EFFECTS OF SMALL MOLECULES ON CARTILAGE TISSUE REGENERATION

by
Tansu Turan Gökçen

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2022

EFFECTS OF SMALL MOLECULES ON CARTILAGE TISSUE REGENERATION

APPROVED BY:

Prof. Dr. Gamze Torun Köse ……
(Thesis Supervisor)
(Yeditepe University)

Assoc. Prof. Dr. Fatih Kocabaş ……
(Yeditepe University)

Assist. Prof. Dr. Ayşe Ceren Çalıkoğlu Koyuncu ……
(Marmara University)

DATE OF APPROVAL: ..../..../2022

I hereby declare that this thesis is my own work and that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I have fully cited and referenced all material and results as required by these rules and conduct, and this thesis study does not contain any plagiarism. If any material used in the thesis requires copyright, the necessary permissions have been obtained. No material from this thesis has been used for the award of another degree.

I accept all kinds of legal liability that may arise in case contrary to these situations.

Name, Last name Tansu, Turan Gökçen

Signature …..…….……..

ACKNOWLEDGEMENTS

First and foremost, I would like to present my sincere gratitude to my thesis advisor Prof. Dr. Gamze Torun Köse for accepting me in her reseach group and gave me the chance of studying cartilage tissue enginerring. I am appreicated her endless support, guidance, patient, kindness and knowledge throughout all my master reseach and thesis writing period. It is an honour for me working with her in my thesis project.

I would like to appriciate to jury members Assoc. Prof. Fatih Kocabaş and Asst. Prof. Dr. Ayşe Ceren Çalıkoğlu Koyuncu for their significant contributions in this study.

I also would like to thank to Asst. Prof. Dr. Onur Cem Namlı for supporting and guding in the mechanical tests of this study.

I also like to thank my labmates in Yeditepe University Tissue Engineering Group (YUTEG): Nisa İrem Büyük, Ahmet Alperen Tuncer, Kardelen Tüfekçi, Didem Aksu, Gülnihal Bozdağ, Saime Bengü Kaptanoğlu and Emine Afra Demirci. I am grateful to all of them because of their valuable support and precious advices during my reseach.

I would also like to give special thanks to The Scientific And Technological Research Council Of Türkiye (TÜBİTAK) Directorate of Science Fellowships and Grant Programmes (BİDEB) for their financial support by providing scholarship during my Master's thesis study.

I would like to thank to my dear familiy Gülşen and Cavit Turan and my lovely sister Gizem Turan for supporting me in any event throughtout my entire life. Without their endless support, patience and encourgement, this thesis would not have been possible.

Above all, I would like to thank my dear husband Burak Gökçen who gave me endless moral support throughout my thesis. He has been always with me and helped me to overcome all obstacles by giving him kindly and patiently support.

ABSTRACT

EFFECTS OF SMALL MOLECULES ON CARTILAGE TISSUE REGENERATION

Every year millions of people suffer from cartilage defects. Although, there are some traditional treatment strategies to heal defects, they are inadequate to repair full thickness defects and they cannot give its native mechanical endurance and function to cartilage back. Therefore, cartilage tissue engineering studies provide new treatment methods to overcome limitations of traditional methods. In this study, effects of small molecules on chondrogenic differentiation of RBMSCs were examined to evaluate their cartilage tissue regeneration potential. Three different small molecules (KGN, ADZ and SKF) were used in chondrogenic induction, RBMSCs were seeded into fibrous polylactic acid (PLA) scaffolds prepared with wet spin technique. While kartogenin (KGN) is already known in literature with its chondrogenic induction and chondroprotection properties; ADZ and SKF are recently found molecules having proliferation enhancing feature on MSCs, but their potential effects on chondrogenic induction has been never studied. Chondrogenic differentiation effects small molecules of ADZ and SKF were examined on differentiated RBMSCs throughout 14 days of incubation compared to KGN molecule via Alcian Blue-PAS staining, MTS cell proliferation assay, RT-PCR methods. Although, ADZ had a better proliferative effects on RBMSCs, there was reduced ECM deposition during chondrogenic differentiation. Therefore, rest of the study was carried on with small molecule SKF and its combination with KGN. Alcain Blue-PAS staining, MTS assay and RT-PCR assays were conducted on stem cells. Higher amount of cartilagous ECM deposition, higher proliferation rate and higher expression of chondrogenic marker genes were observed in groups induced with small moleule SKF compared to controls. Results of the study showed that small molecule SKF had chondrogenic induction potential to use in cartilage tissue engineering.

ÖZET

KÜÇÜK MOLEKÜLLERİN KIKIRDAK DOKU YENİLENMESİNE ETKİSİ

Her yıl milyonlarca insan eklem hasarlarının sıkıntısını çekmektedir. Hasar tedavisinde bazı geleneksel tedavi yöntemleri olmasına rağmen, bu yöntemler tam kat eklem kıkırdak hasarlarının onarılmasında ve doğal kıkırdağın mekanik dayanıklılık ve fonksiyonunu geri kazandırılmasında yetersiz kalmaktadır. Bu sebeple, geleneksel metotların kısıtlamalarını aşabilmek amacıyla kıkırdak doku mühendisliği yeni tedavi yöntemleri sunmaktadır. Bu çalışmada, küçük moleküllerin (KM) sıçan kemik iliği mezenkimal kök hücreleri (SKİMKH) üzerinde kondrojenik farklılaşmaya olan etkileri ve kıkırdak doku yenilemesindeki potensiyel faydaları araştırılmıştır. Üç farklı KM (KGN, ADZ ve SKF), kondrojenik farklılaşmayı indüklemek için kullanırken, SKİMKHler ıslak eğirme yöntemi ile hazırlanmış polilaktik asit (PLA) hücre iskeleri üzerine ekilmiştir. Kartogenin (KGN), kıkırdak farklılaşması indüksiyonu ve kıkırdak koruyucu özelliği ile literatürde bilinen bir KM iken; ADZ ve SKF küçük molekülleri yakın zamanda kök hücre çoğalmasını arttırıcı etkileri gözlemlenmiş fakat kıkırdak farklılaşmasına olan potensiyel etkileri araştırılmamış moleleküllerdir. SKİMKH üzerinde yapılan bu çalışmada, kök hücreler KGN, ADZ, SKF küçük molekülerinin indüksiyonunda 14 gün boyunca kıkırdağa farklılaştırıldı ve alsiyan mavisi boyaması, MTS hücre çoğalma testi ve gerçek zamanlı PCR yöntemleri kullanılarak karşılaştırılmıştır. Hücre çoğalmasında ADZ küçük molekünün daha yüksek etki göstermesine rağmen, kıkırdak farklılaşması sırasında hücrerler arası madde (HAM) sentezini düşürdüğü gözlemlendi. Bu yüzden çalışma çalışmanın devamında, SKF KM ve onun KGN ile kombinasyonu kullanılmıştır, PLA hücre iskeleleri üzerine ekilmiş SKİMKHler KMler ile kıkırdak farklılaşmasına indüklenmiştir. Alsiyan mavisi boyaması, MTS hücre çoğalma testi ve gerçek zamanlı PCR yöntemleri hücreler üzerinde uygulandı. Kontrol gruplarına kıyasla daha yüksek miktarda kıkırdaksı HAM birikimi, daha fazla hücre çoğalması ve daha yüksek kıkırdak spesifik gen ifadesi SKF KM verilen gruplarda gözlenmiştir. Bu çalışmanın sonucunda SKF küçük molekülünün kondrojenik indüksiyon özelliği ile kıkırdak doku mühendisliği çalışmalarında kullanım potensiyeli olduğu gösterilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND/ABBREVIATIANS

ACAN	Aggrecan
ADSCs	Adipose derived stem cells
ADZ	Adezmapimod
BMP	Bone morphogenic factor
BMSC	Bone marrow mesenchymal stem cell
CM	Chondrogenic media
COL1A1	Type I collagen
COL2A1	Type II collagen
DCM	Dichloromethane
dH_2O	Distilled water
DMEM	Dulbecco's Minimum Essential Medium
ECM	Extra cellular matrix
FBS	Fetal bovine serum
GM	Growth media
IGF	Insulin-like growth factor
KGN	Kartogenin
MAPK	Mitogen-activated protein kinase
MEM α	Minimum Essential Medium α
mM	Millimolar
MSC	Mesenchymal stem cell
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
OM	Osteogenic media
PBS	Phosphate buffered saline
PCLC	Poly(L-lactide-co-ε-caprolactone
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGA	Polyglycolic acid
pH	Negative log of hydrogen ion concentration
PLA	Poly-lactic acid
PLGA	Polylactic-co-glycolic acid

RBMSC	Rat bone marrow derived mesenchymal stem cell
RT-PCR	Real time polymerase chain reaction
RUNX1	Runt-related transcription factor 1
SKF	SKF-96365 hydrochlorine
TGF-β	Transforming growth factor-β1, human

1. INTRODUCTION

Every year millions of people suffer from cartilage defects. While technological improvements increase, sedentary life style becomes increasingly common. Obesity, absence of physical activity, ageing are the main factors of cartilage diseases [1]. Cartilage tissue is an avascular tissue with a limited regeneration ability when deformed [2]. Although there are traditional methods for treatment of cartilage defects, they are still unsatisfactory in terms of fully restoration and bringing tissue function back. Cartilage tissue engineering provides new treatment methods for regeneration of damaged cartilage via gather molecular biology and materials engineering [3].

Cartilage tissue engineering includes cells, scaffolds and growth factors for treatment of defects. Cell source can be autologous chondrocytes or differentiated stem cells and scaffold is 3-D material supporting defect mechanically, enabling cell migration, proliferation, differentiation and ECM organization [4]. Growth factors are inducing factors that enhance proliferation, differentiation and ECM production in tissue engineering constructs [5]. The goal of cartilage tissue engineering is regeneration of damaged cartilage and creation of functionally and mechanically close tissue with native one [6]. There are numerous cartilage tissue engineering studies that combine different cell sources, scaffolds and growth factors to accomplish this goal, but investigations still continue to get on ideal cartilage treatment technique.

This study aimed cartilage regeneration via small molecule induction on chondrogenically differentiated RBMSCs. Fibrous PLA scaffolds were used as a 3-D support material for differentiated RBMSC. Two small molecules Adezmapimod (SB203580) (ADZ) and SKF-96395 hydrochlorine (SKF) were used as inducer and their effects on chondrogenic differentiation were determined. In previous studies, it was proven that these two molecules enhances proliferation of MSCs [7,8]. In this study, effect of ADZ and SKF on chondrogenic induction was researched. Small molecules ADZ and SKF were compared with another small molecule KGN which is known as chondrogenic inducer having chondroprotective features on chondrocytes. Small molecules were introduced to RBMSCs seeded on PLA scaffolds and cartilage tissue regeneration potential of the constructs were determined.

1.1. CARTILAGE

Cartilage is a component of the skeletal system and has important roles in body such as providing flexibility, resistance to compressive forces and mechanical resilience of the bones for the movements of the body [2]. Cartilage is an avascular tissue that has no blood or lymph vessels for nutrient, oxygen and metabolite transfer from circulation. Cartilage takes nutrients and growth factors from synovial fluid found between tissues by passive diffusion. The lacking of the vascularization causes slow turnover rate in nutrients and growth factors which leads to no damage repairing ability in cartilage tissue [9].

There are three types of cartilage found in the body as hyaline cartilage, fibrous cartilage and elastic cartilages. Hyaline cartilage, abundant type of cartilage in the human body is composed of proteoglycans and type II collagen. It is found in respiratory tract, nose, and growth plates of long bones [10]. Smooth and moist surface structure of the hyaline cartilage gives resilience to the bones against compressive forces [11]. The second cartilage type, fibrous cartilage is found in the tendon and ligaments and interverbal discs. It is composed of type I collagen instead of type II collagen, it is more rigid than hyaline cartilage and found in areas exposed of high amount of compression and tension [12]. The thirdly, the elastic cartilage consists of elastin fibers, proteoglycans and type II collagen found in ear, larynx and esophagus and it is the most elastic cartilage type among the three cartilage types [13].

1.1.1. ARTICULAR CARTILAGE

Articular cartilage is the special type of hyaline cartilage found in synovial joints and covers the ends of the bones. Articular cartilage has a huge stress resistance capacity because of its highly organized extracellular matrix (ECM) structure that gives the load bearing ability to the joint up 20 times of body weight [14]. ECM production and organization of the cartilage tissue are maintained by chondrocytes that is the only cell type found in the articular cartilage [15]. Water and ECM proteins including proteoglycans, collagens and noncollagenous proteins are main contents of the articular ECM (Figure 1.1).

Proteoglycans are large macromolecules consisting of a protein core and glycosaminoglycan chains. Negatively charged proteoglycans provide high water retention capacity in ECM and give resisting ability against compression forces in the cartilage tissue. There are various types of proteoglycans including aggrecan, fibromodulin, decorin, biglycan and aggrecan is predominant among them [16]. Beside of the water holding capacity of the proteoglycans, they are also important in protection of the collagen networks from degradation. Aggrecan monomers consisting of three globular domains bind to hyaluronan molecules and constitute aggregates. The aggregates fill around of the type II collagen networks and protect them against proteolytic cleavage [17].

Collagens are another main protein group found in the articular cartilage ECM. While type II, type IX and type XI are cartilage specific collagen types, type II is found abundantly in the cartilage tissues. Fibrils of type II collagens attach to each other's via type IX collagen and form a network structure providing basic 3-D shape to ECM [18]. The collagen networks provide mechanical integrity and protect the cartilage from tension forces. Together with aggrecan, type II collagen is predominant molecule in articular cartilage ECM and they are used as a marker in cartilage tissue studies [19].

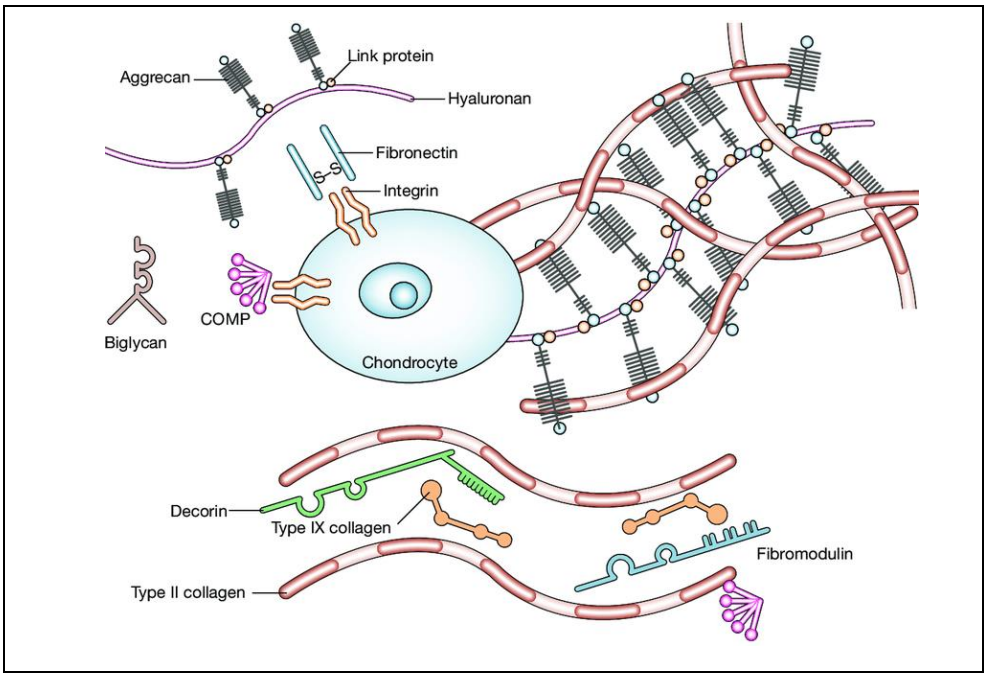

Figure 1.1. Articular cartilage ECM structure [15]

Articular cartilage is found in joints of two bones and covers ends of both bones two provide lubrication and prevent friction between bones (Figure 1.2). Articular cartilage is covered by synovial fluid and membrane and this structure is named as articular capsule [18]. When the articular cartilage cross section is investigated, four zone (superficial zone, middle zone, deep zone, calcified zone) of cartilage from articular surface to bone is seen and bone next to cartilage is called subchondral bone [19]. The chondrogenic and ECM organization change along with cartilage zones. While chondrocyte number is higher on top layers of cartilage tissue and decreases with down layers, ECM matrix amount increases in down layers.

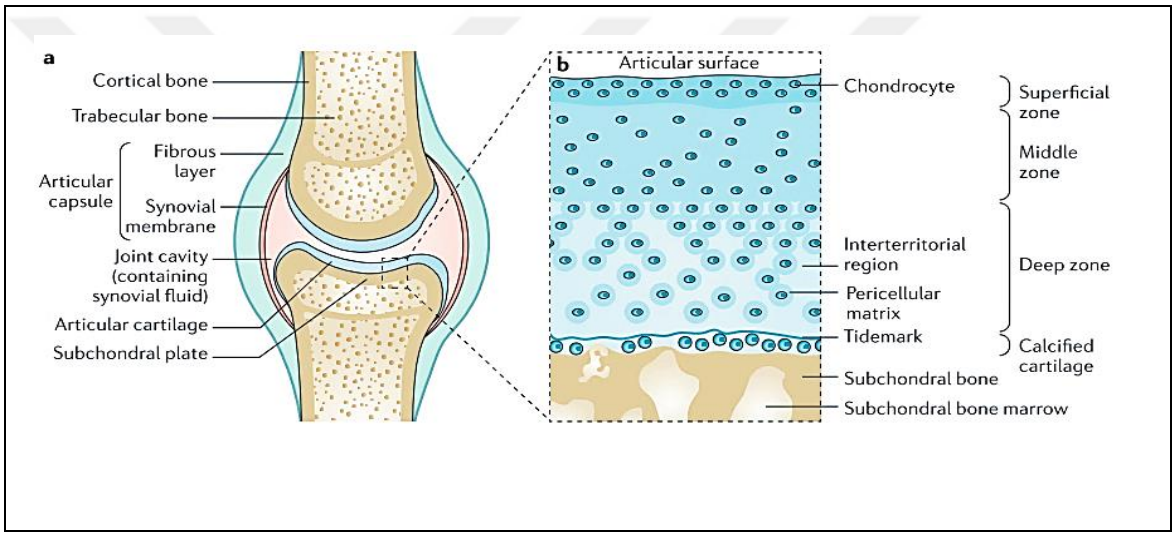

Figure 1.2. Articular cartilage anatomy (A) illustration of joint between two bone and two articular cartilage (B) cross section of a articular cartilage [21]

1.2. CARTILAGE TISSUE ENGINEERING

Osteoarthritis and traumas are main reasons of the cartilage defects [22]. Osteoarthritis is the most common reason of cartilage defects. It is slowly developing disease with age, and causes degeneration of cartilage such as knee, hips and hands. Obesity, age, physical activity, genetic factor are risk factors on progress of osteoarthritis [1]. On the other hand, cartilage trauma injuries are seen mostly in articular knee cartilage and resulted from load bearing actions such as jumping down, twisting or falling on knee [23].

Drug prescriptions, microfracture surgery, prosthetic replacement, autologous chondrocyte transplantation are traditional treatments of cartilage defects [24]. The treatment technique

is chosen according to the thickness of defect on cartilage. Defects found in articular surface and superficial zone and middle zone are named as partial thickness defect [25]. Defects extended along all cartilage until subchondral bone are named as full thickness cartilage defect. In partial thickness cartilage defects losing weight, drug prescription and rehabilitation are chosen for treatments, while for full thickness defects, surgical treatments such as micro fraction, mosaicplasty, autologous chondrocyte implantation are used [26].

The traditional treatment strategies generally aim relieving of symptoms. Surgical strategies have limitations on forming mechanically functional cartilage tissue mimicking native cartilage [24]. Donor site morbidity, dedifferentiation of autologous chondrocytes after implantation, maintaining chondrogenic organization on implants are the main problems in conventional treatment method [6]. Cartilage tissue engineering, multidisciplinary field, is on intersection of biology and materials engineering and it address problems of conventional methods.

There are three elements of cartilage tissue engineering which are cell source, scaffold and growth factors. The autologous cell source is taken from patient, expanded to bigger amounts in cell culture and given back to patient to restore cartilage defects [22]. The scaffold is used for mechanical support in defected area and provides migration, proliferation, matrix production of the cells. Growth factors are used for proliferation and chondrogenic differentiation of the cells in both *in vitro* culture conditions during cell expansion and during new tissue formation on cartilage defect of patient. In the next section, these three elements of cartilage tissue engineering will be determined in details.

1.2.1. Cell Sources

Even though only scaffolds can be used for regeneration of cartilage defects, using the cell sources with scaffolds makes more improved repairing results. There are various cell sources used in cartilage tissue engineering, but ideal one is still unclear. Easiness of isolation, less donor site morbidity, high cell density, expansion *in vitro*, high chondrogenic differentiation capacity and high production of chondrogenic ECM are the requested features in choosing of the cell source. Chondrocytes, stem cells, fibroblast are three main groups of cell sources in cartilage tissue engineering studies [6].

According to type of donor organism, cell sources can be classified as autologous, allogenic and xenogeneic cell sources. In autologous cell sources, both donor and recipient are the same organism. Chondrocytes, stem cells or fibroblasts are obtained from patient, expanded in cell culture and introduced to defected cartilage of patient for induction of cartilage regeneration [27]. Unlike autologous cell sources, allogenic cell sources are obtained from genetically non-identical donor but same species with the recipient. When patient has healthy donor tissue for autologous translation, using allogenic cell sources can be advantageous. Obtaining autologous cells is a challenge especially in patients at later ages having health problems, and allogenic cell sources are advantageous to overcome this challenge [28]. On the other side, use of allogenic cell sources can cause immune rejection in patient or diseases can be transferred from donor to recipient [29]. Xenogeneic sources are an alternative to autologous and allogenic cell sources. Donor organisms are different species from recipient organism in xenogeneic sources. Because of ethical concerns and immunogenicity, xenogeneic cell sources are least preferred one among the other cell sources.

1.2.1.1. Chondrocytes

Chondrocytes are the only cell type found in cartilage tissue; this makes it first cell source to come in to mind in cartilage tissue engineering. The chondrocytes are isolated from the cartilage of the patient at low compression bearing sites and expanded to large numbers *in vitro* conditions. Then, the chondrocytes are seeded into scaffolds to create 3-D culture environment enhancing ECM matrix production of the cells, and finally cartilage graft is implanted to the defect side of the patient (Figure 1.3) [25]. Although, their ECM matrix production seems like appropriate option for cartilage defects, there are numerous disadvantageous of using chondrocytes. Donor site morbidity, invasive cell harvesting procedure, limited cell number availability, low expansion capacity in *in vitro* conditions, loss of function because of rapid dedifferentiation of chondrocytes are the main disadvantageous. Therefore, other cell sources having high expansion capacity and lower donor site morbidity are more attractive in cartilage tissue engineering studies [30,31].

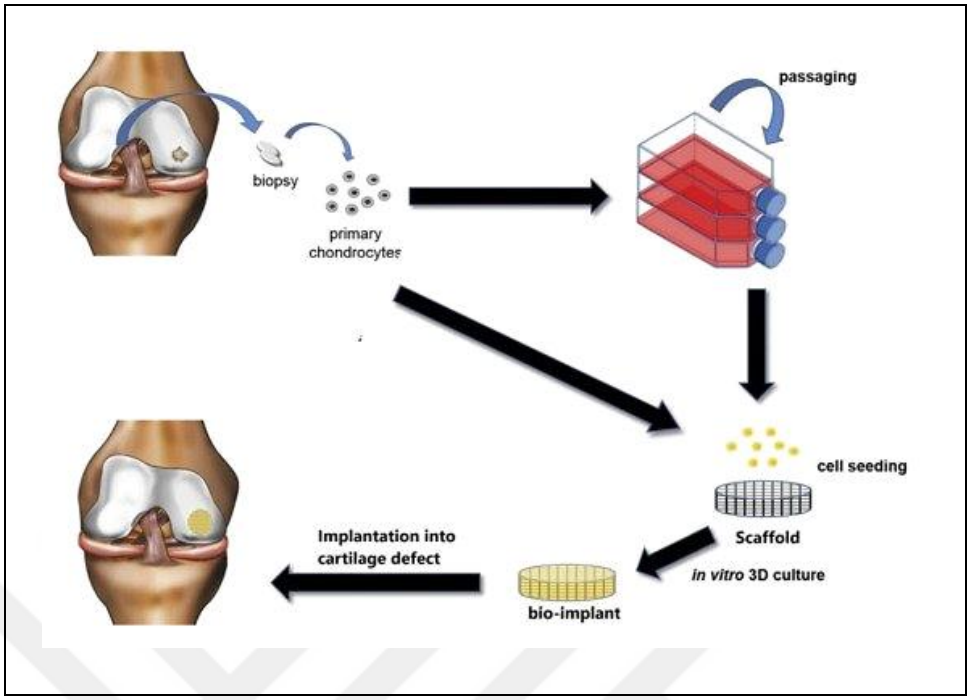

Figure 1.3. Implantation of autologous chondrocyte with scaffold [25]

1.2.1.2. Stem Cells

As an alternative to autologous chondrocytes, stem cells are good option as a cell source with their high expansion rate in *in vitro* conditions and differentiation capacity into the chondrocytes. Mesenchymal stem cells (MSCs) can be isolated from various tissues such as bone marrow, adipose tissue, synovial and periosteal membranes, umbilical cord, placenta and embryos [32].

Bone marrow derived stem cells (BMSCs) are extensively used stem cells source in tissue engineering studies. TGF-β, a growth factor, is a necessity for chondrogenic differentiation and it is used in media supplements in *in vitro* cultures. Even though only differentiated BMSCs can be used in treatment of cartilage defects, the addition of scaffolds and inducing the environment with the molecules enhance the renewal of cartilage (Figure 1.4.) [33]. Three-D culture environment provided by scaffold positively affects the chondrogenic differentiation degree and ECM matrix production amount *in vitro*. Although BMSCs are advantageous due to their high proliferation capacity, there are some problems in obtaining of BMSCs from donors. Studies show that the proliferation rate of the BMSCs obtained from patients is significantly lower than the ones coming from health donors, also chondrogenic and adipogenic differentiation capacities of the BMSCs are reduced with diseases and older

age in donors. Therefore, age and disease of the patients are limiting factors on usage of BMSCs in therapies [34].

