USAGE OF BACTERIAL CELLULOSE-COLLAGEN SCAFFOLDS INCORPORATED WITH IGF-I AND TGF-β1 GENES FOR CARTILAGE REGENERATION

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ABSTRACT

USAGE OF BACTERIAL CELLULOSE-COLLAGEN SCAFFOLDS INCORPORATED WITH IGF-I AND TGF-β1 GENES FOR CARTILAGE REGENERATION

Ultimate goal of tissue engineering and regenerative medicine is to develop substitutes to help the guidance of the growth of new functional tissue using biological, mechanical and structural cues.

Scaffolds used in tissue engineering applications have primarily been incorporated with the growth factors in the form of recombinant proteins to boost the therapeutic response. However, using growth factors for that purpose is associated with a number of distinct disadvantages such as the requirement of large doses, the need for repeated applications, poor distribution, expense and especially short half-life. The healing potential of scaffolds for tissue engineering can be enhanced by incorporating them with the genes. The scaffolds can be used as gene delivery vehicles in this technique. Scaffold mediated gene delivery is an advantageous strategy for gene transfer due to localized delivery of a therapeutic gene. DNA delivered from the scaffold is principally taken up by the seeded cells, therefore limiting unwanted exposure in other areas.

Bacterial cellulose-collagen composites can be used as a scaffold for genes in cartilage tissue engineering due to their biocompability, high waterholding capacity and biodegradability.

Rat bone marrow mesenchymal stem cells are preferable cell source for the tissue regeneration. Rat bone marrow mesenchymal stem cells (RBMSCs) can be easily isolated, expanded and cryo-preserved. Moreover, these cells are able to differentiate into osteo-, adipo- and chondrogenic cells.

In this study, rat bone marrow stem cells (RBMSCs) seeded bacterial cellulose-collagen scaffolds that were crosslinked with DHT and/or Genipin were used to investigate the

effect of scaffold mediated gene therapy on chondrogenic differentiation process of RBMSCs. The bacterial cellulose-collagen scaffolds were incorporated with phrGFP-II-I plasmids which contain IGF-I and TGF-β1 genes to induce chondrogenic differentiation process of RBMSCs. For this study, MTS cell proliferation assay was used to learn about the cytotoxic effects of untreated and crosslinked scaffolds. For the characterization of RBMSCs, flow cytometry analysis was carried out. Besides, Alcian blue staining was applied to analyze chondrogenic potentials of RBMSCs. In addition, to assess the effect of gene application on protein production of cells, total protein concentration of samples were compared. Confocal microscopy study was carried out to show the presence of cartilage specific proteins. The results of these studies demonstrated that RBMSCs were proliferated and differentiated into chondrogenic cells on crosslinked bacterial cellulose-collagen scaffolds that were incorporated with phrGFP-II-I plasmids which contain IGF-I and TGF-β1 genes better than the others.

The combination of an efficient IGF-I and TGF-β1 gene transfer system residing in bacterial cellulose-collagen scaffold that was crosslinked with different crosslinkers provided better chondrogenesis. Ultimately, the application of a DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds containing IGF-I and TGF-β1 genes can be a promising scaffolds for cartilage repair to provide efficient sustained protein expression within a defect without the side effects of the current recombinant protein based technologies.

ÖZET

KIKIRDAK YENİLENMESİ İÇİN IGF-I VE TGF-β1 GENLERİ YÜKLENMİŞ BAKTERİ KAYNAKLI SELULOZ-KOLLAJEN DOKU İSKELELERİNİN KULLANILMASI

Doku mühendisliği ve rejeneratif tıpın temel amacı biyolojik, mekanik ve yapısal uyarıcılar kullanarak yeni ve işlevsel doku oluşumuna yardımcı olmak amacıyla bileşenler geliştirmektir.

Doku mühendisliğinde kullanılan doku iskeleleri, terapötik yanıtı artırmak amacıyla çoğunlukla rekombinant protein formundaki büyüme faktörleri ile birleştirilir. Fakat, bu amaçla büyüme faktörü kullanmanın, yüksek doz ihtiyacı, tekrarlı uygulama gereksinimi, yetersiz dağılım, maaliyet ve özellikle kısa yarı ömür gibi çok sayıda dezavantajı vardır. Doku mühendisliği için kullanılan doku iskelelerinin iyileştirme potansiyeli onların genlerle birleştirilmesiyle artırılabilir. Doku iskeleleri ekilmiş hücrelere gen aktarım aracı olarak kullanılabilirler. Doku iskelesi aracılı gen aktarımı, terapötik genin bölgesel transferi bakımından avantajlı bir stratejidir. Doku iskelesinden salınan DNA, ekilmiş olan hücreler tarafından alınmakta ve böylece istenmeyen yerlere dağılım sınırlanmaktadır.

.

Bakteri kaynaklı selüloz-kolajen kompozit polimerleri, kıkırdak doku mühendisliğinde biyouyumlulukları, yüksek su tutma kapasitesi ve biyobozunurlukları nedeniyle doku iskelesi olarak kullanılabilirler.

Sıçan kemik iliği mezenkimal kök hücreleri, doku yenilenmesi için tercih edilen hücre kaynağıdır. RBMSC'ler, kolayca izole edilip, çoğaltılabilir ve dondurulup saklanabilir. Ayrıca, bu hücreler kemik, yağ ve kıkırdak hücrelerine farklılaşabilmektedirler.

Bu çalışmada, doku iskelesi aracılı gen terapinin, sıçan kemik iliği mezenkimal kök hücrelerinin kıkırdağa farklılaşma sürecindeki etkisini araştırmak amacıyla DHT ve/veya Genipin ile çapraz bağlanmış bakteri kaynaklı selüloz- kollajen doku iskelelerine ekilmiş sıçan kemik iliği mezenkimal kök hücreleri kullanılmıştır. Bakteri kaynaklı selüloz-

kollajen doku iskeleleri, sıçan kemik iliği mezenkimal kök hücrelerinin kıkırdağa farklılaşmasını sağlamak amacıyla IGF-I ve TGF-β1 genlerini taşıyan phrGFP-II-I plasmidler ile yüklenmiştir. Bu çalışmada, çapraz bağlanmış ve bağlanmamış doku iskelelerinin hücreler üzerindeki sitotoksik etkilerini öğrenmek amacıyla MTS hücre çoğalma testi kullanıldı. Bu hücrelerin karakterizasyonu için, akış sitometri analizi yapıldı. Bunun yanında, sıçan kemik iliği mezenkimal kök hücrelerinin kıkırdağa farklılaşma potansiyellerinin analizi için alcian mavi boyaması uygulandı. Ek olarak, gen uygulamasının hücrelerin protein üretimi üzerindeki etkisini ölçmek için, örneklerin toplam protein konsantrasyonu karşılaştırıldı. Kıkırdağa özel proteinlerin varlığını göstermek amacıyla konfokal mikroskopu çalışması yürütüldü. Bu çalışmaların sonuçları, sıçan kemik iliği mezenkimal kök hücrelerinin, phrGFP-II-I plazmidler ile yüklenmiş çapraz bağlı bakteri kaynaklı selüloz-kollajen doku iskelelerinin üzerinde diğerlerine göre daha iyi çoğaldığını ve kıkırdak hücrelerine farklılaştığını gösterdi.

Çeşitli çapraz bağlayıcılarla bağlanmış bakteri kaynaklı selüloz-kollajen doku iskelesi içine yüklenmiş IGF-I ve TGF-β1 gen transfer sistemi kombinasyonunun daha iyi kıkırdak oluşumu sağladığı görülmüştür. Sonuç olarak, DHT/Genipin uygulanarak çapraz bağlanmış IGF-I ve TGF-β1 genlerini içeren bakteri kaynaklı selüloz-kollajen doku iskeleleri, hasarlı bölgelerde kullanılan rekombinant protein kaynaklı teknolojilerin yan etkileri olmaksızın sürekli protein anlatımı sağlayarak kıkırdak onarımına yardımcı olacak, umut verici malzemelerdir.

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LIST OF ABBREVIATIONS

ACI	Autologous Chondrocyte Implantation
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared Radiation
ADSC	Adipose Derived Stem Cell
BC	Bacterial Cellulose
BMP……...Bone Morphogenic Protein	
CC……..Calcified Cartilage	
CMP	Collagen Mimetic Peptide
COMP	Cartilage Oligometric Matrix Protein
DHT	Dehydrothermal Treatment
DS	Dermatan Sulfate
DZ	Deep Zone
ECM……...Extra-cellular Matrix	
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EGF	Epidermal Growth Factor
ERK-1	Extracellular Signal-Regulated Kinase-1
ESC	Embryonic Stem Cell
FACIT	Fibril Associated Collagens with Interrupted Triplehelices
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GA	Glutaraldehyde
GAG	Glycosaminoglycan
HA	Hyaluronan
HAS	Hyaluronan Synthase
IGF-I	Insulin-like Growth Factor-1
IL-1β	Interleukin-1Beta
KS	Keratan Sulfate
MACI	Matrix Assisted Autologous Chondrocyte Implantation
MAPKs	Mitogen-Activated Protein Kinases
MMP	Matrix Metalloproteases

MSC……..Mesenchymal Stem Cell
NSAIDs Non-sterodial Anti-inflammatory Drugs
NiPAAm Poly(N-isopropylacrylamide)
OA Osteoarthritis
PBT Polybutylene tetraphtalate
PDGF Platelet-derived Growth Factor
PEG Polyethylene glycol
PGA……...Poly(glycolic acid)
PHBV Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)
PLA……....Poly(lactic acid)
PMMA Polymethylmetacrylate
PVA Polyvinyl alcohol
PU Polyurethanes
RBMSC Rat Bone Marrow Mesenchymal Stem Cell
SRLP Small Leucin Rich Proteoglycan
SEM Scanning Electron Microscopy
SZ Superficial Zone
TGF-β Transforming Growth Factor Beta
TNFα Tumor Necrosis Factor Alpha
TZ Transitional Zone

1. INTRODUCTION

Cartilage is a vertebrate connective tissue that forms part of the skeletal system [1]. During the embryonic development cartilage is derived from ectoderm or mesoderm. Developmentally cartilage always arises from mesenchyme, in advance of cell differentiation, some of cells aggregate to form blastema. Then these cells start to produce cartilage specific cartilage matrix which are known as chondroblasts. In the process of development, chondroblasts continue to increase their number and produce extracellular matrix that separate cells at last. The cells attached in highly specialized matrix are termed as chondrocytes [2].

Cartilage tissue is devoid of common tissue systems for nutrition and healing such as blood vessels, nerves and lymphatic system [3]. Consequently, chondrocytes exchange their nutrient/waste through the diffusion, thus cells have low oxygen tension environment [4]. Cartilage has limited self-regeneration ability since mesenchymal stem cells and macrophages cannot access the avascular cartilage [5]. Extracellular matrix (ECM) of cartilage is composed of mainly type II collagen, proteoglycans and water [6]. Due to interactions of these components, cartilage is able to withstand tension, compression and shear forces [7]. Cartilaginous tissues are found in the trachea, bronchi, nose, ears, larynx, and intervertebral disks and on articular surface of long bones in the human body [8].

In mammals, depending on appearances of their matrices, cartilage can be subdivided into three categories such as hyaline cartilage, elastic cartilage and fibrocartilage [9]. Elastic cartilage exists in flexible parts of the external ear and parts of the larynx. Major activity of elastic cartilage is to arrange patency of lumina of the tubes that are surrounded by variety of this cartilage. Fibrocartilage is found between fibrous tissue and cartilage, it is located in the junctions between the large tendons, menisci of the knees and intervertebral discs. Fibrocartilaginous menisci are able to absorb the shock and adequately diminish the stress applied to cartilage and underlying bone [10]. Fibrocartilage contains type I, type II collagen and aggrecan within the matrix. Apart from the other types of cartilage, hyaline

cartilage is the predominant type of cartilage found in and around joints [4,6]. Hyaline cartilage is able to bear compressive loads due to relative distribution of its main components, collagen type II and aggrecan [11]. Hyaline cartilage is notably specialized tissue and there are regional zones by virtue of their organization of collagen network.

1.1. ARTICULAR CARTILAGE

Highly specialized type of hyaline cartilage that covers smooth surfaces of diarthrodial joints is called “articular cartilage”. Blood vessels, nerves and the lymphatic system are absent in articular cartilage [12].

Articular cartilage is a connective tissue that contains 65-80% fluid, 25-35% ECM and highly specialized sparse population of chondrocytes [13]. Interaction between these components allows cartilage to withstand mechanical forces [7]. Water content provides nutrition and medium for lubrication as well as it gives elasticity and load bearing capacity to cartilage [2].

Chondrocytes which have spheroidal shape are scattered through the well-structured ECM. Due to its unique distribution of ECM, there is no interaction between chondrocytes. The cells are responsible for the synthesis and maintenance of stiff and viscoelastic ECM. Chondrocytes transport their nutrition from synovium through the ECM by diffusion. Thus chondrocytes have limited metabolic and proliferative abilities [14].

ECM is composed of type II, as well as types III, VI, IX, X, XI, XII and XIV collagens, glycosaminoglycans, proteoglycans (aggrecan, biglycan, decorin, fibromodulin, lumican), hyaluronic acid and noncollagenous proteins (COMP and link proteins) [15].

Collagens account for 10-20% of wet weight of the articular cartilage. Type II collagen is 425 kDa homotrimer helical glycoprotein serves as a scaffold of the ECM. It gives tensile strenght to the articular cartilage [16]. This protein supports chondrocyte adhesion and induces phenotypic differentiation of the cells. Besides these functions, it also enables cartilage to withstand shear forces. Type VI collagen that is found in the pericellular matrix

of ECM attach chondrocytes to the matrix [2]. Collagen type IX is a 222 kDa disulphide-bonded heterodimer protein that is a member of subfamily of collagens termed FACIT (termed fibril associated collagens with interrupted triplehelices) [4]. Type IX collagen attaches collagen fibrils with the other macromolecules in macrofibril structure of the ECM. Crosslinker activity of Type IX increases tensile properties of the articular cartilage and inter-fibrillar connection. Type X collagen is maintained by hypertrophied cells in calcified cartilage layer [2]. This protein supports the structure of ECM and helps in cartilage mineralization. Type XI collagen form as heterotypic fibril with type II and IX collagens [17]. Although it is expressed by non-chondrogenic tissues, this protein is mainly present in cartilaginous tissue [4].

GAG molecules are made up of disaccharide molecules, mainly chondroitin sulphate, keratan sulphate and little amount of dermatan sulphate chains. Due to their charged sulphate and carboxyl groups, GAGs covalently bind to the protein cores of proteoglycans [18].

Proteoglycans contain a core protein glycosaminoglycans (GAG) chains are bonded [19] (Figure 1.1). Proteoglycans are found in ECM as aggregates formed by the non-covalent association of proteoglycan with a hyaluronic acid and a link protein. Proteoglycans has an important function to maintain the fluid and electrolyte balance and load-bearing mechanism in the articular cartilage [20]. Negatively charged sulphate and carboxylate groups of proteoglycans attract inorganic ions such as sodium within the matrix. This function of proteoglycans creates osmotic swelling pressure in the articular cartilage and cations (Na, K) can attach to dissolved plasma proteins of chondrocytes. Result of Donnan effect gives the compressive stiffness to cartilage tissue. Moreover, water intake increases into cartilage because of osmotic imbalance between cartilage and surrounding tissues playing main role in biphasic lubrication mechanism [21].

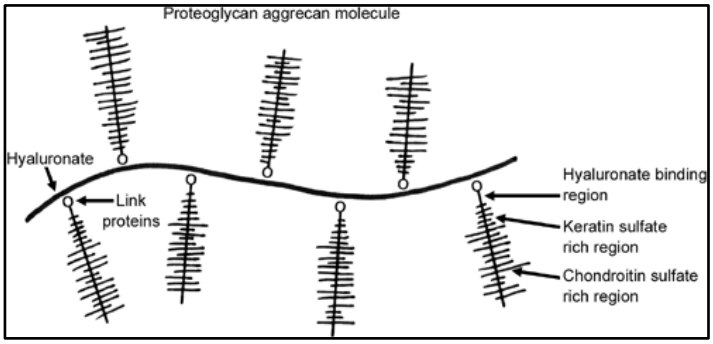

Figure 1.1. Schematic of proteoglycans [22].

Aggrecan is cartilage specific large aggregating proteoglycan and absent in bone. It contains approximately 100 chondroitin sulfate chains which have 200 kDa each [23]. Three globular domains (G1, G2 and G3) and three extended domains (IGD, KS and CS) are found in aggrecan (Figure 1.2). Due to their cysteine residues, each globular domain is able to make disulphide bond with the other molecules. The G1 domain is found in the amino terminus site of aggrecan. This domain possesses three disulphide bonded functional region (A, B1 and B2) that are responsible for the interaction with the link protein (A) or hyaluronan (B1 and B2). The interaction of aggrecan with hyaluronan provides structural ability to withstand compression and link protein stabilize this interaction. Short inter globular domain (IGD) is found between G1 and G2 region, GAG attachment site separates G2 and G3 regions [24]. Aggrecan molecules are found within the extracellular matrix as proteoglycan aggregates. Each aggregates possesses central hyaluronan filament with up to 100 aggrecan molecules. Charged groups of chondroitin and keratan sulfate chains cause an intake of water into cartilage that lead to swelling and expansion of aggrecan rich matrix network. This highly hydrated ECM acts like stiff elastic polymer that can withstand sudden and sustainable loads and lubricate our joints [25]. As a constitutinal ECM protein, aggrecan also mediates chondrocyte-chondrocyte interactions and modulates cell adhesion to the matrix.

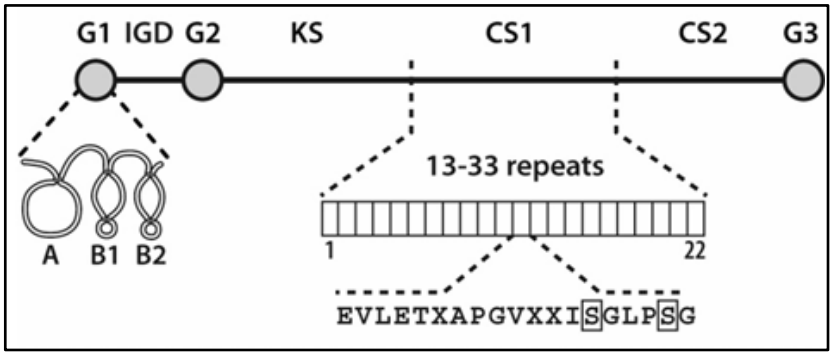

Figure 1.2. The structural domains of aggrecan [23].

SRLPs (small leucin rich proteoglycans) have N-linked oligosaccharide chains and leucin rich repeats. Some members at SRLPs such as Fibromodulin and lumican possess keratan sulphate (KS) chains. Other members, decorin and biglycan have KS and DS within amino terminus of their core proteins. Core proteins of SRLPs interact with collagen fibrils and act as framework of cartilage. This interaction prevents binding of collagenases to collagen molecules. Therefore, SRLPs protect collagens from proteolytic damage. Also GAG chains of SRLPs are able to interact with growth factors including EGF, TGFβ and TNFα and help accumulation of growth factor within the matrix. In this manner, SRLPs have role in differentiation of chondrocytes by allowing growth factor access to the cells [23].

Hylauronan (HA) is the copolymer of glucoronic acid and N-acetylglucosamine and forms non-sulphated GAG. This molecule has high molecular mass and found in extracellular matrix of the connective tissue [26]. HA is synthesized by hyaluronan synthase (HAS) at the plasma membrane of cells and extruded into the extracellular space. HA has a high turnover rate by the action of hyaluronidases or free radicals [23]. It has a main role during cell differentiation. Due to its function in water and plasma protein homeostasis, HA plays a significant role in the functional and structural integrity of connective tissues [27].

Articular cartilage is a strong anisotropic tissue from the surface of the tissue towards the cartilage-bone interface. Histologically, there are four zones and each zone has different morphology depending on the composition, structure and function of chondrocytes (Figure 1.3). In addition, due to their collagen fibril orientation, each zone has different mechanical properties. Architectural structure of the cartilage is subdivided into four zones, superficial

zone (SZ), transitional zone (TZ), deep (radial) zone (DZ), calcified cartilage (CC), from top to bottom [28].

Superficial zone is the thinnest layer among the other layers of cartilage. Lubricin covers the outer surface of this zone and provides smooth surface to the articular cartilage [2]. Chondrocytes in superficial zone have ellipsoid shape and align parallel to the joint surface. There is high concentration of collagen and low concentration of proteoglycans in this zone. SZ has the highest mechanical properties because of its parallel collagen orientation [28].

Transitional zone has a lower concentration of chondrocytes which are mainly spherodial in shape and embedded in extensive ECM. Arrangement of collagen is random and the highest aggrecan content is found in this zone [2].

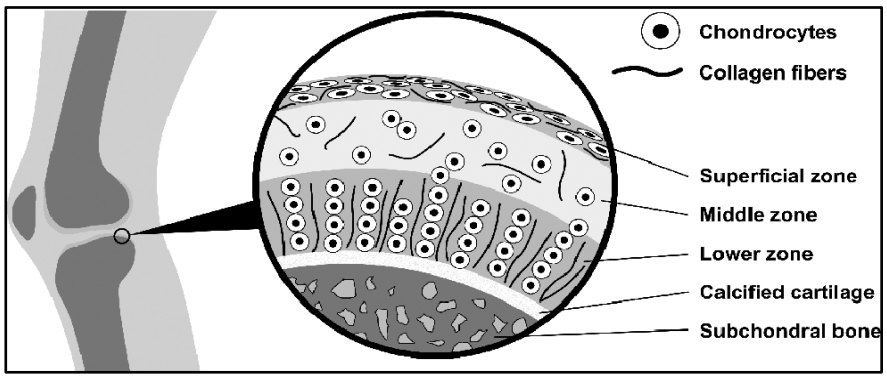

Figure 1.3. Structural layers of cartilage [29]

Apart from the other zones of articular cartilage, middle zone has the lowest cell number. Arrangement of cells is vertical to the surface of articular cartilage [2]. Unlike cell concentration, size of collagen fibrils and proteoglycan contents are maximal in middle zone. Due to the low concentration of collagen, middle zone has lower tensile modulus than the superficial zone [28].

Calcified cartilage zone comprises calcified matrix and low number of cells. Chondrocytes in this zone are hypertrophic and can produce type X collagen to mineralize the ECM [28]. Besides its important function in structural integrity, Type X collagen is able to absorb

mechanical loads along with the subchondral bone. The observable line between MZ and CC zone is called ‘tidemark’ and it acts as main transition to the subchondral bone [2].

1.2. Articular Cartilage Defects

Articular cartilage that covers the surfaces of bone in diarthrodial joints is faced with strains, pressures and stresses daily. Although the function of articular cartilage is to minimize friction and wear when it is subjected to loads, repetitive minor trauma or major explicit injuries can cause defects on cartilage [30].

In case of injury, the tissue response generally follows a cascade of necrosis, inflammation, repair and scar modelling [2]. The most significant factor of healing is vascularization of the site where necrosis occurs. Because of avascular nature of articular cartilage, stem cells cannot reach this tissue so it lacks regeneration ability [31]. Cartilage injuries can occur in case of direct mechanical trauma to the matrix without damaging the chondrocytes or mechanical destruction of cells and matrix. The second situation can generally be seen in clinical practice due to the limited self-regeneration ability of articular cartilage [2].

In response to sudden or repetitive trauma, degeneration of chondrocytes and apoptosis is observed in articular cartilage. Trauma also leads to destruction of ECM by activating gene expression of inflammatory mediators, cartilage degrading proteases and stress response factor [32]. In addition, trauma causes water intake of cartilage and inhibit synthesis of proteoglycans by the cells [2].

