REPUBLIC OF TURKEY
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME
INSTITUTE

GENERATION OF BONE-CARTILAGE INTERFACE IN MICROFLUIDIC SYSTEM

HAMDULLAH YANIK

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCES THESIS

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Supervisor: Assoc. Prof. Dr. Sinan GÜVEN

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Dokuz Eylul University Izmir International Biomedicine and Genome Institute Department of Molecular Biotechnology, Molecular Biology and Genetics Program Master of Science candidate Hamdullah YANIK has successfully finished his thesis as tilted '**GENERATION OF BONE-CARTILAGE INTERFACE IN MICROFLUIDIC SYSTEM**' on 28/08/2018

Assoc. Prof. Dr. Sinan GÜVEN
DEU IBG Dept. of Mol. Biotech.

Prof. Dr. Alper BAĞRIYANIK, MD
DEU IBG Dept. of Mol. Biotech.

Dr. Ozan KARAMAN, PhD
IKCU Dept. of Biomed. Eng.

Dr. Gerhard WINGENDER, PhD
DEU IBG Dept. of Mol. Biotech.

Dr. Umit Hakan YILDIZ, PhD
IYTE, Department of Chemistry

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Abbreviations

ADMSC……adipose derived mesenchymal stem cell

PEG……polyethyleneglycol

PGA……polyglycolic acid

PLA……..akrilonitripolylactic acid

GELMA……gelatin methylacrylate

PEGDA…… poly(ethylene glycol) diacrylate

HA……...hyaluronic acid

UV…….ultra violet

PVA…….polyvinyl alcohol

PCL…….poly- ε -caprolactone

MSC……...mesenchymal stem cell

TGFB1……..…….transforming growth factor beta one

PBSC……....peripheral blood stem cells

IPS……...induced pluripotent stem cells

FDA……..food and drug administration

IGF1……..insulin growth factor

EGF…….epidermal growth factor

VEGF……..vascular endothelial growth factor

hMSC……..human mesenchymal stem cells

PDMS…….Polydimethylsiloxane

PMMA……Poly(methyl methacrylate)

SLA…….steriolithograhy

FSM……...fused supettering model

ABS……...butadiene sterine

Re……...….reynolds number

IBMX……..methylisobutylxatine

DSAT……..double side adhesive tape

PBS…….phosphate buffer saline

DMEM……...dulbecco's modified eagle's medium

BSA……..bovine serum albumin

PFA……..paraformaldehyde

ECM……..extracellular matrix

OM……osteogenic media

CM……...chondrogenic media

IHC……immunohistochemistry

IF……...immunofluorescence

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GENERATION OF BONE-CARTILAGE INTRFACE IN MICROFLUIDIC SYSTEM

Hamdullah YANIK, Dokuz Eylul University, Izmir International Biomedicine and Genome Institute, Department of Molecular Biotechnology, 35340, Inciralti, İzmir

ABSTRACT

Bone-cartilage interface is one of the most affected areas in bone and cartilage disorders. In this regard, tissue engineering approaches have been extensively utilized to regenerate the damaged skeletal tissues. Despite the significant progress in clinics, there is still unmet need for *in vitro* osteo-chondral models to study the mechanisms of these diseases. In their native environments, cells are spatially organized within complex structures known as the extracellular matrix. Current *in vitro* 2D culture platforms have limited capacity to recapitulate physiological clues. Bioengineered systems with 3D cell culture potential provide new venues for generating in vitro disease models. Microfluidic systems enable scientists to mimic native niche of organ and tissues in microscale. Such engineered systems are perfect platforms to examine the natural responses of the tissues, like their native microenvironments. Some of advantages using tissue and organ models as, i) minimizing the number of animals used in research, ii) identification novel therapeutics, iii) defining the drug administration techniques.

This study, aims to generate a novel in vitro disease model for bone-cartilage defects by combining 3D cell culture systems with stem cell technologies. Here, we design a microfluidic system that allows for the direct differentiation of ADMSCs in hydrogel-based 3D micro environment. The capacity of the novel microfluidic system provides simultaneous formation of osteogenic and chondrogenic tissues in the same microenvironment.

Key words: microfluidics, tissue engineering, osteochondral interface, mesenchymal stem cells, biomaterials

MİKROAKIŞKAN SİSTEMLERDE KEMİK-KIKIRDAK ARAYÜZÜNÜN GELİŞTİRİLMESİ

Hamdullah YANIK, Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Moleküler Biyoteknoloji Anabilim Dalı, 35340, İnciraltı, İzmir

ÖZET

Kemik-kıkırdak ara yüzü, eklem hastalıklarında en çok etkilenen alanların başında gelmektedir. Doku mühendisliği zarar görmüş bu dokuların tamiri veya yenilenmesinde yenilikçi yaklaşımlar sunar. Klinik çalışmalar için bu kadar önemli olmasına rağmen, var olan doku ve organ modelleri bu hastalığın çalışılmasında yetersiz kalmaktadır. 3B hücre kültürü sistemleri ile bu sorun aşılıp daha gerçekçi hastalık modelleri üretilmesi amaçlanır ve bu mikroakışkan sistemler içerisinde üretilen mikro yapılar, doku ve hastalık modellerinin, uyaranlara karşı verdikleri tepkileri incelemek için uygun bir platformdur.

Bu çalışmada biyomühendislik yaklaşımları kullanılarak kemik kıkırdak dokusunun mikroakışkan çipler içerisinde modellenmesi amaçlanmıştır. Mikroakışkan çiplerin sağladığı dinamik hücre kültürü ortamı sayesinde 2B sistemlerde karşılaşılan sorunların aşılması amaçlanmaktadır. Geliştirilen kemik-kıkırdak ara yüzü ile 3B hücre kültürünün sağladığı yararlarına ek olarak kontrollü bir mikroakışkan teknolojisiyle dinamik koşulların kök hücrelerin osteojenik/kondrojenik farklılaşması üzerindeki etkisi incelenmiştir.

Anahtar kelimeler: Mikroakışkan sistemler, doku muhendisliği, kemik-kıkırdak arayüzü, mezenşimal kök hücre, biyomalzeme

1. Introduction and Goals

Bone and cartilage tissues, are among the most affected areas of bone and bone diseases related with the articular interface. This interface is complex in terms of position of the cells and composition of the ECM in both sides, Because of this complexity, current studies on recapitulate human in vitro conditions are limited. The aim of this project is to mimic bone-cartilage tissue in microfluidic system using tissue engineering approach. Since nutrient and gas exchange cannot be done in large-scale tissue engineering constructs. There are necrotic cores, these cores are clusters of dead cells because of the insufficient nutrient, gas exchange. This occurs in the interior part of the constructed tissue and limits its functionality. On the other hand, many of the in vitro studies done in static environments which does not represent native physiology. This novel system solves the problem that is lack of dynamic stimuli in vitro models due of its dynamic cell culture environment provided by microfluidic flow. Both cartilage and bone differentiation have been achieved at the same time by using the laminar flow, one of the basic principles of the fluid mechanics, can flow more than one liquid in the same channel without mixing. This is because of the straight flow pattern of the fluids. The generated bone-cartilage interface will use to study the effects of dynamic conditions on bone-cartilage differentiation of adipose derived mesenchymal stem cells, as well as benefits of 3D cell culture and controlled microfluidic environment.

2. Literature

2.1 Tissue Engineering

Tissue Engineering is an interdisciplinary field that allow us to recapitulate native tissue in vivo. The ability to mimicking these nature environments enables us to generate tissue and disease models with their nature response. These tissues and disease models can replace with the malfunctioned damaged or missing part of the tissue [1]. The present requests for transplant organs and tissues is far overtaking the supply, and all way of projections demonstrate that this hole will keep on widen. Cell or artificial tissue transplantation have been proposed as an substitute treatment to entire organ transplantation [2]. In the process of making of an autologous embed transplantation, first donor tissue is isolated and this tissue dissociated small pieces in order to isolate the cells; then the cells are expanded in vitro and after the reaching certain number of cells, they loaded into a suitable substrate where it is re-implanted on the desired site of the organism. Since many freshly isolated cell populaces can be expanded in vitro utilizing cell culture strategies, a few contributor cells can ready to implant. In any case, it is trusted that all of the isolated cells cannot frame new tissues without any external stimulus. Most essential progenitor cells are accepted to be anchorage the surface and require particular factors that regularly incorporate with supporting material to go about as a layout for development of the tissue. The success rate of the cell transplantation highly depends on the suitable biocompatible materials that have been used in these tissue models and also do not have the immunological reaction with the host [3]. In addition to the material that used, progenitor cells, and soluble factors like cytokines and hormones, are also essential for the tissue engineering application. Furthermore, with the help of microfluidic systems the engineered tissues can be turned into more complex structures in a larger scale.

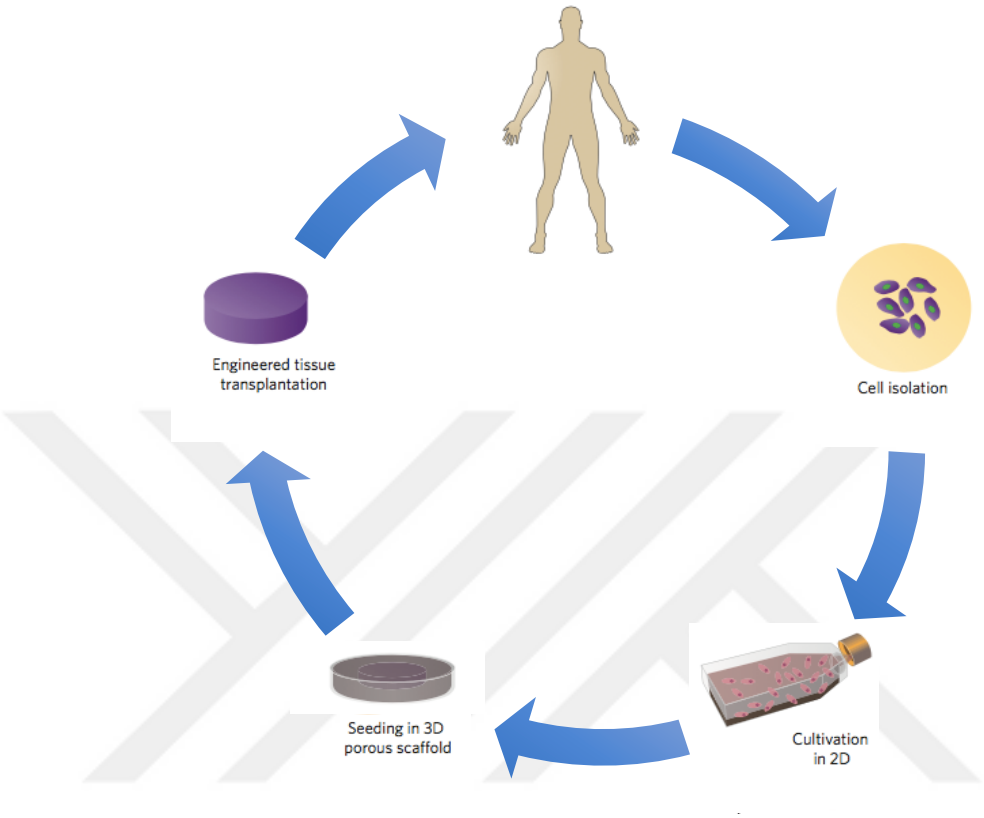


Figure 1. The basic principle of tissue engineering starts with the isolation of the cells from patient. Then, the cells culture in 2D until it reaches desired number and now the cells now ready to load into the scaffold as a carrier material. After short time of the culturing the cells, engineered tissue is ready to implant back again into the patient's body.

2.2 Biomaterials

An introduction to materials in medicine comes with properties and application of these materials these can be natural or synthetic, and these materials are used in directly contact with the organisms' biological system. These biomaterials can be classified in terms of their physical, chemical, and biochemical properties. At the beginning, these biomaterials should be biocompatible means that they do not cause any toxic or immunologic response when they are used with suitable model organisms [4]. The purpose of using these biomaterials in tissue engineering application should help to mimic the native environment of the organism. In addition

its biocompatible property, this biomaterial construct should have porous and fibril structure that allows them to exchange the gas and nutrient through the artificial construct [5]. For all of these reasons, biomaterial that is used in applications has a critical role during the application of these artificial construct [6].

Biomaterials are characterized as natural materials (collagen, gelatin, alginate) or synthetic materials (polytene glycol (PEG), polyglycolic acid (PGA), poly-L-lactic acid (PLA)) and these properties are important when constructing artificial organs or prostheses [7]. Biomaterials can be produced from directly nature or engineering by utilizing an assortment of chemical approaches to achieve the desired goal. They are frequently utilized or potentially adjusted for a restorative application, and in this manner, it contains entire or part of a structure of living organism for biomedical application which performs, enlarges, or replaces a characteristic capacity. These characteristics properties might be favorable for specific cases, for example, being utilized for recovering a bone section or hydroxyapatite covered hip inserts. Although biomaterials mainly biomedical application, they can be also used for expanding of the cells in vitro, in order to analyze the blood sample in forensic science application, fertilization of the cattle with an insert. For this purpose, these biomaterials can assemble in different ways. For example, biomaterials are generally formed via self-assembly or a spontaneous aggregation of the particles without any external force or influence. Large groups of such particles assemble themselves into more stable state thermodynamically.

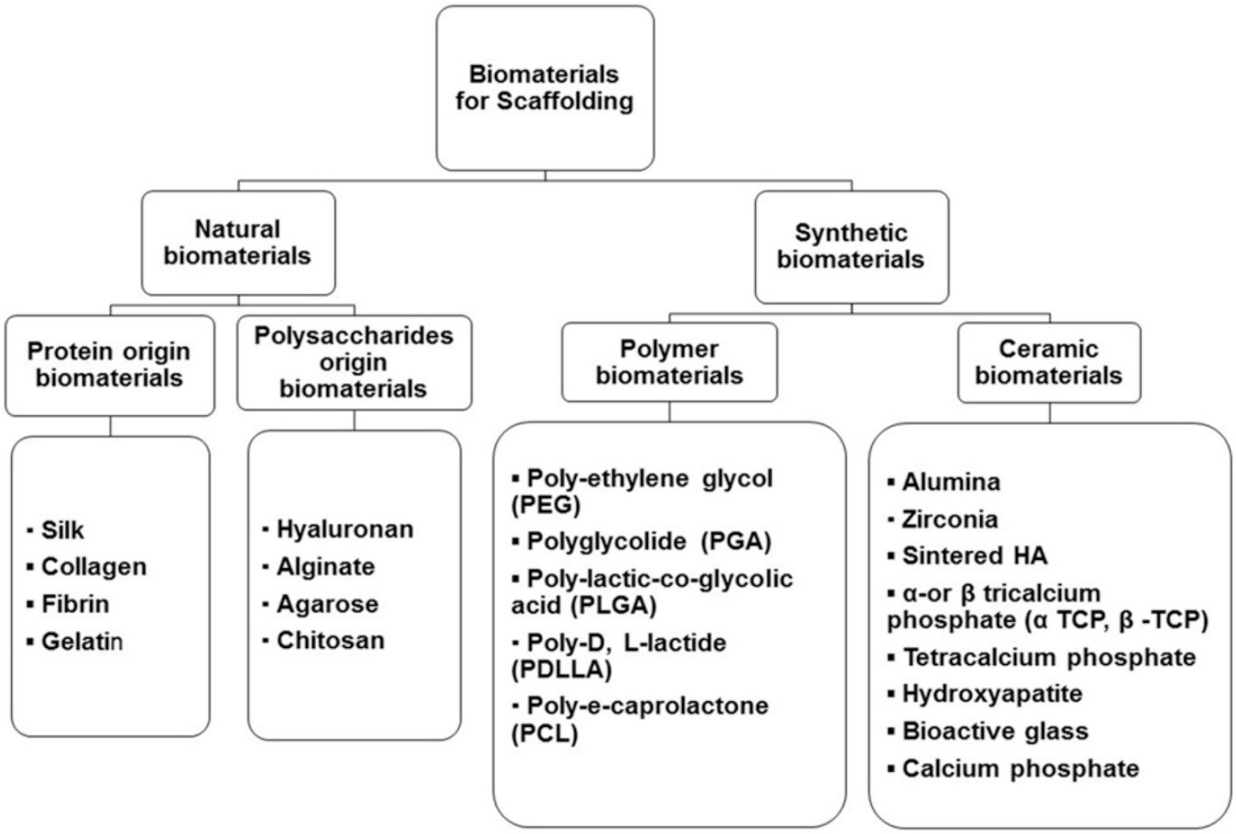


Figure 2. Classification of the biomaterials based on their origin [8].

