Effects of substrate and co-culture on neural progenitor cell differentiation

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Introduction

In recent years the study of stem and progenitor cells has moved to the forefront of research. Since the isolation of human hematopoietic stem cells in 1988[1] and the subsequent discovery of a self renewing population of multipotent cells in many tissues, many researchers have envisioned a better understanding of development and potential clinical usage in intractable diseases. Both these goals, however, depend on a solid understanding of the intracellular and extracellular forces that cause stem cells to differentiate to a specific cell fate. Many diseases of large scale cell loss have been suggested as candidates for stem cell based treatments. It is proposed that replacing the function of the damaged or defective cells by specific differentiation of stem or progenitor cells could treat the disease. Before cells can be directed to specific lineages, the mechanisms of differentiation must be better understood. Differentiation *in vivo* is an intensely complex system that is difficult to study. The goal of this research is to develop further understanding of the effects of soluble and extracellular matrix (ECM) cues on the differentiation of neural progenitor cells with the use of a simplified *in vitro* culture system.

Specific research objectives are to study the differentiation of neural progenitor cells in response to astrocyte conditioned medium and protein substrate composition and concentration. In an effort to reveal the mechanism of the conditioned medium interaction, a test for the presence of a feedback loop between progenitor cells and astrocytes is presented along with an examination of conditioned medium storage temperature, which can reveal enzymatic dependencies. An examination of protein substrate composition and concentration will help to reveal the role of any ECM interactions on differentiation.

This thesis will be organized into a literature review covering recent advances in use of external modulators of differentiation such as surface coatings, co-culture, and soluble factors present in the medium in stem and progenitor cell research followed by a chapter covering the effects of astrocyte conditioned medium and protein substrate composition and concentration on progenitor cell differentiation. Future work will be discussed and an appendix presented.

Chapter 1.

Literature Review

Portions of the following literature review have been adapted from a review published in The Journal of Biomaterials Science, Polymer Edition. (Jones and Mallapragada, Directed growth and differentiation of stem cells towards neural cell fates using soluble and surface mediated cues, in press)

Introduction

Stem and progenitor cells have become an active research focus in the last twenty years due to their potential to shed light on the complex process of mammalian development and as treatment for currently intractable diseases. They have been proposed as potential treatments for Parkinson's disease[2], spinal cord injury [3], autoimmune disease [4], and neurological disorders [5], plus regeneration of damaged cardiac tissue [6], cartilage [7, 8] and liver [9] among many others. For them to become useful for research or clinical treatments, however, the process of differentiation to specific lineages must be better understood[10]. Embryonic stem cells (ESCs) are highly sought after for their great diversity of cell fates. They have the potential to become any cell type found in the body and may possess some immune-privilege[11], although they would not likely be directly injected due to teratomagenic properties. They also offer a valuable window into the process of development. However, there are many ethical issues surrounding the extraction of ESCs from blastocyst stage embryos[12] and political restrictions have been applied to funding of experiments involving their use and derivation[13]. Adult stem and progenitor cells (ASPCs) have restricted lineage potential, but have far fewer non-scientific issues clouding their usage as they can be extracted from tissue biopsies without harm to the donor. They have been found in many tissues, including brain [14], blood and bone marrow [15], endothelial

tissues[16], muscle [17], dental pulp [18] and fat [19]. Initial studies indicated that ASPCs could only differentiate into cells of their specific type, i.e. neural progenitor cells could only differentiate into neural cell lineages. Since then, some evidence has shown that ASPCs may retain plasticity, or the ability to differentiate into tissues other than their own, but due to culture impurities and cell fusion events the extent of this plasticity is currently unclear [15, 20, 21]. Another attractive feature of ASPCs is that they may be extracted from, expanded, and used in treatment on a single individual thus avoiding immune response to cell treatments. Stem and progenitor cells in the adult brain are concentrated in specific niches including the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus in the hippocampus[14]. The surface properties of these stem cell niches are still under study. An ECM molecule found in high concentration in the SVZ of the developing and adult mouse is Tenascin-C (TNC). Experiments with TNC null mice showed delayed expression of EGF receptors, altered numbers of stem cell populations, and increased neurogenesis when compared to wild type mice[22]. Cell-cell interactions are also known to contribute to the maintenance of the stem cell population. Rat ESCs grown on collagen coated substrates differentiate to a predominantly neural lineage, but when cells are co-cultured with glial or endothelial cells found in the SVZ this affect is attenuated. These results suggest that endothelial cell-stem cell contact induces proliferation, and as the number of stem cells increases the environment changes to a stem cell-stem cell contact, inducing differentiation[23]. This review focuses on neural stem and progenitor cells. Strategies for the directed differentiation and studies of cells derived from embryonic stem cells on two and three-dimensional substrates will be discussed, followed by current investigational

techniques into adult neural stem and progenitor cells. First, a brief description of various patterning techniques for proteins is described.

1. Patterning proteins

A common method used to pattern proteins on substrates is soft lithography based micro-contact printing. Most patterning techniques are derived from photolithography methods originally developed for the microcircuit industry. While these techniques are highly specialized for patterning, the equipment and conditions used are not always compatible with biological materials. "Soft lithography" refers to patterning techniques using elastomeric polymers[24]. A master mold is made from a silicon wafer using conventional photolithographic techniques and a soft polymer stamp, usually poly(dimethylsiloxane) (PDMS), is made to transfer a desired protein to a cell culture surface[24]. Often the stamped protein is an ECM component. PDMS is elastomeric, durable, inexpensive, non-toxic, and can be made more hydrophilic or bonded to another surface with plasma treatment. Protein transfer can be made directly in two ways; through contact-mediated physisorption [25] or through flow through microfluidic channels [26]. Using the same stamp, these two techniques would produce opposite patterns as shown in Figure 1. With modifications to the basic technique, microcontact printing can be done on gold, silicon, cell culture surfaces such as polystyrene and glass [27], and on biological tissues such as lens capsule [28]. Both permissive and inhibitory molecules can be patterned to direct cell adhesion and outgrowth.

Using oxygen plasma etching to treat polymeric substrates can enhance micro-contact printing of proteins. Plasma surface modification introduces oxygen containing functional groups to the polymer surface and sterilizes through reaction with oxygen radicals. Wang et

al. used oxygen plasma treatment to functionalize and sterilize chitosan films, tissue culture polystyrene (TCPS) and poly(methyl methacrylate) (PMMA) films for printing of laminin with PDMS stamps[29]. The contact angle of a water droplet on the surface of the film was decreased after oxygen plasma treatment for all substrates, indicating increased hydrophilicity. Master molds for stamping were made from SU-8 photoresist, which was patterned with masks and UV light, and Sylgard 184 PDMS was used to create a soft stamp. Schwann cells were cultured at varying cell densities and good alignment with the stamped pattern was noted up to 1000 cells/cm^2 on patterned TCPS and chitosan. Unpatterned TCPS and patterned PMMA showed random outgrowth, possibly due to lower affinity of laminin binding on the PMMA substrate. At higher cell densities, Schwann cells aggregated into tissue like structures and lost alignment to the pattern.

Conventional polymers have found wide use in microcontact printing applications, from PDMS as a stamp to PDMS, polystyrene, and other polymers as substrates. New techniques allow the use of biocompatible hydrogels in addition to these standard polymers. Hynd et al. have stamped patterns of biotinylated fibronectin, laminin, and laminin IKVAV epitope onto hydrogel surfaces[30]. Previously, it was difficult to maintain long term patterning on hydrogel surfaces due to their hydrophilic properties. This problem was solved by forming a hydrogel of 50% acrylamide and 50% poly(ethylene glycol) with a small amount of streptavidin conjugated acrylamide added during the photo-polymerization step. Biotinylated proteins were then inked on PDMS stamps and transferred to the hydrogels. Cell culture tests were conducted with an astroglioma cell line and primary hippocampal neurons. In both cases, cells adhered specifically to the delineated grid pattern for up to 4 weeks in culture. Active synapses were found only on neurons adhered to protein pattern.

Coq et al. have utilized hydrogels in the other half of the microcontact printing equation, forming stamps from hydrogels[31]. The advantage of using a hydrogel over PDMS is that PDMS is a hydrophobic polymer, leading to problems with surface wetting and nonhomogenous drying. These hydrogel stamps were composed of hydroxylethylacetate with poly(ethylene glycol) diacrylate (PEGDA) as a cross linking molecule. For a hydrogel with 10% water and 18% PEGDA, Young's modulus, a measure of the stiffness of a material, of the stamp is comparable to PDMS. Therefore, the mechanical properties of the hydrogel stamp are similar to those of PDMS but the hydrophilicity of the hydrogel leads to improved buffer holding capacity for stabilizing large sensitive biological molecules.

Cell response to both 3D and 2D substrates has been monitored, but Charest et al. have combined the two in a single culture environment [32]. First polymer substrates were given a 3D grooved topography using hot-embossing imprint lithography, a technique in which high temperature masters are impressed into a thermoplastic polymer. After the polymer cooled, fibronectin stripes were laid across the mesas in an orthogonal configuration. Osteoblasts were then cultured on the substrate. Even though the grooves were only 4 μm deep, the mechanical pattern dominated alignment. Cells were located both on grooves and in mesas, and did not restrict themselves to the fibronectin pattern. Due to the order of patterning, the protein pattern is discontinuous. In a previous study with continuous chemical patterning, it was found that the cells aligned with the protein rather than the topographical cues[33]. This leads to the hypothesis that the direction of migration controls the direction of alignment.

Microfluidic devices and controlled release polymers can be used to create gradients of soluble factors for testing the response of cells to medium components. Most microfluidic

devices are also made of PDMS through similar construction methods. In this case, however, inlets and outlets are provided for flow. Multiple inlet channels may converge to form fairly discrete parallel flows [26] or more complicated structures may mix multiple inlets to form a fluid gradient in a cell growth chamber [34]. Solutions of cell culture medium with or without specific factors such as cytokines and transcription factors flow over adherent cells without disturbing them. In this way, the cell response to the presence or absence of certain factors as well as the response to the presence of a gradient can be tested.