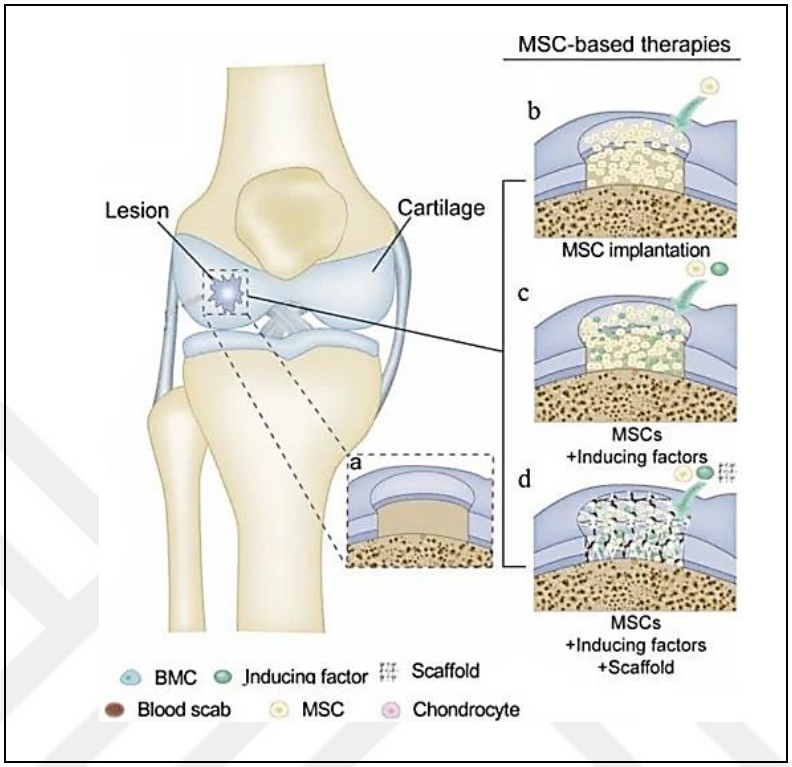

Figure 1.4. MSC usage in cartilage defect therapy (A) defected cartilage area, (B) only MSCs, (C) MSCs and inducing factor, (D) MSCs and inducing factor and scaffold implantation together to the defected area [25]

Adipose derived stem cells (ADSCs) are another stem cell sources which are frequently used in tissue engineering studies because of easiness during isolation than the BMSCs and chondrocytes. While the ADSCs in a large amount can be obtained from the liposuction surgeries, low morbidity in donor tissue is seen together with less discomfort in patient [35]. The growth factor requirements of the stem cells for chondrogenic differentiation vary depending on the stem cell source. While BMSCs need supplementation of growth factor TG-β, ADSCs require BMP-6 for aggrecan and collagen type 2-II ECM production during differentiation. The ADSCs can be expanded more extended time because of their low cell senescence rate during cell growth, on the other hand, the ADSCs have low osteogenic and chondrogenic differentiation capacity compared with BMSCs [31].

Embryonic stem cells (ESCs) are pluripotent stem cells having high proliferative capacity and can be differentiated into all somatic cell types. Although, their superiority in

proliferation and differentiation, studies and clinical applications of ESCs are limited because of ethical issues [37]. Also, antigenicity, purity and chondrogenic differentiation differences between the ESCs line are the other problems in ESCs [38]. Induced pluripotent stem cells are alternative to ESCs with their pluripotency and having no ethical issues, but tumorigenicity risk limits their application [39].

1.2.1.3. Fibroblasts

Fibroblast are good alternative cell source to chondrocytes and adult stem cells to use in cartilage tissue engineering. Relatively large number of dermal fibroblast cells in skin can be easily isolated from patients with minimal invasion. Studies show that fibroblast can produce chondrogenic ECM containing aggrecan and type II collagen, and also they produce TGF-β1 and secrete to environment which, induces the articular ECM production in defect side [40,41].

1.2.2. Scaffolds

Scaffolds are an important elements of cartilage tissue engineering because of their 3-D structure, which gives an appropriate environment for cartilaginous ECM production. Biocompatibility, controlled biodegradability, mechanical resistivity, nontoxicity and causing not immunological and inflammatory response in host organism are requested features of scaffolds [6]. A scaffold must have proper porous size enabling to attachment and migration of cells, diffusion of nutrients, oxygen and growth factors [42]. Scaffolds can be classified according to their material types as natural and synthetic, and also can be divided according to their forms as fibrous and porous scaffolds. In the next section, natural and synthetic polymers used in scaffold preparations will be explained.

1.2.2.1. Natural Materials

Collagen is an abundant protein found in ECM (90 percent of the dry weight of the articular cartilage) that provides mechanical strength and architectural structure to cartilage tissue [43]. Because of its high biocompatibility, collagen is used both *in vitro* and *in vivo* cartilage tissue engineering studies as a scaffold material. By using enzymatic degradation and salt leaching methods, collagen is isolated from animals such as swine and bovine [44]. After reducing immunogenicity of the collagen, it is used in scaffold preparations. Although both

type I collagen and type II collagen can be used in cartilage tissue engineering, the collagen type I is the most preferred type because of its capability to induce redifferentiation of chondrocytes. A commercial type I collagen gel product Atelocollagen was examined *in vitro* and increasing ECM production of chondrocytes was observed [45]. On the other hand, in clinical trials 93 percent success rate was achieved in patients and biomechanical similarity was seen when compared with healthy cartilage. Integration of implanted cartilage to the host tissue is an important challenge in cartilage tissue engineering. Collagen has overcome this challenge with its controlled delivery feature. In one of the *in vivo* study including a collagen scaffold/chondrocyte implant, chondrocytes were seeded into two sides of the collagen scaffolds, then cultured between the defected two cartilage pieces, at the end of 40 days, integration between implanted scaffold and cartilage tissue was observed [46].

Chitosan is a polysaccharide obtained via deacetylation of chitin that is naturally found in seashells. With linear cationic structure, chitosan is an analog of the glycosaminoglycans found in ECM of chondrocytes [47]. Therefore, it gets attention in tissue engineering with its biocompatibility. Chitosan hydrogels have a similar structure of hydrated collagen network in chondrogenic ECM that allows enhanced proliferation and growth of chondrocytes on chitosan scaffolds [48]. Also, crosslinking ability of chitosan provides easy modification and combination with other biomaterials and inducing growth factors. In a study, Arginylglycylaspartic acid (RGD) peptide was encapsulated with chitosan and obtained microcarriers were given to the adipose derived stem cells within N-methacrylate glycol chitosan hydrogel matrix. At the end of this study, enhanced chondrogenesis and articular cartilage repair were observed [49].

Hyaluronic acid is a glycosaminoglycan that is found in articular cartilage, it has a role in linking aggrecans and giving lubricant property to cartilage via immobilization of water [50]. While hyaluronic acid is an important polysaccharide in maintaining of viscoelastic features of cartilage, hyaluronic acid injections are applied in osteoarthritis treatment [51]. Like chitosan, hyaluronic acid can be chemically modified by crosslinking reactions and hyaluronic acid containing hydrogels can be obtained from crosslinks. Hydrogels of hyaluronic acid were used in encapsulation of chondrocytes and injected into cartilage defects. Hyaline cartilage formation and tissue integration between the new formed cartilage and host tissue were observed [52]. Even tough hyaluronic acid has a great biocompatibility in cartilage treatments, its mechanic features are not enough to support chondrogenesis in

defected area. However, usage of the hyaluronic acid together with other materials could be the solution. In one of the study, tripolymer of hyaluronic acid, gelatin and chondroitin was used for delivery of chondrocytes to a cartilage defect. Enhanced matrix synthesis and successful cartilage regeneration was observed [53].

Alginate is a natural polymer obtained from brown algae. It has a linear anionic polysaccharide structure and can be dissolved in water in the presence of calcium chelating agents. The alginate gels are mostly used in cartilage tissue engineering for encapsulation and injectable delivery of chondrocytes to cartilage defects. Also, alginate beads were used for *in vivo* and *in vitro* studies for growth of BMSCs and differentiation into chondrocytes [54,55]. On the other hand, alginate can immunogenic *in vivo* conditions and can induce severe foreign body giant cell reactions, therefore, there is limited number of clinical research on alginate based cartilage implants [56].

1.2.2.2. Synthetic Materials

Although natural materials have great a biocompatibility, integrity and nontoxicity futures due to their chemical similarity with native articular ECM, they are mechanically weak for cartilage tissue engineering applications. Synthetic polymers are alternative to natural materials with slow degradation rates, easy processability and mechanical strength. There are various FDA approved synthetic polymers for medical applications such as polyglycolic acid (PGA), polylactic acid (PLA), polyethylene glycol (PEG), polylactic-co-glycolic acid (PLGA), polyurethane etc. [57]

Combination of two materials, composite materials provide better mechanical properties than their components. In scaffold prepared from polyvinyl alcohol graphene composite, increased elastic and compression modulus, electrical conductivity and toughness of composite material were found when compared with the single materials [58]. Also, biocompatibility can be enhanced by combining synthetic polymers with natural ones. In one of the study, chondrocytes were seeded into PCL/type I Collagen scaffolds and increased hyaline like ECM production was observed *in vivo* study in nude mice model [59]. In another study, PCLC (poly(L-lactide-co-ε-caprolactone) was combined with chitosan and pig articular chondrocytes were seeded and cultured on porous composite scaffolds. It was observed that chondrocyte attachment and proliferation, type II collagen and aggrecan synthesis were increased with chitosan compared with only PCLC scaffolds [60].

Polylactic acid (PLA) is a biodegradable thermoplastic mostly used in fiber, film and nonwoven fabric production such as disposable cup, plastic bottle, teabag, diaper and sanitary pad [22]. As seen in Figure 1.5, PLA is composed of lactic acid monomers and it is produced from polymerization of organic lactic acid sources such as sugar cane, corn starch etc. [61]. Because of biocompatibility, slow and controllable degradation rates of PLA, it is widely used in medical applications. Bone and cartilage tissue engineering grafts, sutures, prostheses, medical screws, spinal cages, drug carriers, microneedle patches are example of PLA usage in medical product and treatments [62].

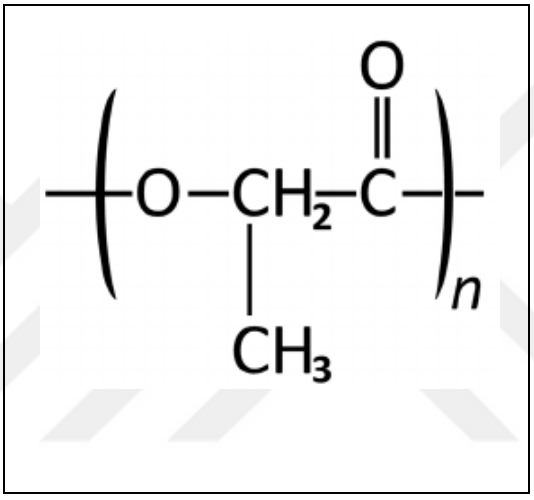

Figure 1.5. Chemical structure of polylactic acid [61]

When PLA is degraded, L-lactic acid monomers become the hydrolysis product. The L-lactic acid monomers are nontoxic molecules and can be easily removed out of body by kidneys [63]. Although L-lactic acid monomers cause pH decrease in the tissue, the slow degradation rate of PLA provides tolerable pH decrease. Slow degradation property of PLA is also important to use it as a drug carrier. PLA is approved by FDA for delivery of numerous drugs such as anticancer, anti-inflammatory, antipsychotics [64].

Even though, PLA is a good scaffold material with its slow degradation rate and mechanical properties supporting tissue regeneration, its hydrophobicity and low cell attachment properties are disadvantages in tissue engineering applications. However, PLA surface can be modifiable by using cross-linking reactions or combined with other materials to enhance its biocompatibility [65]. There are numerous studies with PLA scaffolds used for cartilage tissue engineering. In these studies, proliferation and aggrecan containing ECM production

were observed in chondrocytes seeded on scaffolds [66–68]. Histological, morphological and biochemical integration were observed between native tissue and grafted scaffold in *in vivo* study [69]. Type II collagen synthesis and hyaline like ECM formation were observed in PLA constructs and full thickness cartilage defect in rabbit knees was repaired and new cartilage formation was observed.

1.2.3. Growth Factors

Third element of the cartilage tissue engineering is the growth factors used for inducing chondrogenesis of MSCs, enhancing proliferation, cell adhesion and hyaline like ECM formation [70]. TGF-β, IGF (insulin-like growth factor), BMP (bone morphogenic factor), FGF (fibroblast growth factor), PDGF (platelet derived growth factor) are the growth factors that have enhancing and inducing effect on chondrogenic differentiation and cartilage formation [4,71]. Growth factors not only induce chondrogenesis, but also down regulate synthesis of type I collagen that relates with hypertrophy and reduce enzymatic activity of MMPs (matrix metalloproteinases) degrading ECM matrix proteins [20].

Growth factors can be introduced to cells directly by adding to culture medium or using scaffold delivery systems or using nanoparticle carriers. Adding growth factors to the culture media is the easiest way of delivery but half-life of growth factor is short and the amount of growth factor with time is variable when compared with other delivery methods. In Park et al. study, TGF-β was loaded into microparticles and mixed with hydrogel composites and used for chondrogenic differentiation of MSCs [72]. Significantly increased amount of glycosaminoglycan and type II collagen synthesis was observed in microparticles containing group compared with controls. This study showed that encapsulated growth factors remain viable for a longer period in culture and increase efficiency of growth factors.

Growth factors have different effects when applied independently or combined together. While BMP-7 induces chondrogenic differentiation, it also stops cell proliferation [73]. But, when TGF-β is added together with BMP-7, cell proliferation continues with chondrogenic differentiation. In another study, TGF-β and IGF combination were examined. Chondrocytes were induced with TGF-β and IGF, increased cell proliferation, enhanced matrix synthesis were observed compared with individual TGF-β and IGF groups and controls [74].

Small molecules are low molecular weight molecules (lower than 900 Daltons) used in medical applications for treatment, diagnostic and prevention of diseases [75]. Small molecules are predominant among pharmaceutical products for example, aspirin and diphenhydramine are well known small molecule drugs. Unlike growth factors, small molecules can easily pass-through the cell membrane [76]. Also, small molecules are advantageous due to their stability and non-immunogenicity. Therefore, small molecules recently get attention in tissue engineering researches. There are numerous studies showing that small molecules can induce cell proliferation and chondrogenic differentiation in MSCs. Honokiol, Rosmarinic Acid, Cycloastragenol, Kartogenin are some of these small molecules and they were proved to promote chondrogenic differentiation of stem cells in preclinical researches [77–80].

1.2.3.1. Kartogenin

Kartogenin (KGN) is a small molecule used in chondrogenic differentiation and chondroprotection in cartilage regeneration Its chemical name is 2-[(4-phenylphenyl) carbamoyl] benzoic acid (Figure 1.6). KGN has several advantageous to protein growth factors since some growth factors like TGF-β and BMP have problems of immunogenicity, instability and short *in vivo* half-life [24]. It was discovered in 2012 by Johnson et al, and they proved chondrogenic potential of Kartogenin [81]. According to the study, KGN interacts with core binding factor (CRFβ), acting binding protein filamin A (FLNA) and RUNX1 transcription factor that stimulates the chondrogenic differentiation of MSCs. Increased synthesis of chondrogenic markers such as type II collagen, aggrecan, lubricin and SOX9 was observed upon application of KGN on MSCs when compared to control group. Also hypertrophy and calcification were examined on MSC and no significant difference was observed on induced MSCs with KGN. These results showed that KGN both induces chondrogenic differentiation of MSC and protects the cartilage ECM from degradation.

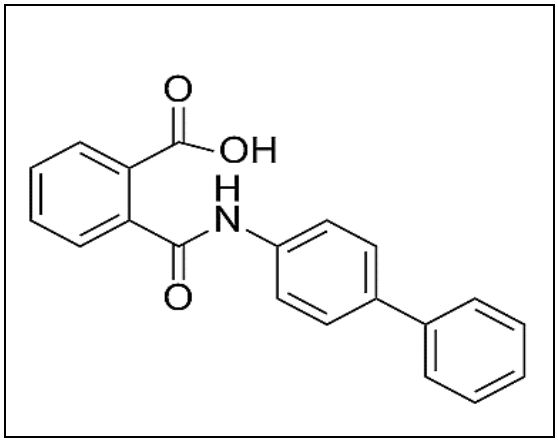

Figure 1.6. Chemical structure of KGN [82]

There are numerous studies about cartilage regeneration effects of KGN. Combination of KGN significantly increases synthesis of lubricin which is chondroprotective glycoprotein and decrease in osteoarthritis [83]. Another study revealed that KGN is a chondroprotective under pathological conditions that were simulated on cartilage explants [84]. Decreased nitric oxide production and increased glycosaminoglycan secretion were observed on KGN induced group. KGN also has synergistic effects when combined with other growth factors like TGF-β and BMP-7, lubricin and glycosaminoglycan secretion amount increases on differentiated BMSCs when compared with groups induced with only growth factors.

1.2.3.2. Adezmapimod (SB203580)

Adezmapimod (ADZ) or SB203580 is a small molecule containing two phenyl rings and an imidazol ring as seen in Figure 1.7. Its chemical name is 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole. It is selective inhibitor of p38 mitogen-activated protein kinase (MAPK) that is responsive to stress stimulis such as heat, UV, osmotic pressure, inflamation etc. There are four types of p38 MAPK in mammalians: p38α, p38β, p38γ and p38δ, that involve various pathways during cell cycles such as differentiation, autophagy, apotosis. Therefore, there is various literature study showing the effects of inhibiton of p38 MAPK via induction of SB203580. In one of the literature study, SB203580 was applied on rats with middle cerebral artery occlusion and significant reduction in infarct volume was shown in this study [85]. In another study, effects of p38 MAPK inhibition via SB203580 treatment was investigated on osteogenically differentiated

muscle derived stem cells While cell proliferation increased in the stem cells, lower expression osteogenic differentiation markers were observed [86].

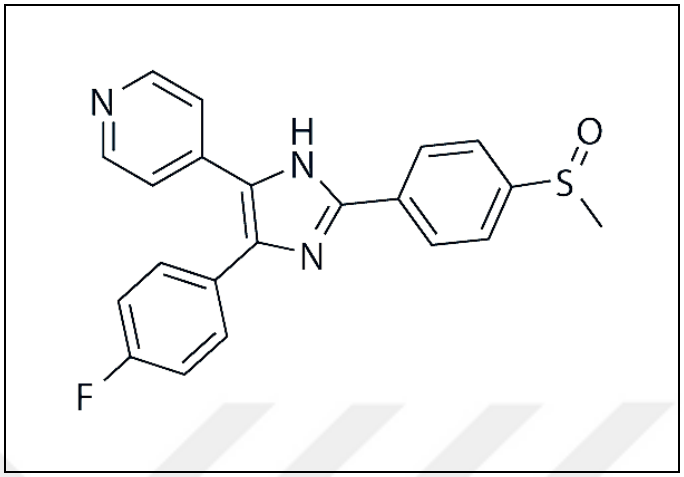

Figure 1.7. Chemical structure of SB203580 [87]

1.2.3.3. SKF 96365

SKF 96365 (SKF) is a small molecule containing two phenyl ring and one imidazole ring like SB203580 as seen in Figure 1.8. Its chemical name is 1-[β-(3-(4-Methoxyphenyl) propoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride, 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy] ethyl] imidazole [88]. It is an inhibitor of transient receptor potential channels (TRPCs) which are a receptor family found in mammalians. There are seven different types of TRPCs in mammalians, over expression of TRPC6 is seen in Alzheimer's disease, TRPC1 and TRPC3 are upregulated in cardiovascular disease. In a study, cell growth of glioblastomas (found in brain tumors) was inhibited by SKF 96365 *in vitro* experiments [89]. In another study, increased apoptosis in cardiomyocytes and high sensitivity to injuries were observed on transgenic mice that showed an overexpression on of TRPC3 in myocardial cells, and SKF 96365 treatment resulted in enhanced viability of cardiomyocytes [90].

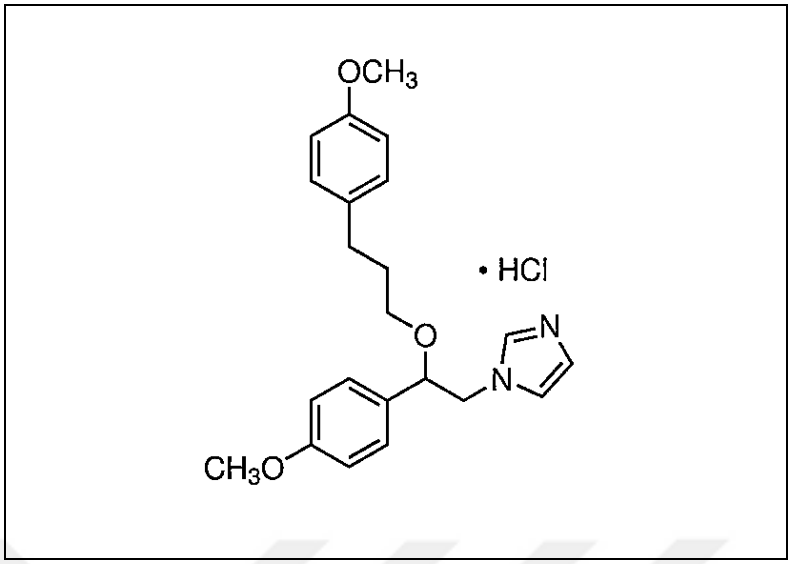

Figure 1.8. Chemical structure of SKF 96395 hydrochlorine [91]

1.3. AIM OF THE STUDY

The aim of this study was to provide cartilage tissue regeneration via small molecule induced RBMSCs. Small molecules ADZ and SKF are known with proliferative inducers of MSCs [8]. In this study, chondrogenic induction potential of ADZ and SKF molecules were examined and compared with KGN small molecule which is already known as chondrogenic differentiation and chondroprotection inducer molecule in literature [81,84,92]. PLA fibrous scaffolds spun via wet-spin technique was used as a 3-D support material to establish mechanical strength and articular ECM formation of differentiated RBMSCs. Cartilage tissue constructs were prepared by seeding RBMSCs into fibrous PLA scaffolds and differentiated into chondrocytes via using chondrogenic differentiation medium supplemented with small molecules ADZ, SKF and KGN. *In vitro* evaluations of small molecules were carried out on tissue constructs.

2. MATERIALS

2.1. CHEMICALS AND REAGENTS

- Absolute ethanol (Isolab, Turkey)
- Absolute isopropyl alcohol (Isolab, Turkey)
- Alizarin-Red Solution (Merck-Millipore, Germany)
- Corning™ ITS Premix Universal Culture Supplement (Fisher Scientific, USA)
- Dexamethasone (Sigma-Aldrich, Germany)
- Dichloromethane (Merck Millipore, USA)
- Dimethyl Sulfoxide (DMSO), Sterile (Bioshop, Canada)
- Distilled water
- Dulbecco's Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, USA)
- Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4500 mg/L) Medium (Gibco-Thermo Fisher Scientific, USA)
- Fetal Bovine Serum (FBS) (Gibco-Thermo Fisher Scientific, USA)
- Formaldehyde (Fluka, Switzerland)
- Human Transforming Growth Factor β-1 (TGF β -1) (PRP100190) (Abbkine, USA)
- Indomethacin (Chem Cruz- Santa Cruz Biotechnology, USA)
- Insulin solution human (Sigma-Aldrich, Germany)
- Kartogenin (SML0370) (Sigma-Life Science, Germany)
- L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Santa Cruz Biotechnology, USA)
- L-Proline (Sigma-Aldrich, Germany)
- MEM α, GlutaMAX™ Supplement, no nucleosides (Gibco-Thermo Fisher Scientific, USA)
- Oil Red O (Sigma-Aldrich, USA)
- Penicillin-Streptomycin-Amphotericin (PSA) Sigma-Aldrich Corporation, Germany)
- Primers for COL2A1, ACAN, SOX9, RUNX1, COL1A1 (Macrogen, South Korea)
- SB203580 (ADZ) (PHZ1253) (Gibco-Thermo Fisher Scientific, USA) was kindly donated by Yeditepe University Regenerative Biology Research Laboratory

- SKF 96365 Hydrochloride (Small Molecule SKF) (1147) (Biotechne, USA) was kindly donated by Yeditepe University Regenerative Biology Research Laboratory
- Sodium azide (Sigma-Aldrich, Germany)
- Trypsin-EDTA solution (Sigma-Aldrich, Germany)
- Tween® 20 (AppliChem, USA)
- β-Glycerophosphate disodium salt hydrate (Sigma-Aldrich, Germany)

2.2. KITS

- Alcian Blue-PAS Stain Kit (Atom Scientific, UK)
- iScript cDNA synthesis kit (Bio-Rad, USA)
- Maxima SYBR Green, ROX qPCR Master Mix, 2X (Thermo Fisher Scientific, USA)
- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA)
- RNA Spin Total RNA Isolation Kit (Intron Biotechnology, Korea)

2.3. INSTRUMENTS

- Nanodrop Spectrophotometer (Thermo Scientific, USA)
- Scanning Electron Microscope (Carl Zeiss EVO, Germany)
- Laminar Flow Cabinet (ESCO Lab. Culture Class II Biohazard Safety Cabinet 2A, Singapore)
- CO_2 Incubator (ESCO Lab., Singapore)
- Brightfield Microscope (Carl Zeiss, USA)
- Centrifuge (Hettich micro 22R and Sigma 2-5 centrifuge, Germany)
- pH Meter (Hanna, Germany)
- ELISA Plate Reader (BioTek ELx800, USA)
- CFX Touch™ Real Time PCR Detection System (Bio-Rad, USA)
- Shaking Water Bath (Memmert, Germany)
- Freeze Dryer (Savant Modulyo Freeze Drying Systems, Thermo Fisher Scientific, USA)
- Vortex (Biostellar, China)

- Countess Cell Counter (Thermo Scientific, USA)
- Inverted Microscope (Carl Zeiss, USA)
- ExplorerPro Analytical Balance (Ohaus, USA)
- Universal Testing Machine (3382, Instron, USA)

3. METHODS

3.1. PREPERATION AND CHARACTERIZATION OF SCAFFOLDS

PLA (Poly Lactic Acid) was pre-dried for 6 hours at 100 ºC before use in wet spinning. Then it was dissolved in DCM at 20 percent w/v ratio and stirred on magnetic stirrer at 120 rpm overnight. Wet spinning set up was prepared as seen in (Figure 3.1). Coagulation bath was filled with 90 percent cold isopropanol and put onto the turntable. The blunt end 21-gauge needle was fit onto the 10 mL syringe. The syringe was filled with dissolved PLA and placed into the syringe pump, which was set to 50 µL/min flow rate. After wet spinning, the spun PLA was incubated in coagulation bath at +4 ºC overnight. Next day, spun PLA was washed with dH_2O several times, and frozen at -80, and freeze-dried overnight. Dried spun PLA was cut via a steel punch with a 0.9 cm diameter. PLA fibers were cut as circles (6 mm diameter) by using steel punches and placed into 48 well plate. The scaffolds were sterilized with 70 % ethanol solution. The plate was kept on +4 ºC for 3 hours, then scaffolds were washed three times with PBS buffer.

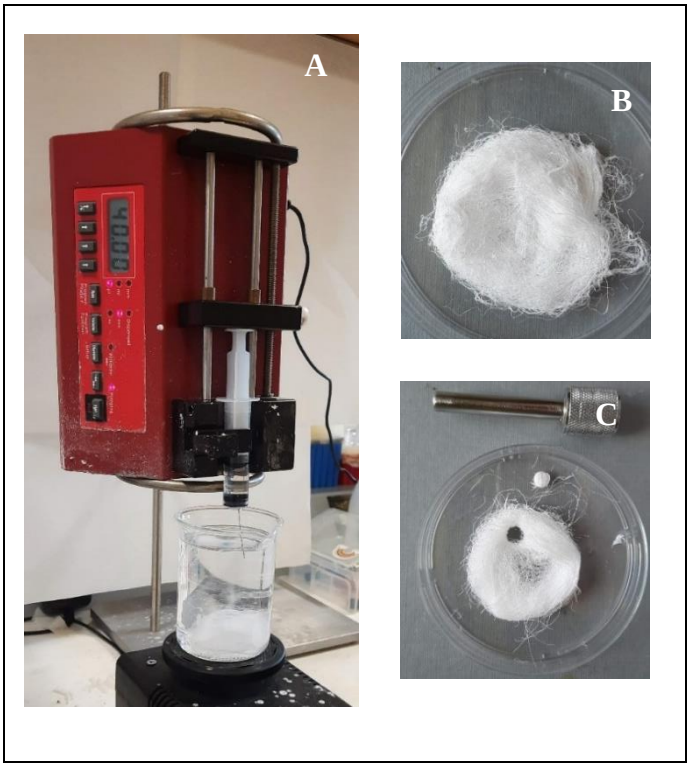

Figure 3.1. Wet spinning set up (A) dried fibrous PLA spun (B) and final form after cutting with steel punch (C).

