Comparative Study of Condylar Cells of the Goat TMJ

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The Temporomandibular Joint (TMJ) is the unique ginglymo diarthrodial synovial joint of the body, and a vital component of the stomatognathic system. Despite the limited regenerative ability of cartilage, recent studies on goats have demonstrated that the TMJ cartilage, which is histologically defined as fibrocartilage, possesses an innate regenerative ability in vivo. These studies have also determined that the fibrocartilage subchondral interface of the TMJ condyle contains a heterogeneous population that includes stem cells/progenitor cells. This study aims to compare biological activities, such as changes in cell number over time, space filling capability and osteogenic potential of 3 types of condylar cells in the mandibular condyle of goat TMJ. It aims to go further on understanding the tissue biology of the condylar compartment. This work seeks to better understand the cells involved in subchondral bone remodeling and fibrocartilage regeneration in the context of TMJ tissue engineering. Condylar cells from the surface and cartilaginous layers were compared with cells from the subchondral bone layer and bone marrow cells for different biological activities. To assess changes of cell number overtime, condylar cells were cultured in different vessels and counted after 7 days. The growth curves of condylar cells were compared. For space filling ability, the time it took the cells to occupy a void space created by a silicone block on the culture dish was measured, as well as the distance from the leading edge to the furthest cell. For osteogenic potential, cells were cultured for 21 days in media containing inorganic phosphate and stained for Alizarin red, Von Kossa and Alkaline Phosphatase.

The results showed that all condylar cells differed with respect to all biological activities. To highlight, all three types were able to deposit copious amounts of mineral. We demonstrate that the fibrocartilage and subchondral bone interface compartment of the TMJ condyles contain a heterogeneous cell population that includes progenitor cells. These populations showed different cell numbers over time, space filling activity and mineral deposition after cultured under osteogenic condition. We verified that condylar cell populations can be harnessed through tissue engineering to guide fibrocartilage and subchondral bone regeneration.

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1.0 Introduction

The regeneration of osteochondral tissues has been extensively recognized as one of the more challenging processes in the area of tissue regeneration. This is primarily due to cartilaginous tissues lacking an intrinsic or innate regenerative ability, and the complex architecture of multilayer tissues. Cartilage, both hyaline and fibrocartilage, is a structural and functional component of synovial and cartilaginous joints. The temporomandibular joint (TMJ) is a diarthrosis and one of the most complex joints of the body. The cartilage of the TMJ, which is histologically defined as fibrocartilage, has cellular and molecular characteristics, and biomechanical properties that remarkably distinguish it from the hyaline cartilage of other diarthrodial joints. Furthermore, it has been postulated that the fibrocartilage of the TMJ possesses an innate potential capacity of regeneration in vivo, unlike the hyaline cartilage (Embree, Chen et al. (2016). For these reasons, the regeneration of the TMJ cartilage is an objective of constant and extensive research in the field of tissue regeneration and bioengineering. Recent work suggest that the superficial zone or layer harbors a pool of progenitor cells that maintain the homeostasis of condyle by eventually differentiate into osteoblasts in the deep cartilage zone (Embree, Chen et al. (2016). This work aims to explore the osteogenic potential of the TMJ cells, especially in the fibrocartilage and subchondral spaces, and support the studies that hypothesize viable and potential tissue regeneration of the TMJ bone through bioengineered materials and cell therapies. Cells of the articular disc tissue, synovial fluid, and fossa articular surface are excluded from this study.

As aforementioned, the TMJ is a complex and very specialized joint characterized by articular fibrocartilage. It is a synovial ginglymo diarthrodial bilateral joint that articulates the mandibular condyles with the glenoid fossa and the articular eminence of the temporal bone. Each mandibular

condyle of the mandible is covered by a unique and specialized fibrocartilage layer. TMJ condyles are the moving parts of the system. The other articulating surface of the TMJ is the fossa, which remains static (Rees, et al. (1954). A fibrocartilaginous disc, also a moving part of the joint, is located between both articulating surfaces, specifically oriented between the condyle and fossa eminence zone. The TMJ disc and its fibrous attachments and ligaments divide the joint space into superior and inferior compartments (Rees, et al. (1954). The distances between the disc and the articular surfaces are congruent and filled with synovial fluid. The TMJ disc allows congruity between two differently shaped and sized articular structures. The disc covers the condylar head and together with its attachments and a surrounding joint capsule, forming a closed space delimiting intra articular and extra articular environments (Rees, et al. (1954).

TMJ mandibular condyles are bone structures containing blood vessels in medullar spaces covered with articular cartilage, histologically defined as fibrocartilage (Van Bellinghen, Idoux-Gillet et al. (2018). Structurally, there are four distinguishable compartments or layers in the interface between subchondral bone and the articular surface of the TMJ condyle. The first layer is a fibrous upper layer uniquely made up of fibroblasts and contains abundant type I collagen, while collagen type II is minimally present. Underneath this superficial fibrous zone, a fibrocartilage layer is described which can be subdivided schematically into proliferative and hypertrophic zones. The proliferative zone is rich in type I collagen and chondroitin sulfate-based proteoglycan. The hypertrophic zone is rich in chondrocytes, in aggrecan, and in collagen type II. Collagen types I and X are also detected. Next the subchondral bone, which contains osteoblasts, osteocytes, and osteoclasts. The extra cellular matrix of the condylar bone is characterized by collagen type 1, III, VI, and IX and it is rich in chondroitin sulfate and dermatan sulfate (Van Bellinghen, Idoux-Gillet et al. 2018). The TMJ cartilage remarkably differs from the other hyaline

articular cartilages. TMJ condylar fibrocartilage contains less glycosaminoglycans (GAGs) than hyaline articular cartilage (Delatte, Von den Hoff et al. (2004). Cellular heterogeneity and macromolecular compositions of the subchondral fibrocartilage area drive further study of this complex. Subchondral bone – fibrocartilage is a unique interface, and it is critical to better understand its biology to advance on TMJ regeneration. Promoting chondrogenic and osteogenic maturation, a matrix synthesis, and a molecular gradient of functionalization through a 3D tissue engineered system is particularly challenging. This work endeavors to study TMJ condylar cells to contribute to the TMJ regeneration research based on bioengineering.

Regeneration of TMJ cartilage meets a clinical need since patients who suffer from TMJ pathologies, specifically those which affect the articular surface, have no options for regenerative treatment. Generally, these patients live with pain until the condylar cartilage degenerate, and they can no longer function (Lowe and Almarza (2017). The fibrocartilage of the TMJ does not heal once a degenerative process is present. This process is characterized by deterioration and abrasion of articular surface, local thickening, and remodeling of the subchondral bone (Zarb and Carlsson (1999). These features are accompanied by inflammatory infiltration in the joint. The most common TMJ disorder related to the articular cartilage is internal derangement, which is defined as an abnormal positional relationship of the articular disc relative to the mandibular condyle and the articular eminence (Zarb and Carlsson (1999). It is currently unclear if internal derangement causes degeneration or if it is caused by degeneration (Zarb and Carlsson (1999). It is important to mention that TMJ cartilage degeneration can be described as osteoarthrosis, in which intra articular structural abnormalities are present. Morphological and functional deformities in the osteoarthrosis fibrocartilage can lead to a crippling disease of the TMJ. There is no treatment that offers a long-term solution for a late stage of degeneration of the TMJ. It has been proposed that

the TMJ cartilage degeneration is the result of a dysfunctional articular remodeling and a decreased adaptative capacity of the articular structures in response to functional demands. This may be concomitant with an excessive physical stress that also exceeds the adaptive capacity of the TMJ structures (Arnett, Milam et al. (1996). Factors that affect the host's general condition, such as age, systemic diseases, and hormonal imbalances, can cause a decreased ability of articular tissues to withstand normal forces or biomechanical stress within a physiologic range. Abnormal forces regarding direction, magnitude and time of application can be an etiology for the degeneration of articulating surfaces. Abnormal joint loading due to trauma, parafunction, and unstable occlusion can also be responsible for the progressive degeneration of TMJ cartilage. Trauma has been reported to change the properties of the articular disc over time, as well as leading progressive condylar resorption and deformation (Arnett, Milam et al. (1996). So, considering the nature of the TMJ fibrocartilage pathologies and the clinical necessity, it is imperative to develop cell therapies and tissue engineering biotechnologies to regenerate the fibrocartilage and condylar bone of the TMJ.

