ENGINEERED N-CADHERIN SUBSTRATES FOR STEM CELL DIFFERENTIATION

BY

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THESIS

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Abstract

In the case of injury, aging, or disease, tissues in the body may have a limited capability to regenerate. As a result, great interest have been put into tissue engineering as a way to assemble functional constructs that restore, maintain, or improve those tissues. Certain success was made to modulate neural differentiation of stem cells using soluble factors and cell adhesion matrix properties. These differentiation studies are often dependent on the density of cells plated on the substrate, which implies that there is an important role of cadherin-modulated cell-cell adhesion in regulating stem cell differentiation levels. As a result, the goal for this research is to develop a hydrogel platform which integrates the effects of N-cadherin and matrix stiffness to modulate cell differentiation. To create this system, Fc-tagged N-cadherins are attached to alginate gels of varying stiffness through Protein A, which is chemically bound to the surface. Different cells known to express N-cadherin were seeded onto the gels, with the bone marrow stromal cells (BMSCs) showing the best adhesion properties. This study thus demonstrates that N-cadherin substrates can be used to promote cell adhesion, and may be useful for differentiation studies with BMSCs.

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Chapter 1: Introduction

The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve weakened tissues. Unlike many other tissues in the human body, the tissues in the nervous system have a limited capability to regenerate in response to injury or disease. In addition, even though skeletal muscles have robust regeneration, damage to the skeletal muscles is common due to strains during sports activities and crush trauma due to accidents. In these instances, the muscles would not be able to regenerate, and the muscles would lose functionality. Cell transplantation has thus been seen as a promising way to replace damaged tissues and to stimulate growth of new neurons. Furthermore, *in vitro* platforms have been developed in which to model the interactions of potential transplanted cells with different surfaces and to assess their differentiation and proliferative properties.

Of the many different cells, bone marrow stromal cells, in particular, have emerged as a clinically relevant cell source for regenerative medicine due to their accessibility and potential to differentiate into various mesenchymal lineages.[1,2] Various methods have been developed, many of them through a combination of different cell types, soluble factors, and scaffolds. In order for differentiation to occur, these methods depend on the density of cells coated on the substrate, as cell-cell contact is important for signaling between the cells. N-cadherin, in particular, is one of the cell adhesion molecules that is prominently expressed in various cell types including myoblasts and neuron precursor cells, and plays an important role in regulating cell differentiation.

Previous studies have looked at the effects of N-cadherin on neural, embryonic, and induced pluripotent stem cells on neural differentiation, as well as bone marrow stromal cells on myogenic differentiation; however, no research thus far has looked into the effects of substrate stiffness on differentiation. This is mainly due to the lack of hydrogel platforms that are available to address these conditions. As a result, the goal of this study is to develop a novel hydrogel platform, which integrates the effects of N-cadherin and matrix stiffness to promote first cell adhesion for differentiation studies. The hypothesis is that cells which express N-cadherin would be able to adhere to the gels immobilized with Fc-tagged N-cadherin on the surface. Furthermore, N-cadherin would be able to modulate the level of stem cell differentiation through intracellular signaling at different levels dependent on the stiffness for which the cells are adhered to.

To test this hypothesis, hydrogels with stiffnesses mimicking the brain, muscle, and collagenous bones would be developed and the surface would be conjugated with Fc-tagged N-cadherin. Collagen, which is an extracellular matrix protein commonly used for cell studies, was used as a control to compare the adhesion and differentiation properties of the cells on top of a collagen versus an N-cadherin surface. The ability to use this platform to study N-cadherin and matrix stiffness interactions on cell adhesion and

later cell differentiation provides the field of tissue engineering with a unique way of modulating cell differentiation. Not only would be it useful for clinical studies regarding tissue regeneration, but it would also be useful for fundamental studies to look at how cells respond to N-cadherin and matrix stiffness *in vitro*.

Chapter 2: Literature Review

The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve injured or damaged tissues. For instance, the tissues in the nervous system, unlike other tissues in the human body, have a limited capacity to generate new functional neurons in response to injury or disease. As a result, there is great interest in the ability to repair the nervous system through alternative cell sources that can be transplanted and be used to replace damaged neurons and to stimulate the growth of new neurons. Various stem cell sources such as neural stem cells, embryonic stem cells, mesenchymal stem cells, and olfactory ensheathing cells, have been explored as candidates for the treatment of injuries and treatment of diseased tissues of the central nervous system.[3] Research in tissue engineering seek to provide a readily available cell source to repair and regenerate dead or damaged tissues through a combination of cells, soluble factors, and scaffolds. The types of cells, growth factors present, matrix stiffness, and extracellular matrix proteins have all been shown to influence the proliferative and differentiation properties of the cells. This chapter will be a literature review on work that has been done in relation to neural differentiation. This covers alginate gels as tissue engineering scaffolds, the effects of matrix stiffness on stem cell fate, the role of N-cadherin on neural differentiation, and bone marrow stromal cells as a cell source to study neural differentiation.

2.1 Hydrogels as scaffolds for tissue engineering

Soft hydrogels are often used for fundamental biological studies and tissue engineering as they have structural and mechanical properties close to the extracellular matrix (ECM) *in vivo*. As a scaffold, the hydrogels play the role of an artificial extracellular matrix, which can provide a platform for which cells can grow. For instance, hydrogels can be functionalized with ligands or amino acid sequences which can specifically bind to the receptors on cells and support cell adhesion and control cell function.[4] Additionally, bioactive molecules such as drugs or growth factors can be incorporated and released from the hydrogel, which can further promote and regulate new tissue development.[5] The mechanical properties of the hydrogels can also be altered to mimic the natural microenvironment in the body. By controlling the relevant mechanical and chemical properties, hydrogels are widely used to model ECMs to study cell-material interactions and cellular processes *in vitro*.