2. Embryonic Stem Cells

Embryonic stem cells (ESCs) are pluripotent cells with the capability to differentiate into any cell in the body under the right conditions. They are derived from the inner cell layer of the blastocyst[35]. ESCs are able to reproduce indefinitely *in vitro* without changes to karyotype. They show telomerase activity[35] and express the transcription factors Oct-3/4[36], SSEA-3/4 and TRA-1-60 and TRA-1-80 as well as a characterized set of genes known to be involved in the maintenance of the undifferentiated state[37].

a. Soluble Factors

Soluble factors in the medium have commonly been used to direct the growth and differentiation of embryonic stem cells. Retinoic acid is one of the earliest and best known factors influencing neural differentiation of embryonic stem cells[38]. Recently, the effect of cannabinoids, specifically HU210, on the proliferation but not differentiation of neural stem and progenitor cells has been demonstrated[39]. Rat neural stem/progenitor cells (NS/PCs) were exposed to 10 nM to 1 μM concentrations of HU210. This exposure caused a significant increase in cell proliferation with or without growth factors such as EGF and bFGF present in the medium. The CB1 receptor agonist AM281 blocked this increase,

indicating that HU210 acts on the CB1 receptor specifically. Ratios of differentiated neurons as measured by TuJ1 were unchanged. *In vivo* studies of both adult and newborn rats showed that chronic exposure to HU210 caused an increase in neurons incorporating bromodeoxyuridine (BRDu), a marker of cell division. Newborn neurons were found in the SVG and mature BRDu positive neurons were found throughout the dentate gyrus. Since proliferation was increased and differentiation was unaffected, the net result was more newborn neurons[39]. The mechanistic pathway of this response has not yet been found. It has been shown, however, that μ and □opioids act through the ERK/MAP kinase pathway to induce early differentiation of mouse ESCs [40]. Receptors for μ and □opioids were found on embryonic stem cells and ESC derived nestin-positive neural progenitors. The receptors were proved to be functional via detection of opioid-induced regulation of ERK/MAP kinase pathways. ERK activation induced limited proliferation in ESCs, but more importantly caused asymmetric division to increase the number of nestin positive NPCs while reducing self renewal of ESCs. Opioids decreased proliferation of RA-induced NPCs. It is hypothesized that there are competing mechanisms in undifferentiated ESCs; an ERK-independent self renewal program and an opioid activated ERK dependent pathway that leads to differentiation.

Docosahexaenoic acid (DHA) is one of the main structural lipids in the mammalian brain. It has previously been shown to be essential for development of the brain and retina[41]. Recently the effect of DHA in the medium on the differentiation of rat stem cells has been analyzed. Neuronal differentiation was shown to increase 1.4 fold for neurospheres cultured in the presence of DHA at both 4 and 7 days. BRDu incorporation was decreased, but the total number of cells was unchanged from controls. Furthermore, the number of

apoptotic cells was decreased in DHA exposed cultures when compared to controls. These *in vitro* studies show that DHA increases neuronal differentiation while decreasing proliferation and cell death. Neurons in DHA cultures also had more multi-polar, branched neurites, showing an accelerated rate of maturation as shown in Figure 2. When DHA was orally administered to adult rats, the number of BRDu and NeuN positive cells in the dentate gyrus was increased, demonstrating that DHA induced neurogenesis is not limited to cultured embryonic stem cells but occurs *in vivo* as well[42].

In the process of development, neural cells appear before glial cells[43]. Radial glia first appear at the peak of neuronal development, when neurons are forming networks. Radial glia appear morphologically similar to and are traditionally classified as astrocytes, but have been shown to have the potential to generate neurons as well[44]. A novel secreted protein has been discovered in murine neural and glial progenitor cell cultures that changes the differentiation state of glial progenitor cells, eliciting astrogenesis. The protein, termed meteorin, was discovered through sequencing cDNA that was upregulated upon exposure of ESCs to retinoic acid. Meteorin can be found during development *in vivo* co-localized with glial cell markers such as protein zero and radial glia markers such as GLAST[45]. *In vitro* studies have shown that meteorin is correlated with extensive neurite outgrowth from DRG, but only in high density cultures. An important note is that DRG cultures are not purely neurons, but contain other cells as well. Since neurite outgrowth was only noted in high density culture, Nishino concluded that meteorin does not have a direct affect, but acts through a secondary signal, either by affecting non-neuronal cells such as satellite glia or in combination with another factor released by non-neuronal cells. Medium conditioned by satellite glia exposed to meteorin showed the same long neurite extension, whereas medium

from control satellite glia did not. This secondary signal that causes neurite outgrowth is most likely therefore a protein secreted by satellite glia. Cerebellar astrocytes exposed to meteorin were converted to radial glia as assessed by morphological changes and expression of radial glia specific brain lipid-binding protein. Neurospheres of nestin-positive progenitors showed increased astrocyte differentiation when differentiation medium was supplemented with meteorin, but no increase when meteorin was added to growth medium containing EGF. Differentiation of Map2 positive neurons and O4 positive oligodendrocytes was unaffected. This indicates that meteorin selectively affects cells already committed to the astrocyte lineage.

In the treatment of specific diseases such as Parkinson's, growth of generic neurons is not sufficient. Specific subtypes of neurons are required. It has been shown that dopaminergic neurons can be produced from embryonic stem cells via co-culture with a feeder layer of stromal cells [46]. A procedure for immobilizing neural inducing factors (NIFs) produced by mouse PA6 stromal cells on culture dishes has been developed, avoiding direct contact of human stem cells with mouse cells. It was reported that PA6 stromal cells lost their ability to induce neural differentiation when treated with heparin [46]. It was expected then, that these NIFs could be collected by treatment with heparin and immobilized[47]. Mouse ESCs were cultured with NIF stock solutions added to the medium and on NIF immobilized dishes, both with heparin supplemented in the medium. Differentiation was assessed with TuJ1 and TH, an antibody specific to dopaminergic neurons. When NIF was added to the medium, the combination of NIFs and 100 μg/mL heparin had the greatest effect on differentiation, with 34+12% of colonies expressing TuJ1/TH. When cells were grown on NIF immobilized dishes, colonies formed at

concentrations of 1 and 10 μg/mL heparin, but did not form on dishes with NIF and 100 μg/mL heparin. TuJ1 and TH+ cells were found on NIF immobilized dishes, but at a lower frequency than when cells were cultured with soluble NIF in the medium. This indicates a dual effect on cell proliferation and differentiation, wherein the proliferative capability of stromal cell-produced NIFs is retained after immobilization on culture dishes but the differentiative ability is not. The number of colonies expressing TH was smaller on NIF immobilized surfaces than in co-culture with PA6 cells.

The effect of co-culture with additional cell types has also been investigated. A three stage differentiation procedure involving co-culture with primary cultures of astrocytes has been developed which obtained a population of greater than 90% nestin positive cells from mouse ESCs[48]. Stage 1 involves incubation with all-trans retinoic acid for 2 days. Stage 2 of the procedure co-cultured ESCs with an astrocyte feeder layer for 4 days. Stage 3 removes the astrocytes and changes the treated ESCs to NSC promoting medium. After five passages in NSC medium, 91% of cells were shown to be nestin positive. During the differentiation procedure, Oct-4 expression decreased as nestin increased as measured by RT-PCR. Neurospheres formed from induced cells could replicate and differentiate into neurons, astrocytes, and oligodendrocytes as measured by ICC. The authors concluded that this method was an effective way to obtain populations of neural progenitor cells from murine embryonic stem cells,

b. Three-Dimensional Substrates

There have been many investigations into three-dimensional scaffolds for the growth of cells[49, 50]. Three-dimensional scaffolds have also been investigated for regrowth of axons in the nervous system with positive results[51]. From these previous studies, it is a

natural progression to the use of scaffolds in research into the growth and differentiation of embryonic stem cells towards neural cell fates[2].

When embryonic stem cells are grown to confluence and allowed to aggregate, they begin to spontaneously differentiate even in the presence of feeder cells. Several methods have been developed to control ESC aggregation during culture using microwell substrates. Poly(dimethylsiloxane) (PDMS) microwell culture substrates that control the size and number of human ESC clusters in co-culture with murine embryonic fibroblasts (MEFs) have been developed both for co-culture with MEFs[52] and for use with MEF-conditioned medium[53]. In both cases, PDMS molds bearing indentations from 50 to 200 μm across and 120 μm deep were created from silicon masters. Human ESCs were localized to the depressions in the substrate by novel seeding methods[52] or by inactivating the surface around the depression using self-assembled monolayers of alkanethiols on gold[53]. ESCs became nearly confluent within circular wells and showed much greater homogeneity of aggregate size compared to uncontrolled 2-D cultures. Cell aggregates were able to be passaged from substrates and formed more homogeneously sized embryoid bodies[52]. Human ESCs fed MEF conditioned medium in square wells maintained an undifferentiated phenotype for 20 days in microwell culture as assessed by Oct-4 immunoreactivity. Cells retained pluripotency after culture on microwells[53].

Scaffolds may be formed from non-degradable polymers, degradable polymers, or biologically obtained polymers such as collagen and fibrin. When human ESC derived neurons are grown on a 3-D poly(styrene/divinylbenzene) scaffold manufactured by polymerization in high internal phase emulsions, protein coating is needed for cells to adhere. Poly-lysine allows cells to adhere to the scaffold, but laminin adsorption causes much greater

neurite extension, up to 500 μm in some cases. The largest amount of cell adhesion was achieved when both poly-lysine and laminin were adsorbed to the scaffold surface[54]. Nondegradable polymers are convenient for *in vitro* study, but are not suitable for *in vivo* applications. Furthermore, cells grown on them are restricted by the initial dimensions of the scaffold. Some of these problems can be ameliorated by the use of scaffolds made of biodegradable polymers.

Biodegradable scaffolds have been made from poly lactic and glycolic acids using salt leaching methods. A PLA/PGA mixture of 50/50 with pores from 250-500 μm was found to degrade in an appropriate time scale and to support the ingrowth of cells[55]. Human H9 clone embryoid bodies were dissociated and seeded in scaffolds in control medium or medium supplemented with retinoic acid (RA), neurotrophin-3 (NT-3), and nerve growth factor (NGF) alone or in combination. Neural rosettes were formed over two weeks in culture in all culture conditions, but more and better defined rosettes were found in cultures supplemented with neurotrophins. NGF and NT-3 caused the greatest effect on 4 day old embryoid bodies while NT-3 and NT-3+RA had the largest increase in rosettes for 9 day old bodies. Retinoic acid enhanced rosette numbers in 9 day old embryoid bodies, but not 4 day old bodies. Significantly higher TuJ1 staining was observed in 9 day old embryoid body cultures with NT-3, RA+NGF, and RA+NT-3, but not seen in any 4 day old embryoid body cultures[56]. This result highlights the importance of cell age and timing in signaling and development.