The field of musculoskeletal tissue engineering focuses on creating tissue replacements with biomechanical and functional properties using biomaterials and scaffolds, in which cell interactions and biological signaling can occur. Thus, the science of tissue engineering works based on a triad formed by a cellular source, signaling biomolecules, and biocompatible supporting materials or scaffolds. The goal of tissue engineering is to use natural or synthetic materials combined with competent cells and signaling biomolecules to induce the restoration or regeneration of tissues mimicking morphology, structure, and function (Brown, Chung et al. (2011).

First, cell source is a fundamental part because it is the biological base of the regeneration process. Tissue engineering, tissue regeneration, and cell therapies utilize a clinically feasible cell source. The choice of the cell source is critical in the tissue engineering process. Cells should be abundant, healthy and leave little donor site morbidity (Johns, Wong et al. 2008). When it comes to osteochondral tissue regeneration, there is fortunately a myriad of alternatives of cell source that can be used for this process. These cell sources are: native autologous cartilage cells from the site, cartilage cells from a different joint, and mesenchymal stem cells or MSCs. MSCs, characterized by their multipotency, can be obtained from different sources, such as bone marrow, fat, muscle, and dental tissues (Johns, Wong et al. (2008). The regenerative nature of the osteochondral tissue of the TMJ is closely related to the neighboring bone cell populations. Consequently, it is of prime importance to understand these cell populations of the cartilaginous tissues and condylar bone, as they are related to the biomechanical and structural properties of this joint, in which these populations reside. A good understanding of cartilage, fibrous, and bone cells in terms of their phenotype, interactions, their molecular biology, biological activities such as migration, differentiation, and functional tissue formation will enable to progress on the hampered path of the TMJ cartilage regeneration.

The second fundamental part of the triad in tissue regeneration or bioengineering systems is biological signaling. Cell signaling can be made up of proteins, growth factors, hormones, small molecules, amino acids, and others. Growth factors are the most common signals used in tissue engineering. The functions of bio signals are to stimulate cell migration into the site, the proliferation of cell source and the secretion of specific products necessary for the synthesis of the extracellular matrix of the tissue to regenerate. The function of biological signaling in bioengineered systems is to activate the molecular activity in extracellular matrix of healthy native

tissues. Furthermore, genetic engineering is also used to activate the expression of cellular factors or agents within cells to enrich the extracellular matrix. This is particularly important for the regeneration of the osteochondral tissues, as a precise biological signaling on the cells will lead to the production of the correct architecture of the matrix (Allen and Athanasiou (2006).

Last, the third part of the bioengineering triad is represented by scaffolding materials. The function of biomaterials in tissue engineering is to serve as physical and biochemical support for cellular interactions, proliferation, and differentiation to occur. It is also to facilitate the synthesis of a suitable extra cellular matrix for biological activities. Polymers have been proven to be the choice in tissue engineering to regenerate cartilage and bone. A good example, polyglycolic acid (PGA), has shown encouraging results for cartilage regeneration (Hu and Athanasiou (2006). In the same way, Poly-L-lactic-glycolic acid (PLGA) is a synthetic polymer approved by the FDA for clinical applications which is interesting for cartilage regeneration. The versatility of its structure allows also a modulation of mechanical properties of the scaffold. PLGA scaffolds promote colonization and differentiation of MSCs in vivo (Kinoshita and Maeda (2013). PLGA has been seen to interact positively with chondrocytes and other resident cells of the TMJ disc to regenerate in- vitro. Gelatin, derived from the lysis of collagen, is also appropriate for osteochondral regeneration. Gelatin scaffolds with chitosan have shown their capacity to support chondrogenic differentiation in vitro and in vivo (Kuo, Wang et al. (2010). This research seeks to advance understanding of the regenerative potential of condylar fibrochondrocytes, specifically in gelatin hydrogels utilized in bilayered scaffolds.

Despite the well-known limited ability of cartilaginous tissue to regenerate, previous studies have reported that fibrocartilage, a type of cartilaginous tissue, possesses a regenerative capacity better than hyaline cartilage. Fibrocartilage is a structural component of vital joints such

as the knee, vertebrae, and the TMJ (Lowe and Almarza (2017). Experiments on goats have stated that fibrocartilage of the TMJ condyles harbor regenerative potential. Furthermore, these cells are believed to have the ability to form and maintain cartilage (Embree, Chen et al. (2016). TMJ fibrocartilage regeneration is currently an objective of constant research in bioengineering and tissue regeneration. In this sense, it is necessary to investigate in depth the biological characteristics of the cells of the TMJ articular fibrocartilage and adjacent areas to design bioengineered systems that enhance osteochondral regeneration. In line with this new direction, the objective of this study is to contribute to the understanding of TMJ cell populations, specifically in the fibrocartilage, and in neighboring bone compartments. This study aims to further the understanding of TMJ condyle tissues and to help to elucidate the regenerative potential of TMJ cells. It aims to give more evidence of the tissue source from which the regenerative cells originated and investigate the cellular properties of the TMJ cell populations associated with fibrocartilage and bone regeneration. The future of this is to use bioengineered scaffolds utilized to regenerate cartilaginous tissue of the TMJ want to mimic the native fibrocartilage - bone architecture. This work intends to complement the research, which has demonstrated that TMJ fibrochondrocytes can form cartilage in vivo. Bilayered scaffolds utilized in that research consist of an upper layer of gelatin and a second layer of polyethylene glycol, gelatin, and heparin (PGH) which carry TGF β-1. This model tries to recapitulate the fibrous - cartilage- bone interface of the mandibular condyle, to stimulate the infiltration of progenitor cells in the fibrocartilage layer, and to promote osteogenesis in the subchondral layer. Therefore, it is important to better understand cell populations, especially fibrochondrocytes, which will interact with bilayered scaffolds in the tissue compartments of the TMJ condyle and promote bone regeneration of the bilayer design.

1.1 Specific Aims.

Based on previous studies, which has shown that TMJ condylar cartilage(fibrocartilage) of goat has an innate regenerative potential in vivo, remarkably different than hyaline cartilage of other diarthrodial joints, this work aims to probe this potential by comparing cell populations in surrounding tissues with fibrocartilage cells. Furthermore, we seek to advance the understanding of the potential progenitor pool of cells in the fibrocartilage (fibrous and cartilage layers) and subchondral bone compartments of the TMJ. For this purpose, the biological activities of isolated cells from goat TMJ will be compared, such as changes in cell number over time, space filling capability and differentiation. This work seeks to contribute to the TMJ research to advance the understanding of the regenerative capability of the cell layers and compartments of the TMJ, particularly the fibrocartilage layers. These aims are part of the investigation to probe the regenerative potential in the TMJ in the context of the tissue regeneration utilizing soft polymer scaffolds and biomaterials. Bone marrow derived cells (BMcs) will be used as a control to osteogenic potential.

Aim 1: Assess space filling capability of 3 types of condylar cells of TMJ and BMcs of goat. Rather than the scratch wound assay on the cell monolayer, an adherent silicone barrier of 0.25 cm2 and semi rounds of 3 inches of diameter approximately will be set on the bottom of a tissue culture plastic (TCP) to create an empty void. Next, previously isolated TMJ cells (fibrous and cartilage and subchondral cells) and BMcs will be plated at confluent concentration (10,000 cells/cm2) on TCP with barriers. After 24 hours plating, the barrier will be removed to assess the space filling activity by measuring the space and the distance occupied of the furthest cell from the leading edge, as well as assessing the number of cells beyond the border.

Aim 2: Determine the osteogenic potential of condylar fibrocartilage TMJ cells, subchondral bone cells and BMScs of goat. Previously isolated TMJ cells will be seeded at 10,000 cell/cm^2 and maintained under osteogenic and regular growth media for 21 days on TCP. Next, the cell mono layers will be stained with Alizarin, Von Kossa, and Alkaline Phosphatase activity staining to evaluate mineralization and osteogenic activity. Alizarin red staining will be quantified via dye extraction with acetic acid and measurement of optical absorbance at 405 nm to compare Alizarin red concentration among condylar cells and BMCs of goat.

Aim 3: Quantify the change in cell number over time of TMJ cells from the fibrous, cartilage and subchondral condylar layers and BMcs of goat. Previously isolated fibrous cells (upper fibrous layer cells) cartilage layer cells and subchondral bone layer cells will be seeded at 1000/cm^2 in 6 well plates in regular growth medium. Condylar cells mentioned above and BMcs will be counted every day up to 7 days after trypsinization by hemacytometer. The growth curve will be obtained for each cell type to evaluate changes in cell number. In addition, each cell type will be seeded in three different vessels (different areas) and counted after 7 days in growth medium by hemocytometer to compare changes in cell number in different areas and evaluate cell expansion capability.

