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Optimization of Fibrin Scaffolds for Differentiation of Murine Embryonic Stem Cells into Neural Lineage Cells

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Abstract

The objective of this research was to determine the appropriate cell culture conditions for embryonic stem (ES) cell proliferation and differentiation in fibrin scaffolds by examining cell seeding density, location, and the optimal concentrations of fibrinogen, thrombin, and aprotinin (protease inhibitor). Mouse ES cells were induced to become neural progenitors by adding retinoic acid for 4 days to embryoid body (EB) cultures. For dissociated EBs, the optimal cell seeding density and location was determined to be 250,000 cells/cm^2 seeded on top of fibrin scaffolds. For intact EBs, three dimensional (3D) cultures with one EB per 400 μL fibrin scaffold resulted in greater cell proliferation and differentiation than two dimensional (2D) cultures. Optimal concentrations for scaffold polymerization were 10 mg/mL of fibrinogen and 2 NIH units/mL of thrombin. The optimal aprotinin concentration was determined to be 50 μg/mL for dissociated EBs (2D) and 5 μg/mL for intact EBs in 3D fibrin scaffolds. Additionally, after 14 days in 3D culture EBs differentiated into neurons and astrocytes as indicated by immunohistochemisty. These conditions provide an optimal fibrin scaffold for evaluating ES cell differentiation and proliferation in culture, and for use as a platform for neural tissue engineering applications, such as the treatment for spinal cord injury.

Keywords

nerve tissue engineering; cell spreading; cell culture; progenitor cells

1. Introduction

In the order to successfully engineer tissues using embryonic stem (ES) cells, three-dimensional (3D) biocompatible scaffolds must be developed that can provide a permissive environment for cell proliferation and differentiation. The environment surrounding ES cells plays a major determinant in selecting the fate of these cells and must be carefully designed to promote an optimal response by mimicking the extracellular cues present during development [1,2]. An ideal scaffold should contain sites for cell adhesion, as well as cues to promote cellular

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differentiation. A wide range of potential scaffolds for ES cell culture have been characterized for their ability to promote cell survival, proliferation, and differentiation [2-8].

These scaffolds consist of biocompatible polymers that serve as substrates for cell adhesion, proliferation and differentiation. For example, human ES cells can grow and differentiate into a variety of tissues when implanted in poly(lactic-*co*-glycolic acid)(PLGA)-poly(L-lactic acid) (PLLA) scaffolds and exposed to various cues [3]. The presence of neurotrophic factors induced the differentiation of human ES cells into neural structures when seeded on such scaffolds [2]. In a different study, Yim and Leong investigated the potential use of a cellulose acetate fibrous scaffold containing covalently immobilized nerve growth factor (NGF) for differentiation of ES cells into neurons [5]. These studies suggest that additional signals, such as growth factors, can be used with such scaffolds to achieve differentiation. Differentiation of murine neural stem cells into neurons has also been achieved using amphiphillic nanofibers functionalized with the laminin-derived peptide IKVAV as a scaffold [4]. Harrison *et al.* analyzed the effect of using scaffolds consisting of four different poly(α-hydroxy esters) on the viability of murine ES cells and found that PLGA provided the most suitable environment for ES cell colonization. They also observed that treatment of such polymer scaffolds with 0.1 M potassium hydroxide to improve the hydrophilicity significantly increased the survival of ES cells contained in such scaffolds, suggesting a relationship between hydrophilicity of the polymer surface and cell adhesion and proliferation [6].

A second possibility for tissue-engineered scaffolds involves the use of biologically-dervived proteins, such as collagen or fibrin, to encapsulate ES cells. Studies by Ma *et al.* showed that murine neural stem cells can form functional neuronal circuits when seeded onto 3D collagen scaffolds [7]. Additional studies explored the effects of the collagen matrix composition on murine ES cell differentiation. Specifically, collagen concentration affects the ability of embryoid bodies (EBs), aggregates of ES cells, to differentiate inside of the scaffold [8]. At high concentrations of collagen, the cells could not migrate and became apoptotic, indicating an optimal concentration of matrix for cell migration and cell-cell contact is required for stem cell survival and differentiation.

Fibrin has also been shown to provide a permissive environment for cell growth and can be covalently modified to incorporate growth factors [9] and other cellular cues [10,11]. Cardiac myoblasts have been shown to survive when injected in the myocardium with fibrin glue [12,13]. Additional studies have shown that fibrin-based scaffolds can be used to treat both central and peripheral nerve injury *in vivo* by promoting neural fiber sprouting [14-17]. The material properties of fibrin scaffolds can be modulated by altering the concentration of fibrinogen, Ca^{++} and thrombin present during polymerization. Another important consideration is the use of aprotinin [18], a plasmin inhibitor, as a means of slowing down the degradation of the fibrin scaffold. The concentrations of these components of fibrin scaffolds affect how cells proliferate, migrate, and differentiate inside fibrin [19-24]. Optimal fibrin scaffold conditions were previously determined *in vitro* for nerve cell culture using chick dorsal root ganglia (DRGs) [21,25], fibroblasts [19,20], and mesenchymal stem cells [22]. These results suggest fibrin scaffolds need to be optimized for each cell type to limit the amount of cell death and to facilitate cell adhesion and migration.

The overall goal of this research was to optimize fibrin scaffold conditions to promote the proliferation and differentiation of neural progenitor cells derived from ES cells, and to determine the appropriate cell seeding conditions. ES cells, induced with retinoic acid, were examined in different cell culture conditions to observe how the cells would grow and differentiate on fibrin scaffolds. Different factors, such as the concentration of fibrinogen, thrombin, and the presence of aprotinin, were also tested to determine their effect on ES cell migration, proliferation, and differentiation. The effect of two dimensional (2D, on top) versus

three dimensional (3D, inside) culture of ES cells on/within fibrin scaffolds was also explored. These studies create a framework for further investigation of the environmental cues necessary to form neural tissues using fibrin-based scaffolds.

2. Materials and Methods

2. 1 Stem Cell Culture Conditions

Undifferentiated Stem Cells—All studies were performed using RW4 and CE3 [26] (express green fluorescent protein (GFP) under a β-actin promoter) ES cell lines were grown in complete media consisting of Dubecco's modified eagle media (DMEM; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Invitrogen), 10% new born calf serum (NBCS; Invitrogen) and 0.3 M of each of the following nucleosides: adenosine, guanosine, cytosine, thymidine, and uridine (Sigma, Saint Louis, MO). The cell lines were used at a ratio of 10:1 (RW4: CE3), except in the fibrinogen degradation and Live/Dead experiments where only RW4 cells were used and the cell seeding experiments where only CE3 cells were used. Undifferentiated ES cells were cultured in the presence of 1000 U/mL of leukemia inhibitory factor (LIF, Chemicon, Temecula, CA) and 10^{-4} M β-mercaptoethanol (BME; Invitrogen) in T25 flasks (Fisher, Pittsbugh, PA) coated with 0.1% gelatin solution (Sigma). Cells were passaged at a ratio of 1:3 or 1:4 every 2 days.

Embryoid Body Formation—The undifferentiated RW4 and CE3 cells were induced to form neural progenitor containing EBs using the 4-/4+ retinoic acid treatment protocol as previously described [27]. ES cells were cultured in complete media without LIF and β-mercaptoethanol for 4 days in 100 mm Petri dishes (Fisher) coated with 0.1% agar (Midwest Scientific, Saint Louis, MO) to form EBs. During the next 4 days, the cells were exposed to 500 nM retinoic acid (Sigma) to induce neural differentiation. During this eight day process, the media was changed every other day.

For experiments involving liquid EB culture, 2 mL of 4-/4+ EB culture was placed in a 15 mL conical tube (Fisher) and allowed to settle. The media was aspirated and the EBs were resuspended in 20 mL of complete media. For the experiments testing different fibrinogen concentrations, 1 mL of this mixture was added to each scaffold. For all other experiments, individual EBs were selected using a pipettemen (total liquid volume: 5 μL) and placed on individual fibrin scaffolds.