Alginate is a naturally occurring anionic polysaccharide copolymer which contains irregular blocks of mannuronic acid (M black) and guluronic acid (G block) residues. It is commonly used for many biomedical applications, due to its low immunogenicity, biocompatibility, relatively low cost, and ease of functionalization.[6] Alginate gels can be ionically cross-linked with the addition of divalent cations such as calcium,[7] or they can be chemically cross-linked with diamine cross-linkers such as adipic acid

dihydrazide (AAD). Since cells generally do not adhere to alginate gels, peptides and proteins can be easily coupled to the alginate backbone. Arginine-glycine-asparatic acid (RGD), for instance, is a tripeptide sequence from fibronectin that is crucial for cell adhesion through integrin, and can be coupled to the alginate backbone through carbodiimide chemistry.[8] Due to the number of free hydroxyl and carboxyl groups distributed along the backbone, various methods to chemically modify alginate are also possible.[9]

2.2 Matrix stiffness regulates stem cell fate

Typically, due to simplicity and cost, stem cells are usually cultured on rigid polystyrene tissue-culture plastic; however, these culture conditions are very different from the environment cells are exposed to *in vivo*. Matrix stiffness has been shown to play an important role in the regulation of stem cell fate. Engler, et al. cultured mesenchymal stem cells on collagen-coated surfaces with different elastic modulus, and differentiated them in the appropriate induction medium.[2] On soft, collagen-coated gels that mimic the elasticity of the brain (0.1-1 kPa), mesenchymal stem cells (MSCs) that had adhered and spread resulted in a highly branched morphology similar to primary neurons on matrigel. However, on gels that mimic muscle elasticity (8-17 kPa), the differentiated cells were similar in shape to C2C12 myoblasts. On stiff surfaces, the morphology of the MSCs was similar to osteoblasts. Furthermore, transcriptional profiles showed that the stiffness corresponds to increases in their specific markers. With neural stem cells, on softer matrices, neuronal differentiation is preferred; however, on harder matrices, glial differentiation is preferred.[10]

2.3 N-cadherins

Extracellular matrices (ECMs) play an important role in regulating cellular behaviors such as cell attachment, proliferation, morphogenesis, and stem cell differentiation.[4] In particular, the formation of neural circuits depends on cues from the extracellular matrix environment and intracellular signaling. In particular cell-cell adhesion is extremely important. Cell adhesion molecules (CAMs) include cadherins, integrins, selectins, and the immunoglobulin (Ig) superfamily of CAMs. They can not only attach cells to each other, but they can also act as mechanotransducers and mediate neurite growth.[11,12]

Of the many cell adhesion molecules, one key neural adhesion molecule expressed during neural differentiation are cadherins. Cadherins are calcium-dependent proteins that play an important role in cell-cell adhesion. Depending on the cell type and the stage of their development, different cadherins can be expressed on the cell. For instance, it has been shown that mouse embryonic stem cells (ES) have been

shown to express high levels of E-cadherin. Upon neural differentiation, ES experiences a downregulation of E-cadherin and up-regulation of N-cadherin. [13]

2.4 Bone marrow stromal cells

Bone marrow stromal cells (BMSCs) are a heterogeneous population of cells, which have the potential to differentiate into osteogenic, chromogenic, myogenic, neuronal, and fibroblastic lineages.[1,2] Due to their multipotent differentiation, they have emerged as a clinically relevant cell source for regenerative medicine. Compared to neural stem cells, BMSCs are more easily harvested and effectively expanded, which makes them an ideal platform for fundamental studies and clinical studies, if they can be successfully differentiated into neurons. While the use of embryonic stem cells (ES) have also been shown to differentiate, there are ethical concerns concerning the use of ES as they are derived from embryos. Meanwhile, several reports have shown that BMSCs could differentiate into neurons and glia in vitro by the presence of growth factors or by simple chemical induction. One study, for instance, showed that mouse and human BMSCs could differentiate to more than 70% neurons *in vitro* after treatment via chemical induction with β-mercaptoethanol (BME) in serum-free media.[14] Another study showed that BMSCs cultured in the presence of epithelial growth factor (EGF) or brain derived neutrophic factor (BDNF) resulted in cultures which expressed glial fibrillary acidic protein (GFAP) and neuron-specific nuclear protein (NeuN).[15] Furthermore, BMSCs that were intravenously administered on rats have also been shown to differentiate *in vivo*, and can be used for the treatment of traumatic brain injury.[16]

Chapter 3: Experimental Section

3.1 Materials

Alginate with a molecular weight of 250,000 g/mol was provided courtesy of FMC Biopolymer. 2-(N-morpholino)ethanesulfonic acid (MES), Hydroxybenzotriazole (HOBt), adipic acid dihydrazide (AAD), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Thermo Fischer.

3.2 Preparation of alginate gel

Alginate gels were formed using carbodiimide chemistry. 2% sodium alginate solution was prepared in 0.1 M MES buffer (pH = 6.5). Then, HOBt, AAD, and EDC was sequentially added to the alginate solution. The molar ratio of uronic acid: HOBt: EDC was kept constant at 2:1:2, and the ratio of AAD to uronic acid was varied from 0.01, 0.025, and 0.5. The solution was polymerized between two glass plates separated by a 1.2 mm spacer. After 1 hour, the gel was cut into discs with 5 mm diameter, and placed in phosphate buffer saline (PBS) overnight at room temperature to remove unreacted chemicals.