2.0 Material and Methods

2.1 Goat condylar cells isolation and cell culture

Harvested goat temporomandibular condyles were dissected from adult spanish boer goats, at slaughter age. Whole condyles were digested with collagenase type II (in cell culture medium), and incubated at 37oC, 5% CO2 on a rocker for 1.5 hours. After digestion of the entire condyle, primary cells from the fibrous articular surface, cartilage layer and subchondral bone layer of the condyle were isolated. First, the fibrous superficial layer was separated from the condyle by peeling off the surface and minced for digestion with collagenase type II and incubated for 3 hours. After peeling off the surface, the next layer beneath, the cartilage layer (middle layer) was shaved off and put into a new dish for digestion with collagenase type II and incubated for 18 hours. After shaving the cartilage layer, the subchondral bone layer was scrapped by a bone rongeur. The bone pieces obtained from the scrapping were placed into a petri dish for digestion with trypsin 0.05% and incubated for 10 minutes. Bone pieces were cultured until osteoblasts migrated out. All digested condylar tissues were filtered with 100um cell strainer.

Donor-matched BMCs were isolated from the red marrow like tissue obtained from goat femur (femur shaft) and bone pieces collected after drilling the bone. Marrow tissue and bone pieces were put in culture medium supplemented with heparin and vigorously vortexed to release the cells. BMCs were isolated from the medium through layers separation using Ficoll.

Cell suspensions of mandibular condylar cells (fibrous, cartilage and bone) and BMCs were cultured at 5% CO2, 37oC in basal medium consisting of MEMα/Glutamax supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin (designed as growth medium)

and was replaced every two days. Osteogenic differentiation and mineral production were induced by osteogenic medium consisting of MEMα/ Glutamax supplemented with 10% FBS, 5% penicillin/streptomycin, 0.1 uM Dexamethasone, Ascorbic acid and 10 uM β-Glycerophosphate. Osteogenic medium was replaced every three days. Cells were detached with trypsin 0.05% and plated at P1, P2 and P3 passages for all the in vitro experiments.

2.2 Space filling capacity assay

Silicone barriers of 15x5x3 mm and semi rounds of 3 inches of diameter were placed on the tissue culture plastic of six wells plate and petri dishes. Then, condylar cells and BMCs were seeded at a concentration of 10.000/cm2 in growth medium. After 24h plating, the barriers were removed to measure migration of cells into the space previously occupied by the barrier. The space and the distance reached by the furthest cell from the leading edge were measured, as well as the number of cells beyond the leading edge were evaluated.

2.3 Changes in Cell Number overtime

Cell number was assessed by cell counting at different time points. Condylar cells and BMCs were cultured on 6-wells plate in growth medium and counted every day for 6 days (daily count growth curves). In addition, these cells were seeded in different vessels 25, 75 and 175 flasks and cultured in growth medium for 7 days.

2.4 Differentiation

Osteogenic differentiation was induced with osteogenic differentiation medium based on standard cultivation medium containing 10% FBS, 5% penicillin/streptomycin, 0,01 uM Dexamethasone, 50uM Ascorbic acid and 10uM β-Glycerophosphate. The medium was replaced every three days. Condylar cells and BMCs were cultured for 12 and 21 days. The osteogenic differentiation capacity was determined using the alizarin red S, Von Kossa staining and Alkaline Phosphatase enzymatic activity staining.

2.5 Staining assays

For Alizarin red staining, adherent cell monolayers cultured in 6-well plates were washed with PBS and fixed with 4% paraformaldehyde for 15 min, rinsed two times with PBS, covered for 20 min at 37 °C with alizarin red S (0.5% in miliq aqua, pH 4.1) and washed with dH2O until the supernatant was transparent. Stained monolayers were visualized by inverted microscope. This process was followed by a quantitative destaining procedure using 10% acetic acid for 25 min at room temperature. The alizarin red S concentration was determined by absorbance measurement at 405 nm. Quantification of Alizarin red was performed for cell cultures in osteogenic medium at 21 days. For Von Kossa staining, adherent cell monolayers cultured in 6-well plates were washed with PBS and fixed with 4% paraformaldehyde for 15 min and rinsed two times with PBS. Then, 2 ml of 5% aqueous silver nitrate solution was added to each well. Six wells plate was exposed to UV light in a dark chamber, checking the change on the wells every 5,10 and 15 minutes. The reaction was stopped by extensively washing. Lastly, 5% sodium thiosulfate solution was added

for 5 minutes at room temperature, for a final rinse with water twice. Stained monolayers were visualized by inverted microscope. For Alkaline phosphatase activity staining, cells were seeded in 1.9 cm2/ well plate at 10.000/cm2 and cultured in growth and osteogenic medium for 7 days. Stained monolayers were visualized by inverted microscope.

3.0 Results

3.1 Space filling capacity

To evaluate the space filling capability of condylar cells a similar version of the scratch wound assay was performed. Instead of scratching the treated surface of the culture dish, silicone barriers were placed on the surface of the dish to create a void space. Two types of silicone barriers were used **(***Figure* ***1*****A).** After removing the barriers, cells seeded at high concentration were evaluated as they filled the space (*figure* 1B). All cells are at most P2 in passage and all from the same donor except for BMSCs (*figure* 2). The fibrous layer cells filled the space on Day 5 (*figure* 2A). The MCC cells seem to be more active on day 5 (*figure* 2B) in filling the space compared to fibrous layer cells, as well as seeming to reach a further distance from the limit. The same behavior was observed in subchondral bone cells group on day 5 (*figure* 2C). Both mandibular condyle cartilage layer cells and subchondral bone cells groups seem to have accumulated greater number of cells visible beyond the limit at day 5. BMSCs (*figure* 2D) was the group that did not seem to have many cells beyond the original limit, and the one that seemed to have reached the shortest distance from the space to be filled.

In the comparison between different donors, the space filling capacity of the fibrous layer cells seems to differ (*figure* 3A), as well as the bone marrow derived cells (*figure* 3B). According these observations, donor has as big an impact on space filling capacity as cell type.

3.2 Differentiation

The osteogenic potential of condylar cells was evaluated with different staining, Alizarin red, Von Kossa and Alkaline Phosphatase staining. All condylar cells and BMCs were cultured in osteogenic medium and growth medium in 6 wells plates for 21 days. When observing culture plates stained with Alizarin red, all groups in osteogenic medium were able to stain red, demonstrating calcium deposits in all groups. In contrast, all groups cultured in growth medium stained very slightly red (*figure* 4). We assume that BMCs, condylar fibroblasts, cartilage, and bone cells might generate small amounts of calcium in growth conditions. Interestingly, fibrous layer cells seemed to have formed more consistent cell monolayers with an intense red staining, compared to other groups. BMCs cell monolayer in osteogenic medium tended to detach from the culture wells and shrink before completing the 21 days of culture (*figure* 4).

Comparable results were seen after Von Kossa staining. All groups cultured in osteogenic medium stained dark evidenced phosphate deposits. In contrast, cultures in growth medium stained slightly brown (*figure* 5). notice note, there was the background staining in the culture wells with fibrous layer cells in growth medium, which stained slightly darker than the rest of groups in growth medium. Microphotographs of cell monolayers at different magnifications showed Alizarin red staining verifying copious amounts of mineral deposits from all condylar cells and BMCs with no distinct pattern (*figure* 7). To infer differences in the production of mineral from different cell types, we quantified the concentration of Alizarin red staining in condylar cells and BMCs. We found that condylar fibroblasts concentrated more staining than the other groups (*figure* 8). In addition to Alizarin red and Von Kossa staining, we did the staining assay for Alkaline Phosphatase activity in condylar cells cultured in osteogenic medium and growth medium. Alkaline Phosphatase is a well-known indicator of osteogenic differentiation. All

condylar cells and BMCs slightly stained purple in growth conditions, which is considered a normal observation. In contrast, all cell types seemed to have significantly stained darker after 7 days in osteogenic medium. Interestingly, subchondral bone layer cells seem to have stained an intense purple, more than the other groups (*figure* 9 and 10). These results seem to suggest that three cell types of condylar cells can produce copious amounts of minerals.