Dissociation of Embryoid Bodies—4-/4+ EBs were removed from the agar coated Petri dishes, placed in a 15 mL conical tube, and allowed to settle. The media containing retinoic acid was aspirated and the EBs washed with DMEM containing 25 mM HEPES (Invitrogen). 5 mL of trypsin-EDTA (0.25%, Invitrogen) was added to the EBs, and the mixture was incubated at 37°C for 15 min to allow dissociation of the cells contained in the EBs. 5 mL of complete media was then added to quench the activity of the trypsin. Cells were isolated by centrifugation and resuspended in complete media. The resulting cells were counted and diluted in an appropriate amount of complete media before addition to the fibrin scaffolds. EBs placed in fibrin scaffolds were cultured in complete medium. Media was changed as indicated. All cultures were incubated at 37°C with 5% CO_2.

2.2 Preparation of Fibrin Scaffolds

Solutions were prepared by dissolving plasminogen-free fibrinogen obtained from pooled human plasma (Sigma) in distilled water followed by dialysis for 24 hours against 500 fold excess of tris buffered saline (TBS, pH 7.4) [10,11,28,29]. The resulting fibrinogen solution was then sterile filtered and diluted to the appropriate concentrations. Fibrin scaffolds (total volume: 400 μL) were made by combining fibrinogen at a concentration of 10 mg/mL, 2.5 mM

$CaCl_2$, and 2 NIH units/mL of thrombin (all from Sigma except where indicated). Six different concentrations of fibrinogen were tested: 4, 6, 8, 9, 10 and 12.8 mg/mL. Fibrinogen labeled with AlexaFluor 488 (Invitrogen) was incorporated into each scaffold at a final concentration of 0.156 mg/mL in the quantitative fibrin degradation studies.

For the qualitative fibrinogen concentration studies, four scaffolds at each concentration were polymerized into 24 well plates (2 cm^2/well) and 1 mL of liquid EB culture was added to the top of each scaffold. The scaffolds were observed for 3 days. For the quantitative fibrinogen concentration studies, four scaffolds were prepared at the following concentrations: 4, 8 and 12.8 mg/mL. For both the quantitative and qualitative observation studies, the media was not changed during the course of the study. The amount of degradation was then quantified using a fluorescence-based assay described below.

For cell seeding density experiments, cells were seeded onto the fibrin scaffolds and observed for 3 days to determine how the number of cells affected scaffold degradation and cell differentiation. For this experiment, the media was changed on day 2. After 3 days of culture, cell viability was assessed as described below.

Two different concentrations of thrombin (2 and 4 NIH U/mL) were also tested while holding the fibrinogen concentration constant at 10 mg/mL. Four scaffolds were polymerized at each concentration and the scaffolds were seeded with dissociated or intact EBs (2 scaffolds for each type of cells). The scaffolds were observed for 4 days.

For experiments involving varying the aprotinin (Sigma) concentration, aprotinin was added to the media at the indicated concentrations. Scaffolds treated with aprotinin were observed for 8 days after cell seeding with cell viability being assessed on Day 8.

For the immunohistochemistry experiments, EBs were seeded within fibrin scaffolds in 3D culture. After 3 days, the media was changed to neuralbasal media (Invitrogen) containing B27 supplement (Invitrogen) diluted 1:50. All fibrin scaffolds were polymerized in individual wells of 24 well plates (15.6 mm diameter, Corning, Corning, N.Y.). The scaffolds were then incubated for 1 hr at 37°C, 95% relative humidity and 5% CO_2. For 3D cultures, the bottom layer of the scaffold (300 μL) was polymerized first and allowed to incubate at 37°C for 1 hr. The cells were then added on to the scaffold and a second layer of fibrin (100 μL) was polymerized on top of the cells at 37°C for 1 hr, followed by the addition of 1 mL of complete media to each well.

2.3 Quantification of Scaffold Degradation Using Fluorescently Labeled Fibrinogen

To quantify the amount of fibrin scaffold degradation occurring during the cell culture process, fluorescently-labeled fibrinogen was incorporated into each scaffold at final concentration of 0.3125 mg/mL. 1 mL of complete media with phenol red free DMEM (Invitrogen) was added to each scaffold. The media was collected at the end of the three day experiment. The conditioned media was stored at -20°C until analysis was performed. At the end of the 3 day experiments, fibrin scaffolds were degraded enzymatically using 1 unit of plasminogen activated with urokinase as previously described [15,30]. After the scaffolds were completely degraded, the resulting solution was centrifuged to remove the cells and the supernatant collected for analysis. 200 μL of each sample was applied to a 96 well plate (Corning) and the intensity of each sample was determined using a CytoFluor Series 4000 multiwell plate reader (Applied Biosystems, Foster City, CA) and the amount of labeled fibrinogenwas quantified using a standard curve of known labeled fibrinogen concentrations. This method has been previously used to determine the rate of fibrin degradation both in the presence and absence of cells [31].

2.4 Immunohistochemistry Analysis of Cell Phenotype inside of Fibrin Scaffolds

To confirm ES cell differentiation, immunohistochemistry was performed on EBs after 14 days of culture inside of the fibrin scaffolds. The media was removed from each scaffold and each well rinsed with 1 mL of phosphate buffered saline (PBS, pH 7.4). The cells were fixed inside of the scaffold with 3.7% formaldehyde solution for 1 hour at room temperature. The cells were permeabilized by the addition of 0.1% Triton-X diluted in PBS for 45 minutes. The cells were blocked using 5% goat serum (Invitrogen) diluted in PBS for 5 hours and primary antibody for beta III tubulin (Tuj1, early neuronal marker, mouse IgG, 1:500, Chemicon) or glial fibrillary acidic protein (GFAP, astrocyte marker, rabbit IgG, 1:4, Convance) was applied overnight at 4°C. The next day each well was subjected to three 15 minutes washes with PBS. Secondary antibody (AlexaFluor 555, antimouse IgG, 1:200, Invitrogen or AlexaFluor 488, goat anti-rabbit IgG, 1:200) was then applied for 5 hours. The wells were again washed thoroughly and imaged as described below.

2.5 Assessment of Cell Viability using Live/Dead Cytotoxicity Assay

To assess the cell viability after culture on and inside of fibrinogen scaffolds, a Live/Dead viability/cytotoxicity kit (Invitrogen) was used. Each well was washed with 0.5 mL of PBS (pH 7.4) and then incubated with 1 mL of PBS containing 2 μM Calcein AM and 4 μM ethidium homodimer for 30 minutes at 37°C. Calcein AM is converted to fluorescent calcein by the intracellular esterase activity found in live cells. Ethidium homodimer can penetrate damaged cell membranes of dead cells, where it binds to DNA, which causes a 40-fold enhancement of red fluorescence. For dissociated cell cultures in 2D, representative photos were taken of each well and the number of viable cells was counted. For 3D culture, fluorescent images were taken to qualitatively assess the cell viability of culture.

2.6 Phase Contrast and Fluorescence Microscopy

For Figures 1 through 5, photographs were taken on a Nikon Eclipse TE2000 inverted fluorescent microscope using QCapture software to capture images. For Figure 6 and 7, fluorescent images of the cells cultured in fibrin scaffolds were taken using a CCD camera (MagniFire, Olympus, Melville, N.Y.) attached to an Olympus IX70 microscope (Olympus). The images were analyzed on Adobe Photoshop v 4.0. The photos in Figure 1, 2 and 4 were taken with the 20x objective. The photos in Figure 3 were taken with the 40x objective. The photos in Figure 5A-D were taken with the 20x objective and the photos in Figure 5E-F were taken with 10x objective. The photos in Figure 6A-D were taken with the 2x objective and the photos in Figure 6E-H were taken with the 4x objective. The photos in Figure 7 were taken with the 10x objective.