Defects of articular cartilage can be classified depending on their depth and size. Depth of articular cartilage defect is divided into chondral defects and osteochondral defects (Figure 1.4). Chondral defects can be full thickness or partial thickness that are unable to heal but might be filled with fibrocartilaginous tissue [33]. Since fibrocartilage is weaker than the articular cartilage, it degrades over time. Osteochondral defects, on the other hand extend to the subchondral bone. Bone marrow mesenchymal progenitor stem cells are able to reach to these types of defects. Due to low intrinsic reparative capability of chondrocytes, size of defect is the most significant factor in healing response. Some studies showed that

while defects <3 mm in diameter are able to heal in 9 months, larger defects do not heal completely [34].

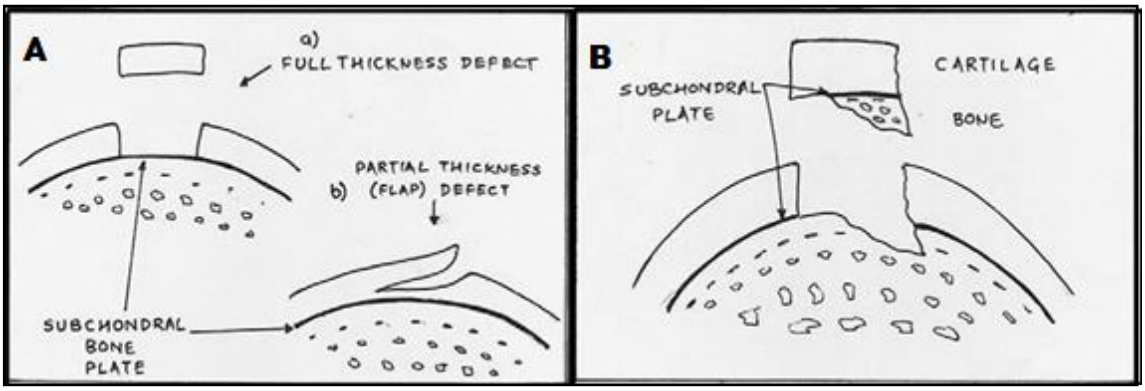

Figure 1.4 A- Full thickness and partial thickness defects of cartilage, B- Osteochondral defects of cartilage [2].

Cartilage disorders as a result of damage or disease disturb both its architectural organization and functional ability of the tissue due to low self-reparative ability of articular cartilage. Common symptoms of chondral injury are effusion, localized or diffused pain, pseudo-locking and catching [35].

Osteoarthritis (OA) is one of the most common cartilage disorder in the aging world population. Degenerative mechanism of OA involves loss of hyaline articular cartilage, bony remodelling, bone marrow lesions, laxity of ligaments, capsular stretching and weakness of periarticular muscles and it can cause functional failure of synovial joints at last [36]. Enzymatic degradation of ECM of articular cartilage leads to development of fissures, gross ulcerations and the disappearance of full thickness surface. In addition, cell death, hypertrophic differentiation of cartilage cells and inflammation of synovial membrane are also observed in osteoarthritic lesions of cartilage [37]. Risk factors for the development of OA can be aging, abnormal mechanical stresses, genetic factors, obesity, diabetes and abnormalities in bone or cartilage [38]. Valdes *et al.* have shown that genetic factors mainly influence development of Type I OA in twin studies [39]. Polymorphism in genes that control chondrogenic differentiation or mutations in genes encoding ECM and signaling molecules may increase susceptibility to OA before the age of 40 [40].

Moreover, OA can also be related to gender for example mutated genes may have different function in males and females and at different body locations [41]. Also Type II OA is related with menopause and this is the evidence that estrogens are important in OA [42]. Obesity is defined as risk factor for initiating and developing OA due to increased load transfer within articular joints [43].

OA can cause some changes in articular cartilage such as gradual proteolytic degradation of extracellular matrix and increase synthesis of matrix substitutes by chondrocytes [44]. Matrix metalloproteases (MMP) which are responsible for cartilage degradation cause OA. Members of three MMP groups including the collagenases, the stromelysins and the gelatinases are known as being increased in OA. Urokinase and plasmin which are the members of plasminogen activation/plasmin family are found in osteoarthritic cartilage and these molecules induce activation of MMP groups [45]. Pro-inflammatory cytokines are also responsible for catabolic processes that occur in osteoarthritic tissues. These cytokines are diffused from synovial membrane to the cartilage. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are the most important catabolic systems involved in joint tissue destruction [46].

Besides cartilage deteriorations, OA also causes some changes in the surrounding bone such as thickening of subchondral bone. Moreover, osteoblasts from osteoarthritic subchondral bone have changed phenotype and increased levels of plasminogen activator and insulin-like growth factor-1 [47].

1.3. Cartilage Repair Strategies

The main aim of the medical therapy for cartilage defects is the reduction of pain starting with paracetamol in mild pain and non-sterodial anti-inflammatory drugs (NSAIDs) in moderate pain [48]. Several therapeutic treatment strategies for cartilage defects are to decrease deterioration progress of cartilage defects. For example, glucosamine which is found in ECM is used for anti-inflammatory drug for the treatment OA and intra-articular injection of hyaluronan can be applied for increasing joint function [49]. Long term effects of these nutritional supplements are not known. Also fibroblast growth factor 18 which

increases cell division rate of chondrocytes and production of proteoglycans are still investigated in phase I and phase II studies [50]. Besides other strategies, reconstructive surgical method is necessary for the improvement joint function and precaution of further damage in defected cartilage. There are many categories for surgical methods of cartilage injury.

1.3.1 Debridement

This technique is nonreparative and nonrestorative. It can be used for smoothing small lesions (<2 to 3 cm^2) in older patients. It involves arthroscopic surgery where small camera and instruments are located through two to three incision to estimate and care for defected area in cartilage [51]. In this technique, loose flaps in defected site that can cause further symptoms are removed so as to avoid conduction of mechanical forces on dense layer of cartilage [52]. Since there are no attempt to restore or repair, debridement is not the best treatment for cartilage lesion. It is only used for patients who have trouble with mechanical activity or load-bearing limitations before other surgical operation such as drilling and microfracture is applied.

1.3.2 Knee Joint Lavage

Joint lavage technique is aimed to remove debris by rinsing joint surface with a physiological fluid [53]. Like debridement, this technique is not reparative and restorative for damaged cartilage. Lavage technique is often used together with debridement and generally applied when other treatment techniques such as debridement and chondral shaving are insufficient [54].

1.3.3. Pridie's Drilling

Drilling into subchondral bone was developed in 1959 by Pridie. It is aimed to induce formation of hyaline cartilage to resurface of subchondral bone by developing blood channels through the bone [55]. In long term follow-up, 85% of patients who were applied this procedure demonstrated improvement. Nevertheless this technique may cause some

undesirable effects such as deterioration of subchondral bone by heat generated during the procedure and hematoma [56].

1.3.4. Arthroscopic Abrasion Arthroplasty

Arthroscopic abrasion arthroplasty is a surgical technique that provides access in vascular system in subchondral bone plate and produce blood clot which can induce repair of fibrocartilaginous tissue [57]. In clinical practice, 50% of patients whose knees treated using arthroscopic abrasion arthroplasty showed improvement in short term but tissue started to degrade after one year follow-up [58].

1.3.5. Microfracture

Microfracture was first applied by Steadman and used for symptomatic chondral defects [59]. Aim of this technique is to create stable hyaline cartilage near the defect site by accessing of marrow elements including progenitor cells and growth factors which induce regeneration process of cartilage. In this technique, arthroscopic awl is used to make multiple fracture perforations in nearly 3-4 mm away from defect site by piercing subchondral bone plate of bone thus bone marrow including platelets growth factors and MSCs is able to reach defect site of cartilage. Components of bone marrow promote healing process of cartilage by filling defect site [59].

Microfracture is modified form of drilling but it is much easier method and there is no over-heating issue. Since microfracture is not time-consuming procedure, it is the most popular treatment strategy among the sportsmen [60].

Hunziker *et. al.* showed that long term follow-up of microfracture technique is 5 years , however another study reported that positive clinical outcomes declined 2 years after the operation [61]. Moreover, Knutsen *et. al.* demonstrated that hyaline cartilage production rate of microfracture is more than ACI technique [62].

1.3.6. Spongialization

Spongialization, developed by Ficat, is modified technique of debridement and drilling [63]. Due to removal of entire cartilage and subchondral bone plate, this technique is more radical when compared to others. Unlike drilling, well innervated subchondral bone plate is removed and spongy bone is exposed. This strategy provides increase in joint motion and 70-80 % pain relief in patients. Disadvantage of spongialization is the potential of thermal necrosis of the neighboring cells that have function in healing process [63].

1.3.7. Mosaicplasty

Mosaicplasty established in 1993, is a popular technique for the treatment of chondral or osteochondral defects [64]. This technique is also known as autologous osteochondral transplantation (OATS) and entails removal of defected cartilage through the subchondral bone by creating small 15 mm deep perforation. Then, cylindrical osteochondral plugs with vertical edges are excised from low-load-bearing site within the knee joint and put in perforation in the defect site. The gap between hole and plug is filled by fibrocartilage tissue. This technique increases function of joint and releases pain whereas some undesirable results such as donor morbidity and chondrocyte death may be observed [64]. Hangody *et. al.* suggest restrictive defect area 1-4 cm^2 to prevent donor site morbidity. Moreover, there is a risk of bone and cartilage collapse and additional defect at donor sites may cause more pain. In clinical practice, 92% patients with defected femoral condyles who were treated by mosaicplasty technique were showed good healing process [65].

1.3.8. Autologous Chondrocyte Implantation

Autologous chondrocyte implantation technique was established by Peterson *et. al* [66]. The aim of autologous chondrocyte implantation technique is to regenerate function of joint and reduce pain in defected cartilage site of knee. In this technique, small piece of cartilage is excised from low-load-bearing site of cartilage and chondrocytes are harvested from this piece. Next, these cells are cultured *in vitro* under proper circumstances. After 2-3 weeks, defect site of joint is opened and covered with periostal flap from upper surface

of patient's tibia or femur. This flap is covered with water tight seal to the edge of defect then cultured cells are injected underneath this flap and after a while cell suspension start to adhere and produce ECM. This procedure is presented by Brittberg *et. al.* and regarded as cell based treatment strategy [67].

In clinical practice, both Knutsen group [68] and Brittberg [69] group were reported that approximately %89 of patients whose knees were treated with ACI technique showed hyaline-like cartilage in defect site. Moreover, long term follow-up of ACI technique was reported as 11 years by Brittberg and his colleagues. In addition Saris group's study of ACI versus microfracture technique showed that ACI showed histologically better structural healing process than microfracture [70]. Besides good results related to this technique, there are some drawbacks of ACI such as risk of chondrocyte dedifferentiation in monolayer culture, long recovery time.

1.4. Cartilage Tissue Engineering

Tissue engineering is a research area aimed to utilize biological substitutes to generate new healthy tissue through combining principles of material science, molecular biology and biomedical engineering [71]. Tissue engineering is the multidisciplinary area since it requires the knowledge associated molecular mechanism of damaged cartilage and mechanics of defect site. Since mature articular cartilage is not able to restore itself completely and other cartilage repair strategies result in fibrocartilage formation or degrade eventually, cartilage tissue engineering is aimed to mimic native structure and durability of articular cartilage to enable long lasting repair of damaged cartilage. MACI (matrix assisted autologous chondrocyte implantation) technique was improved to overcome chondrocyte dedifferentiation issue. MACI which include application of different biomaterials and other cell sources is considered as tissue engineering technique [72]. In tissue engineering there are four important parameters; a suitable cell sources, appropriate scaffolds, mechanical stimulation and optimum signaling molecules [73].

1.4.1. Alternative Cell Sources for Chondrogenesis

Simplicity of isolation and culture, high proliferation ability and maintaining, important ECM molecules such as collagen type II and aggrecan should be considered for choosing an appropriate cell source for cartilage tissue engineering [74].

Autologous articular chondrocytes are mostly used in cartilage tissue engineering to prevent potential immune reaction in donor site of cartilage. However, their isolation and expansion are too hard due to low proliferation ability of chondrocytes and low cell content in the nature of cartilage. In addition, low expansion ability of chondrocytes may cause dedifferentiation of cells *in vitro* conditions. Dedifferentiated chondrocytes start to produce collagen type I instead of collagen type II and aggrecan and eventually change their phenotype after first passage. To overcome these drawbacks, culture conditions of chondrocytes are changed to mimic natural environment of these cells [75]. For example, Narcisi *et al.* used serum-free media [76]. Foldager [77] group reduced oxygen tension of culture and Hardmeier *et. al.* [78] applied mechanical loads through the culture via rotating bioreactor. Unfortunately, none of these attempts showed all desired outcomes of native cartilage. These disadvantages of chondrocytes make them unfavorable cell source for cartilage repair.

Using mesenchymal stem cells (MSCs) for cartilage repair has advantages over the usage of chondrocytes due to their high proliferation ability and capability of multilineage differentiation. MSCs are found in multiple adult tissues such as bone marrow, synovium, periosteum, skeletal muscle, adipose tissue and umbilical cord. Harvested MSCs from these sources are able to differentiate into osteoblasts, myoblasts, chondroprogenitor, hepatocytes and adipocytes *in vitro.* MSCs can be characterized with Flow Cytometry using positive surface markers such as STRO-1, CD73, CD 90, CD105, CD106, CD146, CD 166 and negative markers such as CD45, CD31, CD34, CD11b, CD117. If $CD105^{+}/CD166^{+}$ cells are cultured under the proper conditions, they are able to show osteogenic, chondrogenic and adipogenic potential [79]. These cells are generally found in bone marrow and can easily be isolated. In addition, bone marrow derived mesenchymal stem cells (RBMSCs) are expanded without losing their properties and are easily differentiated into cartilage even after expansion. Chondrogenic differentiation of RBMSCs is induced using proteins or genes from TGFβ superfamily in 3D culture media.

Vinardell *et. al* showed that RBMSCs in chondrogenic differentiation culture led to ECM accumulation and mechanical properties of these constructs were poorer than the chondrocyte derived constructs [80]. Estes and Diekman have reported that adipose derived stem cells (ADSCs) have also chondrogenic potential in the ascorbate, dexamethasone and TGF-β containing media [81, 82]. However, chondrogenic potential of RBMSCs is higher than that of ADSCs. In addition, some studies reported that tissue engineered synovium derived mesenchymal stem cells in chondrogenic media have a similar mechanical properties with articular cartilage. Lepperdinger showed that MSCs lost their proliferation and differentiation capacity with the number of passage and the age of donor [83]. Besides mesenchymal stem cells and chondrocytes, embryonic stem cells can also be excellent source for treatment of cartilage defects since they are able to differentiate into any cell. However, usage of ESCs in cartilage tissue engineering may cause risk of teratoma formation [84].

1.4.2. Scaffolds

The aim of application of biomaterials is to provide the cells with place which induce cells to produce cartilage specific proteins and to regenerate new tissue formation. Scaffolds should be biocompatible and biodegradable for cell viability. When new tissue replaces the scaffold, it should degrade overtime without toxic compounds. Controllable degradation rate of scaffolds should match tissue ingrowth. In addition, surface chemistry of scaffolds should be suitable for cell attachment, proliferation and differentiation. Also scaffolds should have porous structure to provide accessing of nutrients and signaling molecules in the medium. Besides other preferable properties of scaffolds, mechanical competence of the biomaterial is one of the most important parameter in cartilage tissue engineering since mechanical properties of scaffolds should match those of cartilage tissue. Biomaterials are divided into two main categories such as natural and synthetic biomaterials [85].

Most widely used synthetic molecules in medicine include Poly α-hydroxyesters, Poly(ε-caprolactone), Polyanhydrides, Poly(ethylene glycol) (PEG), Polyvinyl alcohol, poly(N-isopropylacrylamide) (NiPAAm), Polyurethane (PU) and Polypropylene fumarate-co-ethylene glycol.

1.4.2.1. Poly α-hydroxyesters

Poly α-hydroxyesters such as polylactides, polyglycolides and their copolymers are widely used in cartilage tissue engineering. Various studies have shown that poly-lactide-glycolide-copolymers induce attachment and proliferation of cells. Both Athanisou group and Freed group have reported that polylactic acid (PLA) and polyglycolic acid (PGA) are able to enzymatically degrade overtime and degradation rate of these copolymers depend on their crystallinity, molecular weight and environmental conditions [86, 87]. Also removal of degradation compounds of these copolymers from the body is done by normal metabolic pathway. Eventhough PLA/PGA copolymers have some advantages, they have low mechanical properties.

1.4.2. 2. Poly (ε-caprolactone)

Poly(ε-caprolactone) is also preferred biomaterial for cartilage tissue engineering. Poly(ε-caprolactone) has longer repeating unit than PGA and this makes Poly(ε-caprolactone) more flexible. However PGA has better thermal and mechanical properties than Poly(ε-caprolactone) scaffolds [88]. Since degradation rate of Poly (ε-caprolactone) is slower than PLA's, Poly(ε-caprolactone) scaffolds are generally used for drug release studies [89].

1.4.2.3. Polyanhydrides

Polyanhydrides has repeating unit of poly (sebacic acid and hexadecadioic acid) with one to one ratio. The polymer rapidly degrades *in vivo* but its aliphatic-aromatic copolymers are used for controlled degradation studies. Due to reaction with free amino groups of drugs, polyanhydrides cannot be used in drug delivery studies [90]. Polyanhydrides have less mechanical and thermal properties than Poly(ε-caprolactone), it has even more $(CH)_2$ than Poly(ε-caprolactone) [91].

1.4.2. 4. Poly ethylene glycol (PEG)

PEG is a member of polyether family and used in cartilage tissue engineering. Various studies have proven that PEG support viability, proliferation and production of ECM. PEG is generally combined with the other biomaterials such as polymethylmetacrylate (PMMA), polybutylene tetraphtalate (PBT) and collagen mimetic peptide (CMP) [92].

Stahl *et. al*. showed that PEG-CMP scaffolds are able to support cell proliferation and increase production of cartilage specific proteins [93].

1.4.2. 5. Poly N-isopropylacrylamide (PNiPAAm)

Ibusuki *et. al.* was the first group that used PNiPAAm for cartilage tissue engineering and their study showed that PNiPAAm has no inflammation and no vascularization effect on cells [94]. Also this polymer induces production of cartilage specific proteins such as collagen type II and aggrecan. In addition, Chen *et. al* showed that PNiPAAm can be an appropriate biomaterial for cartilage regeneration [95]. PNiPAAm is combined with several biomaterials such as chitosan, vinylimidazole and gelatin to increase its mechanical properties. PNiPAAm-hyaluronan and PNiPAAm-chitosan composites are used as injectable hydrogels and they can entrap cells and allow to keep their characteristics within their structure [96]. Studies showed that mechanical properties of cartilage constructs using PNiPAAm-gelatin and PNiPAAm-chitosan copolymers are almost similar with the native cartilage [97, 98].

1.4.2. 6. Polyurethanes

Polyurethanes can form polymer structure in *in situ* conditions and due to ease of production, it can be used in both injectable hydrogels and paste formation. Having high mechanical properties, poly urethanes can be preferred to use in cartilage tissue engineering. Adhikari *et. al.* developed polyurethane co-polymer using dihydroxypolycaprolactone phosphorilcholine and 1,2 dihydroxy N,N-dimethylamino-propane sulfonate polymers to regenerate cartilage substitutes. It was found that this co-polymer support differentiation of cells but show mild inflammatory effect on cells [99].

1.4.2. 7. Polyvinyl alcohol (PVA)

Poly vinyl alcohol is water-soluble and highly adhesive synthetic polymer. Due to its hydrogel structure, Poly vinyl alcohol is able to entrap cells and increase cell interaction. Also this polymer supports proliferation and differentiation of cells. However, this polymer does not completely degrade over the time and withstand mechanical pressures in

bioreactor. Its mechanical properties, especially compressive modulus, are needed to get improved [100].

Several studies have shown that natural biomaterials have some advantages over synthetic biomaterials since they are substitutes of living organisms. Natural biomaterials include agarose, alginate, chitosan, fibrin hydrogels, hyaluronan, gellan gum, poly 3-hydroxybutyric acid-co-3-hydroxyvaleric acid, collagen and bacterial cellulose.

1.4.2. 8. Agarose

Agarose is natural biomaterial and contains galactose monomers. This polymer is used to encapsulate cells and allow cells to keep their phenotype in their structure. Also it has shown that this polymer induces synthesis of glycosaminoglycan in both *in vitro* and *in vivo* [101]. Agarose is one of the most widely used biomaterial for cartilage biomaterial. Awad *et al.* established that in the presence of Transforming growth factor beta 1 (TGF-β1), ADSCs can differentiate into cartilage in agarose scaffolds [102]. In addition Mouw *et. al* showed that agarose is able to induce production of GAG more than the other scaffolds and engineered cartilage using agarose scaffolds has the most similar characteristics with the native one [103]. Tan *et al*. has shown that under mechanical pressures, chondrocytes in agarose scaffolds are able to repair themselves unlike native cartilage. However, it was found out that highly concentrated agarose scaffolds prevent cell migration [104].

1.4.2.9. Alginate

Alginate is produced by Brown algae and due to its high biocompability and water holding capacity, this polymer is generally used in cartilage tissue engineering. It was reported that alginate supports cell migration by interacting with the cell surface receptors [105]. Also alginate induces proliferation and synthesis of cartilage specific proteins such as collagen type II and aggrecan. Cohen *et. al* have reported that *in situ* application of alginate in chondral defects show reparative ability [106]. In addition Tomkoria *et. al* have shown that mechanical properties especially Young's modulus of cartilage constructs using alginate polymer is mostly similar with the native cartilage [107].

1.4.2.10. Chitosan

Chitosan is a deacetylated form of chitin and due to its N-acetylglucosamine and glucosamine contents, chitosan is one of the most widely used biomaterial in cartilage tissue engineering. Chitosan is cheap, biocompatible and inert polymer, however, it doesn't easily take the gel formation so it cannot be applied in *in situ* studies. Recently, application of Chitosan/Poly-3-caprolactone (PCL) in different proportions is becoming popular and studies have shown that mechanical properties of %50 chitosan containing scaffolds are better than %75 ones [108]. Moreover, Alves da Silva *et. al* showed that in the presence of synovial fluid and mechanical load, human mesenchymal stem cells in chitosan- poly butylene tetraphytalate scaffolds are able to differentiate into cartilage and produce cartilage specific proteins such as collagen type II and aggrecan [109].

1.4.2.11. Fibrin Hydrogels

Fibrin gels are the crosslinked form of fibrinogen which is found in blood. Fibrin gels are one of the Food and Drug Administration (FDA) approved biomaterial. Several studies have shown that this biomaterial induce cartilage formation of chondrocytes. Peretti *et. al* used fibrin gels in both swine and mice models and both studies showed that this polymer induce ECM production of chondrocytes [110]. In addition, Rampichova *et. al* used fibrin and hyaluronic acid (HA) to repair cartilage repair *in vivo* and showed that this composite induces production of collagen type II and glycoaminoglycans. Moreover, it has shown that autologous bone marrow stem cells seeded platelet rich fibrin gels were used to fill cartilage defects and 3 of 5 patient showed good results but the success rate depends on the concentration of cells [111].

1.4.2.12. Hyaluronan (HA)

Hyaluronan is one of the GAG molecules which plays crucial role in cartilage ECM. Due to its presence in native cartilage, hyaluronan is a popular biomaterial in cartilage tissue engineering. There are several hyaluronan based scaffolds on the market such as Hyaff-11 (Fidia Advanced Biopolymer, Abano Terme, Italy). This scaffold needs TGF-β1 to enhance chondrogenesis of human mesenchymal stem cells. Also, this scaffold upregulates the production of collagen type II rather than collagen type I. Furthermore, methacrylate form of hyaluronan is used to uniform distribution of cell population and ECM proteins

[112]. However, hyaluronan is expensive, highly degradable and its mechanical properties are very low.

