN immunostaining. The red arrow indicates the neurite and blue arrow indicates the collagen scaffold. Scale bar, 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

through the architecture of collagen sponges. Using laser confocal microscopy analysis revealed that the GFP-NCSCs growing on the surface of the 3-D porous collagen scaffold showed a neurite-like structure (Fig. 2H–J).

To elucidate the growth capacity of NCSCs on the 3-D collagen scaffold and the 2-D collagen coating slides, cell proliferation was measured and quantified using the Alamar Blue dye reduction assay. As shown in Fig. 3, there is a 3-fold increase in proliferation on the 3-D collagen scaffold as compared to the 2-D collagen culture, and an unrestricted expansion was observed from days 3 to 6 of incubation. Therefore, our findings suggest that the 3-D collagen scaffold provides a better architectural structure and space for the proliferation and capacity of growth for NCSCs.

3.2. Enhancement of neurite outgrowth in GFP-NCSCs grown on a 3-D collagen scaffold

To evaluate the morphological structure of NCSCs grown on the 3-D collagen scaffold, we used SEM to scan the fine structures on the surface of the cells seeded on the scaffolds. During cells growth on 3-D collagen scaffolds, NCSCs become assembled into functional networks by growing out neurites (Fig. 4A and B). To further verify if the collagen scaffold promoted neuron cancer stem cell differentiation, the differentiation and neurite outgrowth of NCSCs were evaluated by immunostaining using a neuronal marker, NeuN. NeuN is a nuclear and proximal cytoplasmic marker of the majority of mature neurons, and antibodies to this protein are therefore used to identify neurons. As shown in Fig. 4C, 2-D collagen culture did not change the morphology of the attached NCSCs, and a pale expression of NeuN was observed in the cellular cytoplasm. In contrast, NCSCs in the 3-D collagen culture group revealed strong NeuN expression in a string-like, extended cytoplasm (Fig. 4D). Therefore, our data suggest that 3-D collagen architecture provides a crucial platform for cellular communication due to the complex network of neurite outgrowth. This could potentially promote neural differentiation.

4. Discussion

Collagen is the main structural protein in vertebrates. It plays an essential role in providing a scaffold for cellular support, which thereby affects cell attachment, migration, proliferation, differentiation and survival. Recently, the specific characteristics of collagen have led to the exploration of new applications. These characteristics include biodegradability, affinity to cells and tissues, enhancement of wound healing, tensile strength, and mechanical flexibility. There is also the possibility of chemically modifying collagen in combination with a variety of natural or synthetic materials. Collagen can be used for drug delivery and haemostatic applications. It also plays an important role in numerous approaches involving the engineering of human tissues for medical applications related to the repair and reconstruction of tissue, bone, and skin. [18–20]. A collagen-rich scaffold derived from human peripheral nerve has been studied for tissue-engineered nerve grafts [13]. In this study, we found a porous collagen scaffold that potentially mimicked the extracellular matrix and enhanced the differentiation and neurite outgrowth of neuron cancer stem cells (NCSCs) *in vitro*. Similar to our results, porous scaffolds have been suggested to act as a positive factor that supports connective nerve cell growth [21]. In addition, certain cells that utilize specific glycoproteins, such as collagen-binding integrins, have been shown to interact with the ECM component of collagen and provide directional information for cell attachment, migration, proliferation, differentiation, and survival [22]. Differentiation of neuron cancer stem cells occurs through the transcription factors Oct4 and Sox2, and signal pathway such as p38MAPK, PI3K/Akt and Wnt/β-catenin signaling. This occurs through treatment with All-trans-retinoic acid or miR-340 [23–25]. However, the molecular switch and signaling pathways that affect cell fate upon interaction between cells and collagen scaffolds remain to be addressed. Many challenges remain, including the promotion of neural regeneration on multiple levels and the probable hazards of long term usage of such novel biomaterials on humans [10].

In summary, our findings confirm that the collagen scaffold is highly biocompatible for supporting neurite outgrowth of NCSCs and suggests that the 80 μm porous collagen scaffold is an ideal biomaterial for engineered nerve tissue. This scaffold could be suitable for neuron stem cell therapy in the future.

Acknowledgments

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