The elastic moduli of the alginate gels were calculated from the stress-strain curve obtained with an Instron mechanical tester. The gels were uniaxially compressed at a deformation rate of 1.0 mm/min with a 250 N load cell at 25°C, and the elastic modulus was calculated from slope of the linear portion of the stress-strain curve. The swelling ratio was calculated by measuring the ratio of the hydrated mass to the dry mass of the gel. The hydrogel discs were lyophilized in order to obtain the dry mass of the gels.

3.3 Synthesis of N-Cad-Fc

The extracellular region of chicken N-cadherin fused to a mouse Fc tag (N-Cad-Fc) was stably expressed in HEK293 cells, as previously described.[17] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Invitrogen) and 0.4 mg/ml G418 (Sigma, St Louis, MO) as a selection marker. The N-Cad-Fc fusion protein was filtered and purified from the cell supernatant using a Protein A affinity column (Bio-Rad) followed by gel filtration chromatography.

3.4 Protein immobilization to gel surface

To immobilize N-Cad-Fc to the gel surface, the gels were activated by incubating the gels in 5 mg/mL EDC and 5 mg/mL HOBt dissolved in MES buffer for 20 min. Following three washes to remove

unreacted EDC and NHS, the gel surface was coated with 0.2 mg/mL Protein A (PA) for one hour at room temperature. Following another three washes with PBS, the gel surface was further coated with 0.1 mg/mL N-Cad-Fc at room temperature.

To functionalize the gel surface with collagen, EDC and NHS were also used to activate the gel surface. 0.1 mg/mL bovine collagen (Advanced BioMatrix) was coated onto the cells for 1 hour at room temperature and the unreacted collagen was then rinsed off.

3.5 Cell culture

Mouse bone marrow stromal cells (D1, BMSCs) cells and mouse myoblast cells (C2C12) were purchased from the American Type Culture Collection (ATCC). Mouse cortical stem cells (NSCs) were purchased from R&D systems. The BMSCs and C2C12s were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine horse serum (FBS) and 1% penicillin streptomyocin (PS), and were passaged before confluency using 1 mM EDTA with 0.2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). NSCs were expanded as neurospheres in neurobasal media supplemented with N-2 Plus-supplemented neural cell medium and with epidermal growth factor (EGF) and fibroblast growth factor (FGF) according to the manufacturer's instructions (R&D systems). The neurospheres were partially dissociated into single cells by pipetting. For single cell studies, 10,000 cells per cm^2 were seeded onto the gels. 100,000 cells per cm^2 were seeded onto the gels for differentiation studies. Fresh growth medium was added after 90 minutes. Cells were changed into differentiation media when they are subconfluent or 48 hours after seeding. Neurogenic mesenchymal differentiation media for BMSC was bought from PromoCell. DMEM with 10% horse serum (Gibco) and 1% PS was used for the differentiation of C2C12.

3.6 Immunofluorescence staining and microscopy

After 24 hours, the cells were imaged using a light microscope (Leica DM IL) and then fixed with 4% formaldehyde for 10 minutes, permeabilized with 0.01% Triton X for 20 min, and blocked for non-specific binding with 1% BSA for 30 min. The actin and cell nucleus were fluorescently labeled with Alexa Fluor 488 – phalloidin for one hour and DAPI for 10 minutes, respectively. The cells were visualized using a laser scanning confocal microscope (LSM 700, Carl Zeiss Microimaging).

Chapter 4: Results and Discussion

4.1 Hydrogel characterization

As matrix stiffness has been shown to modulate the level of differentiation, it was important to be able to design a platform that would mimic the matrix stiffness conditions *in vitro*. To do this, alginate was chosen as the ideal substrate. Alginate, when formed into a hydrogel, is biodegradable, has low immunogenicity, and is easy to functionalize. To mimic the matrix environment for tissues *in vivo*, alginate gels of different stiffnesses were made by altering the ratio of the adipic acid dihydrazide (AAD) cross-linker to uronic acid group unit in alginate (Figure 1). Mechanical tests showed that a molar ratio of 0.01, 0.025, and 0.05 formed hydrogels of similar stiffness to that of brain, muscle, and collagenous bone respectively.[2]

4.2 Cell adhesion on N-cadherin substrate

To promote cell adhesion to the gel, the cells were immobilized with either collagen or N-cadherin. Cadherin-mediated intercellular adhesion is dependent on the extracellular domains 1 and 2 (EC12),[18] and in order to optimize the presentation of the cadherins so that the EC12 domains and are able to bind with the N-cadherins on the cells, Fc-tagged N-cadherins were used. Direct chemical conjugations pose a risk in which case the cadherin molecule can be chemically modified and as such, reduce cell adhesion to hydrogels.[19] In this study, the gels were modified with Protein A through carbodiimide chemistry, and then immobilized with Fc-tagged N-cadherins which bind to the protein A through the Fc-tag. (Figure 2). Fluorescent images of Alexa Fluor 555 labeled N-Cad-Fc on the gel showed similar levels of N-cad-Fc regardless of gel stiffness (not shown).

To test for cell adhesion of the gels, different types of cells known to express N-cadherin were seeded onto the gels, and were imaged after 24 hours of seeding. These cells included C2C12 myoblast cells, bone marrow stromal cells, and neural stem cells.

C2C12 myoblast cells exhibit both N-cadherin and M-cadherin on the cell surface. Beads coated with N-cadherin-specific ligands have been shown to facilitate myogenic differentiation, independent of cell plating density.[20] As a result, there is great potential for the use of N-cadherins to enhance the differentiation of skeletal muscles, and it would be interesting to test whether stiffness further modulates myogenic differentiation. C2C12 cells were seeded onto hydrogel surfaces with stiffness similar to muscle tissue, and the cell morphology was monitored at 2 and 12 hours after seeding. (Figure 3) To determine whether the binding is cadherin specific, various conditions were used: no protein was attached to the gel, only Protein A, N-cadherin through carbodiimide chemistry, and N-cadherin with Protein A. It

can be seen that there were little cell spreading within the first 2 hours. At 12 hours, it can be seen that cells had spread on surfaces with both Protein A and N-Cadherin, some spreading can be seen for gels that were chemically conjugated with N-Cadherin, and very little to no spreading can be seen with Protein A and without protein. This shows that cell spreading is specific to N-cadherin, and the N-cadherin hydrogel substrate supports cell adhesion.