3.1.1. Scanning Electron Microscopy

Scaffolds with 6 mm radius were cut from PLA fibers with a steel punch. These scaffolds were attached onto glass slide by using adhesive carbon discs. The sample slide was placed into sputter coater machine, and samples were covered with gold coating. Then, surface characteristics of the scaffolds were determined under the scanning electron microscope.

3.1.2. Degradation Analysis

The weight of each scaffold was measured and put into 15 mL centrifuge tubes. PBS buffer solution that contains 0.1% sodium azide was prepared and added into each of the centrifuge tubes (5 mL). The initial pH of the PBS solution was measured as 7.39. The tubes were placed into 37 ºC shaker water bath and incubated, for 3, 5, 7, 15, 30 and 60 days. At the end of each incubation period samples were taken from water bath, the final pH of the PBS solution was measured to observe pH difference. Then, the scaffolds were taken from the tubes and weighed. After freeze-drying, they were scaffolds weighed again to determine weight loss in degraded scaffolds. Weight loss and water retention percentages were calculated using equations 3.1 and 3.2, respectively. W_i is initial weight, W_w is wet weight, W_f is final weight of dried scaffolds in the following equations.

$$\text{Percent Weight Loss} = \frac{W_i - W_f}{W_i} \times 100 \tag{3.1}$$

$$\text{Percent Water Retention} = \frac{W_w - W_i}{W_i} \times 100 \tag{3.2}$$

3.1.3. Mechanical Analysis

Instron Universal Testing Machine was used for mechanical analysis of PLA fibers. For tensile test, PLA fiber bundles containing 400 fibers were prepared (Figure 3.2.A). The length and diameter of the fiber bundles was measured by using of a electronic caliper as 25 mm to 2.49 mm, respectively. The PLA bundle was placed from the two endings to the machine (Figure 3.2.B). Then, the tensile test was carried on the Universal Testing Machine

with a 5 mm/min displacement rate and the force was measured as Newton (Figure 3.2.C). The test was terminated when the PLA fibers started to break off. The elastic modulus value of PLA fibers was calculated from the data.

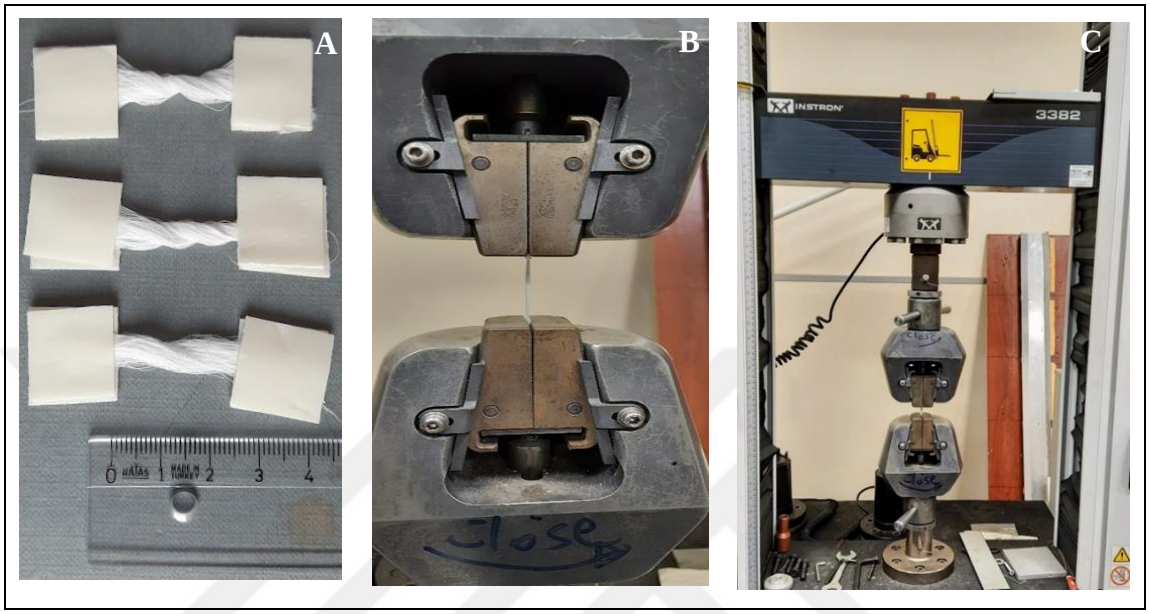

Figure 3.2. Tensile test analysis A) PLA samples before tensile test, B) Tensile grips, C) Tensile set up.

For compression test, PLA fibers were cut as cylinder having 5 mm diameter and 6 mm height. Samples were placed into the cylindrical molds having 5 mm diameter (Figure 3.3.A). The mold was placed into the Universal Test Machine and the compression was applied at 5 mm/min ratio and the force was measured (Figure 3.3.B, C). The test was terminated when the force reached to 100 Newton and the compression modulus was calculated from the data.

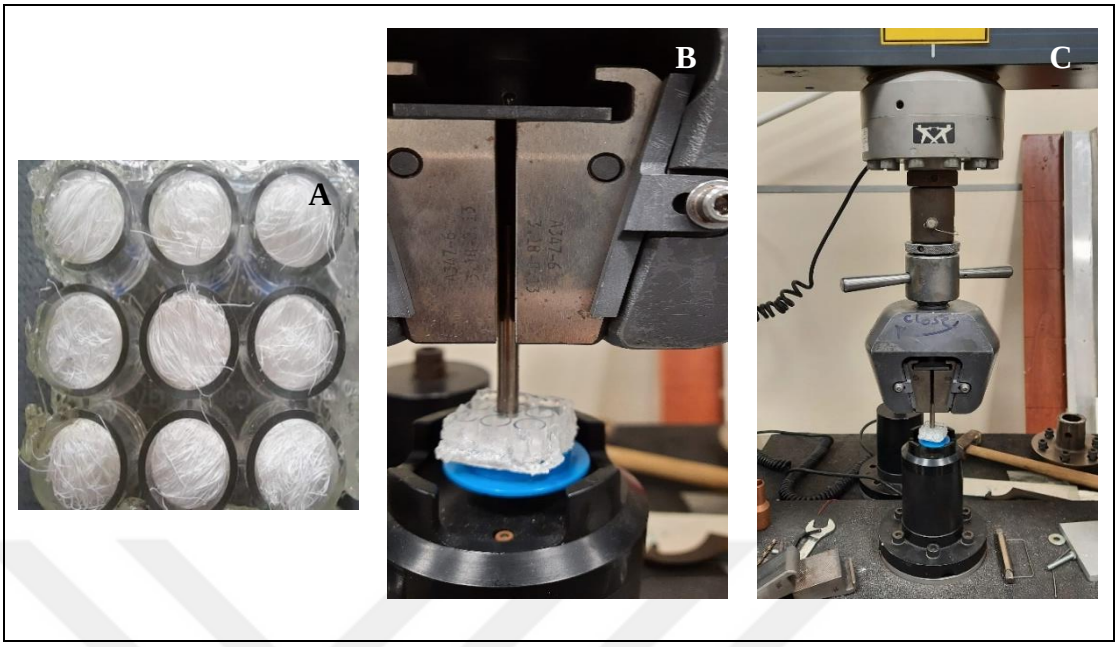

Figure 3.3. Compression analysis (A) PLA samples before compression test, (B) Compression grids, (C) Compression test set up.

3.1.4. Cell Proliferation on Scaffolds

After degradation and mechanical analysis of PLA fibers, their suitability for cell attachment and growth were determined by using cell proliferation (MTS) assay. RBMSCs were trypsinized, seeded onto the sterilized scaffolds in 20 µL growth medium (20,000 cell per well), and incubated at 37°C, in 90 percent humidity and 5 percent CO_2 on incubator. After 2 hours of cell seeding, 250 µL cell growth medium was slowly added into each well and placed again into the cell culture incubator. The media were replaced twice a week with fresh growth media.

After 1, 7 and 14 days of incubation, cell proliferation on the scaffolds were measured by MTS method. Fort this, CellTiter 96® AQueous One Solution was diluted with growth media at 1:6 ratio. The cell seeded scaffolds were put into wells of the new plate and 250 µL MTS solution was added onto each scaffold and blank containing wells. The plate was incubated in cell culture incubator for 2 hours. Then, the absorbance of samples was measured at 490 nm by using ELISA plate reader and cell proliferation rate was calculated by using MTS standard curve.

3.2. *IN VITRO* CELL CULTURE STUDIES

3.2.1. Isolation of Stem Cells

RBMSCs were isolated from 6–8-week-old Sprague Dawley rats. The ethical permission was received from Yeditepe University Experimental Animals Ethical Committee. The femoral and tibial bones were removed from sacrificed rats and put into harvest medium that contains 10 precent (v/v) PSA (Penicillin-Streptomycin- Amphotercin) in low glucose DMEM.

Muscles and connective tissues were cleaned from the femur and tibia via a steril gauze under the laminar flow hood. The ends of bones were cut with bone scissors and the bone marrow was flushed by using a 10 mL syringe and collected in a 50 mL centrifuge tube. The extracted bone marrow was centrifuged at 2,000 rpm for 5 min. The cell pellet was suspended in growth media (GM) (Low Glucose DMEM with 10 percent FBS, 1 percent PSA) and seeded into T75 flasks. The flasks were incubated in CO_2 incubator (5 percent CO_2, 90 percent humidity and 37 ºC). After 2 days incubation, culture medium was thrown away and cells were washed with PBS and culture medium was replenished in every 3 days. The cells were incubated until the confluency and trypsinized cells were frozen in 10 percent DMSO containing FBS for the rest of the study.

3.2.2. Characterization of Stem Cells

Stem cell characterization studies were carried out, when the cells reached to passage 3. Osteogenic, chondrogenic and adipogenic differentiation capacity of the stem cells were examined by Alizarin Red, Alcian Blue PAS, Oil Red O stainings, respectively.

Osteogenic differentiation medium was prepared by supplementing the low glucose DMEM with 10 percent FBS, 1 percent PSA, 50 µg/mL ascorbic acid and 100 nM dexamethasone. For chondrogenic differentiation medium, high glucose DMEM was supplemented with 1 percent ITS premix, 1 percent PSA, 50 µg/mL ascorbic acid, 40 µg/mL L-proline, 10 µg/mL sodium pyruvate, 10 ng/mL TGF-β and 100 nM dexamethasone. Adipogenic differentiation

medium was prepared by supplementing the high glucose DMEM with 100 µM indomethacin, 500 µM IBMX, 10 µg/mL insulin and 100 nM dexamethasone.

RBMSCS were seeded into 24 well plates at 10,000 cell per well ratio. Osteogenic, chondrogenic and adipogenic differentiation media were added onto the related differentiation groups and GM was added onto the control cell wells. Characterization stainings were done at 7, 14 and 21 days of differentiation study. Before staining, cells were washed with DPBS and fixed with 3.7 percent formaldehyde solution (v/v) in DPBS for 45 min at +4ºC. Fixed cells were washed 3 times with dH_2O and then stainings were conducted on the samples.

3.2.2.1. Alizarin Red Staining

Alizarin Red staining was carried out to show osteogenic differentiation in order to observe calcium depositions. Alizarin Red staining solution (0.5 mL) was put into the wells and incubated at RT for 45 mins. After that samples were washed several times with dH_2O and cells were observed under the brightfield microscope and photographed with Zen software.

3.2.2.2. Alcian Blue-PAS Staining

Alcian Blue-PAS staining was conducted for chondrogenic differentiation in order to observe extracellular matrix deposition. Alcian Blue staining solution (0.5 mL) was put into the wells and waited for 10 min at RT. Then the wells were washed twice with dH_2O and periodic acid solution was dropped into the wells and waited for 5 mins. Wells were washed with dH_2O twice, Schiff reagent was put into the wells and incubated for 20 mins. The Schiff reagent was thrown away and the wells were washed under running tap water. They were stained with Haemalum Mayer for 1 min and washed several times with dH_2O. The samples were observed under the brightfields microscope and photographed with Zen software.

3.2.2.3. Oil Red O Staining

Oil Red O staining stock solution was prepared by dissolving the powdered Oil Red O in absolute isopropanol with 3µg/mL (w/v) ratio and incubated for 10 min at RT and then filtered through a 0.45 µm syringe filter. The stock solution was diluted with dH_2O at 3:2 (v/v) ratio right before the staining and incubated at RT for 10 mins. The Oil Red O staining was done for adipogenic differentiation. Fixed cells were incubated with 60 percent isopropanol solution for 5 mins. Then the wells were stained with Oil Red O solution for 30

min at RT then the wells were washed several times with dH_2O. The cells were observed under the brightfield microscope and photographed with Zen software.

3.2.3. Effects of Small Molecules on Chondrogenic Differentiation

3.2.3.1. Alcian Blue-PAS Staining

RBMSCs at passage 3 were seeded into 24 well plates (10,000 cell per well). After 5 days of incubation in complete growth medium, chondrogenic differentiation media and small molecules were added to the related groups. Growth medium and chondrogenic differentiation medium was prepared as indicated in sections 3.2.1 and 3.2.2. There was eight different group in this part: Control Growth Media (Control GM), Control Chondrogenic Media (Control CM), Kartogenin containing Growth Media (KGN GM), Kartogenin containing Chondrogenic Media (KGN CM), ADZ containing Growth Media (ADZ GM), ADZ containing Chondrogenic Media (ADZ CM), small molecule SKF containing Growth Media (SKF GM) and small molecule SKF containing Chondrogenic Media (SKF CM). The medium of the cells was replaced twice a week and small molecules were introduced to the media to related groups at 150 nM concentration.

On days 1, 7 and 14, cell fixation and Alcian Blue-PAS staining were carried out as indicated in 3.2.2.2 and samples were observed and photographed under the brightfield microscope.

3.2.3.2. Cell Proliferation (MTS) Assay

The effects of small molecules on proliferation of differentiated RBMSCs was determined by MTS cell proliferation assay. MTS assay was done on the chondrogenicly differentiated cells after 1, 7 and 14 days of incubation. The medium of the wells was removed and 500 µL of CellTiter 96® AQueous One Solution in growth media (1:6) was added into each well. The plate was incubated for 2 hours in cell culture incubator and the absorbance was measured at 490 nm by using ELISA plate reader.

3.2.4. Effects of Combination of Small Molecule SKF with KGN

In previous section, effect of ADZ and SKF on differentiated RBMSCs were compared. Since much better results were obtained with the small molecule SKF, next coming experiments were carried on with only this small molecule. To observe the effects of combination of small molecule SKF with KGN, Alcian Blue-PAS staining and MTS assay were applied onto RBMSCs. There was two control and 6 experimental group as: control growth medium (Control GM), control chondrogenic media (Control CM), KGN in growth medium (KGN GM), KGN chondrogenic media (KGN CM), KGN+SKF growth medium (KGN+SKF GM), KGN+SKF chondrogenic media (KGN+SKF CM), SKF growth medium (SKF GM) and SKF chondrogenic media (SKF CM). While the same protocol was followed for Alcian Blue-PAS staining and MTS assay (section 3.2.3). RT-PCR was also performed to determine the gene expression during the chondrogenic differentiation.

For the RNA isolation of control and chondrogenic differentiation groups, RBMSCs at passage 3 were seeded onto 12 well plates (20,000 cell per well). The cells were grown in growth media 5 days after seeding. Then, the chondrogenic media (1 mL) was added to the Control CM, SKF CM, KGN CM and KGN+SKF CM groups while growth media (1 mL) was given to the Control GM, SKF GM, KGN GM and KGN+SKF GM groups. At days 7, 14 and 21 RNA isolation was carried out from the cells by using RNA Spin Total RNA Isolation Kit and its protocol. The obtained RNA was measured via nanodrop spectrophotometer.

cDNA was synthesized from the total RNA samples using the iScript cDNA synthesis kit. Variable volume of nuclease free water (NFW) and RNA template were used to obtain equal concentration of the final cDNA of each sample. They put into nuclease free PCR tubes, then 4 µL of reaction mix and 1µL of the reverse transcriptase enzyme was added into the tubes which were spin down for equal mixing. The reaction tubes were put into the ProFlex PCR machine and reaction protocol was applied as priming 5 min at 25 ℃, reverse transcription for 20 min at 46℃, RT inactivation at 96℃ and final hold at 4℃ for 20 µL total reaction volume. At the end of the reverse transcription, samples were diluted with 80 µL NFW and used following RT-PCR protocols.

Then, RT-PCR method was performed by using SYBR Green Maxima Master Mix Kit and primers that are listed in Table 1. For each primer a master mix was prepared by using the components except the cDNA (Table 2). Nuclease free 96 well PCR plate was put on the ice and 2 µL of cDNA of related group was put into each well. Then, 23 µL of master mix was added into each well. The plate was centrifugated at 130 x g at 4℃ for 1 min for spinning down of reagents and the plate was placed on the CFX Touch™ Real Time PCR machine. The gene amplification procedure was conducted as seen in Table 3. Gene expressions of the experimental groups were calculated from the $2^{-\Delta\Delta Ct}$ values of the genes. β-actin gene was used as a reference gene and the CG sample was the control sample in the fold change calculations.

Table 3.1. Primer sequences and melting temperatures used in RT-PCR

Gene Name	**Primer Sequence (5' → 3')**	**Product Length**	**Tm (℃)**
β-actin	F: CCCGCGAGTACAACCTTCTT	481 bp	60℃
	R: AACACAGCCTGGATGGCTAC		
SOX9	F: AGTACCCGCATCTGCACAAC	358 bp	60℃
	R: ACGTCTGTTTTGGGAGTGGT		
COL1A1	F: GATGGACTCAACGGTCTCCC	580 bp	60℃
	R: CAGGATCGGAACCTTCGCTT		
COL2A1	F: CACCGCTAACGTCCAGATGAC	276 bp	59℃
	R: GGAAGGCGTGAGGTCTTCTGT		
AGGRECAN	F: CATTCGCACGGGAGCAGCCA	133 bp	61℃
	R: TGGGGTCCGTGGGCTCACAA		
RUNX1	F: CCAAACTCTGAAAGCGAGGC	300 bp	60℃
	R: CTGGCATCTACGGGGATACG		

Table 3.2. Volumes of the reagents used in RT-PCR

Reagent	Volume
SYBR Green Maxima Master Mix	12.5 µL
Nuclease Free Water	9 µL
Reverse Primer (from 10µMprimer solution)	0.75 µL
Forward Primer (from 10µMprimer solution)	0.75 µL
cDNA	2 µL
Total volume	25 µL

Table 3.3. Thermal cycling conditions of the RT-PCR

Step	Temperature	Time	Number of Cycles
Pretreatment	50℃	2 min	1
Initial Denaturation	95℃	10 min	1
Denaturation	95℃	15 sec	55
Annealing	60℃	30 sec	
Extension	72℃	30 secs	

3.3. *IN VITRO* CELL AND SCAFFOLD INTERACTIONS

The interactions between RBMSCs and PLA scaffolds were determined by using Alcian Blue-PAS staining, MTS assay and RT-PCR. RBMSCs were trypsinized at passage 2 and seeded (30,000 cell per well for Alcian Blue PAS staining and MTS assay, 50,000 cell per well for RT-PCR) into PLA scaffolds. The cells were incubated for 5 days with complete growth medium in cell culture incubator. Then, chondrogenic medium was introduced to the differentiation groups and small molecules (150 nM) were added into related groups. There were three different experimental groups as KGN Differentiation Medium (KGN CM),

KGN+SKF Chondrogenic Differentiation Medium (KGN+SKF CM), SKF Chondrogenic Differentiation Medium (SKF CM) and Control Chondrogenic Differentiation Medium (Control CM). Chondrogenic differentiation was performed and different analysis methods were carried out cell seeded scaffolds at days 7, 14, 21 and 28.

3.3.1. Alcian Blue-PAS Staining

The cell seeded scaffolds were washed twice with PBS and fixated with 3.7% formaldehyde solution in PBS. Alcian Blue-PAS staining was applied on fixed cells by using Alcian-Blue PAS Stain Kit and the stained scaffolds were photographed by using a camera.

3.3.2. Cell Proliferation (MTS) Assay

Cell proliferation was determined by using MTS assay. The medium was removed from the wells and diluted CellTiter 96® AQueous One Solution in growth medium (250 μL) was added onto the scaffolds. After 2 hours of incubation, the absorbances of media were measured in 490 nm.

3.3.3. RT-PCR

RBMSCs (50,000 cell per well) were seeded into scaffolds after passage 2. After cell seeding, chondrogenic differentiation was started by introducing chondrogenic media to Control CM, KGN CM, KGN+SKF CM and SKF CM groups together with the related small molecules. Also, there was a growth media control for gene expression calculations. At the end of days 7, 14, 21 and 28, RNA isolation was carried out on scaffolds. The medium was removed from wells, the scaffolds were put into 2 mL nuclease free centrifuge tubes. After that, the cells lysed with lysis buffer (650 μL) of RNA Spin Total RNA Isolation Kit, and other steps were followed from kit protocol. cDNA synthesis and RT-PCR were applied with the same protocol and materials as indicated in section 3.2.4.

3.4. STATISTICAL ANALYSIS

Statistical analysis was performed by two-way ANOVA test on experimental data via Prism GraphPad software. Data was $p < 0.05$ accepted statistically significant.

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF SCAFFOLDS

Cartilage tissue engineering has three main elements: scaffold, cell source and growth factors. Scaffolds do not only provide mechanical support to cartilage grafts during tissue regeneration, also give 3-D architectural structure for cell proliferation, migration, ECM matrix deposition, nutrient and growth factor diffusion between tissue implant and native tissue [93]. Controlled biodegradability, biocompatibility, mechanical resistance, proper porosity and nontoxicity are important parameters required for scaffolds in cartilage tissue engineering study [70]. Therefore, prepared fibrous PLA scaffolds were characterized by SEM, degradation, mechanical and cell proliferation analyses.

4.1.1. Scanning Electron Microscopy

Morphological and architectural features of the fibrous PLA scaffolds were determined by SEM analysis. Disk shaped PLA fibers were cut with punch, they were attached onto glass slide and coated with platinum. PLA scaffolds were observed with SEM at 100X and 1000X magnification (Figure 4.1). The diameter of PLA fibers was observed between 45-110 μm from microscopy images. Literature studies show that well defined structure of fibrous scaffolds are desirable for cell attachment and migration [94,95]. Our PLA fibers seemed proper for cell attachment with their continuous fibrous structure without breaks.

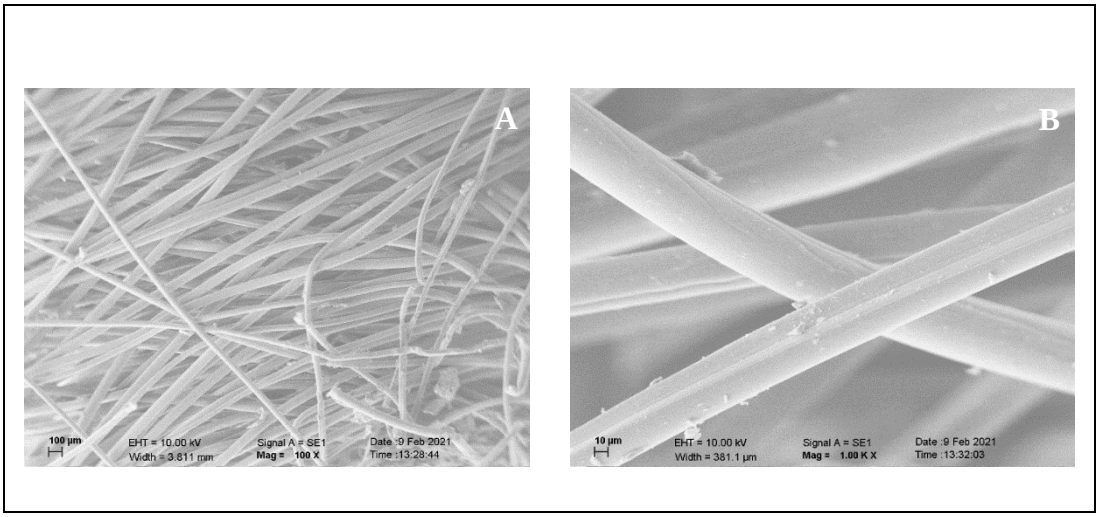

Figure 4.1. SEM images of fibrous PLA scaffold at different magnifications (A) 100X (B) 1000X

4.1.2. Degradation Analysis

Degradation features of scaffolds are important, since it directly affects cell differentiation, ECM matrix production and tissue regeneration in cartilage tissue engineering [43]. When cartilage tissue construct is placed into cartilage defect of a patient, ECM matrix is produced by chondrogenic cells seeded on scaffolds and tissue regeneration comes through via replacement of ECM matrix with degraded scaffold [96]. Therefore, scaffold degradation must be slow enough to support cartilage regeneration. Fibrous PLA scaffolds were incubated in PBS solution at 37 ℃ in mechanical shaker throughout 60 days to determine degradation properties. Weight and pH of scaffolds at day 3, 7, 15, 30 and 60 were measured to determine weight loss, water retention capacity and pH change during incubation.

While weight loss was determined by comparing initial weights of scaffolds with final dry weights of scaffolds, water retention capacity was calculated via measuring wet and dried weights of scaffolds after incubation period (Figure 4.2 A, B). Although fast weight loss of PLA (12 %) was recorded after day 7, PLA scaffolds lost their weights gradually from day 3 to day 60 and 17 % weight loss was calculated at the end of 60 days of incubation. Similar weight loss profiles were presented in literature in a way that PLA scaffolds rapidly degrade at first 7 days and degradation rate decreases from day 7 to 28 days [97]. On the other hand, water retention capacity revealed fluctuation during 60 days of incubation. It increased from

day 3 to day 15, decreased at day 30 and then increased again at day 60. Water retention capacity is related with hydrophilic feature of scaffold. It effects attachment of cells to scaffold surface, therefore high water retention capacity is a desirable feature [70]. In water retention results, increase in water retention capacity was seen during degradation.

Change of pH is another important parameter during scaffold degradation. PLA is a polymer composed of lactic acid monomers and its degradation results in a pH decrease in an environment due to lactic acid release from a polymer. Although lactic acid is not toxic in human body and does not cumulate in organs, sudden pH changes in tissues may lead to inflammation and tissue regeneration on implanted tissue constructs cannot be possible [97]. In our PLA scaffolds, pH decreased from 7.4 to 7.3 during first 30 days, then decreased to 6.81 at day 60 (Figure 4.2 C). In one of the study, PLA scaffolds caused pH change from 7.4 to 5.1 after 28 days of degradation [98]. In another study, fibrous PLA scaffolds degraded throughout 20 days and pH was changed from 7.3 to 6.8 [99]. Degradation characteristics of our PLA scaffolds in term of weight loss, water retention and pH change seem similar with the studies in the literature, and the fibrous PLA scaffolds can provide proper environment for cell growth when used as a cartilage tissue engineering construct.