3.3 Changes in Cell Number over Time

We cultured condylar cells (MCC, Scb and Fibroblasts) and goat BMCs from the same donor at same concentration (1000 cm2) for 7 days in 6 well plates, in growth medium, and counted them every day to create a growth curve. We started the daily counting from 48 hours to count cells of each well until 7 days. Numbers from daily cell counting generated growth curves for each cell type. When we compare the growth curves that we obtained from daily cell counting, we noticed that fibrous layer cells group shows a higher increase in numbers before day 4. Fibrous layer cells and BMCs show an exponential phase reflecting a sustained phase, whereas subchondral bone and cartilage cells show a decline phase in their curves by day 6 (*figure* 11). Finally, after counting condylar cells and BMCs cultured in different vessels (3 different areas) for 7 days in growth medium. There did not seem to be significant differences in totals number except for the biggest vessels ($75cm^2$). We noticed that fibrous layer cells logged a significant difference in count numbers only for these containers. Whereas subchondral bone layer cells were the ones that counted the least numbers for each culture vessel, likely reflecting less proliferation (*figure* 12). These cell count results give us an idea that fibrous layer cells and BMCs have a greater capacity for proliferation and expansion under physiological conditions within the condylar space.

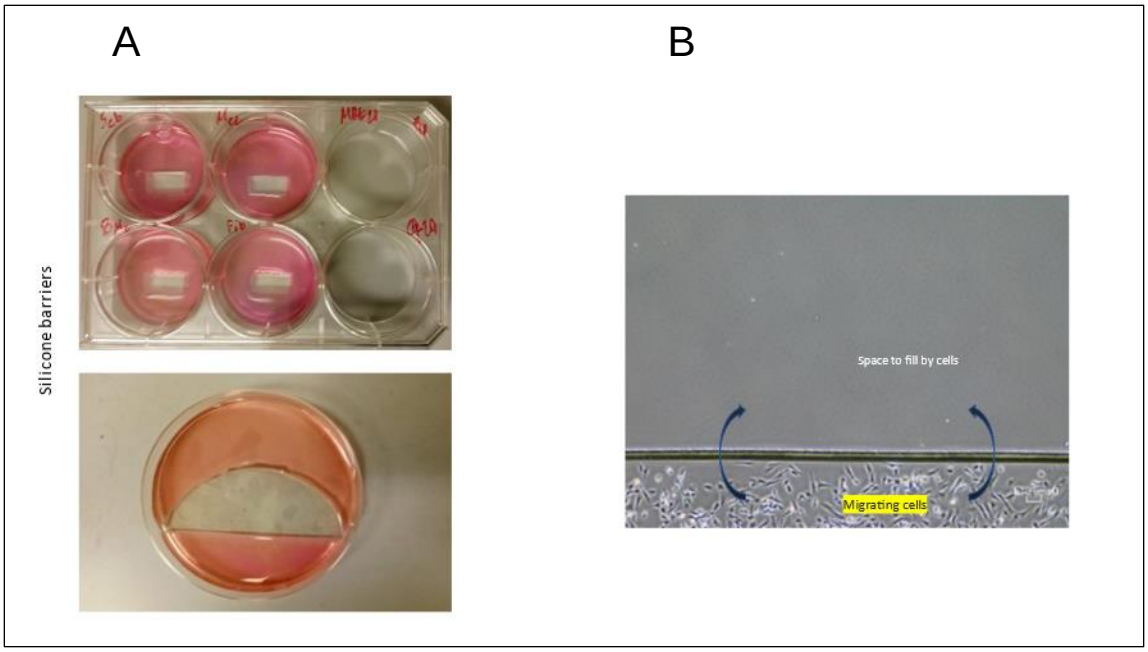


Figure 1: (A)Two types of Silicone Barriers for space filling assay. (B) Silicone barriers make a void space for the cells to move in.

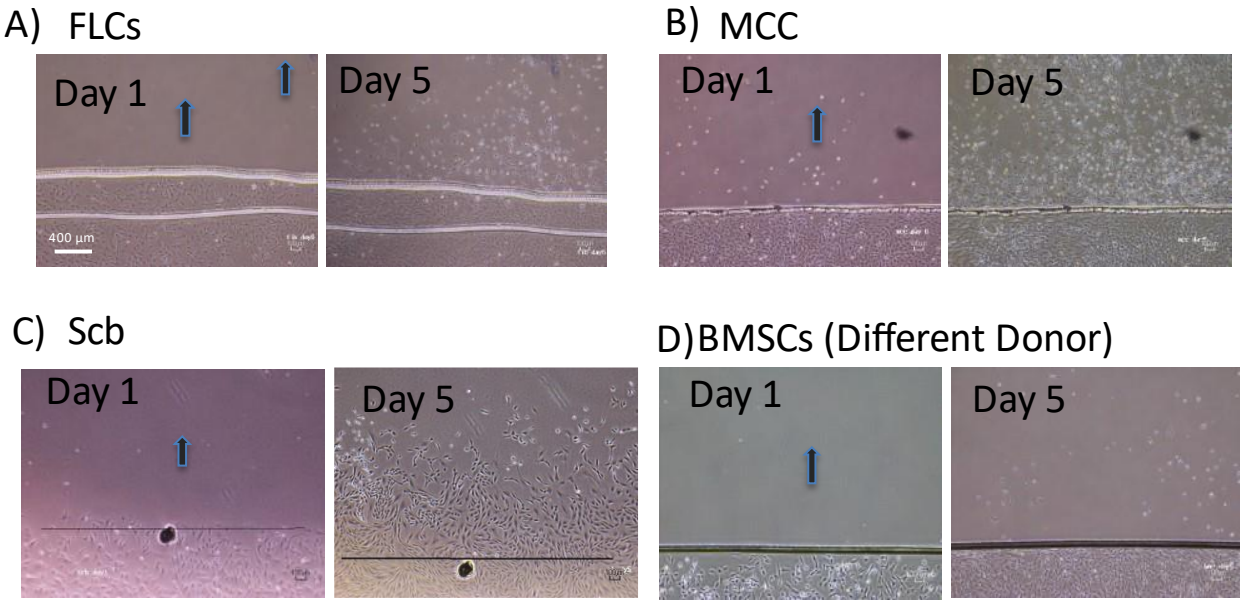


Figure 2: (A)Space filling assay at Day 1 and Day 5 for fibrous layer cells, (B) mandibular condyle cartilage layer cells, (C) subchondral bone layer cells, (D) and bone marrow derived cells (different donor)

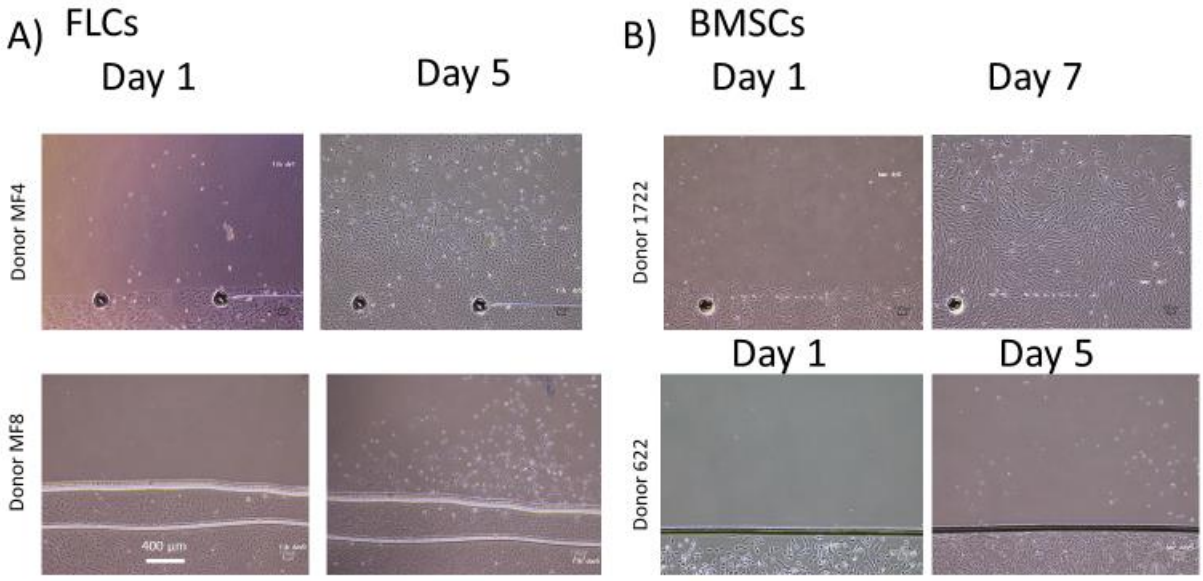


Figure 3: (A Space filling capacity assay showing different potential based on donor for fibrous layer cells at Day 1 and Day 5 for two donors; and (B) bone marrow derived cells at Day 1 and 7 for donor 1722, and Day 1 and 5 for donor 622.

