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EFFECTS OF DIFFERENT MICROENVIRONMENTAL CONDITIONS ON THE GROWTH AND DIFFERENTIATION OF DENTAL PULP STEM CELLS

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EFFECTS OF DIFFERENT MICROENVIRONMENTAL CONDITIONS ON THE GROWTH AND DIFFERENTIATION OF DENTAL PULP STEM CELLS

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ABSTRACT

Human teeth are very complex structures that are susceptible to many different pathologies due to poor dental health. Currently, there are many restorative methods to reestablish some of the function that teeth have, but the materials used in these methods all have drawbacks and cannot fully mimic the native teeth. Tissue engineering research groups have begun to explore regenerating bone or dental tissue using mesenchymal stem cells derived from the bone marrow. However, our group focuses on regenerating dental tissues using multipotent stem cells from dental pulp.

Dental pulp stem cells (DPSCs) have shown similarities to bone marrow stem cells in in that they can differentiate into many cell types. Also, stem cells in general have shown that differentiation can be induced with microenvironmental factors such as growth factors and substrate properties. If enough is known about the cues that cells receive that induces differentiation, tissues could be engineered using the constructs and growth conditions necessary.

To determine the effect of substrate stiffness on human DPSCs, cells were placed on polyacrylamide gels of varying stiffness and in varying growth factor conditions. The cells were then observed with light and confocal microscopy, and the amount of alkaline phosphatase (ALP) activity was measured. These tests gave an indication of growth and differentiation. It was seen that the growth patterns were different on the gels than they were on a glass control, but there was little difference between the two gels. Also, the growth factors did not appear to have a significant contribution to differentiation.

Much work has been done to determine the effects of mechanical compression muscleoskeletal tissues, such as cartilage and bone. Dental tissues are also subject to loading throughout the day. Therefore, it was hypothesized that if dental pulp stem cells are compressed with pressure similar to that seen physiologically, it will induce differentiation to a bone or tooth-like lineage.

To determine the effects of static compression on dental pulp stem cells, a custom compressive device was fabricated. The device was tested for usability and it was deemed acceptable for use. ALP assays were performed similar to the previous studies. Preliminary results showed that that after only 1 day of culture time, the compression did not have much of an effect on dental pulp stem cells, while it did have an effect on osteoblasts. More work is to be done to determine the effects of compressive forces on dental pulp stem cells.

DEDICATION

This work is dedicated to my friends and family for their continued support. To my parents, Richard and Anne, thank you for always pushing me to be the best and for showing me the importance of education. To my fiancé, Emily, thank you for always supporting me and showing me how proud you are of me. To my friends, thank you for holding me accountable for having balance in my life.

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CHAPTER 1: Oral Anatomy and Physiology

Almost every adult has dealt with some sort of dental pathology over time. With the poor dental health and lack of natural restorative mechanisms in teeth, clinicians are forced to replace or repair teeth in an attempt to restore the function. Replacement with synthetic materials has been a long-established dental procedure, but these replacements fail in time. At best, they can only afford some of the original functionality of the tooth. Research is being done to assess the possibilities for stem cell tissue engineering in dental applications to replace teeth with native dental material.

It is necessary to understand the biological and functional oral and tooth anatomy to be able to reconstruct or repair any issues within the systems. This is important so that a suitable replacement can be created. The following chapter gives an overview of oral and tooth anatomy, pathologies within the systems, and natural repair mechanisms for the pathologies. Also, a comparison between dental tissue and bone tissue will be discussed for tissue engineering applications.

1.1 Oral Cavity Anatomy and Physiology

The oral cavity, or mouth, belongs in the digestive system as it is the entryway to the gastrointestinal system. It begins the digestion process by mechanical and chemical means and also facilitate speech and sensory reception. The oral cavity proper includes organs such as the teeth, the gingiva, the tongue, the hard and soft palates, the uvula, the

tonsils and the salivary glands. Every structure in the oral cavity except the teeth is lined by a mucous membrane.(Shier, Butler, & Lewis, 2007)

Teeth develop within the alveolar processes of the mandibulary arch (lower jaw) and maxillary arch (upper jaw). The gingivae, or gums, cover the alveolar processes of jawbones and surround the necks of the teeth. This covering is composed of dense, irregular connective tissue with an overlying nonkeratinized stratified squamous epithelium. The lips are also connected to the internal surfaces of the lips.(McKinley & O'Loughlin, 2008) The tongue occupies the floor of the oral cavity and is an accessory digestive organ. It is composed of mostly skeletal muscle fibers that run in several directions to aid in mixing food particles during chewing and compressing them into a bolus. A mucous membrane covers the tongue, and the superior surface of the tongue has rough projections called papillae that function to provide friction and contain taste buds. The hard and soft palates combine to form the upper barrier of the oral cavity to separate it from the nasal cavity. The hard palate is part of the maxilla and functions to assist the tongue in manipulating food particles, while the soft palate is composed mostly of skeletal muscle and functions with the uvula to prevent food from entering the nasal region. The uvula is a projection from the soft palate. The tonsils are organs of the lymphatic system that act as a first line of defense against ingested antigens. They monitor the ingested food and drink and can initiate an immune response if necessary.(McKinley & O'Loughlin, 2008) The salivary glands can be divided into different categories, but they all include serous cells, mucous cells, and ducts to perform the function of secreting 1.0-1.5 liters of saliva per day. Saliva is 99.5% water and has

many functions. First, it begins chemical digestion with enzymes as well as moistening food to make it easier to swallow. Also, the pH of saliva (6.5-7.5) and its buffering capabilities shield teeth from acidic environments from foods. Saliva also acts as a solvent for food particles to dissolve so that they may be tasted. Lastly, saliva lubricates the mucous membranes throughout the oral cavity and cleanses the oral cavity structures by maintaining a moist environment in the mouth.(Shier et al., 2007)

The structures of the oral cavity can be seen in Figure 1.1 below. The figure includes the anatomical features discussed as well as a greater distinction of the teeth. The anatomy and physiology of the teeth, also called the dentition, will be discussed further in the next section. Overall, the oral cavity functions in many of the aspects of early digestion utilizing the teeth and saliva to achieve that function.

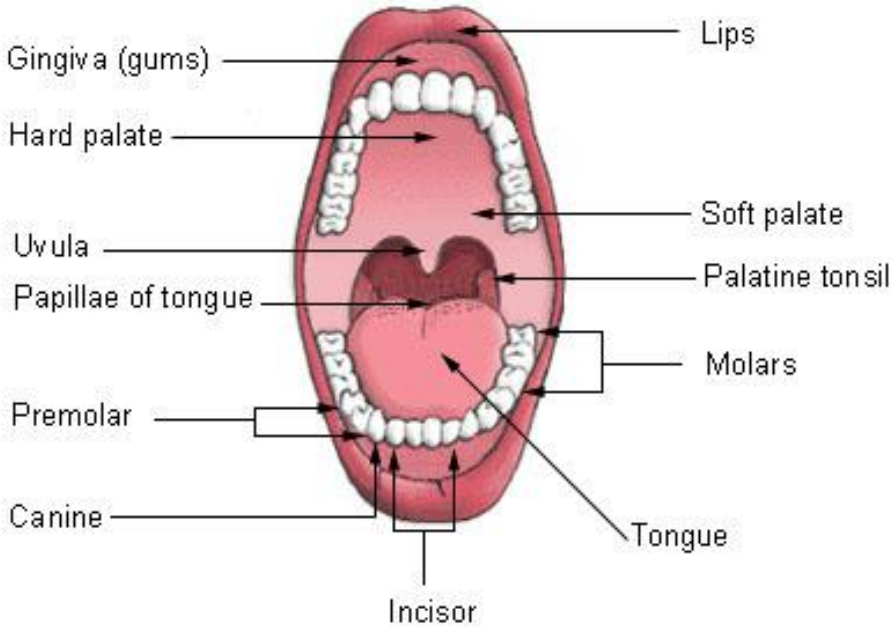

Figure 1.1: Oral anatomy (Wikipedia, 2012a)

1.2 Tooth Anatomy and Physiology

During a lifetime, there are two sets of teeth in the human mouth, primary and secondary. The primary (deciduous) set consists of 10 teeth in each jaw, and these teeth are fewer in number and smaller than the teeth in the permanent dentition. The primary teeth usually erupt through the gingivae at regular intervals between around six months to two to three years of age. The teeth function until the child reaches the age range of six to eight years and then begin getting replaced by the secondary (permanent) teeth in the same order they appeared. From age 6 or 7 to about age 12, the dentition is usually mixed between the primary and permanent teeth. The permanent set of teeth consists of 16 teeth in each jaw, and these teeth are larger than the primary teeth. By age 12 or 13, most humans have their full set of permanent teeth. The lack of tooth replacement or renewal after age 12 shows the need for a suitable replacement. Table 1.1 below shows the types and numbers of primary and secondary teeth.

Table 1.1: Primary and Secondary Tooth Distribution Summary

Primary Teeth (Deciduous)		Secondary Teeth (Permanent)	
Type	**Number**	**Type**	**Number**
Incisor, Central	4	Incisor, Central	4
Incisor, Lateral	4	Incisor, Lateral	4
Cuspid (canine)	4	Cuspid (canine)	4
		Bicuspid, First	4
		Bicuspid, Second	4
Molar, First	4	Molar, First	4
Molar, Second	4	Molar, Second	4
		Molar, Third	4
TOTAL	**20**	**TOTAL**	**32**

Teeth are the hardest structures in the body, and they develop within the alveolar processes of the mandibular (lower) and maxillary (upper) bones. They are anchored to the alveolar processes of the jaw by the periodontal ligaments. Each tooth consists of two main sections, the crown and the root, which are joined by the neck. The root is ensheathed with a hard material called cementum and is anchored to the alveolar process. The root provides the vasculature to provide nutrient exchange for the cellular component of the tooth. Also, the root contains the nerves that allow sensing in the tooth. The neck of the tooth, marked by a narrowing of the tooth, marks the transition area from the root to the crown. The crown is the exposed portion of the tooth that extends beyond the gum

and is active is mastication (chewing) for digestive purposes.(McKinley & O'Loughlin, 2008) Figures 1.2 and 1.3 show the anchoring of the tooth as well as the gross tooth anatomy.

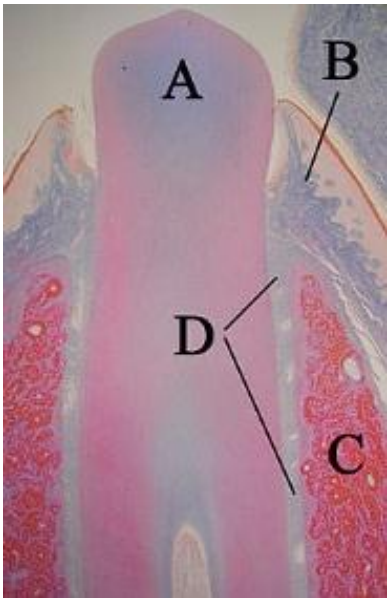

Figure 1.2: Histology of the tooth showing the (A) erupted tooth, (B) gingiva, (C) alveolar bone, and (D) periodontal ligament. Note the distinct organization of the tooth and its surrounding structures. (Wikipedia, 2012b)

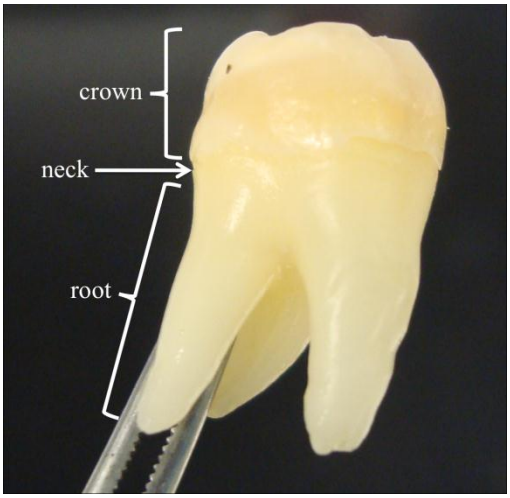

Figure 1.3: Gross anatomy of a molar showing the root, neck, and crown.(Datko, 2011)

The crown of the tooth is layered in three sections: the enamel, dentin, and pulp. Enamel is a glossy, white, a-cellular layer that consists mainly (94%) of inorganic calcium salts and completely covers the crown of the tooth. The most abundant inorganic mineral is hydroxyapatite, a calcium phosphate that almost makes up the majority of the inorganic component in bone, dentin, and other osseous tissues. There is a small organic portion of the enamel, mostly enamelin. If it is worn away or injured, enamel cannot be replaced. This is because it is formed during a process called amelogenesis, and the cells that perform that process are not found after the developmental stages of life. The main function of the enamel is protection as it is the hardest biologic tissue in the body. However, this makes it brittle and susceptible to fracture. The inorganic components give the enamel these properties.(Avery, 2000; McKinley & O'Loughlin, 2008)

Moving medially from the enamel, one finds a transition region to the next layer of the crown(dentin) called the dentinoenamel junction. Underneath the dentinoenamel junction is the living, cellular tissue called dentin. Dentin makes up the primary mass of the tooth, and it is very similar to bone. Dentin is composed of roughly 70% inorganic minerals (mostly hydroxyapatite). The hydroxyapatite crystals in dentin are smaller than those found in enamel. Also, instead of enamelin, the major organic component of dentin is collagen, so dentin is less stiff and more elastic than enamel. This elasticity is due to the structure and composition of dentin.

Dentin can be characterized as primary, secondary, or tertiary. Primary dentin is the first type of dentin that develops near the dentinoenamel junction during dentinogenesis. Secondary dentin develops more slowly than primary dentin, and makes

up most of the volume of dentin in the tooth. It doesn't develop until after the crown has reached clinical functionality. Dentin has both tubular and globular structures, but secondary dentin contains mainly tubular components. The tubular structure allows for fluid movement. Finally, tertiary (or reparative) dentin only is produced when an injury occurs. Dentin is formed by odontoblasts during dentinogenesis. Upon completion of dentinogenesis, odontoblasts can only be found along the dental pulp edge. Odontoblasts are post-mitotic cells that secrete and mineralize dentin and are sensitive to heat and mechanical stress. It is thought that dentin is formed as mineralized nodules that fuse together to form the mass of dentin.(Hao et al., 1997; M. Liu et al., 2012; Magloire, Couble, Thivichon-Prince, Maurin, & Bleicher, 2009)

The center of the tooth is a pulp cavity that contains dental pulp, a connective tissue. The pulp houses blood vessels and nerves which are fed through tubular root canals through an opening called the apical foramen. Also, the pulp contains most of the cellular component of the tooth. As previously stated, odontoblasts live along the edge of the pulp with connections to both the dentin and pulp. However, other cells types such as fibroblasts and undifferentiated stem cells are also present in the pulp. The dental pulp allows the tooth to communicate with the rest of the body via the system of nerves and blood vessels mentioned above. This allows the body to respond to stimuli felt by the teeth such as temperature or pressure. Also, cell damage stimuli can be received to spur a restorative cellular response.(Avery, 2000)

The root of the tooth is similar to the crown in that it has three layers of tissue, but the outer layer is not the same. Where the crown has enamel, the root has the cementum

previously mentioned. The cementum is a bone-like material that seals dentin and also serves as an attachment point for the periodontal ligaments.(McKinley & O'Loughlin, 2008) The different layers of the tooth can be seen in Figure 1.4.

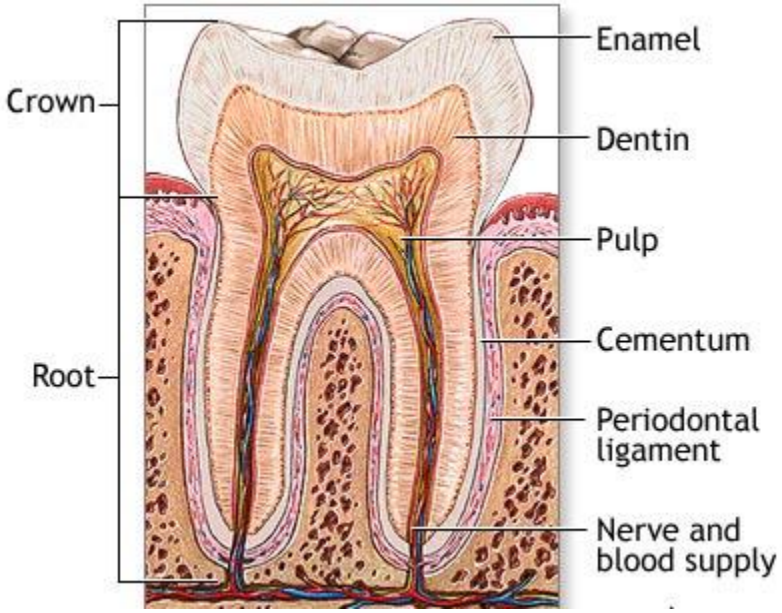

Figure 1.4: Deep anatomy of the tooth.(NIH NLM, 2010)

Teeth function to break food into small pieces to help with digestion. The different shapes of teeth are present to handle foods in different ways. For example, the incisors are chisel-shaped as they can bite off pieces of food while the molars are flattened for grinding food particles.(Shier et al., 2007) The different types of teeth can be seen in Figure 1.5.