Another example of this assembles, molecular self-assembly is found primarily in biological systems and it provides the basis for a wide variety of complex biological structures. It is these self-assembled biological structures which are most sought in biomaterials and how to replicate their design since naturally derived composites to date are still the most efficient when it comes to stimulating, initiating or inducing regenerative processes [7]. These self-assembling polymers contains specific peptide sequences which can be produced not only from protein-derived sequences but also from de novo sequences that are designed to self-assemble under certain physiological conditions. These protein structures tend to have bulky, have hydrophobic side groups and include amino acids such as valine, isoleucine, and phenylalanine [9]. On the other hand, some other 3D crosslinked structure of the hydrophilic polymer chains, they are capable of holding large amounts of water, hence hydrophilic chains hydrogel swells in the water.

Water makes up a high percentage of the human body, thus making hydrogels promising tools for bioengineering application [10]. As expected, naturally formed hydrogel is not enough to mimic the native environment of the tissue. With suitable chemical modification of the hydrogels, their mechanical and chemical properties can be tuned for the environment. In addition to this process, by modifying the polymer chains with radical functional groups, the properties of hydrogel become inducible with heat, light, magnetic fields [11]. One of the most common modifiable polymers is gelatin, which is fragmented collagen, cocktail of proteins and peptides that extracted from skin, bone and connective tissue of the bovine or porcine. It has Arginyl-glycyl-aspartic acid (RGD) groups these are cell binding groups, and MMPs. These MMPs have sensitive degradation sites that required for the degradation of the polymer by cells. In a 3D environment, crosslinking of the gelatin is crucial for long-term integrin binding sites need to binding of the cells. For this purpose, methyl acrylated gelatin, GelMA, is used. This polymer inject in liquid forms and polymerizes under UV light [12]. These biomaterials can be classified as natural and synthetic based on their source of production.

2.2.1 Natural Biomaterials

2.2.1.1 Collagen

Medical applications of collagen have entered a new era in the last decade. The potential use of collagen-based materials in medicine has increasingly been appreciated as the science and technology advances. For these applications, collagen is naturally found in connective, muscle, and chondrogenic tissue [13]. This biomaterial contains non-dissolvable molecules that are most abundant in the ECM. These molecules construct scaffold very similar to a DNA helix. In addition to this helix structure, biodegradable, non-toxic, and non-immunogenic properties allow us to have a suitable environment for long-term cell culture while constructing artificial tissues. For example, one of the these is a chondrogenic tissue that cannot regenerate itself. In clinical trials, stem cells are taken from the suitable cell (blood, adipose tissue or bone marrow) source that is taken from the patients to expand in vitro. Then, it is loaded into collagen type I and III as two layers of gel, and after that it is placed to the missing part of the patient tissue. Finally, the

study results with a high yield in terms of regeneration in the missing part of the tissue when compared with the other type of biomaterials that is used in this assay [14].

2.2.1.2 Hyaluronic Acid

Another example of the natural biomaterial that has been used most commonly in biomedical application is hyaluronic acid. Hyaluronic acid (HA) is one of the most abundant molecules in the connective tissue. It composed of GAGs and has important role in wound healing. HA degrades with the help of hyalorinase enzyme and this enzyme can be isolated from human blood [15]. Because of the limited mechanical properties of this polymer, it cannot be used in connective and bone tissue engineering efficiently. HA generally uses a combination with different type of biomaterial in order to increase its mechanical stability [16]. Chondral tissue engineering is one of the most suitable study fields for this biomaterial. To construct this chondral tissue, stem cells are expanded on petri dish and differentiated in HA based biomaterials, and with this biomaterial loaded cells tend to be a more chondrogenic fate. HA based biopolymer can be modified with chemical groups to become UV cross-linkable hydrogel. Then, this is easily printed into the missing part of the bone and cartilage with specific growth and differentiation factors [17]. In addition to these biomedical application, HA is suitable for the regeneration of the soft tissue [18].

2.2.1.3 Alginate

Alginate, which is an also natural biopolymer, is a biomaterial isolated from marine algae. Biocompatibility, cost, and easily modifiable structure make it one of the most preferred biomaterials in tissue engineering applications [19]. Alginate can be cross-linked in more than one way to provide mechanical integrity. For example, solutions containing calcium ions will polymerize by allowing carboxyl groups in alginate to bind. Another method of cross-linking is partial oxidation. In this manner, in liver tissue engineering application, hepatocyte cells embedded in alginate-based 3D structural skeletons were 90% spread [20]. In addition, alginate implantation results in positive results in liver injuries and cardiac interventions [21].

2.2.1.4 Chitosan

Chitin is the second most important natural polymer in the world. The main sources exploited are two marine crustaceans, shrimp and crabs. One of the most important derivatives of chitin is chitosan. Depending on the method of its production and the source from which it is obtained, the size of the molecule can range from 300 to 1000 kD. This characteristic of chitosan allows the material to play in its physical structure without additional chemical. Chitosan allows bio-ceramics to form hybrid materials and to obtain effective results in bone tissue engineering [22].

2.2.1.5 Gelatin

Another example of natural polymer is Gelatin. Gelatin is a biomaterial obtained by breaking heat sensitive triple helix bonds of the collagen. Compared to collagen, production is cheaper and it is less antigenic means that low capacity of causing inflammation in organism. Due to its high biocompatibility, easy polymerization and controlled biodegradation, it is more feasible than other natural biomaterials for the biomedical applications [3]. Gelatin, which has high capability of chemical and physical modification rates, is frequently used as cell carrier structures in tissue engineering applications. Recent studies have shown that growth and vascularity have at higher rates of cells where placed in gelatin-based hydrogels which is also containing growth factor and cytokines [23]. In this study, we utilized gelatin-based hydrogels as cell carriers and base for an artificial tissue. GelMA is modified version of gelatin with methacrylate that allows us to polymerize our hydrogel with UV light, and the cells can be printed into desired pattern with help of photomask.

2.2.2 Synthetic biomaterials

2.2.2.1 Polyvinyl alcohol (PVA)

Polyvinyl alcohol (PVA) is a biomaterial obtained from the reaction of polyvinyl acetate. This synthesis process involves the steps of hydrolysis, alcoholysis, and aminolysis. These biomolecules react with different ratios of glutaraldehyde to yield products with different mechanical properties. Because of its high bioprocessing time, PVA proves to be a good candidate for cartilage tissue engineering [24]. In addition to these, PVA-modified aliphatic polyesters also show surface erosion degradation mode rather than the bulk mode found in aliphatic polyesters with nonmodified characteristic. Ionic groups such as sulfobutyl and dimethylamino amine were further incorporated into the PVA-modified aliphatic polyesters to generate either anionic or cationic charges for potential drug delivery application.

2.2.2.2 Poly- ε -caprolactone (PCL)

Another synthetic polymer example is Poly- ε -caprolactone (PCL). It is an aliphatic polyester [22]. It is frequently used in the development of carrier scaffolds for surgical sutures, drug delivery systems, and various tissue engineering techniques. The commercial advantages of PCL are: i) approval by the US Food and Drug Administration (FDA) to use it in humans, ii) biodegradability, iii) capability of combination with many other polymers, iv) and can be shaped into many different forms, v) easy melting due to high thermal stability, and vi) relatively inexpensive [25].

2.2.2.3 Polyethylene glycol (PEG)

Another synthetic biopolymer that has been frequently used in medical application is a poly (ethylene glycol) (PEG). It is a synthetic and hydrophilic polymer that has been modified with many functional groups and it is frequently used in tissue engineering and bioengineering

applications due to its low toxicity and biocompatible properties. PEG easily overcomes its drawbacks of not supporting cell adhesion by binding to various peptide groups using its structure. Furthermore, PEGDA becomes cross-linkable by UV light because of methacrylate groups and photoinitiator that are added to the polymer chains, and thus is well suited for in situ applications [26]. These aliphatic polyesters are favorable for gene therapy, angiostatin delivery via stent, plasmid TGF-β delivery for promoting diabetic wound healing. Due to the increasing hydrophilicity, those PEG-aliphatic polyesters hydrolytically degrade at faster rates than unmodified aliphatic polyesters.

2.3 Stem Cells

Stem cells are a type of undifferentiated cells that have capacity of differentiate into different cell types based on their origin. In general, stem cells can be isolated two different sources. One of them, embryos that form during the blastocyst phase of embryological development (embryonic stem cells), and the other one is adult tissue (adult stem cells). Both of them are generally identified by their potency, or potential to differentiate into different cell types (such as muscle, skin, bone, etc.) [27]. These cells are classified as totipotent, meaning that they have the ability to generate a whole organism by itself, or pluripotent, meaning they have the capacity to differentiate almost all of the cell types and have important role in construction of three germ layer which are mesoderm, endoderm and ectoderm. In addition to these cell types, multipotent ones have less differentiation ability compared to the others; in this stage multipotent cells are almost specified. After developmental stage of the organism, these cells have crucial role in injuries. One of the most interesting findings is the migration of the stem cells to the site of injury in order to speed up the regeneration of the damaged tissue [28]. Because of regenerative abilities of the stem cells, they are strong candidate for tissue engineering and therapeutic applications.

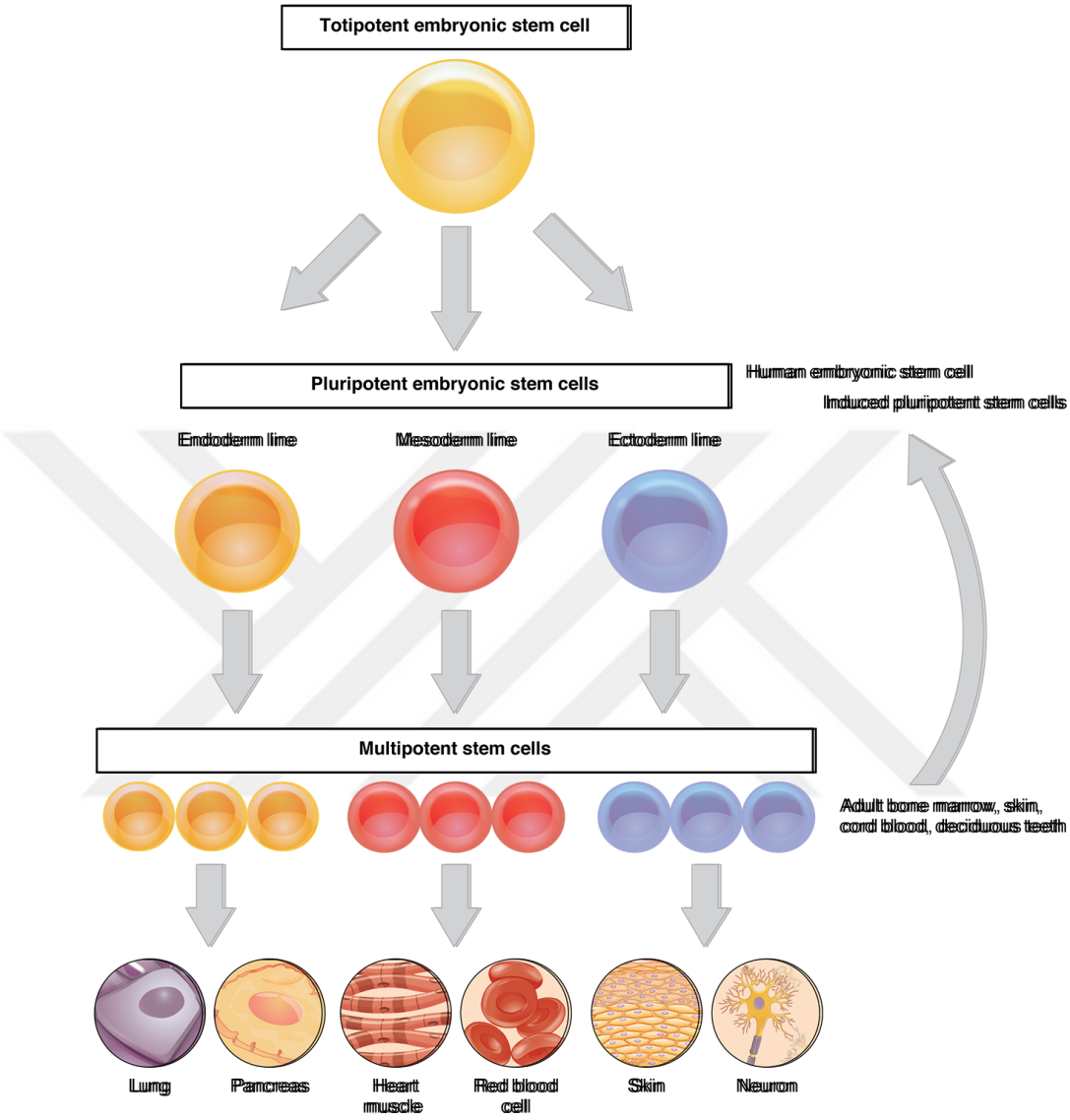


Figure 3. This figure shows us the multilineage properties from a single cell to more than 200 different cell types [29].

These cells can be identified with their specific surface markers, such as STRO-1, SB-10, SH3, and SH4 antigens. On the other hand, MCS show negative for the hematopoietic markers CD34, CD45, and CD14. SH3, SH4, and STRO-1 and identify the antigens that are already specific for the MSCs, but not for the hematopoietic cells. In addition to this, the specific surface markers are not only essential for the MSCs, but can also observe with the other type of cells. Up

to date, there is no specific surface marker known making it challenging during the process to isolate specific MSCs from a mixed cell culture. For this purpose, a combination of more than one antibody is used. Also, there is a lack of information about characterization of specific cell markers. This characterization goes with the proliferate to self-renew and undefined state with the signal taken, and so differentiate capacity with different cell stimulus [30].

2.3.1 Sources of MSCs

The one of the most common tissue sources of the MSCs is bone marrow. In addition to this, other tissues like periosteum, adipose, synovial membrane, deciduous teeth, pericytes, articular cartilage, immobilized peripheral, synovial membrane, cord blood and placenta. Endogenous function of the MSC is still uncertain. The cells are also known as the key part of local tissue repair [31].

2.3.1.1 Bone Marrow Derived Mesenchymal Stem Cells

There are many advantages of using BMDMSCs in clinical use, the first advantage is the isolation of the cells easy and expand in higher rate. Due to these characteristics, these cells can easily isolate from patient and is stored bio-banks for further usage. In addition to storage of these cells, BMDMSCs are also strong candidate while studying recovery of bone [32] and cartilage [32] defects. Recent studies show that, BMDMSCs with osteochondral defects are recover with a scaffold that contains TGF-β 1 with higher yields [33] . Other advantage of using BMDMSCs, ability of the cells migrating to the injured zone with their T-cell suppressive cytokines [34], these have crucial role in rejection of allotransplantation and xenotransplantation [35]. Recent study about cartilage defects, BMDMSCs specifically targets the lesion area of the cartilage and bone with the help of releasing suppressive, proliferative, and regenerative factors. The factors provide a perfect microenvironment for regeneration of the bone and cartilage tissue [36]. Despite the promising application with the BMDMSCs, there are still some parameter the think of such as the number of cells that is required for the cartilage repair if there is necessary one or more injection to the lesion site for observation of the desired effect [37].

2.3.1.2 Adipose Derived Mesenchymal Stem Cell (ADMSC)

Adipose derived mesenchymal stem cells can be identified as the most suitable stem cells source with their non-immunogenic, anti-inflammation properties [38], and their less ethical concerns related when it compared with stem cells which are isolated from different source. Expansion and differentiation capacity of these cells are less likely to be changed with person's age [39]. For example, ADMSCs use to cure abnormal cartilage pathology was treated with injection of cells into osteochondral interface of elderly patients that ranged from 65 to 80 with arthroscopic lavage. The result of the study is reduced pain, improved physical function, and also cartilage regeneration [40].