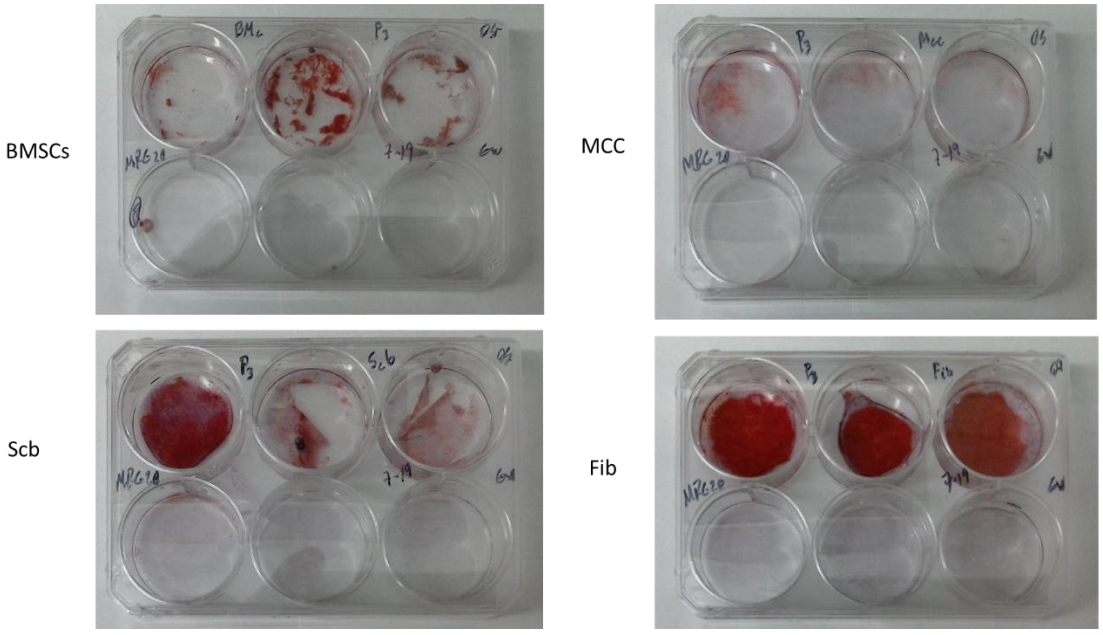


Figure 4: Differentiation assay with Alizarin red staining for bone marrow derived cells, mandibular condyle cartilage cells, subchondral bone cells, and fibrous layer cells. BMSCs from a different donor. The upper 3 wells are in osteogenic media, and lower 3 wells are in growth media.

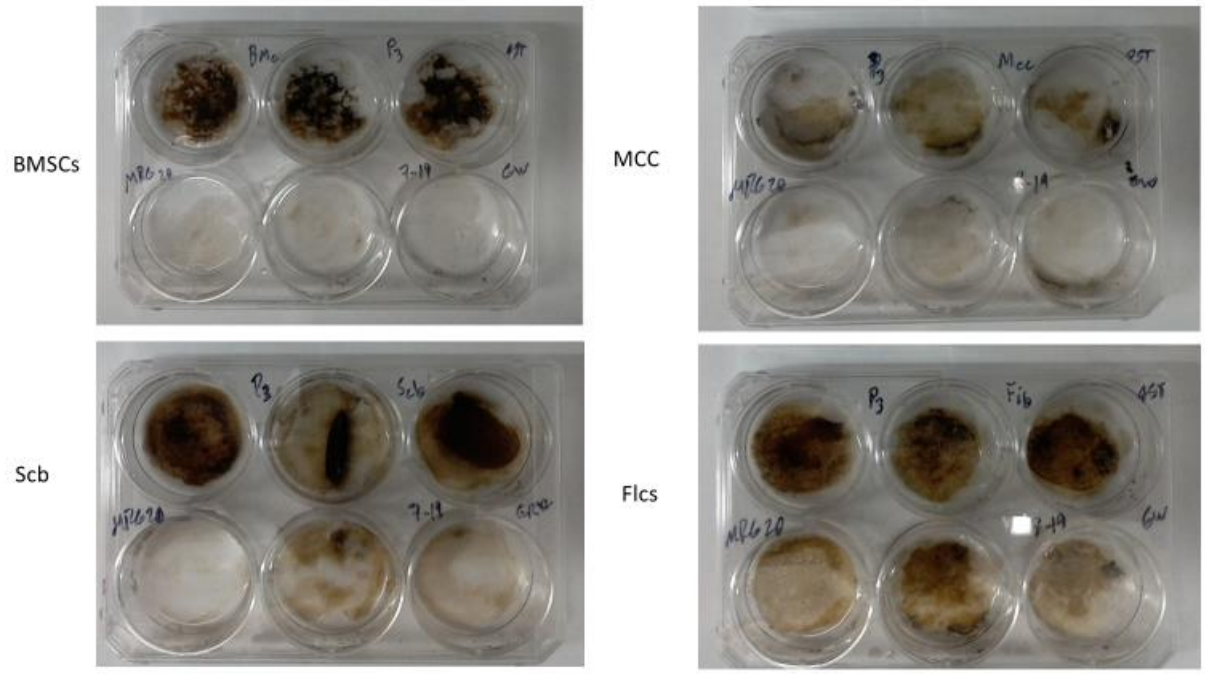
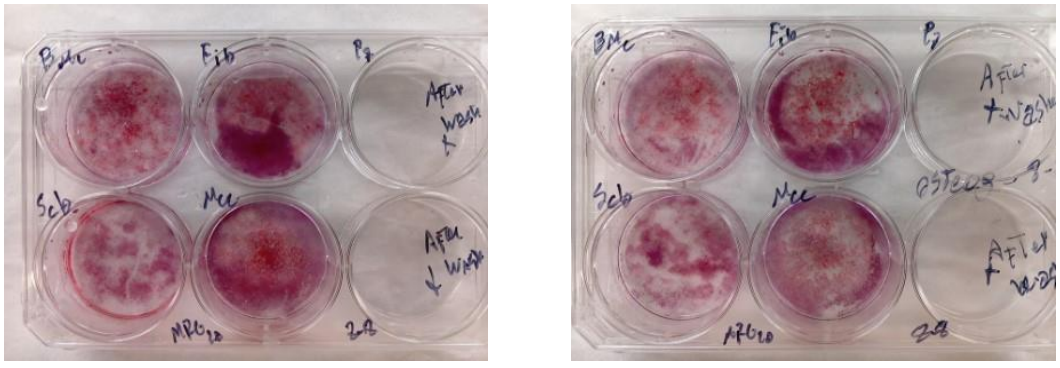


Figure 5: Differentiation assay with von Kossa staining for bone marrow derived cells, mandibular condyle cartilage cells, subchondral bone cells, and fibrous layer cells. BMSCs from a different donor. The upper 3 wells are in osteogenic media, and lower 3 wells are in growth media.



BMSCs from different donor

Figure 6: Differentiation assay with Alizarin red staining. Comparing the staining of 4 cell types in the same plate. BMCs from different donor

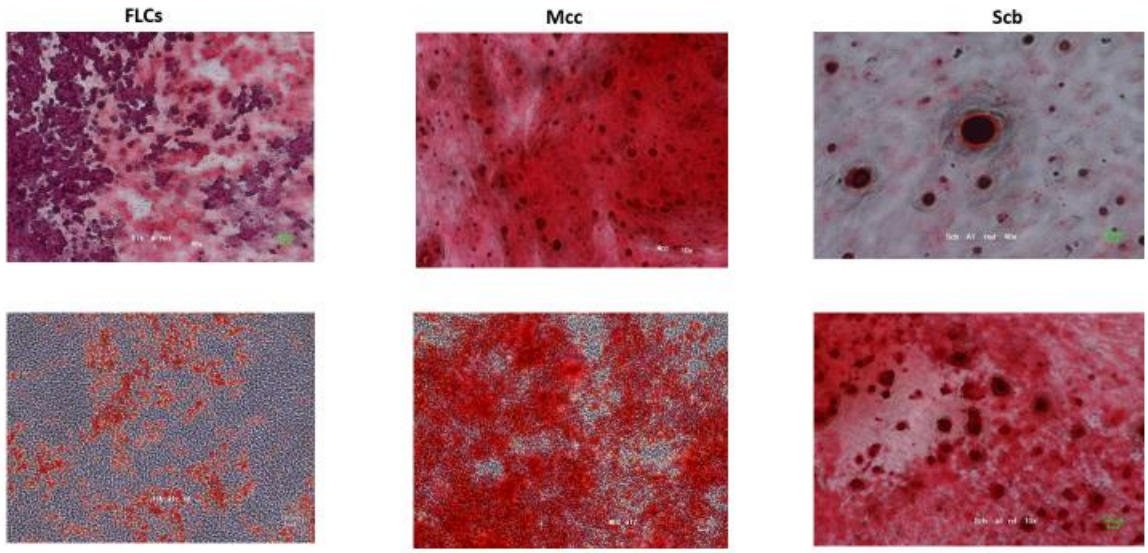


Figure 7:Zoom in of Alizarin red staining for the different condylar cell types.