Poly-lactic and glycolic acid scaffolds may degrade over weeks, but fibrin scaffolds degrade even faster. Fibrin scaffolds have been used to optimize the differentiation of human ESCs to neural lineages. These scaffolds are made by combining thrombin, calcium

chloride, and fibrinogen. Fibrin scaffolds degraded completely in only a few days of *in vitro* culture, so aprotinin, a plasma inhibitor, was added to slow degradation. After addition of aprotinin, some scaffold remained after eight days in culture, compared to complete degradation in four days without. Embryoid bodies (EBs) were exposed to retinoic acid for four days and then either dissociated or placed intact into fibrin scaffolds. Intact embryoid bodies showed greater cell growth and differentiation than dissociated cells, and embryoid bodies imbedded into the scaffolds showed better migration into and through the scaffold than EBs placed on top. Cells differentiated into both neurons and astrocytes as assessed by immunocytochemistry (TuJ1 and GFAP, respectively)[57].

3. Adult neural stem and progenitor cells

Neural stem and progenitor cells in the adult brain are a relatively new concept, as traditionally it was thought that the brain had no capacity for self-renewal, with neuronal proliferation ceasing before or shortly after birth. The discovery of mitotic cells that produced mature neurons was therefore unexpected and exciting[10]. In 1992 the first reports of proliferating, differentiating cells from the adult brain were published[58]. Since then techniques for extraction and culture of adult neural progenitor cells have been refined. These adult NPCs express nestin and can differentiate into neurons, astrocytes, and oligodendrocytes[14]. Techniques used in the study of ESCs may be adapted for investigation into adult neural stem and progenitor cells.

a. Substrate Bound Cues

A commonly used cell-adhesion substrate is the effective and economic poly-lysine. While this substrate has only a single amino acid residue for a repeating unit, lysine-alanine sequential polymer (LAS) has alternating units. It has been reported that growth of rat neural

stem cells is enhanced on LAS under serum-free medium conditions[59]. These effects have since been better characterized. When rat-derived neurospheres are plated on LAS substrate at low densities, cells did not migrate out to cover the substrate but instead sent out long, thin neurite-like processes to the area around the neurosphere. Length of neurite outgrowths was significantly increased on LAS as compared to poly-lysine after 1, 2, and 4 days of culture. When differentiation was assessed with immunocytochemistry, there was a significantly higher ratio of neuronal to astrocytic differentiation on the LAS substrate compared to the poly-lysine substrate under serum free conditions. In contrast, when neurospheres were plated at high density, neurospheres broke down and cells migrated out. GFAP positive astrocytes were present on the LAS substrates, but MAP2 positive neurons and O4 positive oligodendrocytes were not, demonstrating the importance of external conditions on cell behavior and differentiation. Finally, when cells were plated at low density but with serum in the medium, cells migrated away from the neurospheres and formed a confluent monolayer. A large majority of these cells were GFAP positive, although MAP2 and TUJ1 positive cells were also found. Astrocytes exhibited both fibrous and protoplasmic morphologies when serum was present, but not otherwise[60].

Uncoated polymer substrates have differing effects on stem cell growth as well. The growth of rat embryonic cerebral cortical progenitor cells was compared on standard glass coverslips coated with poly-lysine, uncoated poly(vinylalcohol) (PVA), and uncoated poly(ethylene-co-vinylalchohol) (EVAL). Cell growth results on glass were comparable to literature results, with neurospheres adhering and cells migrating out and differentiating. On PVA films, cells did not adhere either as neurospheres or single cells. They aggregated and died within 2 days. Similar results were found with primary cultures of neurons on EVAL,

but nestin positive progenitors were able to attach to non-modified EVAL. Cells plated at varying densities from 3,125 to 100,000 cells/mL did not appear to proliferate or differentiate, remaining in a quiescent state over four days of culture. The majority of cells remained nestin positive, while around 25% were positive for GFAP and only 8% displayed neural markers. When neurospheres were cultured at low density on EVAL, the spheres attached to the substrate and neurites extended from the neurosphere body but cells did not migrate out, similar to results on LAS polymer presented previously[60]. High density neurospheres did not attach to the EVAL substrate. Cells did continue to proliferate however, with the number and size of neurospheres steadily increasing over four days in culture. At the end of four days the number of neurospheres had tripled, and virtually all the cells contained therein were positive for nestin indicating an undifferentiated state[61]. It is thought that when cells are dissociated, cell-substrate effects dominate and the cells remain in a quiescent state. When neurospheres are plated at low density, cell-cell interactions are important but cell-substrate interactions are present as well. Thus neurospheres attach, but send long processes out to establish communication with other neurospheres. When neurospheres are plated at high density, cell-cell interactions dominate and the cells proliferate in neurospheres. These results show the importance of and delicate balance between cell-substrate interactions and cell-cell contact interactions.

It is well known that the extracellular matrix (ECM) *in vivo* has strong effects on the growth, migration, and differentiation of cells. Patterns of extracellular matrix proteins can be made with soft lithographic techniques such as PDMS stamping and cell growth can be guided via patterning[62, 63]. While many experiments have been conducted with rodent cells, recently efforts have been made to understand the impact of ECM proteins on human

progenitor cells[64]. Human neural progenitor cells were seeded on poly-ornithine (a positively charged molecule), laminin, fibronectin, or Matrigel™, a combination of ECM proteins. Motility was assessed via outgrowth from neurospheres. Outgrowth on all the ECM substrates was greater than on poly-ornithine. Among the ECM substrates, laminin and Matrigel™ supported more spreading than fibronectin. Differentiation was assessed by plating equal numbers of viable cells on each type of substrate. After 20 days, there were more MAP-2 positive neurons on pure laminin than on either fibronectin or Matrigel™. Cell density was similar between laminin and Matrigel™, but 2.4 times more neurons formed on the laminin substrate, as shown in Figure 3. GFAP expression was similar on Matrigel™ and laminin but both remained higher than fibronectin. Neurons on laminin had longer neurites and more well developed networks. Number of primary neurites and branches per unit length were not different, indicating the main difference between substrates was neurite elongation. In all cases, responses were similar between rodent and human progenitors, indicating conservation of function.

b. Three-dimensional substrates

Three-dimensional patterned substrates have also been used to investigate growth and differentiation of neural progenitor cells[65]. The scale of the scaffold does not have to be many times that of the cell body. Grooved polystyrene substrates with dimensions of 16 μm wide and 4 μm deep were cast from etched silicon wafers and coated with laminin. Rat adult hippocampal progenitor cells (AHPCs) were seeded on the grooves and assessed for alignment and differentiation. AHPCs displayed a tendency to develop bi-polar phenotypes in which the cells aligned with groove edges. Over 75% of AHPCs were aligned with the groove pattern, whereas AHPCs on smooth surfaces had a radial shape and a random

orientation. Differentiation was not significantly different between cells grown on patterned and smooth surfaces. It was also shown that astrocytes aligned with the pattern[66]. Previous studies have indicated that neurites align with an aligned substratum of cells[67]. When AHPCs were co-cultured with aligned astrocytes on the grooved substrates, alignment of AHPCs was observed but there was also significantly more neuronal differentiation as assessed by TUJ1 immunoreactivity on the grooved surface, when compared to co-culture on smooth surface and AHPCs alone on patterned surface. TUJ1 expressing AHPCs in AHPC/astrocyte co-culture also appeared more mature than in the other culture conditions, with longer and more elaborate neurites that aligned with the astrocyte sublayer formed. Astrocyte and oligodendrocyte differentiation was not significantly changed between any of the aforementioned culture conditions[68]. These results showed the synergistic effect of combined physical and biological cues on differentiation.

Most previous studies involving scaffolds involved pre-made polymeric or gel surfaces for cell growth. An additional method for construction of cell directing structures is self-assembly of amphiphilic peptides. A molecule was synthesized containing the active cell binding sequence from laminin, isoleucine-lysine-valine-alanine-valine (IKVAV)[69], and a hydrophobic tail[70]. When an aqueous solution of this peptide is introduced to a neural progenitor cell suspension in medium, self-assembly occurs and a gel of nanofibers encapsulates the cells. Murine neurospheres encapsulated in the IKVAV gel sent out neurites and migrated out into the surrounding nanofibers. When encapsulated in a gel with identical structural properties but a random peptide sequence, cells remained viable but did not leave the neurosphere. After only one day in IKVAV gel culture, 35% of cells expressed a neuronal phenotype. In contrast, astrocytic phenotypes were rare after seven days in culture.

Nanofibers were also formed in 2-D sheets and similar differentiation profiles were found. When the IKVAV peptide alone was immobilized on flat culture dishes, any immunoreactivity was difficult to find even after seven days in culture, indicating that the high epitope density achieved in the nanofiber structure is more important than the shape factor. Importantly, self-assembly was found to occur when peptide solutions were injected *in vivo* as well, allowing for localized assembly of scaffold without diffusion away from the site or large incisions required for the implantation of traditional polymeric scaffolds[70].

It has been shown that somatic cells such as neurons and schwann cells tend to align with fibrous scaffolds[71], and that fibrous scaffolds can enhance axonal regeneration[72]. One method for making a fibrous scaffold is electrospinning, which allows microscale fibers to be aligned with each other. Poly-L-lactic acid biodegradable polymer was electrospun in either aligned or random fashion. By varying the composition of the polymer solution, fibers of 300 nm (aligned nano-fiber, ANF) and 1.5 μm (aligned micro-fiber, AMF) were formed. Random fibers had larger average diameters than aligned fibers of the same polymer composition, most likely due to the spinning disc collection plate used to align the fibers. Murine neural progenitor cells attached to the fibrous substrate and extended processes parallel to the fibers; they showed bipolar morphologies in the aligned case and multiple processes in the random structure as shown in Figure 4. More cells showed neurofilament immunoreactivity, indicating differentiation, on the nanoscale scaffolds than on the microscale scaffolds regardless of alignment. This showed the size of the fiber had more effect than the alignment. Average neurite length was higher on ANF than on random nanoscale or either microscale substrate[73]. Electrospun nanofibrous scaffolds show promise as a neural tissue engineering substrate.