2.3.1.3 Peripheral Blood Stem Cells (PBSCs)

Osteogenic and chondrogenic differentiation capacity of these cells from different sources has also been studied such as autologous PBSCs are one of the most favorite source of stem cells for clinical applications [41]. A clinical study about osteochondral interface recovery in patients aged from 18-50 years with knee chondral lesions treated with arthroscopic subchondral drilling followed after an operation on articular loading of HA and another trial was done with using PBSCs contains hydrogels. The result indicates these hydrogels with PBSCs has a higher rate of regenerate the defect area in the tissue.

2.3.2 Multilineage Differentiation of MSCs

MSC differentiation is strictly controlled under the many factors such as growth factors, hormones, extracellular matrix molecules, and signaling pathway molecules. For the proper musculoskeletal tissue engineering, cartilage formation can be done by growing the cell under the 3D culture condition as a scaffold with the presence of the TGF-β1, TGF-β2, or TGF-β3 or bone morphogenetic proteins (BMPs). An addition of dexamethasone in this culture, causes the

chondrocyte phenotype. This chondrogenic fate can also be observed with the expression of the gene for cartilage matrix formation, such as aggrecan, collagen type II and IX and COMP (cartilage oligomeric matrix protein). On the other hand, osteogenesis can be described as the bone formation which is stimulated by supplying glucocorticoid dexamethasone, β-glycerophosphate, ascorbic acid, and 1,25-dihydroxyvitamin D3 [31]. Furthermore, growth factors also play crucial role at osteogenic differentiation. For example, the culture of the MSCs with the insulin-like growth factors (IGF-1), epidermal growth factors (EGF), and vascular endothelial growth factor (VEGF) surge the activity of ALP, and this also cause mineralize the extracellular environment. Recent studies show that Wnt signaling pathway also has an important role in the hMSC osteogenesis. Cysteine-rich glycoproteins regulate this differentiation. These glycoproteins are regulatory factors for the plasticity, differentiation, and maintenance of the stem cells. Blockage of Wnt pathway will affect the osteogenesis of MSCs which result with deficient bone formation. There is the possibility that the loss of function can cause the loss of bone mass, or the strength of it. On the other hand, stimulation of the cells with this Wnt3a-conditioned medium or overexpression of the ectopic Wnt3a restricts osteogenesis form the MSCs [42].

2.3.3 Plasticity of MSCs

Plasticity or trans differentiation can be identified as the process of the cell type choosing a path for differentiation in order to committed different cell fates. This programing of the cells is strictly under control of the genes. The terminally differentiated cells change their fate under the suitable environment with correct stimulations [42].

2.3.4 MSC Heterogenicity

Isolated colonies of the MSCs do not have homogeneous nature while they cultured in terms of their capacity of proliferation and differentiation potential into distinct cell types. For example, only a few of the population have less than over 20 different subpopulations. There are

also other studies these are evidence that only one of the third of these cells have capacity of the differentiate to the other 3 types of the cells osteogenic, chondrogenic, and adipogenic fate. Another study reports that only the one the non-immortalized cells have the ability of differentiate into three cell fate, osteogenic, adipogenic, or chondrogenic, while the other ones only have bi-linage, osteogenic, chondrogenic, or only uni-linage potential, osteogenic ones. Even with the cells that come from a single cell, the heterogeneity can be observed during the time of expansion of the cells [43]. Differentiation of the MSCs occur in several steps and in different ways; if this signal comes from the environment, the outside of the cells is called paracrine regulation; if its source is the cells itself is called autocrine signaling. In addition, both of the signaling is highly related to developmental position of the cells. The determination of the cell fate can be estimated by looking at the position of the cells. For this purpose, *C.elengans* is used for observing of the stem cells during the developmental stage.

2.3.5 MSC Isolation and In Vitro Culture

The process of MSCs isolation in the bone narrow is very rare with frequency of 0.001-0.01% within the all of the nucleated cells. Many protocols can be applied for the isolation of MSC cells, some of them are tissue specific. Human bone narrow can be easily obtained by simple direct seeding method. After the seeding these cells, nonadherent cells will be washed away when the media has changed. By doing this, cells are selected with time and medium change. On the other hand, the MSCs are observed as small, adherent, spindle-shaped cells. After psychological classification, surface markers will be used for the positive selection, and the negative selection can be done with the magnetic activated cell sorting, or florescence activated cell sorting. After selection of desired population, hMSCs could be used as a cell line. Nevertheless, hMSCs cannot produce telomerase, and this telomerase activity will be lower when the cell becomes older. In addition to this, telomerase knockout mice are not able to differentiate their cells into adipocytes and chondrocytes, and through time, the telomere region is completely lost. hMSCs suppresses the telomerase by using reverse transcriptase, and this can go on for about a measurement of 80 or 240 populations [44].

2.3 Microfluidic Systems

Microfluidics is a multidisciplinary field intersecting engineering, physics, chemistry, biochemistry, nanotechnology and biotechnology with practical applications to the design of systems in which low volumes of fluids are processed to achieve multiplexing, automation, and high- throughput screening of the desired molecules and cells. Main features of microfluidics are a) capacity of working with small volumes b) being in small sizes and c) showing effects of the micro domains.

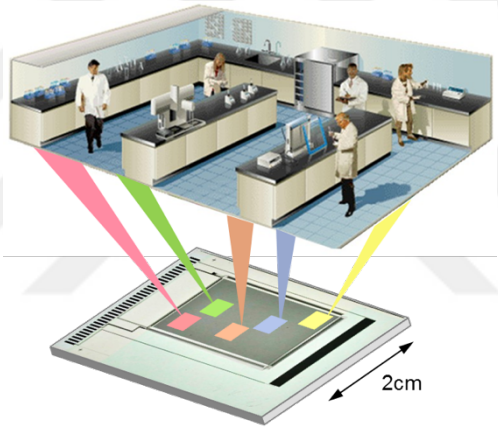


Figure 4. Representative illustration of the microfluidic system which is like miniaturized laboratory [45].

2.3.1 Fabrication of Microfluidic Systems

The fabrication of microfluidic systems requires the combination of disciplines such as material science, fluid mechanics, and bioengineering. The properties of the materials used have a significant effect during the developing phase, the production technique is also important in the design and application stage [46]. Generally, microfluidic systems use polymer, glass, and paper materials as a production material.

2.3.2 Materials for Fabrication of the Microfluidic Systems

Materials used in determine the properties of microfluidic chips, so that the material selection has an important role in microfluidic systems. Glass, polymer, and paper are the most commonly used microfluidic system materials [47]. For example, capillary electrophoresis and solvent separation are used in application. Using proper material for the application results with better via glass based on microfluidic systems when this is compared with other material. In addition to these optical properties, they provide convenience in microscopic examinations. However, the production of glass microfluidic chips is relatively difficult and expensive. On the other hand, polymeric materials enable us to produce rapid prototype with low cost, relatively complex, parallel fluid manipulation, and also microchannel 3D cell culture. For this reason, polymers are the most favorable materials in the production of microfluidic systems. Instead, Paper is a cellulose-based material with an excessively porous matrix, which is very useful when microfluidic chips combine to capture the liquids. It is also possible to impart hydrophobic properties of the paper in order to pass fluids through the desired regions of the microfluidic system. Paper-based micro-porous chips are portable and very cheap systems for biological analysis. The material to be selected in microfluidic chip technology is very important for the application to show accurate results. Each of the glass, polymer, and paper systems consists of many materials in its own right, each providing different advantages. In addition to these, it is also possible to produce composite materials and devices with advanced features, such as a synthesis of surface engineering, functional materials, and microfluidic technology.

2.3.3 Production Techniques of Polymer Based Microfluidic Systems

Polymers are macromolecules with their high molecular weight and composed of repeating monomeric units. Polymer chain structures can be linear, cross-linked, and branched, they melt at varying temperature range due to differences in chain lengths. The low cost, low electrical, thermal conductivity, their suitability for surface treatments, and biocompatibility make them attractive for use in microfluidic technology. There are four ways to produce

microfluidic systems from polymers include injection molding, casting, laser cutting and thermoforming.

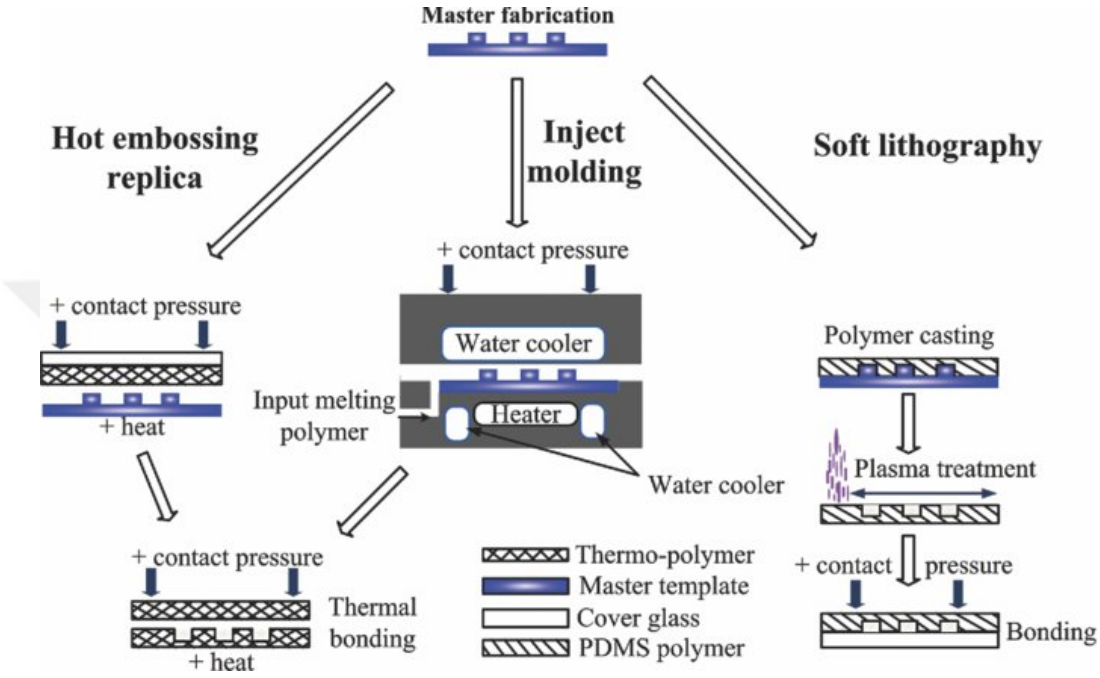


Figure 5. This figure shows the different ways to fabricate the microfluidic device with polymers that have different chemical properties. In the hot embossing technique thermally, the responsive material printed desired is shaped with master mold. In injection molding, the polymer injects to the mold, then polymerize. Finally, it is put in the soft lithography master mold that is prepared with UV lithography; next PDMS polymerize on is placed top of the master mold; then it separated from the molds and bonds to the glass surface with plasma treatment.

2.3.3.1 Injection Molding

Injection molding is one of the first techniques used in the production of microfluidic systems from polymeric structures. Generally, amorphous thermoplastic polymers such as PMMA are removed on glassy transition temperature and then softened and processed for production.

Injection molding the heated polymeric material is injected into the mold. Once the mold cavity is filled, it is allowed to cool and then the mold is opened to obtain the product. The quality and type of the mold greatly influences the properties of the mold surface. Injection molding is very suitable for industrial applications since it obtains products within minutes.

2.2.3.2 Casting

Casting is a way of obtaining a mold-shaped product by casting a liquid material. The liquidated polymer is poured into a mold and solidified. After solidification, the product is removed from the mold. Thermoset and elastomer chips can also be produced in this way. The thermoset polymers (e.g., PDMS) are poured in liquid form by mixing it with the accelerator and crosslinked by heat or UV light. However, it is difficult to adjust the proportions of both components very well, to adjust homogeneous mixing and cross-linking. The casting process is longer than injection molding. Because of these properties of the material, it can be easily applied in the laboratory environment without any expensive equipment, it is a preferred method in academic researches.

2.2.3.3 Assembly of PMMA

Rapid production is preferred both in laboratory investigations and in commercial microfluidic system production. Laser cutting gives efficient and reproducible results in terms of cost, quick and easy production and precision. Specifically, thermo-rubber polymers such as PMMA are produced within minutes by this method. The method uses laser beams to precisely cut the solid polymeric material to form parts of the microfluid system. In order to achieve the desired designs, the laser beam can move in 2D and pieces in 2D assemble with the help of DSA and produce the desired patterns in 3D. While 2D visual design platforms such as AutoCAD and CorelDRAW are available for chip design, it is also possible to convert the extension of the folder to use on other platforms. The chips produced by laser cutting devices consist of more than one component, and are then manually assembled. Opening the guide ports near the edge of the chip makes it easier to do this correctly. The pressure for the fluid flows are provided by the

syringe or peristaltic pumps. In order to provide fluid flow, it is necessary to open the inlet and outlet ports which vary according to the diameter of the syringes and pipes used in the dimensions of the chip. The distance between the guide ports and the edges of the section where the flow is made is critical, and if the pair does not have enough distance, it can leak. The production of thermoplastics in a manner similar to injection molding uses thermoforming or pressure casting.

2.2.3.4 3D Printers

Generally, microfluidic devices are produced by photolithographic techniques from elastomeric polymers such as poly (dimethyl siloxane). However, this process requires high levels of labor, specialized devices, and institutional competencies. With the help of 3D printing techniques and simple and easy-to-use devices, it is possible to produce micro-structured fibers within a few hours [48]. Compared to other techniques, the institutional infrastructure is an alternative way of reducing costs associated with equipment installation and maintenance. Recent developments in 3D printing technologies have resulted in highly complex microfluidic devices that can be fabricated in a single step, quickly and cost-effectively, making microfluidic technology more accessible to users [49].

2.3.4 Production Techniques of Glass Based Microfluidic Systems

Glass is very suitable for biological applications with its chemical inertness, insulation, and optical properties. However, the production techniques of microfluidic glass chips are very costly and are a difficult process due to the anisotropic and amorphous nature of the glass. Passing the microfluidic chip design to the glass surface by means of chemical etching of solid glass carries out chip production.

2.3.4.1 Photolithography

Photolithography is the technique most commonly used to produce photonic transfer of integrated circuits and other microstructures to one surface. In the field of bioengineering, the position of the cells is also used in a controlled manner on the desired surface, drug and toxic biosensors, tissue engineering applications, and basic biology studies. Precision modeling at submicron levels makes the use of photolithography widespread in the semiconductor industry. In microfluidic systems, glass-based materials are produced by this method. Photolithography begins with preparation of the surface for processing and includes three basic elements: photoresist, photomask, and light source. Moisture and other residues on the surface are cleaned with chemicals which is then formed by a spin coating technique on the surface of a light-sensitive layer of material called photoresist. Various adhesion promoters are also used to strengthen the substrate-photoresist interface. It is most important that the photoresist is formed homogeneously on the surface the photoresist or under the light source. At this stage, the photoresist is polymerized under the UV light to take the pattern on the photomask. In order to transfer to the shaped surface on the photoresist, etching process is usually done by using chemicals. After the pattern is obtained, the photoresist layer is thermally, physically, and chemically stabilized so that it is not affected by the photoresist. By cleaning the surface of the system exposed to the rays through the light source, the portions of the photoresist are exposed to the light are removed and the desired design is obtained. Pattern transfer can be done in two different ways depending on whether the mask is positive or negative. Microfluidic chip production serves two purposes: laboratory research or commercial purposes. While laboratory researches require sensitive and fast chips, commercial cost is most important. The development and optimization of new materials and production techniques, reduction of the cost of existing techniques, and increasing chip sensitivity are among the main objectives of microfluidic chip technology [37].

2.3.4.2 Microfluidic patterning / Liquid Phase Printing

The channels of the microfluidic systems can be used for selective surface patterning as well as providing suitable conditions for desired application. In the microfluidic patterning method, depending on the geometry of the channels, it is possible to render the desired liquids on

the surface by delivering the desired liquids. This technique is similar to the microcontact printing technique, where the printing material is transported to the surface via microchannels instead of stamps. In this way, the desired molecules are delivered to the desired region in successive and different combinations. In addition, this method allows to simulate signal pathways using similar gradient mechanisms, allowing for gradient formation, using lamina flow and controlled diffusion mechanisms [51].