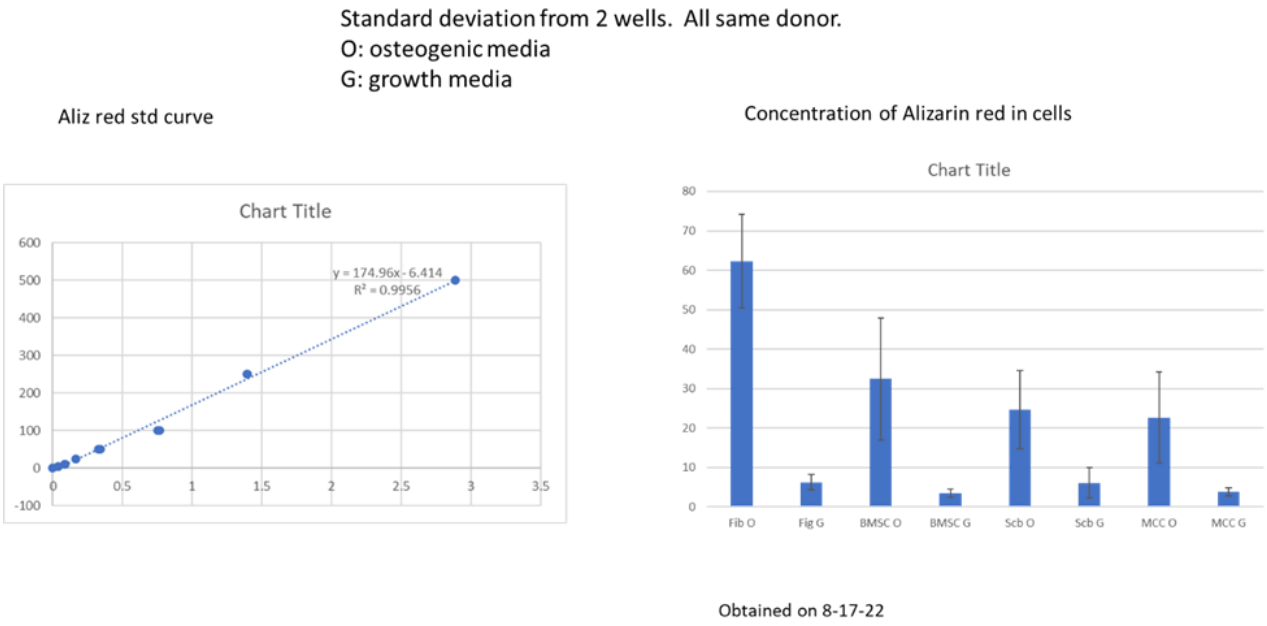


Figure 8: Quantification of Alizarin red staining for the different cell types (Fibrous layer cells, cartilage layer cells, subchondral bone layer, bone marrow derived cells). Standard error for three technical replicates.

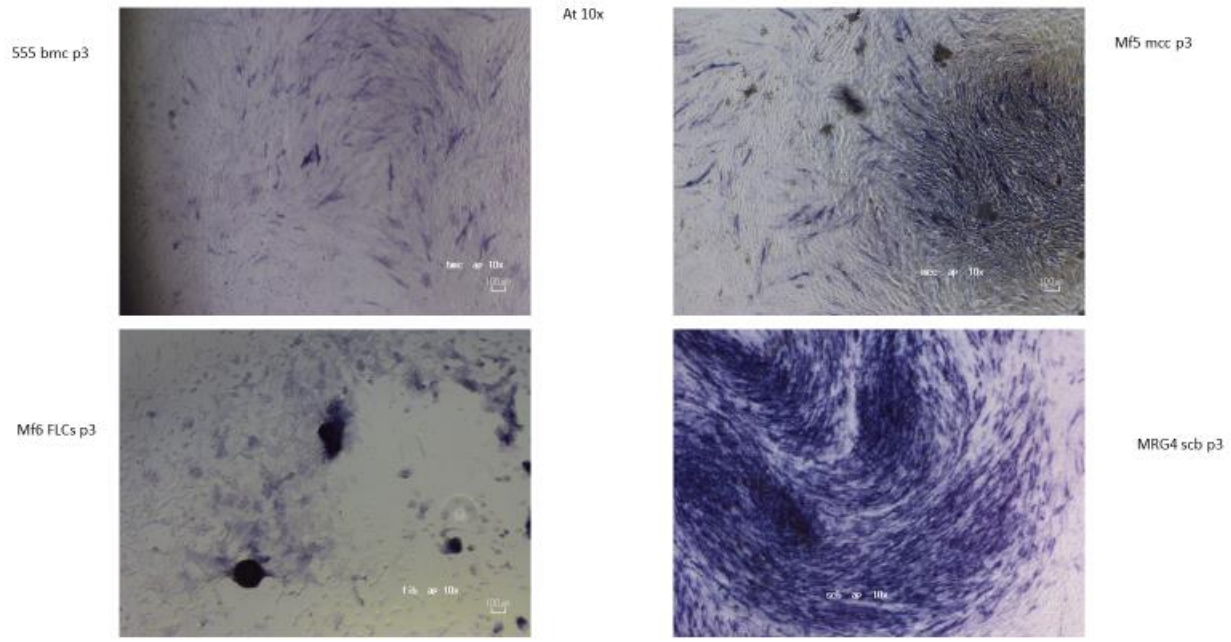


Figure 9: Differentiation assay with alkaline phosphate staining for bone marrow derived cells, mandibular condyle cartilage cells, subchondral bone cells, and fibrous layer cells. BMSCs from a different donor. In osteogenic media.

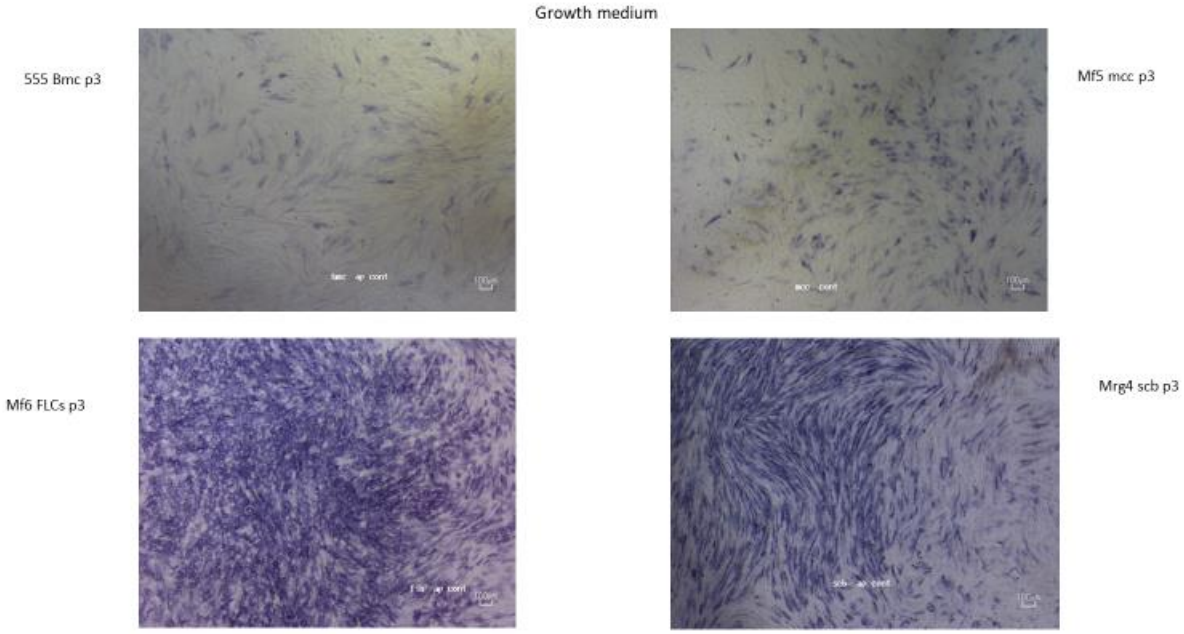


Figure 10:Differentiation assay with alkaline phosphate staining for bone marrow derived cells, mandibular condyle cartilage cells, subchondral bone cells, and fibrous layer cells. BMSCs from a different donor. In growth media.