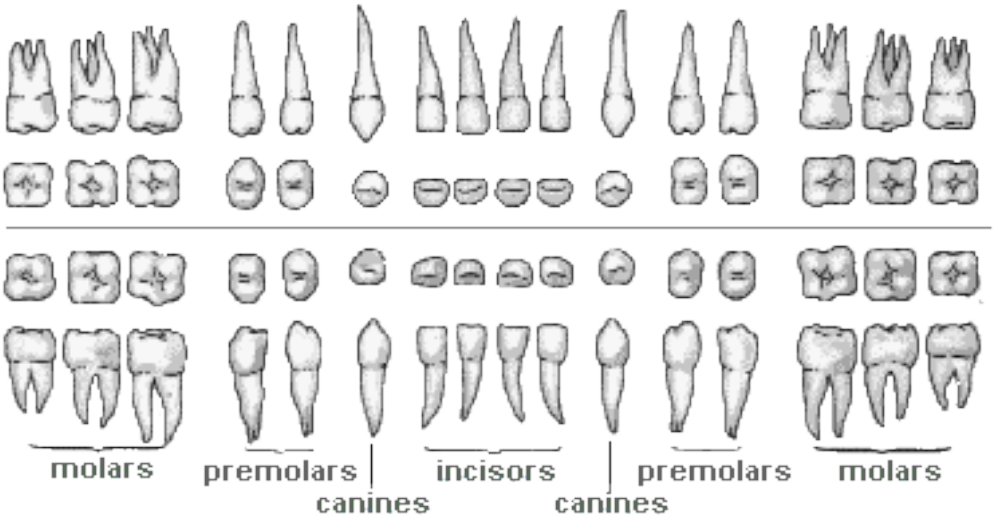

Figure 1.5: Shapes of the different types of teeth.(Web Dental Office, 2009)

1.3 Dental Pathologies

Tooth damage and eventual tooth loss can be caused by several things, but they are most often associated with diseases of the gums (gingivitis) and the dental pulp (endodontitis). Other common pathologies associated with teeth are periodontitis and caries. **All of the pathologies listed are preventable, but untreated conditions will lead to teeth that cannot be easily saved**.(Shier et al., 2007) As stated previously, teeth have poor repair mechanisms, and permanent loss can affect digestive processes and speech.

Gingivitis is one of the most common forms of dental disease, and it involves inflammation of the gingiva, or gum. It is caused by the prolonged presence of plaque, a sticky material composed of bacteria, mucus, and food debris that collects on teeth. If plaque if allowed to remain on a tooth, it hardens and becomes trapped at the base of the

tooth transforming into tartar. Plaque and tartar irritate the gums as the bacteria produce toxins that cause the gums to become infected, swollen, and tender. This effect on the gums is labeled gingivitis. It can be prevented with regular tooth brushing and dental cleaning. Gingivitis can be reversed, left untreated, it can become a more serious disease called periodontitis.(Shafer, Hine, & Levy, 1974)

In periodontitis, the infection caused by the plaque spreads below the gingiva into the periodontal ligament and the bones of the jaw. This happens because the inflammation experienced by the gingiva causes it to pull away from the tooth allowing the bacteria access to the underlying structures. If this condition remains untreated, the bacteria continue to infect the bone and ligament until the tooth is lost. (Shafer et al., 1974) An image of periodontal disease can be seen in Figure 1.6

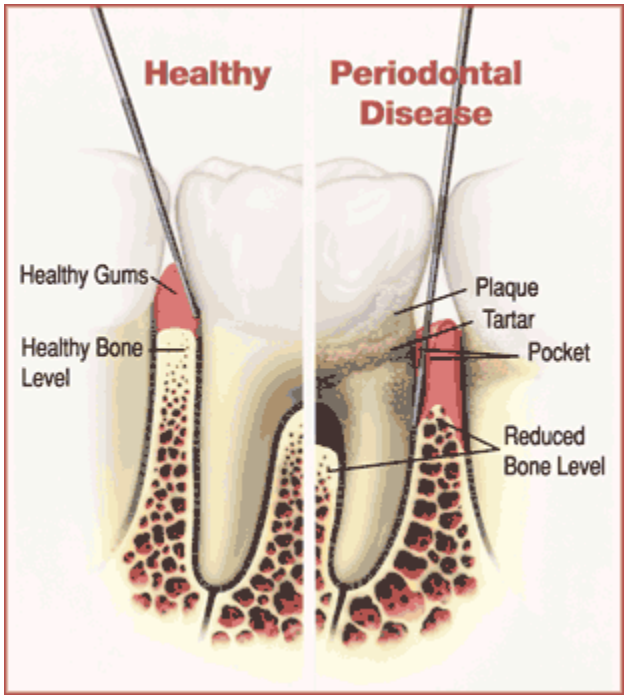

Figure 1.6: Comparison of a healthy tooth and a tooth infected with periodontal disease. Note the bone degradation and irreparable damage on the tooth root. (American Academy of Periodontology, 2011)

Tooth decay, also called dental caries or cavities, is the second most common dental pathology in humans behind gingivitis. This condition is caused by the same plaque and bacteria that cause gingivitis. Bacteria such as *Actinomyces, Streptococcus mutans,* and *Lactobacillus* metabolize food lodged in between teeth at on the gum. The metabolic by-products of these bacteria are acidic, and these acids can degrade the enamel and later the dentin of the teeth. The bacteria also produce sticky substances to ensure they stay in place to continue to cause damage. However, the bacteria and plaque can be prevented and treated by normal brushing and dental visits. Also, the drinking of fluorinated water is helpful in preventing dental caries as fluoride is incorporated into the chemical structure of enamel to help strengthen it and protect it from acids and decay.(Shier et al., 2007)

1.4 Natural Repair Mechanisms of Teeth

When a tooth is damaged, a limited amount of natural repair of the tooth can occur. Dentin can be restored to some extent, but enamel cannot. This is because enamel is produced by ameloblasts which under desmolysis (destruction and disintegration) after enamel maturity. Therefore, if an injury causes damage to enamel, no biological repair occurs. If the injury extends into the dentin, natural restoration of the dentin can occur if there is at least 0.25mm of intact dentin remaining. The dentin that is formed in response to injury is tertiary dentin as previously discussed. This dentin is unorganized in structure and mechanically weak compared to primary or secondary dentin due to the fact it is

produced extremely rapidly. This is analogous to vasculature formation in cancer tumors or scar tissue formation being disorganized and mechanically weak. If less than 0.25 mm of dentin remain after injury, little tertiary dentin is formed and the injury will need to be treated clinically. If the injury extends into the dental pulp, a majority of the dentin-producing cells odontoblasts are most likely injured as they reside on the junction between the dentin and pulp. The undifferentiated dental pulp stem cells migrate to edge of the pulp tissue to take the place of the damaged odontoblasts.(H. Liu, Gronthos, & Shi, 2006; Yen & Yelick, 2011) This migration and differentiation leads to odontoblastoid cells which produce tertiary dentin similar to the previous example. This dentin is very rapidly deposited as its primary goal is to protect the pulp.(Murray & Garcia-Godoy, 2004; Shafer et al., 1974)

It should be noticed that the mechanisms for repair of dental tissue are, at the very best, acceptable to protect the tooth from further damage. However, clinical methods of treating dental tissue damage do not achieve a much better outcome. The methods of dental repair will be discussed in the Chapter Two along with an extensive discussion about dental pulp stem cells.

1.5 Comparisons Between Tooth and Bone

While they are similar in appearance, mechanical properties, and composition, teeth are not considered to be bones and are not included in the skeletal system. Bone and teeth are analogous in that they both have layers of tissue that have different compositions and functions, but those layers are the not the same. Unlike teeth whose

outer layer is a hard enamel, bone's outermost layer is either a cartilage layer or the periosteum. Both are very different from enamel.(McKinley & O'Loughlin, 2008; Shafer et al., 1974; Shier et al., 2007; Wikipedia, 2012b)

Bone and the dentin component of a tooth both have a majority of mineralized tissue composed mostly of hydroxyapatite, but their organic components differ. For example, there are multiple types of proteins that are found specifically in dental tissue and not found in bones. Some of these proteins include enamelins, amelogenins, and dentin sialophosphoproteins. Also, bone contains more collagen, so it is not as hard as dentin. Comparisons have been drawn between bone marrow and dental pulp due to the presence of the undifferentiated stem cells present in both. However, the tissues remain very different, for bone marrow produces blood cells. Lastly, the regenerative capabilities of bone and teeth are very contrasting. Bone, even if fully broken, can heal itself in a matter of months. The dentin and enamel components of tooth have very limited regeneration capabilities.(Shier et al., 2007)

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CHAPTER 2: Restorative Dentistry

With the prevalence of dental pathologies and the lack of natural healing in teeth, clinicians and scientists have been searching for a suitable solution to restore function and appearance in teeth after damage. Synthetic materials have been the main choice, but these materials often fail. Even when failure is not present, they can only provide some of the functionality of the original tooth as the living component is removed. Research is being done to assess the possibilities for stem cell tissue engineering in dental applications, and the research will be discussed in the following chapters.

2.1 Market for Restorative Dental Procedures

The market for dental restoration procedures is not as high as it once was due to better education and the fluoridation of water in many communities around the country. In a 2004 statement, the US Surgeon General Richard H. Carmona, M.D. was quoted saying:

> Scientific studies have found that people living in communities with fluoridated water have fewer cavities than those living where the water is not fluoridated . . . An economic analysis has determined that in most communities, every $1 invested in fluoridation saves $38 or more in treatment costs. Fluoridation is the single most effective public health measure to prevent tooth decay and improve oral health over a lifetime, for both children and adults.(Carmona, 2004)

However, there are still many complications associated with oral health in the United States today. A Gallup poll indicated that 34% of Americans did not visit a dentist at all in the previous year.(Marcus, 2009) Also, according to the Nation Institute of Dental and Craniofacial Research (NIDCR), 23% of children aged 2-11 have never been to the dentist while 12% of adults aged 20-64 haven't visited a dentist in over five years.(NIDCR, 2011) This shows that the deficiency of oral health is significant for all ages and gives an indication of the prevalence of dental pathologies. The lack of dental care and other factors has led to a large number of dental caries and other oral health conditions. Some of the NIDCR statistics are as follows:

- 21% of children ages 6-11 have had tooth decay (dental caries)
- 59% of adolescents ages 12 to 19 have had tooth decay, and 23% of adolescents continue to have untreated decay.
- 92% of adults 20 to 64 have had tooth decay, and 23% of adults continue to have untreated decay.
- Adults have an average of 3.28 decayed or missing teeth and 13.65 decayed and missing surfaces.
- Approximately 5% of adults have no teeth.
- 8.52% of adults 20 to 64 have periodontal disease, and 5.08% have moderate or severe periodontal disease.

It can be seen that dental restoration procedures have a large market even with the better oral education and increase in fluoridation of water.(NIDCR, 2011)

The dental industry provides treatments to restore the form, function, and appearance of teeth, and most of the research is focused on improving techniques, equipment, and materials. This is because the constant numbers of restorative procedures demand a high price tag for the best technologies. In the US, the dental market reached $12.7 billion in 2010, and it is expected to increase to $14.4 billion by 2016. This money is split between equipment/supplies and consumer dental care. The split along with projections can be seen in Figure 2.1.

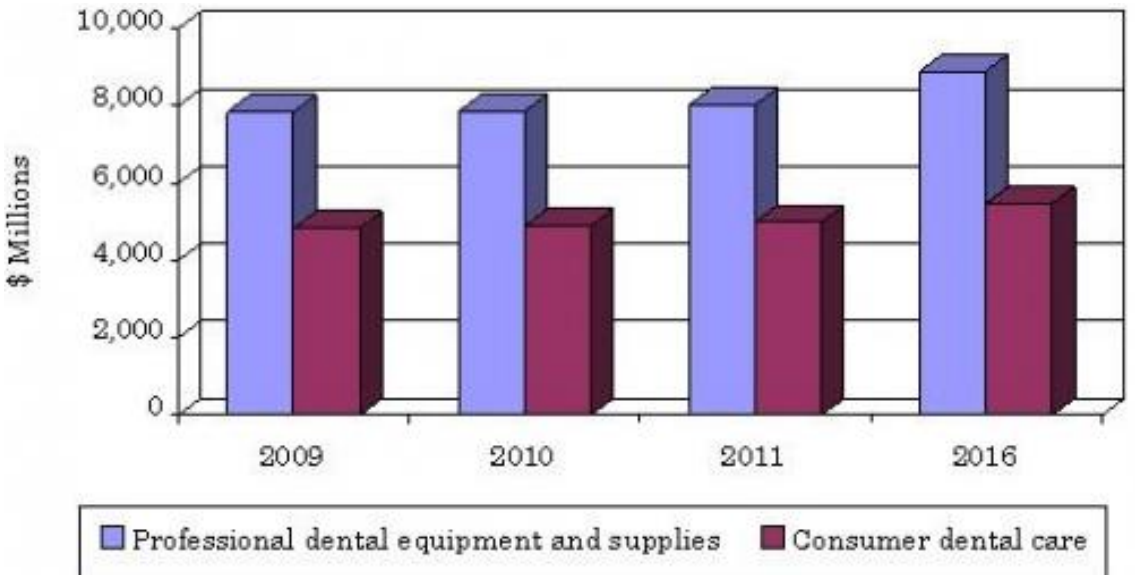

Figure 2.2: US Dental Market Analysis by Major Product Segment. This graph was reprinted without permission. (Research, 2012)

Also, in global markets, the value of dental consumables and prosthetics was $15.9 billion in 2010 and is projected to be $18.5 billion in 2015. The supplies, materials, and prosthetics market accounts for the largest share of that at $9 billion, and it is driven by the sales generated for crowns, bridges, and dental implants. In the materials market,

filling materials account for a majority of the sales totaling $5.8 in 2010. This shows that both globally and in the United States, dental products are extensively used and can be very profitable. Therefore, finding a better replacement dental material to supplant the current materials would be a huge breakthrough in the dental health field.

2.2 Current State of the Art

2.2.1 Fillings

Fillings are a common treatment for tooth decay where the dentist removes decayed sections of the tooth and restores the original tooth shape and function with a synthetic material. This stops continued decay and also prevents further decay by closing the spaces that bacteria can enter. The materials used for fillings include metals (gold, amalgam (a mixture of silver, tin, zinc, copper, and mercury)), composites, and porcelain. None of these restorative materials has the ideal set of properties, and dentists select the filling type bases on the specific patient case. For example, gold fillings have an extremely long life because they are durable and do not corrode, but gold has an extremely high cost. Another consideration is appearance. Gold does not match the color of teeth and is easily noticeable, so porcelain may be used as it is tooth-colored. Yet, porcelain is brittle so shouldn't be used for load-bearing surfaces, etc. There are many other considerations to make before choosing a dental material.(Colgate, 2012; Dental Association,) Figure 2.2 shows the range of filling appearance when used to restore a human tooth.

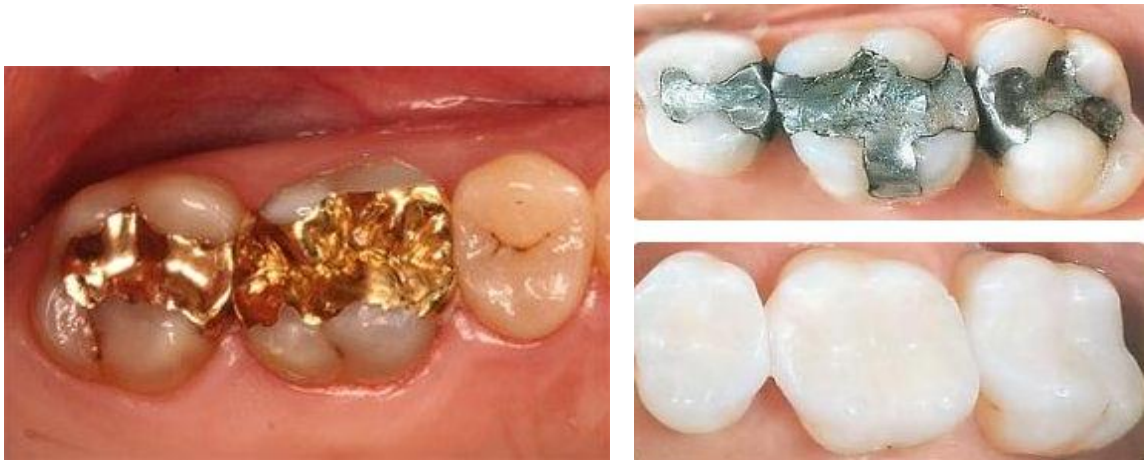

Figure 2.3: Comparison of Gold (left), Amalgam (top right), and composite material (bottom right). Note the visual blending of the composite with natural tooth as opposed to the stark contrast in gold and amalgam. These photos were reprinted without permission. (Heritage Dental Centre, 2009)

Amalgam is the most common filling material, but recent concerns of the safety of amalgams due to their mercury content is leading government and health organizations to recommend ceramic and composites in their place. Gold is still preferred, but the high price of a gold filling leaves it out of reach for most consumers. Replacing amalgam with composites and porcelain is acceptable in some applications, but amalgams are used in load-bearing surfaces, such as molars, where composites and porcelain could fracture. They are more suited to incisors and canines due to good wear resistance.(Colgate, 2012; Dental Association,)

2.2.2 Crowns

Crowns are similar in materials to fillings, but the application is different. A crown is simply a cap that goes on top of an existing tooth or dental implant. If a tooth is too weak or damaged for a filling or even fractured, crowns can be used to protect what is remaining of the tooth, specifically the dental pulp. The damaged tooth is reduced in size

and a mold is made of the remaining structure. Then, the fitted crown is applied and cemented into place. Figure 2.3 shows this process. The materials used in crowns are similar to fillings, but the preferred material is porcelain bonded to a metal shell because it is both strong and attractive.(Colgate, 2012)

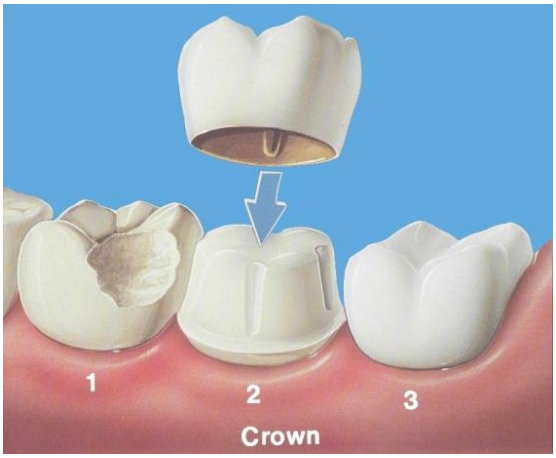

Figure 2.4: A diagram of the crowning process for dental restoration.(Dolce, 2012)

In both crowns and fillings, a discrepancy between the material properties of the replacement material and the natural tooth can lead to failure. Almost all dental restoration procedures need revisions at least once, while the average time frame of functionality is 5-12 years for non-gold materials. Research is currently being done to find a suitable replacement material that can mimic the tooth better in terms of strength and resistance to damage. It is probable that the only suitable material would be the natural dental material itself. Guiding stem cells with tissue engineering techniques could be an ideal solution to solving this material shortage.