2.3.5 Fundamental concepts of microfluids

2.3.5.1 Laminar Flow / Turbulent Flow

Reynolds number (Re) is the ratio of inertia forces to viscosity forces, independent of the size of a fluid. Re is inversely proportional to the characteristic velocity of the liquid and to the liquid viscosity, which is directly proportional to the length scale of the system. The flow mechanics of liquids with high Re values are turbulent and causes liquid to mix and flow. In microfluidic systems, Re is mostly in the laminar flow range. This ensures that flow mechanics are often computable [50].

2.3.5.2 Surface and interface stress

Surface tension defines the tendency of a liquid on the surface to reduce its free energy by shrinking at the surface-air interface. Interfacial tension is a similar concept but it is usually applied to non-intermixing fluids. These forces are more oppressive on a micro scale, as opposed to the more dominant gravitational force on a macro scale.

2.3.5.3 Capillary forces

Capillary action discloses the movement of a fluid as it passes through a narrow structure (a narrow channel or porous structure). The capillary action, which is dominant in micro scale, allows liquids to move against the gravity.

2.4 Microfluidic Systems for Tissue Engineering Applications

The ultimate goal of tissue engineering is to produce tissue and organs on a large scale and to produce sustainable and continuous solutions in the field of regenerative medicine and organ transplant alternatives as well as to develop appropriate platforms for diagnosis and monitoring at the same time. It is aimed at the produced tissues, achieving the correct morphology and its functionality. Since tissue engineering is applied in 3D systems, it is necessary to overcome problems such as directing factors, removing waste while achieving sustained oxygen and nutrient with continuous flow, and providing near-realistic physical conditions [51]. Bioreactors are engineered systems that provide a dynamic and realistic environment for a biologically active environment. Bioreactors may be of different sizes and structures. Microfluidics systems are micro-sized bioreactors. Microfluidic systems have the potential to provide solutions to the problems faced in the creation and control of the cell microenvironment of traditional cell culture system. While these constraints can be solved by micro-scale controllable flow mechanics and 3D cell culture systems, a culture medium close to the true in vitro conditions can be provided [39]. The first approaches to tissue engineering are top-down tissue engineering studies that produce viable carrier scaffolds for cell cultures and then rely on in vivo implantation. These initial studies have been successful in creating simple tissue structures (in mm^3 scale), but are insufficient to construct complex vessel and neural network structures in large (cm^3 scale) constructions. The transfer of these experiences from biomaterials and tissue engineering to microfluidic bioreactors has gained momentum in recent years. Microfluidic systems are concerned with the behavior, control and manipulation of fluids in nano and micro-volumes. Microfluidic systems generally include microfabricated channels, reservoirs, cell culture fields, stimulation electrodes, temperature controllers, sensors and detectors. The

greatest advantage of microfluidic systems over macrometric technologies is that their interaction at the cellular and molecular level can be easily controlled. In order to be able to form tissues and organs, microenvironmental conditions that can be controlled by well-defined architects, sharp control over molecular and chemical signals and appropriate intercellular matrices are required [52]. The microfluidic interventions used for tissue engineering are mostly molecular and cellular and adopt the "next-generation" tissue engineering approach, similar to the early developmental phase of the body. With this approach, cells or micro / nano building units can be placed in desired patterns by biopsy and joining methods. Examples include, but are not limited to, the formation of intercellular matrices at predetermined locations for cell attachment, the printing of different groups of cells side by side to mimic the architecture of the tissues, the establishment of vascular structures for the removal of toxic wastes while providing continuous nutrient and essential factors, it can be considered that the signals are delivered at the desired time in the spatial mode. As a result, controls and manipulations that cannot be achieved using traditional methods can be achieved using microfluidic technologies. The creation of the intercellular space has a highly critical design for tissue engineering studies in microfluidic systems. The distribution of these signals along with the shape, size and compositional changes on the surfaces of the cells, fatigue media, soluble biochemical signaling factors, neighboring cells and their distribution over time significantly affect the characteristics of the cells such as survival, functionality, proliferation and differentiation. The first step in establishing this controllable microenvironment is through the distribution of cells and intercellular matrix proteins mimicking the underlying organism in a realistic way [53]. Following this step is the creation of appropriate circulation and delivery of the biochemical signals in the fluid medium to the locations needed at the right time in the spatial environment.

3. Materials and Methods

3.1 Type of Study

All experiments were done in *in vitro* conditions.

3.2 Location and Time of Study

All experiments were performed between September 2016 - August 2018 at Izmir Biomedicine and Genome Center.

3.3 Material of Study

Adipose derived mesenchymal stem cells were used in this study.

3.4 Variable Parameters of the Study

Independent variables: Expanding Medium (DMEM:F12 (Thermo Scientific 31330 - 038), %10 FBS (Thermo Scientific 43595 - 40) (Thermo Scientific 43595-40), %1 Penicillin / streptomycin (Thermo Scientific 11320033), 5 ng/ml FGF-2 (PepProtech 100 - 21 - 10UG)), chondrogenic induction media (DMEM (Thermo scientific 41965-039), %10 FBS (Thermo Scientific 43595 - 40), %1 Penicillin / streptomycin (Thermo Scientific 11320033), 0.1 M ascorbic acid (Sigma A8960-5G), 10^{-7} M dexamethasome (Sigma D4902 - 25MG) , 10 ng/ml TGFB1 (PepProtech 121 – 32- 10UG), Osteogenic Induction media (Low Glucose DMEM (Thermo scientific 41965-039) , %10 FBS (Thermo Scientific 43595 - 40) (Thermo Scientific

43595 - 40), %1 Penicillin / streptomycin (Thermo Scientific 11320033), 0.1 M ascorbic acid (Sigma A8960-5G), 10^{-8} M dexamethasome (Sigma D4902 - 25MG), 0.01 M beta-glycerophospate (Sigma G-9891), 10 ng/ml BMP2 PepProtech (1115255 - 10UG)

Dependent Variables: Specific linage markers (aggrecan, osteocalcin, runx2, sox2, col I), expression of specific marker genes (osteocalcin, osterix, aggrecan, col I, sox2)

3.5 Data Collection Tools

3.5.1 Culture of ADMSCs

ADMSCs were cultured in cell culture dishes with high-glucose DMEM:F12 (Thermo Scientific 31330 - 038) supplemented with 10% FBS (Thermo Scientific - 16140071), (Thermo Scientific - 15070063) (Thermo Scientific - 15070063) and 5 ng/ml FGF2 (ProProtech – 100-21-10UG). The following the seeding day, the cells were replaced with fresh medium and resuspended according to cell density. These processes were continued until the cells reach the confluency about 90-80%. For sub-culturing the cells, firstly media was aspirated, then rinsed with 1x PBS (Thermo Scientific - 10010023) in order to remove the cell debris and serum. Cells were treated with 5 ml of Trypsin/EDTA solution (Thermo Scientific - 25300054) in a 37°C incubator for 5-7 minutes. Then, plate was observed to ensure the complete detachment of cells. After being sure all cells detached from the surface of the flask double amount of serum containing medium was added in order to stop the activity of trypsin. cells were seeded for expansion at 4000 cell/cm^2 and for differentiation 8000 cell/ cm^2 density.

3.5.2 Differentiation of ADMSC

3.5.2.1 Osteogenic Differentiation of ADMSCs

Three weeks of osteogenic differentiation was performed with osteogenic induction media composed of DMEM – Low Glucose that media supplemented with 0.1 M ascorbic acid

(Sigma - A8960), 10^{-8} M dexamethasome (Sigma 2915), 0.01 M beta-glycerophospate (Sigma G - 9891), 10 ng/ml BMP2 (PepProtech -1115255-10UG), %10 FBS (Thermo Scientific 43595 - 40), %1 Penicillin/Streptomycin (Thermo Scientific - 15070063). The culture media was changed with 2 days intervals. During the media change only half of the volume the media was aspirated and replaced with fresh media.

3.5.2.2 Chondrogenic Induction of the ADMSCs

Three weeks of differentiation was performed with chondrogenic induction media composed of DMEM – Low Glucose that media contains 0.1 M ascorbic acid (Sigma - A8960), 10^{-7} M dexamethasome (Sigma – D4902 25MG), 10ng/ml TGFB2 (PepProtech - 100tgv), %10 FBS (Thermo Scientific 43595 - 40), %1 Penicillin / streptomycin. The culture media was changed with 2 days intervals. During the media change only half of the volume the media was aspirated and replaced with fresh media d.

3.5.2.3 Adipogenic Induction of the ADMSCs

21 days of differentiation was done with adipogenic induction media with DMEM – Low Glucose that media contains, 10ug/ml IBMX (methyl-isobuthyl xantine, SIGMA - I-5879), 5 ng/ml Insulin (ACTRAPID HM Apotek: 9007083, 0.1mM Indomethacin (SIGMA-I-7378), 10^{-6} M dexamethasome (Sigma - 2915), 10ng/ml TGFB1 (PepProtech - 100tgv), %10 FBS (Thermo Scientific 43595 - 40), %1 Penicillin / streptomycin. The culture media was changed with 2 days intervals. During the media change only half of the volume the media was aspirated and replaced with fresh media

3.5.2 Synthesis of GelMA

Firstly, 5 grams of Gelatin (Sigma - G6144 - 100G) measured and poured into 1x PBS solution on heater set to 50 $^{\circ}$C. In order to compete solving of the gelatin, powder of the gelatin should be added slowly in to PBS and mixed with magnetic stirrer for 1h. After there was no

gelatin particle inside the solution, Methacrylate was added slowly to the solution in droplets with (w/v) ratio 1:2. After 4 hours of incubation at 40 °C, dilution of solution was made with (v/v) ratio 1:5 with 1x PBS. After centrifugation of the diluted solution at 3600 g for 10 minutes, dialysis bag was filled with supernatant.

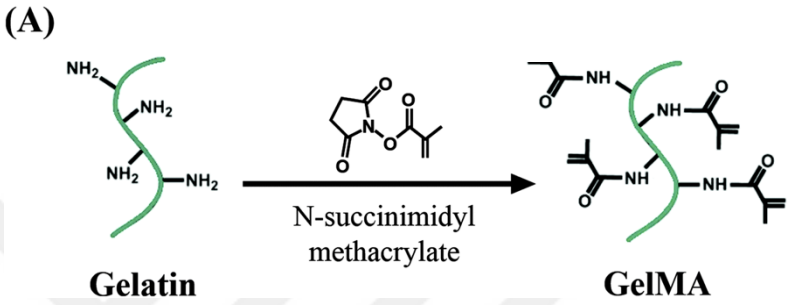


Figure 6. Modification of gelatin with MA groups after 4 hours of incubation at 50 °C [54].

Dialysis was performed in plastic bucket at 40 C° in 5L ultrapure water. This process was continued until there is no MA inside the solution.

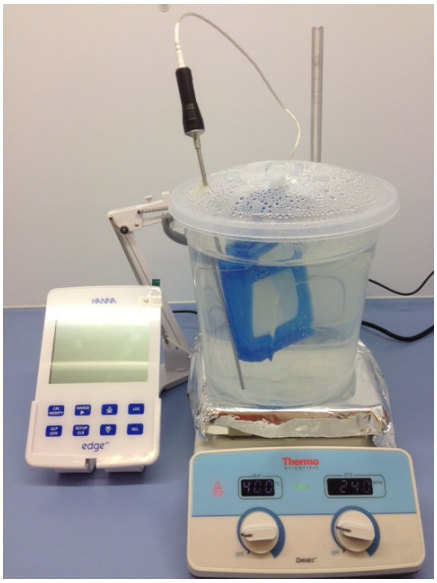


Figure 7. Experimental setup of the dialysis of the unreacted MA, the aluminum foil at the top of the bucket indicator for MA. If the aluminum foil turns blackish or brownish color, there is still

unreacted MA inside the dialysis bag. Dialysis should be done until there is no color change on the aluminum foil.

3.5.3 Fabrication of Microfluidic System

The design of the chip was determined according the parameters for the H filter system, which already described at the introduction part. After designing of the chip using CorelDRAW (licensed software installed with the Epilog Mini60). After cutting of the PMMA pieces were assembled with the help of DSA (Double Side Adhesive Tape). Precise assemble of the chip the stage with reference points was used that prepared with syringe needles and petri dish.

Throughout the study, various microfluidic chips were designed and tested using engineering software in order to provide a platform that resembles the natural environment for differentiation of adipose derived mesenchymal stem cells. Microfluidic chips were designed and fabricated from PMMA sheets and double-sided adhesive tapes, which are cut in high precision with laser cutter and manually assembled.

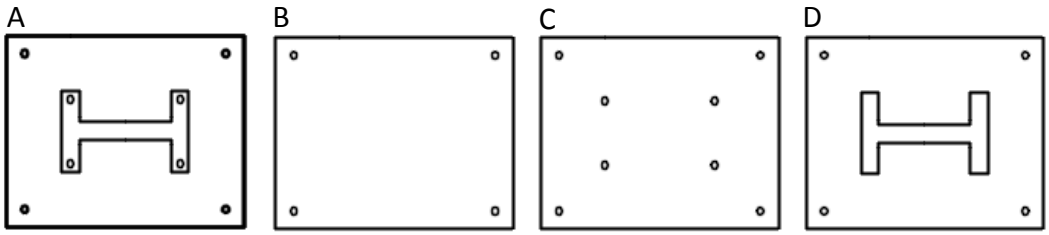


Figure 8. Fabrication of the H- filter device. At the figure A, the assembled version of the overall system. Figure 3B, bottom of the chip, C indicate the cover of the system and finally D is the most important part that give the system unique properties.

3.2.5 Encapsulate and differentiate MSCs in 3D microfluidic system

3.5.4 Encapsulation of ADMSC for Static 3D Differentiation

Adipose derived mesenchymal stem cell-encapsulated 3D hydrogels were generated using methacrylated gelatin (GelMA) as a prepolymer solution. ADMSCs were resuspended in 5% (w/v) GelMA prepolymer solution containing 0.3% (w/v) 2-hydroxy-1- (4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone photo initiator (Sigma- 480896) with the cell densities of $1x10^6$, $5x10^6$ cells/ml. The mixture of the cell-prepolymer solution were placed between 150 µm-thick spacers and covered with a glass coverslip. The geometry of the hydrogel units were shaped by a photomask with motifs fitting the microchannel geometry, placed on top of the glass coverslip. The prepolymer solution with cells were crosslinked with UV light (1 mW/cm^2) for 45 seconds, and the excess uncrosslinked polymer solution was washed out with 1x PBS. The resulting cell encapsulating 3D hydrogels were cultured in culture media.

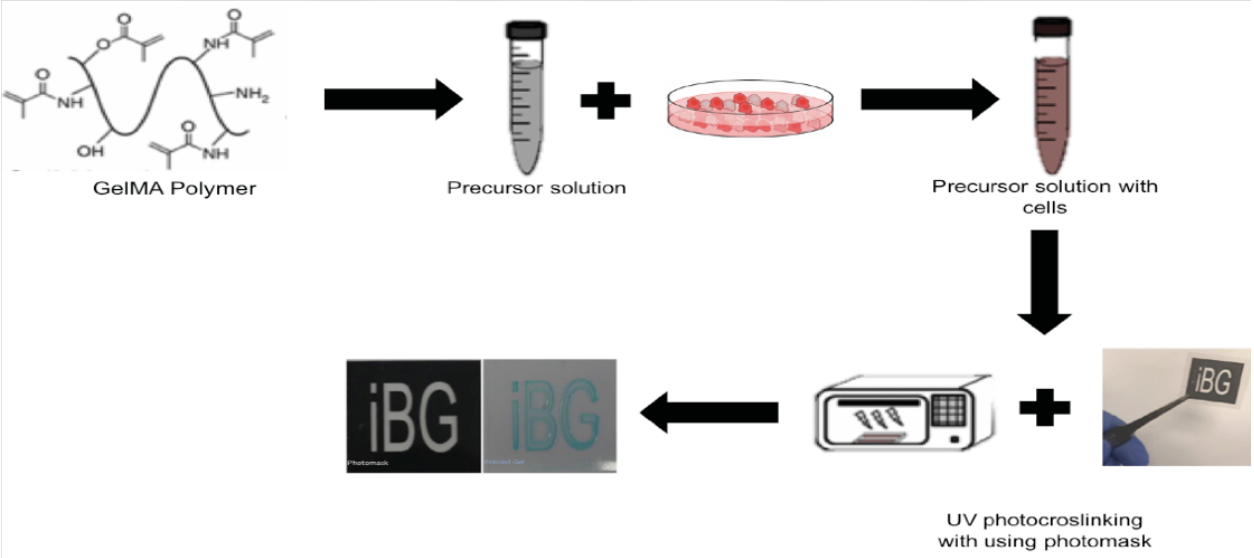


Figure 9. Experimental setup starts with synthesis of the GelMA that is UV-croslinkable gelatin-based polymer, next this polymer is mixed with cells and with the help of photomask cells printed with desired pattern.