Cell viability was determined by observing cell morphology as described in the following scale: - = dead cells can be seen floating in media, + = few cells are alive, but fluoresce weakly, ++ = many cells are alive and some bright fluorescent cells are observed, and +++ = the majority of cells appear to be healthy and strong fluorescence is observed. The scoring scale for cell infiltration and spreading is as follows: - = cells remain on top of the scaffold and display rounded phenotype, + = slight cell spreading is observed, but scaffold is not infiltrated, ++ = some cells have entered scaffold and spreading is observed, and +++ = the majority of the cells have infiltrated the scaffold and spreading is observed. The cell cultures were also scored based on their differentiation state. The following scale was used: - = cells remain undifferentiated along with rounded phenotype is observed, + = few cells have begun to change shape and extend neurite like protrusions, ++ = some cells have begun to change shape and extend neurite like protrusions, and +++ = the majority of the cells have begun to change shape and extend neurite like protrusions.

3. Results and Discussion

3.1 Determining optimal concentrations of fibrinogen and thrombin for ES cell viability and differentiation

To assess their effect on ES cell growth and differentiation in fibrin scaffolds, various concentrations of fibrinogen and thrombin were tested. Six different fibrinogen concentrations seeded with EBs in 2D culture were evaluated over a 3 day time course. The range of fibrin concentrations tested was selected with the lowest concentration (4 mg/mL) being optimal for the culture of neurons [28-30,32] and the upper range selected based on the literature on stem cell seeded in fibrin [33] and the solubility limit of fibrinogen (based on supplier) in solution. When both CE3 and RW4 cells were used, only the scaffolds made from 10 and 12.8 mg/mL of fibrinogen remained at day 3 and scaffolds at these concentrations showed similar levels of degradation. Scaffolds made from lower concentrations were almost completely degraded by day 3 and the cells began to differentiate in the bottom of the well as opposed to inside of the scaffold. The time course of scaffold degradation induced by cells migrating out from the EBs is shown in Figure 1 for scaffolds made with 10 mg/mL of fibrinogen. The cells began to infiltrate and degrade the scaffold by day 2. Since scaffolds made at these two concentrations (10 and 12.8 mg/mL) showed similar levels of degradation, 10 mg/mL of fibrinogen, which is easier to prepare, was selected for use in the remaining studies.

Quantitative analysis of scaffold degradation also supported these observations as seen in Figure 1D. The rate of scaffold degradation was reduced for all concentrations tested when only RW4 cells were used. The CE3 cell line divides more rapidly than the RW4 cell line and these differences may be responsible for this observation. The scaffolds made from 12.8 mg/mL of fibrinogen degraded more slowly than the scaffolds made from other concentrations. The scaffolds made from the lowest concentration (4 mg/mL) showed the fastest degradation rate when compared to the other 2 concentrations, consistent with the previous qualitative observations. Control scaffolds without cells present retained 95% of the fibrinogen initially present.

The effect of changing the thrombin concentration in the fibrin scaffold polymerization mixture on ES cell adhesion and growth on the scaffold was also examined. It was hypothesized that increasing the thrombin concentration would result in a more highly cross-linked fibrin scaffold, which would degrade more slowly [19]. To test this hypothesis, two different thrombin concentrations were evaluated: 2 and 4 NIH U/mL. Higher thrombin concentrations have been shown to inhibit cell migration inside of the fibrin scaffolds [34] and recent studies have shown that varying thrombin concentration has a lesser effect on cell behavior than altering the fibrinogen concentration [35,36]. Fibrin scaffolds were polymerized at these two thrombin concentrations and seeded with two types of cells in 2D culture. One set of scaffolds was seeded with 500,000 cells (250,000 cells/cm^2) from dissociated EBs and a second set of scaffolds was seeded with one intact embryoid body on each scaffold. ES cell growth and differentiation was monitored for 4 days and the results are shown in Figure 2. There was no difference observed in cell behavior and scaffold degradation when using a higher thrombin concentration. Since the higher concentration did not offer any additional benefit and because this concentration has been previously used for DRG cultures, 2 NIH U/mL was used for the remaining studies.

3.2 Determining the optimal cell seeding number when using dissociated embryoid bodies on fibrin scaffolds in two dimensional culture

Five different cell seeding densities were tested for dissociated CE3 EB culture to determine the optimal number of cells to be seeded onto fibrin scaffolds in 2D culture. The effect of cell seeding density on scaffold degradation and cell differentiation is listed in Table 1. At lower

cell concentrations ranging from 47,875 to 187,500 cells per well (23,938 - 93,750 cells/cm^2), the cell viability was lower and less proliferation and differentiation was observed. The cells also did not appear to degrade the fibrin scaffold significantly. At the higher concentrations, more cell growth and differentiation was observed. The effect of cell density on cell differentiation is shown in Figure 3. Undifferentiated cells were observed to fluoresce and only produced small extensions at lower cell seeding densities (Figure 3A and B). Extensive differentiation and neurite extension was observed at the higher seeding densities (Figure 3C and D). At a seeding density of 750,000 cells per well (375,000 cells/cm^2), no fibrin scaffold remained after 3 days. However, fibrin scaffold did remain when cells were seeded at 375,000 cells per well (187,500 cells/cm^2). Additionally, quantitative cell viability data (Figure 3E) showed that cells seeded at 750,000 per well had increased viability when compared to lower seeding densities. Due to the low number of cells seeded, no quantitative data could be obtained for the lowest two cell seeding concentrations. From these results, the optimal seeding density was determined to be approximately 500,000 cells per well (250,000 cells/cm^2) in 2D culture.

3.3 Testing the effect of two dimensional versus three dimensional cultures on both intact and dissociated embryoid bodies on cell viability and differentiation

To determine whether dissociated or intact EBs should be used for 3D studies, 2D and 3D culture systems were tested with both cell preparations. In 2D cultures, cells were seeded on top of the fibrin scaffold as done previously. For 3D culture studies, the cells were placed on top of a 300 μL scaffold and an additional layer (100 μL) of fibrin was added after cell seeding. The goal was to determine which cell culture method produced the greatest cell viability and differentiation in the 3D culture system. For all studies using dissociated EBs, scaffolds were seeded with 500,000 cells per well.

The effect of 2D versus 3D cultures on cell growth and differentiation is shown in Figure 4. Some of the dissociated cells reformed EB-like aggregates (Figure 4A and B) on top of the fibrin scaffold in 2D cultures, and cells contained inside of these aggregates began to differentiate. The cells not contained in the aggregates showed little to no differentiation. When seeded inside of the scaffold (Figure 4C and D), the dissociated cells show poor viability and do not form the EB-like structures observed in two dimensional culture. These results suggest that dissociation of the EBs does not seem to be an effective method of culturing stem cells in 3D scaffolds, which would be most useful for *in vivo* applications.

For the intact EB cultures (one intact EB per well), the cells proliferated and differentiated to a greater extent inside of the 3D fibrin scaffolds (Figure 4E and F) when compared to 2D cultures (Figure 4G and H). By the three days post seeding, cells from the intact EBs embedded in the scaffold were touching the bottom of the well, showing that fibrin scaffold allowed for cell migration out from the EB. Comparatively, the intact EBs seeded on top of the fibrin scaffold showed little cell migration into the scaffold. The intact EBs embedded inside of the fibrin scaffold seemed to have good viability and the cells in the EBs were able to differentiate. The dissociated EBs grew optimally when seeded on top of the scaffolds, not inside of the scaffold. However, such 2D cultures do not translate well to *in vivo* applications.

3.4 Testing the effect of varying aprotinin concentration on cell viability and differentiation

During the previous studies, a high rate of fibrin scaffold degradation was observed for some culture conditions. To slow the rate of fibrin degradation, scaffolds were cultured with various concentrations of aprotinin, a plasmin inhibitor, in the media. A balance between allowing localized matrix degradation to permit cell migration and spreading, but preventing global scaffold degradation in less than 4 days was required Varying aprotinin concentrations were tested for dissociated and intact EBs in 2D and 3D cultures. An 8 day time course was analyzed

using concentrations of aprotinin ranging from 0 to 50 μg/mL. The observations made for the dissociated EBs cultured inside of fibrin scaffolds over the course of the experiment are contained in Table 2. This study showed that the optimal aprotinin concentration for dissociated EBs embedded inside of a fibrin scaffold was 50 μg/mL. The differentiation of the stem cells inside scaffolds with this concentration of aprotinin is shown in Figures 5A-D. The cells extended protrusions towards other cells, indicating differentiation. At this concentration, the cell could migrate into the scaffold by locally degrading the matrix while the overall integrity of the scaffold remained intact for 8 days. Cell viability for these conditions was also assessed using Live/Dead staining. As seen in Figure 6E-H, cells cultured in 50 μg/mL aprotinin showed increased viability when compared to cultures without aprotinin.