2.3 Regenerative Medicine in Dentistry

The terms tissue engineering and regenerative medicine have been used since the 1980's, and in the early 1990's, Langer and Vacanti defined tissue engineering as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue function."(Ricci & Terracio, 2011) Tissue engineering is a very intriguing area of research, and many research groups are focused on regenerative medicine applications. The problems seen in dental restoration procedures are evident in every medical field- materials that exactly mimic the body's natural forms and functions are desired. Some areas of the body are already being treated by tissue engineered constructs.

Dentistry has long tried to restore the functions of native teeth, and one source argues that dentistry has always been at the forefront of regenerative medicine, even before the term was used, as the goal of any treatment all along was to restore tissue and organ function in teeth. However, the stem cell based tissue engineering approaches are coming along as well. While a full tooth may never be grown (some argue that it wouldn't be useful even if it was possible), using stem cells to regenerate dental pulp to save teeth. Also, stem cells could be used to regenerate the periodontal ligament and the underlying alveolar bone as those are also damaged in oral diseases.(Ricci & Terracio, 2011)

The source of these stem cells remains an issue. While a lot of press has been given to embryonic stem cells, studies have begun to show that they are susceptible to forming cancerous tissues.(Li et al., 2011) Work is being done with induced pluripotent

stem cells (iPS cells) and also multipotent stromal cells. Some of these stem cells come from human teeth while others come from bone marrow, adipose tissue, or adult muscle.(Gimble, Grayson, Guilak, Lopez, & Vunjak-Novakovic, 2011; Gronthos et al., 2002; Gronthos, 2011; Murray & Garcia-Godoy, 2004)

Multipotent stromal cells have shown promise in studies as many groups have been able to regenerate almost all tissues in the body including bone, periodontal ligament, cementum, and dentin in animals.(Shi et al., 2005; Yen & Sharpe, 2008; Yen & Yelick, 2011)

The results of many studies are promising, but guiding the stem cells down a specific path reproducibly can be difficult. It is known that cells during development respond to numerous different and complex cues to ensure that each cell is directed down the correct lineage. This process is not fully understood, but directed stem cell commitment in the laboratory has been focused on exposing stem cells to soluble factors in growth media (growth factors, cytokines, serum proteins, etc.) and properties of the biomaterial substrate (surface energy, roughness, chemistry, elasticity,composition, etc.). Alterations in these factors have been shown to change a cell's adhesion, morphology, and/or proliferation.(Engler, Sen, Sweeney, & Discher, 2006; Phillips, Petrie, Creighton, & Garcia, 2010)

To understand and be able to manipulate odontogenic differentiation (dentin-forming cells), osteogenic differentiation (bone-forming cells) can be used as a model as they are similar and more work has been done on bone. Bone morphogenic proteins (BMP) are cytokines that, as the name would suggest, induce differentiation down an

osseous lineage. Also, dexamethasone has been successful in the same goal.(Chaudhary, Hofmeister, & Hruska, 2004; Jo, Lee, Suh, & Kim, 2007; Zhang, Walboomers, Shi, Fan, & Jansen, 2006)

Also, RGD peptides, a cellular recognition molecule found in numerous ECM proteins, have been used to promote proliferation and migration or differentiation in stem cells. RGD peptides interact with the cells via integrin receptors and can be easily conjugated to multiple types of scaffolds.(Comisar, Kazmers, Mooney, & Linderman, 2007; Moore, Lin, Gallant, & Becker, 2011) When paired with BMP and patterned on a surface, differentiation down an osteogenic pathway was observed. This was important because there was no supplemental osteogenic growth media present, so the differentiation is believed to be from the signaling molecules attached to the substrate. Also in this study, it was noted that early osteogenic differentiation and mineralization of the bone marrow stem cells was strongly correlated with cell density, attachment and proliferation.(Moore et al., 2011)

Knowing the effects of different molecules on cell guidance is important, but just as important is understanding the effects of substrate matrix properties. In studies done by Engler, et al and Tse, et al, the matrix mechanical properties were varied to determine the mechano-transduction signaling pathways in stem cells. The resistance that a cell feels when attempting to deform its substrate (in the body, the substrate is extracellular matrix) is measured by the elastic modulus, E. It was found that mesenchymal stem cells were far more responsive to the matrix elasticity than differentiated cells. It was also noted that differentiation is enhanced on stiffer substrates (25-50 kPa). On less stiff substrates (0.1-

10 kPa), the cells were guided towards a soft tissue or neuron-like lineage.(Engler et al., 2006; Tse & Engler, 2011)

Studies like the examples above begin to demonstrate the complexity of the development of the human body. The mechanisms of formation and direction may never fully be understood, but if enough information is gained about the factors that we do understand, great strides can be made in tissue engineering.

2.4 Dental Pulp Stem Cells

The idea of tissue engineering for dental applications is to cultivate stem cells with odontogenic induction signals to program the stem cells to adopt dental lineages and, with the help of scaffold/extracellular matrix, to become part of a tooth. For dental applications, it is intuitive that dental pulp stem cells should be the cell type studied as they naturally have a connection to a dental lineage. Also, it is known that teeth contain stem cells based on the natural repairing ability of dentin after injury suggesting that cells in a fully developed tooth can still function as odontoblasts.(Yen & Sharpe, 2006; Yen & Sharpe, 2008)

2.4.1 Characterization

Many cell sources are available within teeth. Human dental pulp stem cells (DPSCs) have been derived from wisdom teeth (third molars) and have been shown to form odontoblast-like cells that can produce dentin-like materials when cultured in

mineralization-enhancing conditions. Another source of stem cells is primary teeth that have been exfoliated naturally during development around 6-10 years of age. These cells are recognized as "stem cells from human exfoliated deciduous teeth" or SHED. The cells isolated from both of these locations have shown multipotent differentiation, expression of stem cell markers Stro-1 and CD146, dentin regeneration *in vivo*, and the ability to form colonies *in vitro*. While these two populations of stem cells are similar, they are considered different as SHED can produce dentin and bone, but not dentin-pulp complexes like DPSCs are able to in immunocompromised mice.(Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Yen & Sharpe, 2008) Also, periodontal ligament stem cells have been isolated from periodontal tissue, but the ligaments must be intact for this isolation.(Liu, Gronthos, & Shi, 2006) These types of stem cells will all be referred to as dental pulp stem cells for the purposes of this thesis. Having many sources for these stem cells can be beneficial as a large number might be necessary for successful tissue engineering.

The DPSCs mentioned can be characterized under the broad heading of mesenchymal stem cells (MSCs). A mesenchymal stem cell is any undifferentiated cell from the mesenchyme, a type of loose connective tissue derived from the mesoderm during development. The first mesenchymal stem cells discovered and used were bone marrow mesenchymal stem cells (BMMSCs), and they are still the gold-standard for comparison of potency.(Huang, Gronthos, & Shi, 2009) When compared to bone marrow, dental pulp contains stem cells that produce many of the same markers that indicate multipotency: Stro-1, CD73, CD90, CD105, and CD146.(Gronthos et al., 2000; Gronthos

et al., 2002; Liu et al., 2006; Perry et al., 2008; Shi et al., 2005; Yen & Sharpe, 2008) In addition to being similar to BMMSCs, the DPSCs have markers similar to osteogenic cells as well: alkaline phosphatase, collagen I, osteonectin, osteopontin, osteocalcin, bone sialoprotein, and matrix extracellular phosphaglycoprotein (MEPE). (Gronthos et al., 2002)

In differentiation studies, DPSCs have been tested with different growth factors known to induce guidance down certain lineages. For example, BMP and dexamethasone for bone-formation as previously mentioned. In the studies, DPSCs were successfully induced into differentiating down osteogenic, neurogenic, adipogenic, myogenic, endothelial, and chondrogenic pathways.(d'Aquino et al., 2007; Laino et al., 2005) It was noticed that osteogenic differentiation was the easiest to achieve which makes sense intuitively. The DPSCs were able to form mineralized nodules *in vitro* after a few weeks in a culture environment with even low levels of inductive growth factors.(Zhang et al., 2005)

Dental Pulp Stem Cells can also be characterized based on factors not associated with their stem cell potential. It has been found that DPSCs are highly proliferative (30-50 times the rate of BMMSCs) and can survive cryopreservation well. These are important traits for tissue engineering applications.(Huang et al., 2009; Li et al., 2011; Zhang et al., 2006)

2.4.2 Regenerative Medicine Applications

As previously stated, dental structures can be formed using DPSCs that are transplanted into immunocompromised mice, but the tissue is poorly organized and multiple tooth-like structures form. This is an unacceptable end product for tissue engineering or regenerative medicine applications. However, the concept that these cells can form tooth-like structures is very promising.(Yen & Sharpe, 2008)

In the previous experiment, DPSCs were seeded onto a scaffold and transplanted into the immunocompromised mice. This technique illustrates the traditional tissue engineering theory to combine cells, osteogenic factors, and an engineered construct in a bioreactor to promote cell growth in the form of the scaffold. The cell-scaffold combination is then implanted into the patient.(Yen & Sharpe, 2008)

Another experiment combine the DPSCs with endothelial cells on the scaffold to more closely mimic the environment that the DPSCs are in within the patient. Upon implantation, only one odontogenic structure formed, which is desired to regenerate tooth tissue.(Chai & Slavkin, 2003) One last recent experiment showed that using a gelatin scaffold, DPSCs showed differentiation into osteoblasts in 14 days in culture. The cell-scaffold construct was then implanted into nude mice, and the cells retained their osteoblastic phenotype and began to form bone-like tissue. This group suggested that DPSCs could be a suitable autologous seed cell type of bone tissue engineering due to the following advantages: osteogenic differentiation potential, obtainability from a common dental practice (wisdom tooth removal), high proliferation rates, and ease of culturing. However, it is noted that a vascularized tissue-engineered bone material has not been

accomplished yet, and this step is key to complete integration into the host.(Li et al., 2011)

Overall, dental pulp stem cells have a high potential for bone and tooth tissue engineering applications, but that potential has not been reached yet. DPSCs have many desirable characteristics that make them an attractive source for tissue engineering, and they even seem to have advantages over cells derived from bone marrow, currently the gold-standard. Research to explore the vast possibilities with DPSCs under different conditions needs to be completed to have a better understanding of the mechanisms and factors that affect DPSCs differentiation and growth.

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CHAPTER 3: Determining the Effect of Substrate Stiffness on the Growth and Differentiation of Dental Pulp Stem Cells

This chapter will outline previous work completed by myself and my laboratory mates. This work is similar to the work done by Engler, et al. but DPSCs are used instead of bone-marrow derived stem cells. The cells were exposed to substrates with different mechanical properties to observe the differences in growth patterns.

3.1 Background

3.1.1 Mechanical Properties of the Substrate

It is known that many different chemical, mechanical, and electrical cues can induce differentiation and cell phenotypic changes. A well-documented cue that seems to have an effect on almost all cells is substrate stiffness. For differentiated cells such as fibroblasts, their anchorage dependency is better understood and documented. For example, softer substrates induce different morphological changes than stiffer substrates due to the focal adhesion interactions with the substrate. Multipotent stromal cells derived from bone marrow have been shown to have an effect as well. Stem cells derived from dental pulp have not been studied extensively in terms of the effect of matrix elasticity or stiffness.(Engler, Sen, Sweeney, & Discher, 2006)

In previous work by Engler, et. al, polyacrylamide hydrogels were polymerized with different ratios of acrylamide to acrylamide-bis concentrations according to a protocol from Wang and Pelham.(Wang & Pelham, 1998) Polyacrylamide gels are easily tunable to have

different mechanical properties by changing the ratio of monomers in the polymer solution, so they are often chosen as a substrate.(Tse & Engler, 2010) Figure 3.1 below diagrams the polymerization of a polyacrylamide.

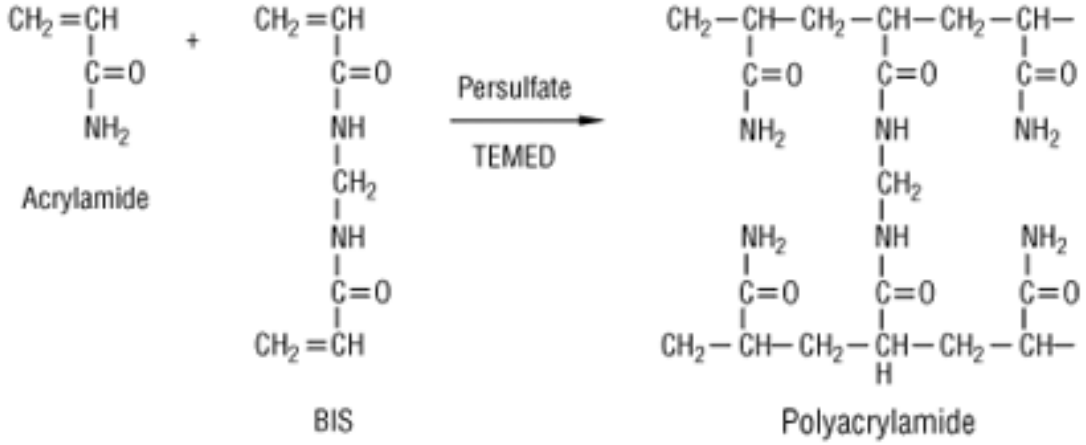


Figure 3.5: The chemical reaction to form a crosslinked polyacrylamide hydrogel.(Thermo Scientific, 2012)

Increasing the percentage of bis-polyacrylamide in the mixture will lead to a greater degree of cross-linking during polymerization, leading to a gel with a greater stiffness. Also, according to the Wang protocol, the glass surface that the gel is on top of needs to be activated by an amino group (3-Aminopropyltriethoxysilane (APTES) so that some of the acrylamide monomers bind to the surface. This step is done so that the gel can be polymerized between two thin, flat glass plates to create a uniform thickness, and the gel will remain adhered to only the bottom glass plate when the top plate is removed. The last step to prepare for cell seeding is to attach ECM proteins such as collagen or fibronectin to the surface of the gel to enhance cell attachment.(Engler et al., 2006; Tse & Engler, 2010)

It should be noted that while polyacrylamide has valuable properties as a easily manipulated substrate as far as stiffness is concerned, there are many questions about the toxicity of polyacrylamide and its monomer, acrylamide. The FDA approves polyacrylamide for many applications, and it has been suggested that polymerized and crosslinked polyacrylamide is not toxic in low doses. However, the monomer acrylamide has been shown to be a neurotoxin. Therefore, if any monomer is present within the polymer or if the polymer is degraded in the body, toxicity will become an issue.(King & Noss, 1989) So, for the experiments outlined below, polyacrylamide is suitable, but for the translation to a clinical application a different substrate would need to be used.

3.1.2 Dental Pulp Stem Cells' Differentiation Potential

As previously written, mesenchymal stem cells, as well as dental pulp stem cells, have multipotent differentiation potential under the correct environmental conditions. As the tissue desired is bone-like in form and function, the environments necessary to induce osteogenic differentiation in bone marrow stem cells have been studied. The common soluble growth factors used to induce osteogenic differentiation are dexamethasone, BMPs (mostly BMP-2 and BMP-7), L-ascorbic acid 2-phosphate (Vitamin C), and β-glycerophosphate.(Kadar et al., 2009; Laino et al., 2005; Zhang et al., 2005) Also, a stiff substrate has been shown to induce osteogenesis.(Engler et al., 2006) As the growth factors need to be constantly supplied to the tissue engineered construct, they are less practical for translation to clinical applications. Therefore, if the substrate properties

could be adjusted to induce osteogenic differentiation, the cells would be able to receive those signals post-implantation as well.

3.1.3 Comparison to Bone Marrow Stromal Cells

Most of the background information has been focused on bone marrow stromal cells, as they are the most studied. However, the cells have shown differences to dental pulp stem cells that should be addressed. First, dental pulp stem cells have been easily differentiated into osteogenic and neurogenic cells, but not myogenic, adipogenic, or chondrogenic. Differentiation has been successful towards most cell types, but it was done with a very strong dose of growth factors and a long culture time. It is thought that since nerves and bone-like tissue are present in/near the pulp tissue, those dental pulp stem cells are predisposed to becoming those types of cells.(Zhang, Walboomers, Shi, Fan, & Jansen, 2006)

Using the data from Engler, et. al showing the differentiation potential for bone marrow stem cells gives an insight into how dental pulp stem cells should be expected to react.(Engler et al., 2006) Also, the fact that dental pulp stem cells have been shown to form a structure similar to the dentin-pulp complex, while hardly any other types of stem cells have shown that, is important.(Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Shi et al., 2005) Combining the information, if the correct conditions could be created, there is great promise for dental tissue regeneration.

3.1.4 Testing Methods

The first method used for testing the cells in this experiment was microscopy. Both light microscopy and confocal fluorescence microscopy were performed. In fluorescence microscopy, immunocytochemistry is used to stain the cells. First, the cells are killed and fixed before they are permeabilized and incubated in an antibody solution that attaches to the protein or molecule of interest. After that, the cells are incubated in another antibody that is tagged with a fluorescent molecule, and that antibody only attaches to the primary antibody. In this way, the protein or molecule of interest is effectively fluorescently tagged and will show up in fluorescence imaging.

The second method to test cells was using alkaline phosphatase (ALP) and bicinchoninic acid (BCA) assays to determine the specific activity of the enzyme alkaline phosphatase, a common enzyme found in many cells. Elevated ALP levels indicates that there could be active bone formation occurring as ALP is a byproduct of osteoblast activity.(Duplomb, Dagouassat, Jourdon, & Heymann, 2007)
In these assays, a colormetric test is done to see how much ALP is present and also how much total protein is present. The data is then normalized to find specific activity. The alkaline phosphatase reaction is shown below in Figure 3.2. Note that the yellow color produced by the product (p-Nitrophenolate) is measured to indicate the amount of ALP present.