1.4.2.13. Gellan Gum

Gellan gum is a polysaccharide which is produced by *Sphingomonas paucimobilis.* Oliveira *et. al* is the first group used this biomaterial in cartilage tissue engineering [113]. Gellan is water soluble biomaterial and become easily take the gel form. Moreover, this biomaterial is biocompatible and shows adequate rheological properties in the tissue culture. However, this biomaterial cannot withstand mechanical loads.

1.4.2.14. Poly 3-hydroxybutyric acid-co-3-hydroxyvaleric acid (PHBV)

Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) is a natural biomaterial in origin. Due to its adequate biodegradability, PHBV becomes popular in cartilage tissue engineering field. PHBV is the combination of PHB (polyhydroxybutyric acid) and PHV (polyhydroxyvaleric acid) polymers. Malm *et. al* reported that PHB is an appropriate biomaterial for repairing defects in calves and this polymer show mild foreign body reaction [114]. G.T. Köse *et. al* established that chondrocyte seeded Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) containing 8% by mole of 3-hydroxyvalerate polymers are able to support hyaline like cartilage regeneration [115].

1.4.2.15. Collagen

Collagen is the main component of cartilage ECM and due to its presence in most tissue such as bone, cartilage, skin and tendon, collagen is one of the most abundant biomaterial in the world. Collagen has been used in widespread applications such as surgical sutures, wound healing, corneal shields, cosmetics and tissue engineering. Several studies demonstrated that even if collagen is a biocompatible biomaterial, it can be a reason for mild foreign tissue response in host tissue when donor and host of collagen are from different organisms [116, 117]. Due to its biomimetic interface for cells and high water holding capacity, collagen is an attractive biomaterial for cartilage regeneration. In addition, collagen is bioactive and highly adhesive biomaterial but also low mechanical characteristics of collagen limit its application in cartilage tissue engineering. There are

various attempts to increase mechanical properties of collagen such as electrospinning, chemical and physical crosslinking methods.

Electrospinning is used to both increase scale of collagen fibers from nano to micro and align collagen fibers to create biomimetic zone for cells. Whereas electrospinning method increases mechanical properties of collagen, it is still unstable in aquaeous environment and need further attempts to create an adequate scaffold for cartilage tissue engineering [118].

To overcome these drawbacks, several crosslinking agents such as glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide and genipin can be used to stabilize collagen fibers (Figure 1.5). However, several studies showed that while these chemicals especially GA and EDAC are increasing mechanical properties of collagen scaffolds, they may reveal cytotoxic effect on cells and decrease hydration rate of collagen [119, 120]. Besides other crosslinking agents, genipin can show notably less cytotoxic effect on cells and increases swelling behaviour of collagen. Genipin is extracted from fruits of *Gardenia jasmoinides Ellis* and animal studies showed that genipin causes no abnormal effect on kidney, liver and blood [121]. In the presence of genipin, primary amine groups on lysine and arginine residues of collagen attract C-3 atom of genipin by nucleophilic attack afterwards oxygen atom replace with nitrogen atom in the six membered ring of genipin.

Physical crosslinking methods such as photopolymerization and dehydrothermal treatment (DHT) can also be applied to increase the mechanical characteristics of collagen scaffolds. In DHT technique, collagen is subjected high temperature (>90 ^{0}C) under the vacuum. In this method esterification or amide formation between collagen molecules is provided by removing water from them. DHT is known as advantageous method since it doesn't need any cytotoxic reagent to make bridge between the molecules and also this method sterilizes scaffolds. O'Brien *et. al* showed that increased rate of mechanical properties of collagen-GAG scaffolds depends on the temperature and duration of DHT technique [122].

On the other hand, collagen is used to make copolymer with the other polymers such as GAG, bacterial cellulose, PVA. In these copolymers, collagen generally increases biocompability and elasticity of other polymers [123].

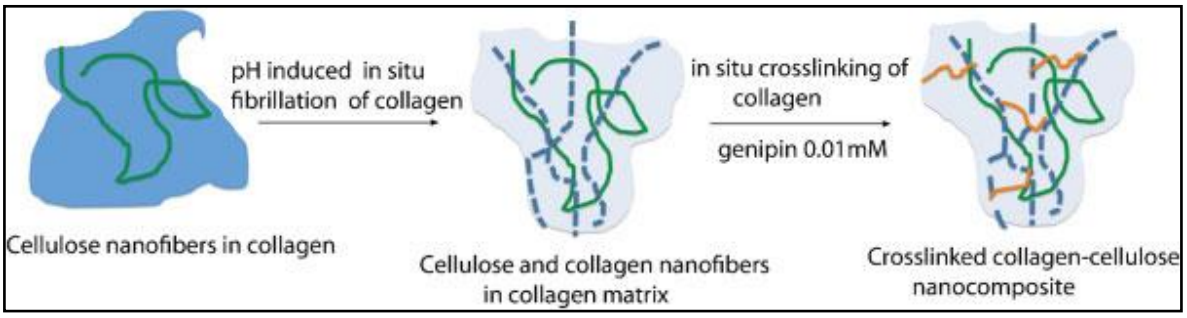

Figure 1.5. Schematic of crosslinked bacterial cellulose-collagen biomaterials using genipin [124].

1.4.2.16. Bacterial Cellulose

Bacterial cellulose (BC) is the most abundant and renewable biomaterial in the world. BC is secreted by *Acetobacter xylinus* as its component of ECM. Unlike plant cellulose, bacterial cellulose consists only branchless glucose chains with β (1→ 4) glycosidic bonds (Figure 1.6). Bacterial cellulose has high load bearing and water-holding capacity and ultrafine fibre network. Also, its large scale production and process are so easy and cheap. In addition, various studies showed that BC shows less cytotoxicity than the other polymers because it doesn't have organism specific structure like collagen [125, 126]. BC presents features which make it an attractive biomaterial in several tissue engineering fields such as bone, cartilage, vascular and skin tissue engineering. Due to absence of cellulose hydrolizing enzymes in mammalian cells and its high crystalline structure, BC doesn't degrade easily and low degradation rate restricts its utility in tissue engineering. To address this challenge, BC is generally combined with the other polymers to increase its degradation rate.

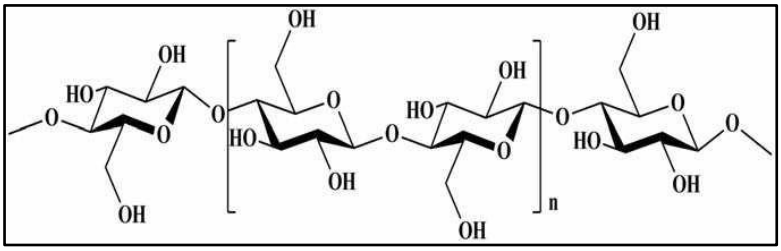

Figure 1.6. Chemical structure of bacterial cellulose [127].

Several studies established that BC-collagen copolymer is beneficial scaffold for cartilage tissue engineering [128, 129]. While collagen provides biomimetic environment and gives elasticity, bacterial cellulose increases mechanical properties of this copolymer. In addition, both collagen and bacterial cellulose have high water uptake capacity, biocompatible residues resulting degradation and are easily shaped into 3D structure. Freeze-drying technique is generally used to give 3D structure of collagen-BC scaffold [130]. In this technique, first liquid which dissolves collagen and BC mix is freezed and then the liquid is lyophilized to create porous 3D structure. Also this technique makes hydrogen bond between hydroxyl (-OH) groups of glucose molecules by removing water from them and increases crystallinity and mechanical properties of bacterial cellulose [131].

1.4.3. Signaling Molecules

Several studies have shown that growth factors, cytokines and hormones act essential role in catabolic and anabolic processes of chondrocyte and mesenchymal stem cells such as proliferation and differentiation [132, 133]. The main aim of using signaling molecules combined with cell seeded scaffold is to regenerate large cartilage defects and create tissue constructs like native cartilage. Various studies showed that transforming growth factor β (TGFβ) isoforms [134], insulin-like growth factor (IGF-I) [135], bone morphogenetic proteins (BMPs) [136], fibroblast growth factor (FGFα) [137], platelet-derived growth factor (PDGF) [138] and epidermal growth factor (EGF) [139] are used to stimulate chondrogenesis and ECM production of MSCs in cartilage tissue engineering.

IGF-I is known as crucial anabolic factor in hyaline cartilage. IGF-I is found at 50 ng/ml concentration in synovial fluid where chondrocytes transport their nutrition. Also chondrocytes keep IGF-I at 10 ng/ml concentration in their ECM [140]. IGF-I is a mitogenic protein and induce proliferation and growth of chondrocyte. Yoon and Fisher [141] reported that IGF-I can be used to promote chondrogenesis pathway of MSCs. In addition, *in vitro* studies have shown that this protein is able to stimulate both collagen type II and proteoglycan synthesis and inhibits ECM degradation by upregulating tissue inhibitors of matrix metalloproteinases [142, 143]. Due to its important anabolic effects and presence in native cartilage, IGF-I is generally added in culture medium to create physiological equivalent environment with the native hyaline cartilage.

To date, several studies reported that TGF-β is responsible for controlling cell differentiation by stimulating different types of cascades such as MAPKs, ERK-1, p38 [144]. TGF-β involves in various cellular processes such as proliferation, differentiation, apoptosis and inflammatory responses. TGF-β plays an important role in cartilage development and repair [145]. TGF-β 1, 2 and 3 isoforms are able to induce proliferation rate and ECM synthesis of chondrocytes [146]. In addition, it is known that TGF-β 1 and 3 promote chondrogenesis pathways of MSCs. Although many studies showed positive effects of TGF-β on chondrogenesis, other studies are established that TGF-β impairs collagen synthesis and inhibits growth of MSCs and chondrocytes [147, 148]. It is suggested that these controversial results depend on cell cycle stage of cells.

Bone morphogenetic proteins (BMPs) are found in bone, cartilage and connective tissues in the body [149]. It has been established that BMP signalling is crucial for differentiate of MSCs into cartilage tissue. BMP isoforms such as BMP-2 and BMP-7 are able to induce cartilage repair [150]. Moreover, BMP-4 promotes chondrogenesis of muscle-derived stem cells in rats [151]. Also, BMP-4 and BMP-2 transduce human mesenchymal stem cells into cartilaginous tissue [152].

Because of different effects of every signalling molecule on cartilage differentiation process, combination of these molecules is generally used in cartilage tissue engineering. Also this is an advantageous method to mimic the natural environment of cartilage. It has

shown that administration of IGF-I and TGF-β1 in culture medium is increased GAG and collagen type II synthesis of MSCs [153]. Kim and Im [154] compared chondrogenic effect of TGF-β2 with members of BMP family (BMP 2, 6, 7) on MSCs and it has shown that TGF-β2/BMP7 combination has the most chondro-inductive effect among other combinations. In addition, several studies showed that different combinations of TGF-β3 with BMP 2, 4, 6 and IGF-I are able to promote chondrogenesis of MSCs [155, 156].

Various studies showed that growth factors used in culture medium have short half-lives [157, 158]. For example, IGF-I has 10-20 min and TGF-β1 has 15 min half-life [159]. However, to mimic native cartilage physiology, growth factors must be released in culture medium over the time course of regeneration. Moreover, these growth factors should be found at localized area to bind their receptors and activate their pathways in cell. To address these challenges, tissue engineering is combined with gene therapy strategies since these strategies provide prolonged protein expression in cells.

1.5. Gene Therapy

Gene therapy is the method that delivers genetic material into cells using vectors. The main aim of this technique is to change cellular genetic information. Viral and non-viral vectors are used to transfect cells. Due to natural transfection ability of viruses, viral vectors are more effective technique than non-viral vectors to deliver genes into cells. Although the encoding proteins of the genes that cause pathogenic effect on cells are removed, viral vectors have still safety issues because their integration ability into genome may cause mutagenesis and malignancies in cellular processes [160]. Because of its low toxicity and immune response, gene transfection using non-viral vector is considered as a safe method. Recently several studies are aimed to increase transfection ability of non-viral vectors by changing their vector system [161, 162]. Moreover, polycations and liposomes are widely used to increase transfection efficiency of non-viral vectors. Some of these biomaterials are still on the market for example Lipofectamine $^{2000\ TM}$ (Invitrogen) and FuGene (Roche).

Recently, scaffold mediated gene therapy technique incorporating non-viral vectors is used in tissue engineering. This application combines cells, scaffolds and signalling molecules

which encode from desired genes and support tissue regeneration. Scaffold mediated gene therapy provides both 3D environment which induce interaction between cells and prolonged release of growth factors which promote differentiation of cells [163]. In addition, scaffold acts as a reservoir for genes and provide continual production of signalling molecules. Also scaffold mediated gene therapy limits unwanted exposure of genes and their proteins in the other areas [164]. Capito and Spector showed that chondrocytes are able to induce GAG and collagen type II synthesis in the cell-seeded scaffold combining IGF-I genes [165].

1.6. Objective of The Study

In this study, 3-D bacterial cellulose-collagen scaffolds that were crosslinked with Genipin, DHT and DHT/Genipin incorporated with IGF-I and TGF-β1 genes were used to promote chondrogenesis of rat bone marrow mesenchymal stem cells.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Scaffold Preparation

2.1.1.1. Production of Bacterial Cellulose

- *Gluconacetobacter xylinus*
- D-Glucose (Riedel-de-Haen)
- Yeast extract (Fluka)
- Peptone (Fluka)
- Acetic acid (Sigma)
- Deionized water

2.1.1.2. Preparation of Bacterial Cellulose-Collagen Scaffolds

- Bacterial cellulose
- Collagen isolated from Spraque-Dawley rat tails
- Pure acetic acid (Sigma)
- Distilled water

2.1.1.3. Crosslinking of Bacterial Cellulose-Collagen Scaffolds

- Freeze-dryer (Thermo Scientific)
- Vacuum oven (Becton Dickinson)
- Genipin (Sigma)
- DMSO (Sigma)
- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)

2.1.2. Characterization of Bacterial Cellulose-Collagen Scaffolds

2.1.2.1. Scanning Electron Microscopy of Cell Seeded Bacterial Cellulose-Collagen Scaffolds

- Cacodylic Acid Sodium Salt Trihydrate (AppliChem, Germany)
- Glutaraldehyde Solution, Grade I, 25% (Sigma-Aldrich Corporation, Germany)
- Deionized water
- Sputter Coater (Bal-tec SCD 005, Germany)
- Scanning Electron Microscope (Carl Zeiss EVO, Germany)

2.1.2.2. ATR- FTIR of Bacterial Cellulose-Collagen Scaffolds

- Sodium hydroxide (Sigma)
- Deionized water
- Freeze-dryer (Thermo Scientific)
- ATR-FTIR (Thermo Scientific Nicolet is900)

2.1.2.3. Degradation of Bacterial Cellulose-Collagen Scaffolds

- Sodium Azide
- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)
- Deionized water

2.1.3. Gene Therapy Studies

2.1.3.1. Competent Cell Preparation for E.coli DH5α Strain

- LB Medium (Acumedia)
- Deionized water
- $CaCl_2$ (Sigma)
- Waterbath (Grant SUB Aqua 12 Plus)
- Centrifuge (Eppendorf 5810 R)

2.1.3.2. Transformation and Cloning of phrGFP-II-I Plasmid to DH5α Competent Cells

- LB Medium (Acumedia)
- LB agar (Acumedia)
- *Escherichia coli* DH5α strain
- Kanamycin (Fisher Scientific)
- Shaker (Sartorius Stedim Biotechnology Certomat® IS)
- Incubator (Binder)

2.1.3.3. Preparation of Plasmid Constructs

- phrGFP II-I plasmid (Agilent Technologies, USA)
- Hind III-HF restriction enzyme (New England Biolabs, England)
- Kpn-I-HF restriction enzyme (New England Biolabs, England)
- EcoR-I-HF restriction enzyme (New England Biolabs, England)
- EcoR-V-HF restriction enzyme (New England Biolabs, England)
- T4 DNA Ligase (New England Biolabs, England)
- IGF-I (GenScript, USA)
- TGF-β1 (GenScript, USA)
- PureLink Plasmid isolation kit (Invitrogen, USA)
- PCR and Gel Recovery kit (Macherey-Nagel, USA)

2.1.4. *In vitro* Cell Culture Studies

2.1.4.1. Isolation and Culture of Rat Bone Marrow Stem Cells

- *Rattus norvegicus*-Spraque-Dawley rats
- Dulbecco's Modified Eagle Medium (DMEM – 4.5g/liter glucose) (Gibco - Invitrogen, USA)
- Penicillin
- Fetal Bovine Serum (Gibco-Invitrogen, USA)
- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)
- T75 Tissue Culture Flask (Orange Scientific, Belgium)
- 24-well Cell Culture Plate (Corning)

- Laminar Cabinet (Telstar, Bio-II-A, Spain)
- Inverted Microscope (Nikon Eclipse TC 100, USA)

2.1.4.2. Characterization of Rat Bone Marrow Stem Cells

- CD 45 anti-rat conjugated antibody (BD Bioscience)
- CD 11a anti-rat conjugated antibody (BD Bioscience)
- CD 90 anti-rat conjugated antibody (BD Bioscience)
- CD 29 anti-rat conjugated antibody (BD Bioscience)
- CD 34 anti-rat conjugated antibody (BD Bioscience)
- CD 14 anti-rat conjugated antibody (BD Bioscience)
- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)
- Flow Cytometry Calibur (BectonDickenson)

2.1.4.3. Optimization of Transfection Efficiency of Rat Bone Marrow Stem Cells

- Dulbecco's Modified Eagle Medium (DMEM – 4.5g/liter glucose) (Gibco - Invitrogen, USA)
- Lipofectamine ^{2000TM} (Invitrogen)
- Fluorescent Microscope (Zeiss Axio Observer)

2.1.4.4. Cell Seeding on Bacterial Cellulose-Collagen Scaffolds

- Rat bone marrow stem cells from *Rattus norvegicus* Spraque-Dawley rats
- Dulbecco's Modified Eagle Medium (DMEM – 4.5g/liter glucose) (Gibco - Invitrogen, USA)
- Primocin (Invivogen)
- Fetal Bovine Serum (Gibco-Invitrogen, USA)
- Trypsin-EDTA (10X) (Gibco-Invitrogen, USA)
- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)
- T75 Tissue Culture Flask (Orange Scientific, Belgium)
- 24-well Cell Culture Plate (Corning)
- Laminar Cabinet (Telstar, Bio-II-A, Spain)

- Inverted Microscope (Nikon Eclipse TC 100, USA)
- Haemocytometer (Hausser Bright-Line, USA)

2.1.4.5. Effect of Crosslinkers on Cell Viability

- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA)
- Dulbecco's Modified Eagle Medium (DMEM - 1g/liter glucose) (Gibco - Invitrogen, USA)
- 96-well Cell Culture Plates (Orange Scientific)
- Elisa Plate Reader (Bio-Tek, El x 800)

2.1.4.6. Differentiation of Rat Bone Marrow Stem Cells

- Dulbecco's Modified Eagle Medium (DMEM – 4.5g/liter glucose) (Gibco - Invitrogen, USA)
- ITS-Premix (Becton Dickinson)
- Ascorbic acid-2-phosphate (Santa Cruz Biotechnology)
- Sodium pyruvate (Sigma)
- Dexamethasone (Sigma)
- L-Proline, non-animal source (Sigma)
- Primocin (Invivogen)
- Insulin-like Growth Factor 1 *Rattus norvegicus*, transcript variant 3 (GenScript)
- Transforming Growth Factor-β1 *Rattus norvegicus* (GenScript)

2.1.4.7. MTS Assay

- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA)
- Dulbecco's Modified Eagle Medium (DMEM - 1g/liter glucose) (Gibco - Invitrogen, USA)
- 96-well Cell Culture Plates (Orange Scientific)
- Elisa Plate Reader (Bio-Tek, El x 800)

2.1.4.8. Alcian Blue Staining

- Alcian blue-PAS staining kit (Atom Scientific)
- Deionized water
- Lysine coated slides (Thermo)
- 10 % Neutral Buffered Formalin (Sigma)
- Tissue Freezing Medium (Biostain)
- Cryostat (Leica)

2.1.4.9. Confocal Microscopy Studies

- Collagen Type II anti rat antibody (Santa Cruz)
- Aggrecan anti rat antibody (Santa Cruz)
- Fetal Bovine Serum (Gibco)
- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)
- Microscope slides and coverslips (Thermo)
- Formaldehyde (Fluka, Switzerland)
- Tween® 20 (AppliChem, Germany)
- Confocal Microscope (Leica)

2.1.4.10. Extraction and Purification of Protein from Bacterial Cellulose-Collagen Scaffolds

- Phosphate Buffered Saline (D-PBS, 10 mM, pH 7.4) (Gibco - Invitrogen, USA)
- RIPA Buffer
- PMSF (Sigma)
- Sodium orthovanadate (Na_3VO_4) (Sigma)
- Smart™ micro BCA protein assay kit (Invivogen)
- Trichloroacetic acid (Sigma)
- HPLC grade-Acetone (Sigma)

2.2. Methods

2.2.1. Scaffold Preparation

2.2.1.1. Production of Bacterial Cellulose

Medium which contains glucose 10 g/L, yeast extract 10 g/L, peptone 7g/L, acetic acid 1,5 mL/L and deionized water was prepared and then autoclaved. The day before *Gluconacetobacter xylinus* was cultured in medium. Inoculum was overnight cultured and added into culture medium at 5 % for 7 days. Bacterial cellulose was harvested from culture medium at day 7 and immersed in 1M NaOH for 2 h for sterilization. Then, it was kept in the deionized water for adjusting pH to 7 for 24 h and freeze-dried for 24 h.

2.2.1.2. Preparation of Bacterial Cellulose-Collagen Scaffolds

Collagen type I was dissolved in pure acetic acid on magnetic stirrer until solution became homogenous. Also, dried bacterial cellulose was crashed in distilled water using Heidolph Silent crusher until solution became homogenous. Then bacterial cellulose and collagen were mixed using vortex at 75%-25% ratio (w/w). Mixed solution (3 mL) was put into each well at 24 well plates. Molded scaffolds were freeze-dried for 24 h.

2.2.1.3. Crosslinking of Bacterial Cellulose-Collagen Scaffolds

To crosslink collagen molecules physically, scaffolds were subjected to dehydrothermal treatment at 105 ^{0}C for 24 h using vacuum-oven. To crosslink collagen chemically, 500 µL genipin solution was put onto scaffolds for 24 h at RT and checked whether color of the solution turned blue. Genipin (5 mg) was dissolved in 40 µL DMSO and the solution volume was adjusted to 10 mL with PBS. Scaffolds were washed several times with dH_2O for the removal of Genipin residues and dried using freeze-dryer for 8 h.

2.2.2. Characterization of Bacterial Cellulose-Collagen Scaffolds

2.2.2.1. Scanning Electron Microscopy of Bacterial Cellulose-Collagen Scaffolds

Cacodylate buffer was prepared using Cacodylic Acid Sodium Salt Trihydrate and deionized water. Glutaraldehyde Solution, Grade I, 25% solution was 1:10 diluted using cacodylate buffer. Cells on the bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers were fixed after 1 day of incubation using 2.5% glutaraldehyde for 1 hour. After that, scaffolds were washed with cacodylate buffer and left overnight for drying. Dry scaffolds were coated with 15 nm gold by sputter coater (Bal-tec SCD 005). Samples were observed using Carl Zeiss EVO Scanning Electron Microscope operated at 10.00 kV accelerating.