Astrocytes have been previously found to enhance neuronal differentiation of rat adult neural progenitor cells[74], but it has been found that they are also able to affect the differentiation of more committed progenitors. Rat oligodendrocyte precursor cells (OPCs) had been viewed as committed to the glial lineage. In the absence of extracellular cues, they differentiate into oligodendrocytes. When serum is added to the medium, they differentiate into astrocytes. The growth factors EGF and bFGF are known to cause proliferation of nestin positive neural progenitor cells and when they are removed the progenitors differentiate into neurons, astrocytes, and oligodendrocytes. OPCs were exposed to EGF and bFGF in the medium, but were unresponsive to EGF and no neuronal immunoreactivity was seen, indicating that they are more differentiated and thus responsive to different signals than neural progenitor cells. When OPCs were plated on a purified population of hippocampal astrocytes, however, 30% of cells expressed Map2ab. To test whether this guidance was contact-mediated or precipitated by a soluble factor, OPCs were cultured with hippocampal astrocyte conditioned medium (HACM). The neuronal differentiation was preserved. A clonal analysis revealed that single OPC cells had the potential to differentiate into oligodendrocytes or neurons when treated with HACM. Neurotransmitter immunoreactivity and calcium signaling were observed in differentiated neurons. From this, it is concluded that OPCs could be a potential source of neurons[75].

While specific types of astrocytes were shown to enhance rat neuronal differentiation in 2002[74], the specific factor causing the increase has only recently been found. When gene expression was assessed by microarray analysis and quantitative real time PCR it was found that neurogenesis promoting and inhibiting astrocytes showed different gene expression patterns. A luciferase reporter system was used to analyze neurogenesis

promoting factors. Both the EGF domain of neuregulin (NRG/ED) and interleukin-6 (IL-6) increased neuronal differentiation in rat AHPCs as measured by Neuro-D1 promoter activity, but both caused less differentiation than the positive control, retinoic acid. NRG/ED also increased proliferation. When assessing differentiation only, IL-6 and IL-1□promoted neural differentiation at 20 ng/mL. A combination of IL1□ IL-6, VCAM-1, IP-10, cathepsin S, and TGF-□2 caused the greatest increase on neuronal differentiation. It is important to note that VCAM-1, IP-10, cathepsin S, and TGF-□2 did not have any effect when applied individually. The combination of factors did not have a synergistic effect in combination with RA, showing that the combination may act through the same pathway[76]. This result is somewhat surprising, since it is thought that inflammatory cytokines such as IL-6 inhibit neurogenesis[77]. Further experiments revealed that concentration of IL-6 had an important effect on the results obtained. In the presence of retinoic acid at 50 ng/mL, IL-6 inhibited neuronal differentiation. When presented alone at 20 ng/mL, IL-6 promoted neurogenesis[76]. This result, along with the combined effect of multiple factors, highlights the importance of context in cell signaling.

Further studies of combined signaling cues were carried out by Soen et al [78] using printed microarrays. Different mixtures of ECM proteins, cell adhesion molecules (CAMs) and known cell signaling molecules were printed in an array. Initial tests indicated that ECM proteins were necessary for long term cultures, as cells on spots containing CAMs or signal molecules only did not adhere for more than a few hours. The ECM proteins laminin, fibronectin, vitronectin, and Matrigel™ were tested. On fibronectin, cell-cell interactions dominated and cells were found tangled together which made analysis difficult. The other three ECM backgrounds allowed monolayer cultures to form, and laminin was chosen as the

common ECM substrate for the second set of experiments. In this second experiment, exposure to CNTF and Notch ligands increased the ratio of GFAP expression, while WNT-3 increased the proportion of TUJ1 expressing cells. Combinations of signals were also tested. In some cases, the combined result was not intuitive from the individual results. When gliogenic Jagged-1 (a notch ligand) and neurogenic WNT-3 were combined, the result was overall low levels of differentiation indicating that both differentiation pathways may have been repressed. A sample array seen in Figure 5 shows the changes in differentiation ratios and cell spreading when different signaling molecules are present. TUJ1 staining appears green, GFAP staining appears red, and BRDu incorporation appears blue. The top left image is the base laminin case in which no additional signaling molecules are present [78].

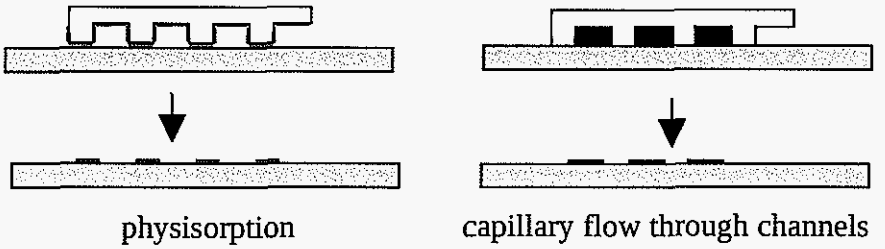

Figure 1. Two methods of patterning solution on a substrate using the same stamp.

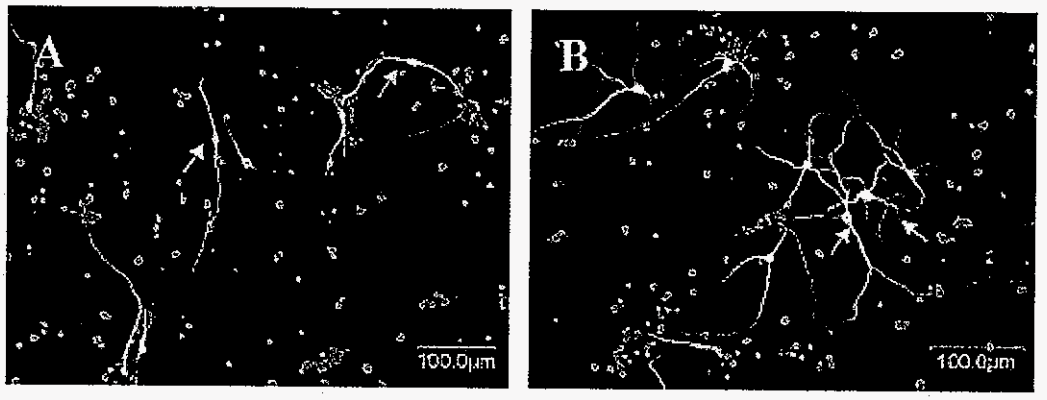

Figure 2. NPCs were cultured in the presence (right) or absence (left) of DHA and stained with TUJ1 (white) and PI (gray). Neurons in the DHA culture show a more mature morphology than neurons in the control(arrows)[42].

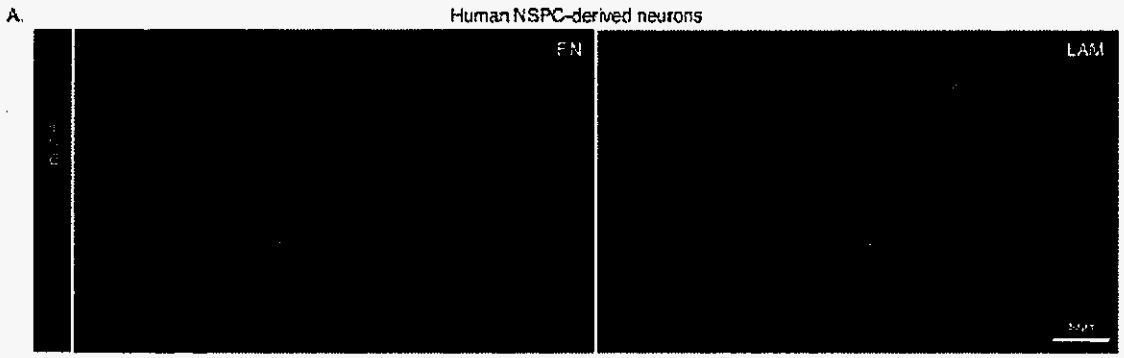

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Figure 3. Human NSPCs (SC27) were differentiated for 20 days on fibronectin (left) or laminin (right). More neuronal differentiation as assessed by MAP2 immunoreactivity is detected in the laminin culture. Nuclei were stained with Hoechst.

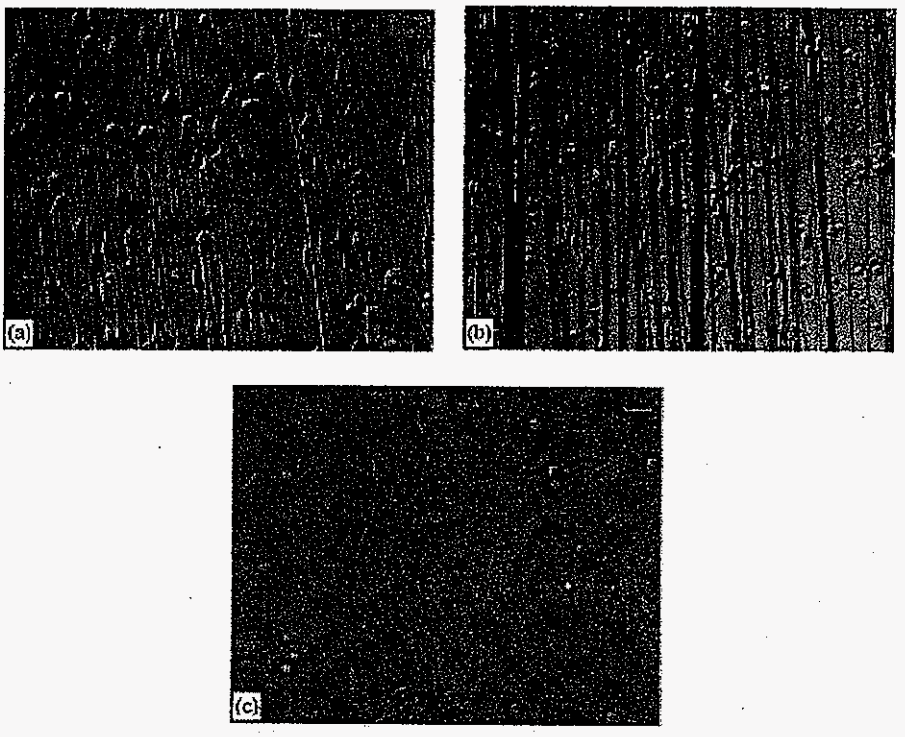

Figure 4. NPC attachment and growth on (a) aligned nanofiber, (b) aligned microfiber, and (c) random nanofiber after one day of culture[73].