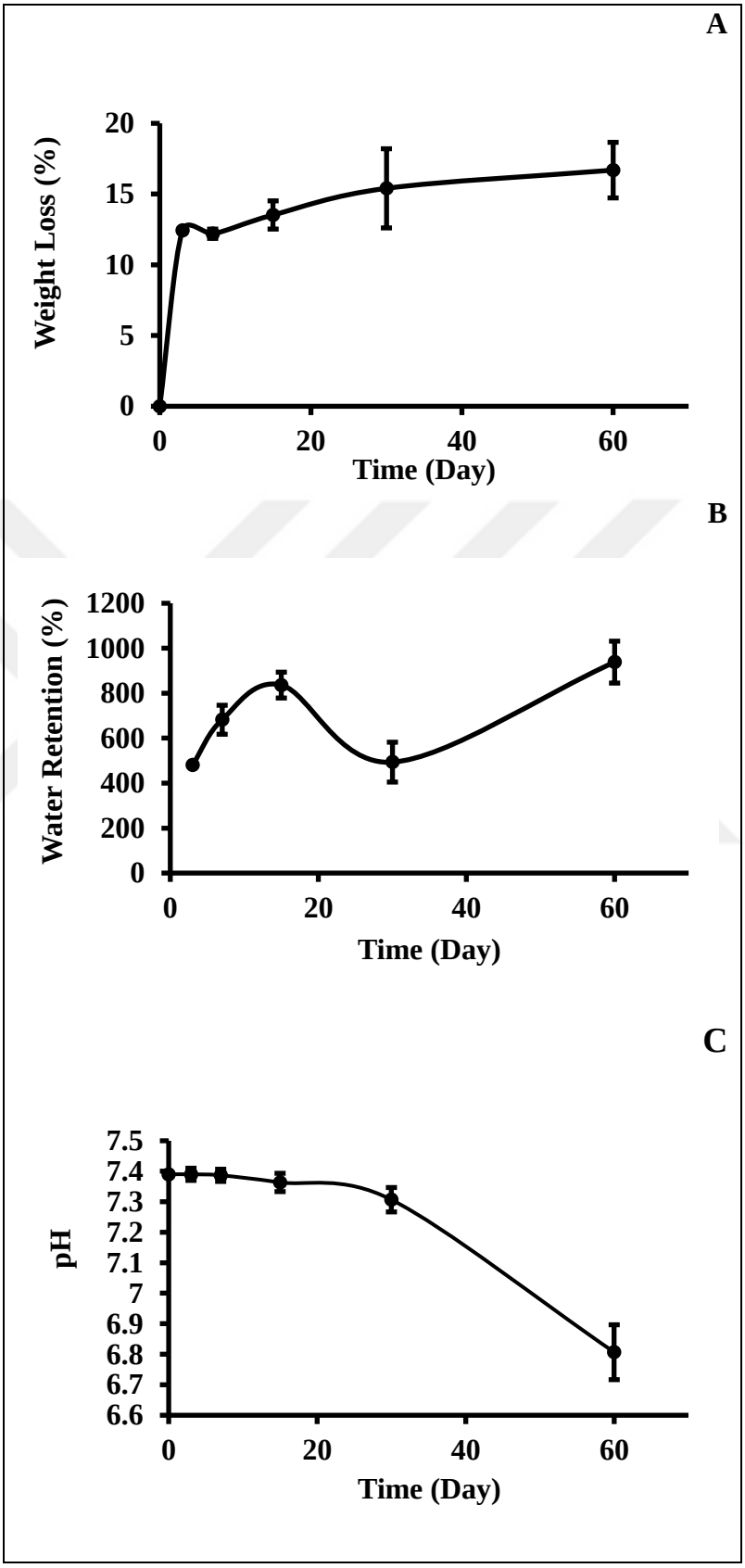

Figure 4.2. Degradation analysis of fibrous PLA scaffolds (A) % weight loss, (B) % water retention, and (C) pH change of scaffolds throughout 60 days of incubation

4.1.3. Mechanical Analysis

Mechanical properties of the scaffolds should show the resemblance with the original tissue. Therefore, mechanical resistance of scaffolds to elastic and compressive forces must be examined. Mechanical analysis of materials is based on measuring their stress and strains values when they expose to tension or compression forces to find out Young's modulus of material. During mechanical tests, dynamic force is applied on an object to achieve constant movement of an object throughout test time. While pulling force is applied in tension test, elastic Young's modulus of object is determined. On the other hand, compressive force is applied on an object in compression test and compression Young's modulus is determined. The Young's modulus (E) is defined by dividing stress that is applied force (F) on an object through cross sectional area (A) to strain that is a ratio of amount of change of material length (ΔL) to initial length ($L_{\circ}$).

$$E = \frac{F/A}{\Delta L/L_{\circ}} \tag{4.1}$$

The PLA fibers were tested via Instron Universal Testing Machine. Tension and compression tests were conducted on fibers. In tension test, fiber bundle was placed into tension machine. During the test, dynamic force was applied on fiber bundle to stretch it 5 mm/sec and stress-strain curve of tension test was drawn from data (Figure 4.3 A). The elastic modulus of the PLA fibers was calculated as 30.82 ± 0.93 MPa from the curve. In one of the biomechanics study, human knee joint tensile modulus was determined and it was generally under 30 MPa, although it can be raised to 105 MPa in some samples [100]. Our scaffolds represented close elastic modulus to native cartilage. On the other hand, compression test of PLA fibers was determined via using compression heading of Universal Test Machine. PLA fibers were filled into molds and compressed with 5 mm/min movement rate. Stress-strain curve of compression test was drawn and compression Young's modulus was calculated as 13.61 ± 0.27 MPa from the curve (Figure 4.3 B). In literature, human native articular cartilage compression modulus was reported as 10.60 ± 3.62 MPa [101]. Both elastic and compression Young's modulus of the PLA fibers were found close to modulus of native human cartilage. It indicates that PLA scaffolds are strong enough for

bearing compressive and elastic loads and are able to support human articular cartilage defects.

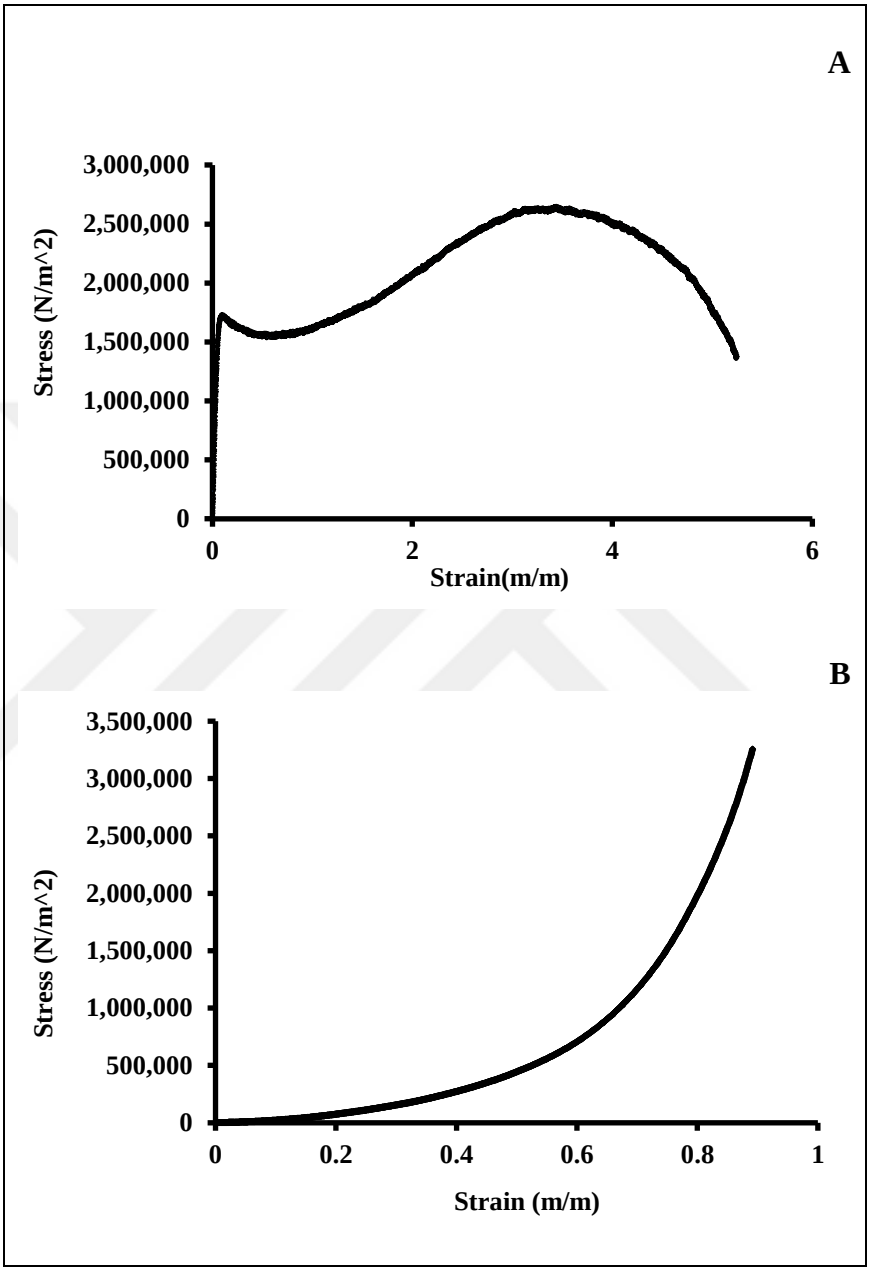

Figure 4.3. Stress-strain curves of PLA fibers (A) tensile and (B) compression tests.

4.1.4. MTS Assay on Scaffolds

MTS cell proliferation assay is a colorimetric method used for determination of cell viability and proliferation. MTS is a water-soluble tetrazolium salt; with a full name of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).
MTS is converted to formazan in metabolically active cells by NADPH-dependent dehydrogenase enzymes found in mitochondria. Formazan has a maximum absorbance at 490 nm and it is soluble in PBS or culture medium.

RBMSCs were seeded both onto scaffolds (20,000 cell per well) and tissue culture plates (10,000 cell per well) and cultured with growth medium throughout 14 days. MTS cell proliferation assay was conducted on samples to determine the effects of fibrous PLA scaffolds on cell proliferation and cell viability. MTS calibration curve of RBMSCs was prepared to calculate cell numbers (Appendix B.1). Measured values of absorbance at 490 nm with calibration equation were used for calculation of viable cell number and cell proliferation on the scaffolds and the culture plates are represented as only cell (Figure 4.4). Although initial RBMSCs number was 20,000 cells per well for PLA scaffolds and 10,000 cells per well for tissue culture plate, MTS results of day 1 was found similar (10,000 per well) for both scaffolds and only cell samples. That means, 50 percent of the seeded RBMSCs attached onto PLA scaffolds. There are various studies examining cell attachment on PLA fibers. Those studies are consistent with our MTS results and revealed that cell attachment ratio on PLA fiber changes between 10 percent to 60 percent on fibrous synthetic polymer based scaffolds [63,102,103]. At days 7 and 14, increased cell proliferation was observed in the scaffolds like the one in only cell samples. The fibrous PLA scaffolds allowed both RBMSCs attachment and proliferation by providing 3-D cell culture environment.

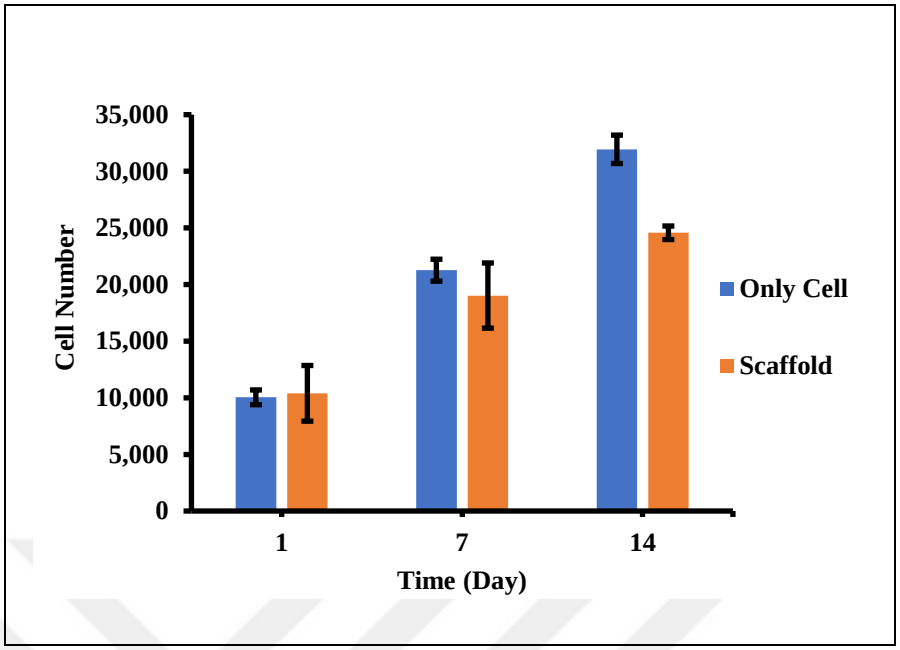

Figure 4.4. MTS cell proliferation assay onto RBMSCs seeded PLA scaffolds throughout 14 days of incubation. Initial cell number was 20,000 cells per well for scaffolds and 10,000 cell per well for cells seeded onto tissue culture plate (only cell).

4.2. *IN VITRO* CELL CULTURE STUDIES

In this part, RBMSCs and fibrous PLA scaffold interaction was investigated. For that purpose, RBMSCs were isolated from 8 weeks old rat, then evaluated in term of stem cell multipotency and used in examination of chondrogenic induction via small molecules ADZ, SKF and KGN.

4.2.1. Isolation of Bone Marrow Derived Stem Cells from Rats

RBMSCs were isolated from femoral and tibial bone marrow of 8 weeks old Sprague-Dawley rats and cultured on T-75 flask with growth medium. At passage 2, RBMSCs were observed under brightfield microscope (Figure 4.5). Fibroblastic spindle shape morphology of RBMSCs was observed.

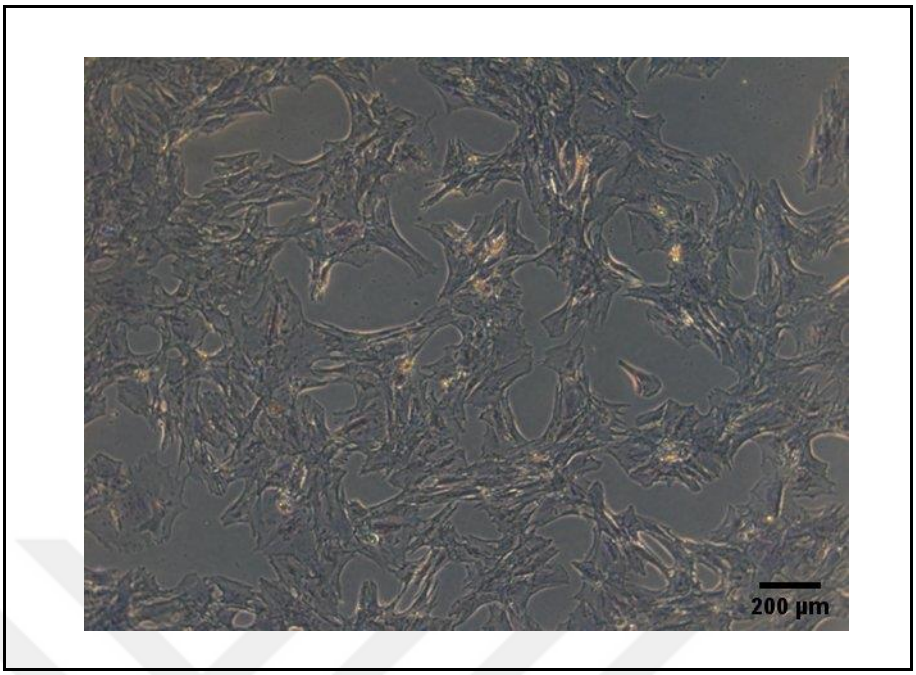

Figure 4.5. RBMSC morphology under bright field microscope with 10X objective

4.2.2. Characterization of RBMSCs

RBMSCs are multipotent stem cells that can differentiate into osteogenic, chondrogenic, adipogenic or myogenic lineages when appropriate growth conditions are provided. To prove multipotency of isolated RBMSCs, osteogenic, chondrogenic and adipogenic media were given and differentiated cells were compared with controls.

4.2.2.1.Alizarin Red Staining

Alizarin Red (3,4-Dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid) is a dye used for identification of calcium depositions of osteogenic cells. It chelates with calcium deposits in tissues and produces red colored stain. To prove osteogenic potential of RBMSCs, they were incubated with the osteogenic differentiation medium throughout 21 days and stained with Alizarin Red and images were taken under bright field microscope at days 7, 14 and 21 (Figure 4.6 A-F). While no staining was seen in controls, increasing calcium deposition with time was observed as red to brown colored stainings in differentiated RBMSCs in osteogenic medium. That means, isolated RBMSCs had osteogenic differentiation capacity.

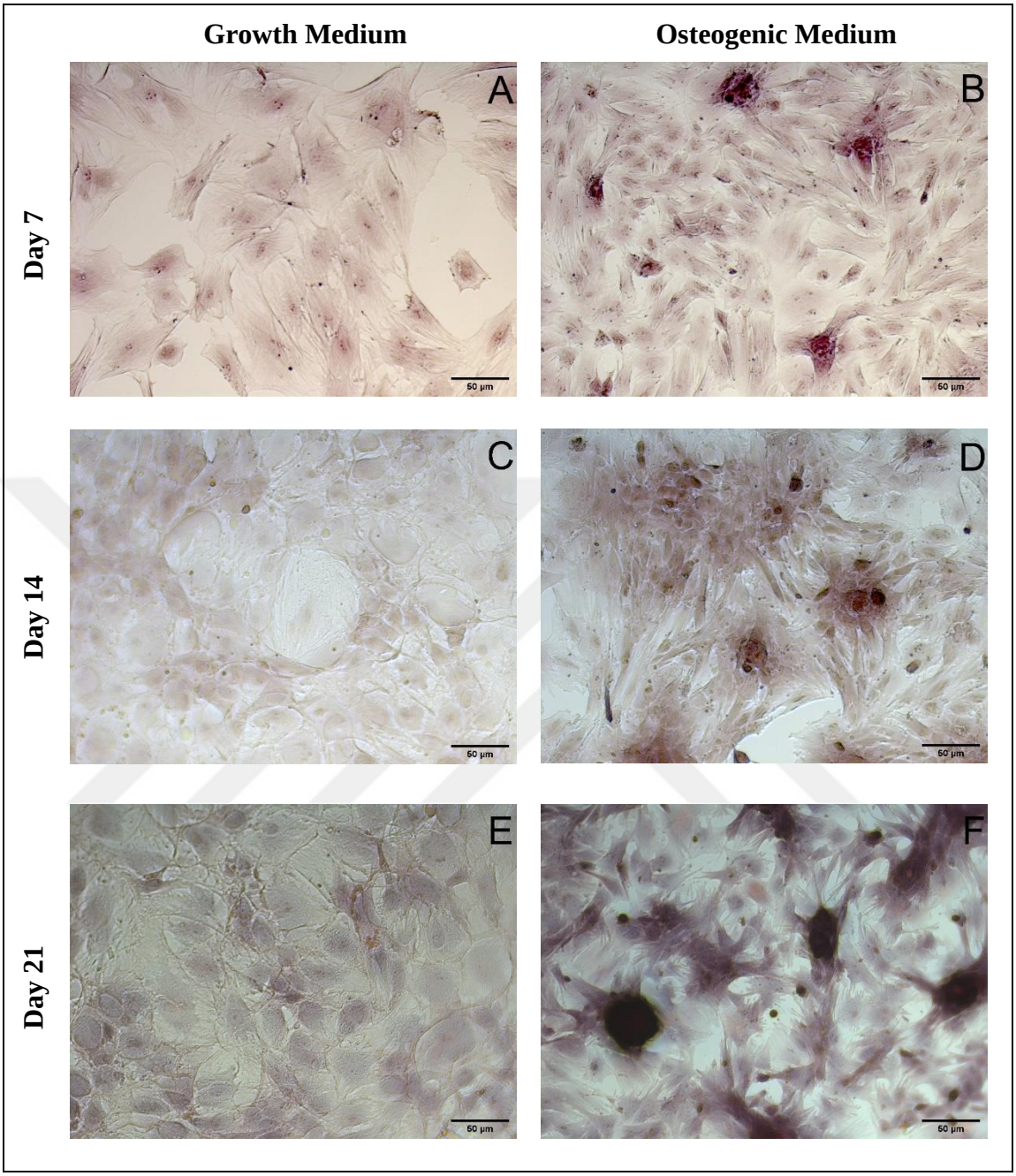

Figure 4.6. Bright field microscope image of Alizarin Red staining of RBMSCs in growth medium (A, C, E) at days 7, 14, 21 and RBMSCs in osteogenic medium (B, D, F) at days 7, 14, 21 at 20X objective, scale bars: 50 µm

4.2.2.2.Alcian Blue-PAS Staining

Alcian Blue-Periodic Acid Shiff is a staining technique that includes multiple stain to observe chondrogenic differentiation. While Alcian Blue stains acidic mucins such as sulfated and carboxylated mucopolysaccharides and hyaluronic acid to blue, Periodic Acid

and Shiff reagent stains neutral mucins like glycogens and neutral glycoproteins to pink. Alcian Blue-PAS staining was applied on differentiated RBMSCs in chondrogenic medium to prove chondrogenic potential of isolated cells and observed under brightfield microscope at 20X objective (Figure 4.7 A-F). At day 7, blue colored acid mucins were observed in chondrogenicly differentiated RBMSCs, purple colored both acid and basic mucins were stained at days 14 and 21. Increasing blue-purple color formation throughout 21 days of incubation was observed, whereas control groups were stained pale purple. Therefore, chondrogenic differentiation capacity of RBMSCs were proven with the Alcian Blue-PAS staining.

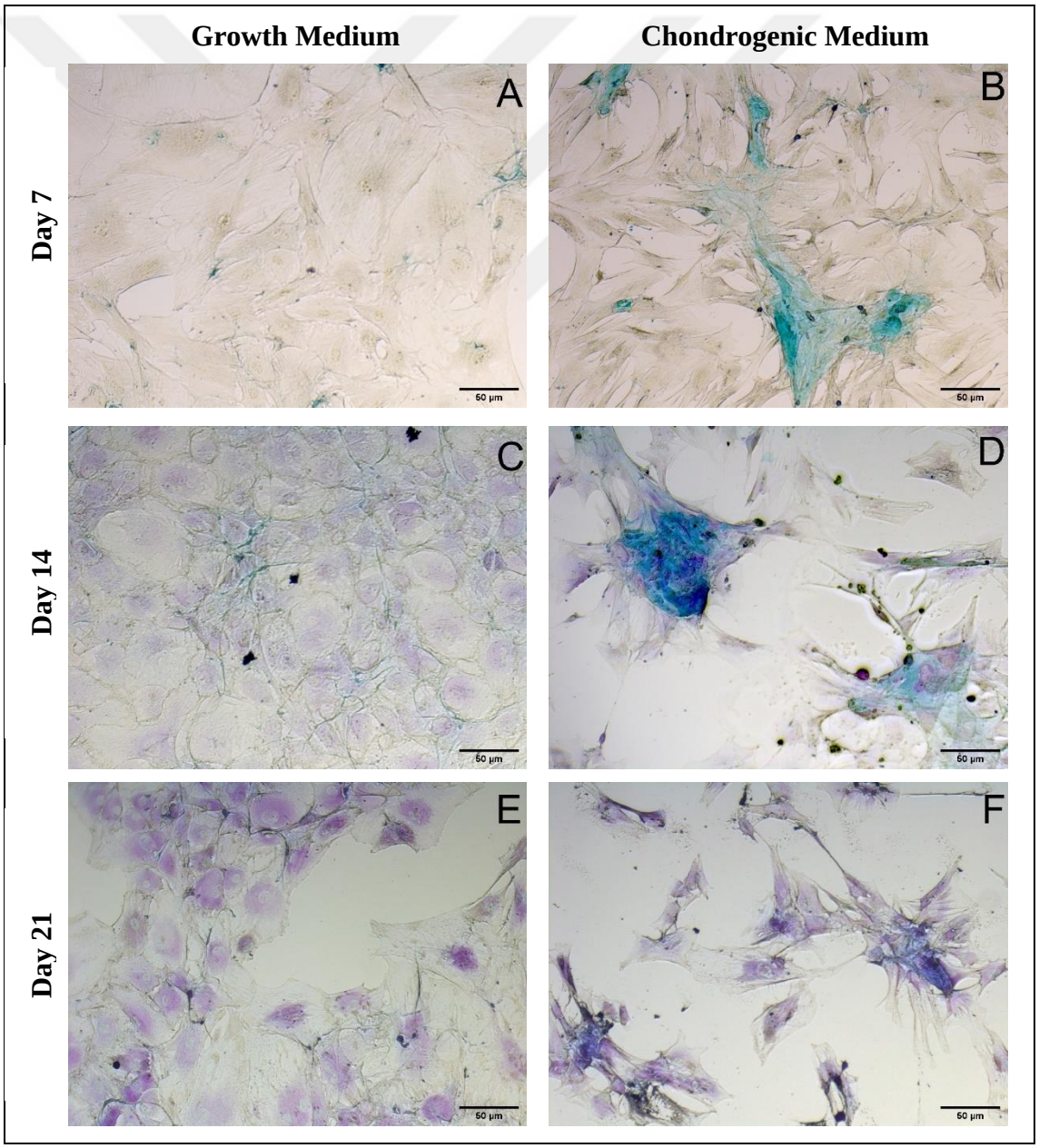

Figure 4.7. Bright field microscope image of Alcian Blue-PAS staining of RBMSCs in growth medium (A, C, E) at days 7, 14, 21 and RBMSCs in chondrogenic medium (B, D, F) at days 7, 14, 21 at 20X objective, scale bars: 50 μm

4.2.2.3.Oil Red O Staining

Oil Red O is a fat-soluble dye that produces bright red color with fats. It is used for staining of lipids and neutral triglycerides to identify fat depositions of adipocytes. While RBMSCs were grown in adipogenic differentiation medium, growth medium was used for control groups throughout 21 days of incubation. Adipogenic differentiation capacity of RBMSCs were determined via using Oil Red O staining at days 7, 14 and 21 and observed under bright field microscope at 20X objective (Figure 4.8 A-F). At day 14, some fat depositions were observed in differentiated RBMSCs. Increased amount of fat deposition was observed at day 21 whereas no staining was observed on control groups. Multipotency of isolated RBMSCs was proved with three different staining Alizarin Red, Alcian Blue-PAS and Oil Red O staining to demonstrate osteogenic, chondrogenic and adipogenic differentiation capacity of the cells.