2.2.2.2. ATR-FTIR of Bacterial Cellulose-Collagen Scaffolds

Harvested bacterial cellulose from culture medium was sterilized by immersing in 1M NaOH for 2 h. Then, pH of bacterial cellulose was adjusted by keeping them in the deionized water for 24 h and freeze-dried for 24 h. Infrared spectra of the bacterial cellulose samples were performed with a Fourier transform infrared (FT-IR) spectrometer at ambient temperature. All spectra were recorded with an accumulation of 64 scans and a resolution of 4 cm^{-1} in the range from 3 400 to 600 cm^{-1}. Harvested bacterial cellulose signals were measured by Thermo Scientific Nicolet is900 FTIR software.

2.2.2.3. Degradation of Bacterial Cellulose-Collagen Scaffolds

Bacterial Cellulose-Collagen scaffolds that were crosslinked with Genipin, DHT and Genipin/DHT were weighed at day 0. In order to evaluate degradation rates, bacterial cellulose-collagen scaffolds were incubated in phosphate buffered saline (20 mL, 0.09% sodium azide 10 mM D-PBS, pH 7.4) at 37 ^{0}C waterbath. After 15, 30, 60, 120 days of incubation, samples were rinsed with deionized water, freeze-dried and weighed. Untreated bacterial cellulose-collagen scaffolds were used as positive control in this experiment. Three specimens were tested for each sample and the averages together with the standard deviations were recorded. The percentage of mass loss rate (ML) was then calculated by the following equation:

$$\%ML = \frac{Mt}{M_0} X\ 100$$

Where M_0 is the initial dry weight of the sample and the M_t is the dry weight of the sample following degradation of each point. Samples, collected at different time points, were freeze-dried for 8h and weighed at the end of the period. At the same time, the pH values in the supernatant were recorded as well. All measurements were expressed as mean standard deviation relative to initial values.

2.2.3. Gene Therapy Studies

2.2.3.1. *Competent Cell Preparation for E.coli DH5α Strain*

For preparing 100 mM $CaCl_2$ solution, $CaCl_2$ dihydrate (2.95 g) was dissolved in 200 mL deionized water and autoclaved before use. All LB medium and LB agar were prepared following commercial instruction of Acumedia Inc. All media was sterilized, *E.coli* DH5α strain was added into 10 mL LB and incubated overnight in shaker at 37 ^{0}C. After incubation, 1 mL of inoculum was put and added into 34 mL LB. This culture was incubated at 37 ^{0}C, 180 rpm for 3h. Then, the culture was centrifuged at 5 500 rpm for 10 min and LB was discarded. After discarding LB, 5 mL cold $CaCl_2$ (100 mM) was added slowly and incubated for 1 h on ice. After incubation, cells were centrifuged at 3 500 rpm for 5 min and lysate was dissolved in 1 mL $CaCl_2$ (100 mM) and put into eppendorf tubes. Competent cells were stored at +4 ^{0}C for 7 days.

2.2.3.2. *Transformation and Cloning of phrGFP-II-I Plasmid to DH5α Competent Cells*

Competent *E. coli* DH5α (50 µL) mixed with 100 ng pUC57 plasmids which contain *Rattus norvegicus* Transforming Growth Factor β1 (RefSeq Accession: NM_021578, 1185 base pair) gene and *Rattus norvegicus* Insulin-like Growth Factor 1, transcript variant 3 (RefSeq Accession: NM_001082478, 492 base pair) gene separately. Tubes which *E. coli* DH5α (50 µL) and pUC 57 plasmids which contain IGF-I or TGF-β1 genes were kept on ice for 15 min. and 90 sec. at 42 ^{0}C in waterbath then 2 min. on ice. After this step, 200 µL LB medium was added into tubes. Tubes with *E. coli* DH5α and pUC57 were shook at 200 rpm, 37 ^{0}C for 1 h. After incubation, samples were put onto 0.1 mg/ml ampicillin containing LB agar plates. Plates were incubated at 37 ^{0}C for 18 h.

2.2.3.3. Preparation of Plasmid Constructs

Rattus norvegicus Transforming Growth Factor beta 1 (RefSeq Accession: NM_021578, 1185 base pair) gene with EcoRI and EcoRV restriction sites at 5' to 3' sites, respectively, in pUC57 plasmid and *Rattus norvegicus* Insulin-like Growth Factor 1, transcript variant 3 (RefSeq Accession: NM_001082478, 492 base pair) gene with Hind III and Kpn I restriction sites at 5' to 3' sites, respectively, in pUC57 plasmid were obtained from GenScript Company (USA). Both genes were cloned into *Escherichia coli* DH5α strain and isolated using PureLink Plasmid isolation kit. IGF-I (1μg) gene was cut using 3 μL of Hind III^{HF} and Kpn I^{HF} restriction enzymes in 50 μL reaction solution and obtained in % 0.8 agarose gel using 1 kb marker. After IGF-I gene was isolated from agarose gel using PCR and Gel Recovery kit, it was first inserted into MCS1 site of phrGFP II-I plasmid using T4 DNA Ligase. Ligation reaction was assessed by agarose gel electrophoresis technique using 1 kb marker. Then IGF-I ligated phrGFP II-I plasmid was cloned using *Escherichia coli* DH5α strain. TGF-β1 gene (1μg) was cut by EcoRI and EcoRV restriction enzymes (3 μL) in 50 μL reaction solution and obtained in % 0.8 agarose gel using 1 kb marker. After TGF-β1 gene was isolated from agarose gel using PCR and Gel Recovery kit, it was inserted into MCS2 site of IGF-I ligated phrGFP II-I plasmid using T4 DNA Ligase. Then, ligation reaction was assessed by agarose gel electrophoresis technique using 1kb marker. phrGFP II-I plasmid which carries both IGF-I and TGF β-I genes was cloned into *Escherichia coli* DH5α strain and isolated using PureLink Plasmid isolation kit for further studies.

2.2.4. In vitro Cell Culture Studies

2.2.4.1. *Isolation and Culture of Rat Bone Marrow Stem Cells*

Abdominal site of *Rattus norvegicus*-Spraque-Dawley rat was cut until their legs. Then, connective tissues were cut between fur and body to remove all fur around the legs. After this step, muscles around femur and tibia were removed. Bones were put into high glucose DMEM which contains 1 000 units penicillin. Bone marrow was flushed into 100 unit penicillin containing high glucose DMEM with syringe. Then, marrow containing tubes were centrifuged at 2 000 rpm for 5 min. After pellet was dissolved in high glucose DMEM with 100 unit penicillin, the cell suspension was transferred to a T75 flask and left

for incubation at 37°C, in 5% CO_2 and 90% humidity. The medium was refreshed every other day until the cells reach to confluency.

2.2.4.2. *Characterization of Rat Bone Marrow Stem Cells*

Flow cytometry analysis was performed to investigate the expression of pluripotency related cell surface markers of RBMSCs. Cells from T75 flask were trypsinized and RBMSCs (40 000 cells/2 mL, passage 2) were taken into flow cytometry tubes after resuspended in 2 mL PBS. Then flow cytometry tubes were centrifuged at 2 200 rpm for 5 min. After discarding supernatant, cell pellet was resuspended in 200 µL PBS and fluorescent conjugated CD 45, CD 11a, CD 90, CD29, CD 34 and CD 14 antibodies were added into corresponding tubes. Cells and antibody mixture was incubated for 1 h at +4 ^{0}C. After incubation, 2 mL PBS was added and centrifuged at 2 200 rpm for 5 min. After discarding supernatant, cells were resuspended in 400 µL PBS. Cell analysis was performed using at least 10,000 events per sample. Data acquisition and analysis were then performed by BD Flow Cytometry Calibur software.

2.2.4.3. *Optimization of Transfection Efficiency of RBMSCs*

phrGFP II-I plasmid which carries both IGF-I and TGF β-I genes was diluted in 50 µL high glucose DMEM and mixed gently. Then, an appropriate amount of Lipofectamine ^{2000} was diluted in 50 µL high glucose DMEM and incubated for 5 min at room temperature. After the incubation, DNA and Lipofectamine^{2000} solutions were mixed together gently and incubated for 20 min at RT. Mixture (100 µL) was added into each well containing rat bone marrow stem cells. To optimize gene transfer in monolayer culture, RBMSCs were transfected using 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5 ratios of DNA (µg)/Lipofectamine^{2000} (µL). In addition, 20 000, 30 000, 40 000, 50 000, 75 000 cells were transfected with 0.5 µg, 1 µg, 2 µg, 3 µg, 4 µg DNA at 1:0.5 ratio of DNA (µg)/Lipofectamine^{2000} (µL).

2.2.4.4. *Cell Seeding on Bacterial Cellulose-Collagen Scaffolds*

Bacterial cellulose-collagen scaffolds were placed into the wells of 24-well cell culture plates in triplicates. Ethanol (1 mL of 70%) was added into each well. Scaffolds were left in 70% ethanol for 2 h and then washed 3 times with PBS that was completely discarded

after third washing step. Scaffolds were left under the laminar cabinet for drying. RBMSCs were counted using hemocytometer under the inverted microscope. Cells (40 000 cells/scaffold) were seeded onto both scaffolds and empty wells. Then, the cell culture plate left for incubation at 37°C, in 5% CO_2 and 90% humidity for 2 h in order to let the cells adhere on the surfaces of both scaffolds and wells.

2.2.4.5. *Effect of Crosslinkers on Cell Viability*

CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine the cell density on the bacterial cellulose-collagen scaffolds. The experiment was performed at 1, 7, and 14 days after seeding the scaffolds with the cells. Low glucose DMEM was mixed with MTS one solution with a ratio of 5:1. Cell seeded bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers were transferred into a new sterile 24-well plate and washed with PBS to remove medium. MTS/media mixture (500 µL) was added into each sample in 24-well plate and incubated for 3 h at 37^0C in a CO_2 incubator. After 3 h of incubation, 200 µL of solution from each well was transferred into a 96-well plate. Absorbance was measured at 490 nm using Elisa Plate Reader (Bio-Tek, Elx800, USA).

2.2.4.6. *Differentiation of Rat Bone Marrow Stem Cells*

To prepare chondrogenic differentiation medium, 1µM ascorbate-2-phosphate (SantaCruz Biotechnology), %1 sodium pyruvate (Sigma) and 40 µg/mL L-proline (Sigma) were added into high glucose DMEM medium (Gibco) and filtered with 0.22 µm filter for sterilization. Then %5 ITS premix (BD Bioscience), 10^{-7} M Dexamethasone and 100 µL primocin were added into this solution. The chondrogenic differentiation medium was added onto RBMSCs seeded scaffolds the day after seeding and replenished twice a week.

In this study, three test groups were evaluated for the RBMSCs differentiation on TCP and scaffolds with different crosslinkers throughout 21 days of incubation. In the first group, Lipofectamine^{2000}-phrGFP-II-I plasmids which carries both IGF-I and TGF-β1 genes (0.5 µg) were incorporated into each scaffolds and RBMSCs (40 000 cells/scaffold) were seeded onto each of them with chondrogenic differentiation medium. In the second group, RBMSCs were seeded onto each scaffold as described above and IGF-I and TGF-β1

growth factors (10 ng/ml each) were directly added into their chondrogenic differentiation medium. In the last group (control), after seeding of RBMSCs, only chondrogenic differentiation medium (500 µL) was added onto each scaffold.

2.2.4.7. *MTS Assay*

CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine the cell density on the collagen-bacterial cellulose scaffolds. The experiment was performed at 1, 7, and 14 days after seeding the scaffolds with the cells. Low glucose DMEM was mixed with MTS one solution with a ratio of 5:1. Cell seeded different cross-linked bacterial cellulose-collagen scaffolds were transferred into a new sterile 24-well plate and washed with PBS to remove medium. MTS/media mixture (500 µL) was added into each sample in 24-well plate and incubated for 3 h at 37 ^{0}C in a CO_2 incubator. After 3 h of incubation, 200 µL of solution from each well was transferred into a 96-well plate. Absorbance was measured at 490 nm using Elisa Plate Reader (Bio-Tek, Elx800, USA).

2.2.4.8. *Alcian Blue Staining*

RBMSCs seeded bacterial cellulose-collagen scaffolds were washed with 1X D-PBS three times and freezed at -80 ^{0}C for 30 min. Freezed scaffolds covered with tissue freezing medium (Biostain) and cut with Leica Cryostat (60 µm) on lysine covered slides. Tissues on slides were treated with neutral buffered formalin for fixation for 10 min. Then, slides were stained with Alcian Blue-PAS staining kit following kit instructions.

2.2.4.9. *Confocal Microscopy Studies*

RBMSCs seeded bacterial cellulose-collagen scaffolds were washed three times with 1X D-PBS. Then, the scaffolds were fixed with 3.7% formaldehyde solution including 0.1% Tween 20 for 30 min at RT. After fixation, scaffolds were washed with 1X D-PBS and kept in the D-PBS including 3% FBS solution as the blocking reagent for 10 min. Then D-PBS including 1.5% FBS solution containing IgG2b Collagen type II antibody and Aggrecan rabbit polyclonal IgG antibody solution at 1:50 ratio was added onto scaffolds and incubated for 18h at RT. After that, scaffolds were washed with 1X D-PBS and incubated in D-PBS including 1% FBS solution containing Alexa Fluor ® 467 goat anti-

mouse antibody and Alexa Fluor ® 488 goat anti-mouse antibody solution at 1:200 ratio for 1h at 37 ^{0}C. Finally, samples were mounted with ProLong Gold antifade reagent and examined using Leica Confocal Microscope.

2.2.4.10. *Extraction and Purification of Protein From Bacterial Cellulose-Collagen Scaffolds*

RBMSCs seeded scaffolds were washed with cold 1X D-PBS and put into 15 mL falcon tubes, RIPA buffer (1 mL) containing 10 µL protease inhibitor, 10 µL PMSF and 10 µL Na_3VO_4 were added onto scaffolds. They were incubated in RIPA buffer for 5 min on ice and the cells were lysed by sonication for 5 min. Mixture was shook gently on ice for 15 min and centrifuged at 14 000 g for 25 min to remove cell debris. After centrifugation step, supernatant was taken into eppendorf tubes. To determine the concentration of protein, Smart^{TM} micro BCA protein assay kit was applied using kit manual and the absorbance of samples was measured at 562 nm using Elisa Plate Reader (Bio-Tek, Elx800, USA).
Extracted proteins from cell seeded scaffolds were mixed with 100 % Trichloroaceticacid (TCA) at 1:10 ratio and incubated on ice for 1 h. Then, protein-TCA solutions were centrifuged at 4 ^{0}C, 12 000 g for 15 min and supernatants were discarded. After that, 800 µL of – 20 ^{0}C HPLC grade pure acetone was added onto pellet and incubated overnight at – 20 ^{0}C. After incubation, protein mixtures were centrifuged at 4 ^{0}C, 6 500 g for 10 min and supernatants were discarded. This step was repeated three times. After the final centrifugation, acetone was discarded and proteins were dissolved in 1X Laemlli Buffer.

3. RESULTS

3.1. Scaffold Characterization

3.1.1. Scanning Electron Microscopy of Bacterial Cellulose-Collagen Scaffolds

Scanning electron microscopy (SEM) gives microstructural information about gold coated biomaterials by attracting electrons onto them. Reflecting electrons from samples provide image of surface structure. Surface images of bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers were taken by SEM (Figure 3.1).

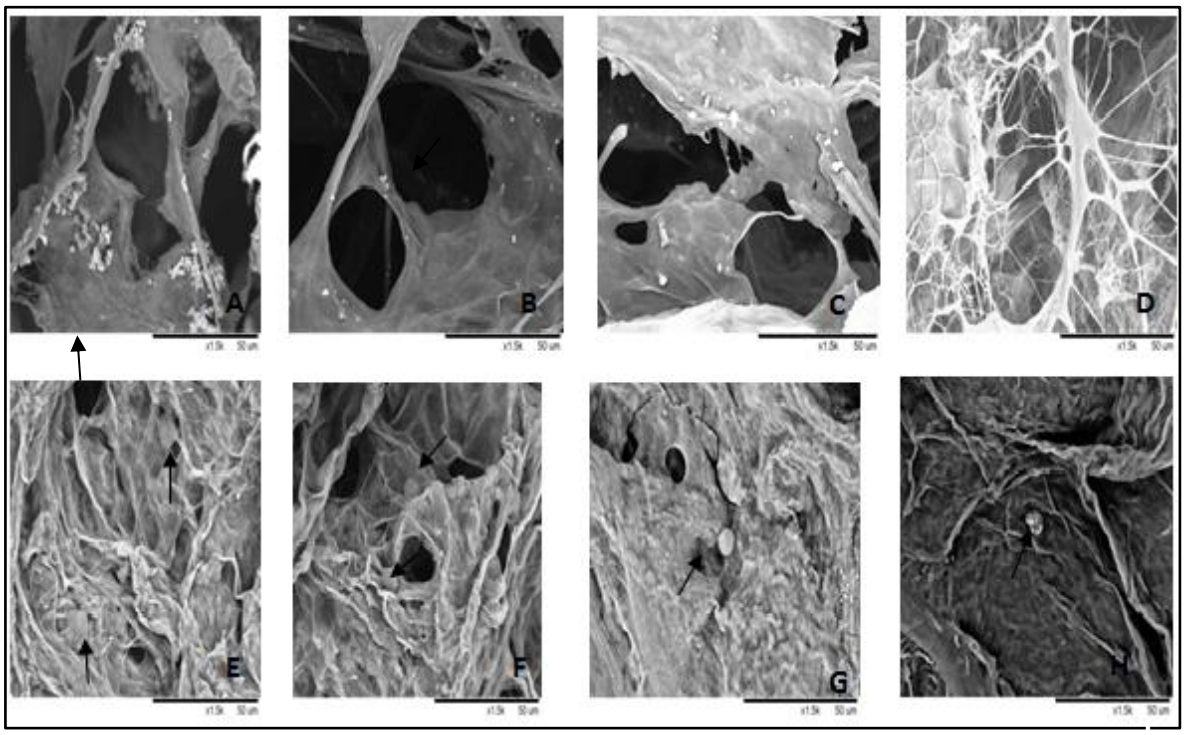

Figure 3.1. SEM images of bacterial cellulose-collagen scaffolds. Unseeded scaffolds A) DHT/Genipin crosslinked, B) DHT crosslinked, C) Genipin crosslinked, D) Untreated scaffolds; RBMSCs seeded scaffolds E) DHT/Genipin crosslinked, F) DHT crosslinked, G) Genipin crosslinked, H) Untreated scaffolds. Arrows show cells on the scaffolds (1500X magnification and scale bar 50µm).

Scanning electron micrographs show that, porous structures of unseeded bacterial cellulose-collagen scaffolds that were crosslinked with DHT technique (Figure 3.1 B) were larger than that of DHT/Genipin crosslinked (Figure 3.1 A) and Genipin crosslinked (Figure 3.1 C) scaffolds. In addition, the pore sizes of DHT/Genipin crosslinked scaffolds were larger than that of Genipin crosslinked ones. It seems that DHT treatment provided bacterial cellulose-collagen scaffolds to keep their original structure when it crosslinked both cellulose and collagen molecules. In comparison to crosslinked scaffolds, untreated ones (Figure 3.1 D) had a loose network structures from cellulose and collagen. Crosslinked scaffolds had denser structures than the untreated bacterial cellulose-collagen scaffolds. Figure 3.1 E-H shows that crosslinked and untreated bacterial cellulose-collagen scaffolds were suitable biomaterials for the cell attachment. Presence of cells can be observed on these scaffolds. Cells easily attached and proliferated on the surfaces of these scaffolds.

3.1.2. Attenuated Total Reflectance (ATR)-Fourier Transform Infrared Radiation Spectroscopy (FT-IR) of Bacterial Cellulose-Collagen Scaffolds

Infrared radiation excites molecular vibrations within a biomaterial. The frequencies of these vibrations, and hence the absorption peaks in the spectrum, are the characteristics of the chemical composition of the specimen. In the ATR technique, sample is just pressed against a crystal ‘window’ (typically diamond) and the infrared beam interacts with the sample at the interface. While the radiation undergoes total internal reflection at the crystal surface, an evanescent wave penetrates the sample to a shallow depth (typically of the order of the radiation wavelength, i.e. a few microns), and absorption of this component produces the infrared spectrum. In this experiment, ATR-FTIR spectra obtained from sterilized and freeze-dried bacterial cellulose from *Gluconacetobacter xylinus* culture are shown in Figure 3.2.

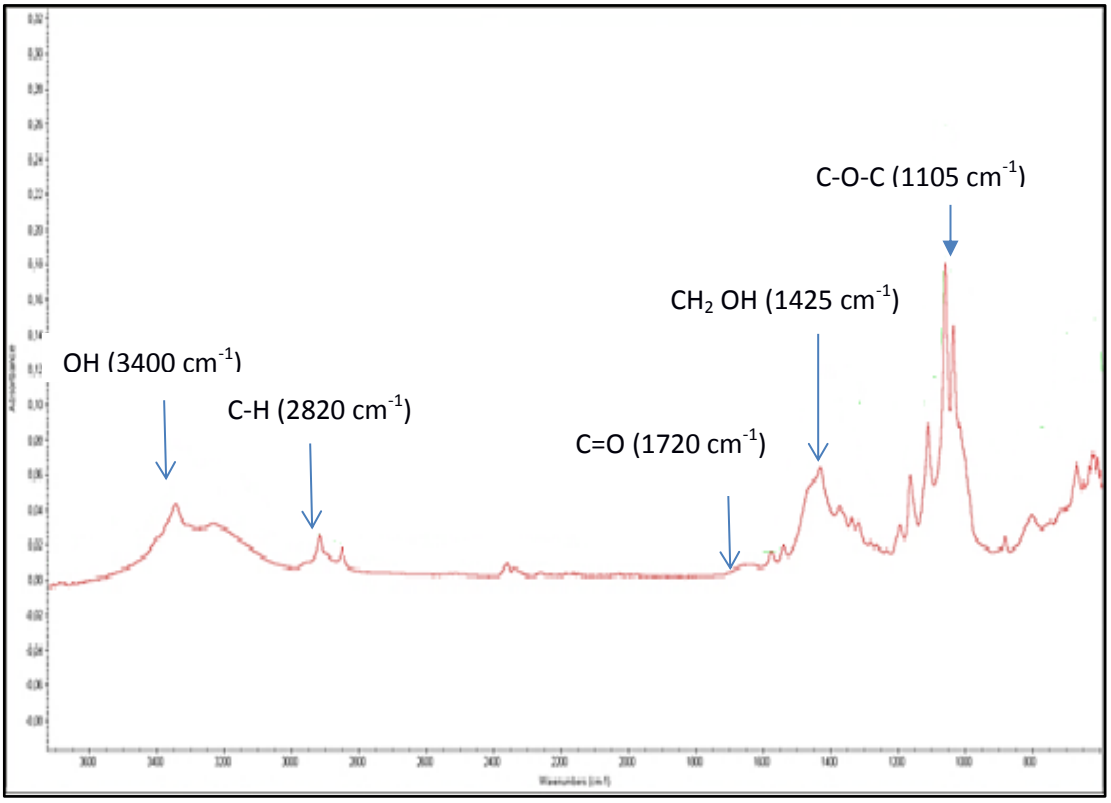

Figure 3.2. FTIR results of harvested bacterial cellulose from Gluconacetobacter xylinus culture. Red line represents bacterial cellulose samples.

Cellulose biosynthesis is characterized by unidirectional growth and crystallization, where glucose molecules are linearly bonded by β(1→4)-glycosidic bond. The union of glycosidic chains forms oriented microfibrils with intramolecular hydrogen bonds. For the harvested bacterial cellulose, a broad band at 3 300 cm^{-1} is attributed to O-H stretching vibration. Band at 2 820 cm^{-1} represents the aliphatic C-H stretching vibration. Absorbance peak at wave number 1 720 cm^{-1}, is attributed to hydrogen-bonded carbonyl stretching vibration. A sharp band observed at 1 080 cm^{-1} is due to the presence of C-O-C stretching vibrations. According to Figure 3.2, signals of bacterial cellulose sample indicate that this sample contains C, H, O atoms as expected. In addition, considering the strong glucose peaks at 1 080 cm^{-1}, 1 425 cm^{-1} and 3 400 cm^{-1}, it can be said that this sample contained glucose molecules.