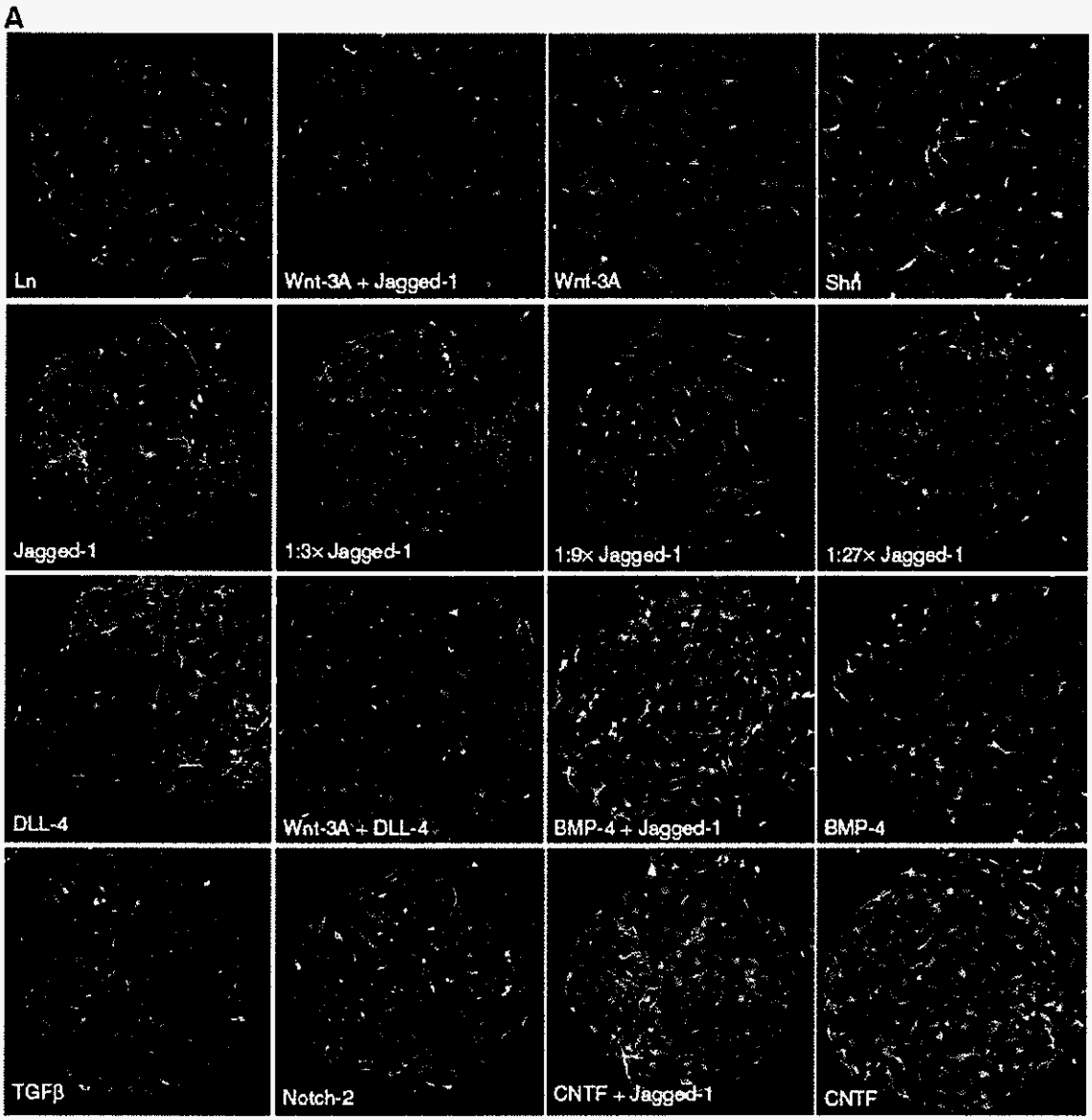

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Figure 5. Human neural precursors were cultured on a printed laminin and signaling molecule array for 70 h under differentiation-promoting conditions. Following the differentiation period, the cells were fixed and stained with GFAP (red), BrdU (blue), TUJ1 (green), and DAPI (not shown). A small portion of the array with 16 different microenvironments is shown, each containing a few hundred cells. The balance between TUJ1 and GFAP staining on the reference laminin spot (top left) was skewed toward preferential expression of the neuronal marker TUJ1. This balance was shifted in a spot-dependent manner by some of the signal-containing spots. In particular, spots containing CNTF (bottom right) and Notch ligands (Jagged, DLL-4; left panels in second and third rows) led to a dramatic shift toward increased GFAP proportions, suggesting a gliogenic

response to Notch stimulation. Dilution series of Jagged-1 (2nd row panels) revealed dose dependent response to Notch stimulation. Combination of some gliogenic signals (e.g. Jagged-1 and CNTF) led to further increase in the gliogenic response. A smaller shift toward increased neuronal proportions was observed on Wnt-3A spots. Typical spot diameter was 400 mm. Fields of view in all panels are identical in size. Wnt-3A-containing spots consistently larger [78].

Chapter 2.

Effects of substrate and co-culture on neural progenitor cell differentiation

Introduction

Stem cells are an active area of investigation in biological research. Their potential to differentiate into any cell in the body has inspired many researchers to investigate their use as treatments for diseases that lack sufficient treatment options, such as multiple sclerosis, Parkinson's disease, and stroke. Before stem cells can be efficiently used in therapeutic treatments, however, the mechanisms behind their differentiation must be better understood. Insight into the differentiation process will also shed light on the development of mature cells.

Stem and progenitor cells have been found in almost every tissue of the body. The discovery of niches containing cells with the capability to self-renew and differentiate in the adult brain disproved the prevailing opinion at that time that the mature brain did not have this capacity. The Gage group at the Salk Institute demonstrated that cells derived from the hippocampus of the adult rat would self-renew when basic fibroblast growth factor was present in the medium and would differentiate to cells of the three neural lineages, neurons, astrocytes, and oligodendrocytes when growth factors were removed [27]. While exploring the role of the environment in control of neural progenitor cells, Song found that mature astrocytes grown in contact with adult hippocampal progenitor cells (AHPCs) on laminin coated substrates were able to instruct them to adopt a neuronal fate [74]. This directed differentiation shows interesting possibilities for regeneration in the central nervous system.

Previously in our group, Miller showed that patterned substrates caused directed growth of peripheral nervous system cells that allowed enhanced recovery from sciatic nerve

injury [79]. Recknor then completed studies showing that patterned surfaces caused both astrocytes and AHPCs to align with the substrate pattern grown alone and in co-culture with astrocytes. In addition, she also showed increased neuronal differentiation as measured by antibody staining of class III □-tubulin, a protein specific to developing neurons, when both astrocytes and three-dimensional pattern were present[68]. The next question asked was whether the observed increased neuronal differentiation was caused by alignment of the cells or whether soluble factors were being concentrated in the grooves of the substrate. To test this hypothesis, non-contact co-culture systems using Transwell Clear permeable culture inserts were devised. These non-contact co-cultures showed a significant increase in neuronal differentiation over even the contact co-cultures, showing that a soluble factor secreted by the astrocytes must be contributing to the increased neuronal differentiation (Oh, under submission).

In this work, to further explore the effects of soluble factors released by astrocytes and to narrow down potential roles and mechanisms of this molecule, conditioned medium cultures were used. Two hypotheses were tested: that the secreted molecule has a short active lifespan, and that AHPC produced factors increase astrocyte production of the secreted molecule, resulting in a feedback loop. The short life hypothesis was tested using time delayed astrocyte culture conditioned medium, in which astrocyte conditioned medium was held for 24 hours at either 37° or 4° C before addition to AHPC cultures. Volumes of 250 μL and 400 μL were tested. The feedback hypothesis was tested using doubly conditioned medium, in which medium was first conditioned by AHPCs and fed to astrocytes, then taken from the astrocytes and given to AHPCs. Setups in which the AHPC source and sink were different and the same were performed.

In addition, an investigation into the effect of extracellular matrix (ECM) proteins on culture surface was conducted. For the co-cultures and many previous experiments, AHPCs were cultured on either laminin or poly-L-lysine and laminin. However, laminin is only a minor component of natural ECM. To investigate the role of ECM proteins on AHPC differentiation, a less purified extracellular matrix extract was used to coat culture surfaces. The hypothesis proposed was that astrocyte mediated differentiation occurred through a mechanism which employed an extracellular matrix molecule. To test this, AHPCs were grown either on laminin or mixed protein coated substrates in a variety of astrocyte co-culture arrangements. The ECM extract used for these experiments was ECL cell attachment matrix (Millipore). Obtained from the same source as purified laminin, ECL contains entactin, collagen, and laminin proteins. Both laminin and collagen are bound by integrin receptors, which are known to be involved in cell signaling. To test the hypothesis the effects of ECM molecule identity and concentration on neural progenitor cell differentiation were examined. Solutions with total protein concentrations of 1 μg/mL and 10 μg/mL were used to coat polystyrene substrates. It was expected that if differentiation was dependent on substrate proteins, differentiation outcomes would change for cells grown on different substrate compositions and concentrations.

Materials and Methods

Adult hippocampal progenitor cells (AHPCs) were donated by F. Gage at the Salk Institute to the D. S. Sakaguchi lab at Iowa State University. They are derived from the hippocampus of adult Sprague-Dawley rats. AHPCs were maintained in complete medium consisting of Hamm's F12 supplemented with N2 and basic fibroblast growth factor (bFGF). AHPCs divide in complete medium, and differentiation is initiated when growth factors are removed. Differentiated cells express markers for neuron, astrocyte, and oligodendrocyte lineages. The AHPCs have been transfected with a lentiviral vector to constitutively express green fluorescent protein (GFP). Cells used in this study were between passages 6 and 10 post receipt. Postnatal type-1 astrocytes were obtained from the cerebral cortices and hippocampi of 2 to 4 day old Sprague-Dawley rats. Astrocytes were purified and maintained in modified minimum essential medium (MMEM), consisting of minimum essential medium supplemented with 10% v/v fetal bovine serum. Astrocytes were limited to three weeks or less in culture for use in the experiments. Since each cell type had its own medium formulation, co-cultures were maintained in a 50% v/v mixture of astrocyte MMEM without FBS and AHPC differentiation medium. Experimental culture chambers were made by adhering PTFE o-rings (Small Parts, Inc.) to 22 x 22 coverslips with Sylgard 184 adhesive (Dow Corning). O-rings had 9/16" inner diameter and 3/4" outer diameter, and 500 μL of medium was used as control volume. A 1 cm x 1 cm smooth polystyrene film was adhered to the coverslip in the center of the o-ring with Silastic medical adhesive (Dow Corning). Substrates were coated with laminin (Sigma Aldrich) solution at 10 μg/mL prior to cell seeding. 300 μL of laminin solution was added to the o-ring and allowed to remain for 20 minutes before removal. Substrates were stored at room temperature.