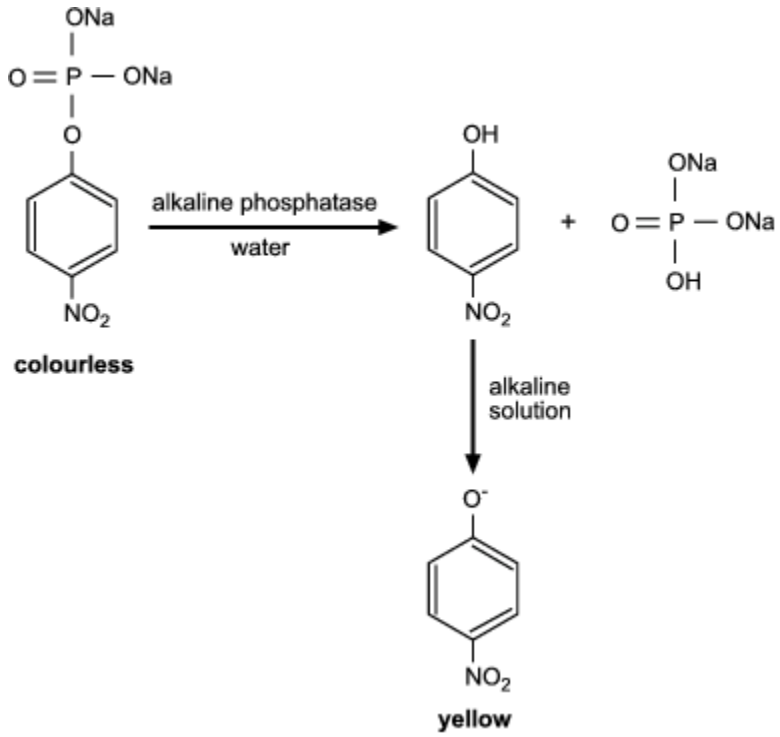


Figure 3.6: The ALP assay reaction. (Napier University, 2007)

3.2 Materials and Methods

3.2.1 Cell Acquisition and Isolation

Dental pulp was obtained from human impacted third molars (wisdom teeth) during a common dental procedure by Dr. Satish Alapati. The dental pulp stem cells were isolated from the pulp tissue using the protocols found in literature.(Huang, Chen, Lin, Shieh, & Chan, 2008; Liu, Gronthos, & Shi, 2006)

First, the extracted teeth were bathed in sterile saline, and the external surfaces of the tooth were cleaned and cleared of bacteria with washes of 70% ethanol and sterile Phosphate Buffered Saline (PBS). Dental instruments were used to expose the pulp

cavity, and the pulp was removed and placed into α-Minimum Essential Medium (α-MEM). The pulp was minced using a scalpel, and the remaining pieces were "digested" using a 3mg/mL solution of collagenase type I and 4mg/mL solution of dispase for 30-45 minutes at 37°C. After digestion, the cells were centrifuged at 1200rpm for 10 minutes to obtain a cell pellet. The supernatant was discarded, and the pellet was re-suspended in mesenchymal stem cell growth medium. The cells then were passed through a 70μm cell strainer to ensure only cells remained. The cells were then cultured in mesenchymal stem cell growth medium at 37°C and 5% CO_2.(Liu et al., 2006)

To purify the cell suspension to only contain DPSCs, it was incubated with STRO-1 (mouse IgM), 3G5 (mouse IgM), or CC9 (mouse IgG2a) antibodies for 1 hour on ice. After washing with 1% BSA/PBS, the cells were incubated with anti-mouse IgG-conjugated Dynabeads for 1 hour at 4°C. The bead-positive cells were selected out using a Dynal MPC-1 magnetic particle concentrator.(Liu et al., 2006) The cells were then cultured as described below.

3.2.2 Cell Culturing

Two different types of cell culture growth media were used in these experiments. The first was Mesenchymal Stem Cell Growth Medium (MSCGM; Lonza) which was "developed for growing large numbers of mesenchymal stem cells without inducing diferentiation."(Lonza, 2012) Later in the experimental stages, MSCGM was formulated by the research lab based on literature, and it contained α-MEM with 15% FBS, 2mM GlutaMAX, 100 μM L-ascorbic acid 2-phosphate, 2.5μg/mL amphotericin B, 100 U/mL

penicillin/streptomycin. The second type of media was Osteogenic Differentiation Medium (ODM). It was formulated similarly to the MSCGM, but the additional components were 1.8 mM KH_2PO_4 and 10 nM dexamethasone.

All cells were allowed to grow in the MSCGM prior to seeding onto the polyacrylamide gels, so they were able to remain stem cells until given a cue to differentiate. The cells were counted, and it was determined that a cell density of 10,000 cells/hydrogel would be used. The cells were transferred to and cultured in standard 6-well cell culture plates on top of the polyacrylamide gels. The appropriate amount of cell solution was added to the gels, and the cells were allowed to adhere in an incubator for 30 minutes before media was added. Each well in the culture plate contained 2-3 mL of media, and the media was removed and replaced with fresh media each 2-3 days. The day when the experimental conditions were applied was defined as day 0, and the cells were analyzed at time points of 0, 1, 3, and 5 days.

Another experimental condition was that half of the cells that were grown in the ODM were also grown in the presence of hyrdroxyapatite (HA) particles. As stated in a previous section, HA is the most common mineral component found in both bone and tooth. The hydroxyapatite particles (MP Biomedicals) were characterized by a lab member, William McCallister, and they had an average particle diameter of 40μm. Before addition of 50μL of HA solution to the samples, the HA particles were diluted to 10mg/mL in de-ionized (DI) water, sterilized using sonication, and vortexed to prevent aggregation.

3.2.3 Polyacrylamide Substrate Polymerization

Using a protocol from the Wang laboratory(Wang & Pelham, 1998), polyacrylamide hydrogels with varying stiffness were polymerized by altering the ratio of the monomers: acrylamide and bis-acrylamide. The gels were formed on #1 glass coverslips of dimensions 22mm x 22mm. The coverslips were prepared and activated according to the protocol in Appendix A. The polyacrylamide hydrogels were polymerized on the activated coverslips to get desired elasatic moduli by preparing solutions with the components listed in Table 3.1 After determining which ratios to use, the protocol was followed again. As a control for the experiments, a glass coverslip (E≈50 GPa) was used as the substrate.

Table 3.1: Composition of the polyacrylamide gels used in these experiments. Note the values for elastic modulus were calculated by the Wang Laboratory.(Wang & Pelham, 1998)

Acrylamide (%) to Bis-Acrylamide (%)	**40% Acrylamide solution**	**2% Bis-Acrylamide solution**	**De-ionized water**	**1 Molar HEPES (pH 8.5)**	**Elastic Modulus, E**
8/0.06	1000 µL	150 µL	3800 µL	50 µL	30 kPa
8/0.10	1000 µL	250 µL	3700 µL	50 µL	100 kPa

A summary of the experimental design can be seen in Figure 3.3 below. Each condition is represented to show all of the different factors that are variables. Note each square cell represents one well in a standard 6-well culture plate. Also, cells were grown in each condition for immunocytochemistry staining.

Control Plates (glass coverslip)		30 kPa gels		100 kPa gels	
MSCGM, day 0	MSCGM, day 1	MSCGM, day 0	MSCGM, day 1	MSCGM, day 0	MSCGM, day 1
ODM, day 0	ODM, day 1	ODM, day 0	ODM, day 1	ODM, day 0	ODM, day 1
ODM + HA Particles, day 0	ODM + HA Particles, day 1	ODM + HA Particles, day 0	ODM + HA Particles, day 1	ODM + HA Particles, day 0	ODM + HA Particles, day 1
MSCGM, day 3	MSCGM, day	MSCGM, day 3	MSCGM, day	MSCGM, day 3	MSCGM, day
ODM, day 3	ODM, day 5	ODM, day 3	ODM, day 5	ODM, day 3	ODM, day 5
ODM + HA Particles, day 3	ODM + HA Particles, day 5	ODM + HA Particles, day 3	ODM + HA Particles, day 5	ODM + HA Particles, day 3	ODM + HA Particles, day 5

Figure 3.7: A visual representation of the experimental conditions to which the cells will be exposed.

3.2.4 Experimental Timeline

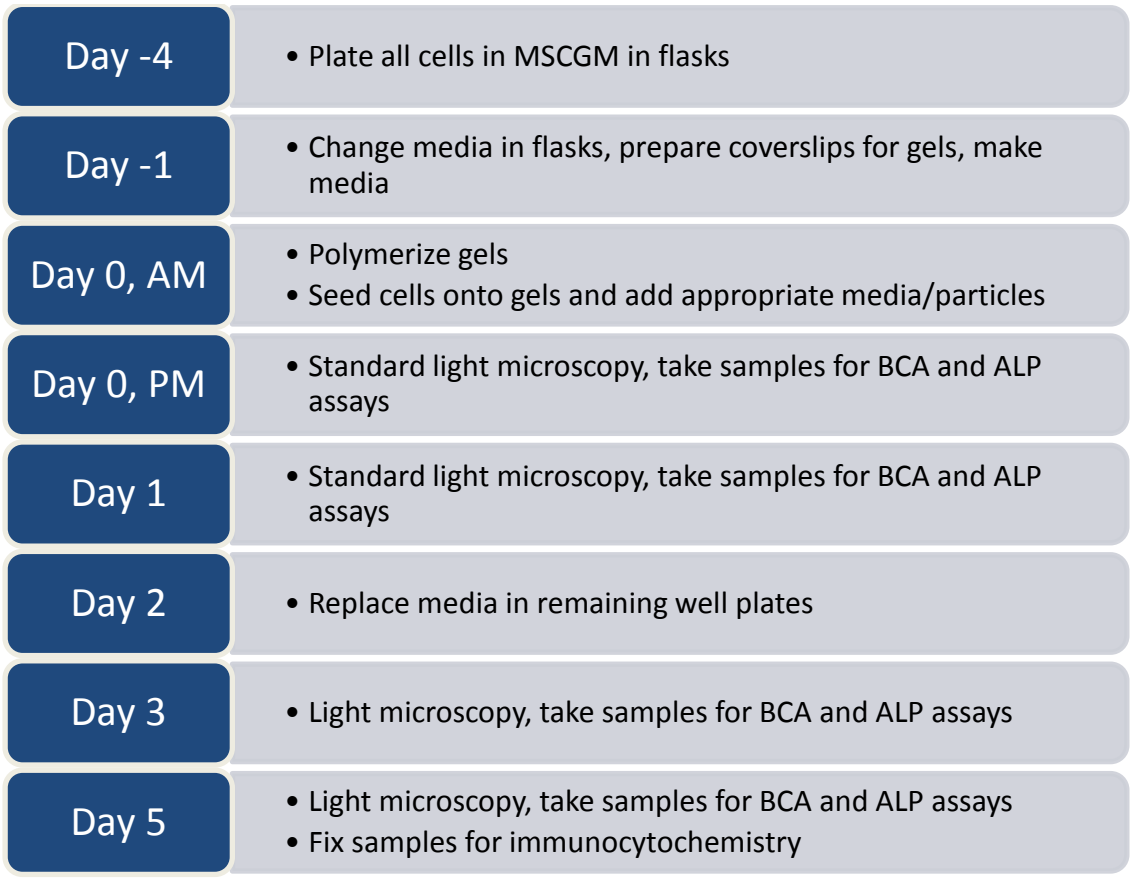


Figure 3.4: Timeline of major events in the experimental procedure.

3.2.5 Imaging

An Olympus IX71 light microscope was used to image the cells almost daily during the experiments. Images were taken to observe morphological changes, and objectives of 4X and 10X were used.

Cells from day 5 were fixed to preserve the cells as close to their natural state as possible and to prevent decay. Fixing was achieved by washing the cells with PBS and letting them sit in 4% paraformaldehyde for 10 minutes. After that time, the cells were washed with PBS again and ready for immunocytochemistry for confocal microscopy. The protocol found in Appendix B was used for the immunocytochemistry. A primary antibody of mouse anti-osteopontin IgG1 (diluted 1:10 in 1% BSA/PBS) was used. This antibody was developed by M. Solursh and A. Franzen and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.(Datko, 2011) The secondary antibody used in the immunocytochemistry was AlexaFluor 647 donkey anti-mouse IgG (diluted 1:100 in 1% BSA/PBS). All other steps in the protocol found in Appendix B were followed. Images were taken with the help of Dr. Terri Bruce on a Zeiss LSM510 Laser Confocal Microscope.

3.2.6 ALP and BCA Assays

At time points on days 0, 1, 3, and 5, alkaline phosphatase (ALP) and bicinchoninic acid (BCA) assays were performed. See Appendix C for the protocol steps

detailing how to obtain standard curves for the assays as well as experimental results and principles of the methods. Note that a BCA Assay Kit (Thermo Scientific) was used.

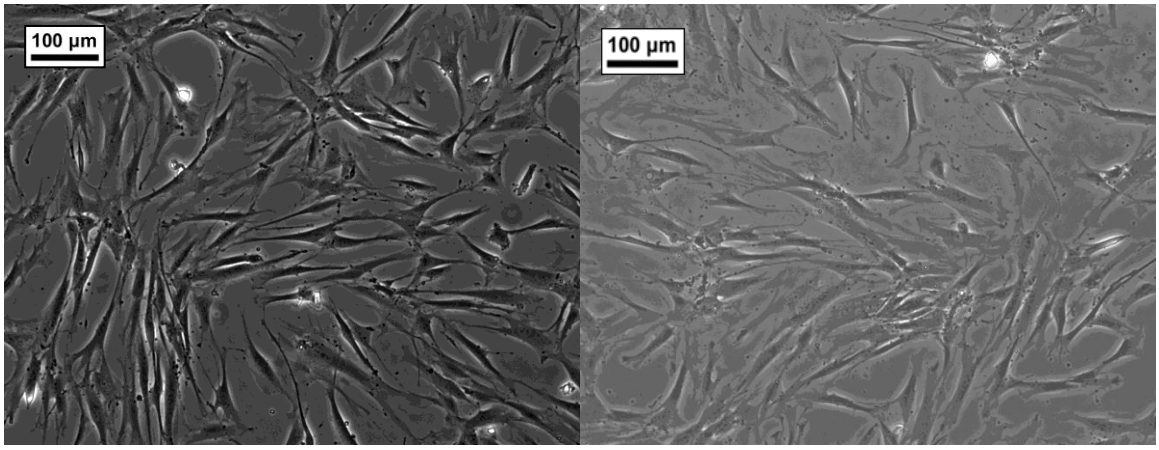
Using the total protein values calculated from the BCA assay and the absorbance values from the ALP assay, specific enzyme activity was calculated using the following steps:

- Using the ALP standard curve, the concentration of pNP formed by the reaction for each sample was calculated in µg/mL.
- That value was converted to µmol/mL formed by dividing by the molecular weight of pNP which is 139g/mol.
- The resulting value is equivalent to the amount of substrate (pNPP) consumed in the reaction in units of µmol S/mL, where S stands for substrate consumed.
- That value was then divided by 30 since the samples were allowed to incubate for 30 minutes, so the substrate consumed can be expressed in µmol S/mL/min.
- To normalize the data for each sample, the resulting value was divided by the total protein concentration for the corresponding sample calculated from the BCA assay leaving the alkaline phosphatase specific activity with units of µmol S/mg protein/min.

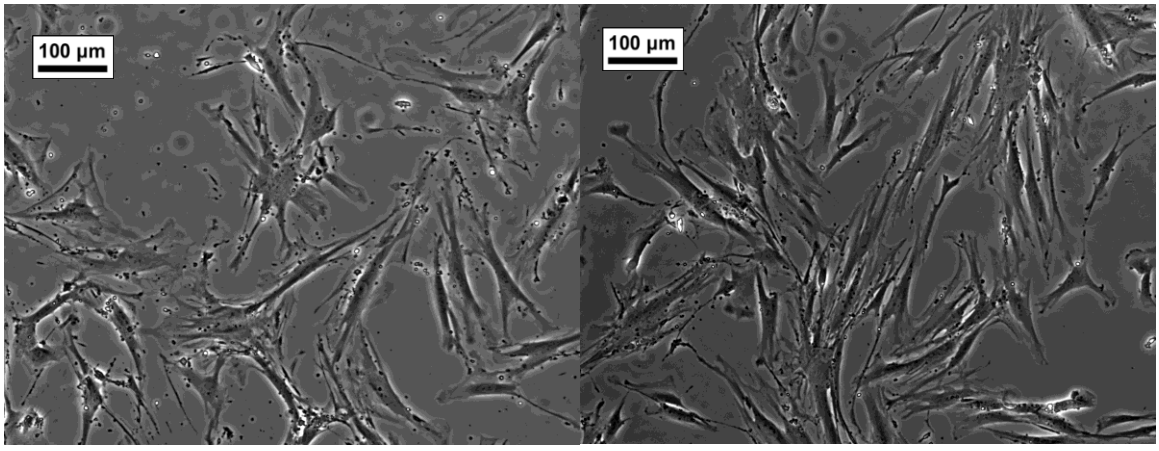
3.3 Results

3.3.1 Basic Imaging

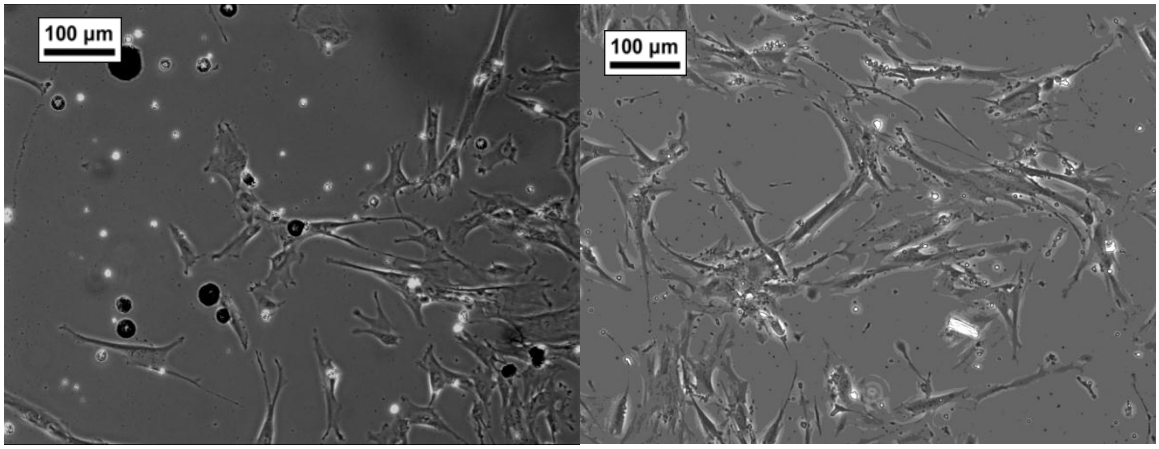
A 10X objective (100X total magnification) was used for each culture condition at most time points. Due to the large amount of pictures, representative images are shown below in Figure 3.5 for each condition on days 1 and 5.