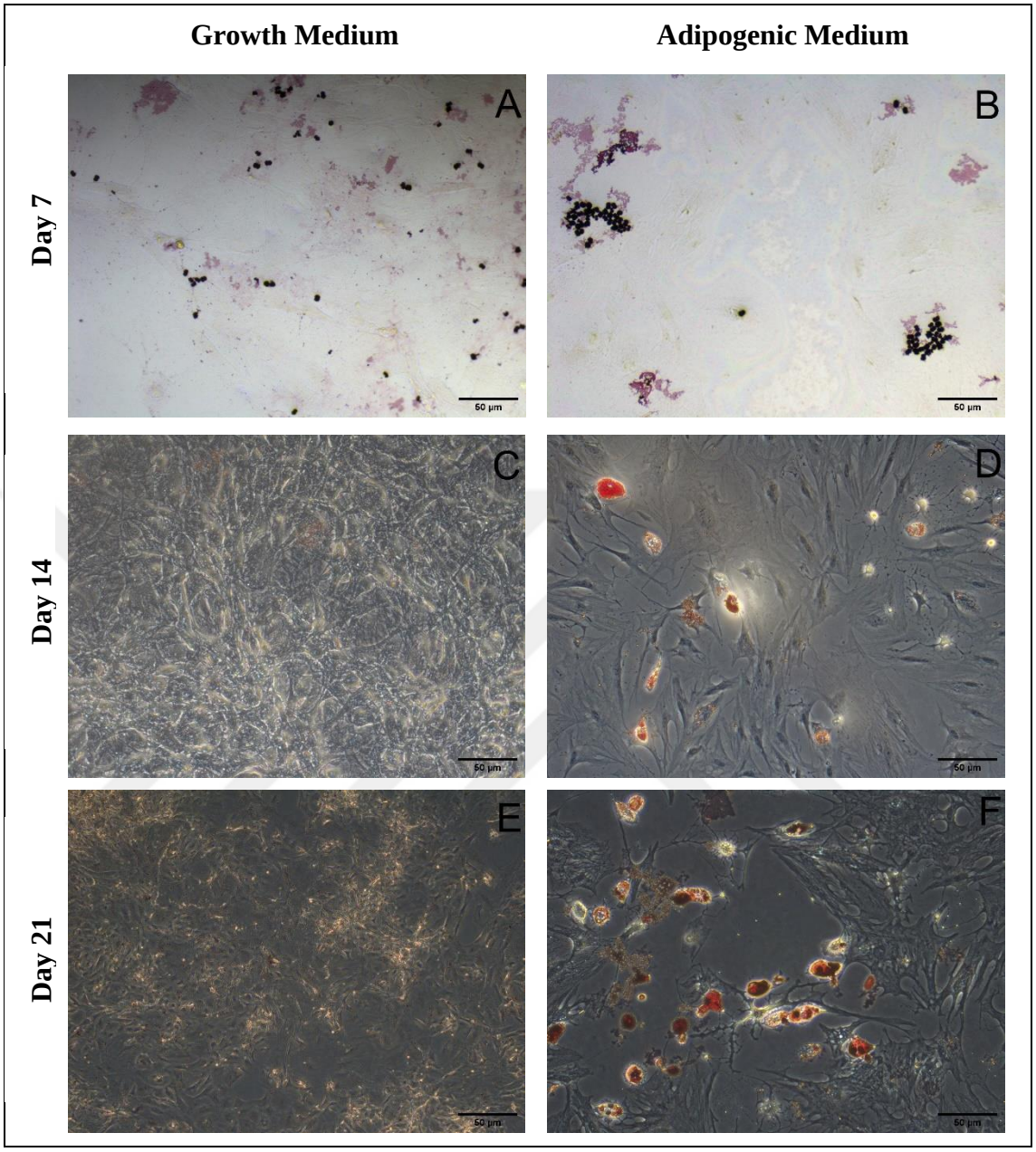

Figure 4.8. Bright field microscope image of Oil Red O staining of RBMSCs in growth medium (A, C, E) at days 7, 14, 21 and RBMSCs in adipogenic medium (B, D, F) at days 7, 14, 21 at 20X objective, scale bars: 50 µm

4.2.3. Effects of Small Molecules on Chondrogenic Differentiation

KGN is a commercially available small molecule known with chondrogenic induction, chondroprotection, enhancing chondrogenic ECM matrix production features on stem cells [24]. On the other hand, small molecules ADZ and SKF are recently produced and

researched molecules that were shown to induce proliferation of MSCs. There is no study in the literature about effects of small molecules ADZ and SKF on chondrogenic differentiation. In this study, Alcian Blue-PAS staining and MTS cell proliferation assay were done to determine the effects of small molecules KGN, ADZ and SKF on chondrogenic differentiation, ECM matrix production and cell proliferation. RBMSCs were cultured in chondrogenic medium for differentiation and control groups were cultured in growth medium. Small molecules at 150 nM concentration were supplemented to these media and the cells were incubated throughout 14 days. MTS assay and Alcian Blue-PAS staining were conducted on RBMSCs at day 1, day 7 and day 14.

4.2.3.1.Alcian Blue-PAS Staining

Alcian Blue-PAS staining was carried out at day 1, 7 and 14 to observe ECM matrix production on differentiated RBMSCs induced with small molecules. KGN, ADZ and SKF were applied on both growth medium (GM) and chondrogenic medium (CM) groups separately, and also one GM control and one CM control groups were cultured without any small molecule treatment. At day 1, it was observed that the appearance of RBMSCs were all the same on undifferentiated groups (Figure 4.9 A-H). Pale blue staining of the cell nuclei and fibroblast like shape of RBMSCs were observed in both GM and CM groups.

Increased cartilaginous ECM production was observed with bright purple and blue colored ECM depositions at day 7 (Figure 4.10 A-H) and day 14 (Figure 4.11 A-H) in all CM groups. Pale purple color was indicating less ECM production in all GM groups, while cell number was higher in GM groups than the ones in CM groups since cell proliferation rate decreases with cell differentiation on RBMSCs.

When small molecules KGN, ADZ and SKF were compared with their controls at days 7 and 14, more purple and blue colored stainings of differentiated RBMSCs were seen in all CM groups than the one in control groups. Also, in GM groups of SKF and KGN, purple and blue stainings were seen higher than the ones in ADZ GM and control GM groups. It may indicate that small molecule SKF had a chondrogenic induction potential even in GM like KGN.

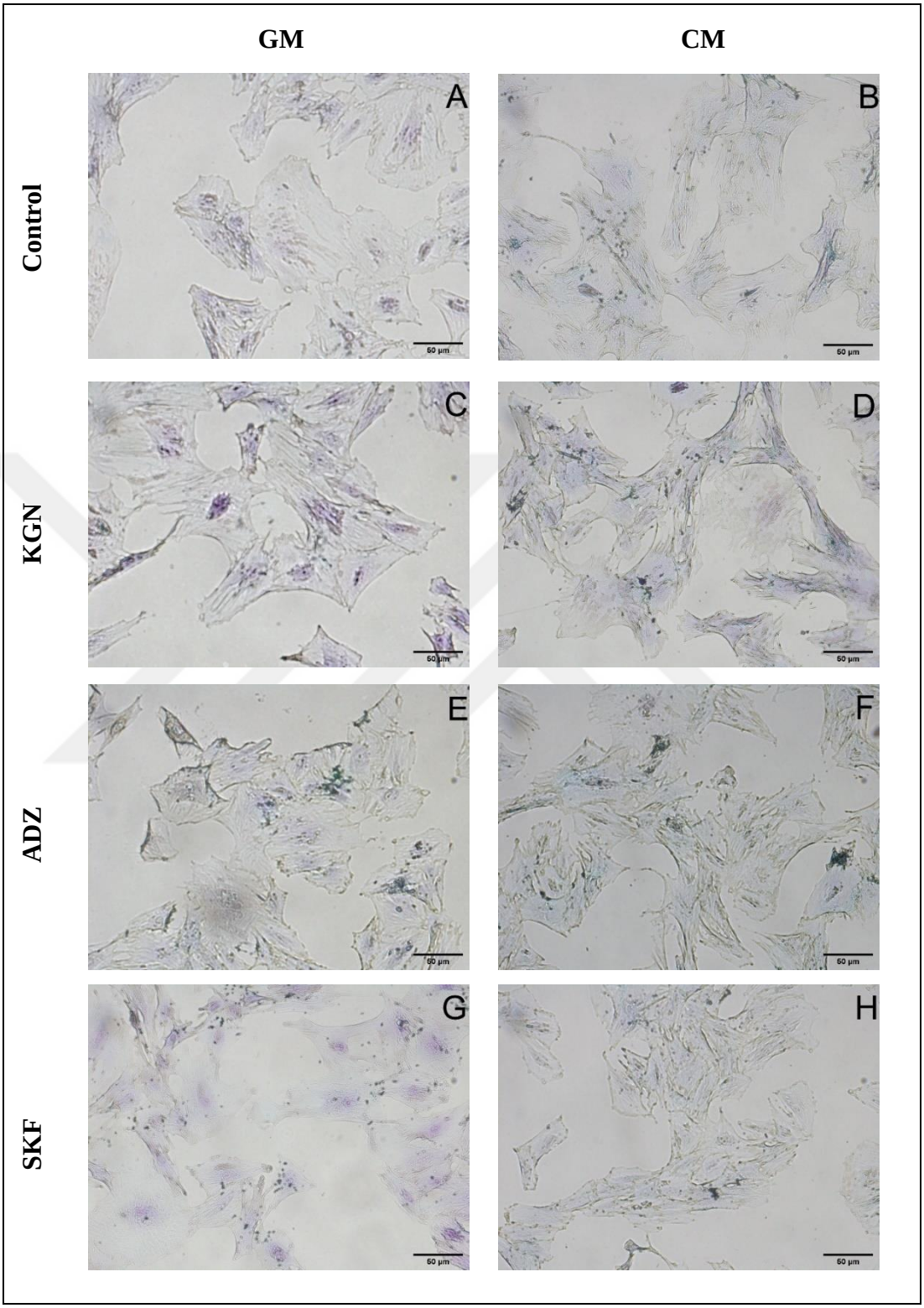

Figure 4.9. Bright field microscope image of Alcain Blue-PAS staining of RBMSCs in growth medium (A, C, E, G), and RBMSCs in chondrogenic medium (B, D, F, H) treated in small molecule KGN (C, D), ADZ (E, F), SKF (G, H) at day 1 (20X objective, scale bars: 50 µm)

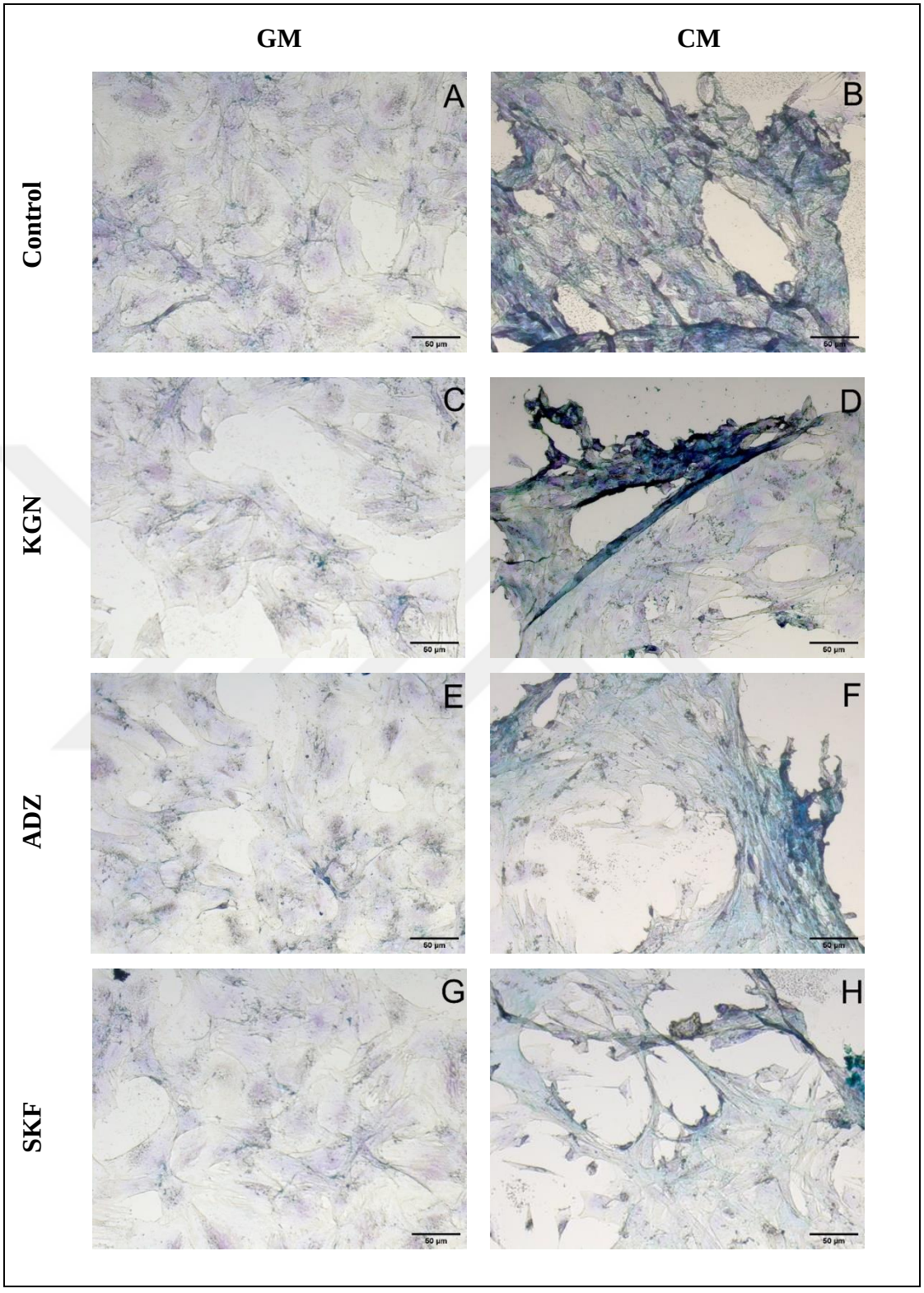

Figure 4.10. Bright field microscope image of Alcain Blue-PAS staining of RBMSCs in growth medium (A, C, E, G), and RBMSCs in chondrogenic medium (B, D, F, H) treated in small molecule KGN (C, D), ADZ (E, F), SKF (G, H) at day 7 (20X objective, scale bars: 50 µm)

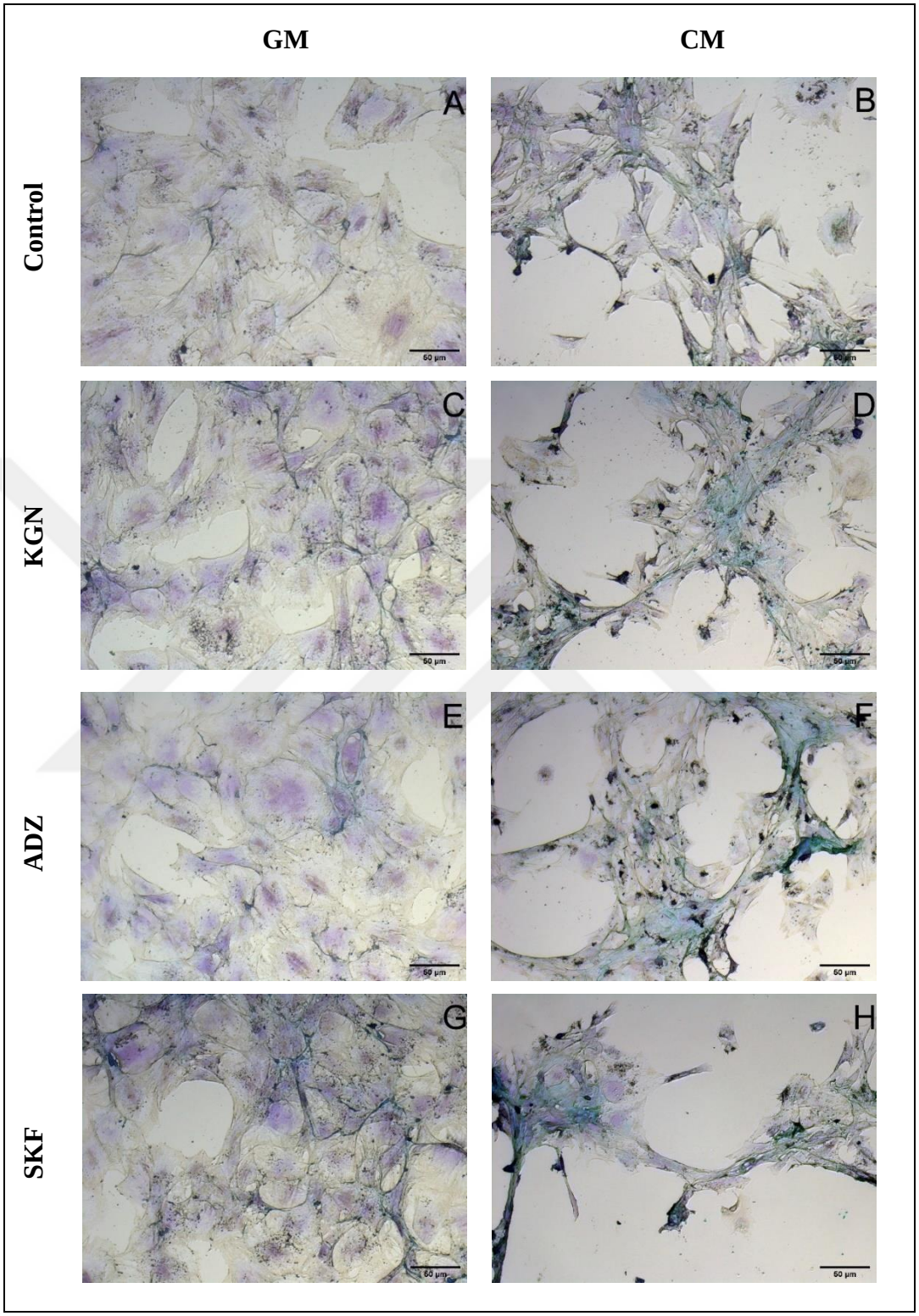

Figure 4.11. Bright field microscope image of Alcain Blue-PAS staining of RBMSCs in growth medium (A, C, E, G), and RBMSCs in chondrogenic medium (B, D, F, H) treated in small molecule KGN (C, D), ADZ (E, F), SKF (G, H) at day 14 (20X objective, scale bars: 50 µm)

4.2.3.2.MTS Assay

MTS is a colorimetric method used for measuring cell proliferation and cell viability. The absorbance recorded at 490 nm is proportional to both cell proliferation activity and viable cell number [104,105]. MTS assay was used to determine the effects of small molecules ADZ, SKF, and KGN on chondrogenic differentiation of RBMSCs (Figure 4.12.). After day 1, lower cell numbers (around 45,000) in CM groups were observed due to cell differentiation. However, it was around 55,000 in GM groups. Even tough, cell proliferation in Control GM, KGN GM, ADZ GM and SKF GM groups increased from day1 to day 7, they decreased at day 14 due to full confluency in the plates, therefore, cells were detached. On the other hand, cell proliferation decreased in Control CM, KGN CM and SKF CM groups throughout 14 days of chondrogenic differentiation. Only cell proliferation increase between CM groups was seen in ADZ CM group from day 1 to day 7, however, it decreased at day 14. Although ADZ enhanced cell proliferation of differentiated RBMSCs at day 7 and day 14, MTS and Alcian Blue-PAS staining results must be evaluated together to find out the effects of small molecules on chondrogenic differentiation. In Alcian Blue-PAS staining, higher ECM deposition was observed in SKF CM and KGN CM groups when compared to ADZ CM and Control CM groups. ADZ induced higher proliferation rates at day 7 and day 14 but this resulted with lower chondrogenic matrix production. By looking at these results, small molecule SKF displayed similar MTS and Alcian Blue-PAS staining results to KGN that was already proven with proliferative and chondrogenic induction ability on MSCs in literature studies [24,81,83]. Therefore, small molecule SKF was found superior to ADZ and the study was continued with only small molecule SKF and its chondrogenic induction features was compared with KGN.

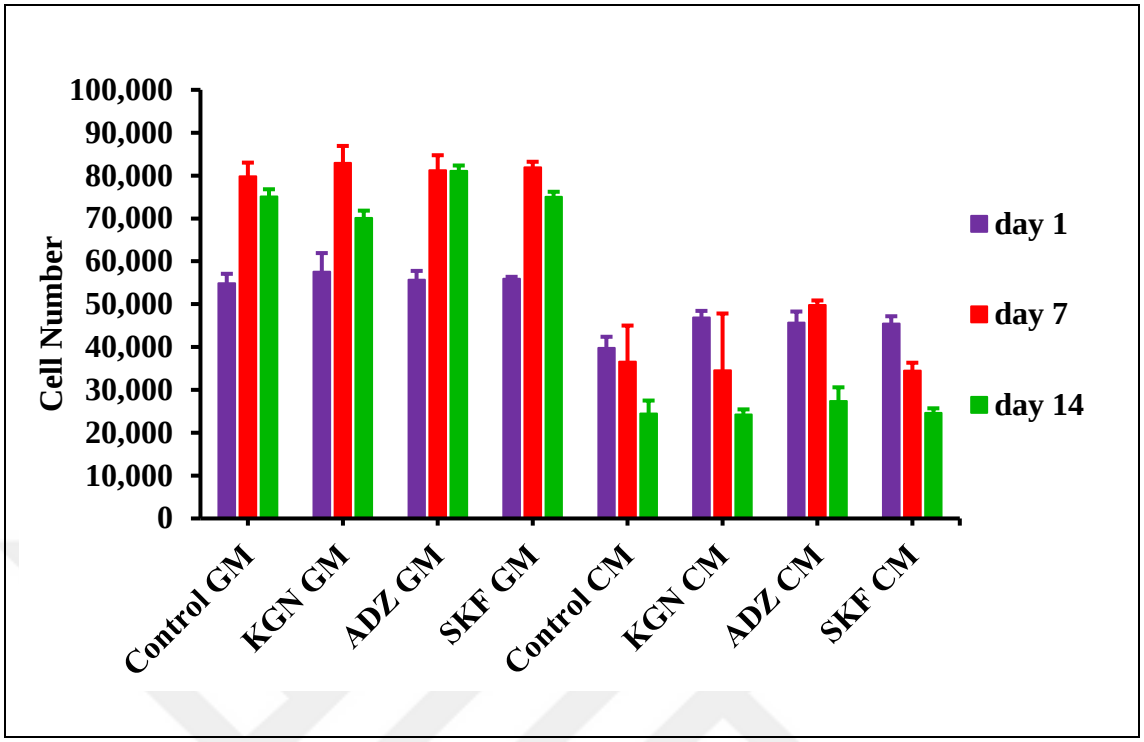

Figure 4.12. MTS cell proliferation assay performed on RBMSCs treated with growth chondrogenic medium and small molecules KGN, ADZ and SKF throughout 14 days of incubation. Initial cell seeding number was 10,000 cells per well.

4.2.4. Effects of Combination of Small Molecule SKF with KGN

In previous section, small molecules ADZ and SKF were compared with KGN and superiority of SKF was seen over ADZ in terms of chondrogenic induction and cell proliferation. Therefore, rest of the experiments were conducted with small molecule SKF and effects of combination of small molecule SKF with KGN were examined. While RBMSCs were differentiated in CM throughout 14 days, they were compared with undifferentiated GM groups. While small molecule SKF was introduced into SKF GM and SKF CM groups, KGN was given to the KGN GM and KGN CM groups. Besides, both SKF and KGN were introduced into KGN+SKF GM and KGN+SKF CM groups. All small molecules were applied at same concentration (150 nM) and no small molecule was applied on the control GM and control CM groups. Alcian Blue-PAS staining, MTS cell proliferation assay and RT-PCR assay were carried out for RBMSC groups at day 1, day 7 and day 14.

4.2.4.1.Alcian Blue-PAS Staining

After 1, 7 and 14 days of incubation, RBMSCs were stained with Alcian Blue-PAS and observed under bright field microscope at 20X objective. Both GM and CM groups were observed pale blue color at day 1 (Figure 4.13 A-H). There was no difference between controls (Figure 4.13 A, B) and small molecule groups (Figure 4.13 C-H). On the other hand, blue stained ECM with Alcian Blue was started to seen at day 7 (Figure 4.14 A-H) CM groups and increased at day 14 (Figure 4.15 A-H) CM groups which indicates that chondrogenic ECM production contains proteoglycans and glycoproteins during differentiation. At day 7, there was no significant difference between small molecule groups KGN CM, KGN+SKF CM, SKF CM and control CM group (Figure 4.14 B, D, F, H). However, more purple color on ECM was observed in KGN+SKF CM and SKF CM groups when compared to KGN CM and Control CM groups at day 14 (Figure 4.15 B, D, F, H). Also, KGN GM, KGN+SKF GM and SKF GM groups showed purple blue colored ECM deposition, indicating that small molecule SKF encouraged chondrogenic differentiation even when it was applied alone without chondrogenic differentiation medium (Figure 4.15 A, C, E, G). Small molecule SKF had a potential to compete with KGN that induced MSCs for production of cartilage like ECM when it was given to growth medium.

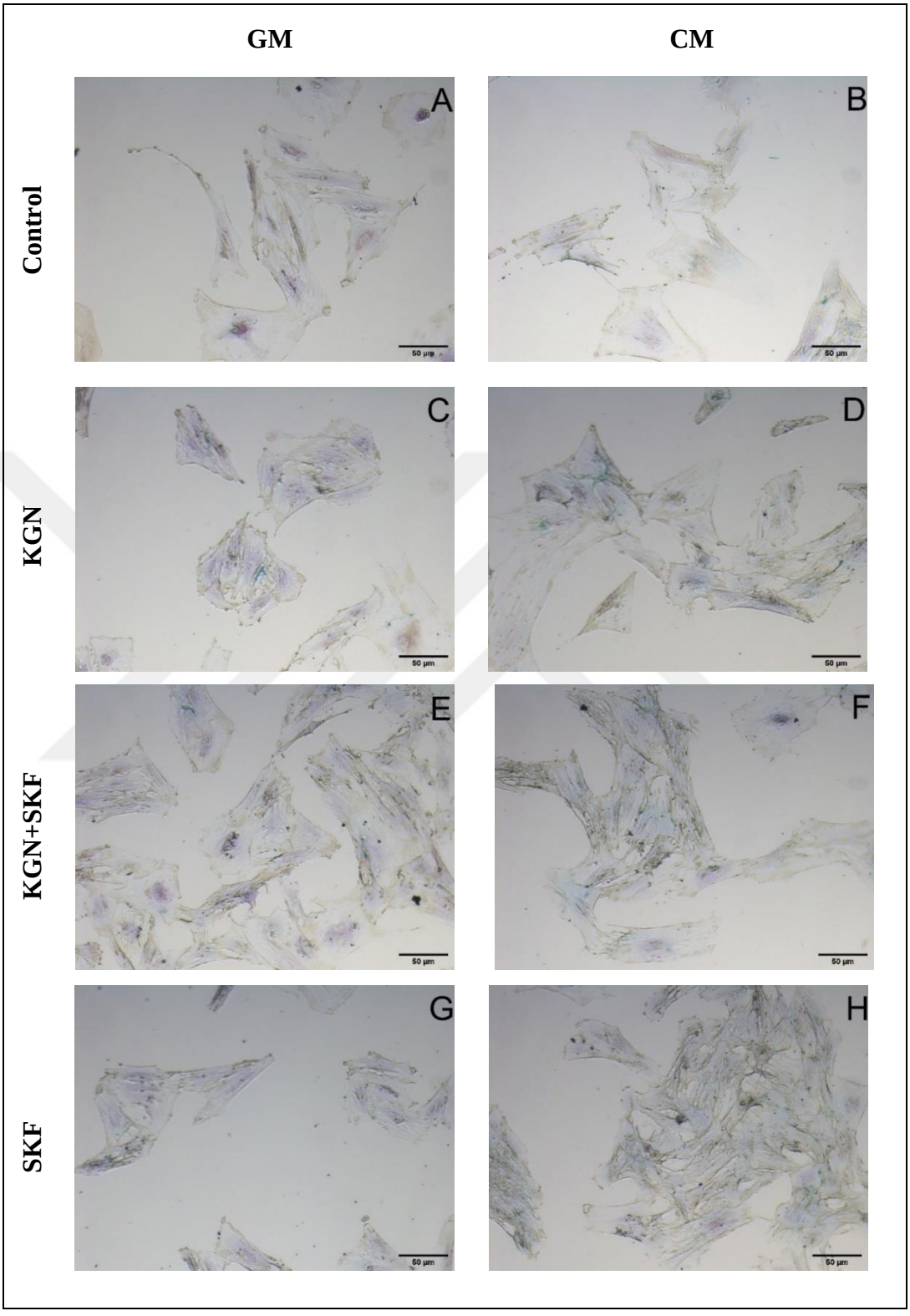

Figure 4.13. Bright field microscope image of Alcain Blue-PAS staining of RBMSCs in growth medium (A, C, E, G), and RBMSCs in chondrogenic medium (B, D, F, H) treated with small molecule KGN (C, D), KGN and SKF (E, F), SKF (G, H) at day1 (20X objective, scale bars: 50 µm)