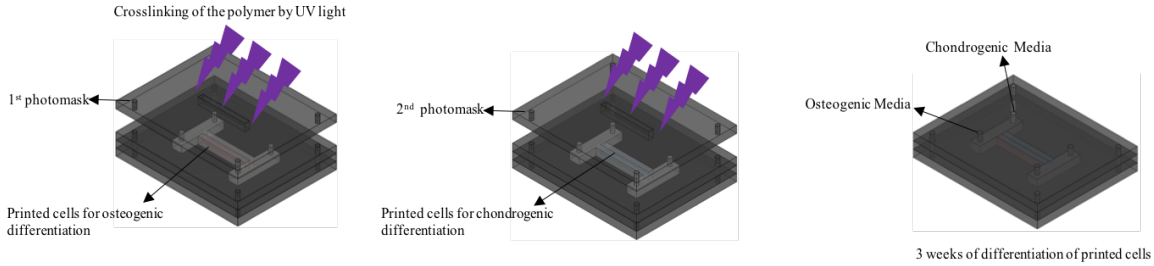


Figure 10. Encapsulation of the ADMS in to a microfluidic channel with help of photomask. The density of the cells is different from each other both osteogenic and chondrogenic compartment.

3.5.5 Encapsulation of the ADMSCs in Microfluidic Platform

ADMSCs were encapsulated in GelMA. These cells were resuspended in 5% (w/v) GelMA prepolymer solution containing 0.3% (w/v) 2-hydroxy-1- (4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone photo initiator with cell densities of $1x10^6$ and $5x10^6$ cells/ml. Next, these precursor solution with density of $1x10^6$ was loaded in the microfluidic system and photomask was replaced with the help of reference points. Precursor solution was polymerized with UV light (1 mW/cm^2) for 45 seconds and the excess uncrosslinked polymer solution were washed out with 1x PBS. After that cell-polymer solution with density of $5x10^6$ was loaded in the microfluidic system. Then again polymerization was done with UV light (1 mW/cm^2) for 45 seconds and the excess uncrosslinked polymer solution were washed out with 1x PBS.

3.5.6 Characterization of generated osteochondral interface

3.5.6.1 Determination of ADSCs by stem cell specific antibodies

Immunofluorescence staining was performed with osteocalcin, osterix, aggrecan, sox2, runx2 and col I antibodies. Cells cultured as $1x10^6$ cell/ml for osteogenic differentiation condition and $5x10^6$ for the chondrogenic differentiation condition on slide were fixed at room temperature with 4% paraformaldehyde for 40 minutes. After fixation, the sample was washed 3 times for 5 minutes with 1x PBS, then blocking and permeabilization (blocking solution, 1x PBS, 1% BSA, 0.3% TritonX100) was performed for 2 hours. After blocking and

permeabilization, the cells were treated with primary antibodies (anti-rabbit osterix 1: 500, anti-mouse aggrecan 1: 100, anti-mouse osteocalcin 1: 100, anti-rabbit runx2 1: 500) for 1 hour. After the incubation period was completed, the cells were washed 2 times with blocking buffer for 10 min and then incubated with secondary antibodies (anti-rabbit Alexa 488 1: 1000, anti-mouse 555 1: 1000) for 2 hours. At the end of the incubation, DAPI was added to stain the nuclei.

3.5.6.2 Quantitative PCR (qPCR)

3.5.6.2.1 RNA isolation

Differentiated cells were washed with 1x PBS three times on ice. After that QIAzol (Qiagen-79306) were used in order to lyse the cells. Further cells were collected with pipetting then 1:5 volume of chloroform was added. Next, cells were centrifuged at 12.000 g for 20 min. After centrifugation there should be three layers on the 2 ml tube. On top there is RNA, in the middle DNA and finally at the bottom there are proteins, lipids and cells debris.

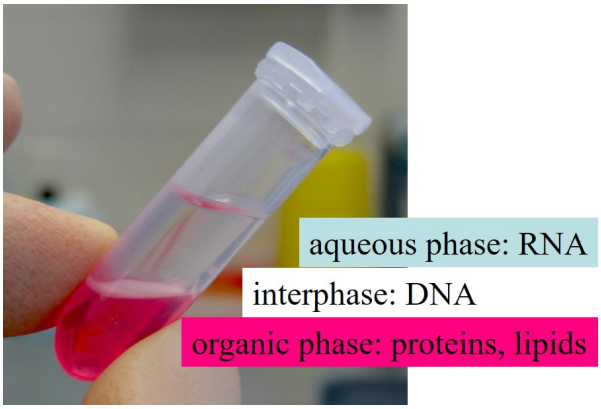


Figure 11. This figure illustrates the aqueous phase of centrifuged sample, there are three different layers can be observed. At the top of these layers clear phase contains RNA and this is our phase of interest. In the middle of these layers there is clear white phase that contains DNA, it is an indicator, if there is thick layer of DNA it means that your sample contains more RNA. As a

final, there is a pink layer at the bottom of the tube, and this layer contains protein, lipids and cell debris.

After the centrifuge to clear phase of the solution where RNA is present was collected to another collection tube, and 1.5X volume of collected phase, was mixed with EtOH. Then the mixture was loaded to the NucleoSpin Filter (Light Blue Ring). After this part, protocol provided by the manufacturer of the RNA isolation kit (Macherey/Nagel- NucleoSpin RNA isolation) was followed. The loaded sample centrifuged at 11.000 g for 30 seconds. Next collection tube was discarded then 350 µl membrane desalting buffer was added at the middle of the membrane then again centrifuged 11.000 g for 1 minute. Meanwhile, DNAase reaction mixture was prepared with 10 µm rDNAase and rDNA reaction buffer. After centrifugation 100 µl mixture was placed again at the middle of the membrane. After 15 minutes of incubation, the RNA was eluted with 60 µl RNAase free water. After the isolation the amount and purity were measured with NanoDrop.

3.5.6.2.2 cDNA Synthesis

cDNA synthesis of isolated RNA was performed by using ABM OneScript, firstly isolated RNAs and other components of the kit thawed on the ice. The reaction mixture that contains RNA, oligo dT, random primer, dNTP mix was added into tube then denaturation of the RNA was done at 65 °C for 5 min. After the incubation, mixture was put on ice about for 1 min. Then the following reagents were added to mixture, 5X RT buffer, RNAaseOFF Ribonuclease Inhibitor, OneScript Plus RTase. Then components were mixed and the synthesis of cDNA started at 25 °C for 10 min, then 50 °C for 50 minutes, and finally reaction end with 85 °C. After the reaction completed the samples were storaged at -20 °C.

3.5.6.3 Histological Staining

3.5.6.3.1 Alizerin Red Staining

The differentiation media was aspirated carefully from each well. Differentiated cells were fixed with iced cold % 70 ethanol for 1 hour at room temperature (RT). After fixation of the cells with ethanol, each well was washed with water 5-10 times. After washing, alizerin red was added enough to cover of each well. The plate was incubated about 30 minutes at RT. After that, stained wells were washed with 1 ml water 5-10 times. Finally, 1x PBS was added to the wells in order not to dry to the wells. The plate is imaged and it can be stored +4 °C for 6-8 months.

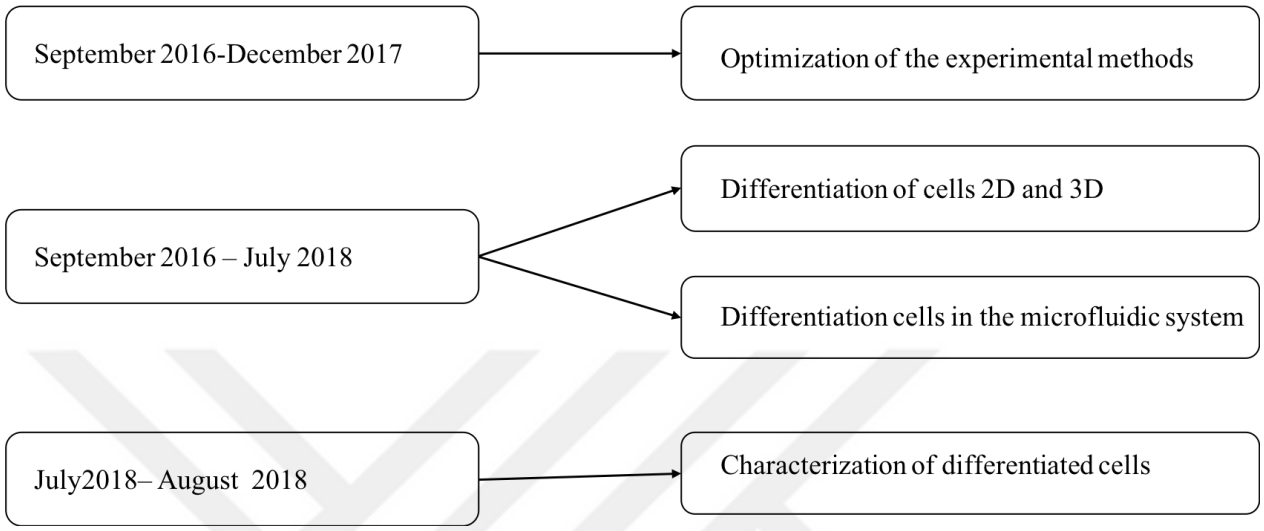
3.5.6.3.2 Alcian Blue Staining

After three weeks of chondrogenic differentiation of the ADMSCs, chondrogenic media was removed from the wells and washed with 1x PBS 5-10 times. Fixation was done % 4 PFA solution about 1 hour. After fixation, each well was washed with 1x PBS. After washing of the wells with 1x PBS, each well was stained with Alcian Blue Solution (%1 Alcian Blue Powder, 0,1 M HCl) for 30 minutes. Then, stained wells were washed with 0,1 M HCl solution 3 times, and finally neutralized with water. Blue staining indicates synthesis of proteoglycans by chondrocytes.

3.5.6.6.3 Oil-o-Red Staining

After three weeks of adipogenic differentiation of the ADMSCs, adipogenic media was removed from the wells and washed with 1x PBS 5-10 times. Fixation was done % 4 PFA solution about 1 hour. After fixation, samples were washed with 1x PBS. After washing of the wells with 1x PBS, each well was stained with Oil-o-Red (Sigma Aldrich) for 30 minutes. Then, stained wells were washed with 1x PBS solution 3 times. Red staining indicates the oil droplets secretes from the adipocytes. Stained samples can be stored at +4 °C in 1x PBS.

3.6 Research Plan



3.7 Analysis of the DATA

Analyzes of the research results were made using the GraphPad Prism 7.0 program. Data were given as mean ± standard error. The Mann-Whitney U test was used to compare groups. Significance level was accepted as $p < 0.05$ in all evaluations.

3.8 Limitation of the Research

If the dialysis of synthesized UV- photocroslinkable polymer GelMA is not done properly cells may die due to existence of metharylic anhydrate.. microfluidic system can leak media during long (21 days) differentiation time due to the change of adhesive properties of the tape inside incubator.

3.9 Ethical Committee Approval

There is no need for ethical committee approval for this study.

4. Results

4.1 Fabrication Microfluidic Platform

4.1.1 Validation of the Microfluidic System with Food Dyes

PMMA components of the microfluidic chips were assembled with double side adhesive tape and tested with food dye for leakage and performance before fitting the tubings. Cole-Parmer Tygon (AAD0291-CP) tubing was used for the controlled continuous flow, which has been fixed with BISON-5 minutes epoxy to the inlets and outlets of the micro channels. After preparing the chip, H-filter has been tested first at high flow rate (100 µl/min), and later with lower flow rate (2 µl/min). The performance of the designed system has been demonstrated in Figure 12 where red and blue colored PBS separately has been administrated at same flow rate and are not mixed in the same channel.

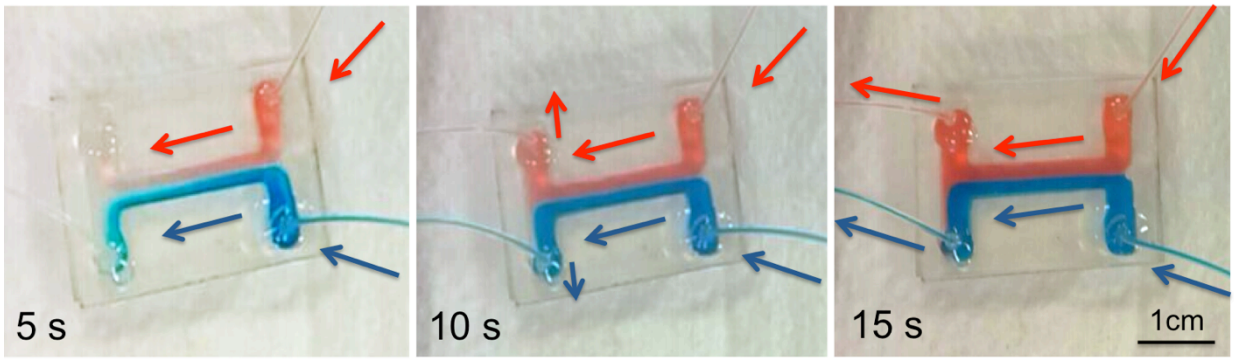


Figure 12. These time lapsed photos show that the validation of the H-filter system with food dye. Red and blue food dye were used to validate the chip if there is any leakage from the system and the interface between the layers.

4.1.2 Validation of the Microfluidic System with NIH 3T3 cells

After validation and leakage checks with the food dye, the system was test with NIH 3T3 cells, before using stem cells. NIH 3T3 cells were seeded as monolayer at density of 50,000

cells/cm^2. After completely attaching of the cells to the surface of the chip, system was set and flow rate start with 100 µl /min and after the channels were filled decreased to 2 µl /minute with serum free media versus complete media. After 2 days of incubation of the chip inside the incubator, H- filter was removed and photos were taken. There are almost no cells on the side of serum free media. On the other hand, the side of complete media the cells look healthy and their typical fibroblastic morphology.

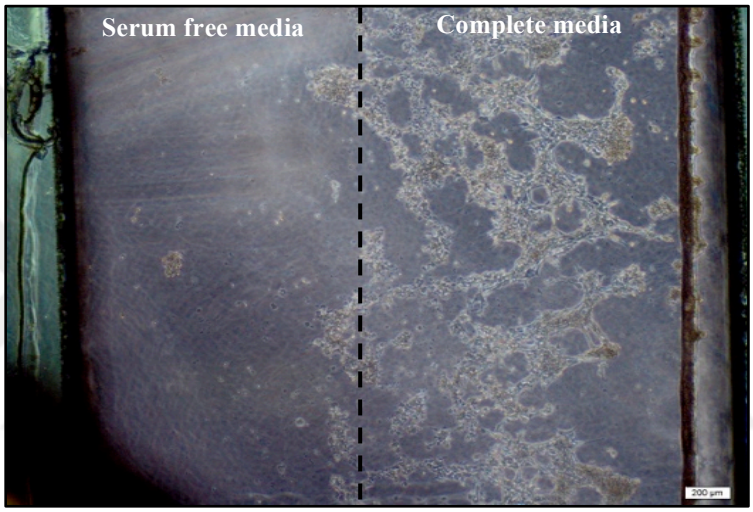


Figure 13. This phase contrast image shows us the cells grow side of the media that have FBS, in contrast that the serum free media part of the chip almost empty after seven days of continuous flow.

4.2 Evaluation of Differentiation of ADMSC in 2D

ADMSC were seeded on six well petri dish and 2 days of interval the media was changed without completely aspirate. After 3 weeks of differentiation of the cells, each cell type was stained with their specific histological dyes. On the osteogenic part calcium deposits of cells were stained with alizerin red. On the other hand, chondrogenic ECM was stained with alcian blue and finally, oil droplets were stained with oil-o-red at adipogenic differentiation (Figure 14).