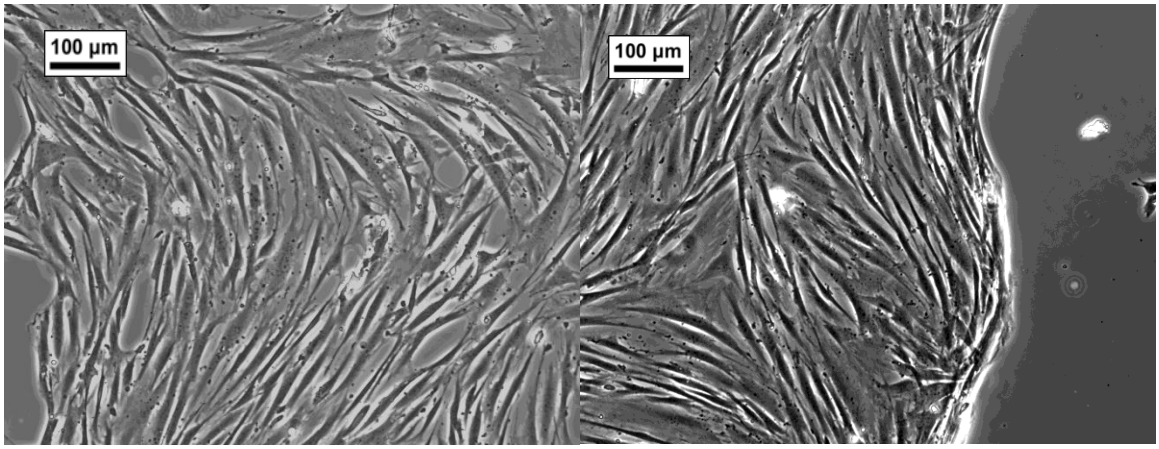
A: Control substrate, MSCGM, day 1 and day 5



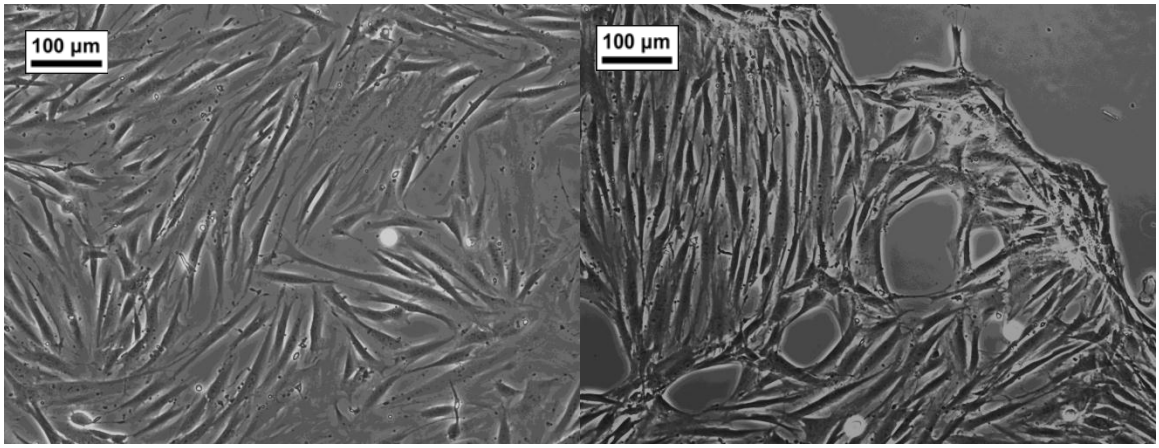
B: Control Substrate, ODM, day 1 and day 5



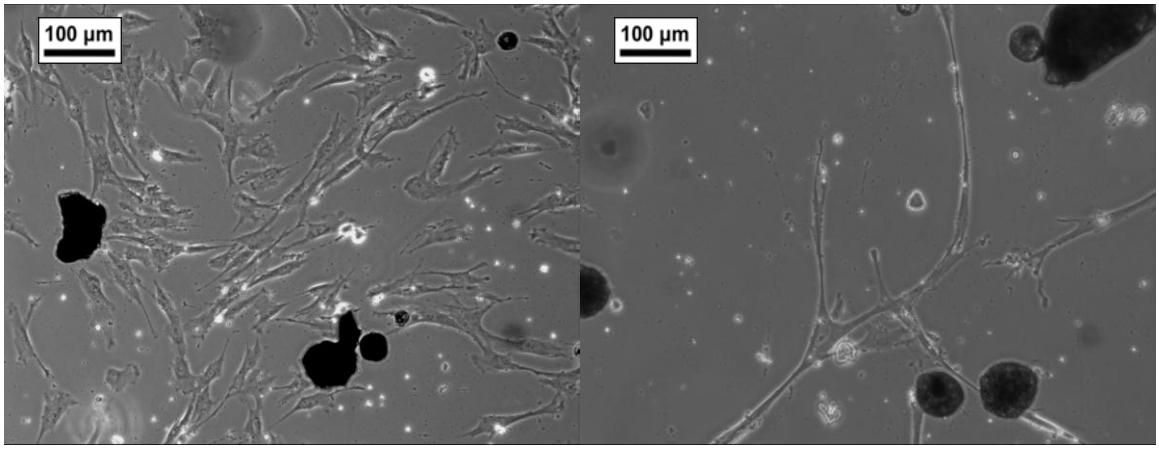
C: Control Substrate, ODM + HA, day 1 and day 5



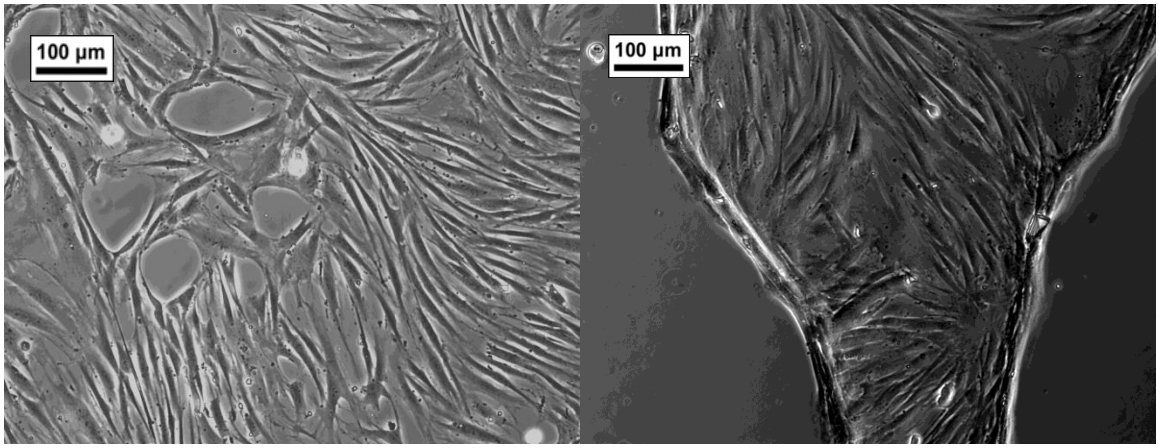
D: 30 kPa gel, MSCGM, day 1 and day 5



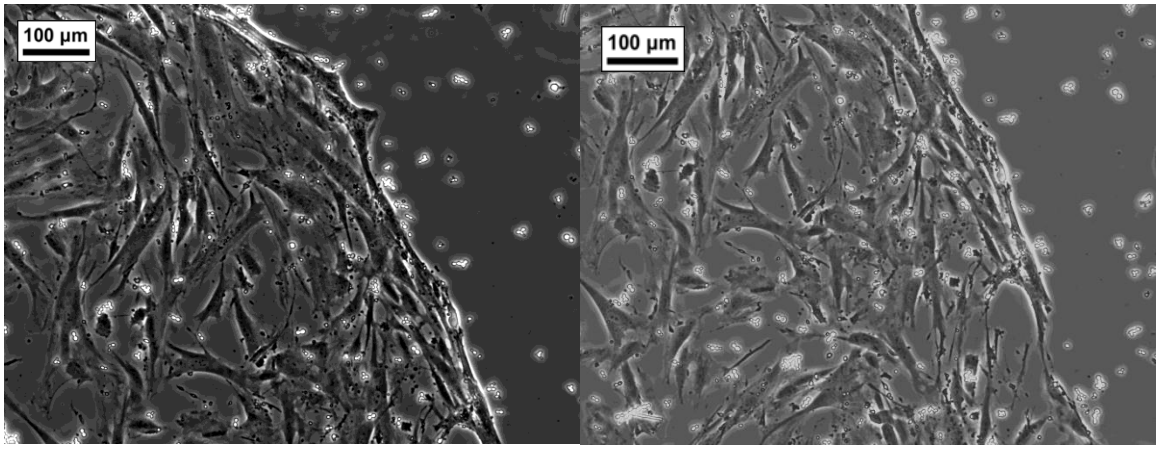
E: 30 kPa gel, ODM, day 1 and day 5



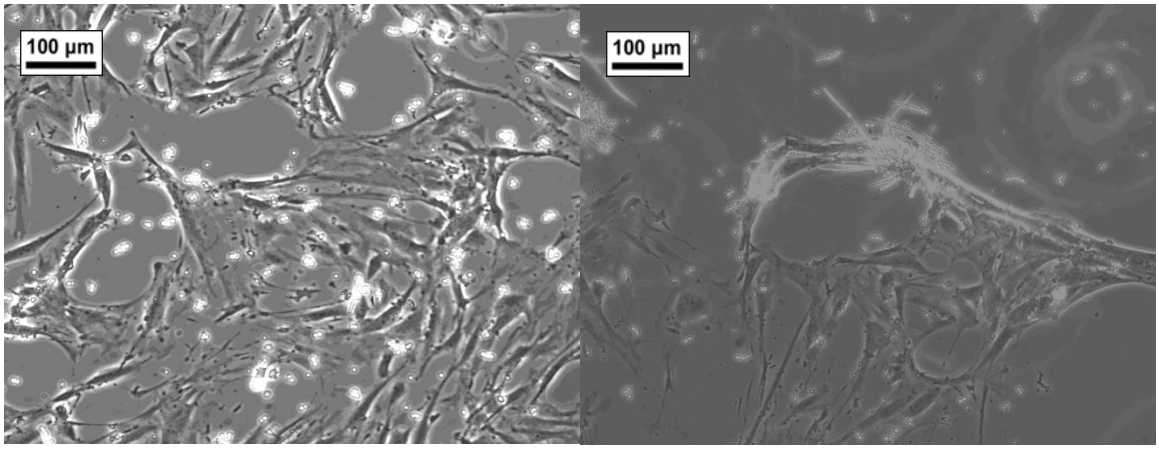
F: 30 kPa gel, ODM + HA, day 1 and day 5



G: 100 kPa gel, MSCGM, day 1 and day 5



H: 100 kPa gel, ODM, day 1 and day 5



I: 100 kPa gel, ODM + HA, day 1 and day 5

Figure 3.5 (above): Representative light microscope images of the cells under each condition and at days 1 (left) and 5 (right). All scale bars are 100 µm.

3.3.2 Immunocytochemistry Imaging

The immunocytochemistry was done to determine the presence of osteopontin (also called bone sialoprotein), a structural protein found in the extracellular matrix. Notably, it is a prominent component of the mineralized extracellular matrices of bones and teeth.(Hao et al., 1997; Shi et al., 2005; Sodek, Ganss, & McKee, 2000) It is commonly involved in many biological processes, but it appears essential in the remodeling and maintenance of bones and teeth.((Sodek et al., 2000)) In the fluorescence images below, osteopontin is labeled purple.

The green filaments seen in the fluorescence images are actin filaments. They are used to get a reference for the cellular outline. They do not show the cell membrane, but

rather the cytoskeleton which gives a fairly accurate representation of the cellular membrane location. Note that all images were taken using a 40X oil immersion lens.

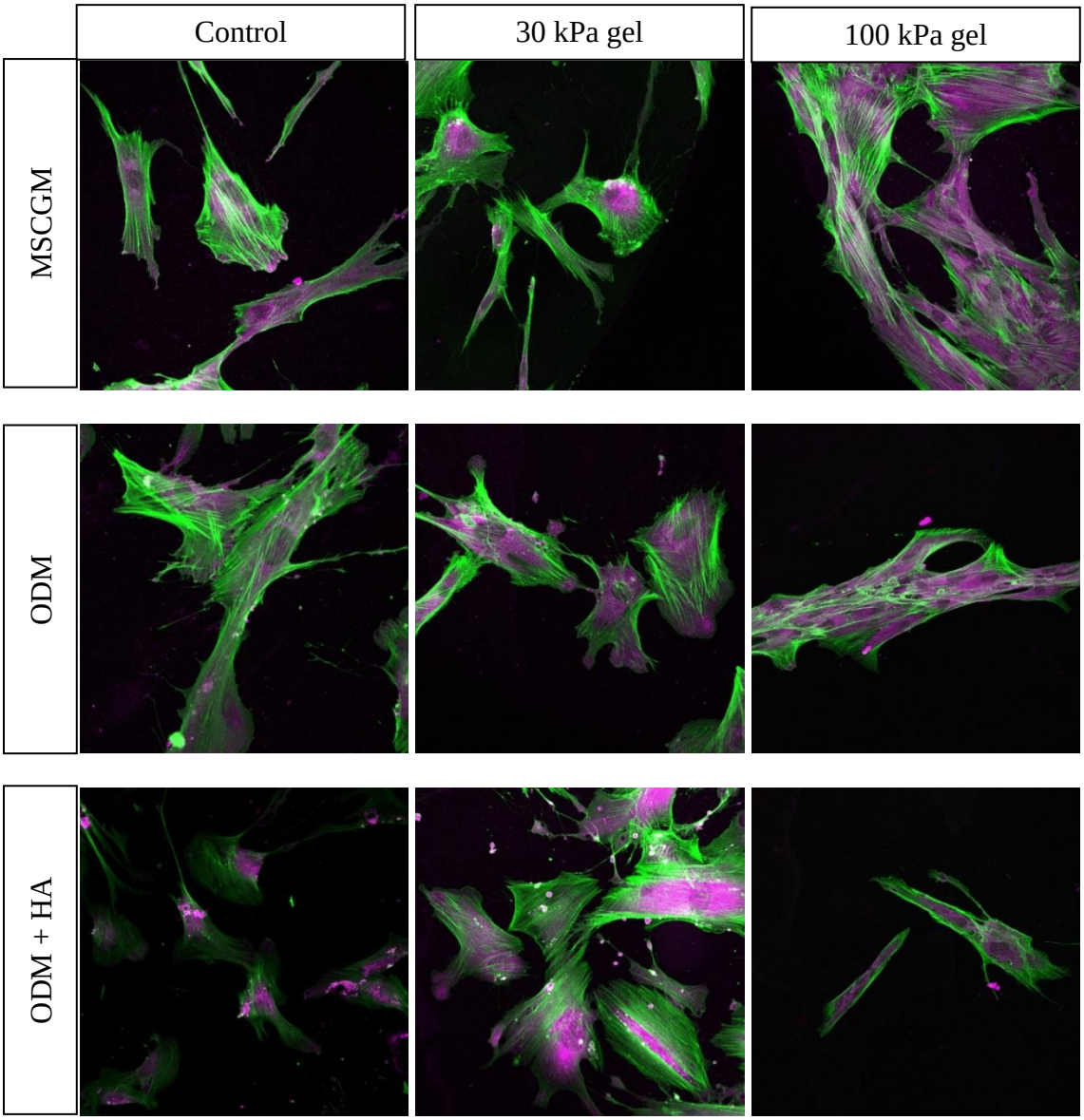


Figure 3.6: Confocal microscopy images of the cells in each condition, labeled on the top and left side of the figure. Note the osteopontin is purple and the actin is green.

3.3.3 ALP and BCA Assays

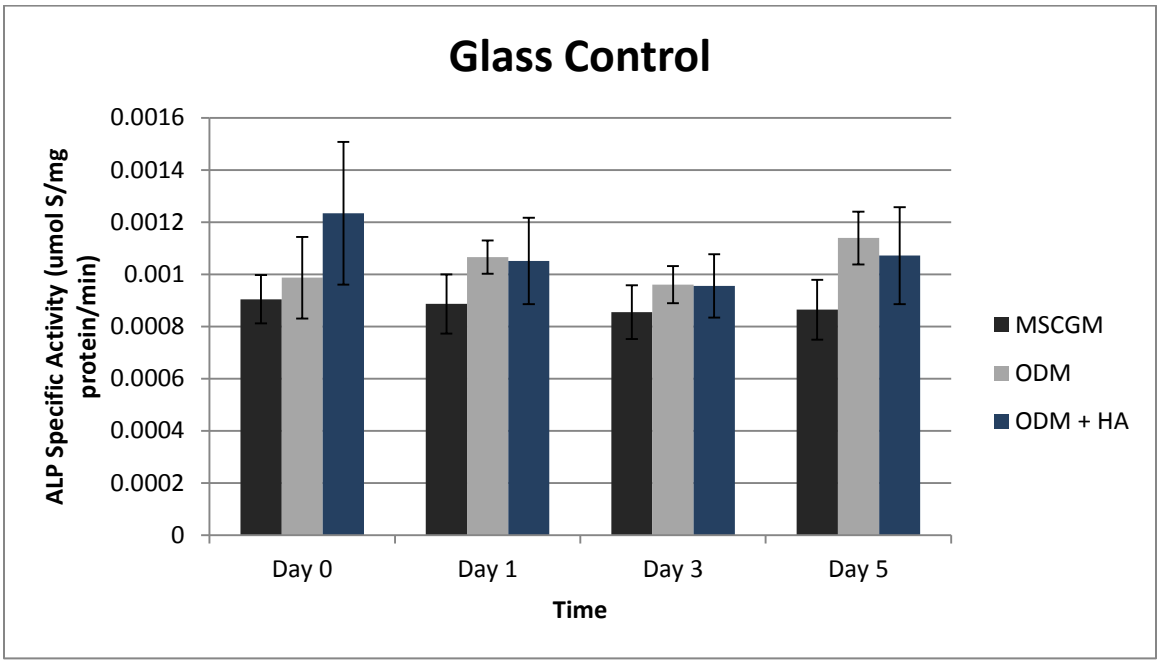


Figure 3.7: ALP Specific Activity under each condition on the glass control substrate.