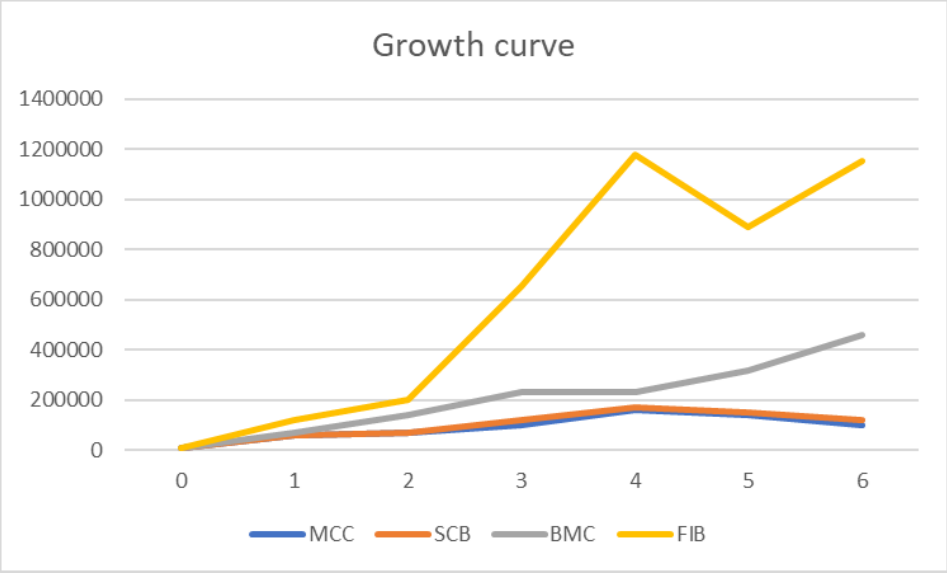


Figure 11:Growth curve (cell number in well plates from Day 0 to Day 6) for condylar cells. Series 1 in blue is mandibular condyle cartilage layer cells, Series 2 in orange is subchondral bone layer, Series 3 in gray is bone marrow derived cells, and Series 4 in yellow is fibrous layer cells (all from same donor).

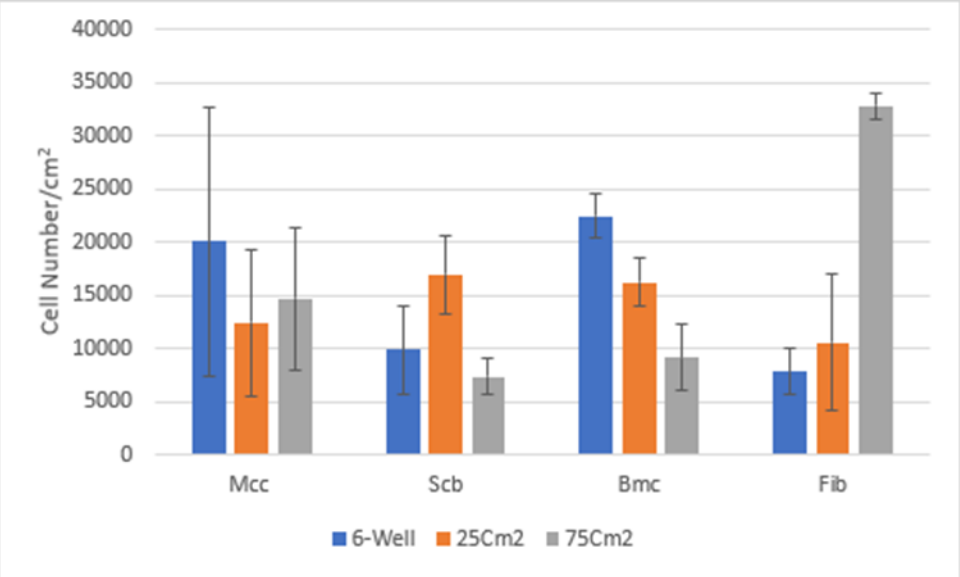


Figure 12: Comparisons of condylar cells in different seeding areas (6-well, 25 cm2, and 75 cm2). The largest surface area container (75 cm2) seems to be different in allowing the fibrous layer cells to expand to higher numbers.

4.0 Discussion

Here in this work, we assess four different cell types in the structure of the TMJ condyles of goat. We showed through our results that condylar cells exhibit differences on cell number overtime, migration, and osteogenic differentiation in vitro. These findings confirm that cell content of the mandibular condyles differ in biological activities, and the condylar structure is histologically well defined. Thereby, we verify the cell heterogeneity in the condylar tissue, its complexity, and regenerative nature. Our results on the biological activities of superficial Fibrocartilage cells, cartilage, and subchondral bone cells of TMJ expand the window for studying the regenerative potential of TMJ fibrocartilage. Our results support the hypothesis that states the innate regenerative potential of the TMJ fibrocartilage to regenerate cartilage and bone tissues. This study enables us to learn on the biological characteristics of the TMJ condylar cells, which will interact with bioengineered materials implanted within the osteochondral compartment to regenerate the condylar cartilage and bone.

Recent studies have reported evidence on the presence of progenitor cells capable of regenerating cartilage in the fibrocartilaginous interface of the mandibular condyle (Embree, Chen et al. (2016). Ruscitto, Scarpa et al. (2020). Furthermore, these cells are also thought capable of regenerating subchondral bone (Bi, Yin et al. (2020). In line with this assertion, our results corroborate that in addition to fibrocartilage cells from the articular surface, cells from the subarticular compartment or cartilage layer, and condylar bone cells substantially produced calcium deposits in osteogenic medium, compared to goat bone marrow cells (BMCs). These results demonstrate that these populations have a remarkable osteogenic potential. We support the hypothesis on the innate regenerative ability of TMJ. This regenerative ability could be due to the

role of progenitor cells residents throughout the TMJ tissue system, including the osteochondral compartment of the condyle. We believe progenitor cells could be part of the populations of each cell layer and the fibrocartilage compartment of the TMJ condyle. We agree with previous reports on the existence of multipotent stem cells or fibrocartilage stem cells (FCSCs) in the superficial fibrocartilaginous layer of the mandibular condyle. Our results showed that these cells produced a higher quantity of mineral deposits compared to other condylar cell populations and BMCs, as seen by alizarin red staining. Nevertheless, we do not rule out the hypothesis that in addition to FCSCs, there are progenitor cells in the cartilaginous space and subchondral bone able of forming bone and cartilage, and all of them participate in the maintenance of TMJ homeostasis. We include bone marrow cells of goat in our study to compare the mineralization potential of TMJ cells. The osteogenic potential of bone marrow cells is well known. Although cell populations of the TMJ condyle showed no significant differences in terms of mineralization capacity in general, it is important to note that previous studies have clearly documented that the extracellular matrix of each cell layer in the mandibular condylar cartilage is different (Embree, Chen et al. (2016). According these studies, FCSCs do not produce aggrecan and collagen II (Col II), which are proteins secreted from mature chondrocytes, but are surrounded by lubricin and collagen I (Col I) It is known that stem cells are differently regulated by the local microenvironment according to the requirements of the host tissue (Bianco and Robey (2001). We are consistent with the concept that these populations exert distinct functions within the maintenance of the TMJ physiology. Further investigation to characterize these cell populations in the osteochondral compartment of the TMJ condyle, as well as determining their roles in TMJ homeostasis is needed.

Accordingly, some ideas and questions come up to be considered regarding osteogenic potential of the TMJ articular cartilage and subchondral cells. For example, could there be

MSCs/progenitors in each cell layer of the condyles? Are there progenitors in each layer? are MSCs type cells in each compartment the ones that produce calcium deposits in an osteogenic environment? Lastly, the characterization of bone forming cells in the subchondral compartments would be the ultimate step to distinguish these cells.