When neural stem cells were seeded onto the cadherin and collagen coated substrate, little to no cell attachment could be seen (Figure 4). Even though neural stem cells are commonly found on neural cells, studies have shown that they have high levels of E-cadherin. Upon neural differentiation, neural stem cells down-regulates the expression of E-cadherin and up-regulates N-cadherin.[21] Interestingly, the neural stem cells also did not adhere to collagen.

When bone marrow stromal cells were seeded on the gels, within two hours, cell adhesion was seen, notably on gels with stiffer substrates. (Figure 5). BMSC adhesion was prevalent on gels on stiffer substrates, with little difference between collagen or cadherin present on the surface. Softer gels showed less cell adhesion and fewer number of cells adhered to the gel. Control gels without collagen or N-cadherin showed little cell attachment. This is different from C2C12 cells, in which most cells remain rounded even at 3 hours (not shown), or have not adhered even following three days in culture for NSCs.

Cell adhesion studies showed that surface proteins affect cell adhesion to gels. C2C12, BMSCs, and NSCs all express N-cadherins; however, the various levels of N-cadherin and the role of N-cadherin in cell regulation for these cells are different. This may therefore contribute to which cells can be maintained on surfaces with only N-cadherin. Of the three cell types, BMSCs showed the best cell adhesion. While C2C12 cells still adhered to N-cadherin substrates, they did not proliferate, which suggests the N-cadherin inhibits C2C12 proliferation. On the other hand, C2C12 cells proliferated on collagen surfaces.

4.3 Single cell analysis

When cells are seeded onto a material, cells undergo cell adhesion. During attachment of the cells to the substrate, cells flatten and spread, and in the process, the actin skeleton is reorganized. For collagen surfaces, the adhesive interaction between the cells and the substrate are driven by integrin-mediated adhesion. However, in the case of cadherin substrates, the initial receptor-ligand pairs are the result of cadherin interactions.

C2C12 myoblast cells were seeded at a low density onto either a cadherin or a collagen coated alginate gel surface with various elastic moduli. (Figure 6) While there were no significant difference in

the nuclear aspect ratio; there were changes in the cell circularity. On soft hydrogels, the cells were more circular with smaller surface area, whereas for stiffer hydrogels, the cell circularity was smaller and there was a larger cell surface area. This was as expected since normally cells adhere better on stiffer substrates.

Similar to the C2C12 cells, bone marrow stromal cells were seeded at a low density onto either a cadherin or a collagen coated alginate gel surface of various elastic moduli. Collagen, which is a commonly used extracellular matrix protein, was used for comparison purposes. After 24 hours, single cells were analyzed for changes in nuclear aspect ratio, cell circularity, and cell area (Figure 7). When cells are initially seeded on the gels, they were circular and had low cell area. Over time, as cells interact with the surface, they can adhere to the gel surface and as a result, cell spreading can be seen, with increases in cell area and decreases in cell circularity. The nuclear aspect ratio showed no significant difference between conditions; however changes could be seen in terms of cell circularity and cell area. For soft and intermediate matrices, the cells on N-cadherin substrates remain mostly rounded, as evidence by their low surface areas and circular features. However, on collagen substrates, the cell area increased, and cell circularity decreased, which shows that it had adhered and spread on soft substrates. Like N-cadherin, the cells were not spread on gels with intermediate stiffness. Overall, BMSCs showed increased spreading on the stiffer gels.

4.4 Stem cell differentiation

After the cells adhere to the gel, it is necessary to test how they differentiate. Both the medium from C2C12 and BMSCs were switched from growth to differentiation media, in order to induce differentiation. Interestingly, while C2C12 cells may adhere to gels with N-cadherin, when differentiation medium was added, cells would peel off the gel in a sheet. Even before differentiation, the C2C12 cells would bunch together (Figure 9 in Appendix). BMSCs showed similar problems; however, morphological changes can be seen in some of the remaining cells that were adhered on the gel. Figure 8 shows the differentiation of BMSCs on soft substrates (2 kPa) and stiff substrates (30 kPa). At day 0. The cells were spread out on both the soft and stiff gels conjugated with collagen. After 2 days of differentiation, many of the cells bunch together and few of them exhibited long spindle-like extension, which is very notable on stiff gels. The cell density for cells adhered to N-cadherin was very low. However, of the cells that were remaining on the gel, cells on the stiff N-cadherin surfaces exhibited very noticeable spindle-like morphologies. The cells on the soft N-cadherin hydrogel, however, still remained circular.

While several studies have shown that the addition of growth factors or simple chemical induction can lead to increased expression of markers for neurons and glial cells,[14] the functionality of the resulting differentiated cells is questionable. One study has shown that cell lines that do not differentiate

into neurons also showed similar morphological changes typical of neural differentiation, which can be due to cellular response to environmental stress.[22] Similarly, patch clamp tests showed that the differentiated neurons using our differentiation protocol did not show electrochemical changes (Figure 10 in Appendix). In a patch clamp assay, a small section of the cells is pinched, which allows for the flow of ions in and out of the device. The device is able to measure the changes in ion flow, and thus allows for the analysis of electrical properties to determine the functional connectivity of neurons. Studies have shown that using the conditioned media of neural stem cells[23] improves the differentiation of bone marrow stromal cells, and might be useful for generating functional neurons.