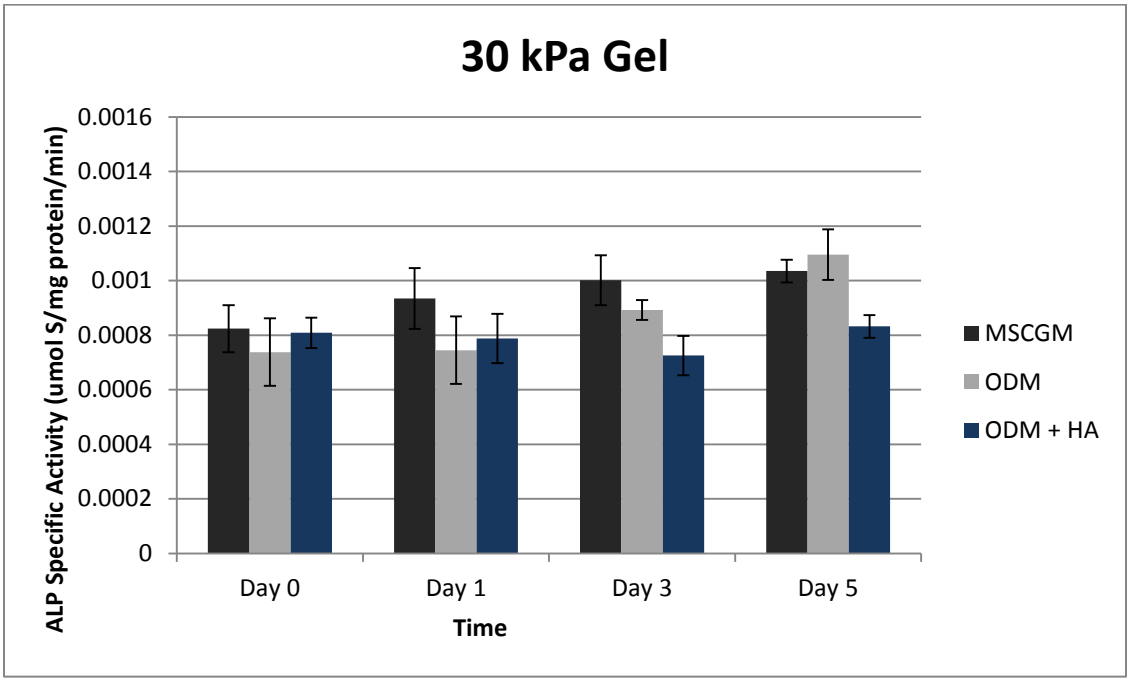


Figure 3.8: ALP Specific Activity under each condition on the 30 kPa gel.

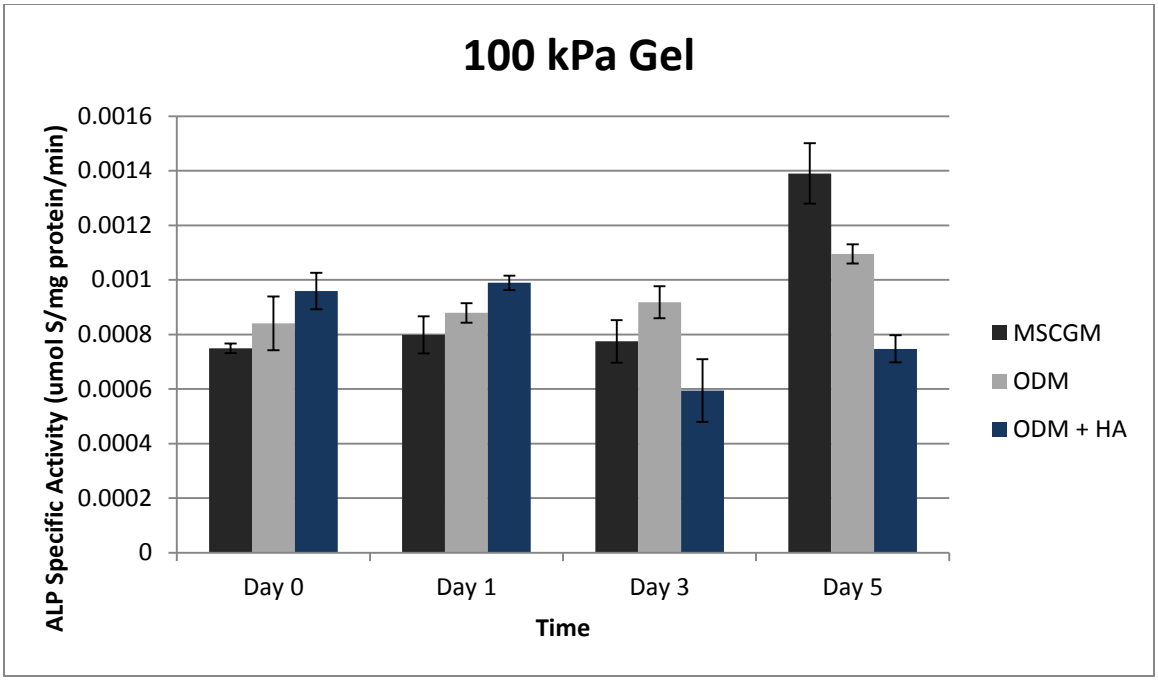


Figure 3.9: ALP Specific Activity under each condition on the 100 kPa gel.

It was seen that the specific activity of alkaline phosphatase was fairly constant over the time period tested in all conditions. It was also evident that the values for the standard deviations were low as the data was consistent within the each sample cell lysate.

3.4 Discussion

3.4.1 Basic Imaging

In Figure 3.5, it was observed that there was a significant difference in the behavior of cells on the glass control substrate as opposed to either polyacrylamide substrate. On the glass coverslips, the cells spread out and grew normally. Neither the osteogenic differentiation media nor the hydroxyapatite particles seemed to have a difference on the morphology of the cells. However, the substrate stiffness had a major effect. On both the 30 kPa and 100 kPa gels, the cells migrated to parts of the gel and formed aggregates that had very defined edges. No morphological differences were seen between the media types, but the HA particles did seem to have a negative effect on the cells. It was noted that seemed to be fewer living cells on the gels with hydroxyapatite on them. In all other conditions, the cells grew and multiplied well.

The clustering of cells on gels could be due to anchorage dependency. It is known that cells need a substrate to attach to, and stiffer surfaces usually are preferred. The surfaces the cells were exposed to might not have been stiff enough. In that case, they migrated to form a rigid aggregate where they could anchor to each other to obtain their firm attachment.(Engler et al., 2006)

Another possible explanation for the aggregation is a defect in the gel. The gels were not always in pristine condition due to the handling of them with a scalpel or forceps, so there could have been defects in the gel. Cells could have migrated towards those defects to settle inside them to have more surface area to attach.

3.4.2 Immunocytochemistry Imaging

Similar aggregates were seen with confocal imaging (see Figure 3.6), but they were not as common. It was noticed that osteopontin (purple in the images) was found in every cell centered around the nucleus. This was expected because osteopontin is not specific to bone-like tissue, it is just more prevalent in osseous tissues. One observation of note was that there were osteopontin-filled nodules stemming from cells only on the gels in osteogenic differentiation media. As it was noted before, it is thought that dentin is formed in nodules.(Hao et al., 1997)The nodules present could an early sign of osteogenic differentiation. In the images, hydroxyapatite appeared to have little to no effect on the cells.

3.4.3 ALP and BCA Assays

The consistency of the specific activity of ALP seen in Figures 3.7-3.9 in almost all conditions is curious. Morphological changes seen in both imaging modalities show that the gel had some effect on the cells, while the ALP data suggest otherwise. One observation is that for the control substrate and the stiffer (100 kPa) gel, the cells in ODM has higher specific activities than the cells in MSCGM. This is expected, as the ODM is supposed to induce osteogenic differentiation which should lead to an increase in activity of alkaline phosphatase. This was not the case for the 30 kPa gel, so the lack of stiffness may have hindered differentiation towards an osseous lineage. In previous experiments testing substrate elasticity, more compliant substrates induced differentiation down a neurogenic or myogenic lineage.(Engler et al., 2006)

In an extension of this study by another lab member, Laura Datko, an increase in ALP specific activity was seen over longer time periods in cells in ODM. Therefore, the short culture times may not have allowed time for enough differentiation to occur to have an effect on the amount of alkaline phosphatase. A longer time study would be needed to determine if this was the case.(Datko, 2011)

The effect of the hydroxyapatite particles could not be determined through these assays. At some time points in some conditions, the HA particles had a positive effect on ALP, and in other samples the effect was negative.

Finally, while the data was steady throughout all conditions and time points, it was seen that the control glass substrate induced the highest amount of ALP activity. This follows the idea that a stiffer substrate (glass is on the order of 1000X as stiff) promotes osteogenic differentiation. However, in contrast to Engler, et al, a stiff hydrogel did not seem to promote differentiation down an osseous lineage.

3.5 Conclusions

The results show promise in that changing the stiffness of the substrate did have an effect on both the growth behavior and alkaline phosphatase activity of human dental pulp stem cells. However, the effect cannot be concluded due to the limited culture time used and lack of volume of data. It can be noted, though, that morphological changes towards a defined lineage were not seen with either imaging modality other than the presence of osteopontin-filled nodules. Also, no significant changes in ALP were noticed

for any of the media conditions. One noticeable change in the cells was the migration and aggregation of cells on gels towards each other or possibly toward a defect in the gel.

In comparison to the study performed by Engler, et al on bone marrow stem cells, the results were not similar. In this study, no definite morphological changes were seen in the cells regardless of substrate, so it appears that dental pulp stem cells behave differently than bone marrow-derive stem cells.

3.6 Future Work

The goal of these studies was to determine the effect of substrate stiffness on differentiation of dental pulp stem cells. Valuable data was obtained, but more data needs to be collected at more time points, especially later time points, for conclusions from the data to be drawn.

The tests performed in these experiments were designed to show signs of differentiation down an osteogenic lineage. However, the overall goal of the research is to determine the odontogenic potential of dental pulp stem cells. Both lineages could show signs of the indicators tested in these experiments, so it is unknown which lineage the cells are actually differentiating towards. In the future, proteins specific to teeth, such as dentin sialophosphoprotein (DSPP) and dentin matrix protein I (DMP-1), will need to be stained for and also Western blotting should be done for these proteins.

To better compare with other literature sources, differentiation down other lineages should be attempted alongside the experiments. For example, neurogenic and

myogenic differentiation media should be used to see if that differentiation down those pathways is possible as well.

3.7 References

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CHAPTER 4: The Effect of Static Compressive Stress on the Growth and Differentiation of Dental Pulp Stem Cells

4.1 Introduction

In 1892, Julius Wolff published a work on bone transformation that is still applicable today. In his work, Wolff explained his model of how a bone's morphology develops. He theorized that a bone changes its external shape and internal architecture in response to the forces and stresses acting on it. Though this has been challenged by some, it is widely accepted as "Wolff's Law."(Team Bone, 2008)

There are forces in the body that constantly push and pull on our bones, cartilage, teeth, and the rest of the body. Much research has been done on the effects of these stresses on cartilage because it is commonly damaged and cannot regenerate. Studies have shown that cartilage is responsive to compressive stresses. In one study, both static and dynamic stresses had an effect on the biosynthesis of proteoglycans in cartilage explants. Proteoglycans are ECM components heavily expressed in cartilage cells.(Nugent et al., 2006)

Also, research has been performed to understand the effects of compressive stresses on mesenchymal stem cells for cartilage regenerative medicine. It has been shown that in the absence of any chondrogenic growth factors, dynamic compressive stresses applied to bone marrow stem cells induce synthesis of proteoglycans, a ECM component heavily expressed in cartilage cells.(Kisiday, Frisbie, McIlwraith, & Grodzinsky, 2009) Also, compressive stress applied to bone marrow stem cells induced

chondrogenic gene expression. Further, if the chondrogenesis had already been induced with transforming growth factor β1 (TGF-β1), the effects were amplified. (Miyanishi et al., 2006) Another study indicated that the same results were consistent for mesenchymal stem cells embedded within hydrogels.(Terraciano et al., 2007)

Up to this point, dental pulp stem cells have not been studied for their response to mechanical stresses and compression. However, it is known that odontoblasts do respond to mechanical stimuli.(Magloire, Couble, Thivichon-Prince, Maurin, & Bleicher, 2009) Odontoblasts are the dentin-producing cells that originate from DPSCs. Also, dental pulp stem cells that are subjected to orthodontic tooth movement forces have been thought to differentiate within hours to form either osteoblasts or osteoclasts depending on the force present. Compression forces induce osteoclastic differentiation, and tension forces induce osteoblastic differentiation.(Sutherland et al., 2004; Zainal Ariffin, Yamamoto, Zainol Abidin, Megat Abdul Wahab, & Zainal Ariffin, 2011)

The aim of this study is to determine the effects of static compression on the differentiation and growth of dental pulp stem cells. This will be achieved by first designing and fabricating a suitable compression chamber in which to test the cells. Then, cells will be cultured under different compressive conditions and analyzed for differentiation markers.

4.2 Experimental Design

4.2.1 Design of a Static Compression Chamber

To determine the effect of compressive stresses on dental pulp stem cells, a suitable compressive device needed to be obtained. No such device was available on the market, so a device was designed using SolidWorks. Requirements of the device were that it would fit onto a standard 6-well culture plate such that it could provide compression to cells seeded into collagen gels in the wells. The design was taken to Clemson Machining and Technical Services to discuss possible design modifications, and the custom compression chamber device was fabricated using 316L Stainless Steel. The device was analyzed and tested for its usability. Figures 4.1 and 4.2 show original design ideas from SolidWorks. The theory was that the cylinders would protrude into the wells of the culture plate and provide the compressive force.

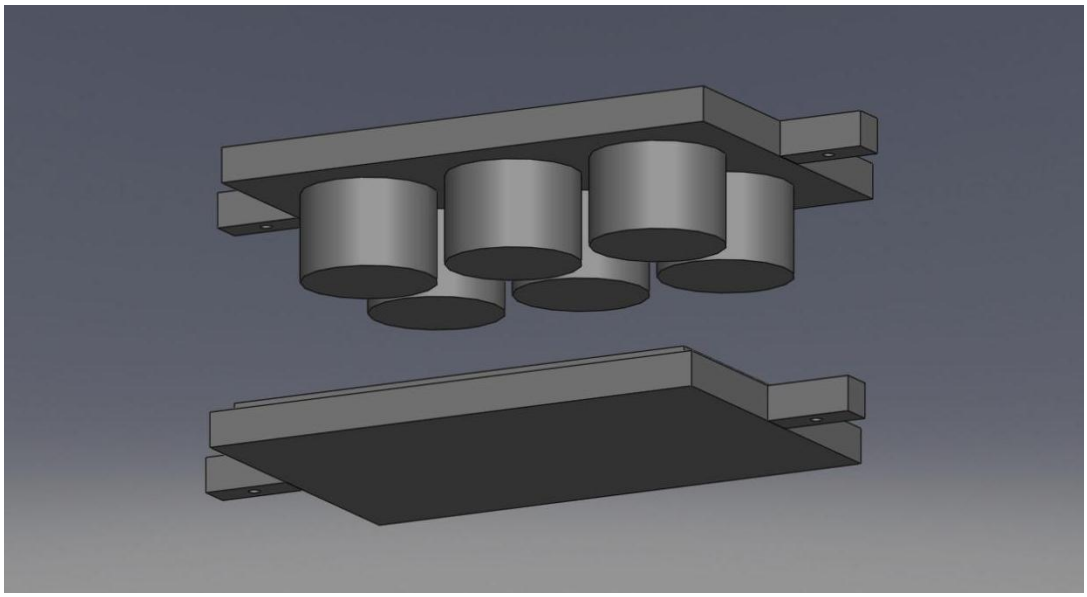

Figure 4.8: Bottom view of the initial SolidWorks design for the compression chamber

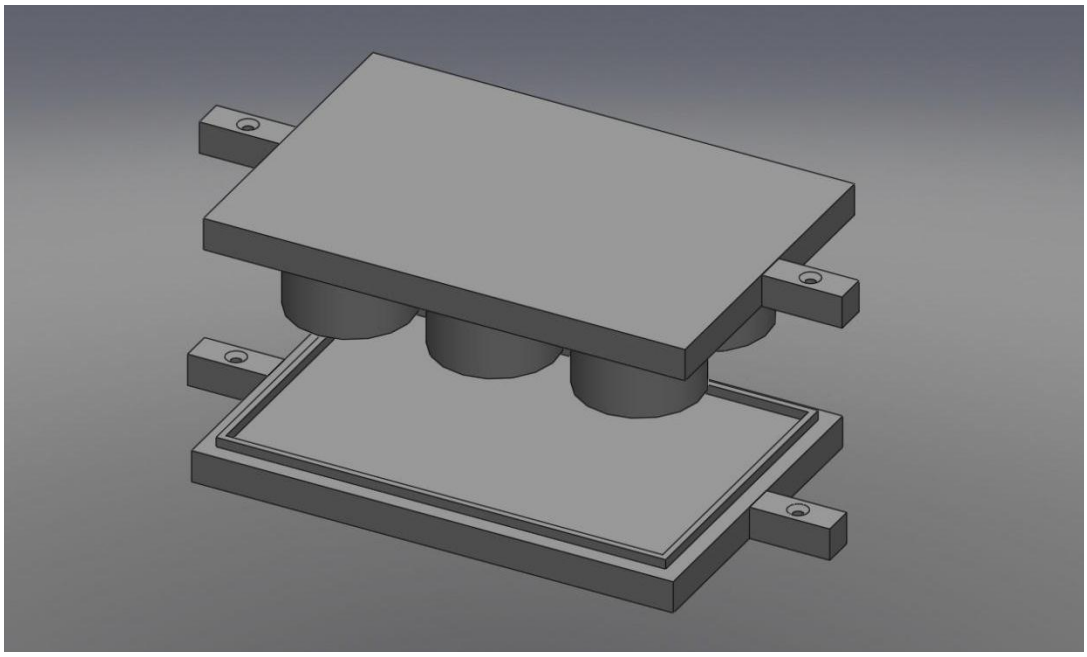

Figure 4.9: Top view of the initial SolidWorks design for the compression chamber

Though the design of the chamber was altered some in processing and fabrication, it ultimately was designed for symmetrical compression to all wells. To test, a pressure sensitive film was obtained (Fujifilm Prescale) and the device was tested for even pressure distribution. This was done by placing the film on a gel and setting the compression device onto the film.