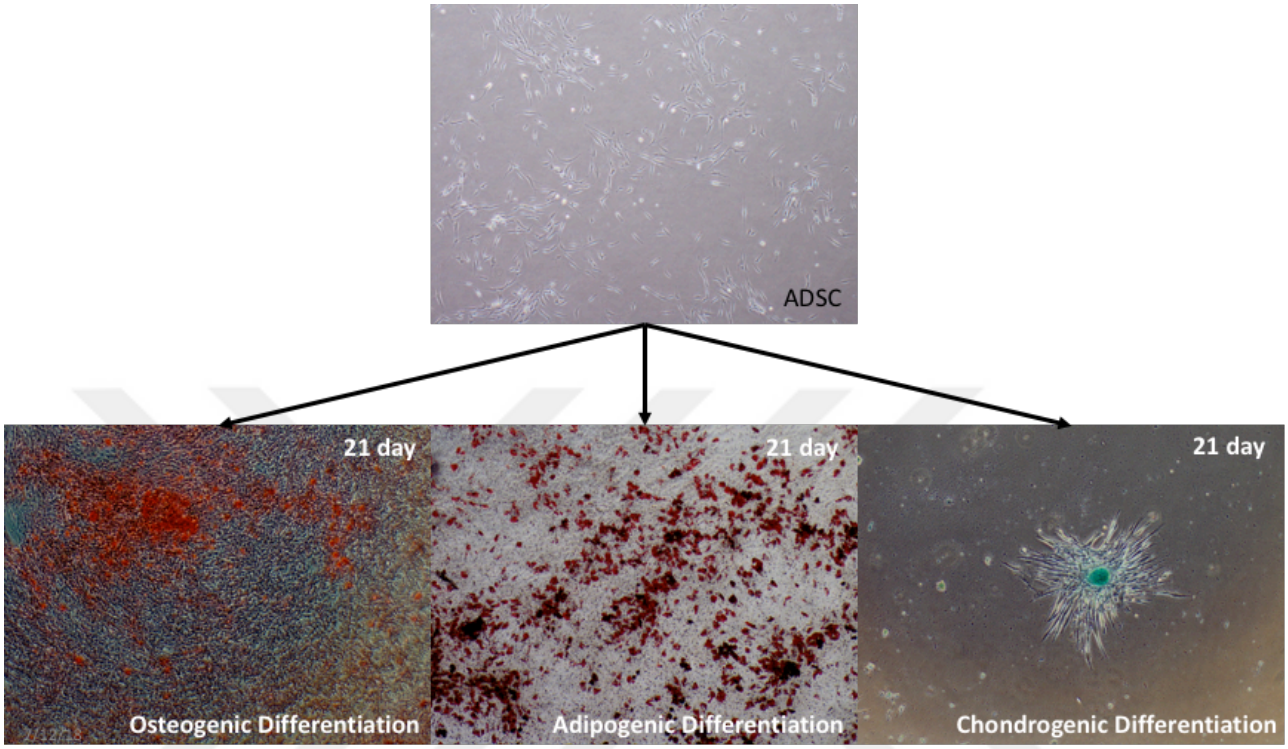


Figure 14. The figure above indicates that the twenty-one days of differentiation of the ADMS in 2D. After differentiation, calcified matrix stained with alizarin red for osteogenic differentiation. Oil droplets were stained with oil-o-red solution for adipogenic differentiation. As a final, GAGs were stained with alcian blue for chondrogenic differentiation.

4.4 Encapsulation and Differentiation of ADMSC

After three weeks of differentiation of ADMSCs, the difference between different differentiation groups was clearly observed. On the chondrogenic cells if the cells inside the hydrogel looks more spherical shape, the cells outside of the cells look like more fibroblastic morphology. In contrast to chondrogenic differentiation at the osteogenic part, the cells looked like more fibroblastic, star like shape and they connected like neural cells.

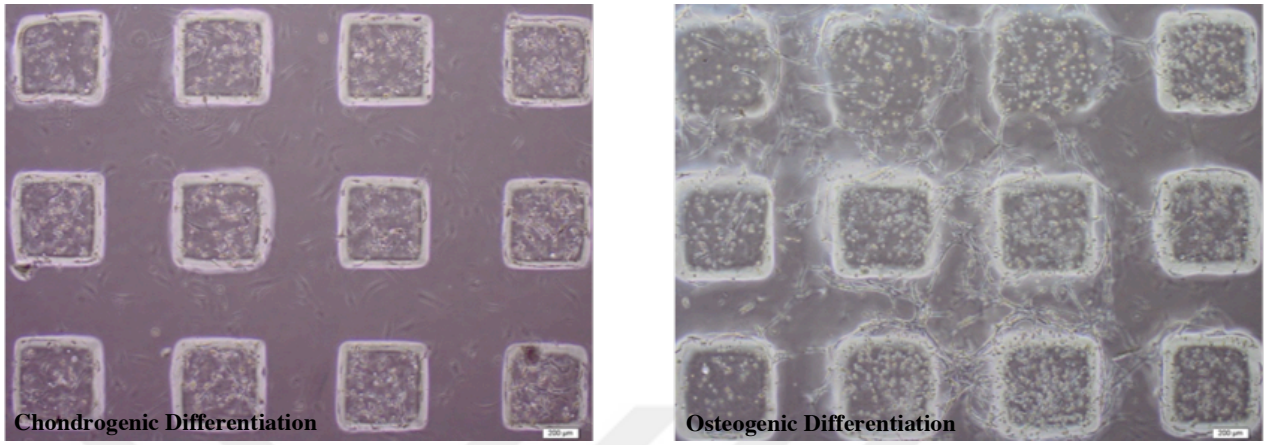


Figure 15. Differentiation of the ADMS after 3 weeks in 3D.

4.5 Printing of the ADMSC Inside Microfluidic system

With the help of UV cross-linkable hydrogel GelMA, ADMSC were printed inside the microfluidic chip. The density of the cells at the chondrogenic compartment were higher than the the osteogenic compartmentt. It can be clearly seen from the dense signal of the AF555 (red) on the chondrogenic compartment and less signal from the osteogenic part from DAPI (blue).

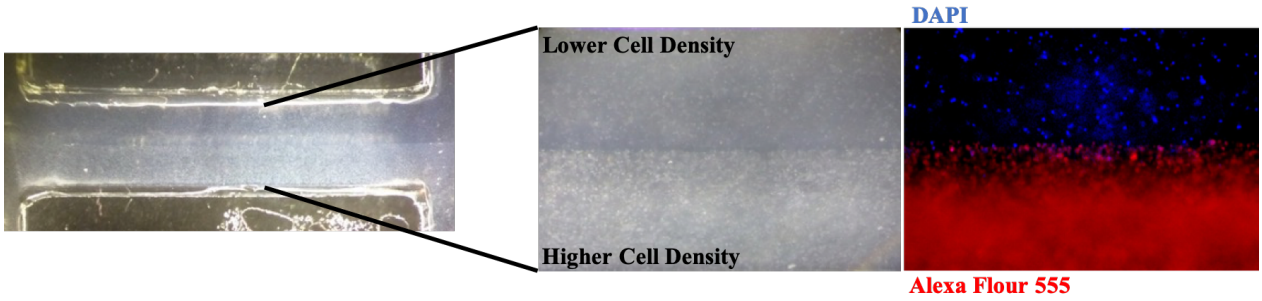


Figure 16. This figure shows that 2 steps of printing of ADMS with different density. There is no mixture of the cells during the printing. It can be clearly observed that there are no DAPI labeled (blue) cells in Alexa Flour 555 (red) labeled side.

4.6 Differentiation of the ADMSC In Microfluidic System

After successfully encapsulation of the cells in the microfluidic channel, system was run at 2 µl/min flow rate for 3 weeks. Osteogenic and chondrogenic cells can be easily distinguished from each other through their very distinct morphology. On the chondrogenic parts the cells look like more spherical shape. On the other hand, the cells, supplemented with osteogenic media looks like more fibroblastic like shape..

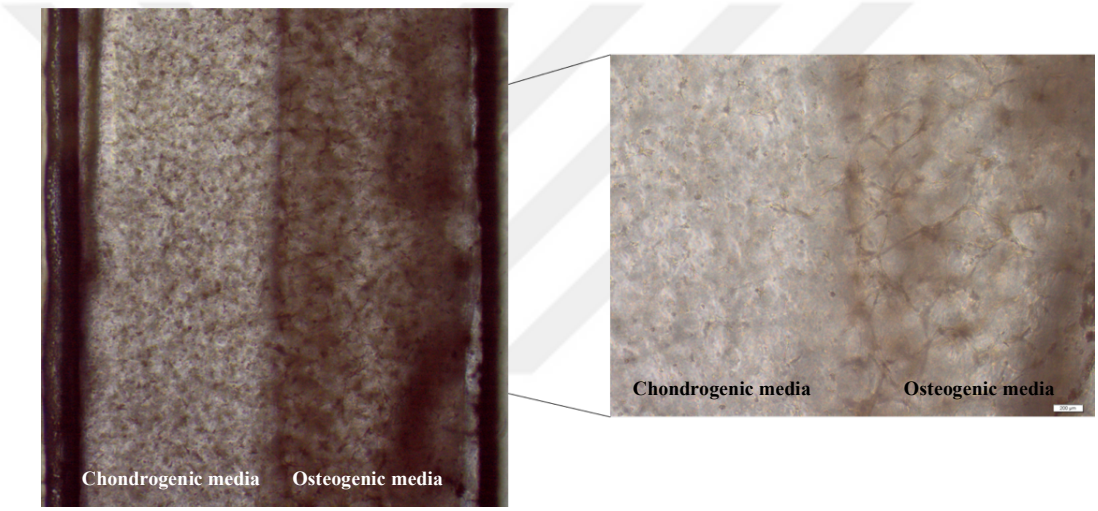


Figure 17. Differentiation of ADMS to osteocyte and chondrocyte under continuous flow in the same channel without any separating compartment.

4.7 Characterization of the 3D Differentiated Cells

4.7.1 Relative gene expressions of differentiated cells

The three weeks chondrogenic and osteogenic differentiation and the formation of bone-cartilage interface tissue has been characterized by immunohistochemistry and validated at the gene expression level. According to this, for cartilage differentiation, Sox9, aggrecan and collagen type 1 genes were compared with bone differentiated osteoblasts. It can be clearly seen that chondrogenic differentiation evolves.

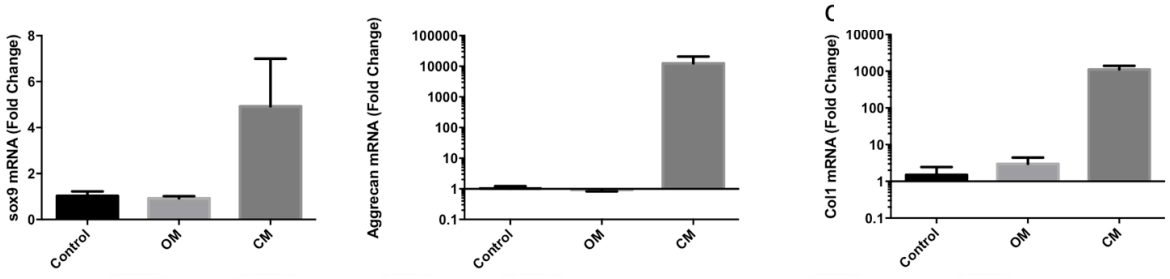


Figure 18. The graphs show us the qPCR result of the ADMS in 3D without flow under chondrogenic media (CM) and osteogenic media (OM) for chondrogenic markers.

Similarly, osteogenic cells obtained as a result of differentiation of bone tissue in 3D structures were compared with chondrogenic cells osterix, osteocalcin, alkaline phosphatase (ALP) and bone sialoprotein (BSP) gene expressions after 3 weeks culture. Accordingly, it was observed that osteogenic differentiation was very clear.

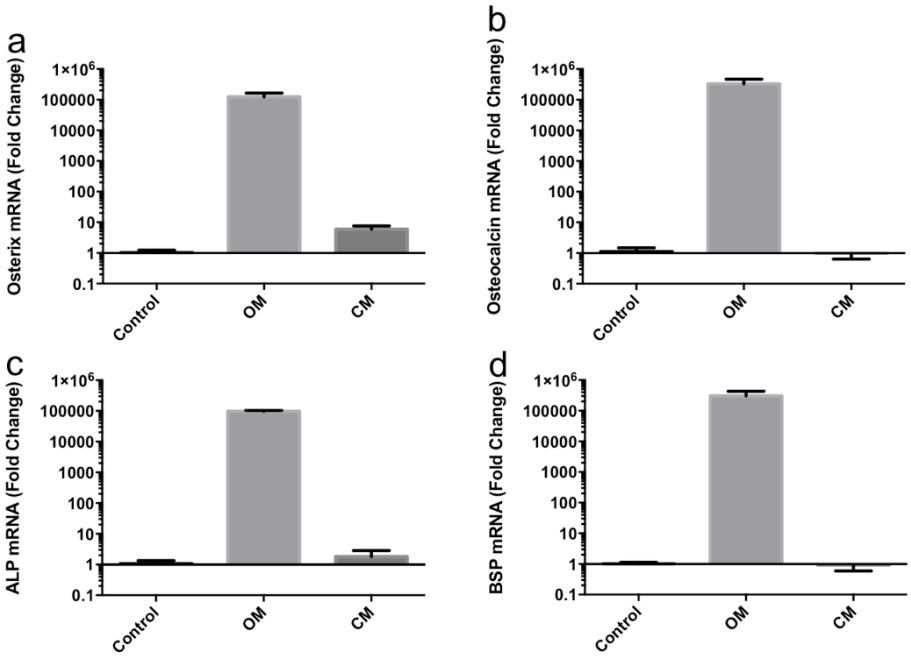


Figure 19. The graphs show us the qPCR result of the ADMS in 3D without flow under chondrogenic media (CM) and osteogenic media (OM) for osteogenic markers.

4.7.2 Relative gene expressions of differentiated cells under the flow

After three weeks chondrogenic and osteogenic differentiation of the bone-cartilage interface tissue characterized by immunohistochemistry was also determined at the gene expression level. This time the experiments were done under 2 µl/min flow. According to this, for cartilage differentiation, Sox9, aggrecan and collagen type 1 genes were compared with bone differentiated osteoblasts.

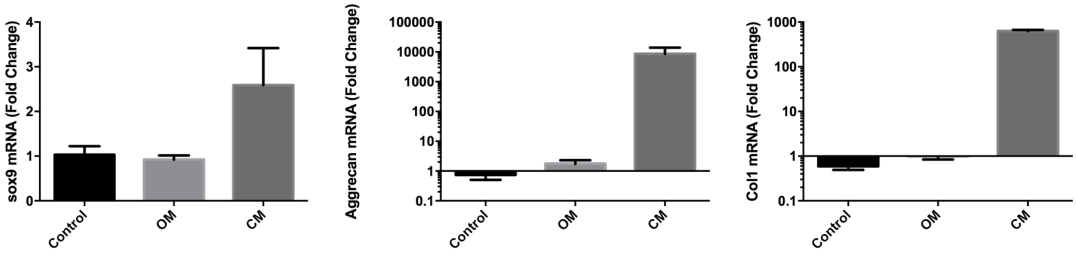


Figure 20. The graphs show us qPCR result of the ADMS in 3D with flow under chondrogenic media (CM) and Osteogenic media (OM) for chondrogenic markers.