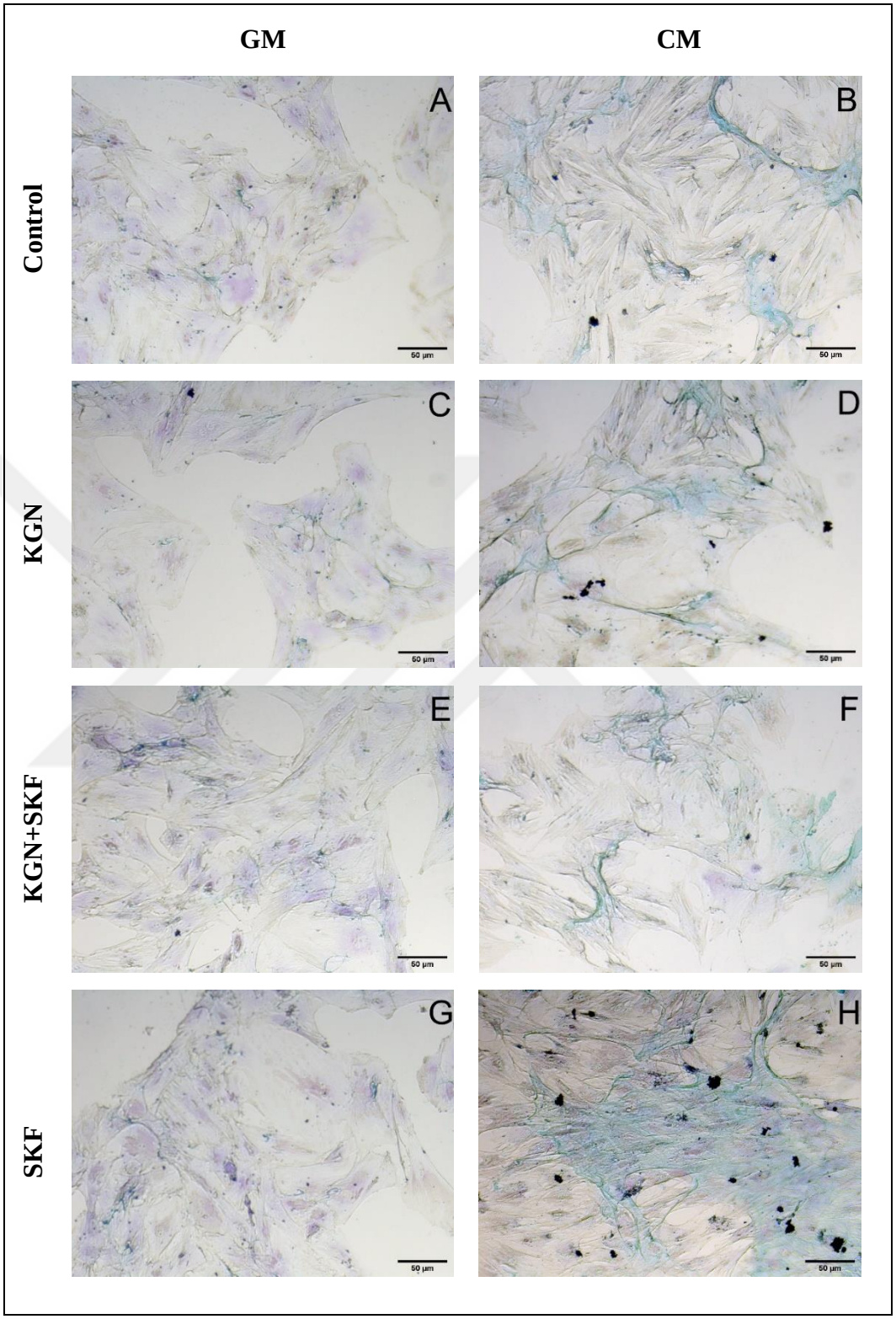

Figure 4.14. Bright field microscope image of Alcain Blue-PAS staining of RBMSCs in growth medium (A, C, E, G), and RBMSCs in chondrogenic medium (B, D, F, H) treated with small molecule KGN (C, D), KGN and SKF (E, F), SKF (G, H) at day7 (20X objective, scale bars: 50 µm)

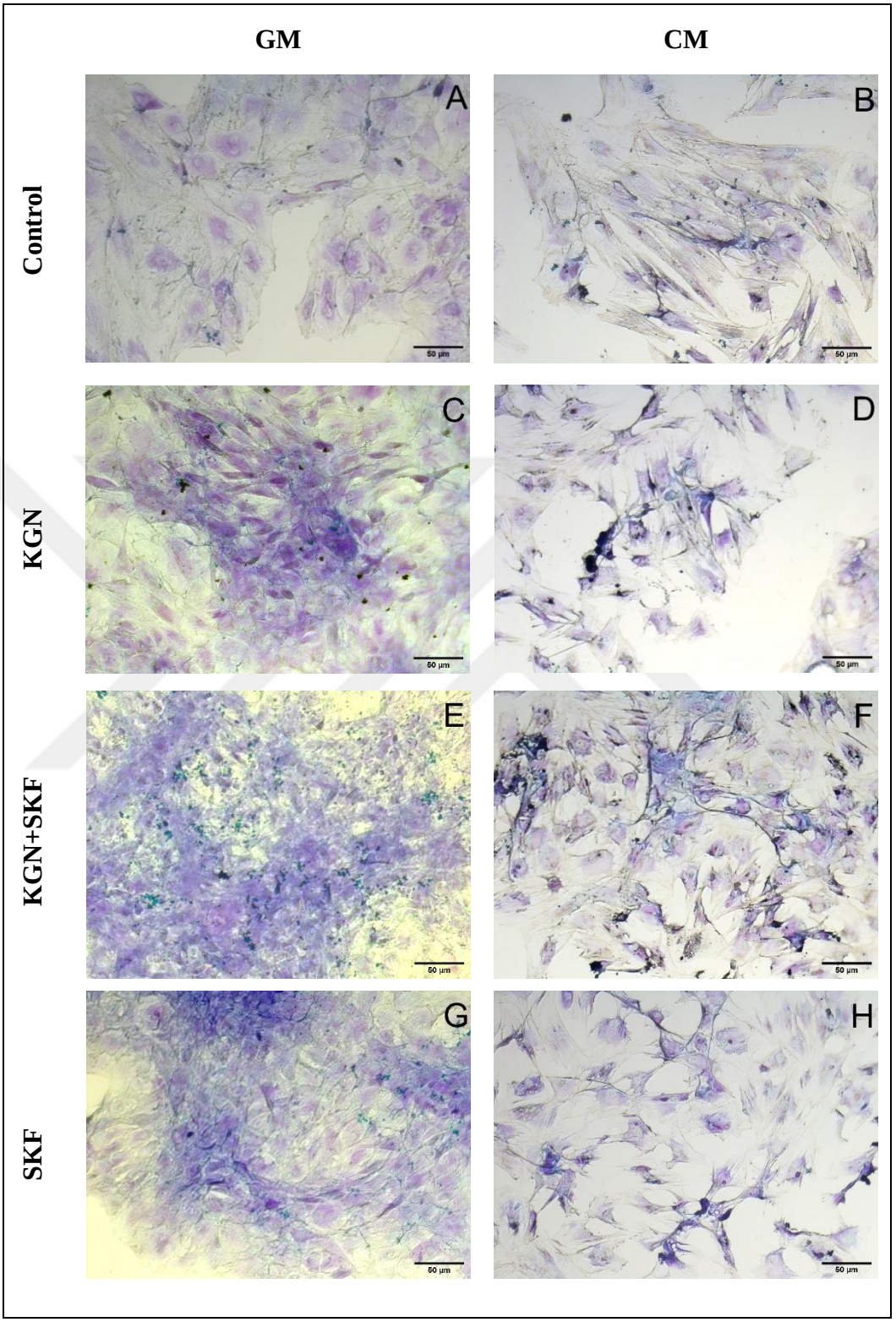

Figure 4.15. Bright field microscope image of Alcain Blue-PAS staining of RBMSCs in growth medium (A, C, E, G), and RBMSCs in chondrogenic medium (B, D, F, H) treated with small molecule KGN (C, D), KGN and SKF (E, F), SKF (G, H) at day14 (20X objective, scale bars: 50 µm)

4.2.4.2.MTS Assay

MTS cell proliferation assay was conducted on RBMSC groups of Control CM, Control GM, KGN CM, KGN GM, KGN+SKF GM, KGN+SKF CM, SKF GM and SKF CM at days 1, 7 and 14 (Figure 4.16). RBMSCs were was seeded into plates (10,000 cell per well) and incubated for 5 days before the treatment. While there were 35,000-40,000 cells in Control GM, KGN GM, KGN+SKF GM and SKF GM groups, there were 17,000-19,000 cells in CM groups, since, chondrogenic differentiation resulted in a decrease in cell proliferation (Figure 4.16).

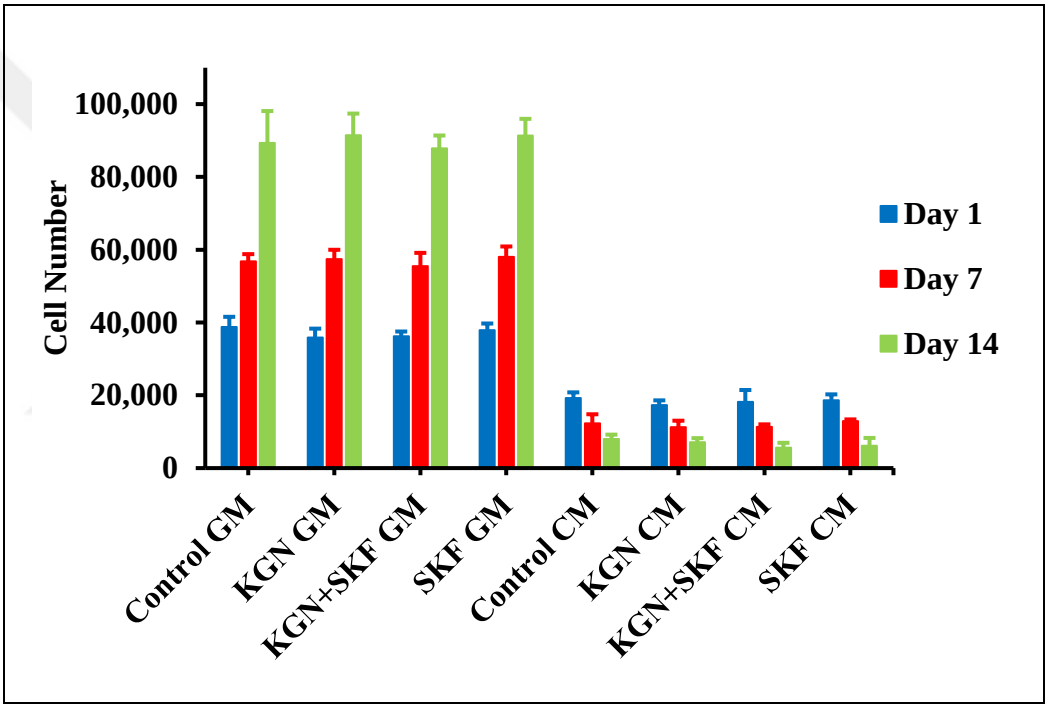

Figure 4.16. MTS cell proliferation assay performed on RBMSCs treated with chondrogenic medium and small molecules throughout 14 days of incubation. Initial cell seeding number was 10,000 cells per well.

4.2.4.3.Real Time Polymerase Chain Reaction (RT-PCR)

RT-PCR analysis was carried out to examine expression of SOX9, RUNX1, COL2A1, ACAN and COL1A1 genes on RBMSCs groups. While CM was used in chondrogenic differentiation groups Control CM, KGN CM, KGN+SKF CM and SKF CM, GM was used in Control GM, KGN GM, KGN+SKF GM and SKF GM groups. Relevant small molecules were introduced into experimental groups and incubated for 21 days. At days 7, 14 and 21,

total RNA was isolated from RBMSCs and cDNA was synthesized. RT-PCR analysis was performed on cDNA samples and gene expression fold changes were calculated with respect to Control GM group via $2^{-\Delta\Delta Cq}$ method (Figure 4.17 A-E).

SRY-box 9 (SOX9) is a transcription factor that plays a key role in upregulation of chondrocyte specific genes COL2A1 and ACAN during chondrogenic differentiation [106]. While COL2A1 gene provides type II collagen protein, ACAN gene provides production of aggrecan protein [107]. Both type II collagen and aggrecan are the main components of cartilage specific ECM and give viscoelasticity, flexibility and mechanical endurance to cartilage tissue [17]. RUNX1 (Runt- related transcription factor 1) is another transcription factor expressed in early stage of chondrogenic differentiation. RUNX1 gene does not only induce chondrogenic differentiation genes like SOX9, COL2A1 and ACAN, but also it blocks the hypertrophy genes COL10A1, RUNX2 and MMP3 that causes pathologic case in mineralization and bone formation in cartilage tissue [108]. RUNX1 gene is suppressed in human MSCs, whereas, KGN induces chondrogenic differentiation by interacting with RUNX1 transcription factor [81]. On the other hand, COL1A1 produced by dedifferentiated chondrocytes another marker is related hypertrophic cartilages, it is highly expressed in tissues has fibrocartilage phenotype [109].

While RUNX1 gene expression increased in CM groups from day 7 to day 14, it decreased at day 21 in all groups (Figure 4.17 B). On the other hand, SOX9 gene expression increased at day 14 and decreased at day 21 (Figure 4.17 A). It is because RUNX1 gene induced expression of SOX9 during chondrogenic differentiation. It is earlier marker than SOX9. The highest fold change of SOX9 gene was observed 44.86-fold in KGN GM group at day 14, while KGN CM, SKF CM, SKF GM and KGN+SKF GM groups expressed 30.41, 32.64 and 21.56 -folds respectively. SOX9 gene expression was significantly lower in KGN+SKF CM (21.56-folds) compared with SKF CM (32.64-folds) and KGN CM (30.41-folds) groups. Applying separately of these two small molecules on chondrogenicly differentiated cells can be better than introducing them together. Results showed that SOX9 expression increased both in CM and GM groups when small molecules were introduced to RBMSCs. Whereas, the highest fold change of RUNX1 gene (5.53-fold) is seen in SKF CM group at day 14. While KGN CM had 4.95-fold and KGN+SKF CM had 4.43-fold and Control CM had 3.95-fold gene expression, gene expression was found around 1 in all GM groups. This means that gene expression of RUNX1 was increased by small molecule induction in CM

groups and gene expression did not change in GM groups. Even though, small molecules enhanced chondrogenic differentiation of RBMSCs, they were not enough for the induction of differentiation when introduced in GM. COL2A1 and ACAN are related genes with ECM deposition and their expressions were expected later than SOX9 and RUNX1 genes. It was observed that gene expression of SKF CM 2.78-fold and KGN+SKF CM 2.89-fold significantly increased at day 21 in COL2A1 gene (Figure 4.17 C). Also, gene expression of ACAN was significantly higher in SKF CM (9.94-fold) and KGN+SKF CM (3.68) compared with control groups. Results showed that small molecule SKF induced chondrogenic differentiation and enhanced ECM production when it was introduced to RBMSCs within CM. COL1A1 gene is a hypertrophy marker for cartilage tissues. Its expression decreased with time throughout 21 days of incubation except in KGN CM and GM groups. Although COL1A1 expression increased at day 14, it decreased at day 21. KGN GM, SKF GM and KGN+SKF GM groups had a higher gene expression COL1A1 compared to CM groups. The lowest COL1A1 expression was observed in Control CM (0.55-fold), but the fold change was approximately 1 in all groups. Results showed that small molecules had no hypertrophic effect resulting in increased COL1A1 expression on RBMSCs.

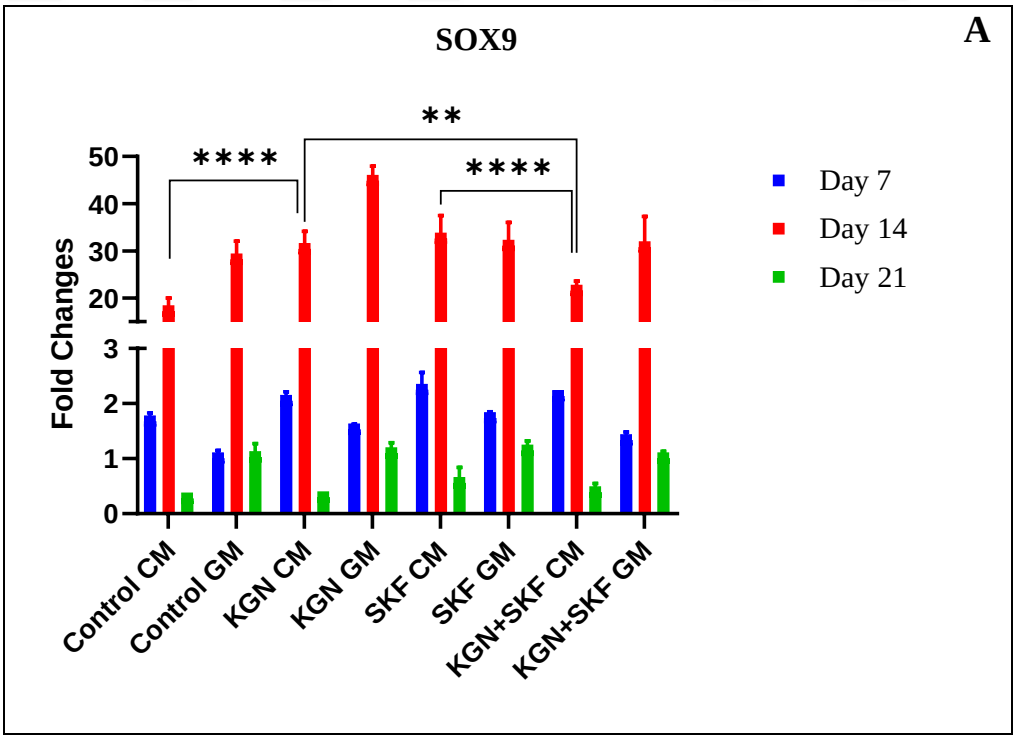

Figure 4.17. Expressions of A) SOX9 B) RUNX1 C) COL2A1 D) ACAN E) COL1A1 genes as fold changes with respect to Control GM groups (**** $P \leq 0.0001$, ***$P \leq 0.0002$, ** $P \leq 0.0021$, * $P \leq 0.0332$)

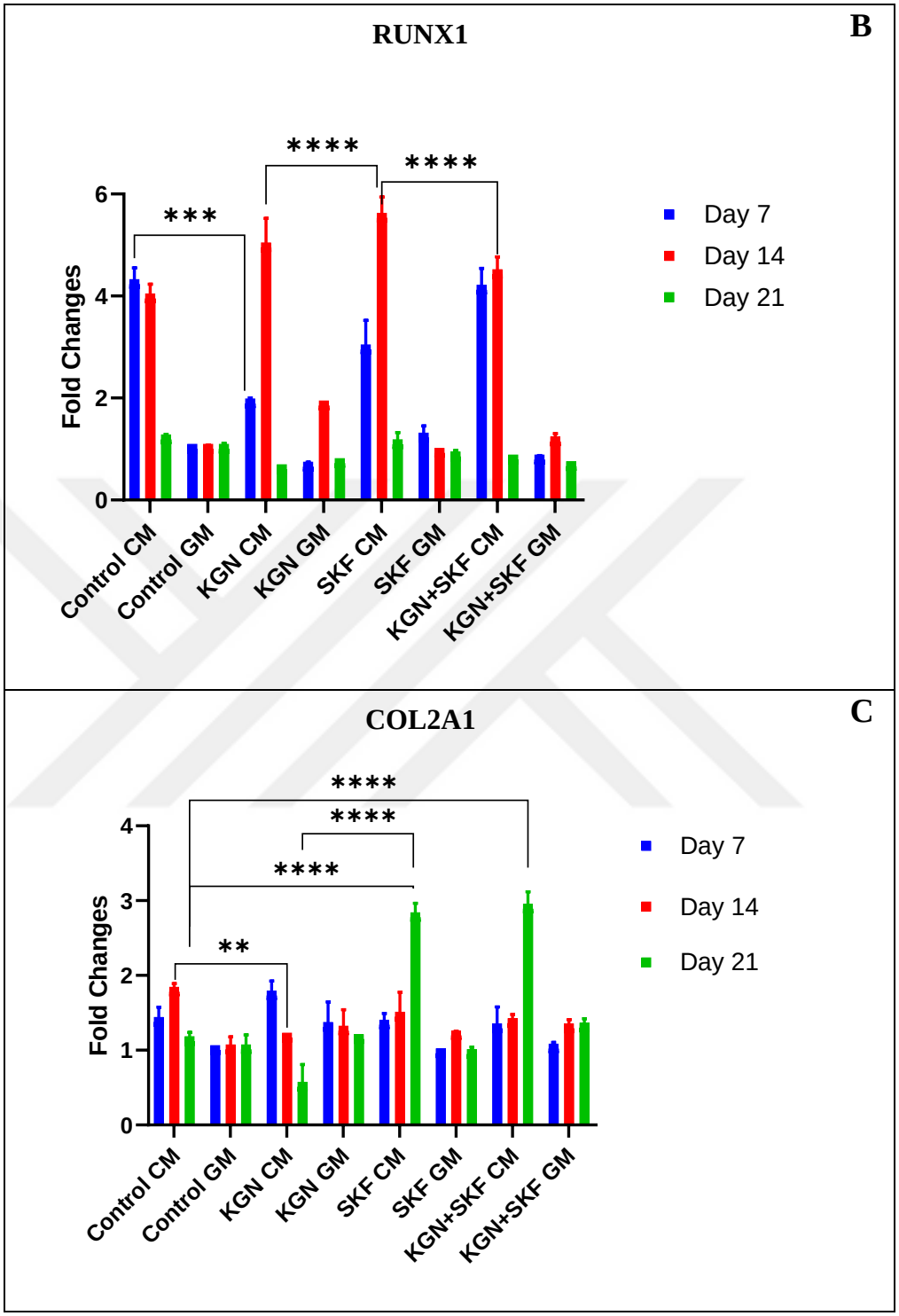

Figure 4.17. Expressions of A) SOX9 B) RUNX1 C) COL2A1 D) ACAN E) COL1A1 genes as fold changes with respect to Control GM groups (**** $P \leq 0.0001$, ***$P \leq 0.0002$, ** $P \leq 0.0021$, * $P \leq 0.0332$)

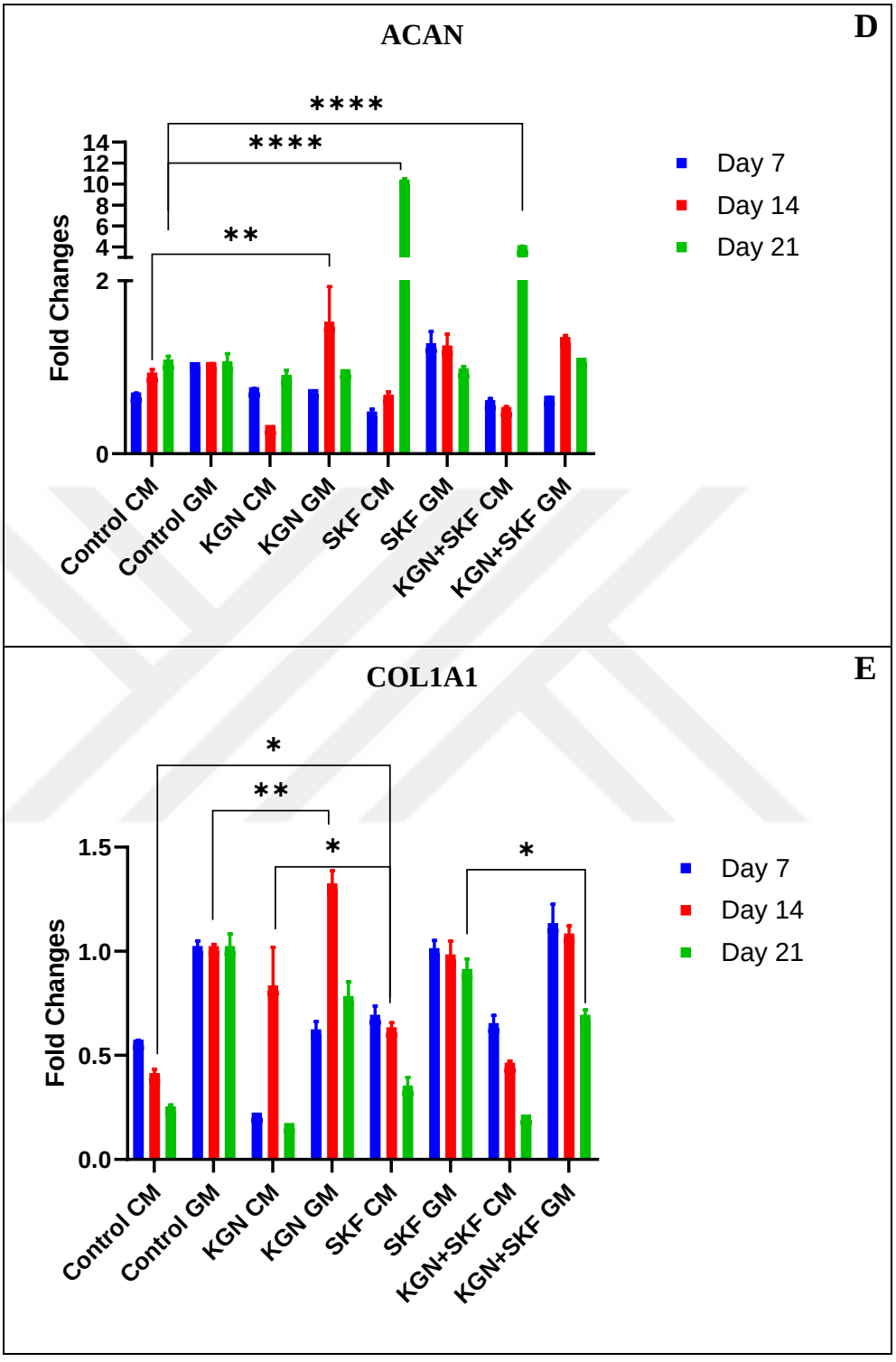

Figure 4.17. Expressions of A) SOX9 B) RUNX1 C) COL2A1 D) ACAN E) COL1A1 genes as fold changes with respect to Control GM groups (**** $P \leq 0.0001$, ***$P \leq 0.0002$, ** $P \leq 0.0021$, * $P \leq 0.0332$)

4.3. *IN VITRO* CELL AND SCAFFOLD INTERACTIONS

Scaffolds provide 3-D environment for cell proliferation and they are important elements of cartilage tissue engineering studies. Literature shows that there are differences between RBMSCs cultured in 2-D culture plates and 3-D scaffolds in terms of cell proliferation, migration, chondrogenic differentiation, hyaline like ECM matrix production. Therefore, effects of small molecules were also evaluated on RBMSCs seeded PLA scaffolds. Alcian Blue-PAS staining, MTS cell proliferation assay and RT-PCR assay were applied on chondrogenicly differentiated and small molecule induced RBMSCs seeded scaffolds.

4.3.1. Alcian Blue-PAS Staining

RBMSCs seeded and empty PLA scaffolds were incubated in chondrogenic medium throughout 21 days. While KGN was given to KGN CM group, small molecule SKF was introduced into SKF CM group and both KGN and SKF were given to KGN+SKF CM group in chondrogenic medium. Alcian PAS staining of scaffolds were carried out at days 7, 14 and 21 (Figure 4.18).