For the intact EBs, the optimal aprotinin concentration in the media was determined to be 5 μg/mL (Table 3). The extent of cell differentiation and migration out from the EB at days 5 and 8 is shown in Figure 5E, F, G and H. The cells extended neurite-like protrusions, suggesting differentiation into neurons. Additionally, Live/Dead staining, as seen in Figure 6A-D, shows increased cell viability when aprotinin is present in the media. The optimal aprotinin concentrations are different for the two types of ES cell preparation (dissociated versus intact EBs). This result suggests that the dissociated EBs digest the scaffold at a more rapid rate, potentially due to the greater surface area in contact with the fibrin. For both preparations, some of the scaffold remains after 8 days of culture with the optimal aprotinin concentration, which was a vast improvement over fibrin scaffolds without aprotinin which degrade completely in less than 4 days. Preventing degradation of the fibrin scaffold allows a longer time frame for cell growth and differentiation and allows the ES cells to secrete their own extracellular matrix.

3.5 Immunohistochemistry analysis of EBs seeded inside of three dimensional fibrin scaffolds at 14 days

The differentiation state of EBs in 3D fibrin scaffolds over 14 day was assessed using immunohistochemistry. For this study, the media was changed after 3 days to neural basal media containing B27 supplement. This media change resulted in less scaffold degradation and increased cell differentiation (data not shown). At the end of the study, fibrin scaffold still remained in each well. To confirm that the ES cells were differentiating into neural phenotypes, EBs were stained for the beta tubulin III (Tuj1, early neuron marker) and glial fibrillary acidic protein (GFAP, astrocyte marker) after 14 days of culture inside of fibrin scaffolds, Figure 7A shows the results of the Tuj1 staining. The staining is localized to cells which display a clear neuronal phenotype, indicated by the cell body with extending, axon-like protrusions. Figure 7B shows the results of staining for GFAP. These cells show an astrocytic phenotype characterized by their star-shaped structure. These results suggest that culture of EBs inside of fibrin scaffolds can produce cells with different mature phenotypes, such as neurons and astrocytes. These results also show that seeding EBs into fibrin scaffolds could provide a method of generating neural cells to replace those lost to SCI.

4. Conclusions

This study provides insight in the factors affecting ES cell growth and differentiation when seeded on top of and inside fibrin scaffolds. For the culture of neural lineage progenitor cells derived from EBs, the optimal fibrinogen and thrombin concentrations were determined for fibrin scaffold polymerization. It was also determined that aprotinin, a plasmin inhibitor, must be added to the media to prevent rapid degradation of the scaffold by the ES cells. The optimal concentration of aprotinin was determined for both dissociated and intact EBs in a 3D culture system. The best cell preparation method for optimal ES cell growth and differentiation in 3D cultures was found to be the use of a single intact EB embedded inside of a fibrin scaffold. After 14 days of culture inside of fibrin scaffolds, these cells were found to differentiate into

neurons and astrocytes. The use of cells from dissociated EBs was also investigated, but these cells did not migrate and differentiate well in 3D culture, which is most similar to the 3D environment present following *in vivo* transplantation. Overall, this study presents a method for culture of ES cells inside of a fibrin scaffold that can be potentially be useful for tissue engineering applications, such as a treatment for spinal cord injury because neurotrophin-3 delivery from fibrin scaffolds has already shown promise by promoting neural fiber sprouting following injury [15,17]. In such a system, the ES cells could provide a way of repopulating neurons that were lost during the injury or replacing lost glial cells. Other potential tissue applications could involve the use of fibrin scaffolds containing ES cells to treat defects in cardiac or bone injuries.

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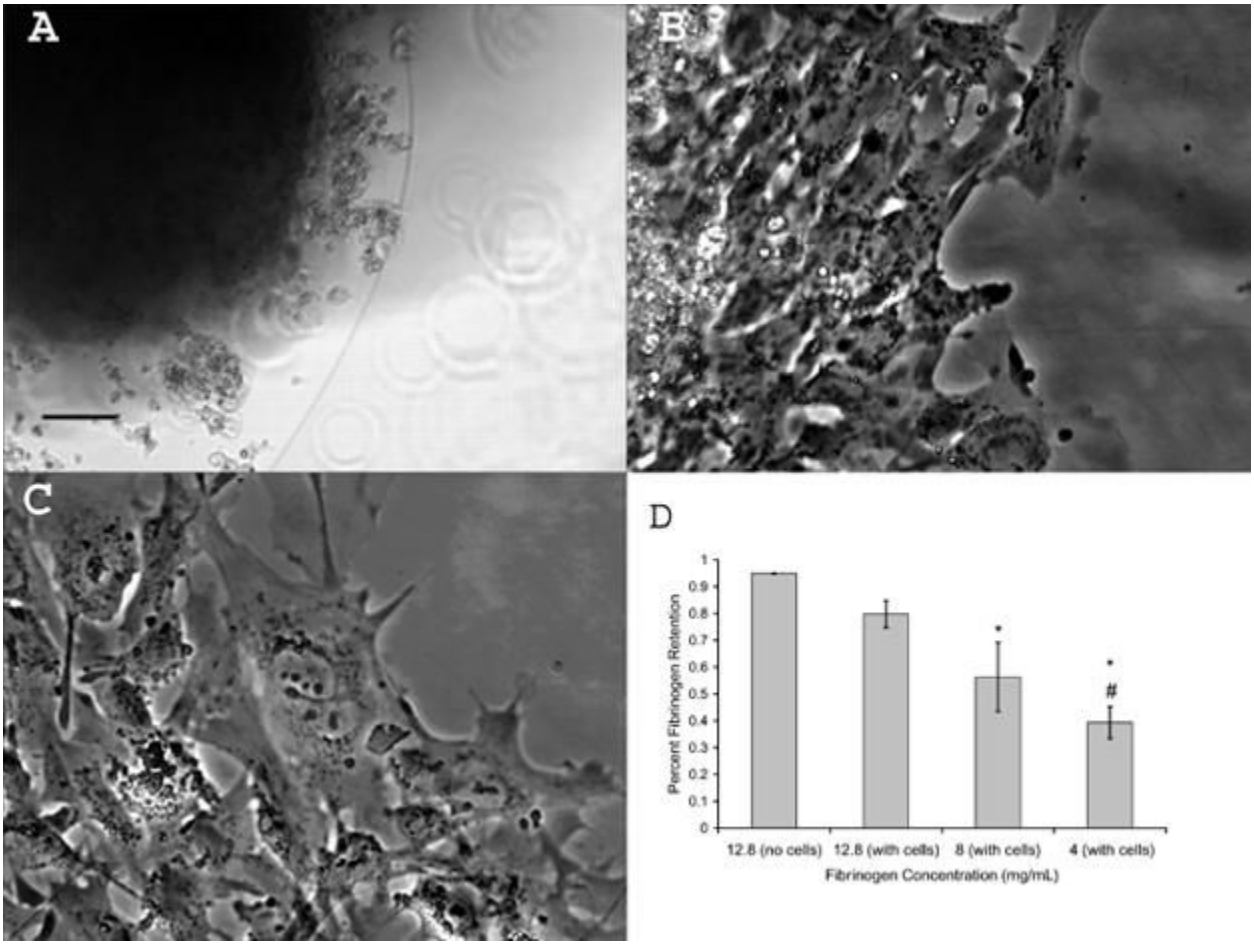

Figure 1.