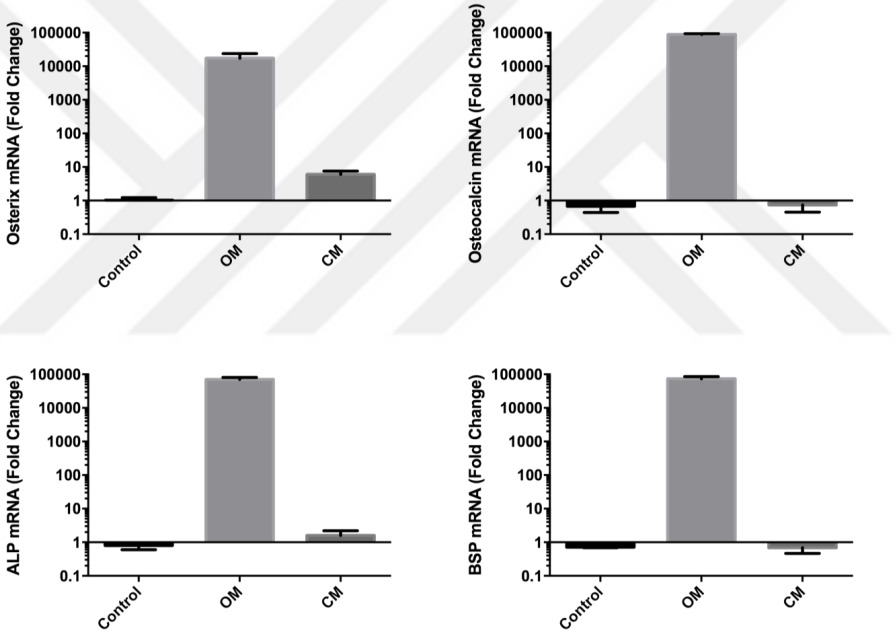


Figure 21. The graphs show us the qPCR result of the ADMS in 3D with flow under chondrogenic media (CM) and Osteogenic media (OM) for osteogenic markers.

4.7.2 Characterization of osteogenicly differentiated cells in 3D

The developed bone tissue was characterized in detail by early and late osteogenic markers. Accordingly, the most commonly used markers in bone differentiation were included in the study. 3D structures were stained immunohistochemically using osterix, Runx2 and osteocalcin, and examined under confocal microscope (Figure 22).

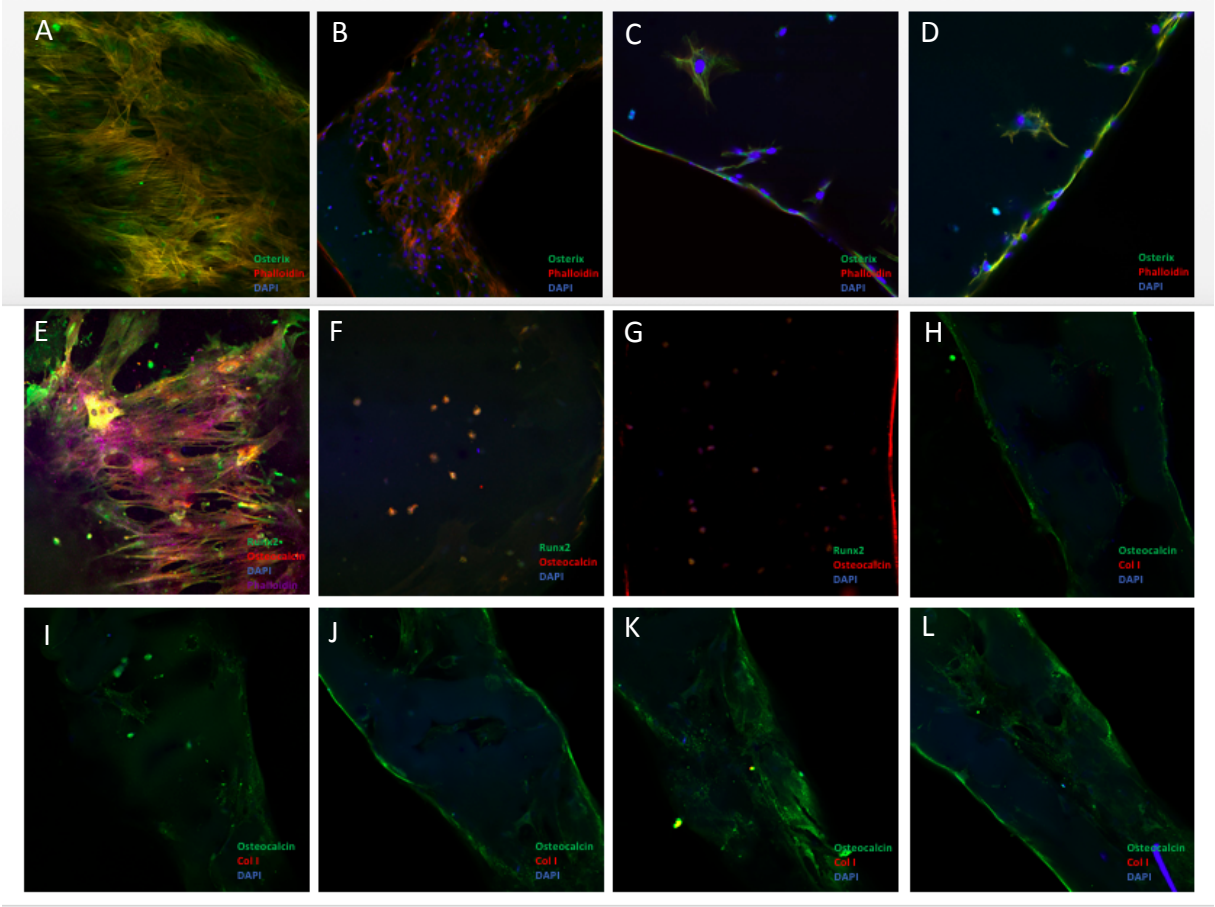


Figure 22. Osteogenic differentiation of the ADMS were characterized with Osterix (green), Phalloidin (red), DAPI (blue) (Figure 20 A, B, C, D) runx2 (green), osteocalcin (red), DAPI (blue) (E, F, H), osteocalcin (green), collagen type I (red), DAPI (blue) (I, J, K, L, M)

4.7.3 Characterization of chondrogenicly differentiated cells in 3D

At the next stage, the bone and cartilaginous regions were characterized seperately in more detail to prevent cross-reactivity of the antibodies. In the chondrogenic analyzes performed, the stem cells in the 3D structure do not overlap osteocalcin, an osteogenic marker, as expected while SOX2 replicates collagen-1 and the contractor proteins (Figure 23).

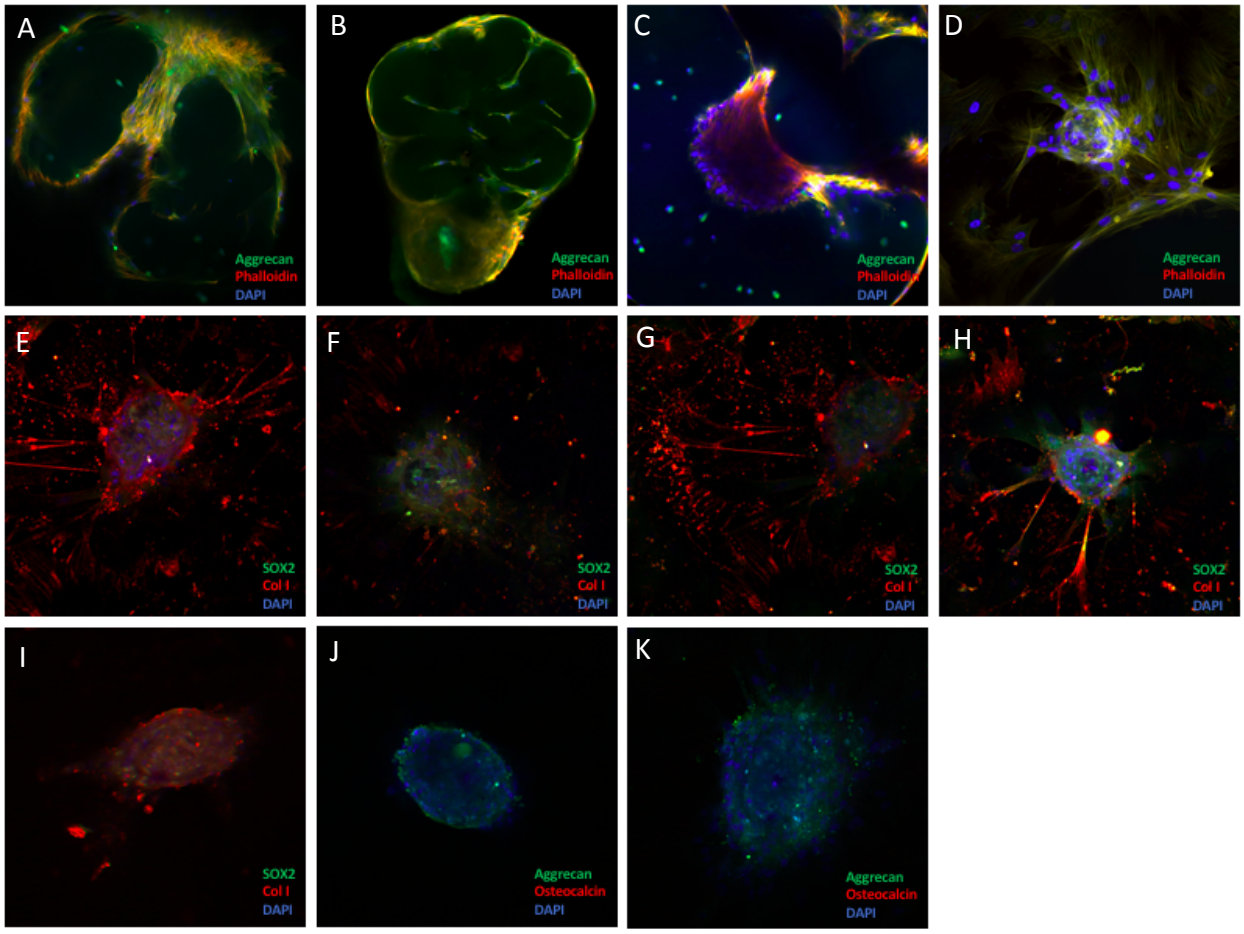


Figure 23. Osteogenic differentiation of the ADMS were characterized with Aggrecan (green), Phalloidin (red), DAPI (blue) (A,B,C,D), sox2 (green), collagen I (red), DAPI (E, F, H, I) aggrecan (green), osteocalcin (red), DAPI (J, K, L)

4.7.4 Histological analysis of generated tissues

The most common method used to determine glycosaminoglycans in the cartilaginous tissue is histochemistry with alcian blue staining (Figure 24 A). The osteogenic matrix secreted by differentiated cells is examined with hematoxylin and eosin staining (Figure 24 B). Analyzes have confirmed that the differentiation of cells to bone tissue has been successfully accomplished.

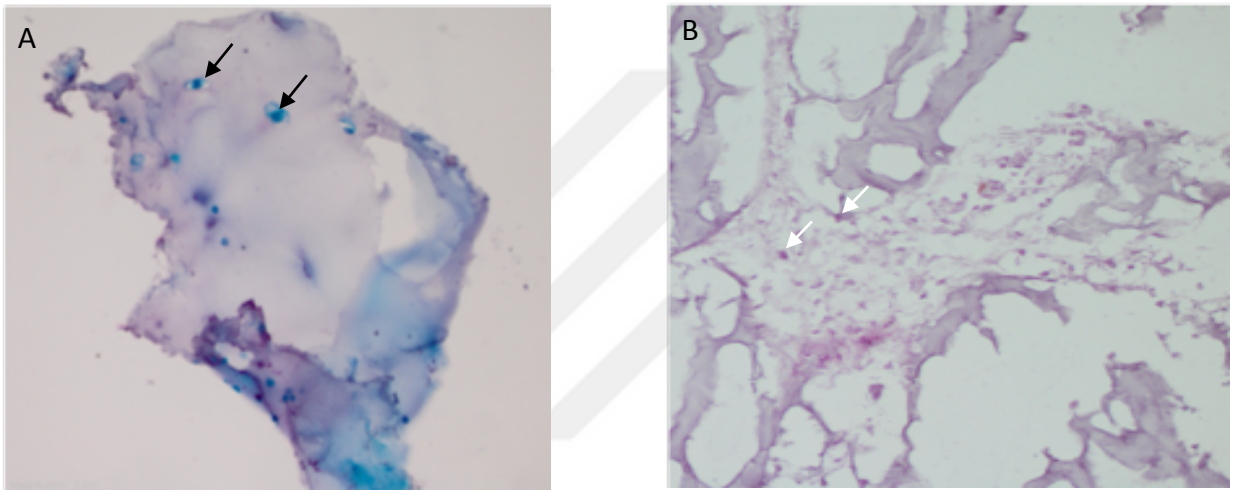


Figure 24. These figure shows that cryosectioning of the 3 weeks differentiation of the ADMS in GelMA. At the first figure sample stained with alcian blue and the black arrows show that chondrocyte. In the second picture the sample stained with alizarin red white arrows show us the osteocytes.

4.8 Characterization of Osteochondral Interface

The 3D structures and mesenchymal stem cells biocompatible in the improved microfluidic system were subjected to both cartilage and bone differentiation for three weeks at the same time, and then they were characterized for immunohistochemical and gene expression. According to this, half of the system is expected to be osteogenic differentiated and the other half to chondrogenic tissue. One of the most important functional markers of cartilage differentiation

is the accumulation of aggregates of chondrogenic cells and the accumulation of matrices. Aggrecan is a proteoglycan, also called cartilage-specific proteoglycan core protein (CSPCP) or chondroitin sulfate proteoglycan 1. The presence of consent is an indication that the mesenchymal stem cells perform chondrogenic differentiation functionally (Figure 25 A). Immunohistochemical staining with aggrecan-specific antibody has been observed to successfully perform cartilage differentiation of the biocompatible cartilage area. The effect of osteogenic differentiation on the engineered tissue was determined by osteocalcin staining specific to bone tissue (Figure 25 B). Bone osteocalcin, also known as gamma-carboxy glutamic acid-containing protein (BGLAP), is secreted by osteoblasts. The presence of osteocalcin is a sign that the mechanism of bone formation works and that calcium ion homeostasis is safe. The regionally correct distribution of aggrecan and osteocalcin staining is the most important indication that the bone-cartilage interface we have developed in the microfluidic system is successful (Figure 25 C).

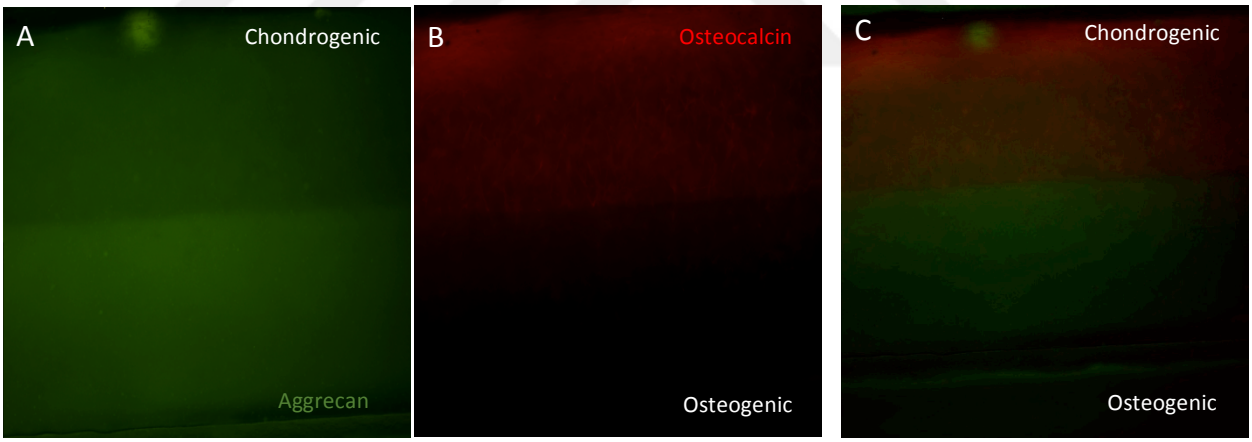


Figure 25. This final figure shows that our overall experimental results ADMS were successfully printed in the microfluidic channel and 3 weeks of differentiation has been accomplished. After differentiation of the cells, generated tissues were characterized with osterix (red) for bone and aggrecan (green) for cartilage matrix.