3.1.3. Degradation of Bacterial Cellulose-Collagen Scaffolds

Degradation profile of bacterial cellulose-collagen scaffolds was determined by checking both pH and weight changes in time. Percentage of mass loss rate was usually used to evaluate *in vitro* degradation of biomaterials. The mass loss curve of the bacterial cellulose-collagen scaffolds that were crosslinked with DHT, Genipin and DHT/Genipin is shown in Figure 3.3.

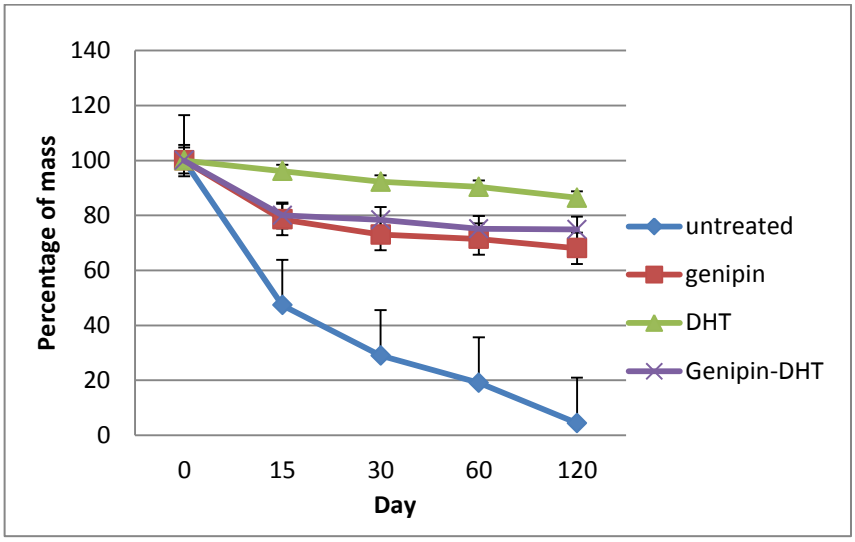

Figure 3.3. Degradation with respect to percentage of mass of bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers at different time points

It was found that untreated bacterial cellulose-collagen scaffolds showed a rapid mass loss in PBS at 37 ^{0}C. However, DHT treated samples were highly stable throughout 120 days of incubation. Genipin treated samples were also found stable with respect to untreated ones but their percentage of mass loss was 18% less than that of DHT treated ones. The mass loss of DHT/Genipin treated samples was found 10% higher than that of Genipin treated and13% lower than that of DHT treated ones.

In order to check the pH difference in time, all bacterial cellulose-collagen scaffolds that were crosslinked with DHT, Genipin and DHT/Genipin were incubated in phosphate buffered saline (10 mM) at 37 ^{0}C for 120 days. The initial pH value of phosphate buffered saline was 7.40. Untreated bacterial cellulose-collagen scaffolds were used as a control in

this experiment. In order to determine the degradation of the scaffolds, the pH of the solution was also measured at each time point (Figure 3.4).

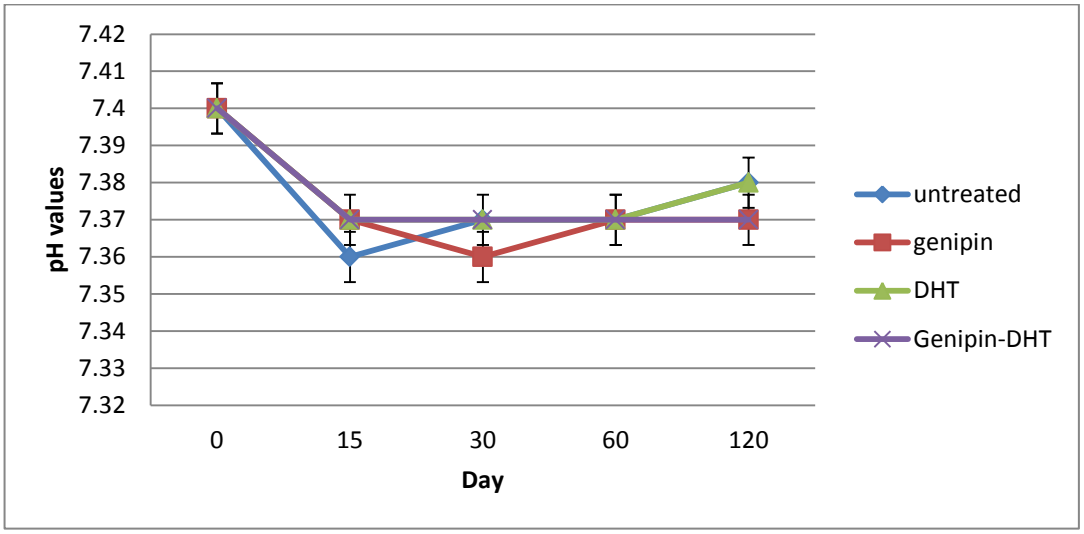

Figure 3.4. pH changes of bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers at different time points.

After 15 days of incubation, the highest pH change from 7.40 to 7.36 was observed in the untreated scaffolds as it was expected. The pH of Genipin crosslinked scaffolds dropped to pH 7.36 after day 30. On the other hand, the pH of the scaffolds that were crosslinked with DHT and DHT/Genipin decreased from 7.40 to 7.38 after day 15 and almost stabilized for the rest of the study. It is important to note that pH changes of all type of bacterial cellulose-collagen scaffolds had an insignificant effect on cell viability.

3.2. Characterization of Plasmid Constructs

In order to transfect the cells with both *Rattus norvegicus* Transforming Growth Factor β 1 (RefSeq Accession: NM_021578, ORF sequence, 1185 bp) gene and Insulin-like Growth Factor 1, transcript variant 3 (RefSeq Accession: NM_001082478, ORF sequence, 492 bp) gene at the same time, they were ligated into two multiple cloning sites containing phrGFP-II-I vector (Figure 3.5 A). Ligated plasmid constructs were cut with EcoRI and EcoRV restriction enzymes for TGF-β1 gene and also cut with Hind III and Kpn I

restriction enzymes for IGF-I gene in phrGFP-II-I vector. In addition, ligated plasmids were cut with Hind III and EcoRV for gene cassette which contains IGF-I, hrGFP II-I and TGF-β1 genes (Figure 3.5 A). Results of the restriction reactions were visualized by ethidium bromide staining on 0.8% TBE agarose gel and in order to obtain gene size, 1 kb and 300 bp markers were loaded in agarose gel as well (Figure 3.5 B).

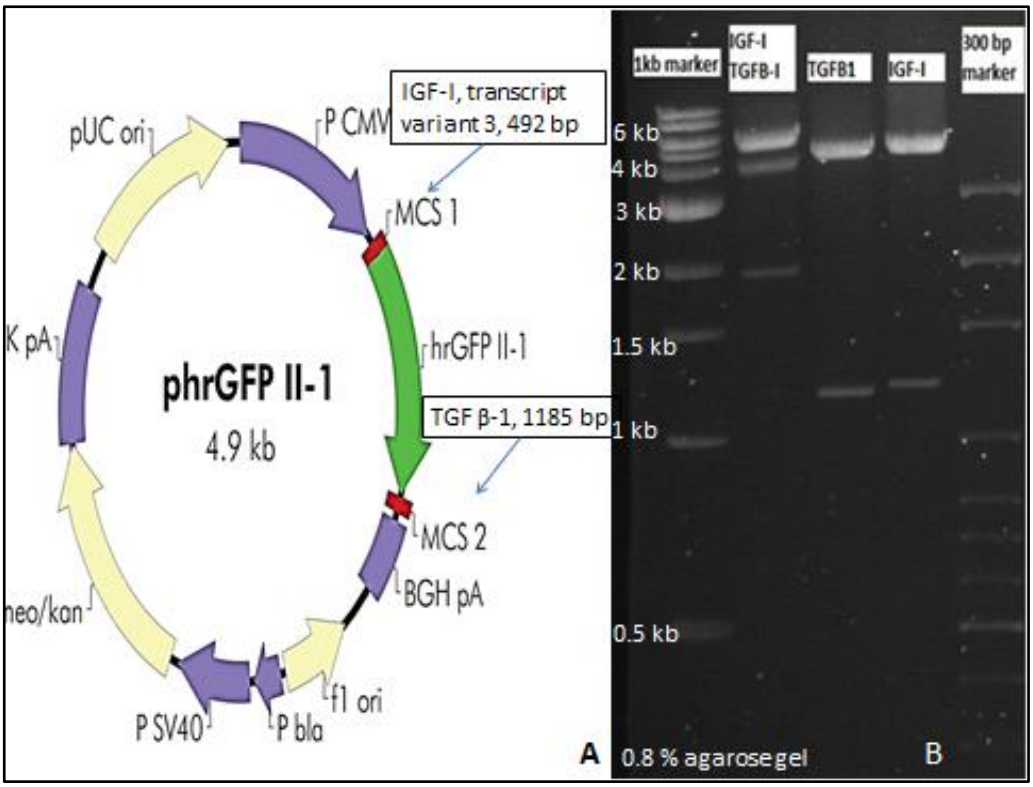

Figure 3.5. (A) Vector map of phrGFP II-I, (B) Agarose gel electrophoresis for IGF-I, TGF-β1 genes ligated phrGFP-II-I vector.

TGF-β1 gene (1 185 bp) was obtained between 1 kb and 1.5 kb band and the rest of plasmid (5 392 bp) which contains IGF-I gene was obtained between 5 kb and 6 kb bands as expected. In addition, IGF-I gene (492 bp) and hrGFP-II-I gene (728 bp) constructs were observed between 1 kb and 1.5 kb bands. Also, expected size of gene cassette which contains IGF-I, hrGFP II-I and TGF-β1 genes (2405 bp) was and obtained near 2 kb band as expected. Due to the presence of uncut plasmids which contain IGF-I and TGF-β1 genes in reaction solution, there were three bands at lane 2 in agarose gel instead of two and this band (7 005 bp) was obtained above 6 kb band.

Figure 3.6. Analyzed chromatogram data of Rattus norvegicus Insulin-like Growth Factor 1, transcript variant 3 (RefSeq Accession: NM_001082478, 492 base pair) gene using DNA Baser software.

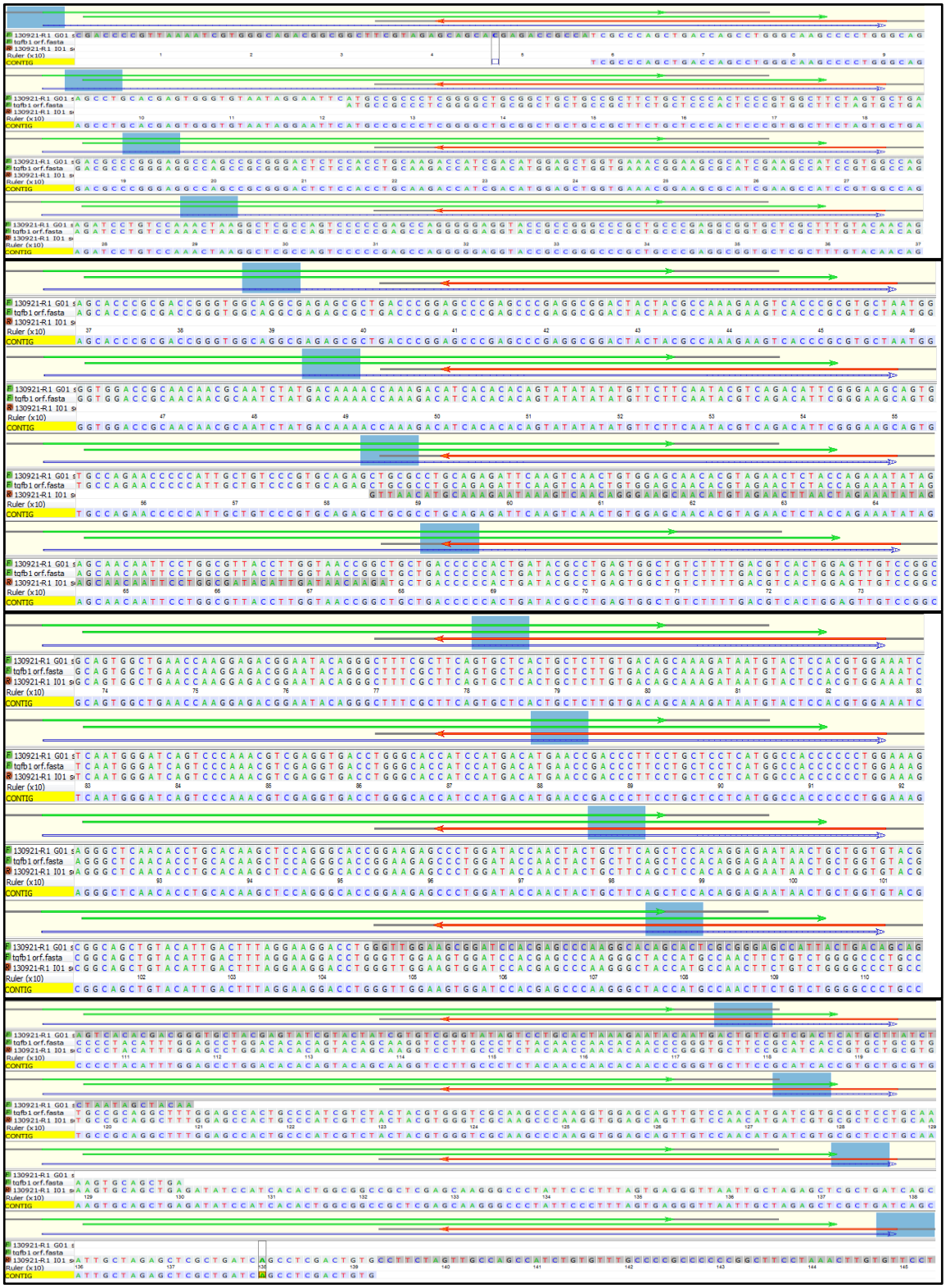

Figure 3.7. Analyzed chromatogram data of Rattus norvegicus Transforming Growth Factor beta 1 (RefSeq Accession: NM_021578, 1185 base pair) gene using DNA Baser software.

TGF-β1 and IGF-I genes in phrGFP-II-I plasmid were sequenced by Macrogen Inc. (Netherland) in order to confirm the presence of these genes in phrGFP-II-I plasmid. Chromatogram data providing from Macrogen Inc. was analyzed using DNA Baser software. As shown in Figure 3.6 and 3.7, Chromatogram results completely matched with the ORF sequences of IGF-I (RefSeq Accession: NM_001082478, 492 bp) and TGF-β1 (RefSeq Accession: NM_021578, 1 185 bp) genes without any mismatches, respectively. Presence of these genes in phrGFP-II-I plasmid was also confirmed.

3.3. *In vitro* Cell Culture Studies

3.3.1. Characterization of Rat Bone Marrow Stem Cells

Flow cytometry was used to characterize stem cells using specific cell surface markers. The isolated rat bone marrow stem cells were stained with FITC-Conjugated mesenchymal markers CD 90 and CD 29 (Figure 8 A, B) and hematopoietic markers such as CD 45, CD 34, CD 14 and CD 11a (Figure 8 C-F).

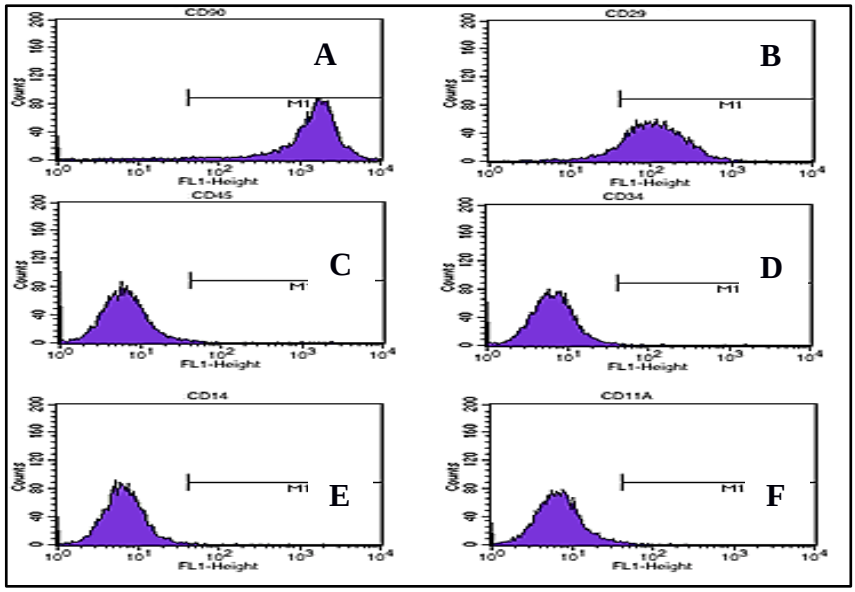

Figure 3.8. Flow cytometry analyses of the cell population isolated from bone marrow of 6 weeks old rats. FITC-Conjugated antibodies A) CD 90, B) CD 29, C) CD45, D) CD 34, E) CD14, F) CD11a.

According to Table 3.1, 97.73 % of cell population isolated from rat bone marrow expressed CD 90 which is one of the important mesenchymal stem cell surface markers. In addition, these cells also expressed CD 29 cell surface protein which is found in both bone marrow and adipose derived mesenchymal stem cells. For the full characterization of cell population isolated from rat bone marrow, expressions of hematopoietic derived cell surface markers such as CD 45, CD 34, CD 14 and CD 11a were also assessed and it was found that isolated passage 2 cells were almost negative to CD 34 (% 0.17), CD 45 (% 0.51), CD 14 (% 0.25), CD 11a (% 0.44) .

Table 3.1. Flow cytometry analysis of RBMSCs (Passage 2).

	Antibody	**Histogram Result**
Positive surface markers for RBMSCs	**CD 90**	**% 97.73**
	CD 29	**% 88.84**
Negative surface markers for RBMSCs	**CD 45**	**% 0.51**
	CD 34	**% 0.17**
	CD 14	**% 0.25**
	CD 11a	**% 0.44**

3.3.2. Optimization of Transfection Efficiency of RBMSCs

To optimize the gene transfer in monolayer culture, RBMSCs were transfected with a range of DNA (µg)/Lipofectamine^{2000} (µL) which were ranging between 1/0.5 to 1/5 plasmid/reagent. The fluorescent green color is an indication of successful transfection with the level of GFP expression that dependens on DNA (µg)/Lipofectamine^{2000} (µL) ratio (Figure 3.9). The best transfection result was obtained at 40 000 cells with 0.5 µg DNA at 1:0.5 ratio of DNA (µg)/Lipofectamine^{2000} (µL). For the rest of the study, RBMSCs were transfected in monolayer culture with 0,5 µg Lipofectamine^{2000}-phrGFP-II-I polyplexes at 1/0.5 plasmid/reagent ratio and cells were imaged the day after transfection using Zeiss Axio fluorescent microscope.

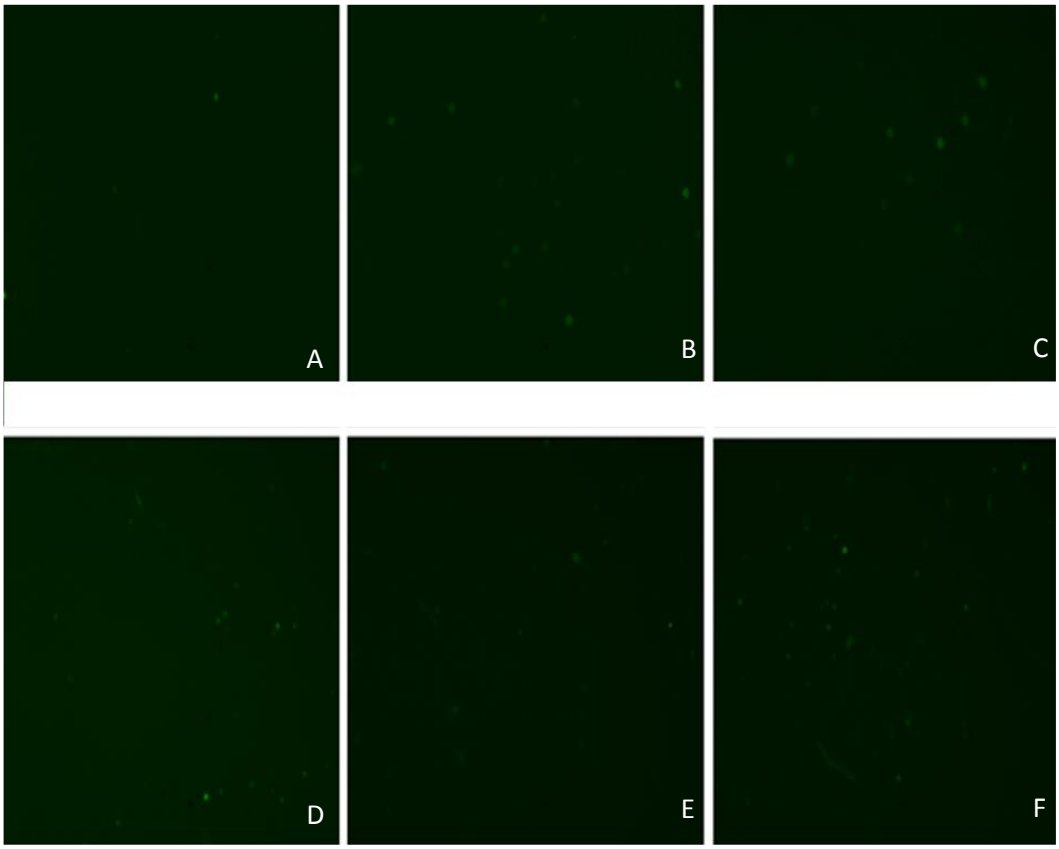

Figure 3.9. GFP expressing cells transfected with phrGFP-II-I vector containing IGF-I and TGF-β1 genes. Plasmid (0.5 µg) application for A) 30 000 cells, B) 40 000 cells, C) 50 000 cells; 1-0.5 plasmid-reagent ratio for D) 30 000 cells, E) 40 000 cells, F) 50 000 cells (4X objective).

3.3.3. Effect of Crosslinkers on Cell Viability

Cell viability and proliferation were studied by MTS cell proliferation assay on RBMSCs seeded bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers after 1, 7, 14 and 21 days of incubation periods. A calibration curve was constructed before with a known number of cells. The cell numbers on the RBMSCs seeded bacterial cellulose-collagen scaffolds and on TCP (Tissue Culture Polystyrene) were calculated according to this calibration curve. Figure 3.10 shows the cell proliferation on the RBMSCs seeded bacterial cellulose-collagen scaffolds. Only cells (OC) that were grown on the wells of the 24 well plate used as positive control in this experiment.