Time course of fibrin scaffold degradation for 2D EB culture. A) On day one after seeding scaffold with EBs, a halo has formed surrounding the EBs indicating scaffold degradation. B) On day two, cells from the EBs have begun to migrate and infiltrate the scaffold. C) On day three, increased cell migration in the scaffold was observed. Fibrin scaffold was prepared with 10 mg/mL fibrinogen and 2 NIH U/mL of thrombin. Scale bar indicates 500 microns. D) Quantitative analysis of fibrin scaffold degradation as determined by the fluorescence based assay. The scaffolds containing 12.8 mg/mL of fibrin showed the least amount of degradation after 3 days in the presence and absence of cells. Error bars indicate standard deviation. * indicates $p<0.05$ versus scaffolds polymerized with 12.8 mg/mL both with and without cells present. # indicates $p<0.05$ versus scaffolds polymerized with 8 mg/mL with cells present.

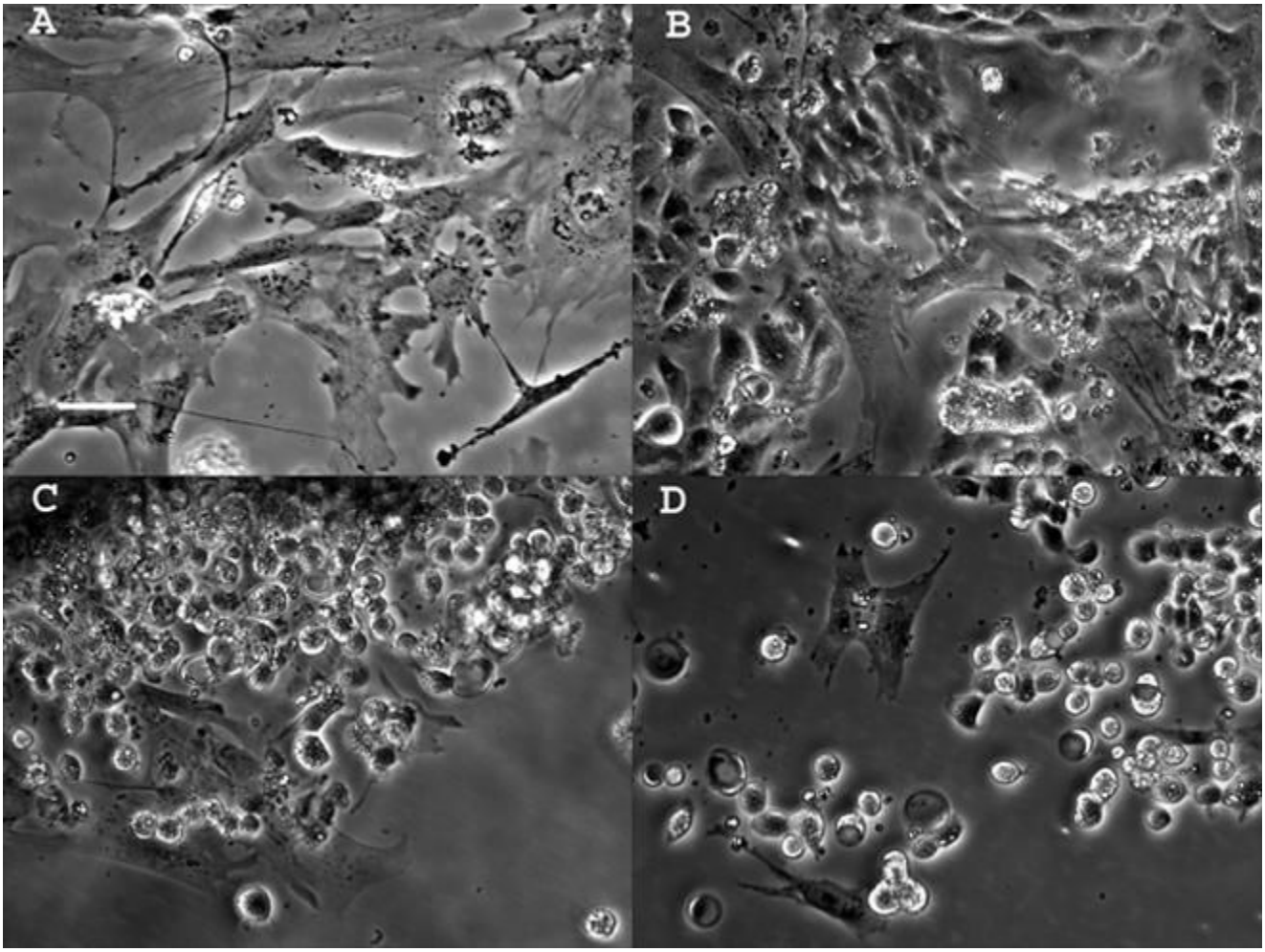

Figure 2.
Effect of thrombin concentration on dissociated and intact EBs in 2D cultures. Images were taken four days after scaffold seeding. A) 2 NIH U/mL thrombin fibrin scaffold containing dissociated EBs. B) 4 NIH U/mL thrombin fibrin scaffold containing dissociated EBs. C) 2 NIH U/mL thrombin fibrin scaffold containing an intact EB. D) 4 NIH U/mL thrombin fibrin scaffold containing an intact EB. Scale bar indicates 500 microns.

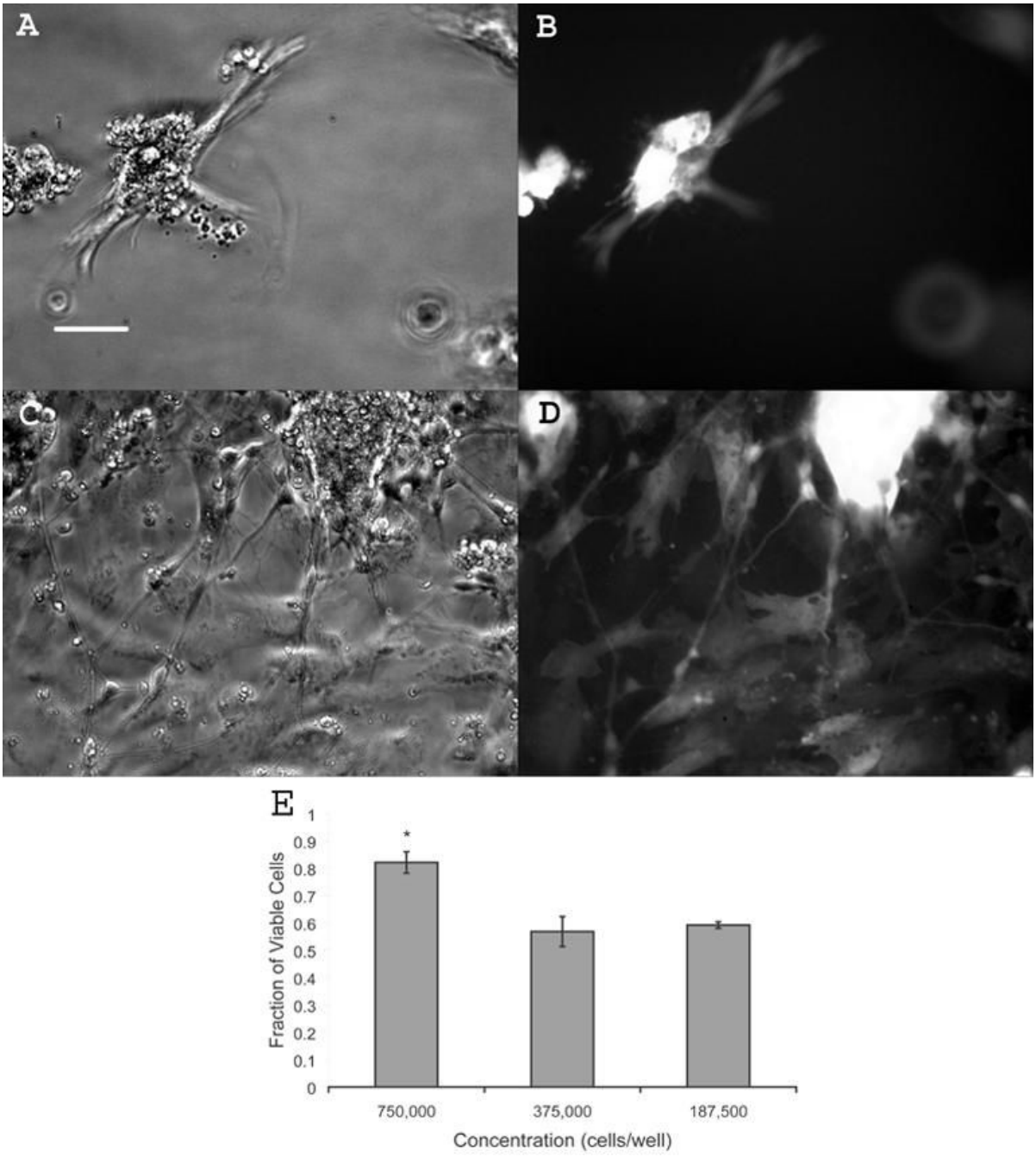

Figure 3.