Conditioned medium experiments

There were several aspects of conditioned medium treatment that were examined, and multiple approaches used. The double conditioned medium experiment sought to reveal any feedback loops in signaling between AHPCs and astrocytes, while the time delayed conditioned medium looked for storage effects due to time and temperature. Table 1 shows the co-culture arrangements.

Similar techniques were used for both experiments but there were some notable differences between the two. For the time delayed conditioned medium, two conditions of astrocyte cultures were used. Both had approximately confluent cell layers, but the small 12.5 cm^2 flask preserved the approximate 0.5 mL medium/cm^2 ratio of the o-rings, while the large 75 cm^2 flask had a much lower ratio of 0.13 mL medium/cm^2. While there may be many factors affecting the growth and secretion of factors by the astrocytes so that any changes may not be easily predicted, these two arrangements provide different conditions. Two days prior to AHPC seeding, a 12.5 cm^2 flask was seeded with approximately 900,000 astrocytes in 6 mL of MMEM. One day prior to AHPC seeding, the 6 mL of MMEM in the seeded flask and the 10 mL in a confluent T-75 flask of astrocytes were replaced with co-culture medium. Co-culture medium consists of a 50% v/v mixture of AHPC differentiation medium (-bFGF) and MMEM without FBS. These two medium-to-area ratios enable investigation of the effect of different astrocyte growth conditions on AHPC differentiation. AHPCs were seeded on gravity cast smooth polystyrene substrates in 500 μL of AHPC differentiation medium and allowed to adhere for 24 hours. Just as with the astrocytes, seeding the AHPCs in their own medium appears to increase cell adhesion and survival. On

the same day that AHPCs were seeded, one half the total volume of the container of each astrocyte conditioned medium was gathered, centrifuged, and stored at either 4 or 37° C. This volume is 250 µl for o-rings, 3 mL for the small 12.5 cm^2 flasks, and 5 mL for the large 75 cm^2 flasks. The following day, conditioned medium treatments were initiated. One half of the total volume of the culture chamber, 250 µl unless otherwise stated, was replaced with astrocyte conditioned medium daily. Medium changes can be seen in Figure 6.

For double conditioned medium, both astrocytes and AHPCs were seeded in o-rings on laminin coated smooth polystyrene films. 75,000 astrocytes were seeded in 500 µl MMEM with FBS. One to two days later, 15,000 AHPCs were seeded in separate o-rings in 500 µL of AHPC differentiation medium. On the same day that the AHPCs were seeded, the astrocytes were rinsed with EBSS and switched to co-culture medium, which is a 50% v/v ratio of astrocyte MMEM without FBS and AHPC differentiation medium. The following day, half the volume of the co-culture medium over the astrocytes was removed and saved in a microcentrifuge tube. Half the volume of the co-culture medium in the designated AHPC source dish was then removed and added directly to the astrocyte o-ring. The medium changes can be seen on the timeline in Figure 7. The medium was not centrifuged in double conditioned medium experiments due to the small volumes involved. At this point, the two different double conditioned medium treatments diverge. For the one dish double conditioned medium, the astrocyte conditioned medium saved in step 1 was returned to the AHPC dish. Every other day 150 µL of additional fresh, unconditioned co-culture medium was added to each dish to maintain a 500 µL volume. In the two-dish double conditioned medium setup, 250 µL of the saved astrocyte conditioned medium was given to dish B and

250 μL of fresh unconditioned co-culture medium was given to dish A. Conditioned medium cultures were fed daily for 6 days followed by fixation and immunocytochemical staining. Experiments were repeated three times for statistical analysis of results.

Substrate concentration experiments

The proteins selected for substrate concentration study were laminin and ECL cell adhesion matrix (Millipore). It is estimated that ECL contains approximately 65% collagen, 34% laminin, and 1% entactin. Both laminin and ECL are derived from the Engelbreth-Holm-Swarm murine sarcoma. Smooth gravity-cast polystyrene substrates in o-rings were coated with solutions of laminin at 10 μg /mL or ECL at concentrations of 1 and 10 μg/mL protein (as described previously). Astrocyte/AHPC co-cultures were set up in three arrangements: contact, non-contact, and conditioned medium. AHPC only substrates were made as a control. All astrocytes were seeded in MMEM + 10% FBS one to two days prior to AHPC seeding. For contact and conditioned medium co-cultures, 75,000 astrocytes were seeded on 1 cm^2 substrates. For non-contact co-cultures, 75,000 astrocytes were seeded in MMEM + 10% FBS on Corning* Transwell-Clear* Permeable Supports (Fisher Scientific). Polystyrene substrates in o-rings received 500 μL of medium. Non-contact co-cultures were seeded in the same way as contact or co-culture medium initially, but were moved to a 6 well plate to facilitate combination with the Transwell inserts. The o-ring was removed from the non-contact co-culture coverslips to allow diffusion in the space below the insert. To maintain a continuous fluid phase through the insert, 2 mL of medium was added to the bottom of the 6 well plate and 1 mL to the Transwell insert. Prior to AHPC seeding, astrocyte cultures were rinsed with Earle's Balanced Salt Solution (EBSS) to remove residual FBS and the medium was changed to AHPC/astrocyte co-culture medium. 15,000 AHPCs

were seeded either on top of astrocytes for the contact co-cultures or on fresh coated substrates for the conditioned medium studies. Non-contact co-culture AHPCs were allowed to attach to the polystyrene substrates for several hours prior to removal of the o-ring, medium exchange, and transfer of culture inserts to the AHPC 6-well plate. After combination of AHPCs and astrocyte substrates into a single 6-well plate, AHPCs were allowed to grow together for 6 days before fixation with 4% para-formaldehyde. TUJ1 antibody was used to stain AHPCs expressing class III beta-tubulin, indicating neuronal differentiation.

Results and Discussion

Previous data indicated that a soluble factor produced by astrocytes increased neuronal differentiation in AHPCs (Oh, under submission). To further investigate this effect, AHPCs were exposed to astrocyte conditioned medium in a variety of arrangements. Double conditioning, holding time, and holding temperature were varied. First we will look at double conditioned medium. The hypothesis under investigation in double conditioned medium cultures was that a feedback mechanism exists between the AHPCs and the astrocytes. In this feedback loop, AHPC exposure would cause astrocytes to further increase AHPC differentiation through stimulation of soluble factor production. To test this hypothesis, medium was transferred from an AHPC dish to an astrocyte dish, allowed to remain for 24 hours, and then transferred from the astrocyte dish to an AHPC dish. When neuronal differentiation was assessed by specific TUJ1 staining, there were no significant differences between the various AHPC cultures; DCM1, DCM2a, and DCM2b (3 replications, 2 plates per replicate). (Table 2)

It is important to note that dish DCM2a received only fresh medium, and was never exposed to any astrocyte produced factors. This is an interesting result because in several previous series of experiments, AHPC only cultures in AHPC/astrocyte co-culture medium averaged approximately 15% TUJ1 expression [68, 80]. So in this experiment there does not seem to be any feedback mechanism documented, nor is there any astrocyte mediated differentiation. However, the aforementioned studies replaced medium every second day, whereas this study replaced media daily. This may indicate that a removal of inhibitory factors is contributing to the heightened differentiation seen in the conditioned medium studies.

The hypothesis for time-delayed conditioned medium studies predicted that conditioned medium cultures would display less neuronal differentiation than non-contact co-cultures due to the decay of soluble factors released from astrocyte cultures. These short lived molecules would quickly decay and not be present in insufficient quantities to activate neuronal differentiation when AHPC cultures were fed once a day as opposed to being in constant diffusion contact as in insert co-cultures. To test this hypothesis, conditioned medium was removed and stored for 24 hours prior to addition to AHPC cultures. To determine whether any changes were enzymatic in nature, medium was stored both at 4° and 37° Celsius. Time delayed conditioned medium was compared to non-delayed conditioned medium from two different astrocyte cultures. Medium changes can be seen on the timeline below.

TUJ1 expression could increase with increasing volume fed if the soluble factor was present at neither too low nor saturated concentrations in the conditioned medium. In addition, soluble factor may be more concentrated in the astrocyte plates that have a 0.13 mL/cm^2 volume ratio than in the plates using a 0.5 mL/cm^2 ratio. This is intuitive, but may not be the case as cell growth and factor production may behave unpredictably in the different culture conditions. A comparison of the refrigerated conditioned medium to that stored in the incubator may reveal enzymatic changes to a soluble factor. If the refrigerated medium gives more neuronal differentiation than the incubated, enzymatic degradation may be occurring. If the refrigerated medium shows less differentiation, there may be an enzymatic activation step in the interaction. Results are shown in Table 3.