We consider as relevant elucidating that cells from the articular surface, cartilaginous layers, and cells of the subchondral bone have osteogenic potential. This work is a contribution to the research for the regeneration of condylar bone through tissue engineering. These cell populations would interact with biocompatible materials or scaffolds, which combined with bioactive molecules seek to regenerate degenerated subchondral bone, as seen in osteoarthritis. In addition to the osteogenic potential of the condylar cells, the capacity assessed in this work, other considerations arise with respect to bone regeneration. It would be important, for example, to evaluate the activity of these cells during bone remodeling and maturation, as well as their interaction with and in scaffolds placed in the subchondral space. Indeed, this would be assessed in an in vivo model. These investigations would further complement the knowledge on the TMJ cells in bioengineered systems to regenerate the subchondral bone of the TMJ.

Knowing these cell populations is important to experiment on cell methods in tissue engineering to regenerate cartilage and bone. These methods for example can be implemented in situ, in which local cells are attracted by the incorporation of an acellular scaffold matrix (to cell homing) guiding the process of regeneration. Another possible method is by an ex vivo cell seeding on the scaffolds, in which enough competent cells are transplanted to orchestrate the regenerative mechanism (Bi, Yin et al. (2020). It has been documented that the cell seeding strategy appears better for TMJ regeneration because of its limited capacities of self-repair and the rapid regeneration. Knowing the resident populations in the TMJ osteochondral area is essential to

predict host response, degradation of scaffold material, and cell interactions. These bioengineered methods seek in general a long-term prevention of ossified or fibrous adhesions, which are the main complications of engineered TMJ replacements (Fan, Cui et al. (2021). Also, pro-regenerative active molecules can be incorporated in scaffolds of engineered TMJ to also prevent any ossifications and any adhesions. Therefore, to support both cartilage and bone regeneration of the TMJ condyle, osteochondral constructs, and scaffolds, with or without cells, and pro-chondrogenic and pro-osteogenic molecules, must positively interact with native cells. So, a comprehensive understanding of TMJ subchondral and fibrocartilage cells in their compartments is critical to the regeneration of the TMJ tissues, as well as to the restoration of their function.

We conveniently harvested TMJ cells from goat for this work. Our work corroborates the benefits seen in previous methods utilizing large animal models to study TMJ tissues. These models are remarkably advantageous on mouse and rat models. TMJ of mouse offer a limit number of cells, especially stem cells like from the condylar articular layer. FCSCs have not been successfully isolated and cultured in vitro due to their small number in the mouse TMJ. Further research is required to optimize the isolation method to harvest mouse FCSCs (Fan, Cui et al. (2021).Nathan, Ruscitto et al. (2018). Because this work is part of a project of tissue engineered regeneration, our experiments utilized TMJ cells from goats as it has been reported as an excellent model for testing biomaterials and scaffolds for the regeneration of cartilage and condylar bone. Adult goats have good TMJ size, it is anatomically similar to human, and it allows easy surgical access to the joint space, which is suitable for implanting materials in the condyles (Almarza, Brown et al. (2018). Goat and sheep have been reported as an appropriate model to relate histologic changes to insults, to evaluate mechanical properties of the joint tissues, as well as degeneration.

These models have been reported to have very few limitations attributed to their mandibular condyles function in translation movement.

The space filling capacity of TMJ condylar cells in vitro was studied to get a sense of the migration capabilities of the cell types. We measure the distance traveled by articular surface fibrocartilages cells, cartilage cells, subchondral bone cells, and goat bone marrow cells on the culture dish. Then, we compared these distances to determine which population migrate farther from a starting point at different time points. If different populations of condylar cells participate in regenerating condylar bone, it is worth learning how these cells differ with respect to this activity during tissue regeneration. Furthermore, to have a better understanding of cell migration towards bioengineered scaffolds with which to interact in the cartilaginous compartment to regenerate condylar tissues. It is significant to learn, for example, how different the migrations of condylar fibroblasts and condylar chondrocytes are. What cells would tend to migrate more during regeneration/repair process? Are the proliferating cells from the sub articular fibrocartilage layer the fastest cells to migrate? Our migration experiment in vitro coincides with previous analysis performing the scratch wound healing assay, in which FCSCs showed a weaker migration capability compared to bone marrow mesenchymal stem cells (Bi, Yin et al. (2020). We observed that both subchondral bone and cartilage cells showed higher space filling capacity on the dish, in comparison to goat bone marrow cells and superficial fibroblasts. Unlike previous assays, we used silicone barriers to create an empty space on the tissue culture plastic. We measured the time it took each cell type to partially repopulate the space, and measured the distance traveled after the barrier was removed 24 hours after plating. We preferred this method instead of the scratch wound assay, which scratches the cell monolayer and mars the bottom of the treated culture dish. At present, there is little documentation on the migration of condylar cells, especially stem cells

towards defect sites during repair in vivo. Our results seek to serve as a reference for the expectations on cell migration towards tissue defects in the TMJ condyle, where biomaterials that promote regeneration have been implanted.

Our work also demonstrated differences in the cell number change over time among fibrocartilage cells, cartilage, subchondral bone cells of the TMJ condyle, and BMCs in vitro. When we compare the expansion of these three types of condylar cells and BMCs in regular growth medium, we observed that both BMCs and superficial fibrocartilage cells exhibit a higher cell division, compared to cartilage and subchondral bone cells. Observations of colony forming cultures and assays of previous research have reported that rat FCSCs formed more colonies than cartilages cells from donor matched (Embree, Chen et al. (2016). However, we found that proliferating cartilage cells and superficial fibrocartilage cells similarly reach confluency on the fifth day of culture, seeded at low concentration. Moreover, recent research has reported a comparable propagation ability and colony forming efficiency between human FCSCs and orofacial bone marrow cells (Bi, Yin et al. (2020). In our study we observed a higher cell number in the cultures of fibrocartilage cells compared to BMSCs of goat. We think that DNA quantification analysis would be a convenient method to assess and confirm differences on cell proliferation of distinct types of condylar cells. However, our results provide an idea that goat fibrocartilage cell proliferation is different from those of mouse and human, when compared to other condylar populations. They differ compared to cartilage cells and BMCs in those species, respectively. We also concluded that condylar cells from the subchondral bone space of goat, presumably osteoblasts and osteocytes, have less proliferation ability in vitro culture, compared to other condylar cell types. Likewise, as mentioned above about other biological properties, it is critical to have an expectancy about cell proliferation of the migrated cells that interact with

bioengineered materials implanted in the condyle head to regenerate condylar cartilage and subchondral bone.

5.0 Limitations and Future directions

The results obtained in this study verified that the condylar tissue of the TMJ contains a heterogenous cell population. Condylar cells showed differences in biological activities, such as space filling capability, cell number variations during culture and osteogenic differentiation. Additional experiments may be performed to further define the cell heterogeneity in the condyle structure, as well as the differences in cell behavior. For example, we know that DNA quantification at different time points would be an ideal method to better see differences in cell proliferation of TMJ condylar cells. Going further in cell analysis, a genetic characterization of the cells of each condylar compartment would be the next step to further distinguish these populations. For example, by single cell RNA seq of condylar cells. This kind of analysis would be a remarkable advance since it has been documented that fibrocartilage stem cells residing in the superficial articular layer express the same typical MSCs surface markers, as those expressed by dental stem cells (DSCs). Looking at the entire structure of the mandibular condyle, it should also be considered that skeletal stem cells, which have been reported to express MSCs surface markers, may reside on the surface or periosteum of the mandibular condyles. Thereby, it would be necessary to explore the expression of these markers by cells in the subchondral bone layer to elucidate the presence of MSCs in all condylar layers.

Another important direction in the investigation on condylar cells must be aimed on the synthesis of the extracellular matrix from condylar cells during osteogenesis and chondrogenesis. Different condylar cells may be distinguished according to their matrix products secreted under osteogenic and chondrogenic conditions, and when combined with active molecules. This would be essential to go further understanding the role of these cells during the tissue maturation of

chondrogenic and osteogenic cells. Ultimately, future works on osteochondral regeneration must be directed to harness local stem cells and promote a separated but synchronized chondrogenesis and osteogenesis utilizing suitable scaffolds implanted in the osteochondral interface of the mandibular condyles.

6.0 Conclusion

This research verified that the fibrocartilage (fibrous and cartilage layers) and the subchondral bone compartments of the temporomandibular condyles contain a heterogenous cell population that includes progenitor cells. These populations showed differences with respect to biological activities, such as cell number over time, space filling ability and osteogenic differentiation. Knowing these activities is important in the context of tissue regeneration, utilizing engineered biomaterials to regenerate the mandibular cartilage and the condylar subchondral bone. Finally, this study supports the claim on the innate regenerative ability of the condylar articular cartilage and the subchondral bone, a process in which condylar cells including stem/progenitors cells play a critical role.

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