Results of FACS analysis on the dose response studies of EBs cultured in fibrin scaffolds for 14 days in the presence of individual growth factors at the following concentrations: 2, 10, and 25 ng/mL. A) EBs seeded into fibrin scaffolds after 14 d of culture in the presence of NT-3. B) EBs seeded into fibrin scaffolds after 14 d of culture in the presence of bFGF. C) EBs seeded into fibrin scaffolds after 14 d of culture in the presence of PDGF. D) Results of FACS analysis performed on EBs seeded into fibrin scaffolds after 14 d of culture in the presence of CNTF. E) Results of FACS analysis performed on EBs seeded into fibrin scaffolds after 14 d of culture in the presence of Shh. The markers examined were SSEA-1 (undifferentiated mouse ES cells), nestin (neural precursors), Tuj1 (neurons), O4 (oligodendrocytes), and GFAP (astrocytes). *

indicated $p < 0.05$ for that marker compared to EBs cultured in fibrin for 14 days with no growth factors present.

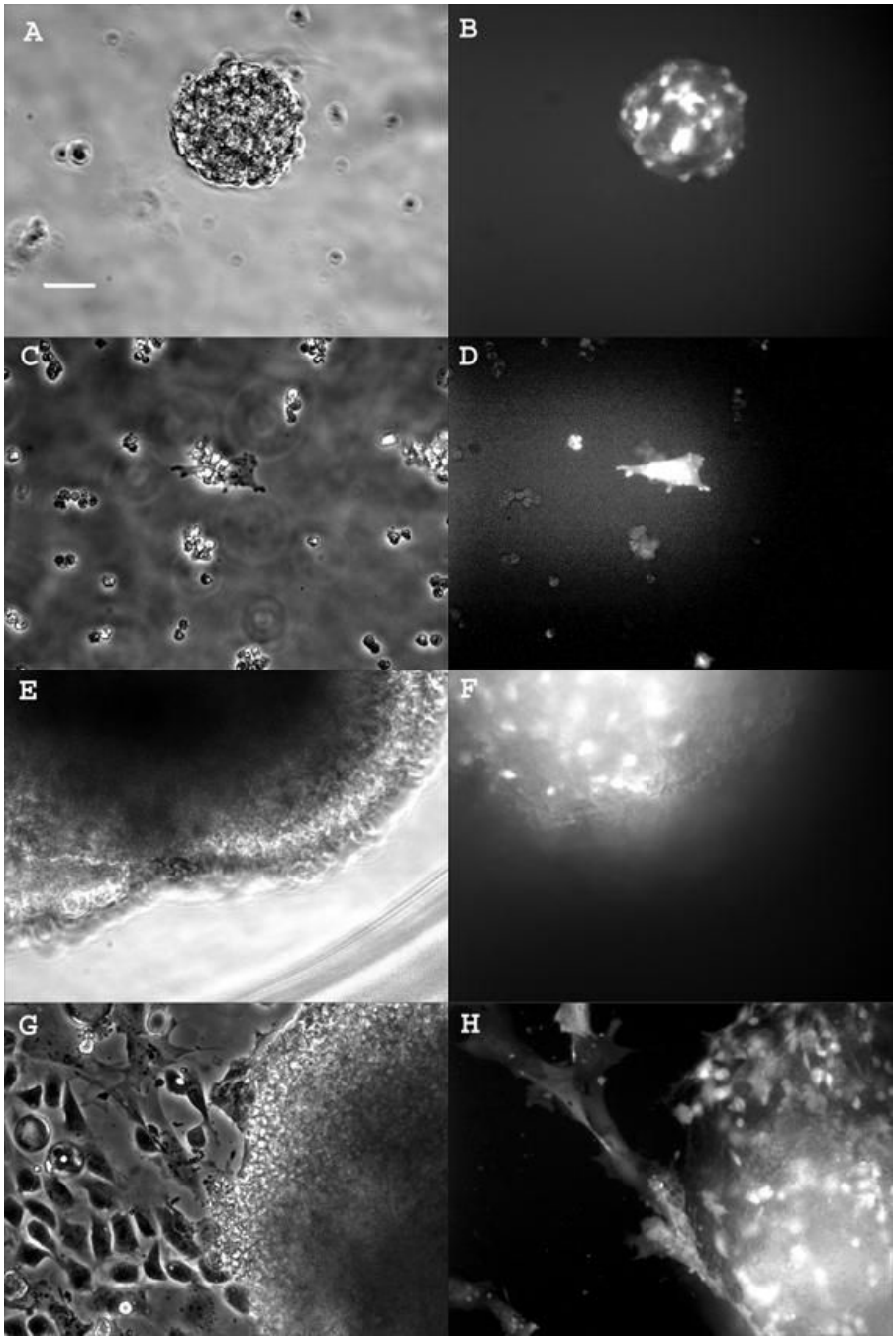

Figure 4.
ES cell growth and differentiation in 2D versus 3D culture. Images were taken at day 3. A) Dissociated EBs seeded on top of fibrin scaffold (2-D). Cells are forming EB like structures. B) Fluorescent image of A. C) Dissociated EBs seeded inside of the fibrin scaffold (3-D). Cells are not differentiating. D) Fluorescent image of C. E) Intact EB seeded on top of the fibrin scaffold (2-D). Cells are not migrating out from the EB. F) Fluorescent image of E. G) Intact EB seeded inside of the fibrin scaffold (3-D). Cells appear to be healthy, differentiating, and migrating. H) Fluorescent image of G. Scale bar indicates 500 microns.