Again, there were no significant differences (□=0.05) between any of the treatments. Among the astrocyte conditioned media stored in the incubator, the mean neuronal expression did increase with volume fed and smaller volume per area, but only by about 5% which is not enough to be statistically significant given the standard deviations common in these experiments. The refrigerated medium (TDRC) had a smaller mean than the incubated medium (TDIC), under the same astrocyte source conditions and volume fed. Both of these had large standard deviations when compared to the other conditions, so it is not possible to conclusively state the presence of an enzymatic activation or degradation from these two conditions. A comparison of the treatments using 250 μL fed and astrocyte source media to area ratio of 0.50 mL/cm^2 reveals weakly significant differences between the time delayed feeds and double conditioned medium cultures, which have no time delay. It appears that if there is an effect on differentiation caused by medium storage, it is either small or not well documented by this series of experiments. (Figure 8)

In conclusion, since there were no differences or very weakly significant differences detected in TUJ1 expression between the different conditions, no feedback mechanism was documented in the double conditioned medium studies and no enzymatic control or molecules with an active lifespan in the time scale of these experiments were detected in time delayed conditioned medium. Double conditioned medium differentiation was similar to single conditioned medium differentiation and also to differentiation in AHPCs that were only used as sources for conditioning astrocyte medium. The levels of differentiation were higher than that seen in previous AHPC only cultures. One possible explanation for this is the fact that these cultures were fed daily, while previous experiments replaced medium only every other day. It is possible that a buildup of inhibitory factors was responsible for the low

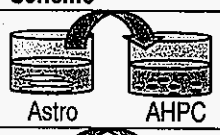
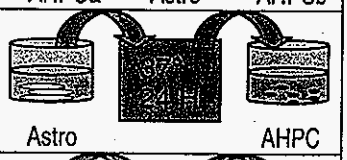
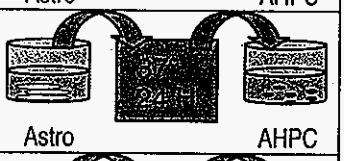
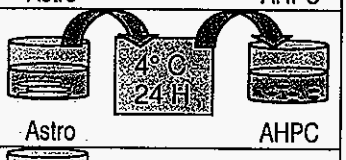
levels of differentiation noted in previous experiments where AHPC only cultures were fed every 48 hours rather than every 24 hours. Another possibility is that a feedback loop exists, but that the time scale is much shorter than the one day turnover used in these studies. If some intermediate molecules are very short-lived, the effect may only be detectable in non-contact co-cultures. Indeed, the differentiation levels in non-contact co-cultures, as seen from previous studies, are much higher than those in the various conditioned medium experiments. Differentiation levels in conditioned medium treatments with and without time-delay were similar. Whether conditioned medium was fed instantly, stored for 24 hours at 37°, or stored for 24 hours at 4°, the differentiation results were not different or very weakly significantly different from each other.

Since evidence shows that a secreted molecule is likely involved in the directed differentiation of AHPCs to neuronal fates, the possibility exists that the mechanism involves an ECM intermediate. In addition, previous work has only used laminin or laminin and poly-lysine as substrates to increase cell adhesion. By varying the concentration and composition of ECM protein substrates we can explore the role of laminin in AHPC differentiation. Four different co-culture arrangements were used; no astrocytes, contact co-culture, non-contact co-culture, and conditioned medium (without any time delay or double conditioning). Results are displayed in Table 3 and graphically depicted in Figure 9.

In contrast to results observed on laminin substrates, there is no significant difference between co-culture arrangements on ECL surface. While AHPC only and contact co-cultures are comparable between laminin and ECL, conditioned medium and non-contact co-cultures are quite different. Non-contact co-cultures on ECL are significantly different from laminin with p-values of 0.010 for 1 μg ECL and 0.020 for 10 μg ECL respectively. It appears, then,

that the neuronal differentiation increase seen on laminin substrates is not observed on ECL. This is an interesting result, since ECL contains laminin as a component. One hypothesis that would explain this disparity is that one of the other components of the ECL protein mixture, such as collagen, blocks or attenuates the activation of neuronal differentiation observed in astrocyte co-cultures. Another is that a certain minimum density of laminin is required for the activation to occur. Tests that can address these hypotheses will be discussed further in the future work section.

Table 1. Co-culture medium treatments and abbreviations.

Name	Astrocytes	Storage and volume	Abbreviation	Scheme
Control conditioned medium	0.5 mL/cm^2	No storage, 250 µl	CCM	
Double conditioned medium, one AHPC dish	0.5 mL/cm^2	No storage, 250 µl	DCM1	
Double conditioned medium, two AHPC dishes	0.5 mL/cm^2	No storage, 250 µl	DCM2a, DCM2b	
Time delay	0.5 mL/cm^2	24 hours in incubator, 250 µl	TDIA	
Time delay	0.5 mL/cm^2	24 hours in incubator, 400 µl	TDIB	
Time delay	0.13 mL/cm^2	24 hours in incubator, 400 µl	TDIC	
Time delay	0.13 mL/cm^2	24 hours in refrigerator, 400 µl	TDRC	
AHPC only	-	-	AHPC	

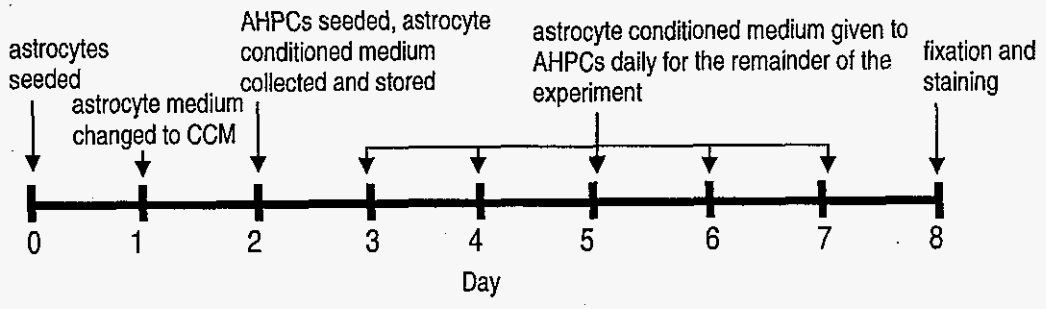


Figure 6. Time-delayed conditioned medium experimental timeline.

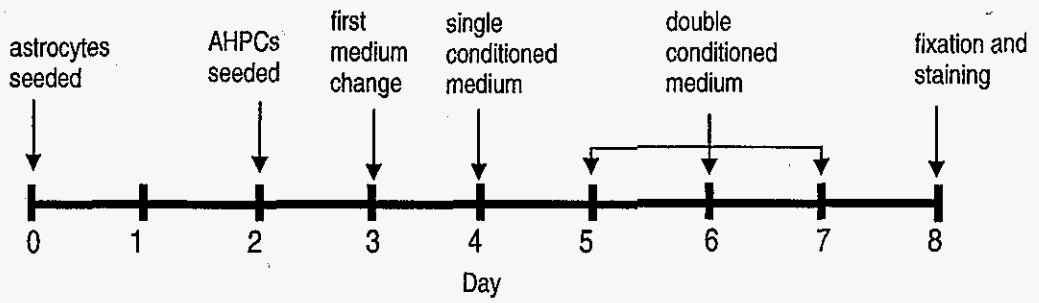
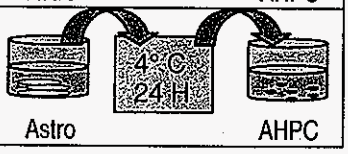


Figure 7. Double conditioned medium experimental timeline.

Table 2. Double conditioned medium results. Standard deviation based on 3 replicates. Bolded text indicates substrate examined.

Abbreviation	Arrangement	Mean TUJ1 expression	Standard deviation	
DCM1	AHPC <-> Astro	36.9%	7.3%	
DCM2a	AHPC -> astro	30.8%	4.2%	
DCM2b	Astro -> AHPC	32.9%	6.2%	

Table 3. Time-delayed conditioned medium results. Mean and standard deviation, three replicates.

Abbreviation	Arrangement	Mean TUJ1 expression	Standard deviation	
TDIA	0.5 mL/cm^2 24 hours in incubator 250 µl fed	22.1%	4.0%	
TDIB	0.5 mL/cm^2 24 hours in incubator 400 µl fed	28.5%	6.4%	
TDIC	0.13 mL/cm^2 24 hours in incubator 400 µl fed	34.0%	7.8%	
TDRC	0.13 mL/cm^2 24 hours in refrigerator 400 µl fed	27.3%	11.6%	

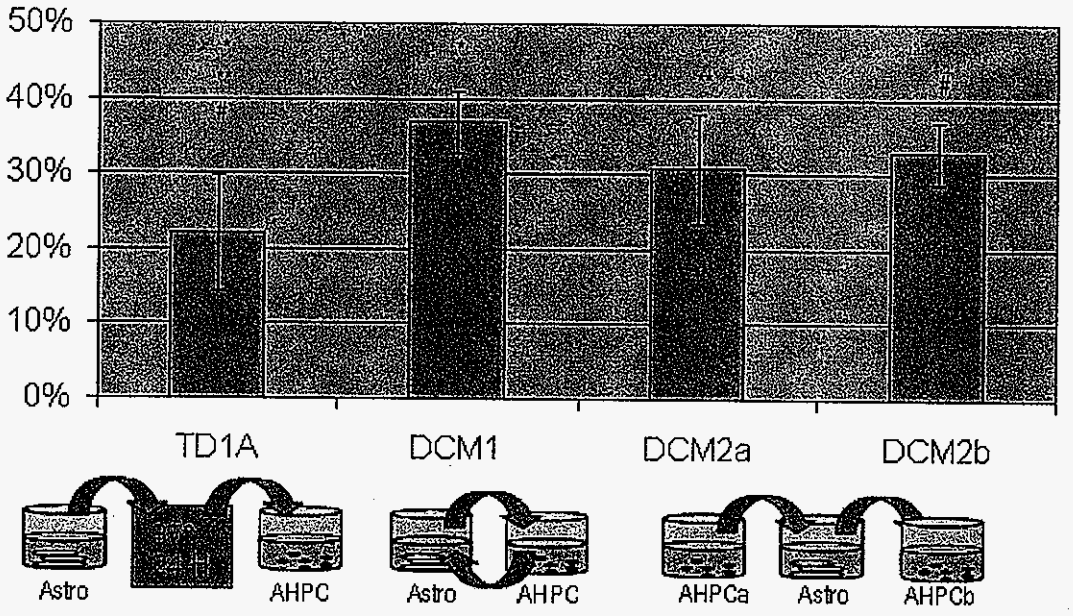
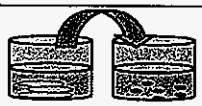


Figure 8. Significant differences in conditioned medium cultures (n=3, □=0.05)

Table 4. Protein substrate results. Mean ± one standard deviation, three replicates.