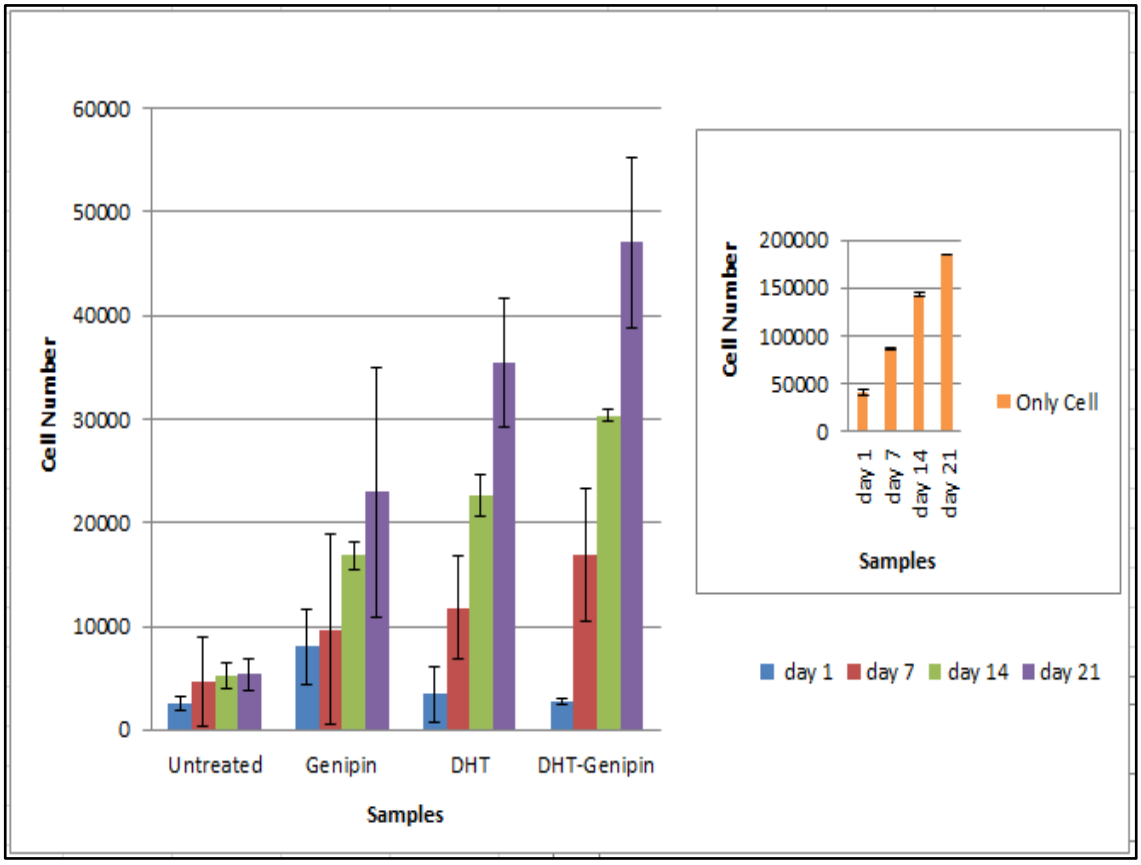

Figure 3.10. MTS assay of RBMSCs seeded on bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers throughout 21 days of incubation. Initial cell seeding was 40 000 cells/per scaffold.

The MTS test was performed to investigate the cytotoxicity of the bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers. Regarding the MTS assay, it was showed that bacterial cellulose-collagen scaffolds did not have any cytotoxic effect on RBMSCs. As shown in Figure 3.10, cell numbers in all scaffolds increased throughout 21 days of incubation. The highest initial cell attachment (8 916 cells) was observed in Genipin crosslinked bacterial cellulose-collagen scaffolds at the day after cell seeding on scaffolds and the minimum (3 375 cells) cell attachment was observed in the untreated scaffolds. After 7 days of incubation, the highest cell proliferation (15 516 cells) was observed in DHT/Genipin crosslinked scaffolds. The cell proliferation behavior on the scaffolds was as follows: DHT/Genipin > DHT > Genipin > Untreated scaffolds. The same behavior was also observed after 14 and 21 days of incubation.

3.3.4. MTS Assay

Cell proliferation on the scaffolds was assessed by MTS assay after 1, 7, 14 and 21 days of incubation. A calibration curve was constructed before with a known number of cells (Appendix A). The cell numbers on the bacterial cellulose-collagen scaffolds which were treated with gene therapy, IGF-I and TGF-β1 growth factors and only chondrogenic differentiation medium were calculated according to this calibration curve. Figure 3.11 shows the RBMSC proliferation behavior on the bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers. Only cells (OC) that were grown on the wells of the 24 well plate used as positive control in this experiment.

According to the MTS assay, it was showed that none of these treatments had any cytotoxic effect on RBMSCs. As shown in Figure 3.11, cell numbers in all scaffolds increased throughout 21 days of incubation. The highest cell attachment (10 057 cells) was observed in phr-GFP-II-I treated RBMSCs seeded on DHT crosslinked bacterial cellulose-collagen scaffolds and the minimum cell attachment (3 915 cells) was observed in only chondrogenic medium added RBMSCs seeded on uncrosslinked scaffolds after day 1. At the end of 7 days of incubation, the highest cell number (15 775 cells) was observed in DHT crosslinked scaffolds. The cell proliferation behavior on the scaffolds was as follows: DHT/Genipin > DHT > Genipin > Untreated scaffolds. The same behavior was also observed after 14 and 21 days of incubation. Also, in all time points cell seeded onto polyplexed scaffolds that were showed higher cell number than the others.

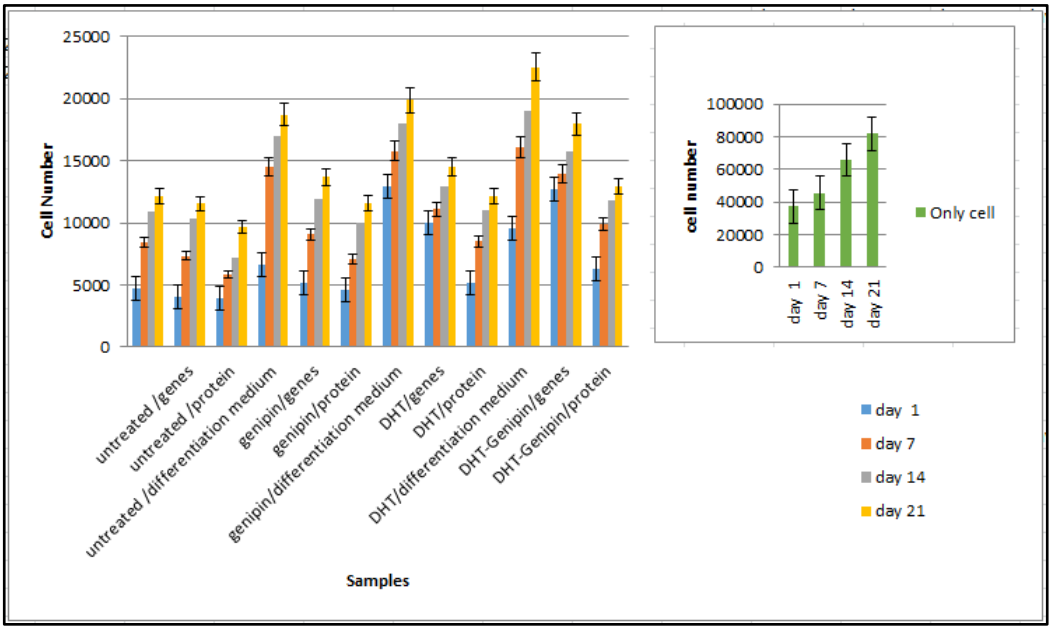

Figure 3.11. MTS assay of differentiated RBMSCs seeded bacterial cellulose-collagen scaffolds with different crosslinkers throughout 21 days of incubation. Initial cell seeding was 40 000 cells/per scaffold.

3. 3.5. Alcian Blue Staining

Alcian blue staining is used to indicate the presence of cartilage specific ECM molecules. This stain attracts acid mucins (such as hyaluronic acid and aggrecan) which are generally found in ECM of cartilage due to its basic nature. Histochemical results for cell seeded scaffolds that were polyplexed, IGF-I/TGF-β1 growth factors added and only chondrogenic medium (control) are shown in Figures 3. 12-14. Dark blue color indicates the presence of GAG, which is mostly found in cartilage tissue. Pale blue indicates the nuclei of RBMSCs due to Haemalum Mayer stain. Neutral mucins and glycogens which are mostly found in other connective tissues are stained by Periodic Acid and Schiff reagents in Alcian Blue-PAS stain kit and the purple color is an indication for a mixture of GAGs and neutral mucins.

As shown in Figure 3.12, at the end of 7 dyas of incubation, all phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes treated samples (A, D, G, J) were rich in cartilage specific GAGs with respect to their blue color. Also, it can be observed that all IGF-I and

TGF-β1 growth factors treated samples (B, E, H, K) produced more acid mucins than the samples treated with only chondrogenic differentiation medium (C, F, I, L) was added. These results indicate that RBMSCs can easily differentiate into cartilage when IGF-I and TGF-β1 growth factors are present in chondrogenic medium. In addition, samples which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes produced more GAG molecules than others due to sustained expression of IGF-I and TGF-β1 genes. If bacterial cellulose-collagen scaffolds are compared with each other, it can be observed that DHT/Genipin crosslinked scaffolds were the most supportive scaffolds with respect to differentiation of RBMSCs than the others regarding the presence of blue color (A, B, C). In addition, RBMSCs seeded DHT crosslinked bacterial cellulose-collagen scaffolds (D, E, F) produced more cartilage specific molecules than that of Genipin crosslinked scaffolds (G, H, I). Because of their instability in culture conditions, untreated bacterial cellulose-collagen scaffolds (J, K, L) were the least supportive scaffolds among the others after 7 days of incubation.

After 14 days of incubation, it was found that DHT/Genipin crosslinked (A, B, C) scaffolds were the most supportive scaffolds with respect to differentiation of RBMSCs than the others owing to the presence of blue color (Figure 3.13). Moreover, RBMSCs seeded on DHT crosslinked bacterial cellulose-collagen scaffolds (D, E, F) produced more cartilage specific molecules than Genipin crosslinked ones (G, H, I). Due to their unstability in culture conditions, untreated bacterial cellulose-collagen scaffolds (J, K, L) were the least supportive scaffolds among the crosslinked ones. In addition, all phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes incorporated samples (A, D, G, J) were rich in cartilage specific GAG molecules with respect to the presence of blue color. Furthermore, it seems that samples that were treated with only chondrogenic differentiation medium (C, F, I, L), produced more neutral mucins than the ones treated with IGF-I and TGF-β1 growth factors (B, E, H, K). In addition, samples which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes produced more GAGs than the others due to the sustained expression of IGF-I and TGF-β1 genes. Additionally, RBMSCs seeded on the bacterial cellulose-collagen scaffolds produced more GAGs at the end of 14 days of incubation than that of 7 days of incubation.

After 21 days of incubation, the same behaviors that were observed in day 7 and 14 were recorded in all samples (Figure 3.14). Also, it is important to note that, RBMSCs seeded on the bacterial cellulose-collagen scaffolds showed prolong release of GAGs during the time course of incubation.

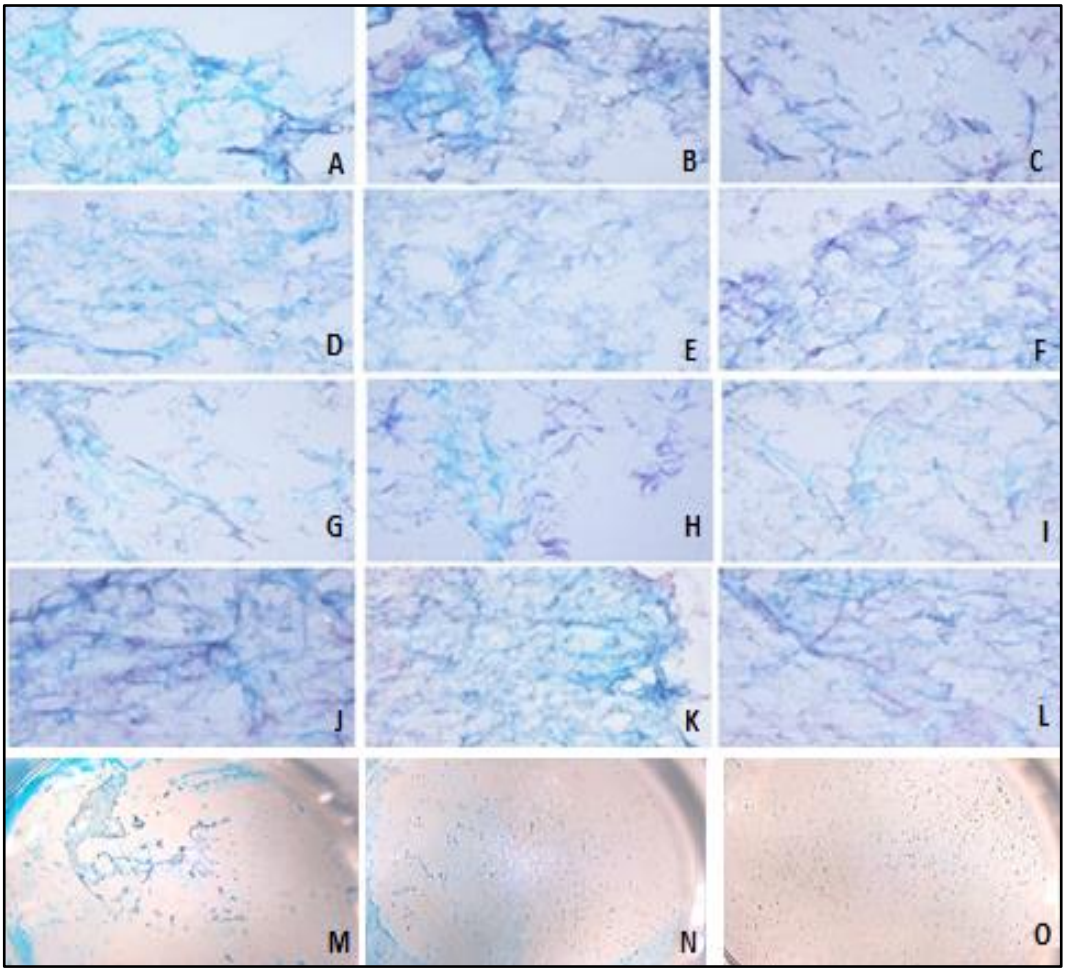

Figure 3.12. Alcian Blue staining after 7 days of incubation. RBMSCs seeded on bacterial cellulose-collagen scaffolds that were crosslinked with DHT, Genipin and DHT/Genipin as follows: DHT/Genipin crosslinked scaffolds with a treatment of A) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, B) IGF-I and TGF-β1 growth factors, C) only chondrogenic differentiation medium; DHT crosslinked scaffolds with a treatment of D) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, E) IGF-I and TGF-β1 growth factors, F) only chondrogenic differentiation medium; Genipin crosslinked scaffolds with a treatment of G) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, H) IGF-I and TGF-β1 growth factors, I) only chondrogenic differentiation medium; Uncrosslinked

scaffolds with a treatment of J) the plasmid containing IGF-I and TGF-β1 genes, K) IGF-I and TGF-β1 growth factors, L) only chondrogenic differentiation medium; RBMSCs seeded on TCP with a treatment of M) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, N) IGF-I and TGF-β1 growth factors, O) only chondrogenic differentiation medium. Stained scaffolds were observed under 40X magnification.

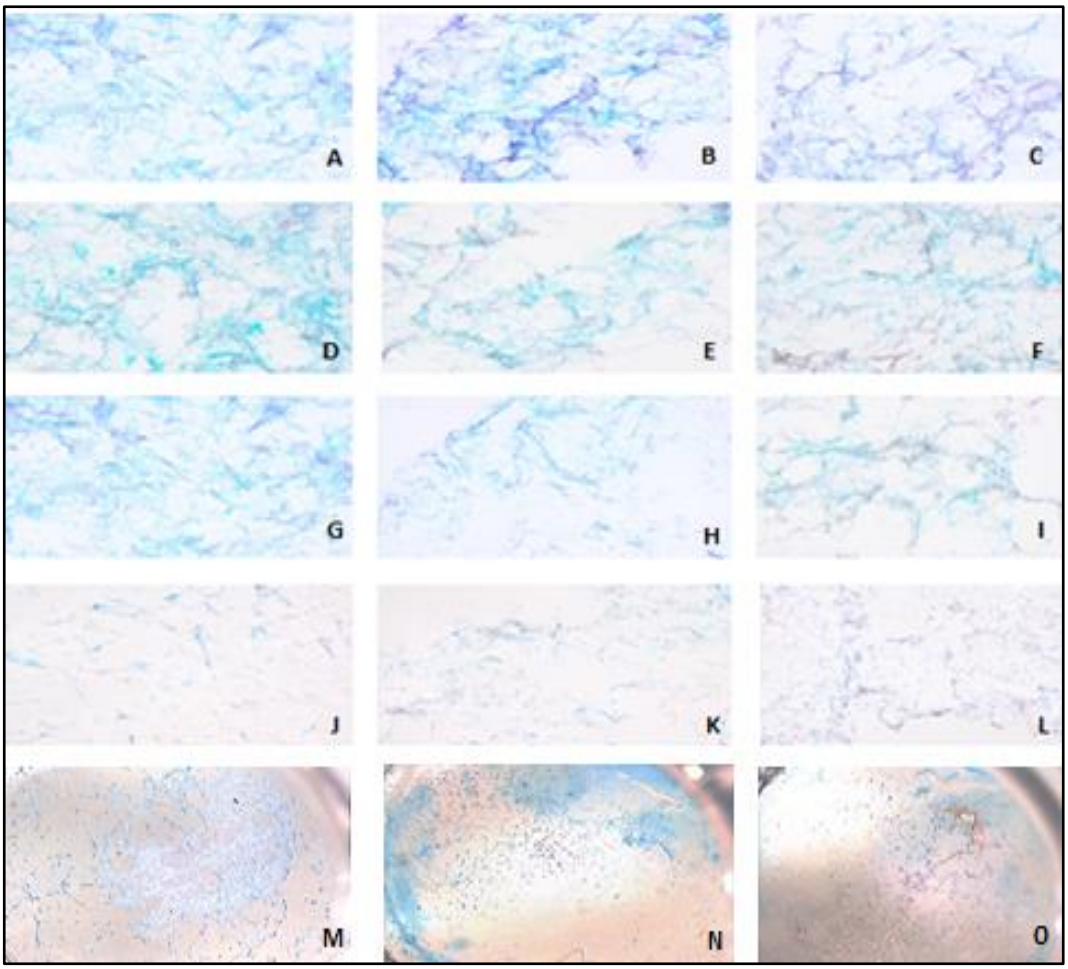

Figure 3.13. Alcian Blue staining after 14 days of incubation. RBMSCs seeded on bacterial cellulose-collagen scaffolds that were crosslinked with DHT, Genipin and DHT/Genipin as follows: DHT/Genipin crosslinked scaffolds with a treatment of A) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, B) IGF-I and TGF-β1 growth factors, C) only chondrogenic differentiation medium; DHT crosslinked scaffolds with a treatment of D) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, E) IGF-I and TGF-β1 growth factors, F) only chondrogenic differentiation medium; Genipin crosslinked scaffolds with a treatment of G) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, H) IGF-I and

TGF-β1 growth factors, I) only chondrogenic differentiation medium; Uncrosslinked scaffolds with a treatment of J) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, K) IGF-I and TGF-β1 growth factors, L) only chondrogenic differentiation medium; RBMSCs seeded on TCP with a treatment of M) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, N) IGF-I and TGF-β1 growth factors, O) only chondrogenic differentiation medium. Stained scaffolds were observed under 40X magnification.

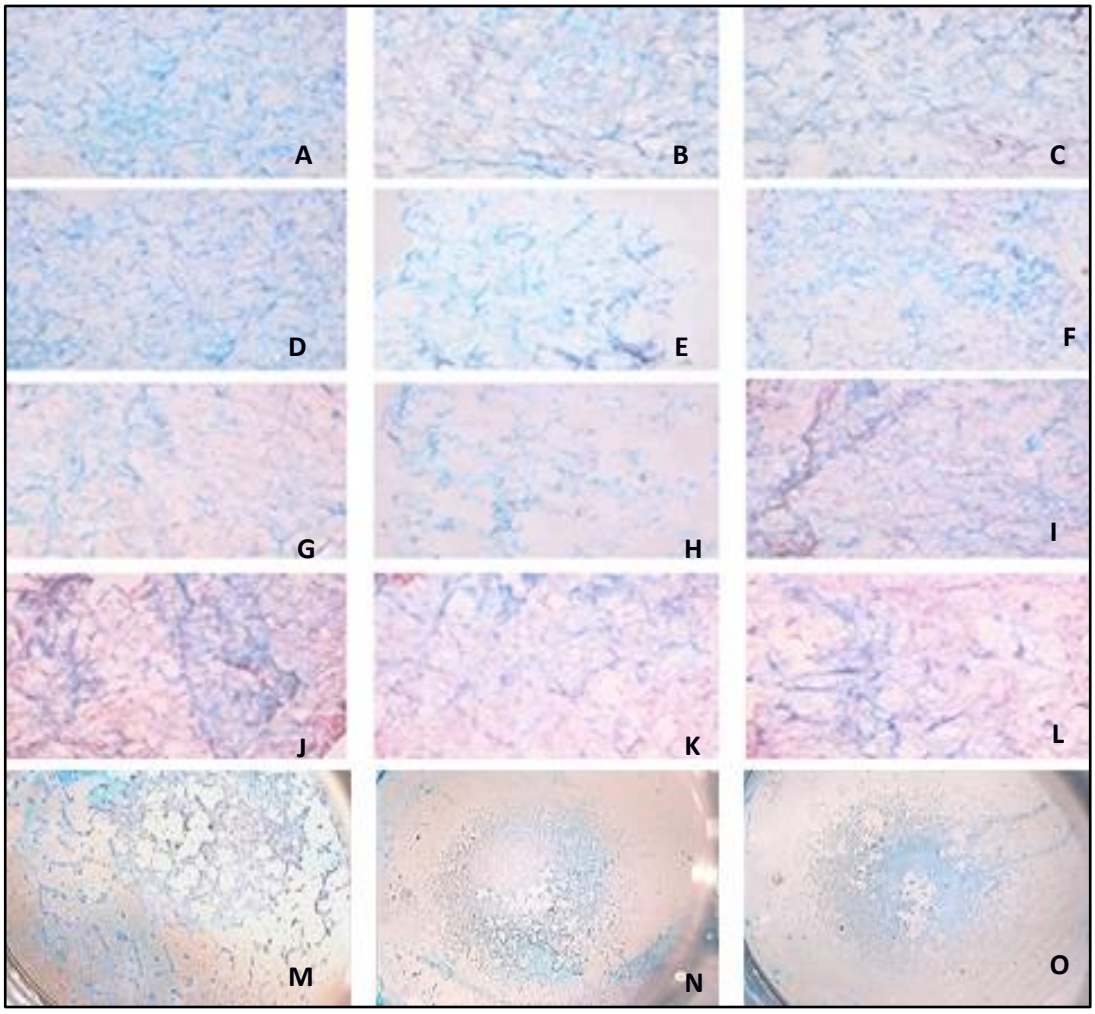

Figure 3.14: Alcian Blue staining after 21 days of incubation. RBMSCs seeded on bacterial cellulose-collagen scaffolds that were crosslinked with DHT, Genipin and DHT/Genipin as follows: DHT/Genipin crosslinked scaffolds with a treatment of A) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, B) IGF-I and TGF-β1 growth factors, C) only chondrogenic differentiation medium; DHT crosslinked scaffolds with a treatment of D) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, E) IGF-I and TGF-β1 growth

factors, F) only chondrogenic differentiation medium; Genipin crosslinked scaffolds with a treatment of G) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, H) IGF-I and TGF-β1 growth factors, I) only chondrogenic differentiation medium; Uncrosslinked scaffolds with a treatment of J) phrGFP-II-I plasmid containing IGF-I and TGF-β1 genes, K) IGF-I and TGF-β1 growth factors, L) only chondrogenic differentiation medium; RBMSCs seeded on TCP with a treatment of M) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, N) IGF-I and TGF-β1 growth factors, O) only chondrogenic differentiation medium. Stained scaffolds were observed under 40X magnification.

3.3.6. Confocal Microscopy Studies

Confocal microscopy is used to investigate the presence of protein which is matched with a light reflecting probe. To assess the effects of treatment with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, IGF-I and TGF-β1 growth factors and only chondrogenic medium on RBMSCs differentiation, samples and the controls were stained with cartilage specific collagen type II (green) and aggrecan (red) molecules and observed under the confocal microscope.