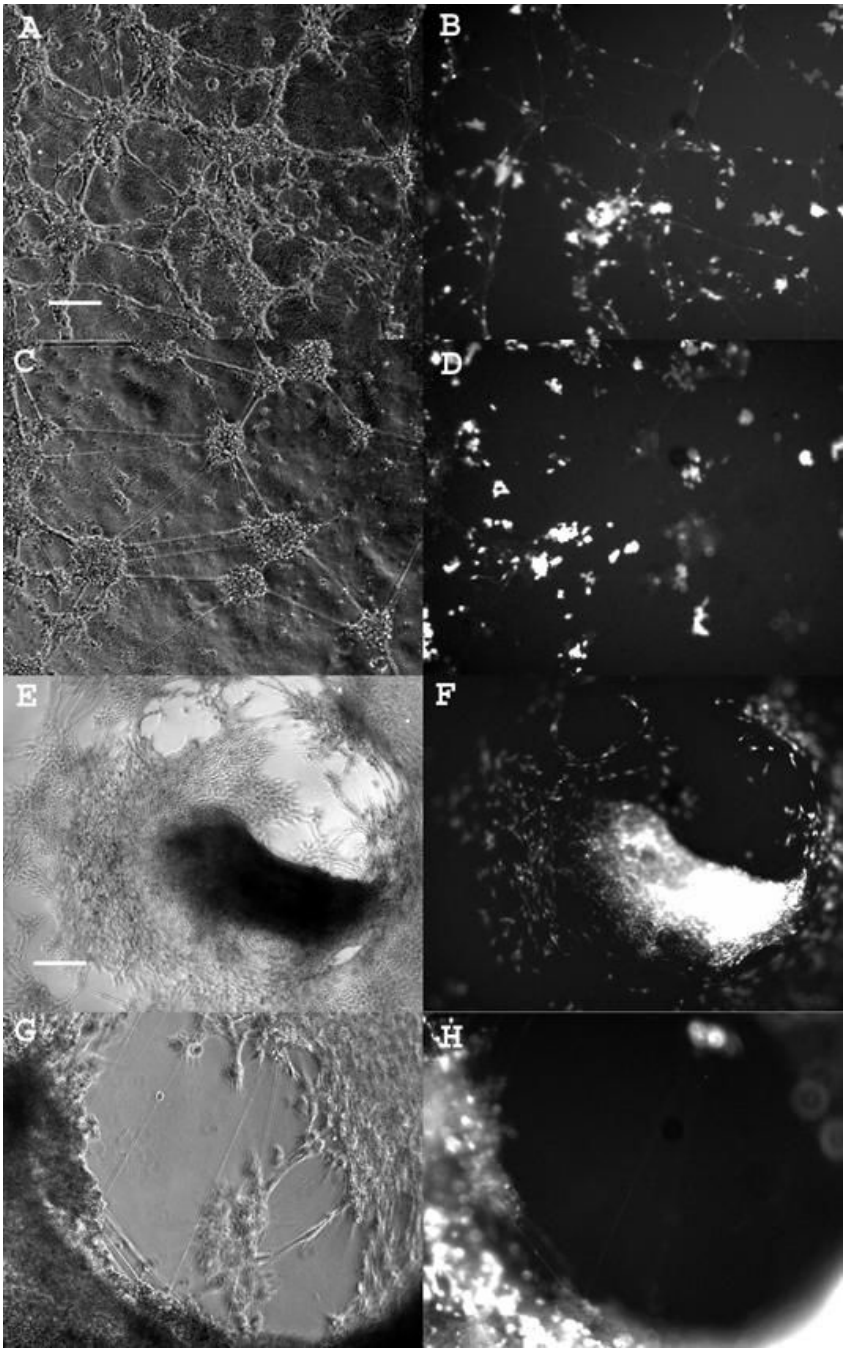

Figure 5.
ES cell differentiation at the optimal aprotinin concentration for each type of cell culture A) Dissociated EBs grown inside of a fibrin scaffold with 50 μg/mL of aprotinin present at Day 5. B) Fluorescent image of A. C) Dissociated EBs with the same scaffold conditions at Day 8 D) Fluorescent image of C. E) Intact EBs grown inside of a fibrin scaffold with 5 μg/mL of aprotinin present at Day 5. F) Fluorescent image of E. G) Intact EBs with the same scaffold conditions at Day 8. H) Fluorescent image of G. Scale bars indicate 500 and 1000 microns respectively.

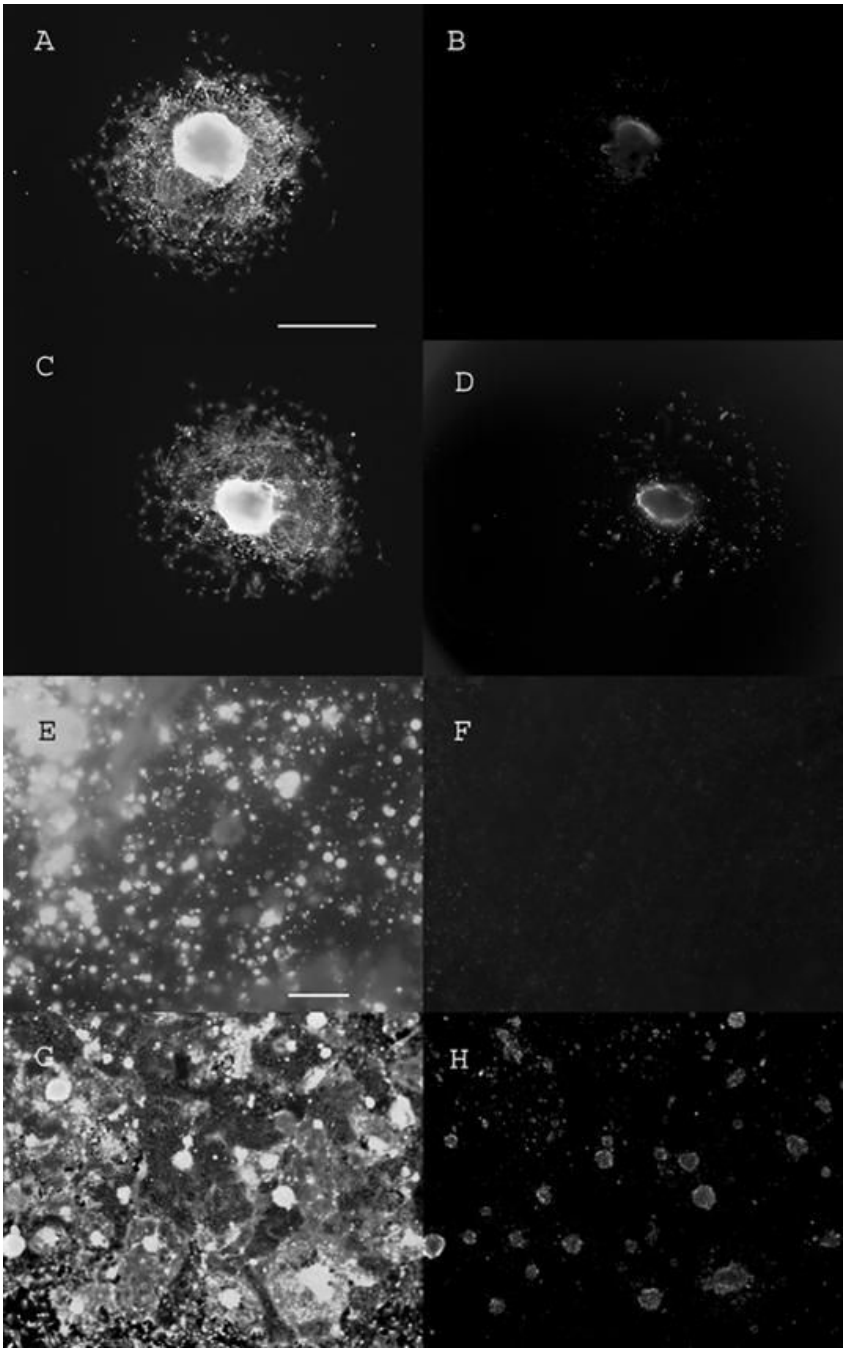

Figure 6.

Cell viability of intact and dissociated EBs in 3D culture as determined by Live/Dead Staining. A) Live cells present in an intact EB surrounded by 3D fibrin scaffold cultured with 5 μg/mL of aprotinin in the media on Day 8. Scale bar indicated 10 millimeters. B) Dead cells present an intact EB surrounded by 3D fibrin scaffold cultured with 5 μg/mL of aprotinin in the media on Day 8. C) Live cells present in an intact EB surrounded by 3D fibrin scaffold cultured with no aprotinin in the media on Day 8. D) Dead cells present in an intact EB surrounded by 3D fibrin scaffold cultured with no aprotinin in the media on Day 8. E) Live cells present in dissociated EBs inside of a 3D fibrin scaffold cultured with 50 μg/mL of aprotinin in the media on Day 8. Scale bar equals 3 millimeters. F) Dead cells present in dissociated EBs inside of a

3D fibrin scaffold cultured with 50 μg/mL of aprotinin in the media on Day 8. G) Live cells present in dissociated EBs inside of a 3D fibrin scaffold cultured with no aprotinin in the media on Day 8. H) Dead cells present in dissociated EBs inside of a 3D fibrin scaffold cultured with no aprotinin in the media on Day 8.