When Alcain Blue-PAS staining protocol was applied on empty scaffolds at days 7, 14 and 21, no color formation was observed. At day 7, scaffolds of Control CM, KGN CM, KGN+SKF CM and SKF CM were stained pale blue and pale purple color at some areas and small purple dots were seen on the surface of PLA fibers. That means that even at day 7, ECM deposition was visible on PLA scaffolds and chondrogenic differentiation of RBMSCs on PLA scaffolds was observed. At day 14, staining on RBMSCs seeded PLA scaffolds was observed as brighter blue and purple especially in SKF CM and KGN+SKF CM groups. Also, dark purple stainings on PLA surface were clearly seen on KGN CM, SKF CM and KGN+SKF CM groups. At day 21, increased ECM production was observed on scaffolds, particularly on SKF CM. The dark purple colored chondrogenic cell layer covered the surface of scaffolds was less in KGN CM, SKF CM and KGN+SKF CM groups. On the other hand, there was a smaller purple staining in Control CM group. Significantly, the higher amount of cartilage specific matrix production was observed in KGN CM, SKF CM and KGN+SKF CM groups compared to Control CM group. Even though, SKF CM and KGN+SKF CM groups stained more than KGN CM, the difference between these groups

was not significant. In conclusion, small molecule SKF and KGN both enhanced the chondrogenic matrix production in RBMSCs groups.

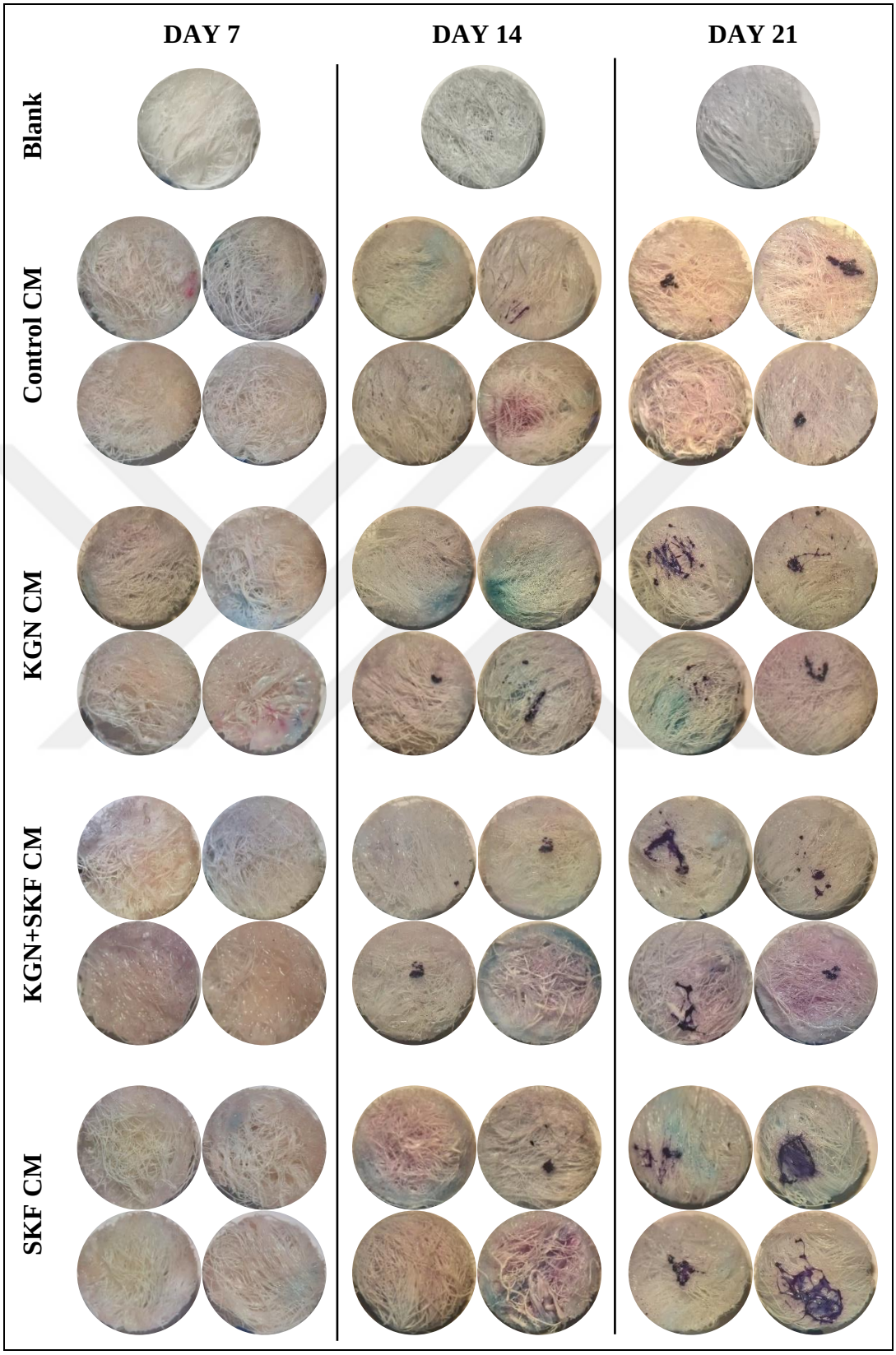

Figure 4.18. Image of Alcian Blue-PAS staining of RBMSCs seeded of fibrous PLA scaffolds and cultured throughout 21 days in chondrogenic differentiation medium containing small molecules

4.3.2. MTS Assay

To determine the effects of PLA scaffolds on cell proliferation, MTS assay was done on RBMSCs seeded PLA scaffolds at days 7, 14, 21 and 28 (Figure 4.19). In control group, higher cell number was observed at day 1 compared to KGN CM, KGN+SKF CM and SKF CM, but this situation changed at day 7 in a way that in small molecule induced groups higher cell proliferation was observed. After day 7, proliferation rate decreased in Control CM, on the contrary of KGN CM, KGN+SKF CM and SKF CM. Although cell proliferation increased in all groups from day 7 to day 14, there was a decrease at day 21 that could be result of chondrogenic differentiation. In the literature, similar decline was observed at day 21 [110,111]. At day 28, cell proliferation increased again in small molecule induced groups: KGN CM, KGN+SKF CM and SKF CM, whereas cell proliferation decrease continued in Control CM. In conclusion, PLA scaffolds provided 3-D environment to RBMSCs and cell proliferation continued during 28 days of chondrogenic differentiation. These results proved the importance of 3-D scaffolds for proliferation of cells in tissue engineering studies.

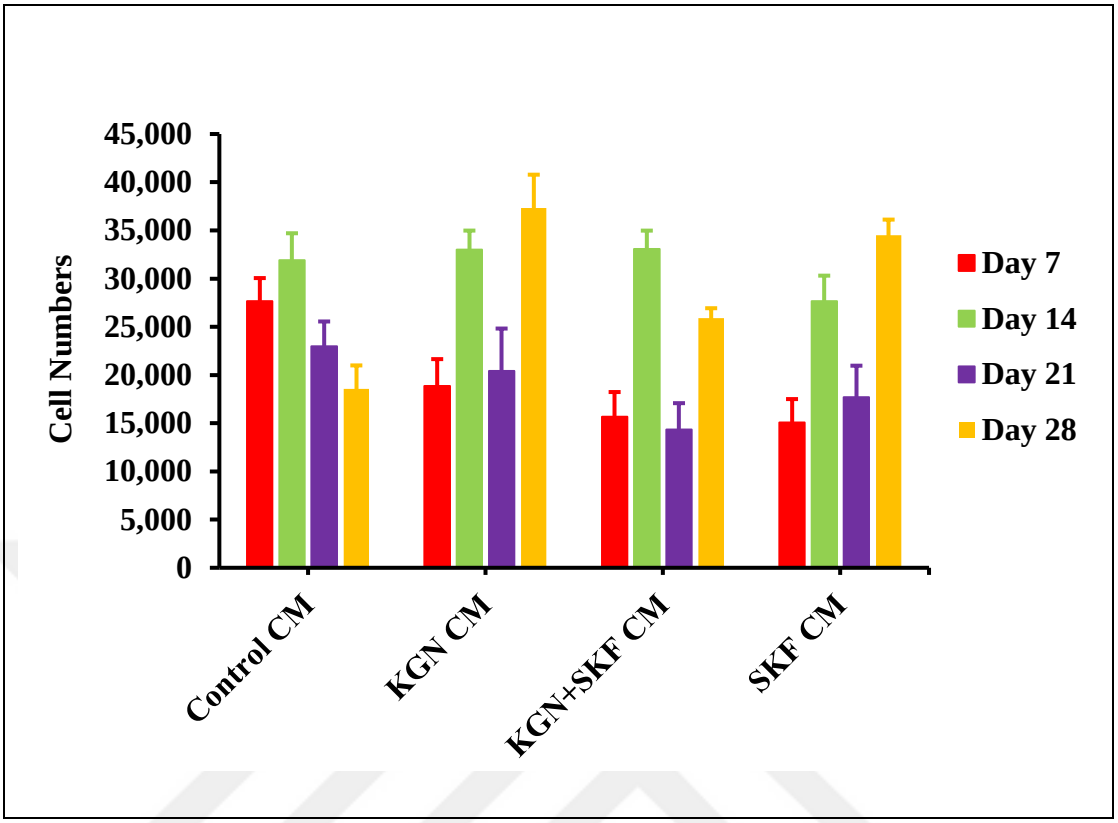

Figure 4.19. MTS cell proliferation assay performed on RBMSCs seeded fibrous PLA scaffolds and treated with chondrogenic medium and small molecules throughout 28 days of incubation. Initial cell seeding number was 30,000 cells per well.

4.3.3. RT-PCR

Gene expression profile of small molecule induced RBMSCs was determined via RT-PCR analysis. To determine the effects of small molecules SKF and KGN, RBMSCs were seeded onto PLA scaffolds and differentiated throughout 21 days with CM and small molecule SKF was introduced to SKF CM, KGN was introduced to KGN CM, and both small molecules were introduced to KGN+SKF CM groups. There were two different control groups; Control CM and Control GM. At days 7, 14 and 21, RNA was isolated from scaffolds, then used for cDNA synthesis. RT-PCR assay was performed on cDNA samples to determine fold changes of SOX9, RUNX1, COL2A1, ACAN and COL1A1 genes.

SOX9 and RUNX1 are transcription factors expressed during chondrogenic differentiation [106]. At day 14, Both SOX9 and RUNX1 genes were expressed in KGN+SKF CM samples with the highest fold changes as 13.53-fold and 30.66-fold, respectively (Figure 4.20 A, B).

Although, SOX9 gene expression increased from day 14 to day 21 in all groups, fold change of groups at day 21 was different from each other in a way that Control CM was 4.65, KGN CM was 7.95, SKF CM was 16.65 and KGN+SKF CM was 17.59. By looking the results, it can be seen that both small molecule KGN and SKF increased SOX9 gene expression during chondrogenic differentiation. Similar gene expression profile in RUNX1 was observed, but this time the highest fold change was seen at day 14. While KGN+SKF CM has highest expression at day 14 (30.66-fold), SKF CM expressed 10.05-fold, KGN CM expressed 4.45-fold and Control CM expressed 5.02-fold RUNX1 with respect to Control GM. From day 14 to day 21, RUNX1 gene expression increased in KGN CM, KGN+SKF CM and SKF CM groups to 6.02, 8.96 and 4.43-folds respectively. Gene expression decrease was only observed in Control CM group (1.38-fold). According to the results, it was observed that RUNX1 gene expression was increased upon induction of small molecule SKF when compared with Control CM.

Expression of COL1A1 and ACAN, chondrogenic marker genes, reveal cartilage specific ECM production in cells [89–90]. COL2A1 increased in KGN CM, KGN+SKF CM and SKF CM groups from day 7 to day 21 (Figure 4.20 C). In Control CM group, there was a decrease from day 7 to day 14 which was followed by an increasing at day 21. The highest fold changes of COL2A1 gene were observed in KGN+SKF CM 15.16-fold and SKF groups 14.70-fold and the lowest one in Control CM group 9.18-fold at day 21. This reveals that chondrogenic matrix production increased when RBMSCs were induced with small molecule SKF compared to KGN CM group. Similar gene expression profile was also observed in ACAN in which gene expression increased during 21 days. While the highest gene expression was seen in KGN CM 7.50 and SKF CM groups 10.47-fold at day 21, the lowest expression was observed in Control CM group 4.40-fold. As a result, small molecule SKF and KGN both significantly increased the gene expression of ACAN is when compared with Control CM. However, gene expression of ACAN was significantly higher on SKF CM (10.47-fold) compared with KGN+SKF CM (8.43-fold). COL1A1 is a hypertrophy marker seen in fibrocartilage tissue regarding with dedifferentiated chondrocytes [109]. During 21 days, a decrease in COL1A1 expression was observed in all groups. At day 7, there was 1.89-fold COL1A1 in Control CM, 1.90-fold in KGN CM and 2.15-fold SKF CM groups, and 2.57-fold COL1A1 in KGN+SKF CM group with respect to Control GM. At days 14 and 21, COL1A1 gene expression decreased to around 1, below 1, respectively, in all

chondrogenic differentiation groups. In both small molecules induced groups KGN CM, SKF CM and KGN+SKF CM, and Control CM group, COL1A1 decreased below 1-fold, showing that small molecules did not induce hypertrophic ECM deposition in any groups. In conclusion, increase in gene expressions of chondrogenic marker genes SOX9, RUNX1, COL2A1 and ACAN was observed upon small molecule KGN and SKF inductions. COL1A1 hypertrophy marker gene expression was not changed with small molecule induction. Small molecule SKF induced chondrogenic differentiation and ECM deposition in RBMSCs and can compete with KGN molecule in this field.

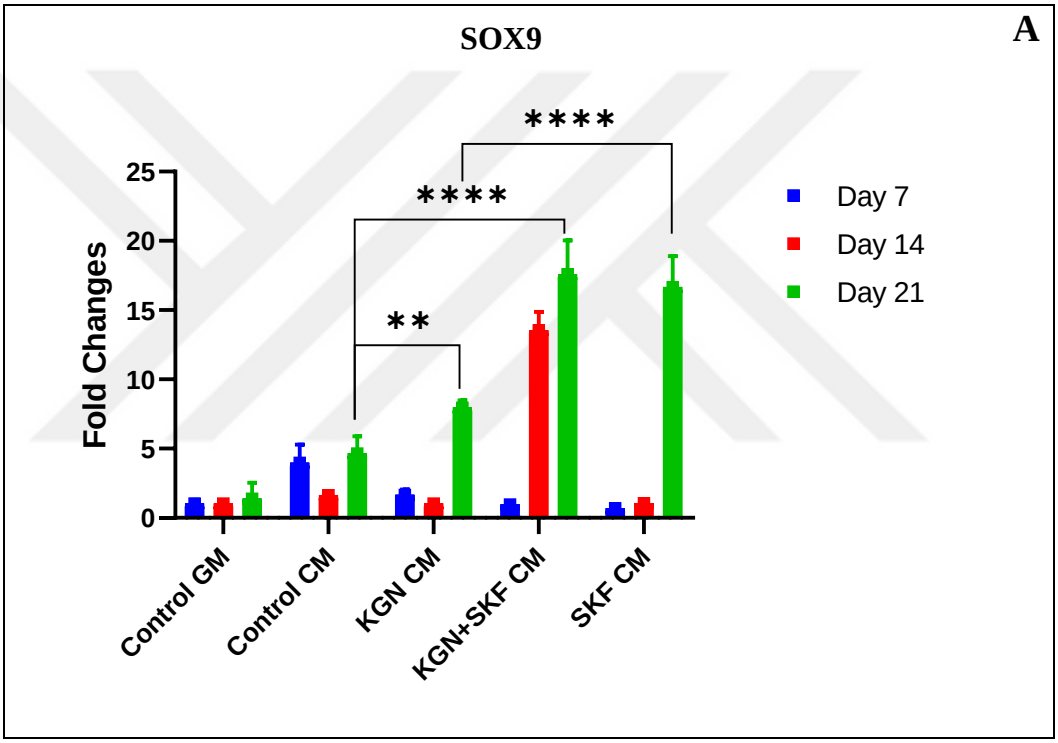

Figure 4.20. Expression of (A) SOX9, (B) RUNX1, (C) COL2A1, (D) ACAN and (E) COL1A1 genes as fold changes with respect to Control GM groups (**** $P \leq 0.0001$, ***$P \leq 0.0002$, ** $P \leq 0.0021$, * $P \leq 0.0332$)

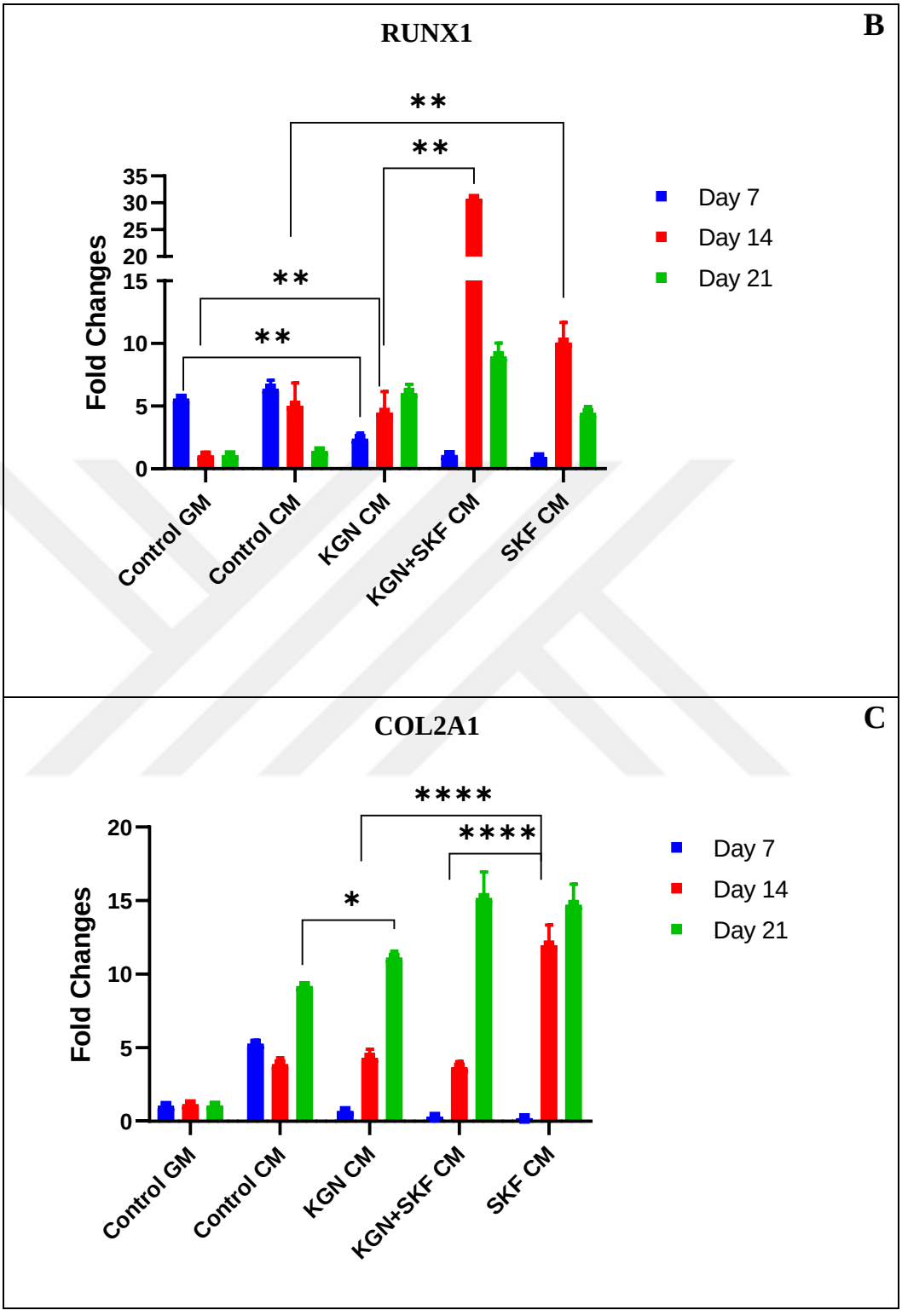

Figure 4.20. Expression of (A) SOX9, (B) RUNX1, (C) COL2A1, (D) ACAN and (E) COL1A1 genes as fold changes with respect to Control GM groups (**** $P \leq 0.0001$, ***$P \leq 0.0002$, ** $P \leq 0.0021$, * $P \leq 0.0332$)

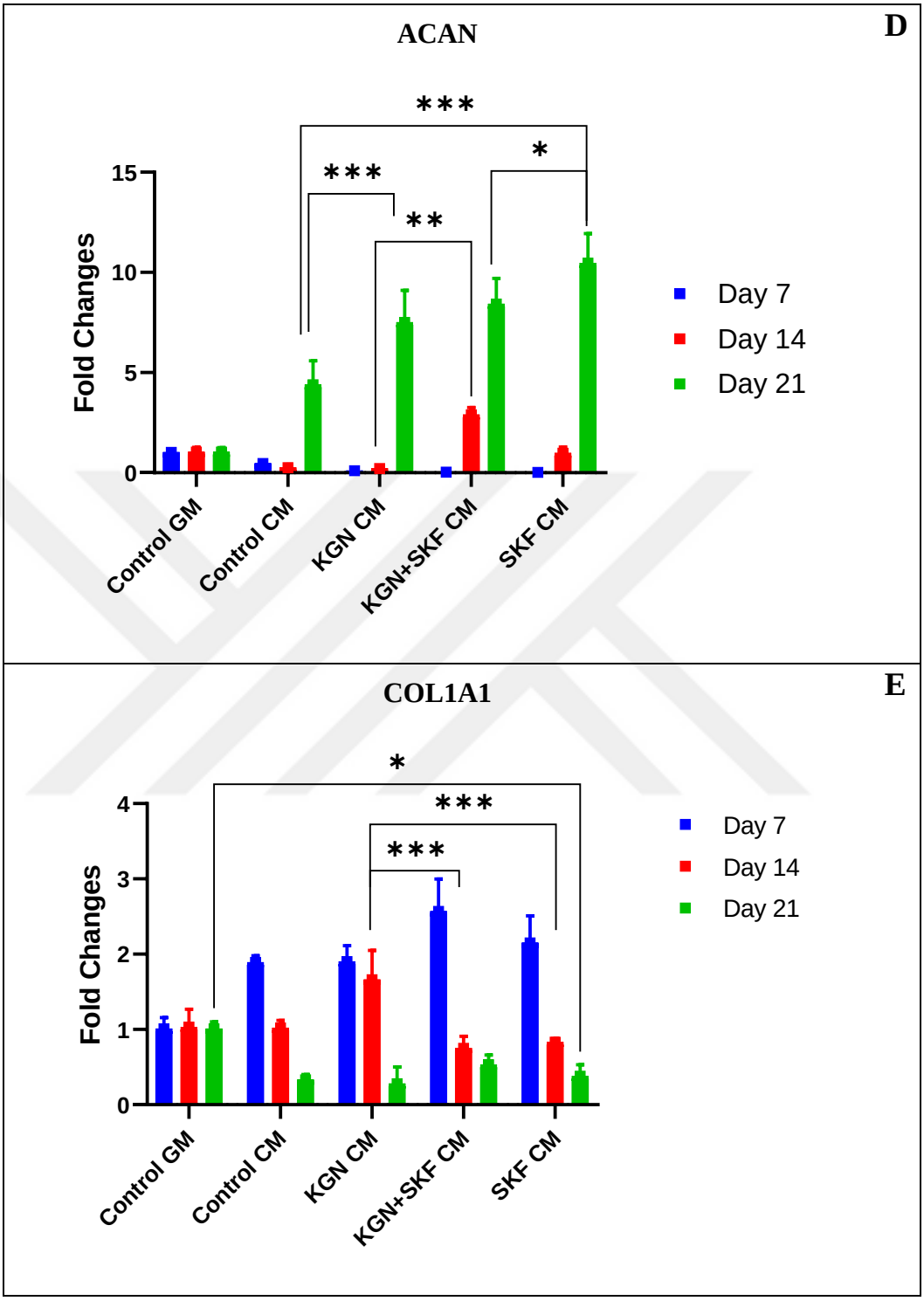

Figure 4.20. Expression of (A) SOX9, (B) RUNX1, (C) COL2A1, (D) ACAN and (E) COL1A1 genes as fold changes with respect to Control GM groups (**** $P \leq 0.0001$, ***$P \leq 0.0002$, ** $P \leq 0.0021$, * $P \leq 0.0332$)

5. CONCLUSION

Cartilage tissue has low a self-renewal capacity due to lack of blood vessels, and treatment is necessary for repairing of cartilage deformations and injuries. Although there are some traditional treatments, they are inadequate in gaining back function and mechanical properties of native cartilage tissue. Therefore, cartilage tissue engineering studies were emerged as a new treatment strategy to achieve fully regenerated of defects. In this study, effects of small molecules ADZ and SKF on chondrogenic differentiation of RBMSCs seeded on PLA fiber scaffolds were examined and compared with KGN. Firstly, fibrous PLA scaffolds were prepared via wet-spinning technique and characterized by using mechanical, degradation and SEM analyses and MTS cell proliferation assay. In terms of mechanical strength, degradation time, water retention, pH change profile, porous PLA scaffolds was successful and proper for cartilage regeneration studies. Then, the study was carried on in comparison of small molecules. While RBMSCs differentiated in chondrogenic medium, relevant study groups were supplemented with ADZ, SKF and KGN throughout 14 days. Alcian Blue-PAS staining and MTS assay were applied on groups and it was revealed that small molecule SKF induced high amount of ECM matrix compared to control group. On the other hand, ADZ increased cell proliferation but it resulted in lower cartilage specific ECM production. Therefore, the study was continued with the small molecule SKF and KGN. Alcian Blue-PAS staining, MTS assay and RT-PCR were applied on RBMSCs differentiated in chondrogenic medium and induced with small molecules SKF and KGN. Increased amount of ECM deposition was observed in small molecule induced groups in Alcian Blue-PAS staining. While increased gene expression of chondrogenic differentiation markers SOX9, RUNX1, COL2A1, ACAN was observed, the expression of COL1A1 gene, marker of hypertrophy, was not increased upon small molecule induction. Then, chondrogenic differentiation of small molecule induced RBMSCs in 3-D environment was determined via seeding them into PLA scaffolds. RBMSCs were differentiated throughout 21 days. Alcian Blue-PAS staining, MTS assay and RT-PCR were conducted on samples at day 7, 14 and 21. Results showed that small molecule induction increased the cartilage specific ECM deposition and gene expressions of chondrogenic markers. In conclusion, using PLA scaffolds were increased cell density and higher ECM deposition was observed, while mechanical support was provided. Also, chondrogenic induction capacity of small molecule SKF was compared with KGN and was found that small molecule SKF can

compete with KGN in terms of chondrogenic ECM production, chondrogenic gene expressions and cell proliferation.

6. FUTURE PROSPECTS

In this study, *in vitro* chondrogenic induction effects of ADZ and SKF were determined and compared with KGN. Chondrogenic differentiation and ECM deposition on PLA scaffolds of small molecule induced RBMSCs were observed. In the future, chondrogenic differentiation effects of small molecules and scaffolds must be observed in extended culture periods to determine their long-term success. Also, *in vivo* experiments must be conducted to evaluate tissue regeneration effects of small molecules on experimental models of cartilage defects. Possible immunogenic and inflammatory responses developed in native tissues against implanted scaffold must be determined. PLA can be blended with different polymers to obtain better cell attachment and mechanical strength in scaffolds. For the treatment of osteochondrogenic defects, PLA fibers can be combined with hydrogels and biphasic scaffolds can be prepared. Small molecules can be delivered to cells by using nanocarrier particles or hydrogel scaffolds for controlled release during differentiation. Finally, osteogenic differentiation effects of small molecules ADZ and SKF can be studied and compared with osteogenic inducer small molecules that are commercially available.