It is known from the literature that while undifferentiated RBMSCs have a fibroblast-like shape, chondroblasts and chondrocytes have a spheroidal shape. As shown in Figure 3.15, RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes (A, B, C) had a round shape while the others were more fibroblast-like after 7 days of incubation on TCP wells. Moreover, unlike growth factors (D, E, F) and only chondrogenic medium treated ones (G, H, I), RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes (A, B, C) started to aggregate. In addition, it seems that RBMSCs treated with only chondrogenic medium had more fibroblast-like shape than the ones treated with IGF-I and TGF-β1 growth actors. In Figure 3.15, green color indicates the presence of collagen type II which is the key component of ECM. Red color indicates the presence of aggrecan which is the key molecules of chondrocytes in culture conditions. It can be observed that all treatment strategies that were applied for chondrogenic differentiation of RBMSCs on TCP wells induced production of cartilage specific molecules after 7 days of incubation. However, RBMSCs which were treated with

phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes (A, B, C) differentiated faster than the others (D-I). RBMSCs treated with both IGF-I and TGF-β1 genes (A, B, C) and growth factors (D, E, F) presented round shape while the only chondrogenic medium treated ones (G, H, I) showed elongated shape on TCP wells during 7 day of differentiation.

However, there was a significant difference between IGF-I and TGF-β1 genes and growth factors treated RBMSCs at 14 day with respect to differentiation (Figure 3.16). While IGF-I and TGF-β1 growth factors treated cells (D, E, F) only increased in cell number, the other cells that were treated with IGF-I and TGF-β1 genes (A, B, C) aggregated on TCP wells Moreover, RBMSCs which were treated with phrGFP-II-I plasmids had more condense structures then the growth factors treated ones. As can be seen in all images of Figure 3.16, RBMSCs produced aggrecan and collagen type II during 14 days of incubation. This result indicates that RBMSCs continued to produce these cartilage specific molecules. In addition, RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes (A, B, C) had more aggregation form than the others. Moreover, RBMSCs treated with IGF-I and TGF-β1 growth factors (D, E, F) increased in numbers during 14 days of incubation and produced collagen type II and aggrecan molecules. Also, cells treated with only chondrogenic medium (G, H, I) started to differentiate during this time period. These results indicate that chondrogenic differentiation process of RBMSCs can be induced in the presence of IGF-I and TGF-β1 growth factors in chondrogenic medium. In addition, considering their blastema formation, RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes differentiated into cartilage faster than the others since transfected cells with IGF-I and TGF-β1 genes provided prolong release of protein product. Additionally, RBMSCs differentiated more into chondrocyte at the end of 14 days of incubation than the cells incubated for 7 days on TCP wells.

As shown in Figure 3.17, RBMSCs continued production of Collagen type II (green color) and Aggrecan (red color) glycoproteins which are the key components of ECM in chondrocytes, this result indicates that RBMSCs continued differentiation after 21 days of incubation on TCP wells. In addition, RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes (A, B, C) had blastema formation which is

one of the important steps of chondrogenic differentiation. Moreover, RBMSCs treated with IGF-I and TGF-β1 growth factors (D, E, F) started to aggregate for extracellular matrix formation. Moreover, cells treated with only chondrogenic medium (G, H, I) increased in numbers. In addition, considering their blastema formation, RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes differentiated into cartilage faster than the others since IGF-I and TGF-β1 genes transfected cells provided prolong release of protein product. Also it is important to note that, RBMSCs incubated for 21 days had better chondrogenic properties than the ones incubated for 14 days in culture.

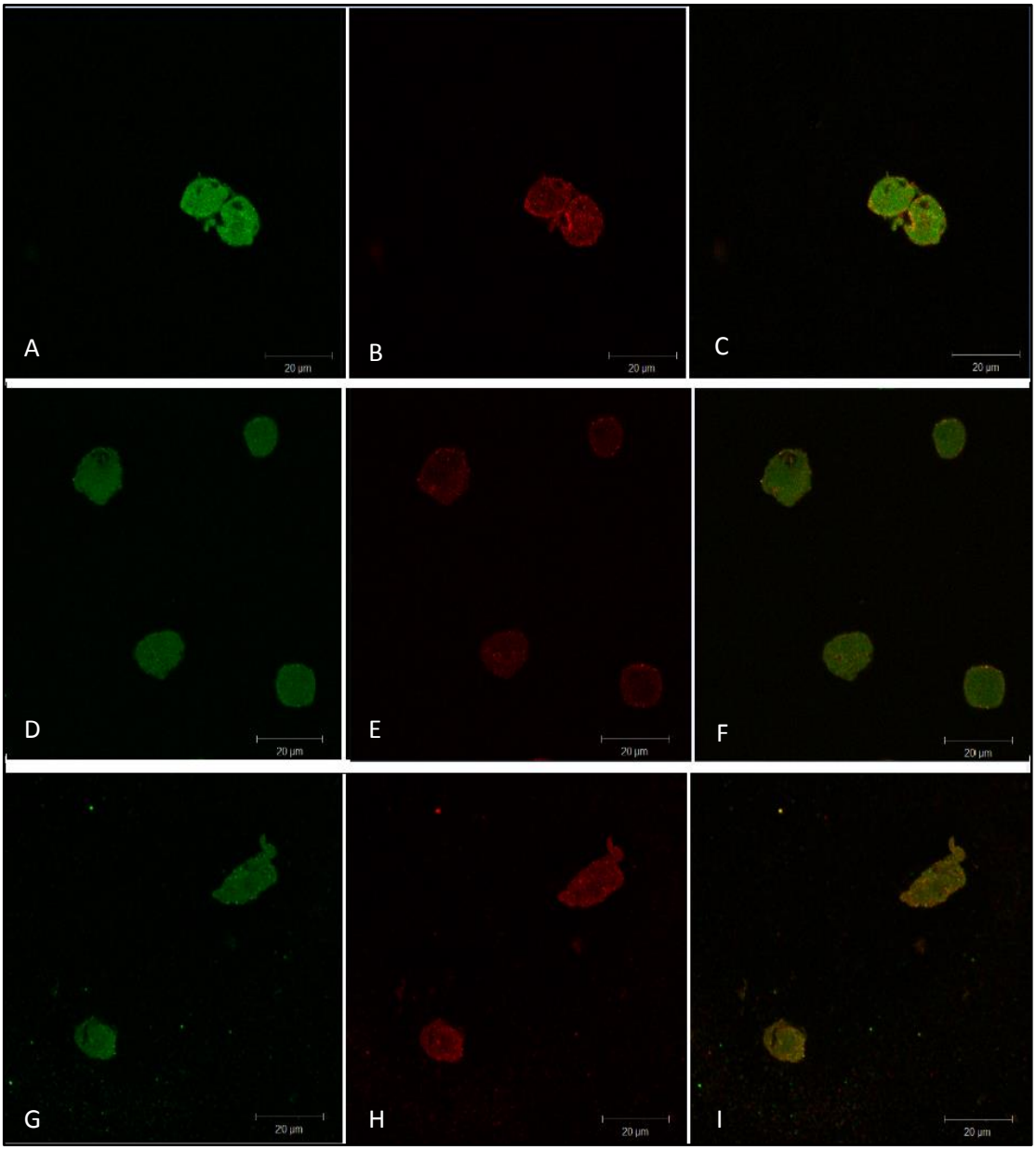

Figure 3.15. Confocal images after 7 days of incubation. RBMSCs seeded on the TCP wells and stained with FITC conjugated-Collagen Type II antibody (Green) and Aggrecan antibody with Alexa Fluor® 647 secondary antibody (Red) as follows: RBMSCs treated with A, B, C) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, D, E, F) IGF-I and TGF-β1 growth factors, G, H, I-) only chondrogenic differentiation medium. C, F and I are the merged images of Collagen type II and Aggrecan antibody stained samples. All images were recorded under 63X magnification and scale bar represents 20 µm.

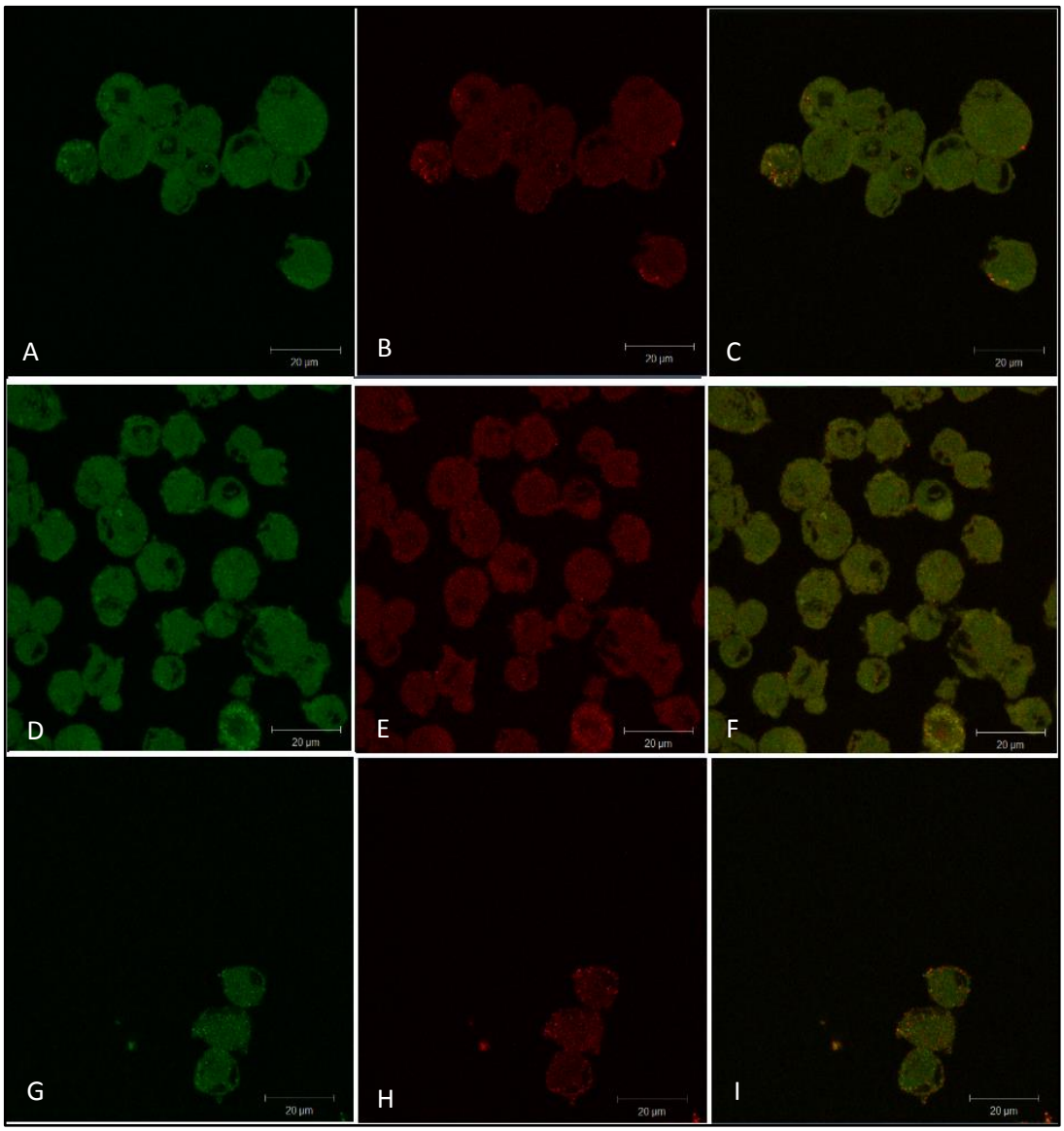

Figure 3.16: Confocal images after 14 day of incubation. RBMSCs seeded on the TCP wells and stained with FITC conjugated-Collagen Type II antibody (Green) and Aggrecan

antibody with Alexa Fluor® 647 secondary antibody (Red) as follows: RBMSCs treated with A, B, C) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, D, E, F) IGF-I and TGF-β1 growth factors, G, H, I) only condrogenic differentiation medium. C, F and H are the merged images of Collagen type II antibody and Aggrecan antibody stained samples. All images were recorded under 63X magnification and scale bar represents 20 µm.

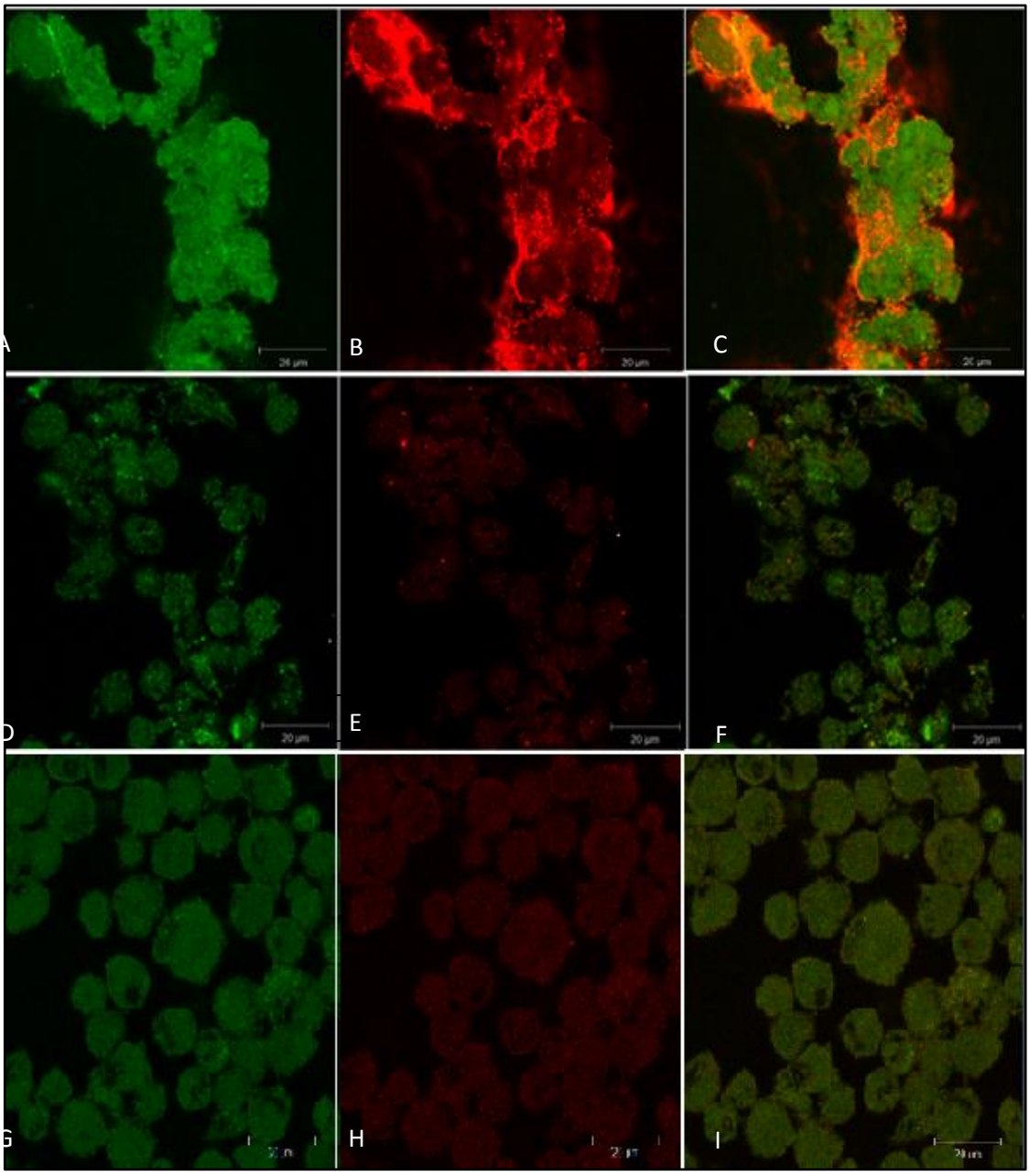

Figure 3.17. Confocal images after 21 days of incubation. RBMSCs seeded on the TCP wells and stained with FITC conjugated-Collagen Type II antibody (Green) and Aggrecan antibody with Alexa Fluor® 647 secondary antibody (Red) as follows: RBMSCs treated with A, B, C) phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, D, E, F) IGF-I and TGF-β1 growth factors, G, H, I) only chondrogenic differentiation medium. C, F and H are the merged images of Collagen type II antibody and Aggrecan antibody stained samples. All images were recorded under 63X magnification and scale bar represents 20 μm.

To assess the effect of bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers on chondrogenic differentiation process of RBMSCs which were treated with phr-GFP-II-I plasmid containing IGF-I and TGF-β1 genes, samples were visualized under the confocal microscope (Figure 3.18). Due to auto-fluorescent property of collagen type I, cells were also stained with nuclei stain DAPI to distinguish cells from the bacterial cellulose-collagen scaffolds.

As shown in Figure 3.18, RBMSCs attached on bacterial cellulose-collagen scaffold produced cartilage specific molecules such as collagen type II (green) and aggrecan (red). To indicate the presence of cells and its ECM, merged images of nuclei, collagen type II and aggrecan molecules were recorded.

It was observed that the number of RBMSCs seeded onto bacterial cellulose-collagen scaffols that were crosslinked with different crosslinkers increased throughout 21 days of incubation. However, this increase of cell was not observed in RBMSCs seeded untreated bacterial cellulose-collagen scaffolds (L). RBMSC which were seeded onto DHT/Genipin bacterial cellulose-collagen scaffolds (C) produced more collagen type II (green) and aggrecan (red) molecules than the others at the end of 21 days of incubation.

Morphology of RBMSCs had spheroidal which is the characteristics of chondrocytes in all samples (C, F, I, L). Moreover, RBMSCs which were seeded onto DHT/Genipin crosslinked scaffolds (C) showed more condense structure than the ones seeded on DHT crosslinked (F) and DHT/Genipin crosslinked (I) bacterial cellulose-collagen scaffolds.

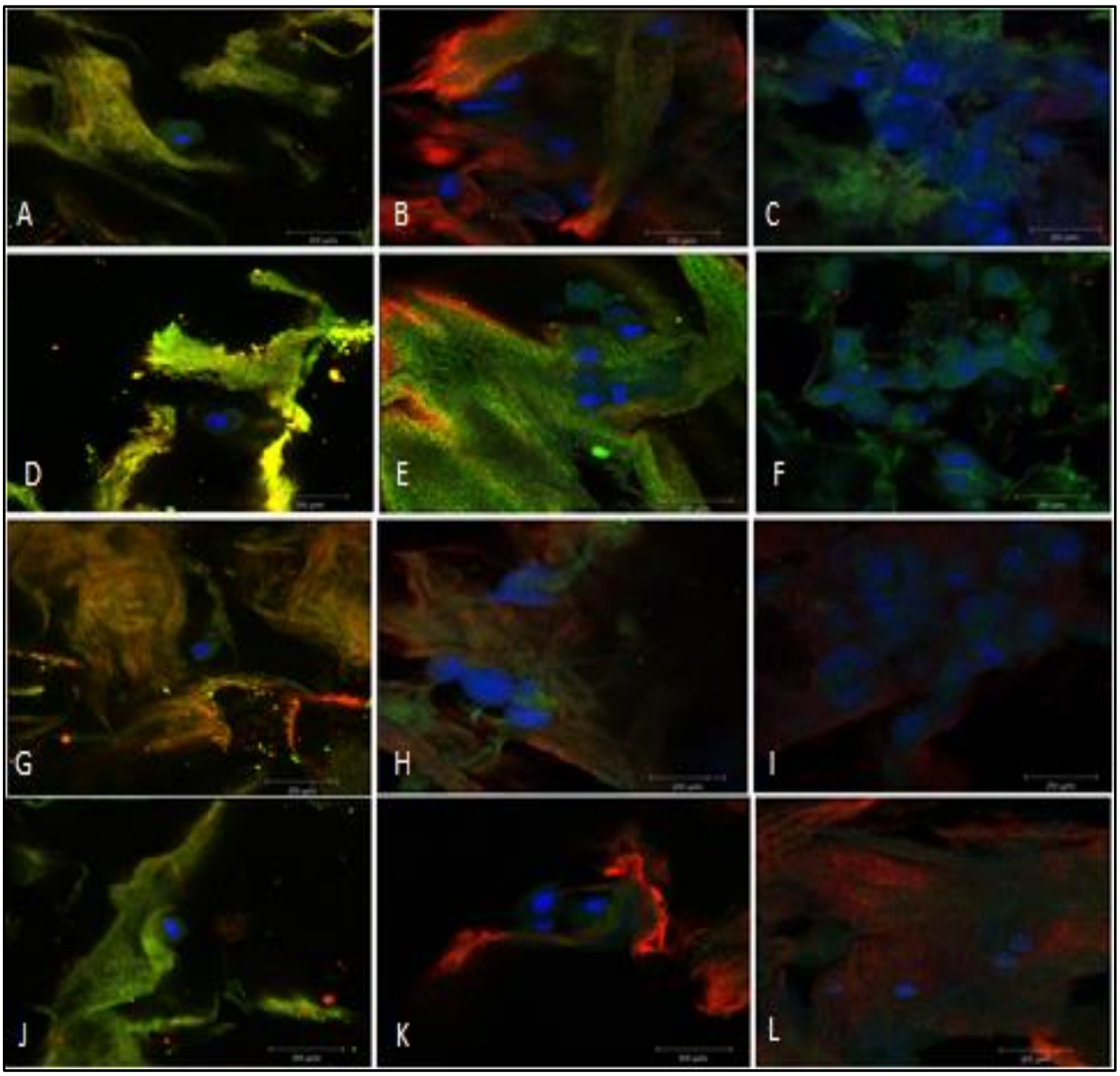

Figure 3.18. Confocal images of RBMSCs seeded on bacterial cellulose-collagen scaffolds with different crosslinkers throughout 21 days of incubation. Samples were stained with DAPI (blue), FITC conjugated-Collagen Type II (Green) and Aggrecan (Red) antibodies with Alexa Fluor® 647 secondary antibody. All cells were seeded onto polyplexed bacterial cellulose-collagen scaffolds with different crosslinkers as follows: DHT/Genipin crosslinked scaffolds A) 7 days of incubation, B) 14 days of incubation, C) 21 days of incubation; DHT crosslinked scaffolds D) 7 days of incubation, E) 14 days of incubation, F) 21 days of incubation; Genipin crosslinked scaffolds G) 7 days of incubation, H) 14 days of incubation, I) 21 days of incubation; Uncrosslinked scaffolds J) 7 days of

incubation, K) 14 days of incubation, L) 21 days of incubation. All images were recorded under 63X magnification and scale bar represents 20 µm.

3.3.7. Protein Production Studies

Several studies have shown that both IGF-I and TGF-β1 growth factors have an effect on metabolic process of mesenchymal stem cells [166, 167]. To investigate the effects of treatment of IGF-I and TGF-β1 genes, IGF-I and TGF-β1 growth factors and only chondrogenic medium on metabolic process of RBMSCs seeded onto bacterial cellulose-collagen scaffolds with different crosslinkers throughout 21 days of incubation, total protein amounts of all samples were determined and compared with each other (Figure 3.19).