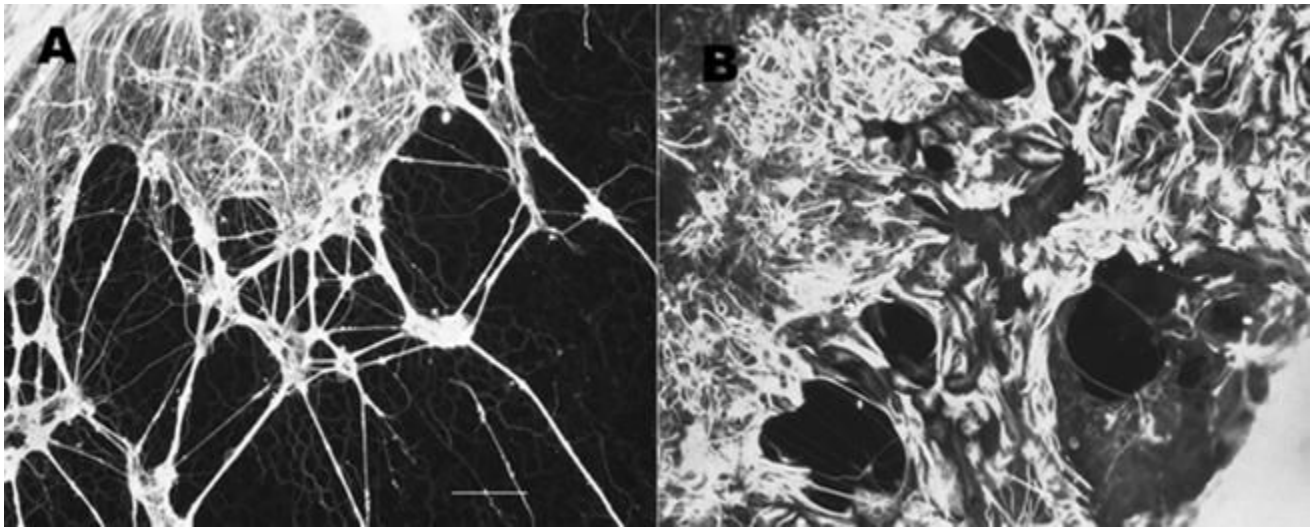

Figure 7.
Immunohistochemistry performed on EBs after 14 days of culture inside of 3D fibrin scaffolds. A) Cells which have migrated out from the EBs stained positive for Tuj1 and possess axon-like processes. B) Cells which have migrated out from the EBs stained positive for GFAP and have the star-like morphology of astrocytes. No double staining of cells was observed. Scale bar indicate 1000 microns.

Table One

Testing the Effect of Cell Density on Scaffold Degradation and Cell Proliferation and Differentiation

Concentration ells seeded per well)		Scaffold Degradation	Cell Viability/Survival	Cell Infiltration and Spreading	Differentiation
0,000	Day 1	++	++	+	-
	Day 2	++	+++	++	+
	Day 3	+++	++	+++	++
5,000	Day 1	+	++	+	-
	Day 2	+	+++	++	+
	Day 3	++	+/-	+++	++
7,500	Day 1	-	+	+	-
	Day 2	+	++	+	-
	Day 3	+	+	++	+
8,750	Day 1	-	+	-	-
	Day 2	+	+	-	-
	Day 3	+	-	-	-
7,875	Day 1	-	+	-	-
	Day 2	-	+/-	-	-
	Day 3	+	+/-	-	-

Scoring Scale for Scaffold Degradation:- = No scaffold degradation was observed; += Less than 50% of the scaffold has been degraded;++ = More than 50% of the scaffold has been degraded; +++ = No scaffold remains

Scoring Scale for Cell Viability and Survival:-: Dead cells can be seen floating in media; + = Few cells are alive, but fluoresce weakly; ++ = Many cells are alive and some bright fluorescent cells are observed; +++ = The majority of cells appear to be healthy and strong fluorescence is observed.

Scoring Scale for Cell Infiltration and Spreading:- = Cells remain on top of the scaffold and display rounded phenotype; + = Slight cell spreading is observed, but scaffold is not infiltrated; ++ = Some cells have entered scaffold and spreading is observed; +++ = The majority of the cells have infiltrated the scaffold and spreading is observed.

Scoring Scale for Differentiation:- = Cells remain undifferentiated. Rounded phenotype is observed; + = Few cells have begun to change shape and extend neurite like protrusions; ++ = Some cells have begun to change shape and extend neurite like protrusions; +++ = The majority of the cells have begun to change shape and extend neurite like protrusions.

Table Two

Determining Optimal Aprotinin Concentration for Dissociated Embryoid Body Differentiation

Concentration (μg/mL)		Scaffold Degradation	Cell Viability/Survival	Cell Infiltration and Spreading	Differentiation
50	Day 1	-	++	++	++
	Day 2	-	++	++	++
	Day 3	-	+++	++	++
	Day 4	+	+++	+++	+++
	Day 5	+	+++	+++	+++
	Day 6	+	+++	+++	+++
	Day 7	+	++	+++	+++
	Day 8	+	++/-	+++	+++
5	Day 1	-	++	+	-
	Day 2	-	++	++	+
	Day 3	+	+++	++	+
	Day 4	+	+++	++	++
	Day 5	++	+++	+++	++
	Day 6	++	+++	+++	+++
	Day 7	++	++	+++	+++
	Day 8	++	++	+++	+++
0.5	Day 1	-	++	-	-
	Day 2	+	++	-	-
	Day 3	++	++	+	+
	Day 4	++	++	+	+
	Day 5	++	++	++	++
	Day 6	+++	++	++	++
	Day 7	+++	++	+++	+++
	Day 8	+++	++	+++	+++
0.05	Day 1	+	++	-	-
	Day 2	++	++	-	-
	Day 3	++	++	-	+
	Day 4	++	++	+	+
	Day 5	+++	++	++	++
	Day 6	+++	++	++	++
	Day 7	+++	++	++	+++
	Day 8	+++	++	++	+++
0	Day 1	+	++	-	-
	Day 2	++	++	-	-
	Day 3	++	++	-	+
	Day 4	+++	++	+	+
	Day 5	+++	++	++	++
	Day 6	+++	++	++	++
	Day 7	+++	++	++	+++
	Day 8	+++	++	++	+++

Table Three

Determining Optimal Aprotinin Concentration for Intact Embryoid Body Differentiation

Concentration (μg/mL)		Scaffold Degradation	Cell Viability/Survival	Cell Infiltration and Spreading	Differentiation
50	Day 1	-	++	-	-
	Day 2	-	++	+	+
	Day 3	+	++	+	+
	Day 4	+	++	++	++
	Day 5	+	++	++	++
	Day 6	++	+/-	++	++
	Day 7	++	+/-	+++	++
	Day 8	++	+/-	+++	++
5	Day 1	-	++	+	+
	Day 2	-	++	++	++
	Day 3	+	+++	+++	++
	Day 4	+	+++	+++	++
	Day 5	+	+++	+++	++
	Day 6	++	+++	+++	+++
	Day 7	++	++	+++	+++
	Day 8	++	++	+++	+++
0.5	Day 1	+	++	-	-
	Day 2	+	++	+	-
	Day 3	++	++	+	-
	Day 4	++	++	+	-
	Day 5	++	++	++	+
	Day 6	++	++	++	+
	Day 7	++	++	++	+
	Day 8	++	++	++	++
0.05	Day 1	+	++	-	-
	Day 2	+	++	+	+
	Day 3	++	++	++	++
	Day 4	++	++	++	++
	Day 5	++	++	++	++
	Day 6	++	++	++	++
	Day 7	++	++	++	++
	Day 8	+++	++	++	++
0	Day 1	+	++	-	-
	Day 2	+	++	+	+
	Day 3	++	++	+	+
	Day 4	++	++	++	++
	Day 5	++	++	++	++
	Day 6	++	++	++	++
	Day 7	+++	++	++	+
	Day 8	+++	++	++	+