4.2.2 Cell Acquisition and Isolation

In this experiment, porcine dental pulp stem cells (pDPSCs) were used instead of human cells as the source of the human cells was no longer available. It is assumed that porcine dental pulp is similar to human dental pulp, and the cells found in the dental pulp would have stem cell characteristics.

Porcine jawbones were obtained from Snow Creek Meat Processing facility in Seneca, SC and transported to Godley-Snell Animal Research Facility at Clemson University. Molars were extracted by peeling away the gingiva and excavating the alveolar bone beneath the molar. Once the root of the tooth was visible and free of attachment to the bone, the tooth was removed using an elevator and dental forceps. The molars were placed in sterile Hank's Balance Salt Solution (HBSS) with 2% anti/anti solution (penicillin/ streptomycin/ amphotericin B). The higher concentration of the anti/anti solution was used because the external surface of the tooth could have had a high amount of bacteria on it.

The teeth were rinsed with 70% ethanol and HBSS before the pulp chamber was exposed. This was accomplished by cracking the root of the tooth with pliers. The pulp was extracted using sterile forceps and placed in a sterile petri dish where the pulp was minced using a scalpel. The pieces were rinsed using sterile HBSS with 1% anti/anti solution. The pulp pieces were "digested" by incubating them in a 3mg/mL solution of collagenase II for 1 hour at 37°C. The solution was then passed through a cell strainer with a pore diameter of 70µm and the remaining solution was centrifuged at 1200 rpm for 10 minutes. The cell pellet was re-suspended in fresh MSCGM and plated onto cell culture flasks. Cells were adherent after 5-7 days. Note that the teeth and pulp were kept separate from each other throughout the whole process to reduce contamination.

4.2.3 Culture and Compression Conditions

To allow the cells to be under compression in a more controlled way, the cells needed to be embedded in a 3-D gel construct. Since the stiffness of the gel would not be altered, polyacrylamide was not the gel material chosen. For clinical translational purposes, gels were made out of rat tail collagen type I (BD Biosciences).

To contain the collagen gel to a consistent shape and height, hollow cylindrical molds that were 12 mm in diameter were formed with a PDMS Sylgard 184 Silicone Elastomer kit (Dow Corning). The molds were then placed onto glass coverslips and set into the wells of a standard 6-well cell culture plate. The molds can be seen in Figure 4.3.

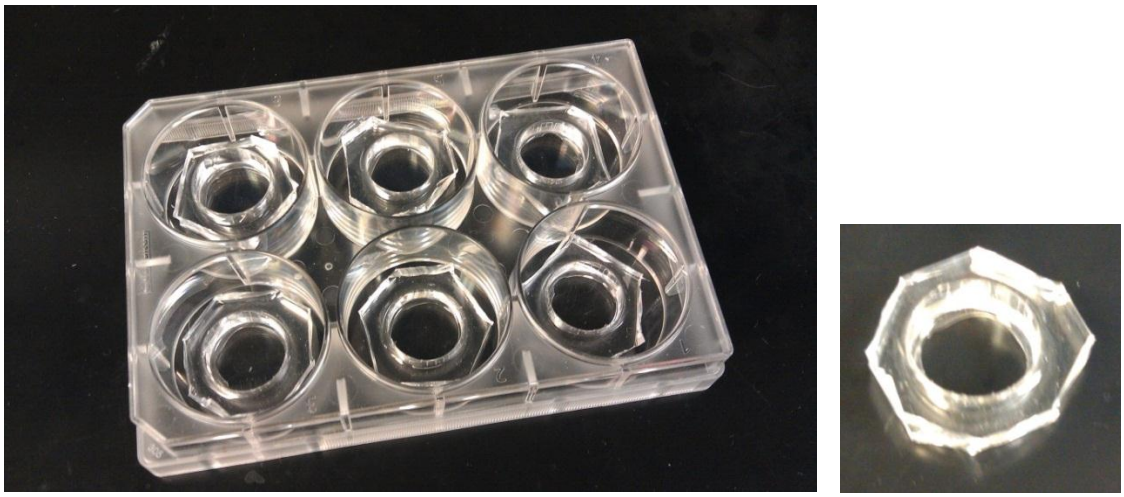

Figure 4.10: The PDMS molds in a cell culture plate (right), and one individual mold (left)

Collagen gels were synthesized within the PDMS molds by mixing a 2mg/mL collagen solution, HEPES, and 10X MEM in an 8:1:1 ratio to get the desired final volume. Then, 600 µL of the collagenous mixture was added to each mold and cells were quickly added before gelling occurred. The collagen-cell mixture was placed in the 37°C

incubator for 30 minutes to allow gelling to occur. The compression chamber piece replaced the original culture plate top, and the collagen gels were observed under compression. To determine usability and form a protocol, 7F2 osteoblasts were used because they are an abundant and well-characterized cell line. To perform the actual tests, both the porcine dental pulp stem cells previously discussed and the osteoblasts were used. The media was varied between MSCGM and ODM.

To test the compression effects, different compressive conditions needed to defined and tested. First, the loading weight could be altered by adding weights onto the top of the compression device. Also, the schedule of time compressed vs. time uncompressed could be varied. Taking all factors into account, a testing plan was devised and it is summarized in Table 4.1 below. Note that the control used for the experiment will be cells grown in collagen gels allowed to freely swell.

	6 hours on/6 hours off	**12 hours on/ 12 hours off**	**24 hours on/ 24 hours off**
No weight added	MSCGM	MSCGM	MSCGM
	ODM	ODM	ODM
Add 1 kg	MSCGM	MSCGM	MSCGM
	ODM	ODM	ODM
Add 2 kg	MSCGM	MSCGM	MSCGM
	ODM	ODM	ODM

Figure 11.4: Experimental Design Conditions.

4.2.4 Testing Analysis

Similarly to before, cells were tested with BCA and ALP assays to determine total protein content and ALP specific activity. However, since the cells were embedded in a collagen gel, the gels first needed to be digested using a collagenase solution incubated at 37°C for 20 minutes. The cells were then strained through a 70 μm cell strainer, and the resulting cells were re-suspended in PBS and the protocol from Appendix C was followed as mentioned in Chapter 3. The control in the experiment was to use gels that were allowed to freely swell (not under compression). Also, ALP and BCA assays were performed on samples from days 1, 3, and 5.

4.3 Results

4.3.1 Compression Chamber

The chamber was fabricated by Clemson Machining and Technical Services, and the final dimensions and measurables of the device are listed in Table 4.1.

Table 4.1: Physical specifications of the custom compression device

Specification	Measurement
Weight	636 grams
Length	5.5 inches
Width	3.75 inches
Cylinder Depth	0.57 inches
Cylinder Diameter	0.5 inches

The device was fabricated from medical grade 316L stainless steel. By calculating the total surface area of all of the cylinders and finding the force exerted by the weight of the device, a theoretical pressure of 9.2 kPa was calculated for the pressure exerted on the collagen gels. Images of the compression device can be seen in Figures 4.5-4.7 below.

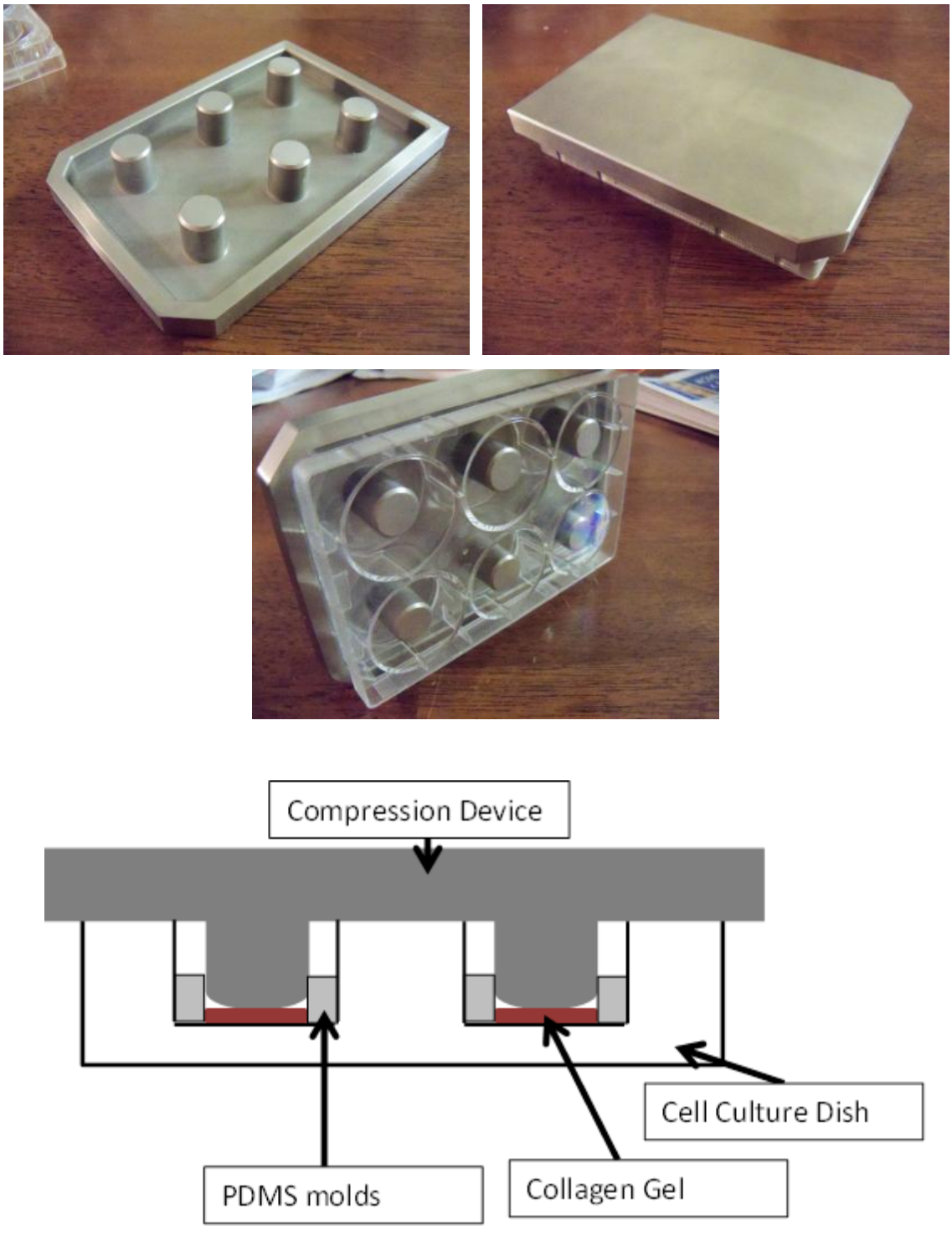

Figure 4.12: Views of the compression device from the underneath (top left), top of the compression device (top right), the device fitting onto a 6-well culture plate (middle), and a diagram of the components of the compression chamber (bottom).

In testing for usability, the compression device passed. It performed all necessary functions as needed. In symmetry tests with the pressure-sensitive film, the device was found to be mostly symmetric with the pressure being distributed almost completely evenly over all surfaces. The pressure film can be seen in Figure 4.6. Note that the film was not quite large enough to fit all the cylinders on it, but enough data was seen to assume symmetry.

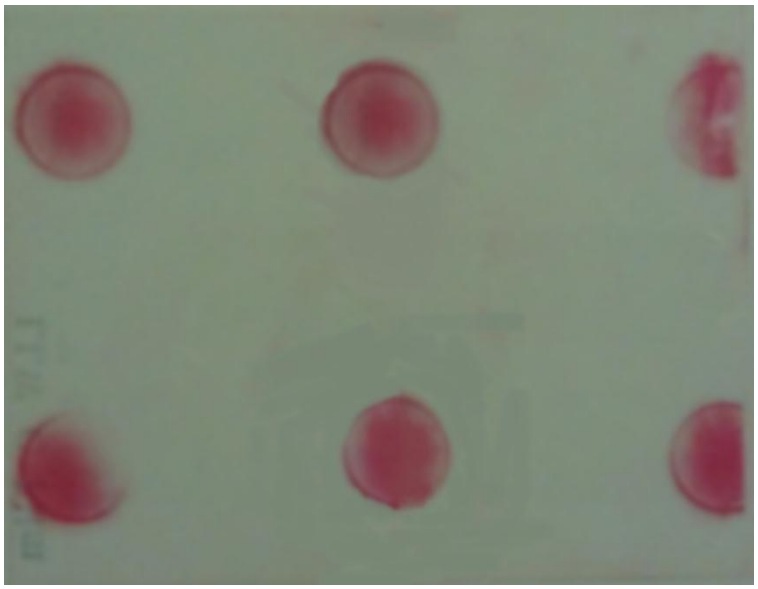

Figure 4.13: The colorimetric pressure sensitive film results.

4.3.2 ALP and BCA Assays

The results presented in this section are preliminary, and not enough data has been obtained due to time constraints and the fact that only one device was available for use. Therefore, only data from day 1 of the experiments was able to be recorded for all conditions. The data for dental pulp stem cells and 7F2 osteoblasts under three

compressive conditions after day 1 are presented below in Figures 4.7 and 4.8. The error bars on the graphs represent the standard deviations of the samples.