4.5 Figures

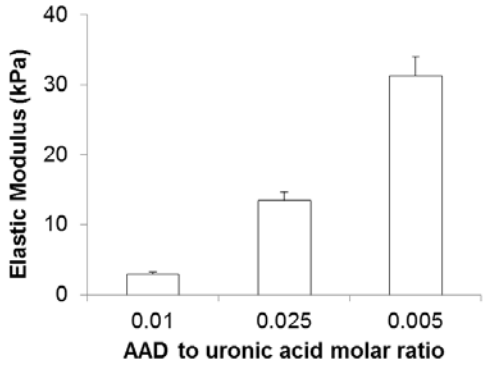

Figure 1: Elastic modulus of the alginate hydrogel with different adipic acid dihydrazide (AAD) cross-linker molar ratios. (n=3)

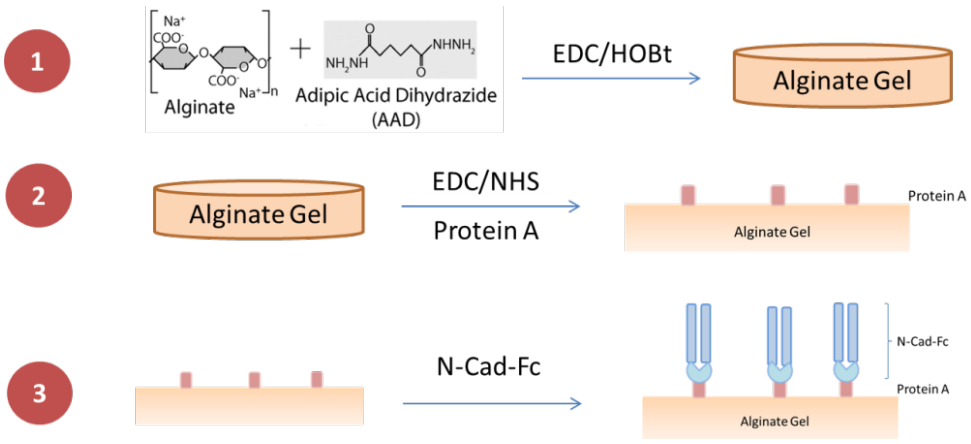

Figure 2: Scheme for the immobilization of N-cadherin onto the gel surface.

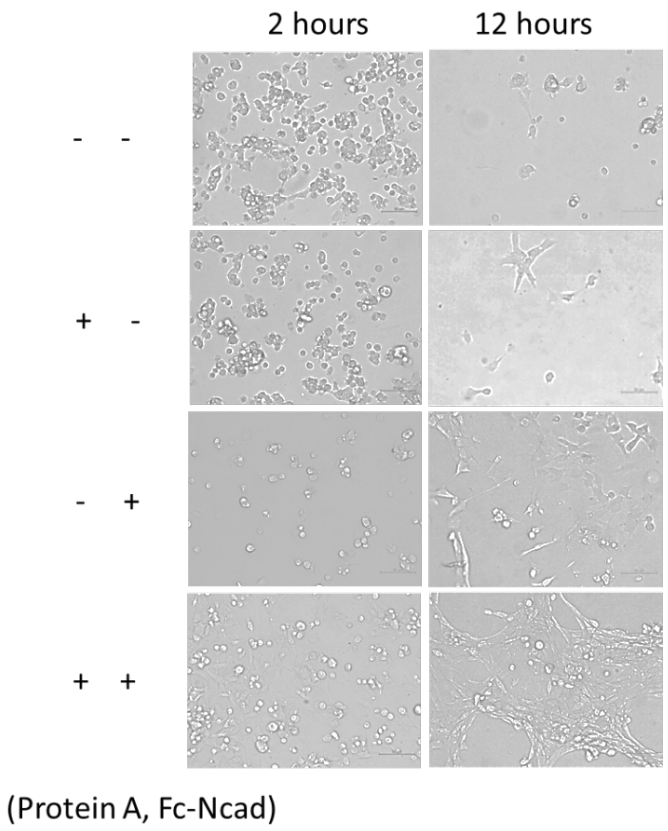

Figure 3: C2C12 myoblast cell adhesion to hydrogels at a stiffness of 10 kPa with different surface proteins after 2 and 12 hours.

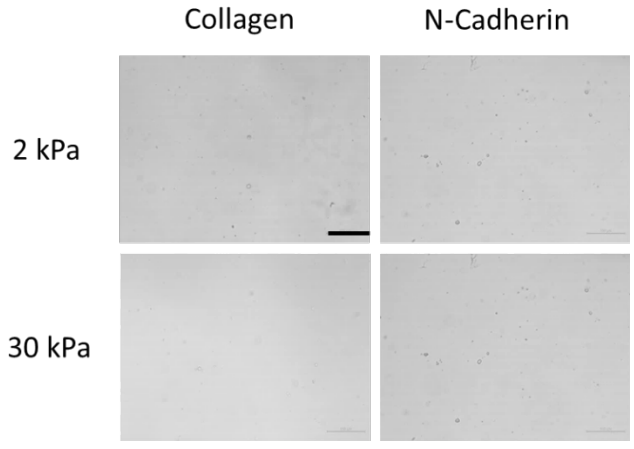

Figure 4: Neural stem cell (NSC) adhesion to hydrogels conjugated with different surface proteins at 24 hours after seeding. (Scale bar represents 100 μm.)