REFERENCES

1. Yusuf E, Nelissen RG, Ioan-Facsinay A, Stojanovic-Susulic V, DeGroot J, van Osch G, et al. Association between weight or body mass index and hand osteoarthritis: a systematic review. *Ann Rheum Dis*. 2010;69(4):761–5.

2. Hall BK. Bones and Cartilage Second Edition.

3. Chung C, Burdick JA. Engineering cartilage tissue. *Adv Drug Deliv Rev*. 2007/10/05. 2008;60(2):243–62.

4. Chen L, Liu J, Guan M, Zhou T, Duan X, Xiang Z. Growth Factor and Its Polymer Scaffold-Based Delivery System for Cartilage Tissue Engineering. 2020;6097–111.

5. Gresham RCH, Bahney CS, Leach JK. Growth factor delivery using extracellular matrix-mimicking substrates for musculoskeletal tissue engineering and repair. *Bioact Mater*. 2021;6(7):1945–56.

6. Bhardwaj N, Devi D, Mandal BB. Tissue-engineered cartilage: The crossroads of biomaterials, cells and stimulating factors. *Macromol Biosci*. 2015;15(2):153–82.

7. Sidal H, Colakoglu Erkan P, Uslu M, Kocabas F. Development of small-molecule-induced fibroblast expansion technologies. *J Tissue Eng Regen Med*. 2020;14(10):1476–87.

8. Alyazici LY, Kocabas F. Identification of Small Molecules That Enhance the Expansion of Mesenchymal Stem Cells Originating from Bone Marrow. In Cham: Springer International Publishing; p. 1–13.

9. Lebaron RG, Athanasiou KA. Ex vivo synthesis of articular cartilage. 2000;21:2575–87.

10. Loy BN, Zimel M, Gowda AL, Tooley TR, Maerz T, Bicos J, et al. A Biomechanical and Structural Comparison of Articular Cartilage and Subchondral Bone of the Glenoid and Humeral Head. *Orthop J Sport Med*. 2018;6(7):2325967118785854.

11. Gupton M, Munjal A, Terreberry RR. Anatomy, Hinge Joints. In Treasure Island (FL); 2022.

12. Chen S, Fu P, Wu H, Pei M. Meniscus, articular cartilage and nucleus pulposus: a comparative review of cartilage-like tissues in anatomy, development and function. *Cell Tissue Res*. 2017;370(1):53–70.

13. Safshekan F, Tafazzoli-Shadpour M, Abdouss M, Shadmehr MB. Viscoelastic Properties of Human Tracheal Tissues. *J Biomech Eng*. 2017;139(1).

14. Stammen JA, Williams S, Ku DN, Guldberg RE. Mechanical properties of a novel PVA hydrogel in shear and unconfined compression. *Biomaterials*. 2001;22(8):799–806.

15. Chen FH, Rousche KT, Tuan RS. Technology insight: Adult stem cells in cartilage regeneration and tissue engineering. *Nat Clin Pract Rheumatol*. 2006;2(7):373–82.

16. Knudson CB, Knudson W. Cartilage proteoglycans. *Semin Cell Dev Biol*. 2001;12(2):69–78.

17. Pratta MA, Yao W, Decicco C, Tortorella MD, Liu R, Copeland RA, et al. Aggrecan Protects Cartilage Collagen from Proteolytic Cleavage *. *J Biol Chem*. 2003;278(46):45539–45.

18. Eyre D. Collagen of articular cartilage. 2002;

19. Responte DJ, Natoli RM, Athanasiou KA. Collagens of articular cartilage: structure, function, and importance in tissue engineering. *Crit Rev Biomed Eng*. 2007;35(5):363–411.

20. Gugjoo MB, Sharma GT, Aithal HP, Kinjavdekar P. Cartilage tissue engineering : Role of mesenchymal stem cells along with growth factors & scaffolds. 2016;(September):339–47.

21. Martel-Pelletier J, Barr AJ, Cicuttini FM, Conaghan PG, Cooper C, Goldring MB, et al. Osteoarthritis. *Nat Rev Dis Prim*. 2016;2.

22. Liu S, Qin S, He M, Zhou D, Qin Q, Wang H. Current applications of poly (lactic acid) composites in tissue engineering and drug delivery. *Compos Part B*. 2020;199(December 2019):108238.

23. Trasolini NA, Lindsay A, Gipsman A, Rick Hatch GF. The Biomechanics of

Multiligament Knee Injuries: From Trauma to Treatment. *Clin Sports Med*. 2019;38(2):215–34.

24. Cai G, Liu W, He Y, Huang J, Duan L, Xiong J, et al. Recent advances in kartogenin for cartilage regeneration. *J Drug Target*. 2019;27(1):28–32.

25. Wasyłeczko M, Sikorska W, Chwojnowski A. Review of synthetic and hybrid scaffolds in cartilage tissue engineering. *Membranes (Basel)*. 2020;10(11):1–28.

26. Walter SG, Ossendorff R, Schildberg FA. Articular cartilage regeneration and tissue engineering models: a systematic review. *Arch Orthop Trauma Surg*. 2019;139(3):305–16.

27. Al Hamed R, Bazarbachi AH, Malard F, Harousseau J-L, Mohty M. Current status of autologous stem cell transplantation for multiple myeloma. *Blood Cancer J*. 2019;9(4):44.

28. Khorraminejad-Shirazi M, Dorvash M, Estedlal A, Hoveidaei AH, Mazloomrezaei M, Mosaddeghi P. Aging: A cell source limiting factor in tissue engineering. *World J Stem Cells*. 2019;11(10):787–802.

29. DuRaine GD, Brown WE, Hu JC, Athanasiou KA. Emergence of Scaffold-Free Approaches for Tissue Engineering Musculoskeletal Cartilages. *Ann Biomed Eng*. 2015;43(3):543–54.

30. Wakitani S, Nawata M, Tensho K, Okabe T, Machida H, Ohgushi H. Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees. *J Tissue Eng Regen Med*. 2007;1(1):74–9.

31. Chen M, Xu Y, Zhang T, Ma Y, Liu J, Yuan B, et al. Mesenchymal stem cell sheets: a new cell-based strategy for bone repair and regeneration. *Biotechnol Lett*. 2019;41(3):305–18.

32. Baghaban Eslaminejad M, Malakooty Poor E. Mesenchymal stem cells as a potent cell source for articular cartilage regeneration. *World J Stem Cells*. 2014;6(3):344–54.

33. Diekman BO, Rowland CR, Lennon DP, Caplan AI, Guilak F. Chondrogenesis of Adult Stem Cells from Adipose Tissue and Bone Marrow: Induction by Growth Factors and Cartilage-Derived Matrix. *Tissue Eng Part A*. 2010;16(2):523–33.

34. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis \& Rheum*. 2002;46(3):704–13.

35. Jurgens WJ, MJ O-V. Helder MN, ZandiehDoulabi B., Schouten TE, Kuik DJ, Ritt MJ and van Milligen FJ: Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: Implications for cell-based therapies. *Cell Tissue Res*. 2008;332:415–26.

36. Im G-I, Shin Y-W, Lee K-B. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthr Cartil*. 2005;13(10):845–53.

37. Raghunath J, Salacinski HJ, Sales KM, Butler PE, Seifalian AM. Advancing cartilage tissue engineering: the application of stem cell technology. *Curr Opin Biotechnol*. 2005;16(5):503–9.

38. Kramer J, Bartsch M, Krug D, Klinger M, Nitschke M, Rohwedel J. Simvastatin modulates mouse embryonic stem cell-derived chondrogenesis in vitro. *Toxicol Vitr*. 2012;26(7):1170–6.

39. Medvedev SP, Grigor'eva E V, Shevchenko AI, Malakhova AA, Dementyeva E V, Shilov AA, et al. Human Induced Pluripotent Stem Cells Derived from Fetal Neural Stem Cells Successfully Undergo Directed Differentiation into Cartilage. *Stem Cells Dev*. 2011;20(6):1099–112.

40. Yates KE, Forbes RL, Glowacki J. New Chondrocyte Genes Discovered by Representational Difference Analysis of Chondroinduced Human Fibroblasts. *Cells Tissues Organs*. 2004;176(1–3):41–53.

41. Lee KH, Song SU, Hwang TS, Yi Y, Oh IS, Lee JY, et al. Regeneration of Hyaline Cartilage by Cell-Mediated Gene Therapy Using Transforming Growth Factor β1-Producing Fibroblasts. *Hum Gene Ther*. 2001;12(14):1805–13.

42. Bhardwaj N, Rajkhowa R, Wang X, Devi D. Milled non-mulberry silk fibroin microparticles as biomaterial for biomedical applications. *Int J Biol Macromol*. 2015;81:31–40.

43. Zhao W, Jin X, Cong Y, Liu Y, Fu J. Degradable natural polymer hydrogels for articular cartilage tissue engineering. *J Chem Technol Biotechnol*. 2013;88(3):327–39.

44. Coleman RM, Case ND, Guldberg RE. Hydrogel effects on bone marrow stromal cell response to chondrogenic growth factors. *Biomaterials*. 2007;28(12):2077–86.

45. Koga H, Muneta T, Nagase T, Nimura A, Ju Y-J, Mochizuki T, et al. Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. *Cell Tissue Res*. 2008;333(2):207–15.

46. Pabbruwe MB, Esfandiari E, Kafienah W, Tarlton JF, Hollander AP. Induction of cartilage integration by a chondrocyte/collagen-scaffold implant. *Biomaterials*. 2009;30(26):4277–86.

47. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthr Cartil*. 2002;10(6):432–63.

48. Kang H-S, Park S-H, Lee Y-G, Son T-I. Polyelectrolyte complex hydrogel composed of chitosan and poly(γ-glutamic acid) for biological application: Preparation, physical properties, and cytocompatibility. *J Appl Polym Sci*. 2007;103(1):386–94.

49. Sukarto A, Yu C, Flynn LE, Amsden BG. Co-delivery of Adipose-Derived Stem Cells and Growth Factor-Loaded Microspheres in RGD-Grafted N-Methacrylate Glycol Chitosan Gels for Focal Chondral Repair. *Biomacromolecules*. 2012;13(8):2490–502.

50. Campo GM, Avenoso A, Nastasi G, Micali A, Prestipino V, Vaccaro M, et al. Hyaluronan reduces inflammation in experimental arthritis by modulating TLR-2 and TLR-4 cartilage expression. *Biochim Biophys Acta - Mol Basis Dis*. 2011;1812(9):1170–81.

51. Knudson CB, Nofal GA, Pamintuan L, Aguiar DJ. The chondrocyte pericellular matrix: a model for hyaluronan-mediated cell-matrix interactions. *Biochem Soc*

Trans. 1999;27(2):142–8.

52. Hollander AP, Dickinson SC, Sims TJ, Brun P, Cortivo R, Kon E, et al. Maturation of Tissue Engineered Cartilage Implanted in Injured and Osteoarthritic Human Knees. *Tissue Eng*. 2006;12(7):1787–98.

53. Chang C-H, Liu H-C, Lin C-C, Chou C-H, Lin F-H. Gelatin–chondroitin–hyaluronan tri-copolymer scaffold for cartilage tissue engineering. *Biomaterials*. 2003;24(26):4853–8.

54. Guilak F, Awad HA, Fermor B, Leddy HA, Gimble JM. Adipose-derived adult stem cells for cartilage tissue engineering. *Biorheology*. 2004;41:389–99.

55. Diduch DR, Jordan LCM, Mierisch CM, Balian G. Marrow stromal cells embedded in alginate for repair of osteochondral defects. *Arthrosc J Arthrosc Relat Surg*. 2000;16(6):571–7.

56. Fragonas E, Valente M, Pozzi-Mucelli M, Toffanin R, Rizzo R, Silvestri F, et al. Articular cartilage repair in rabbits by using suspensions of allogenic chondrocytes in alginate. *Biomaterials*. 2000;21(8):795–801.

57. Haaparanta A-M, Järvinen E, Cengiz IF, Ellä V, Kokkonen HT, Kiviranta I, et al. Preparation and characterization of collagen/PLA, chitosan/PLA, and collagen/chitosan/PLA hybrid scaffolds for cartilage tissue engineering. *J Mater Sci Mater Med*. 2014;25(4):1129–36.

58. Holzwarth JM, Ma PX. Biomimetic nanofibrous scaffolds for bone tissue engineering. *Biomaterials*. 2011;32(36):9622–9.

59. Chen C-H, Shyu VB-H, Chen J-P, Lee M-Y. Selective laser sintered poly-$\upepsilon$-caprolactone scaffold hybridized with collagen hydrogel for cartilage tissue engineering. *Biofabrication*. 2014;6(1):15004.

60. Li C, Wang L, Yang Z, Kim G, Chen H, Ge Z. A Viscoelastic Chitosan-Modified Three-Dimensional Porous Poly(L-Lactide-co-ε-Caprolactone) Scaffold for Cartilage Tissue Engineering. *J Biomater Sci Polym Ed*. 2012;23(1–4):405–24.

61. Sheikh Z, Najeeb S, Sultan Z, Verma V, Baloch H, Glogauer M. Biodegradable

Materials for Bone Repair and Tissue Engineering Applications. *Materials (Basel)*. 2015;8:5744–94.

62. Borkotoky SS, Dhar P, Katiyar V. Biodegradable poly (lactic acid)/Cellulose nanocrystals (CNCs) composite microcellular foam: Effect of nanofillers on foam cellular morphology, thermal and wettability behavior. *Int J Biol Macromol*. 2018;106:433–46.

63. Gutiérrez-Sánchez M, Escobar-Barrios VA, Pozos-Guillén A, Escobar-García DM. RGD-functionalization of PLA/starch scaffolds obtained by electrospinning and evaluated in vitro for potential bone regeneration. *Mater Sci Eng C*. 2019;96:798–806.

64. Arora B, Bhatia R, Attri P. 28 - Bionanocomposites: Green materials for a sustainable future. In: Hussain CM, Mishra AK, editors. New Polymer Nanocomposites for Environmental Remediation. Elsevier; 2018. p. 699–712.

65. Zong E, Liu X, Liu L, Wang J, Song P, Ma Z, et al. Graft Polymerization of Acrylic Monomers onto Lignin with CaCl2–H2O2 as Initiator: Preparation, Mechanism, Characterization, and Application in Poly(lactic acid). *ACS Sustain Chem Eng*. 2018;6(1):337–48.

66. Dounchis JS, Bae WC, Chen AC, Sah RL, Coutts RD, Amiel D. Cartilage Repair With Autogenic Perichondrium Cell and Polylactic Acid Grafts. *Clin Orthop Relat Res*. 2000;377.

67. Honda M, Yada T, Ueda M, Kimata K. Cartilage formation by cultured chondrocytes in a new scaffold made of poly(L-lactide-ϵ-caprolactone) sponge. *J Oral Maxillofac Surg*. 2000;58(7):767–75.

68. Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D. Articular cartilage repair using allogeneic perichondrocyteseeded biodegradable porous polylactic acid (PLA): A tissue-engineering study. *J Biomed Mater Res*. 1995;29(9):1147–54.

69. Valonen PK, Moutos FT, Kusanagi A, Moretti MG, Diekman BO, Welter JF, et al. In vitro generation of mechanically functional cartilage grafts based on adult human

stem cells and 3D-woven poly(ε-caprolactone) scaffolds. *Biomaterials*. 2010;31(8):2193–200.

70. Zhang Y, Liu X, Zeng L, Zhang J, Zuo J, Zou J, et al. Polymer Fiber Scaffolds for Bone and Cartilage Tissue Engineering. *Adv Funct Mater*. 2019;29(36):1–20.

71. Tiwary R, Amarpal, Aithal HP, Kinjavdekar P, Pawde AM, Singh R. Effect of IGF-1 and Uncultured Autologous Bone-Marrow-Derived Mononuclear Cells on Repair of Osteochondral Defect in Rabbits. *Cartilage*. 2013;5(1):43–54.

72. Park H, Temenoff JS, Tabata Y, Caplan AI, Mikos AG. Injectable biodegradable hydrogel composites for rabbit marrow mesenchymal stem cell and growth factor delivery for cartilage tissue engineering. *Biomaterials*. 2007;28(21):3217–27.

73. Goodrich LR, Hidaka C, Robbins PD, Evans CH, Nixon AJ. Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. *J Bone Joint Surg Br*. 2007;89-B(5):672–85.

74. Davies LC, Blain EJ, Gilbert SJ, Caterson B, Duance VC. The Potential of IGF-1 and TGFβ1 for Promoting "Adult" Articular Cartilage Repair: An In Vitro Study. *Tissue Eng Part A*. 2008;14(7):1251–61.

75. Ngo HX, Garneau-Tsodikova S. What are the drugs of the future? *Medchemcomm*. 2018;9(5):757–8.

76. New 2016 Data and Statistics for Global Pharmaceutical Products and Projections through 2017. *ACS Chem Neurosci*. 2017;8(8):1635–6.

77. Wu H, Jia B, Erbe L. Oscillation criteria for second order superlinear dynamic equations with oscillating coefficients. *Appl Math Lett*. 2017;72:29–34.

78. Szychlinska MA, Calabrese G, Ravalli S, Parrinello NL, Forte S, Castrogiovanni P, et al. Cycloastragenol as an Exogenous Enhancer of Chondrogenic Differentiation of Human Adipose-Derived Mesenchymal Stem Cells. A Morphological Study. *Cells*. 2020;9(2).

79. Eo S-H, Kim SJ. Rosmarinic acid induces rabbit articular chondrocyte differentiation by decreases matrix metalloproteinase-13 and inflammation by upregulating

cyclooxygenase-2 expression. *J Biomed Sci*. 2017;24(1):75.

80. Zhou Q, Zhang J-H, Yuan S, Shao J-H, Cai Z-Y, Chen S, et al. A New Insight of Kartogenin Induced the Mesenchymal Stem Cells (MSCs) Selectively Differentiate into Chondrocytes by Activating the Bone Morphogenetic Protein 7 (BMP-7)/Smad5 Pathway. *Med Sci Monit*. 2019;25:4960–7.

81. Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, et al. A stem cell-based approach to cartilage repair. *Science (80-)*. 2012;336(6082):717–21.

82. Zhang S, Hu P, Liu T, Li Z, Huang Y-C, Jinqi L, et al. Kartogenin hydrolysis product 4-aminobiphenyl distributes to cartilage and mediates cartilage regeneration. *Theranostics*. 2019;9:7108–21.

83. Liu C, Ma X, Li T, Zhang Q. Kartogenin, transforming growth factor-β1 and bone morphogenetic protein-7 coordinately enhance lubricin accumulation in bone-derived mesenchymal stem cells. *Cell Biol Int*. 2015;39(9):1026–35.

84. Ono Y, Ishizuka S, Knudson CB, Knudson W. Chondroprotective Effect of Kartogenin on CD44-Mediated Functions in Articular Cartilage and Chondrocytes. *Cartilage*. 2014;5(3):172–80.

85. Piao CS, Kim J-B, Han P-L, Lee J-K. Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult. *J Neurosci Res*. 2003;73(4):537–44.

86. Payne KA, Meszaros LB, Phillippi JA, Huard J. Effect of Phosphatidyl Inositol 3-Kinase, Extracellular Signal-Regulated Kinases 1/2, and p38 Mitogen-Activated Protein Kinase Inhibition on Osteogenic Differentiation of Muscle-Derived Stem Cells. *Tissue Eng Part A*. 2010;16(12):3647–55.

87. Adezmapimod (SB203580) Product Data Sheet. Selleck Chemicals. 2022 [cited 2022 Aug 13]. Available from: https://www.selleckchem.com/products/SB-203580.html

88. Wang H, Cheng X, Tian J, Xiao Y, Tian T, Xu F, et al. TRPC channels: Structure, function, regulation and recent advances in small molecular probes. *Pharmacol Ther*. 2020;209:107497.

89. Song M, Chen D, Yu SP. The TRPC channel blocker SKF 96365 inhibits glioblastoma cell growth by enhancing reverse mode of the Na(+) /Ca(2+) exchanger and increasing intracellular Ca(2+). *Br J Pharmacol*. 2014;171(14):3432–47.

90. Falcón D, Galeano-Otero I, Martín-Bórnez M, Fernández-Velasco M, Gallardo-Castillo I, Rosado JA, et al. TRPC Channels: Dysregulation and Ca2+ Mishandling in Ischemic Heart Disease. *Cells*. 2020;9(1).

91. SKF-96365 Product Data Sheet. Merck KGaA. 2022 [cited 2022 Aug 13]. Available from: https://www.sigmaaldrich.com/TR/en/product/sigma/s7809

92. Zhang J, Wang JHC. Kartogenin induces cartilage-like tissue formation in tendon-bone junction. *Bone Res*. 2014;2(December 2013):12–7.

93. Calikoglu Koyuncu AC, Nayman AH, Telci D, Torun Kose G. Tissue transglutaminase_variant 2-transduced mesenchymal stem cells and their chondrogenic potential. *Biotechnol Bioeng*. 2020;117(6):1839–52.

94. Jafari M, Paknejad Z, Rad MR, Motamedian SR, Eghbal MJ, Nadjmi N, et al. Polymeric scaffolds in tissue engineering: a literature review. *J Biomed Mater Res Part B Appl Biomater*. 2017;105(2):431–59.

95. Cheng K, Kisaalita WS. Exploring Cellular Adhesion and Differentiation in a Micro- / Nano-Hybrid Polymer Scaffold. 2010;

96. Zhang H, Zhou L, Zhang W. Control of Scaffold Degradation in Tissue Engineering: A Review. *Tissue Eng Part B Rev*. 2014;20(5):492–502.

97. Singh D, Babbar A, Jain V, Gupta D, Saxena S, Dwibedi V. Synthesis , characterization , and bioactivity investigation of biomimetic biodegradable PLA scaffold fabricated by fused filament fabrication process. *J Brazilian Soc Mech Sci Eng*. 2019;41(3):1–13.

98. Revati R, Majid MSA, Ridzuan MJM, Basaruddin KS, Rahman Y. MN, Cheng EM, et al. In vitro degradation of a 3D porous Pennisetum purpureum/PLA biocomposite scaffold. *J Mech Behav Biomed Mater*. 2017;74:383–91.

99. Zong X, Bien H, Chung C-Y, Yin L, Fang D, Hsiao BS, et al. Electrospun fine-

textured scaffolds for heart tissue constructs. *Biomaterials*. 2005;26(26):5330–8.

100. Akizuki S, Mow VC, Müller F, Pita JC, Howell DS, Manicourt DH. Tensile properties of human knee joint cartilage: I. Influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res*. 1986;4(4):379–92.

101. Kabir W, Di Bella C, Choong PFM, O'Connell CD. Assessment of Native Human Articular Cartilage: A Biomechanical Protocol. *Cartilage*. 2020;13(2_suppl):427S-437S.

102. Abay Akar N, Gürel Peközer G, Torun Köse G. Fibrous bone tissue engineering scaffolds prepared by wet spinning of PLGA. *Turkish J Biol*. 2019;43(4):235–45.

103. Lin C-C, Fu S-J. Osteogenesis of human adipose-derived stem cells on poly(dopamine)-coated electrospun poly(lactic acid) fiber mats. *Mater Sci Eng C*. 2016;58:254–63.

104. Xin C, Addy MM, Zhao J, Cheng Y, Cheng S, Mu D, et al. Comprehensive techno-economic analysis of wastewater-based algal biofuel production: A case study. *Bioresour Technol*. 2016;211:584–93.

105. Jin G, Prabhakaran MP, Ramakrishna S. Stem cell differentiation to epidermal lineages on electrospun nanofibrous substrates for skin tissue engineering. *Acta Biomater*. 2011;7(8):3113–22.

106. Robins JC, Akeno N, Mukherjee A, Dalal RR, Aronow BJ, Koopman P, et al. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. *Bone*. 2005;37(3):313–22.

107. Tang C, Chen W, Luo Y, Wu J, Zhang Y, Mcvicar A, et al. HHS Public Access. 2021;477(13):2421–38.

108. Blanco FJ, Ruiz-Romero C. New targets for disease modifying osteoarthritis drugs: Chondrogenesis and Runx1. *Ann Rheum Dis*. 2013;72(5):631–4.

109. Jakobsen RB, Shahdadfar A, Reinholt FP, Brinchmann JE. Chondrogenesis in a hyaluronic acid scaffold: comparison between chondrocytes and MSC from bone marrow and adipose tissue. *Knee Surgery, Sport Traumatol Arthrosc*.

2010;18(10):1407–16.

110. Mishra A, Tummala P, King A, Lee B, Kraus M, Tse V, et al. Buffered Platelet-Rich Plasma Enhances Mesenchymal Stem Cell Proliferation and Chondrogenic Differentiation. 2009;15(3).

111. Vaca-González JJ, Clara-Trujillo S, Guillot-Ferriols M, Ródenas-Rochina J, Sanchis MJ, Ribelles JLG, et al. Effect of electrical stimulation on chondrogenic differentiation of mesenchymal stem cells cultured in hyaluronic acid – Gelatin injectable hydrogels. *Bioelectrochemistry*. 2020;134:107536.

APPENDIX A: ETHICAL APPROVAL FORM

T.C.
YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2022-21	28.04.2022	2022/04	2022/04-6	Prof. Dr. Gamze TORUN KÖSE

"Farklı TG2* İsoformlarının Kıkırdak Doku Rejenerasyonu Üzerine Etkisi" isimli projenin isminin "Küçük Moleküllerin Kıkırdak Doku Rejenerasyonu Üzerine Etkisi" olarak değiştirilmesi oy birliğiyle etik açıdan uygun görülmüştür.

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Dr. Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	KATILMADI
Üye	Prof. Dr. Özlem MALKONDU	KATILMADI
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	
Üye	Atakan Mücahit YAVUZ	KATILMADI
Üye	Ahmet ŞENKARDEŞLER	

APPENDIX B: MTS CALIBRATION CURVE

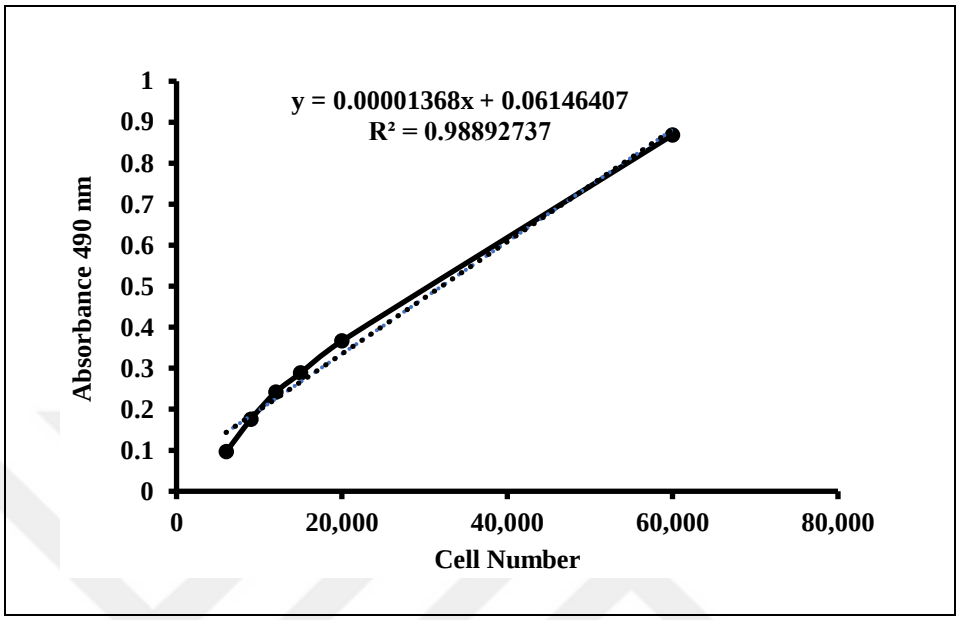

Figure B.1. MTS calibration curve of RBMSCs (P3) seeded on 48 well plate