5. Discussion

The bone-cartilage interface is one of the most affected areas of bone and skeletal diseases. Although this region is complex in terms of cell type, the difference between the bone and cartilaginous tissue matrices strengthens systematic studies. Today, although studies on animal models are quite extensive, studies on simulating human physiology in *in vitro* conditions are limited. The aim of this thesis was to model bone-cartilage tissue in microfluidic chips using a tissue engineering approach. In one study, authors construct the osteochondral interface with 3D printer in order to recover the osteochondral defects [55]. Since nutrient and gas exchange cannot be done in large-scale tissue engineering structures, necrotic cores occur in the interior and functionality is not at the desired level. On the other hand, many *in vitro* studies are performed under in static conditions and are deprived of dynamic stimuli in real tissues. This thesis work addresses such problems because of the dynamic cell culture environment provided by microfluidic chips. Both cartilage and bone differentiation have been achieved at the same time using the principle that the laminar flow, one of the basic principles of the microfluidic physiology, can move more than one liquid in the same channel without mixing. The improved bone-cartilage interface examines effects of dynamic conditions on bone and cartilage differentiation of stem cells, as well as benefits of 3D cell culture, as well as controlled microfluidic technology. During this experimental progress, firstly we optimized the assembly technique, it started with the standard double side adhesive tape that has size with 5 cm diameter, and this tape is really hard to manipulate. Because of its size, it cannot be used in laser cutter. Even if, it used chips were leaky. In addition to size problem this tapes also has plastic carrier and this also cause a thickness for the channel. It is also causing the leakage of the liquid from the chip. After achieving this leaking problem, to the chip now we face with the leaking of the liquid from inlet and outlet part. In order to overcome this problem, different epoxy types were used. The results were the same. After that we made some fittings for the connection of the tubing to the chips and again result was the same. Then finally, we made two layers of epoxy the second one put after one is completely polymerized. Then we synthesize our GelMA and its final step was dialysis of the polymer, however we realize that our detection of methacrylic anhydrite (MA) gave as false positive, because even 6 weeks later, the aluminum foil still turned in to brownish or

black color. It might be we used plastic bucket, and the MA might bind to the surface of the plastic after realize that we wash with detergent each time we change water, and the problem was solved. In addition to these, we know that MA make the environment acidic, we also can detect the MA in water by using pH meter. If it is nearly 7, it can be taught there is no MA inside the water. After successfully, dialysis of the GelMA, now we encapsulate the cells however, concentration of PI is important here. More than 1% has toxic effect for the cells. Even it looks like it works after 5 days of encapsulation cell did not get their fibroblastic morphology. Another key point about the polymerization of GelMA, if you try to polymerization at the top of PMMA you probably fail, because how much you increase the power UV – light it not affected all. PMMA that we used probably scatter the light that is the reason why GelMA did not polymerize properly. The problem solved with giving the light at the bottom of the chip that is made of polystyrene that is standard material used in petri dish, well plate and flasks. After twenty-one days of differentiation of the cells with different induction media, they were characterized with the specific marker genes and confocal microscope used for the imaging of the sample. Because of long acquisition times, samples were drying out. That is why we add drop of 1x PBS on the samples before taking pictures. 1x PBS should not be added during the photo taking because hydrogel might swell because of the 1x PBS. After all of these, ADMSC were successfully differentiated into osteogenic and chondrogenic linage in the same channel with controlled microfluid technology.

6. Conclusion and Future Aspects

In this study, we demonstrate a microfluidic platform for two differentiation media in a single channel for osteogenic and chondrogenic differentiation of mesenchymal stem cells simultaneously. After differentiation of the cells, specific early and late marker genes like osterix, osteocalcin, aggrecan, col I, sox2, runx2 were checked with fluorescence and their localization was detected. After observed of the location of the proteins expression level of these proteins measured with qPCR with the same genes. As we expected ALP, BSP, osterix and osteocalcin highly expressed in osteogenicly differentiated cell. On the other hand, aggrecan, col I and sox9 were highly expressed in chondrogenicly differentiated cells. Such bioengineered platform can be used to mimic the bone-cartilage interface and potentially utilized in pre-clinical tests of therapeutics. In order to increase the efficiently of chondrogenic differentiation of the system, hypoxic media can be used at the chondrogenic part. For hypoxia, copper sulfate can be used as agent, after that HIF-1α should check if there is any hypoxic respond from the cells, it should increase the chondrogenic differentiation rate of the microfluidic system. Another future study about the system, immune cells like T cells, and B cells can be added to the culture system. Because we construct this osteochondral interface in order to mimic a disease model Rheumatoid arthritis (RA), which is an autoimmune disease that caused by attacking the immune cells in joint and this inflammation cause the swelling of the joints. In this study, such problems like lack of nutrient and gas exchange were solved help of the dynamic cell culture environment provided by microfluidic chips. Both cartilage and bone differentiation have been done simultaneously using the principle that the laminar flow, one of the basic principles of the microfluidic physiology, can move more than one liquid in the same channel without mixing. Bone-cartilage interface are examined the effects of dynamic conditions on bone / cartilage differentiation of stem cells, as well as benefits of 3D cell culture, as well as, controlled *in vitro* microenvironment.

As a future study this tissue construct can be modified with the HUVEC (human umbilical vein/vascular endothelial cells) in order to mimic the blood vessel formation for the osteogenic part. It is also known that Wnt signaling important for osteogenic differentiation,

knocking out this pathway cause abnormalities in the bone formation with help of this interface model importance of the Wnt pathway can be understand.

7. References

1. Griffith, L.G. and G. Naughton, *Tissue engineering--current challenges and expanding opportunities.* Science, 2002. **295**(5557): p. 1009-14.
2. Langer, R. and J.P. Vacanti, *Tissue Engineering.* Science, 1993. **260**(5110): p. 920-926.
3. Tabata, Y., *Biomaterial technology for tissue engineering applications.* J R Soc Interface, 2009. **6 Suppl 3**: p. S311-24.
4. Yi, H., et al., *Recent advances in nano scaffolds for bone repair.* Bone Res, 2016. **4**: p. 16050.
5. Lutolf, M.P. and J.A. Hubbell, *Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering.* Nat Biotechnol, 2005. **23**(1): p. 47-55.
6. O'Brien, F.J., *Biomaterials & scaffolds for tissue engineering.* Materials Today, 2011. **14**(3): p. 88-95.
7. Holzapfel, B.M., et al., *How smart do biomaterials need to be? A translational science and clinical point of view.* Adv Drug Deliv Rev, 2013. **65**(4): p. 581-603.
8. Adelöw, C., et al., *The effect of enzymatically degradable poly(ethylene glycol) hydrogels on smooth muscle cell phenotype.* Biomaterials, 2008. **29**(3): p. 314-326.
9. Park, J. and R.S. Lakes, *Biomaterials: an introduction*. 2007: Springer Science & Business Media.
10. Ahmed, E.M., *Hydrogel: Preparation, characterization, and applications: A review.* Journal of Advanced Research, 2015. **6**(2): p. 105-121.
11. Guoqing, P., et al., *Thermo-Responsive Hydrogel Layers Imprinted with RGDS Peptide: A System for Harvesting Cell Sheets.* Angewandte Chemie International Edition, 2013. **52**(27): p. 6907-6911.

12. Milašinović, N., et al., *Hydrogels of N-isopropylacrylamide copolymers with controlled release of a model protein.* International Journal of Pharmaceutics, 2010. **383**(1): p. 53-61.
13. Getgood, A., et al., *Articular cartilage tissue engineering: today's research, tomorrow's practice?* J Bone Joint Surg Br, 2009. **91**(5): p. 565-76.
14. Matthews, B.G., et al., *Enhanced osteoblastogenesis in three-dimensional collagen gels.* Bonekey Rep, 2014. **3**: p. 560.
15. Liu, L.-S., et al., *An osteoconductive collagen/hyaluronate matrix for bone regeneration.* Biomaterials, 1999. **20**(12): p. 1097-1108.
16. Park, Y.D., N. Tirelli, and J.A. Hubbell, *Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks.* Biomaterials, 2003. **24**(6): p. 893-900.
17. Bae, M.S., et al., *Photo-cured hyaluronic acid-based hydrogels containing growth and differentiation factor 5 (GDF-5) for bone tissue regeneration.* Bone, 2014. **59**: p. 189-98.
18. Morozzo, U., et al., *Soft Tissue Reconstructions with Dermal Substitutes Versus Alternative Approaches in Patients with Traumatic Complex Wounds.* Indian J Surg, 2015. **77**(Suppl 3): p. 1180-6.
19. Chan, B.P. and K.W. Leong, *Scaffolding in tissue engineering: general approaches and tissue-specific considerations.* Eur Spine J, 2008. **17 Suppl 4**: p. 467-79.
20. Shteyer, E., et al., *Reduced liver cell death using an alginate scaffold bandage: a novel approach for liver reconstruction after extended partial hepatectomy.* Acta Biomater, 2014. **10**(7): p. 3209-16.
21. Tsur-Gang, O., et al., *The effects of peptide-based modification of alginate on left ventricular remodeling and function after myocardial infarction.* Biomaterials, 2009. **30**(2): p. 189-95.
22. Pok, S., et al., *A multilayered scaffold of a chitosan and gelatin hydrogel supported by a PCL core for cardiac tissue engineering.* Acta Biomaterialia, 2013. **9**(3): p. 5630-5642.
23. Del Gaudio, C., et al., *Induction of angiogenesis using VEGF releasing genipin-crosslinked electrospun gelatin mats.* Biomaterials, 2013. **34**(31): p. 7754-65.

24. Nie, L., et al., *Physicochemical characterization and biocompatibility in vitro of biphasic calcium phosphate/polyvinyl alcohol scaffolds prepared by freeze-drying method for bone tissue engineering applications.* Colloids Surf B Biointerfaces, 2012. **100**: p. 169-76.
25. Zhong, X., et al., *Fabrication of chitosan/poly(epsilon-caprolactone) composite hydrogels for tissue engineering applications.* J Mater Sci Mater Med, 2011. **22**(2): p. 279-88.
26. Jeong, S.I., et al., *Electrospun chitosan-alginate nanofibers with in situ polyelectrolyte complexation for use as tissue engineering scaffolds.* Tissue Eng Part A, 2011. **17**(1-2): p. 59-70.
27. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**: p. 105.
28. I., C.A. and D.J. E., *Mesenchymal stem cells as trophic mediators.* Journal of Cellular Biochemistry, 2006. **98**(5): p. 1076-1084.
29. Farenga, S.J., D. Ness, and M. Hutchinson, *Discussion of Animal Stem Cells in the Classroom.* The American Biology Teacher, 2015. **77**(6): p. 405.
30. Minguell, J.J., A. Erices, and P. Conget, *Mesenchymal Stem Cells.* Experimental Biology and Medicine, 2001. **226**(6): p. 507-520.
31. Bianco, P., *"Mesenchymal" Stem Cells.* Annual Review of Cell and Developmental Biology, 2014. **30**(1): p. 677-704.
32. Quarto, R., et al., *Repair of large bone defects with the use of autologous bone marrow stromal cells.* N Engl J Med, 2001. **344**(5): p. 385-6.
33. Ringe, J. and M. Sittinger, *Tissue engineering in the rheumatic diseases.* Arthritis Res Ther, 2009. **11**(1): p. 211.
34. Krampera, M., et al., *Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells.* Stem Cells, 2006. **24**(2): p. 386-98.
35. Liechty, K.W., et al., *Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep.* Nat Med, 2000. **6**(11): p. 1282-6.
36. Kuroda, R., et al., *Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells.* Osteoarthritis Cartilage, 2007. **15**(2): p. 226-31.

37. Wong, K.L., et al., *Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: a prospective, randomized controlled clinical trial with 2 years' follow-up.* Arthroscopy, 2013. **29**(12): p. 2020-8.
38. Manferdini, C., et al., *Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2.* Arthritis Rheum, 2013. **65**(5): p. 1271-81.
39. Parker, A.M. and A.J. Katz, *Adipose-derived stem cells for the regeneration of damaged tissues.* Expert Opin Biol Ther, 2006. **6**(6): p. 567-78.
40. Koh, Y.G., et al., *Clinical results and second-look arthroscopic findings after treatment with adipose-derived stem cells for knee osteoarthritis.* Knee Surg Sports Traumatol Arthrosc, 2015. **23**(5): p. 1308-16.
41. Filardo, G., et al., *Stem cells in articular cartilage regeneration.* J Orthop Surg Res, 2016. **11**: p. 42.
42. Pittenger, M.F., et al., *Multilineage Potential of Adult Human Mesenchymal Stem Cells.* Science, 1999. **284**(5411): p. 143-147.
43. Bartholomew, A., et al., *Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo.* Experimental Hematology, 2002. **30**(1): p. 42-48.
44. S., i.t.A.P., et al., *Isolation of Mesenchymal Stem Cells of Fetal or Maternal Origin from Human Placenta.* STEM CELLS, 2004. **22**(7): p. 1338-1345.
45. Gilligan, M., *Single cell sequencing: An engineer and business person's perspective on how we got here.* Lab on a Chip, 2017. **17**(20): p. 3349-3350.
46. Bhatia, S.N. and D.E. Ingber, *Microfluidic organs-on-chips.* Nat Biotechnol, 2014. **32**(8): p. 760-72.
47. Ren, K., J. Zhou, and H. Wu, *Materials for microfluidic chip fabrication.* Acc Chem Res, 2013. **46**(11): p. 2396-406.
48. Amin, R., et al., *3D-printed microfluidic devices.* Biofabrication, 2016. **8**(2): p. 022001.
49. Alapan, Y., et al., *Three-Dimensional Printing Based Hybrid Manufacturing of Microfluidic Devices.* J Nanotechnol Eng Med, 2015. **6**(2).

50. Sackmann, E.K., A.L. Fulton, and D.J. Beebe, *The present and future role of microfluidics in biomedical research.* Nature, 2014. **507**(7491): p. 181-9.
51. Andersson, H. and A. van den Berg, *Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities.* Lab Chip, 2004. **4**(2): p. 98-103.
52. Alapan, Y., K. Icoz, and U.A. Gurkan, *Micro- and nanodevices integrated with biomolecular probes.* Biotechnol Adv, 2015. **33**(8): p. 1727-43.
53. Dinleyici, E.C., et al., *Saccharomyces boulardii CNCM I-745 reduces the duration of diarrhoea, length of emergency care and hospital stay in children with acute diarrhoea.* Benef Microbes, 2015. **6**(4): p. 415-21.
54. Lai, T.C., J. Yu, and W.B. Tsai, *Gelatin methacrylate/carboxybetaine methacrylate hydrogels with tunable crosslinking for controlled drug release.* Journal of Materials Chemistry B, 2016. **4**(13): p. 2304-2313.
55. Radhakrishnan, J., et al., *Injectable and 3D Bioprinted Polysaccharide Hydrogels: From Cartilage to Osteochondral Tissue Engineering.* Biomacromolecules, 2017. **18**(1): p. 1-26.

8. Attachments

8.1 Curriculum Vitae

HAMDULLAH YANIK

ID number:	13888510172
Date of birth:	26/05/1992
Contact address:	Kazim Dirik Mahallesi/ Süvari Caddesi no:3/2 Bornova/Izmir
Telephone:	00905354411992
Email address:	yanikhamdullah@gmail.com

EDUCATIONAL BACKGROUND

Country	University	Faculty	Department	Degree	Year of Graduate
Turkey	Dokuz Eylul University	Izmir International Biomedicine and Genome Institute	Molecular Biology and Genetics	MSc	On going
Turkey	Izmir Institute of Technology	Science Faculty	Molecular Biology and Genetics	BSc	01.07.2016

SPECIALTIES

SPECIALTIES
Molecular biology, tissue engineering, microfluidic systems, biomaterials

AWARDS

	Name of the Reward	Organization	Year
	Poster Award	BIOMED	2017

PUBLICATIONS

İ. Erbay,Ali K. Baş, M. Asal, **H. Yanık**, Sinan Güven ,İleri Biyomalzemeler: Biyomalzeme Ve Nanomalzeme Uygulamaları, Bolum 11, 2017

CONFERENCES and ABSTRACT BOOKS

H. Yanık, O.B. Akbulud, A.K. Baş, S. Güven**,** Mikroakışkan Sistemler ile Kemik-Kıkırdak Arayüzü Modellenmesi, 4. Biyomalzeme Günleri, 19-20 September 2017, Yalova

H.Yanik, A.K. Bas, O. Akbulud, S. Guven, Microfluidic Osteochondral Interface, TERMIS-EU 2017, 26-30 Haziran 2017, Davos, İsviçre

H. Yanık, O.B. Akbulud, A.K. Baş, S. Güven, Generation of Bone-Cartilage Interface in Microfluidic System, BIOMED 2017, 12-14 Mayıs 2017, Ankara