Abbreviation on Figure 9	Arrangement	ECL 1 µg/mL	ECL 10 µg/mL	Laminin 10 µg/mL	
AHPC	AHPCs cultured in co-culture media but with no astrocyte contact	16.7% ±3.3%	19.1% ±4.3%	15.6% ±5.8%	 AHPC
CCM	Astrocyte conditioned medium given to AHPCs daily	20.4% ±10.7%	21.7% ±9.5%	38.4% ±10.0%	 Astro AHPC
CONTACT	AHPCs seeded directly on top of a nearly confluent astrocyte layer	23.7% ±11.3%	21.6% ±8.9%	24.8% ±5.1%	 Astro and AHPC
INSERT	Astrocytes seeded in non-contact co-culture with a permeable membrane insert dividing astrocytes and AHPCs	27.7% ±8.2%	26.3% ±12.4%	54.0% ±7.98%	 Astro AHPC

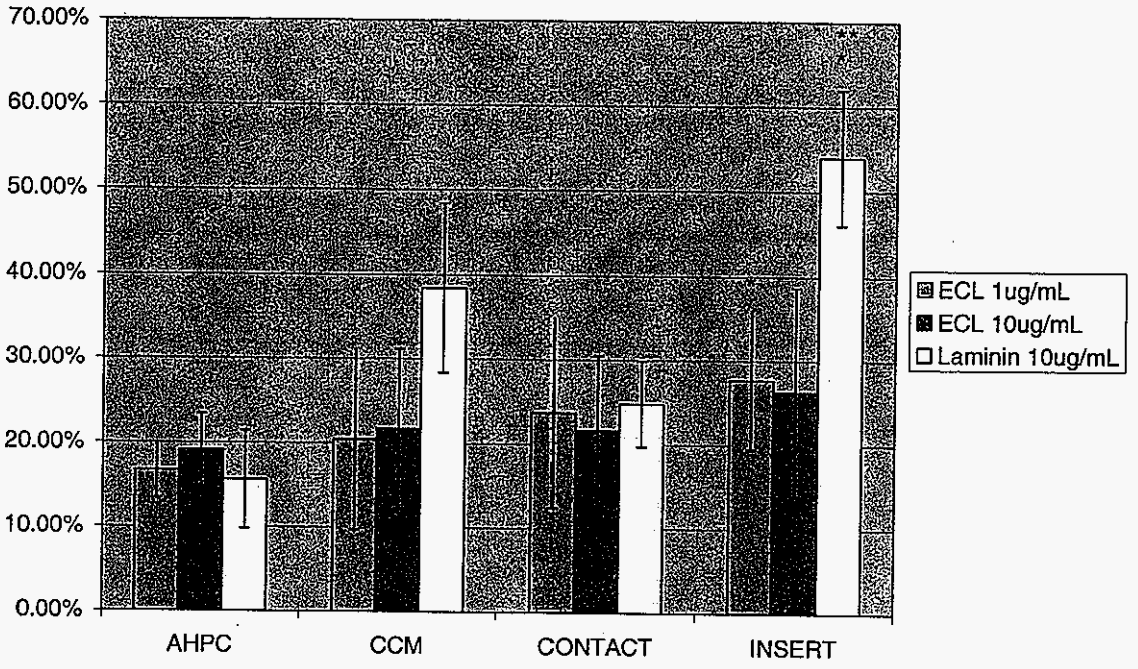


Figure 9. Graphical display of neuronal differentiation on protein substrates. Mean ± one standard deviation, three replicates

Chapter 3.

Conclusions and Future Work

The effects of several variations of astrocyte conditioned medium were tested on AHPCs. Double conditioned medium tests, in which astrocytes were fed AHPC conditioned medium, tested for the presence of a feedback loop in the signaling cascade that activates differentiation in AHPCs. There were no significant differences between any of the conditions, indicating that a feedback loop either does not exist or is not documented in this particular experimental setup. Conditioned medium results were not significantly different from AHPC plates that were not exposed to astrocyte factors, but the AHPC only expression of TUJ1 was higher than previously seen [68, 80]. This may indicate that daily medium change is contributing to the higher differentiation rate seen here. Time-delayed conditioned medium was tested for the effect of storage on decaying factors. There were no differences or very weakly significant differences between time delayed conditioned medium and non-delayed, and no significant differences in neuronal differentiation between storage of conditioned medium at 37° and storage at 4° Celsius.

Since it is possible that a feedback loop between AHPCs and astrocytes exists but is simply not being documented in the experiments, an additional experiment can be designed for the double conditioned medium. The hypothesis to be tested is that there is a short lived molecule in the feedback loop that is the rate limiting reagent. In an effort to bypass any short lived molecules to see the effect, we shall feed conditioned medium as previously described and compare it side by side with medium conditioned by astrocyte-AHPC non-contact co-culture (hereafter referred to as NCCM). The conditioned medium cultures will all be fed daily. This setup will initially allow us to verify that the astrocytes used for all

conditioned medium setups in the experiment do secrete molecules that increase neuronal differentiation in non-contact co-culture by assessment of differentiation in the AHPCs used along with astrocytes to create the NCCM. Once this is established, the presence of a short lived molecule in the signaling chain between astrocytes and AHPCs can be tested. If a short lived molecule acts directly on the AHPCs, no change will be noticed between the astrocyte conditioned medium (as discussed in detail previously) and the NCCM fed AHPCs. If a short lived molecule is present in the signaling chain, but a longer lived molecule acts on the AHPCs, the longer lived molecule may remain in the conditioned medium long enough to affect neuronal differentiation. An increase in TUJ1 expression may then be noted between AHPCs fed the conditioned medium as previously described and AHPCs fed NCCM.

For the protein substrate experiments, an array of substrates can be made that keep the total protein concentration constant but vary the amount of ECL and laminin. The hypothesis to be tested is that a component of the ECL cell adhesion matrix is preventing the increase in neuronal differentiation that has been noted in non-contact co-cultures on purified laminin. A diagram of this type of experiment can be seen in Figure 10. In this diagram, substrate coatings change from 100% laminin to 100% ECL in 20% increments. An additional substrate which combines 10 μg/mL ECL with 10 μg/mL laminin will control for changes in differentiation caused by decreasing amounts of laminin present. By combining pure laminin with ECL, we do not eliminate any of the components of the mixed substrate as possible antagonists for the neuronal differentiation. If changes in differentiation are noted across the substrates, we will have support for our hypothesis and motivation to further break down the components of ECL for individual testing.

In addition, a separate substrate can be set up with increased total protein content. In this substrate, after coating with laminin at 0.10 μg/mL, 0.10 μg/mL ECL will be applied. This combination substrate will have sufficient laminin to activate neuronal differentiation as measured previously but will also contain the additional components found in ECL. Neuronal differentiation on this substrate may remain high as in laminin or be low as observed on ECL, either of which will assist in defining the role of the extra components in ECL on neuronal differentiation in AHPCs.

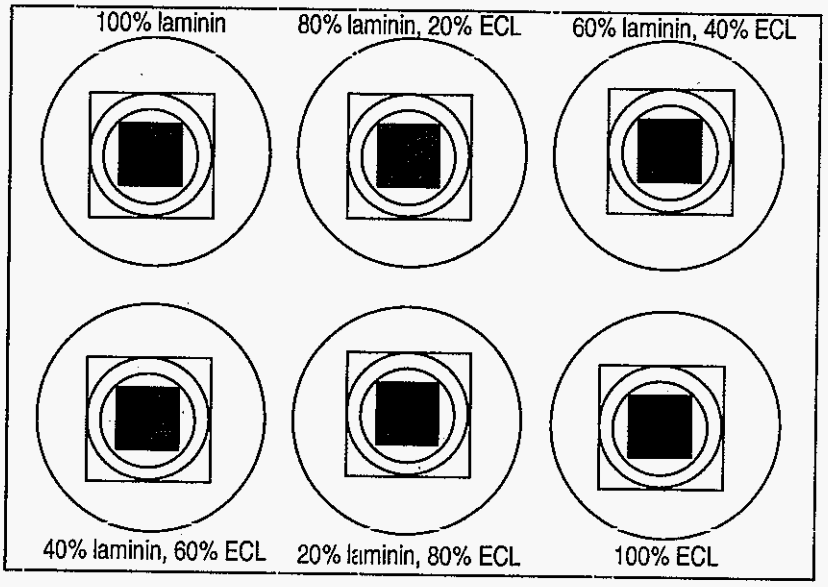


Figure 10. Laminin/ECL array to determine whether ECL components have an inhibitory effect on astrocyte co-culture induced neuronal differentiation.

Appendix. Table of agents and their effects on stem and progenitor cells.

Appendix. Table of agents and their effects on stem and progenitor cells.

Agent	Cell Type	Species	Influence of Agent on Cells	
HU210 (cannabinoid)	ESC	Rat	Increased proliferation of progenitor cells[39]	
μ and δ opioids	ESC	Mouse	Differentiation to neural progenitor cells[40]	
Docosahexaenoic acid (DHA)	ESC	Rat	Increased neuronal differentiation[42]	
Meteorin	Glial progenitor cells in an ESC population	Mouse	Astrogenesis[45]	
Stromal cell produced factors	ESC	Mouse	Increased differentiation to dopaminergic neurons[46]	
Astrocyte co-culture	ESC	Mouse	differentiation to 90% nestin expressing NPCs[48]	
	OPC	Rat	Oligodendrocyte precursor cells differentiate to neurons[75]	
IL-6, IL-10	NPC	Rat	Identified as specific factors produced by astrocytes upregulating neuronal differentiation in NPCs[76]	
ECM molecules	NPC	Human	Fibronectin	Some cell adhesion, outgrowth, and differentiation[64]
	NPC	Human	Matrigel™	More cell spreading and differentiation than fibronectin, similar cell number to laminin[64]
	NPC	Human	Laminin	Increased neuronal differentiation and longer neurite extension than Matrigel™ or fibronectin[64]
Laminin and 3-D pattern	NPC	Rat	Increased neuronal differentiation more than laminin alone[68]	
Poly-lysine and Laminin on a 3-D poly(styrene/divinylbenzene) scaffold	ESC derived neurons	Human	Increased neurite extension[54]	
IKVAV nanofiber	NPC	Mouse	Increased neuronal differentiation[70]	
Neurotrophins (NGF, NT3, and retinoic acid) on a PLA/PGLA scaffold	ESC	Human	More and better defined neural rosettes formed with neurotrophins than without on same scaffold[56]	
LAS sequential polymer substrate	NPC	Rat	Low density neurospheres	Extensive neurite outgrowth, little cell migration, and majority neuronal differentiation[59]
	NPC	Rat	High density neurospheres	Cell migration away from neurosphere, majority astrocytic differentiation[59]
	NPC	Rat	Low density neurospheres with serum	Neurospheres breakdown into confluent monolayer, majority astrocytic differentiation[59]
EVAL substrate	NPC	Rat	Cells remain in a quiescent state[61]	
Electrospun fibrous scaffolds	NPC	Mouse	Increased differentiation on nanoscale fibers as compared to microscale fibers[73]	

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