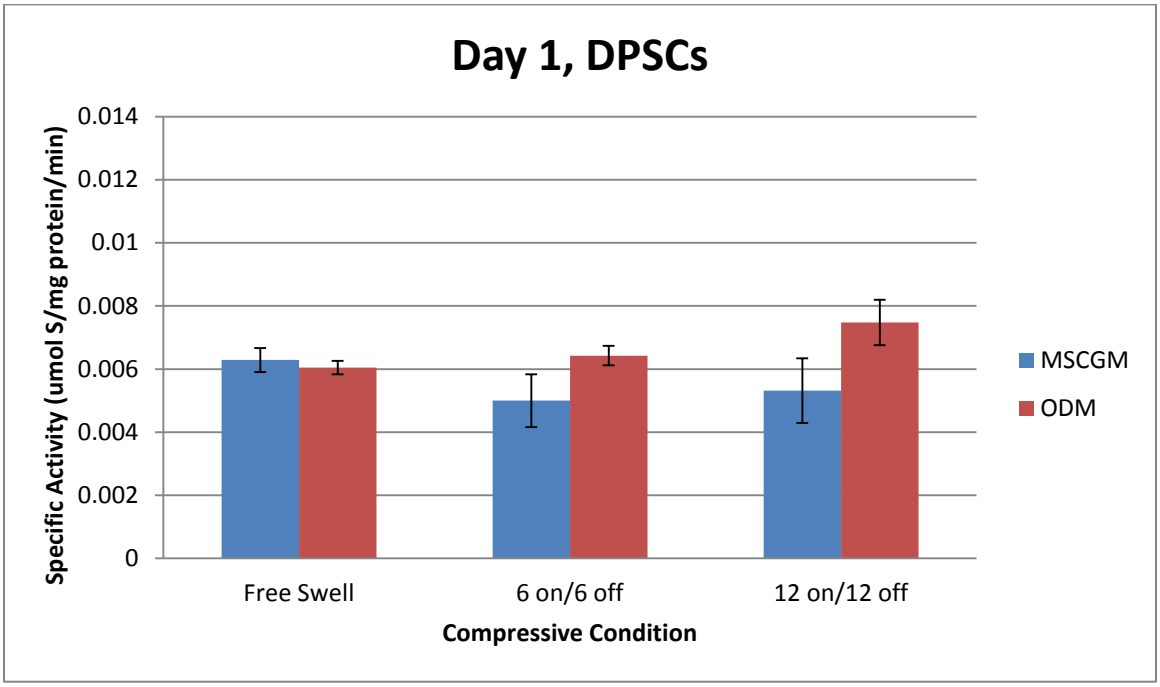

Figure 4.14: Alkaline Phosphatase specific activity for dental pulp stem cells

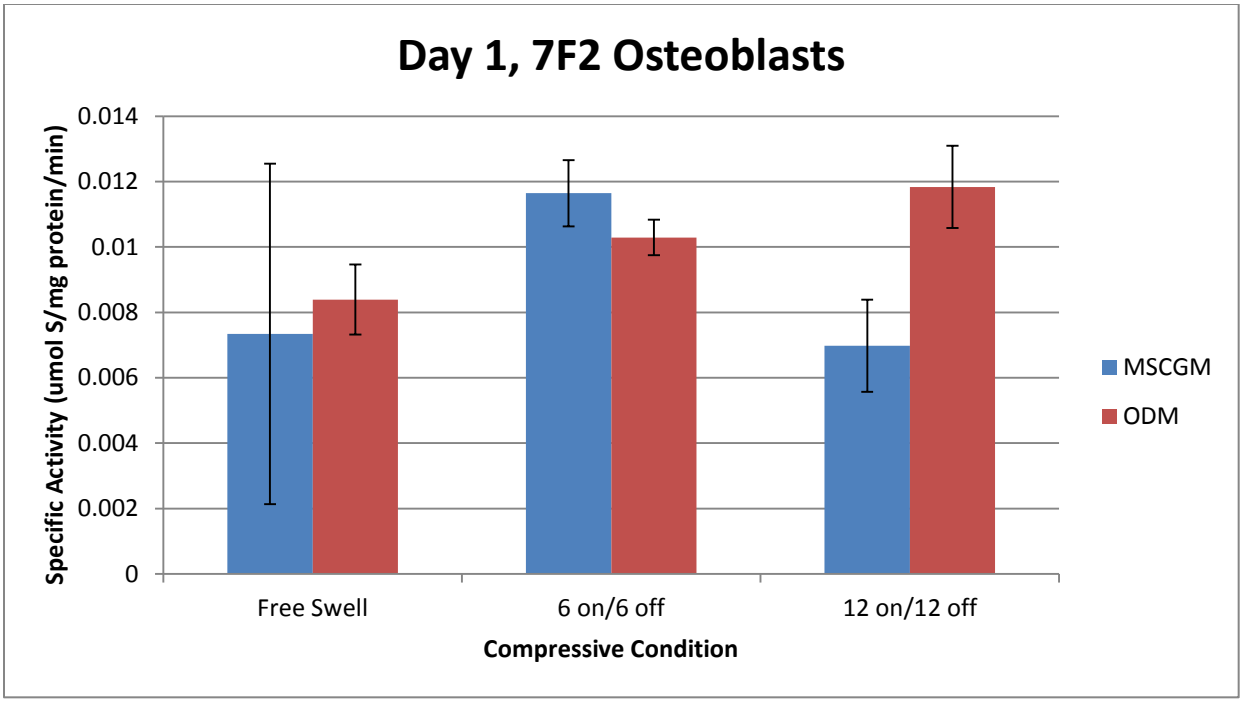

Figure 4.15: Alkaline Phosphatase specific activity for 7F2 Osteoblasts

To draw inferences and conclusions from this data, statistical analysis was performed using Microsoft Excel. A T-Test was performed between the sets of three measurements for each sample. Null (H_o) and alternative (H_a) hypothesis were as follows: H_o: $\mu_1 = \mu_2$, H_a: $\mu_1 \neq \mu_2$. A two-tailed T-Test was used with $\alpha = 0.05$. Table 4.2 below summarizes the results found from the T-Tests.

Table 2.2: Comparison of means for each pair of conditions. Note that when a p-value is less than *α*, the null hypothesis is rejected meaning that the samples are significantly different.

Constant Conditions	Population 1	Population 2	T-Test p-value	Decision
DPSC, ODM	Free Swelling	6 on/6 off	0.158	Fail to Reject H_o
DPSC, ODM	Free Swelling	12 on/12 off	0.029	Reject H_o
DPSC, ODM	6 on/6 off	12 on/12 off	0.081	Fail to Reject H_o
DPSC, MSCGM	Free Swelling	6 on/6 off	0.073	Fail to Reject H_o
DPSC, MSCGM	Free Swelling	12 on/12 off	0.198	Fail to Reject H_o
DPSC, MSCGM	6 on/6 off	12 on/12 off	0.702	Fail to Reject H_o
7F2 OB ODM	Free Swelling	6 on/6 off	0.049	Reject H_o
7F2 OB, ODM	Free Swelling	12 on/12 off	0.022	Reject H_o
7F2 OB, ODM	6 on/6 off	12 on/12 off	0.122	Fail to Reject H_o
7F2 OB, MSCGM	Free Swelling	6 on/6 off	0.232	Fail to Reject H_o
7F2 OB, MSCGM	Free Swelling	12 on/12 off	0.913	Fail to Reject H_o
7F2 OB, MSCGM	6 on/6 off	12 on/12 off	0.009	Reject H_o
Free Swelling, DPSC	MSCGM	ODM	0.401	Fail to Reject H_o
Free Swelling, 7F2 OB	MSCGM	ODM	0.749	Fail to Reject H_o
6 on/6 off, DPSC	MSCGM	ODM	0.051	Fail to Reject H_o
6 on/6 off, 7F2 OB	MSCGM	ODM	0.111	Fail to Reject H_o
12 on/12 off, DPSC	MSCGM	ODM	0.040	Reject H_o
12 on/12 off, 7F2 OB	MSCGM	ODM	0.011	Reject H_o

It is seen in Table 4.2 that most of the conditions yielded similar results, and the p-value for those samples was greater than α (α=0.05). There were some data comparisons that showed p-values less than α, which indicates a statistically significant difference in means. It can be noted that the osteoblasts in ODM have significant differences in both compressive conditions compared to free swelling conditions. Also, it is important to note that the only significant difference in the DPSCs due to compression was seen in ODM when the cells were under 12 hours on/12 hours off of compressive force. Also, the media conditions only seemed to have a significant effect under the 12 hours on/12 hours off condition for both types of cells.

4.4 Discussion and Conclusions

The compression device performed as necessary, and an acceptable protocol was formed for testing cells. The PDMS and collagen gel components also were satisfactory in performing their roles. The pressure exerted on the gels is applicable and is similar to physiological pressures. In other studies, compressive pressures ranging from 1-100 kPa were used(Nugent et al., 2006), so the baseline value of 9.2 kPa is acceptable. Also, the assumption that the theoretical value is correct is supported by the symmetry of the device. While it may not be exactly 9.2 kPa for all gels, the value is close. More weights could still be added to reach higher pressures.

The ALP specific activity data shows very clearly that osteoblasts have more alkaline phosphatase than DPSCs which was expected. Also, it can be seen that in DPSCs, only one mean was shown to be significantly different from another due to

compression. This could indicate that the cells did not have a chance to differentiate yet. This was very likely because the culture time was only 1 day. Also, statistical analysis showed that the only difference due to different media types was seen under compressive conditions, so it could be that the compression and media have an additive effect.

The osteoblasts on average produced more ALP under compression than free swelling conditions. This could mean that the osteoblasts are activated by stress, similar to odontoblasts being activated by dental procedures.(Magloire et al., 2009) The differences in osteoblasts under compression were significant in samples in ODM. It can be inferred that compression was able to have a greater effect on osteoblasts because they were also differentiated into cells that respond to forces. The DPSCs may not naturally respond as readily to forces, as it seems they need more time to differentiate before compression has a significant effect.

Overall, the work presented in this study has shown that there is reason to investigate the effect of compression on dental pulp stem cell growth and differentiation, and this experiment has tested one method of testing static compression.

4.5 Future Work

In the future, testing at more time points should be performed to better understand the differentiation of cells over time. Also, the pressure exerted on the cells in gels should be varied to cover a larger range of physiological pressures and to compare to other experiments. Also, Western blotting should be performed for bone proteins such as osteopontin, but also dental-specific proteins like DSPP mentioned in a previous chapter.

Lastly, this concept needs to be applied to a dynamic compression study to better simulate the pressures associated with chewing and other physiological forces.

4.6 References

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CHAPTER 5: Conclusions

Overall, tissue engineering of teeth is still in its preliminary stages, but the need for a suitable replacement for dental materials is there. With all of the pathologies and no material that can fully mimic the function of teeth, a new dental material is needed that can better replace native dental tissue. Being able to re-grow healthy dental tissue within the tooth would be the holy grail of dental tissue engineering. However, not enough is known about the differentiation of stem cells into dental tissue. Also, what is known is limited in its translational capacity. This research is focused on determining the micro-environmental cues that stem cells need to induce differentiation down a bone or tooth-like path.

Much has been done to characterize and investigate dental pulp stem cells, and the results have been presented above. Among the major points are the observations that growth patterns were affected by substrate stiffness and growth factors. However, the culture times were not long enough to observe differentiation. It was also seen that DPSCs do not behave in the same fashion that bone marrow stem cells do. To continue this research, western blotting and staining for dental-specific proteins such as DSPP and DMP-1 should be done to distinguish whether the differentiation was towards bone or tooth-like tissue. Also, more repetition needs to be done for the results to have statistical significance.

In the current work, it was seen that the setup used allows for testing of static compression on cells seeded in gels. This has promise, but not enough data has been

collected for there to be conclusions drawn. More data will be collected for the cells under different conditions and at more time points to test for statistical significance.

APPENDIX A: Polyacrylamide Gel Protocol

Source: Yu-li Wang Laboratory

Modified Acrylamide Substrate Protocol (by: Matthew Cupelli, Laura Datko, Dr.

Delphine Dean)

Materials:

- 22mm x 22mm square coverslips
- Distilled water
- NaOH pellets
- 3-aminopropyltrimethoxy silane
- 0.5% gluteraldehyde in PBS
- 1M HEPES (pH 8.5)
- 50mM HEPES (500uL of 1M HEPES per 10 mL of PBS, pH 8.5)
- 40% Acrylamide and 2% Bis
- Ammonium Persulfate (10mg in 100µl of PBS – prepare immediately before use)
- TEMED
- Fibronectin (1 mg/mL in H2O)

Coverslip activation:

1. Put several NaOH pellets in a weigh boat and add distilled water. When they dissolve, add coverslips for a few minutes, then remove and let dry on a kimwipe.
2. Coat one side of the coverslips (in a new weigh boat) with STERILE 3-aminopropyltriethoxy silane
3. Incubate at room temperature for 5 minutes
4. Rinse with distilled water and shake for 5 minutes
5. Repeat #4 two more times **make sure to get all the APT off or the coverslips will have a reddish tint when you add gluteraldehyde**
6. Coat the same side of the coverslips with 0.5% gluteraldehyde for 30 minutes at RT
7. Repeat the rinsing process (steps 4 and 5)

Acrylamide preparation:

1. Make acrylamide solution in a 25 mL glass breaker according to the chart at the end of protocol
2. Add 30µl ammonium persulfate and 20µl TEMED to the acrylamide solution; mix gently
3. IMMEDIATELY pipet 20µl onto activated coverslip and quickly place another coverslip on top
4. Leave remaining acrylamide in beaker. (Once this has polymerized, gel on coverslip has also polymerized)
5. Flood the bottom of the dish with ~2mL of 50mM HEPES
6. Remove the top coverslip (with scalpel or forceps). Substrates can now be stored in PBS for up to 2 weeks at 4°C

Final Acryl/Bis	40%Acrylamide	2%Bis	1M HEPES	H_20+Beads	Young's Modulus
8%/0.1%	1000 ul	250 ul	50 ul	3700 ul	100 kN/m2
8/0.08	1000	200	50	3750	75
8/0.06	1000	150	50	3800	30
8/0.05	1000	125	50	3825	23
8/0.04	1000	100	50	3850	17
8/0.03	1000	75	50	3875	14
8/0.02	1000	50	50	3900	10
5/0.12	625	300	50	4025	33
5/0.10	625	250	50	4075	28
5/0.08	625	200	50	4125	24
5/0.06	625	150	50	4175	15
5/0.05	625	125	50	4200	??
5/0.025	625	63	50	4262	7
3/0.10	375	250	50	4325	??

Prepping substrate for cells:

1. Coat gels with 0.2 mg/mL sulfo-SANPAH in H2O for 10 minutes under long wave UV
2. Rinse 2X with 50 mM HEPES (all sulfo-SANPAH waste into designated container in lab)
3. Add 200µl fibronectin (125 ug/mL to get a 5 ug/cm2 coating) to substrate and store overnight at 37°C
4. Rinse and store in PBS at 4°C for up to one week
5. Before plating, UV sterilize substrates for 15-30 minutes

APPENDIX B: Confocal Staining Protocol

Staining Cells for Confocal Imaging

USC School of Medicine, Columbia, SC
Instrumentation Resource Facility
Updated for Dean Lab by Matthew Cupelli

1. 30 min PBS/0.01 M Glycine/0.1% Triton-X
2. 15 min 5% BSA/PBS
3. 15 min 5% Normal Serum (1% BSA/PBS)
4. Overnight *Primary Antibody* (1% BSA/PBS) @ 4°C (1:100)

********** DON'T THROW PRIMARY AWAY!

****

5. 2 x 15 min Rinse with 1% BSA/PBS
6. 15 min 5% Normal Donkey Serum (1% BSA/PBS)

********** COVER WITH FOIL

****

7. 2 hours *Secondary Antibody* (1% BSA/PBS) @ 37°C (1:100)
8. 15 min Rinse with 1% BSA/PBS
9. 15 min Rinse with PBS
10. 1 hour 488 Phalloidin in PBS Shaking at RT (1:50)
11. 2 x 15 min Rinse with PBS
12. Add 1 drop of *SlowFade*® Gold antifade reagent with DAPI
13. Mount hydrogel on a glass slide, hydrogel side down
14. Seal edges with clear nail polish or superglue

APPENDIX C: BCA and ALP Assay Protocol

Protocol for BCA and Alkaline Phosphatase Assays (from Bioengineering Department)

Collecting cell lysates:

1. Remove culture media and wash cells with PBS
2. Add 300 ul Mammalian-Protein Extraction Reagent ™(M-PER) solution to each well
3. Incubate for 20 minutes at room temperature
4. During incubation period, label a microcentrifuge tube for each sample
5. Collect the cell lysates into the tubes (they can be frozen until ready for use)

BCA total protein assay:

1. Prepare working reagent (**WR**) as follows:
    a. Mix in a 15 ml tube 5 ml of **Reagent A** and 100 ul of **Reagent B**
2. Using a stock solution of 1mg/ml of albumin, prepare standard curve as follows (be very careful/accurate when pipetting these volumes as your results will depend on the curve):

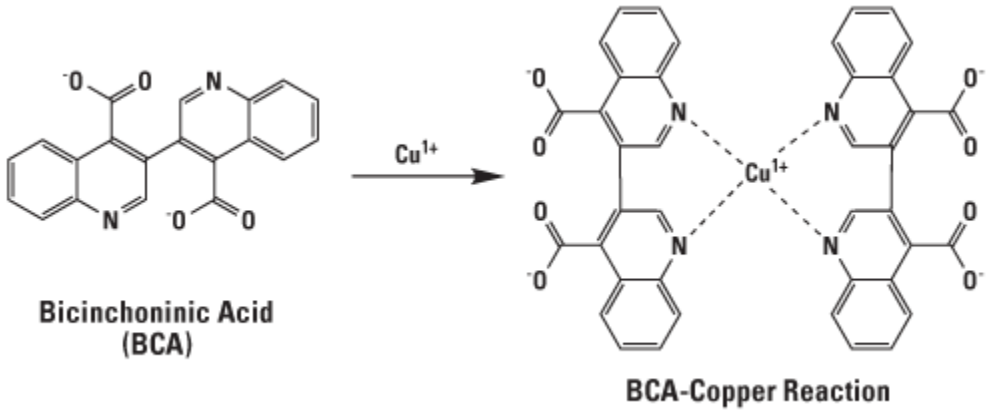
Standard #	Stock Solution (ul)	Water (ul)	Final Concentration (ug/ml)
1	200	200	500
2	100	300	250
3	50	350	125
4	25	375	62.5
5	0	400	0

3. Perform the BCA assay in a 96 well-plate as follows:
    a. Pipette 25 ul of each standard into 5 wells (in triplicate)
    b. Pipette 25 ul of each sample (cell lysates) into separate wells (in triplicate)
    c. Add 200 ul WR to each well
    d. Cover and incubate for 30 minutes at 37 C
    e. Read the absorbance at 562 nm

f. Use Excel to create standard curve with standard #1-5 (Concentration vs. Absorbance scatterplot + trendline) and use the trendline equation to determine the total protein in your samples (ug/ml).

Principle of the method: The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.[1] The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-20,000 ug/ml). The BCA™ method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

(1)



Bicinchoninic Acid (BCA)

BCA-Copper Reaction

Alkaline Phosphatase assay:

1. Prepare reagents:
    a. **Substrate (S)** – In a 15 ml tube, dissolve 1 tablet of p-Nitrophenylphosphate (5mg, PNPP) in 4 ml DI water, and then add 1 ml 5X diethanolamine (DEA) buffer and 50 ul of 10mg/ml $MgCl_2$ solution.
    b. **p-Nitrophenol stock solution** – 400 ug/mL in water
    c. **2 M NaOH** (in water)
2. Make the standard curve:
    a. Label 5 microcentrifuge tubes and make the following dilutions of p-Nitrophenol stock solution:

Standard #	Stock p-NP (ul)	Water (ul)	Final Concentration p-NP (ug/ml)
1	200	200	200
2	100	300	100
3	50	350	50
4	25	375	25
5	0	400	0

    b. Add 25 ul NaOH to each of the 5 microcentrifuge tubes
    c. Pipette 225 ul from each tube in a 96-well plate and read absorbance at 405 nm
3. Perform the ALP assay in a 96-well plate as follows:

a. Label microcentrifuge tubes for each sample and one for a blank (**no enzyme**)
b. Pipette 300 ul substrate (reagent 1) into each tube
c. Add 50 ul DI water to all sample tubes
d. Add 50 ul of your cell lysates to each appropriate sample tube
e. Add 100 ul M-PER to the **"blank" tube**
f. Incubate for 30 min at 37 C
g. Stop the reaction with 25 ul NaOH
h. Pipette 200 ul from each tube into a 96-well plate (in duplicate)
i. Read the absorbances at 405 nm

Principle of the method: Bone Alkaline Phosphatase (B-ALP), a glycoprotein found on osteoblasts and an indicator of bone turnover, catalyses the hydrolysis of p-Nitrophenylphosphate at pH 10.4, liberating p-Nitrophenol and phosphate, according to the following reaction:

p-Nitrophenylphosphate + H2O → p-Nitrophenol + phosphate

The rate of p-Nitrophenol formation, measured photometrically, is proportional to the concentration of alkaline phosphatase present in the sample.