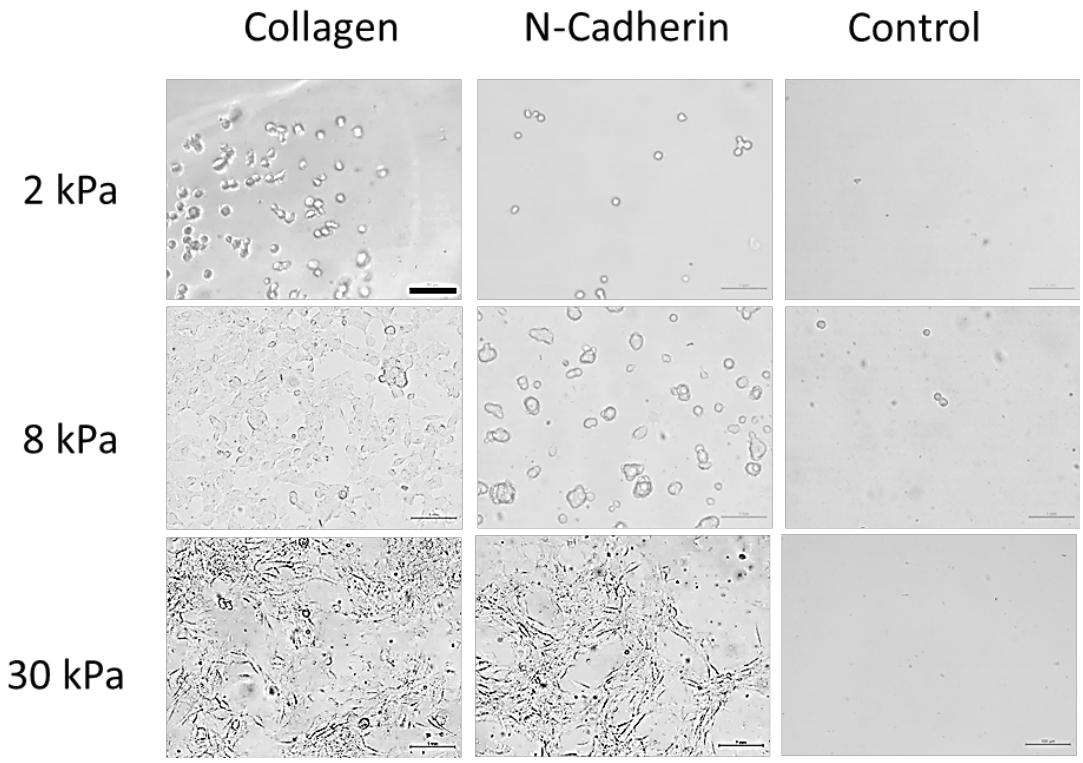

Figure 5: Bone marrow stromal cell adhesion after 2 hours of cell adhesion for gels of different stiffnesses and different surface proteins. Scale bar represents 100 μm.