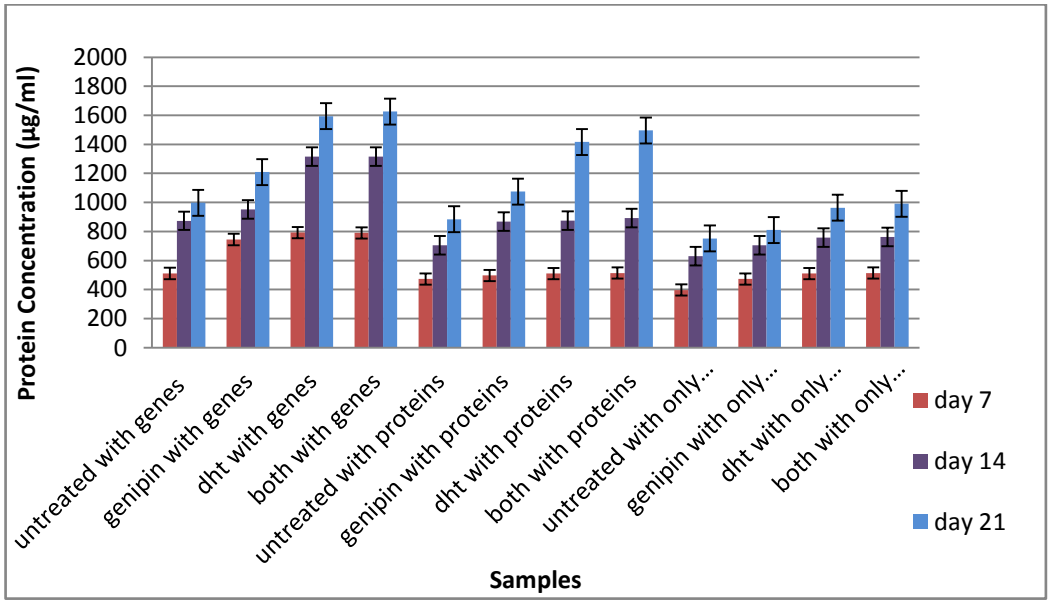

Figure 3.19. Total protein concentration of RBMSCs seeded onto the bacterial cellulose-collagen scaffolds with different crosslinkers throughout 21 days of incubation and treated with IGF-I and TGF-β1 genes, IGF-I and TGF-β1 growth factors and only chondrogenic differentiation medium.

As can be seen in Figure 3.19, total protein amount of RBMSCs treated with IGF-I and TGF-β1 genes were higher than the others throughout 21 days of incubation. In addition,

protein production of IGF-I and TGF-β1 growth factors treated RBMSCs was higher than the ones treated with only chondrogenic differentiation medium. Also, it is important to note that, RBMSCs seeded onto DHT/Genipin crosslinked scaffolds produced more proteins than the others for all chondrogenic differentiation strategies.

4. DISCUSSION

Scanning electron micrographs showed the microstructural morphology of crosslinked and untreated scaffolds. All samples presented a well-organized three dimensional structures. Interconnected cellulose and collagen type I fibrils of untreated scaffolds were also observed. Similarly, Luo *et al*. reported that bacterial cellulose-collagen scaffolds that were not crosslinked with any crosslinker have well interconnected porous structure that has large surface area and this structure is necessary for the cell attachment in biomedical applications [168]. It can be observed from the SEM that crosslinked bacterial cellulose-collagen scaffolds were rougher and denser than the untreated ones. It seems that crosslinking of bacterial cellulose-collagen scaffolds with Genipin, DHT or both increased their surface area. In addition, crosslinked scaffolds had interconnected pores which promoted cellular ingrowth. However, pore size of each crosslinked bacterial cellulose-collagen scaffolds was different. Genipin crosslinked scaffolds had smaller pores than DHT and DHT/Genipin crosslinked ones. Moreover, pore sizes of DHT crosslinked scaffolds were larger than that of DHT/Genipin crosslinked ones. Based on this observation, it can be suggested that Genipin crosslinking decreased pore sizes of bacterial cellulose-collagen scaffolds. Furthermore, DHT technique preserved the original structure of scaffolds. It was also observed from the SEM of all bacterial cellulose-collagen scaffolds were found suitable biomaterials for the cell attachment. Likewise, Zhijiang *et al.* carried out cell adhesion study of bacterial cellulose-collagen scaffolds using 3T3 fibroblast cells and proposed that these scaffolds can be appropriate matrices for the cell attachment [169]. The high surface area and waterholding capacity of all bacterial cellulose-collagen scaffolds provided great advantage.

FTIR spectrum obtained from freeze dried and sterilized bacterial cellulose from *Gluconacetobacter xylinus* culture were also analyzed. Bacterial cellulose have hydroxyl groups on its C atoms and Kim *et. al* reported absorption band assigned to the hydroxyl groups. In this study, hydrogen bonds at 3 200-3 500 cm^{-1} and –OH groups at 3 300 cm^{-1} gave a strong absorption peak [170]. Garside *et. al* reported that the C-C ring breathing band at ~1 155 cm^{-1} and the C-O-C glycosidic ether band at ~1 105 cm^{-1} obtained in

cellulose sample [171]. In this experiment, broad absorption peak at 1 105 cm^{-1} was also observed. This indicates that our sample had pyranose ring structure, which is found in glucose molecules. In addition, Marchessault reported that 1 425 cm^{-1} indicates the presence of β-D-glucose molecules and cellulose I structure which is the non-crystalline form of cellulose [172]. This band was also obtained in the present study and it indicates that our bacterial cellulose sample had non-crystalline structures which are suitable for cell adhesion. Absorption at 1 720 cm^{-1} indicated the presence of water within the sample. Signals of bacterial cellulose sample showed that they contained C, H, O atoms as expected. In addition, strong absorption peaks at 1 105 cm^{-1}, 1 425 cm^{-1} and 3 400 cm^{-1} proved that these samples harvested from *Gluconacetobater xylinus culture* were containing pure cellulose.

After SEM and FTIR studies, the degradation behavior of bacterial cellulose-collagen scaffolds was investigated. A crosslinking reagent is required to improve mechanical strength and degradation properties of biopolymers for tissue engineering since the mechanical properties of a tissue engineering scaffold are critical for preserving structural integrity and functionality during both *in vivo* implantation and long-term performance. In this context, aim of using different crosslinking techniques on bacterial cellulose-collagen scaffolds was to investigate the effects of DHT and Genipin crosslinking on degradation process of the scaffolds. It was observed that majority of all bacterial cellulose-collagen mass loss was obtained in the first 15 day of degradation. Due to rapid mass loss of untreated scaffolds, it can be said that crosslinking activity decreased degradation rate of bacterial cellulose-collagen scaffolds in PBS, as expected. However, degradation profiles of all crosslinked bacterial cellulose-collagen scaffolds were not the same. DHT/Genipin crosslinked scaffolds were found more stable than Genipin crosslinked ones and less stable than DHT crosslinked ones. Based on this observation, it can be suggested that crosslinked bacterial cellulose-collagen scaffolds by DHT is more efficient technique than Genipin crosslinked ones.

In order to show the extent of degradation, pH change in time was also determined in this study. A decline in pH values was obtained in the first 15 day of degradation in all types of scaffolds. This pH decrease is an indication of the degradation extent and therefore, the

sample that leads to the largest pH drop would have been the one that has degraded the most. It was found that pH of all crosslinked and uncrosslinked scaffolds decreased from 7.4 to 7.37 during 15 days of incubation. Collagen degradation might be the reason of this pH change due to acidic nature of collagen. Based on this observation, it can be suggested that DHT and Genipin crosslinking techniques have an effect on degradation of bacterial cellulose-collagen scaffolds. Similarly, O'Brien et al. showed that DHT crosslinking method (105 ^{0}C for 24 h) decreased degradation rate of collagen-GAG scaffolds due to the interaction between collagen molecules [122].

After the characterization of scaffolds, plasmid constructs were also characterized. Clonning vectors can be used to increase copy number of plasmids. Bacteria are used to clone this type of vectors due to their rapid proliferation rate. *E. coli* DH5α strain is one of the most widely used bacteria to clone plasmids due to their endonuclease free nature. In this study, IGF-I and TGF-β1 genes which were in pUC57 plasmid were cloned using *E. coli* DH5α strain. Then, they were cut with restriction enzymes to ligate them into phrGFP-II-I plasmid. In order to increase copy number of these genes and provide their expression in rat bone marrow mesenchymal stem cells, phrGFP-II-I plasmids were used in rest of study. phrGFP-II-I plasmids are the shuttle vectors which allow both cloning in *E. coli* DH5α strain and expression in mammalian cells. In addition, this plasmid contains two multiple cloning sites which can carry two genes in its structure. Because of these properties, phrGFP-II-I plasmids were chosen to transfect cells with two genes at the same time. First, Insulin-like Growth Factor 1 gene, and then, Transforming Growth Factor β 1 gene were ligated into this plasmid. The presence of IGF-I, TGF-β1 genes and IGF-I-hrGFP II-I-TGF-β1 gene construct in phrGFP-II-I vector was shown in agarose gel using appropriate restriction enzymes. Moreover, the presence of these genes in phrGFP-II-I plasmid was also confirmed by sequencing. Analyzed sequencing data confirmed the presence of Transforming Growth Factor β 1 and Insulin-like Growth Factor 1 genes without any mismatches.

For the *in vitro* cell culture studies, first rat bone marrow stem cells were isolated and characterized. Bone marrow is a complex tissue containing stem cells with hematopoietic properties. The hematopoietic stem cells, which are the primary source of blood cells in the

adult body, are regulated within a microenvironment of stromal cells in the bone marrow [173]. In this study, isolated cells (passage 2) from rat bone marrow were stained with both hematopoietic and mesenchymal stem cell markers and analyzed with Flow Cytometry. It was found that isolated cells expressed CD 90 and CD 29 proteins which were found on the surface of the mesenchymal stem cells. Very low amount of CD 45, CD 34, CD 14 and CD 11a, hematopoietic stem cell markers, was also expressed. Flow cytometry analysis results confirmed that these cells isolated from rat bone marrow were mesenchymal stem cells.

The results of MTS assay confirmed viability and proliferative activity of RBMSCs seeded on the untreated bacterial cellulose-collagen scaffolds as well as crosslinked ones. Similarly, Pei *et al.* indicated that cellulose-collagen films were suitable matrices for cell adhesion and growth [174]. All types of bacterial cellulose-collagen scaffolds facilitated the transport of oxygen and nutrients to cells but crosslinked scaffolds were more supportive than the untreated ones. Thus, it can be said that crosslinking activity of DHT or Genipin treatments increased stability of the scaffolds in culture conditions. Yan *et al.* showed that genipin crosslinked collagen-chitosan scaffolds supported attachment of chondrocytes when compared the control groups [175]. On the other hand, it was observed that DHT and Genipin crosslinking methods had different effects on cell viability and adhesion. DHT and DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds were more suitable for proliferation of RBMSCs with respect to the Genipin crosslinked ones during 21 days of incubation. It can be suggested that larger pores of DHT and DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds provided more space for proliferation of cells. Also, Cheng *et al.* indicated that residual genipin in Genipin crosslinked collagen-GAG scaffolds caused cellular toxicity [176]. Considering results of MTS assay, DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds were found the most suitable scaffold for cell growth throughout 21 days of incubation.

Several studies showed that bone marrow stem cells are able to differentiate into cartilage in *in vitro* when suitable culture conditions are applied [177, 178]. In this experiment, RBMSCs were seeded onto bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers. To induce their chondrogenesis pathway, three different study

groups were used. In the first group, cells were treated with phr-GFP-II-I plasmids containing IGF-I and TGF-β1 genes. In the second group, cells were treated with IGF-I and TGF- β1 growth factors and in the last group, only chondrogenic medium was added onto scaffolds as a control. Regarding the results of MTS assay, phr-GFP-II-I plasmids containing IGF-I and TGF-β1 genes loaded scaffolds were found the most suitable matrices for proliferation of RBMSCs throughout 21 days of differentiation. Likewise, Longobardi *et al.* showed that both IGF-I and TGF-β1 growth factors had positive effect on proliferation of mesenchymal stem cells when applied together [179]. Based on these results, it can be suggested that the application of gene therapy provided prolong production of these proteins for the cells and it was found more efficient method than the treatment of IGF-I and TGF-β1 growth factors alone and the control group in which only chondrogenic differentiation medium was used. Additionally, it was observed that cell numbers in growth factor treated samples increased more than the control group where only chondrogenic medium was added. MTS assay also showed that DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds were also the most supportive scaffolds for chondrogenic differentiation process of cells. It might be the result of higher Lipofectamine^{2000}-plasmid holding capacity of DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds due to the gel formation of collagen with Genipin than the DHT crosslinked ones.

For the determination of cell differentiation, Alcian blue staining was used. It can be applied to show the presence and distribution of GAG molecules which are only found in cartilage. During 21 days of differentiation period, RBMSCs were stained with Alcian blue at different time points to assess GAG production of samples. Regarding the color formation on the samples, it was observed that phr-GFP-II-I plasmids treated RBMSCs produced more GAG molecules than the others during 21 days of differentiation. Likewise, Longobardi *et al.* showed that combination of IGF-I and TGF-β1 proteins were able to induce chondrogenic differentiation process of mesenchymal stem cells [179]. Takagi *et al.* indicated that only in the presence of TGF-β3, IGF-I was capable of inducing cartilage specific protein production on mesenchymal stem cells [180]. Based on these observations, it can be said that usage of gene therapy technique on RBMSCs provided more chondrogenic differentiation due sustained expression of growth factors. Corralating with

the MTS data, RBMSCs which were treated with IGF-I and TGF-β1 genes and seeded on DHT/Genipin crosslinked scaffolds produced more GAG molecules than ones seeded on DHT or Genipin crosslinked scaffolds. In addition, chondrogenic differentiation process of RBMSCs seeded on TCP can be easily observed at different time points. Due to their round morphology in the wells of TCP, it can be assumed that RBMSCs had already started differentiation during 7 days of incubation. Poole *et al.* indicated that cartilage is a highly specialized structure that is composed predominantly of extracellular matrix (ECM) and an aggregate-forming proteoglycan, aggrecan, with embedded chondrocytes [181]. Likewise, in the current study, RBMSCs that were treated with IGF-I and TGF-β1 genes connected with each other with their glycosaminoglycans after 14 days of differentiation. Also, due to their more intense alcian blue staining profile, it can be said that RBMSCs had an aggregate formation after 21 days of differentiation. However, IGF-I and TGF-β1 growth factors treated RBMSCs expressed round shape morphology during 14 days of differentiation and connected with each other without aggregate formation. Based on these observations, it can be said that RBMSCs which were treated with phrGFP-II-I plasmids was able to induce chondrogenic differentiation process in shorter time than the others.

Confocal microscopy technique is used to be assure the presence of proteins that are an indicators of differentiation, in the samples using their specific antibodies. Collagen type II and Aggrecan molecules are the important components of cartilage ECM. Using their specific antibodies, Collagen type II and Aggrecan molecules were stained in bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers. Voytik-Harbin *et al.* [182] reported that collagen type I protein has autoflorescence property under the confocal microscopy thus bacterial cellulose-collagen samples were stained with also nuclei stain (DAPI) to show the presence of cells in the scaffolds. Confocal microscopy images showed that RBMSCs seeded on both bacterial cellulose-collagen scaffolds and TCP increased in cell number throughout 21 days of incubation except the ones seeded on untreated scaffolds. Instability of untreated bacterial cellulose-collagen scaffolds in culture conditions may cause this result. Zhong *et al.* showed that chondrocytes had spheroidal shape while undifferentiated stem cells had fibroblast-like shape in the culture [183]. In this study, RBMSCs treated with both IGF-I and TGF-β1 genes and growth factors and seeded on TCP presented round shape while the the ones treated with only chondrogenic

medium exhibited elongated shape during 7 days of differentiation. In the previous studies, it was shown that the presence of IGF-I and TGF-β1 proteins in differentiation medium was able to induce chondrogenic process of mesenchymal stem cells [159]. However, there was a significant difference between phrGFP-II-I plasmid treated and growth factors treated cell with respect to morphology at the end of 14 days of differentiation. Takagi reported that bone marrow stem cells had aggregate formation during their chondrogenic differentiation process [180]. Regarding confocal images of RBMSCs which were seeded on TCP at day 14, cells that were treated with IGF-I and TGF-β1 genes formed aggregated structures while the ones treated with IGF-I and TGF-β1 growth factors only increased in cell number. Moreover, RBMSCs which were treated with phrGFP-II-I had more condense structure while growth factors treated ones seemed to start aggregation at the end of 21 days of differentiation. Based on these observations, it can be suggested that cells which seeded onto polyplexed bacterial cellulose-collagen scaffolds differentiated into cartilage faster than the growth factors treated ones due to their prolong release of IGF-I and TGF-β1 proteins. It was also observed from the confocal microscope that cells seeded onto all types of bacterial cellulose-collagen scaffolds produced cartilage specific collagen type II and aggrecan molecules. Together with Alcian blue staining results, it can be suggested that bacterial cellulose-collagen scaffold were suitable biomaterials for chondrogenic differentiation of rat bone marrow stem cells. Regarding their structure through 21 days of differentiation, RBMSCs that were seeded onto DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds had more aggregate-formation than the ones seeded on the other samples. This result matched with MTS and Alcian blue staining results.

Several studies indicated that IGF-I and TGF-β1 growth factors have an effect on metabolic process of cells [166, 167]. Thus in this study, total amount of proteins from phrGFP-II-I plasmids, growth factors and only chondrogenic medium treated RBMSCs which were seeded on bacterial cellulose-collagen scaffolds. Martins *et al*. showed that IGF-I was one of the most important anabolic factor in chondrocytes [184]. Derynck indicated that TGF-β1 had significant role in differentiation process of stem cells [185]. In addition, Longobardi *et al*. showed that combination of IGF-I and TGF-β1 protein was able to induce chondrogenic differentiation process of mesenchymal stem cells and produced more cartilage specific protein during chondrogenic differentiation process of stem cells

[179]. In this study, phrGFP-II-I plasmids treated RBMSCs which were seeded on bacterial cellulose-collagen scaffolds produced more protein than the others. In addition, DHT/Genipin crosslinked bacterial cellulose scaffolds were more supportive for protein production of cells. This result matched with results of MTS, Alcian blue staining and confocal microscopy studies.

5. CONCLUSION

This study has made a considerable contribution to the field of gene therapy and tissue engineering. So many researches have been reported the application of non-viral vectors to 3D scaffolds. Specifically optimising a vector for targeting cell type and using the refined polyplex doses and compositions in a 3D bacterial cellulose-collagen scaffold that were crosslinked with DHT and Genipin offers a superior bioactive scaffold for tissue engineering applications. Engineering bacterial cellulose-collagen scaffolds using different crosslinkers and incorporation of IGF-I and TGF-β1 genes may cause considerable effects on differentiation process of RBMSCs and it is beneficial in terms of reduction of the amount of growth factors required. This will decrease the cost and the presence of exogenous materials in a defect site. Also, it will eliminate the short half life problem of the growth factors.

The non-viral phrGFP-II-I plasmid-Lipofectamine^{2000} incorporated bacterial cellulose-collagen scaffolds that were crosslinked with DHT and/or Genipin were used to induce chondrogenic differentiation process of RBMSCs. This gene transfer system has enormous scope in genetically directing RBMSCs towards a particular lineage for regeneration. Proliferation and differentiation processes of RBMSCs seeded on phrGFP-II-I plasmid-Lipofectamin^{2000} incorporated bacterial cellulose-collagen scaffolds that were crosslinked with different crosslinkers were faster than that of IGF-I and TGF-β1 growth factors and only chondrogenic medium treated samples. Moreover, the IGF-I and TGF-β1 genes incorporated DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds had a significant effect on the proliferation and chondrogenic differentiation processes of RBMSCs by contributing to prolong elevated levels of gene expression up to 21 days. This suggests that IGF-I and TGF-β1 genes incorporated and crosslinked bacterial cellulose-collagen scaffolds, especially DHT/Genipin crosslinked ones, may have a significant potential for cartilage regeneration process. It can be proposed that these scaffolds might have an immense capability to promote cartilage regeneration and holds great promise for the field of tissue engineering.

6. FUTURE PROSPECTS

This study presents efficacy of combinatorial delivery systems of non-viral polyplexes delivered via crosslinked bacterial cellulose-collagen scaffolds on cartilage regeneration of mesenchymal stem cells. The results of this study showed that scaffold-mediated gene therapy is an advantegous method over growth factor treatment studies for cartilage regeneration.

In vivo experiments are planned to test the functionality and performance of phrGFP-II-I plasmids which contain IGF-I and TGF-β1 genes loaded DHT/Genipin crosslinked bacterial cellulose-collagen scaffolds in Spraque-Dawley rat cartilage defects. Moreover, presence of cartilage specific proteins of engineered cartilage tissue should be confirmed using western-blot technique.

In the future, different composites of bacterial cellulose may be studied. In order to show the effects of combination of growth factors on chondrogenic differentiation process, mesenchymal stem cells may be treated with different growth factor genes. Also, small size vectors can be used in further studies for increasing transfection efficiency of the cells.

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APPENDIX A

Calculation of Calibration Curve

Different amounts (5 000 to 50 000) of six week old Spraque-Dawley rat bone marrow stem cells (passage 2) were seeded on TCP of 24 wells. The day after seeding, MTS/media mixture (500 µL) was added into each sample in 24-well plate and incubated for 3 h at 37^0C in a CO_2 incubator. After 3 h of incubation, 200 µL of solution from each well was transferred into a 96-well plate. Absorbance was measured at 490 nm using Elisa Plate Reader. To determine cell number, calibration curve was prepared using absorbance values for different amounts of cells.

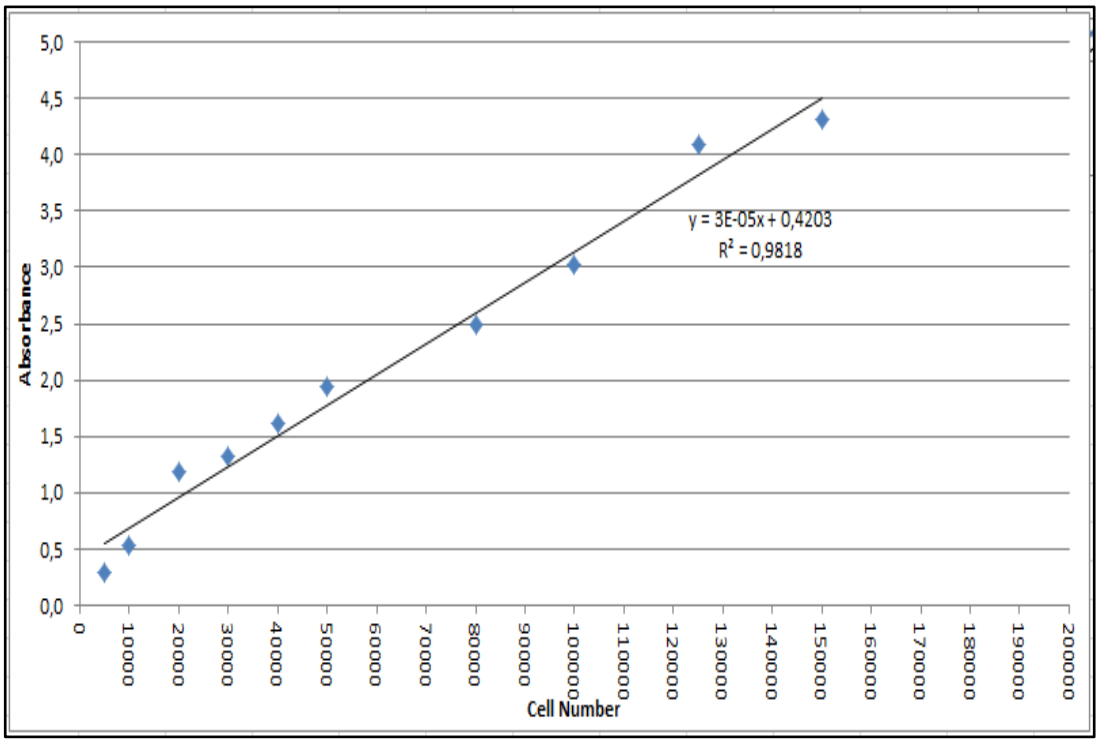

Figure: Calibration curve of six week old rat bone marrow stem cells (Passage 2).