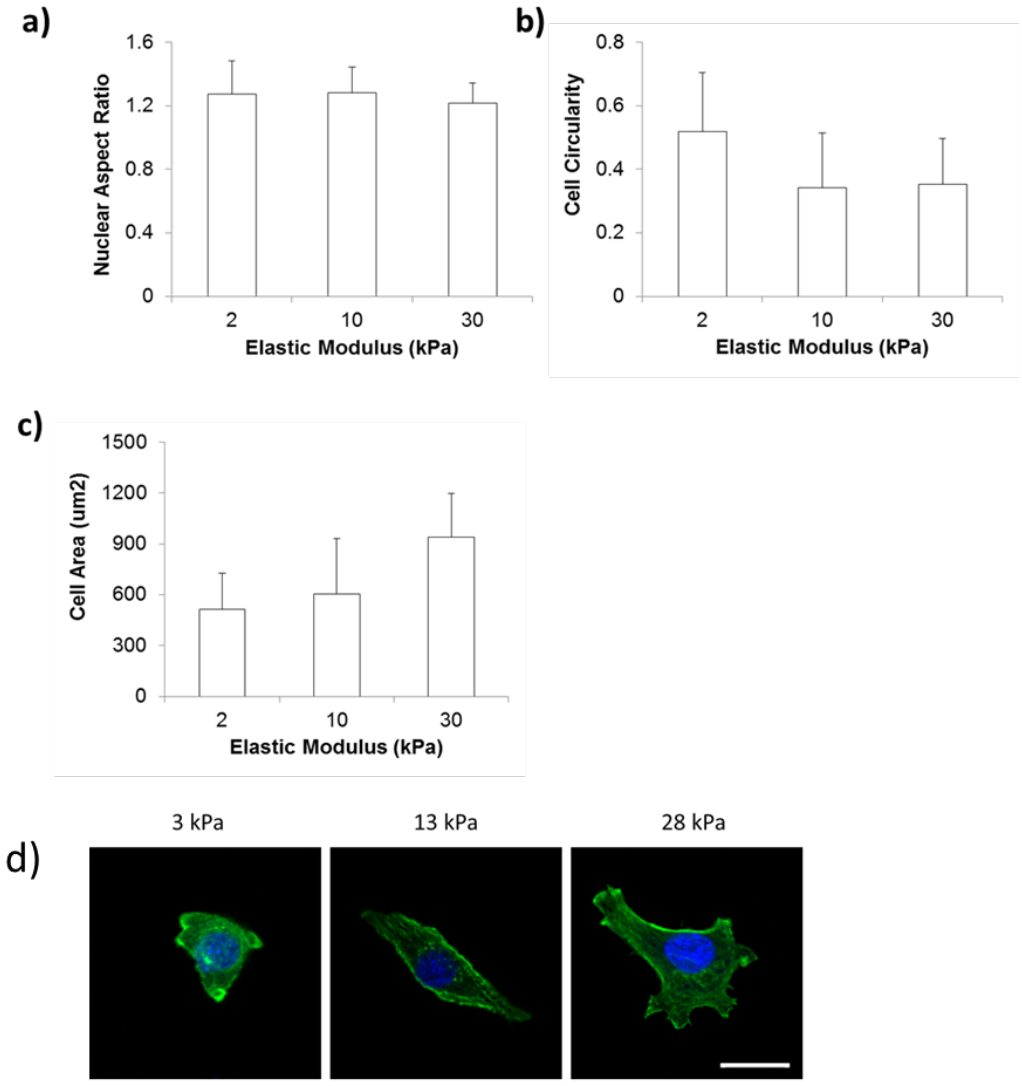

Figure 6: Single cell analysis for C2C12 cells seeded at a low density on ▩ N-cadherin or □ collagen surfaces after 24 hours with regards to (a) nuclear aspect ratio, (b) cell circularity, and (c) cell area. (n=3, >20 cells were analyzed) (d) Representative fluorescent images of the single cells are shown, where blue and green represents the cell nucleus and actin, respectively. Scale bar represents 20 μm. (n=3, >20 cells were analyzed)

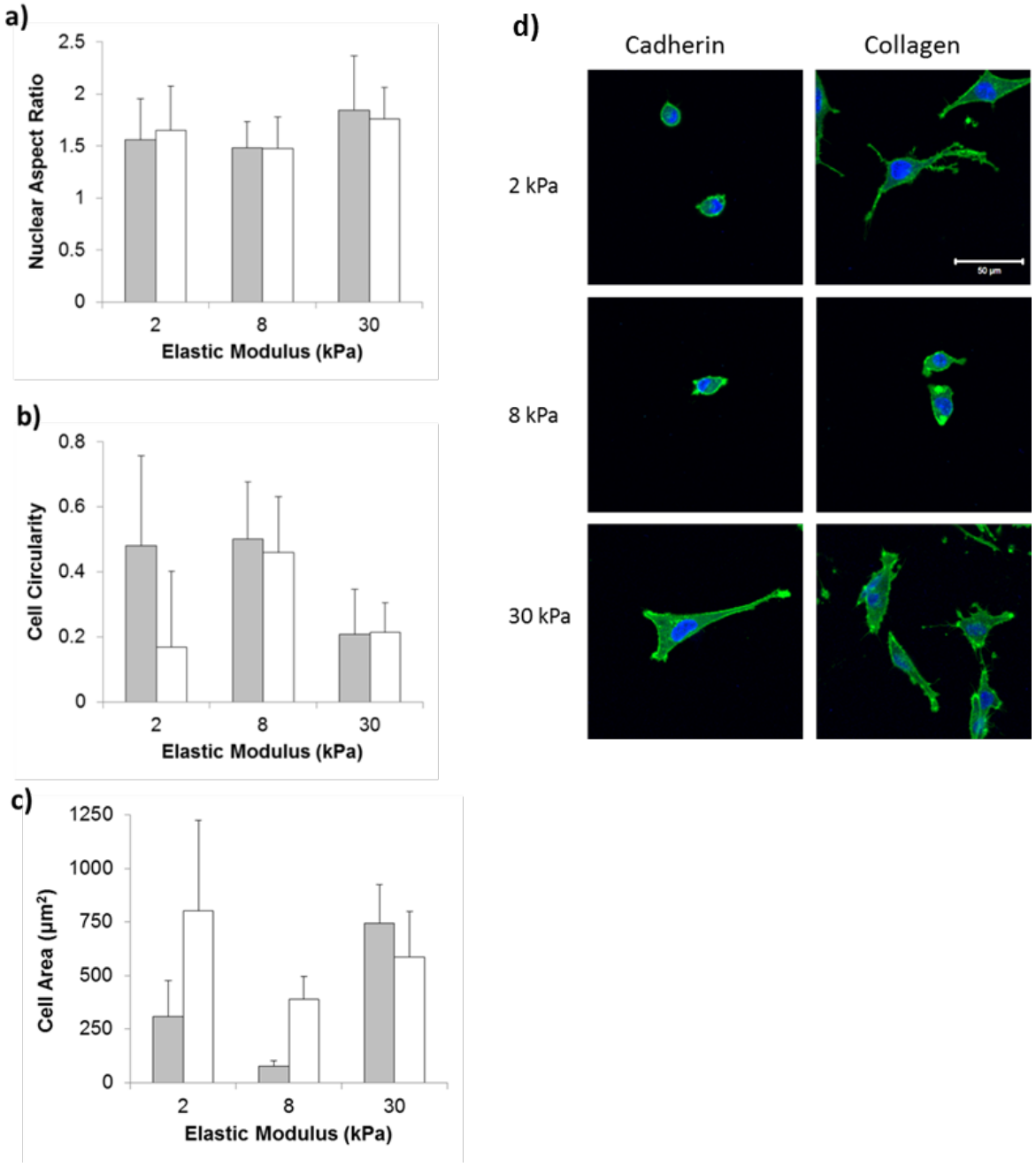

Figure 7: Single cell analysis for bone marrow stromal cells seeded at a low density on ▭ N-cadherin or □ collagen surfaces after 24 hours with regards to (a) nuclear aspect ratio, (b) cell circularity, and (c) cell area. (d) Representative fluorescent images of the single cells are shown, where blue and green represents the cell nucleus and actin, respectively. Scale bar represents 50 μm. (n=3, >20 cells were analyzed)

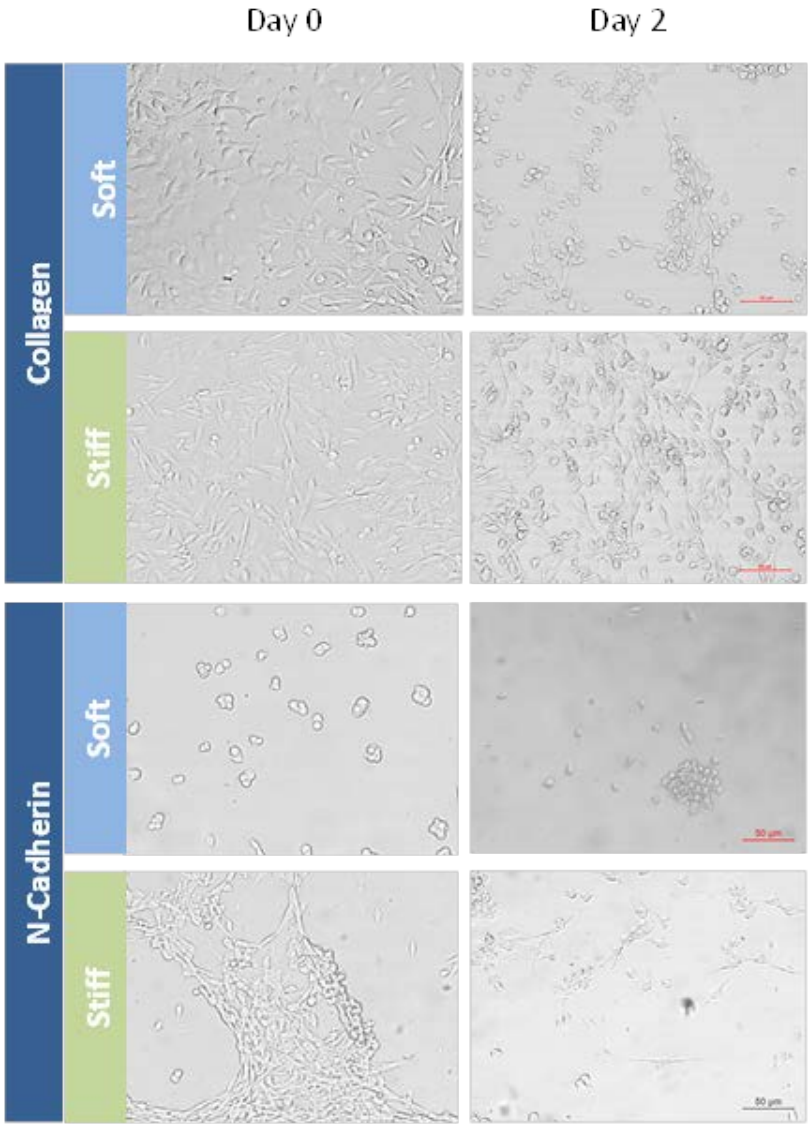

Figure 8: Time course for differentiation of BMSCs on substrates with different proteins and different stiffnesses. Scale bar represents 100 μm.

Chapter 5: Conclusions

In this study, we have developed a platform to adhere cells through N-cadherin interactions *in vitro*, while mimicking the mechanical properties of cells in their natural environment. The cells which can adhere are specific to cells with N-cadherin on the surface. While cells can adhere to the N-cadherin onto the gels with varying levels of cell adhesion, their level of cell adhesion and morphology changes in the presence of differentiation medium. Using this platform it would be possible to study how matrix stiffness and the presence of N-cadherin can be used to modulate stem cell differentiation. Future studies would have to be done to confirm whether there is an increase in expression markers for neural differentiation; however, the results from this study show promise in the use of N-cadherin substrates for neural differentiation.

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Appendix: Supplementary Information

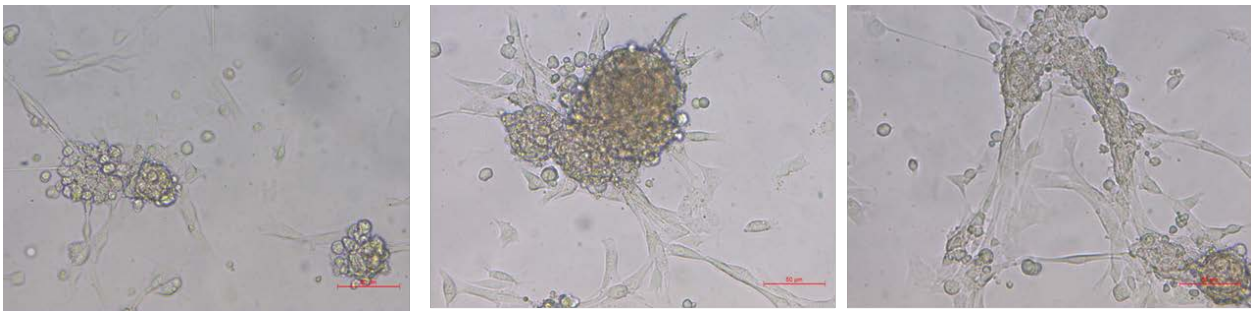

Figure 9: C2C12 cells on day 3 after cell culture.

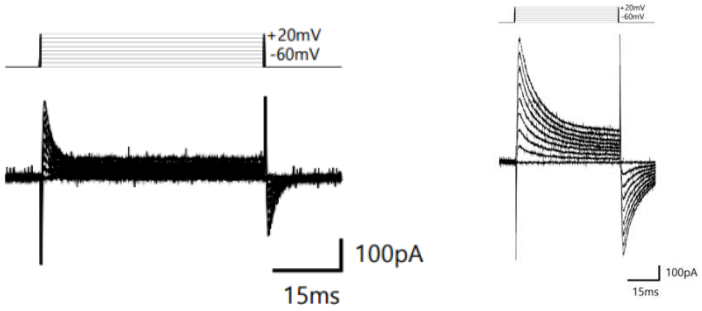

Figure 10: Patch clamp analysis for neural differentiated cells. As there are no changes, this shows that there are no changes in voltage, during an action potential.